

Supporting Information

**An Unexpected Split-Merge Pathway in the Assembly of the
Symmetric Nonribosomal Peptide Antibiotic Closthioamide**

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Experimental Procedures

General methods

Sequencing and oligonucleotide primer synthesis was performed by Eurofins Genomics. Media components were purchased from Sigma, Roth, and Difco. All chemicals were purchased from commercial suppliers (Sigma, Roth, *etc.*) without further purification. Restriction endonucleases were purchased from New England Biolabs. A list of all strains, plasmids, and oligonucleotide primers used can be found in **Table S1**, **Table S2**, and **Table S3**, respectively.

Bacterial strains and culturing conditions

Escherichia coli strains were grown in lysogeny broth (LB) shaken at 160 rpm or on LB agar plates at 37 °C with appropriate antibiotic selection (chloramphenicol, 25 µg mL⁻¹; kanamycin, 50 µg mL⁻¹). All plasmid construction and storage was done with *E. coli* TOP10, while *E. coli* Rosetta (DE3) was used for heterologous protein production.

Ruminiclostridium cellulolyticum DSM 5812 was cultivated under an anaerobic atmosphere (N₂:H₂:CO₂, 85:5:10 vol:vol:vol) in a Whitley A35 anaerobic work station (Don Whitley Scientific) operating at 37 °C. Routine cultivation was conducted in modified CM3 medium with cellobiose (6 g L⁻¹) as previously described.^[1] For the production of CTA, strains were grown in DSMZ medium 165 as previously described.^[2]

Bioinformatic analyses

All proteins discussed in this paper were assigned to a Pfam using HHPred.^[3] The phylogenetic tree of CtaB homologs was generated using MEGA6.^[4] Sequences were aligned using MUSCLE and the phylogenetic trees were reconstructed using the neighbor-joining method with 1,000 bootstraps. The CtaJ and CtaK multiple sequence alignments were generated using Clustal Omega.^[5] The CtaK structural model was made using SWISS-MODEL.^[6]

LC-HRMS

Standard HPLC-HRMS and HPLC-HRMS² measurements were performed with a Thermo Accela HPLC-system coupled to either a QExactive Hybrid-Quadrupole-Orbitrap (Thermo Fischer Scientific) or Exactive Hybrid-Quadrupole-Orbitrap (Thermo Fischer Scientific) mass spectrometer equipped with an electrospray ion source as previously described.^[7] For QExactive measurements, separation was performed with an Accucore C18 column (2.1 × 100 mm, 2.6 µm, Thermo Fisher) operating at a flow rate of 200 µL min⁻¹, with 0.1 % formic acid (solvent A) and acetonitrile + 0.1 % formic acid (solvent B) and the following gradient: 5 % solvent B for 1 min, 5 % to 98 % solvent B over 10 min, hold 98 % solvent B for 12 min. For Exactive measurements, separation was performed with a Betasil C18 column (2.1 × 150 mm, 3 µm, Thermo Fisher) operating at a flow rate of 200 µL min⁻¹, with 0.1 % formic acid (solvent A) and acetonitrile + 0.1 % formic acid (solvent B) and the following gradient: 5 % solvent B for 1 min, 5 % to 98 % solvent B over 15 min, hold 98 % solvent B for 10 min. Sample volumes for injection ranged from 3 to 5 µL.

For the CtaB kinetic assays, the HPLC-HRMS measurements were performed with a Thermo Ultimate3000 UHPLC-system coupled to a QExactive HF-X Hybrid-Quadrupole-Orbitrap (Thermo Fischer Scientific) mass spectrometer equipped with an electrospray ion source. Separation was performed with an Kinetex 1.7 µm, C18, 100 Å, 50 × 21 mm column (Phenomenex) operating at a flow rate of 700 µL, with 0.1 % formic acid (solvent A) and acetonitrile + 0.1 % formic acid (solvent B) and the following gradient: 10 % to 40 % solvent B over 2 min, hold 40 % solvent B for 1 min. Samples were injected in a 2 µL volume.

MALDI-TOF-MS

For MALDI-TOF-MS measurements, samples were quenched by the addition of trifluoroacetic acid (TFA) to a final concentration of 0.2 %. Samples were then mixed with 2 % TFA and 100 mM 2'-5'-dihydroxyacetophenone (DHAP)

at a ratio of 1:1:1 and 0.5 μL of this mixture was spotted onto a MALDI plate. Samples were desalted on the plate by washing twice with 2 μL of 2 % TFA. Samples were analyzed using a Bruker Daltonics UltrafleXtreme MALDI-TOF mass spectrometer. Spectra were obtained in linear positive mode and the instrument was calibrated to a commercially available standard (Protein Calibration Standard I, Bruker) prior to each measurement. Data analysis was performed with flexAnalysis 3.3 (Bruker).

NMR

1D-NMR (^1H , ^{13}C , DEPT) and 2D-NMR (^1H - ^1H -COSY, HSQC, HMBC) data were recorded in deuterated solvents (Deutero GmbH) on a Bruker AVANCE II 300 MHz, a Bruker AVANCE II 500 MHz, or a Bruker AVANCE III 600 MHz instrument equipped with a Bruker Cryo Platform. The chemical shifts are reported in ppm relative to the solvent residual peak (DMSO- d_6 : $\delta_{\text{H}} = 2.5$ ppm, $\delta_{\text{C}} = 39.52$ ppm; D_2O : $\delta_{\text{H}} = 4.79$ ppm; MeOH- d_4 : $\delta_{\text{H}} = 3.31$ ppm, $\delta_{\text{C}} = 49.15$ ppm). For multiplicities of resonance signals, the following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, qt = quintet, br = broad.

Preparative HPLC

Method A: A Shimadzu model (LC-8A pump system) equipped with a diode array detector (type: SPD-M20A) and a Phenomenex Luna C_{18} (10 μm , 250 \times 21.2 mm) column was used for preparative HPLC. The solvents used in the mobile phase were water + 0.1 % TFA (solvent A) and 83 % acetonitrile (ACN, solvent B). The following gradient was used for compound purification: 90 % solvent A, 10 % solvent B for 1 min, ramp to 100% solvent B over 19 min, hold at 100% solvent B for 10 min. The flow rate was 18 mL min^{-1} .

Method B: The system is as mentioned in method A. The following gradient was used for compound purification: 90 % solvent A, 10 % solvent B for 1 min, ramp to 100 % solvent B over 29 min, hold at 100 % solvent B for 10 min. The flow rate was 18 mL min^{-1} .

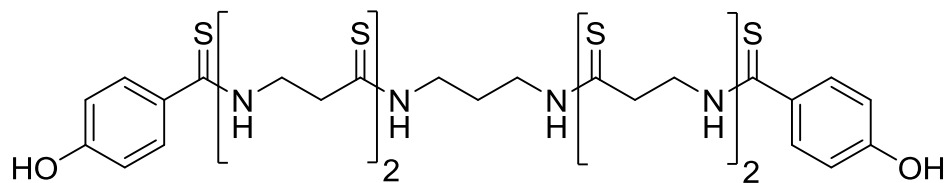
Method C: The system is as mentioned in method A. The following gradient was used for compound purification: 2 % solvent B for 1 min, ramp from 2 % to 10 % solvent B over 4 min and hold for 5 min, ramp to 20 % solvent B over 5 min and hold for 5 min, ramp to 40 % over 5 min and hold for 5 min, ramp to 60 % solvent B over 5 min, ramp to 100 % solvent B over 3 min and hold for 2 min. The flow rate was 20 mL min^{-1} .

Method D: The system is as mentioned in method A. The following gradient was used for compound purification: 90 % solvent A, 10 % solvent B for 1 min, ramp to 100 % solvent B over 9 min, hold at 100 % solvent B for 10 min. The flow rate was 18 mL min^{-1} .

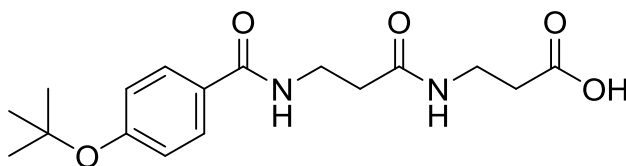
General synthetic methods

The coupling reagents used in the following syntheses were 1-hydroxybenzotriazole hydrate (HOBT), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), and (benzotriazol-1-yloxy)tripyrrolidinophosphoniumhexafluorophosphate (PyBOP). Boc-deprotection reactions were accomplished by the addition of a 95:5 mixture of TFA and water to the respective compound. Following a 15 to 30 minute reaction with stirring, the TFA was removed under nitrogen flow and the compound was lyophilized.

The following compounds were synthesized as previously described: closthioamide (CTA),^[8] and 3-(3-(4-(*tert*-butoxy)benzamido)propanamido)propanoic acid (**8**).^[9]

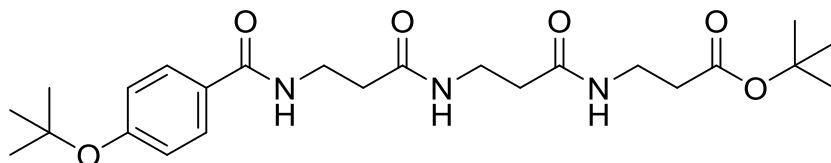


closthioamide (CTA)



(8)

Synthesis of *tert*-butyl 3-(3-(3-(4-(*tert*-butoxy)benzamido)propanamido)propanamido)propanoate (**8**)

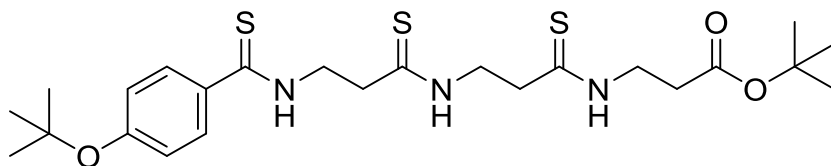


(9)

Compound **8** (131.1 mg, 0.38 mmol), was mixed with HOBT (384.7 mg, 2.8 mmol, 7.5 eq.) and EDCI (121.3 mg, 0.78 mmol, 2 eq.) in 25 ml DMF. After stirring for 30 minutes diisopropylethylamine (DIPEA; 64 μ L, 48.64 mg, 0.38 mmol, 1 eq.) and *t*Bu-3-aminopropanoate (70.2 mg, 0.38 mmol, 1 eq.) in 2 mL of DMF were added. The reaction was stirred for 10.5 hours. The solvent was removed under reduced pressure and the residue was taken up in 15 mL ethyl acetate. The organic phase was washed with 15 mL of 0.2 M HCl, 15 mL of 0.5 M NaOH and 15 mL of brine. The water phase was extracted twice with ethyl acetate. Ethyl acetate of the combined organic phase was removed under reduced pressure. Preparative HPLC (method A) gave the title compound in a yield of 63 %. See **Figures S25–S26 for NMR spectra**.

¹H-NMR (300 MHz, DMSO-*d*₆): δ [ppm] = 8.37 (t, J = 5.26 Hz, 1H), 7.92 (t, J = 4.91 Hz, 2H), 7.75 (d, J = 8.58 Hz, 2H), 7.01 (d, J = 8.58 Hz, 2H), 3.41 (dd, J = 6.83 Hz, J = 5.96 Hz, 2H), 3.25–3.17 (m, 4H), 2.36–2.29 (m, 4H), 2.20 (t, J = 7.09 Hz, 2H), 1.39 (s, 9H), 1.33 (s, 9H). ¹³C-NMR (75 MHz, D₂O): δ [ppm] = 170.61, 170.37, 170.35, 165.73, 157.80, 128.82, 128.33, 122.32, 79.83, 78.66, 36.09, 35.41, 35.38, 35.29, 35.00, 34.76, 28.53, 27.74. HMRS (ESI, positive) m/z calculated for C₂₄H₃₈N₃O₆ [$M+H$]⁺: 464.2755, found: 464.2745.

Synthesis of *tert*-butyl 3-(3-(3-(4-(*tert*-butoxy)phenylthioamido)propanethioamido)propanethioamido)propanoate (10)

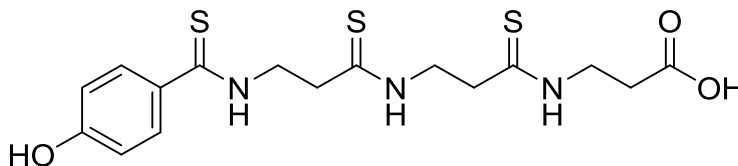


(10)

Compound **9** (46.8 mg, 0.1 mmol) was mixed with Lawesson's reagent (257.1 mg) in 1.5 mL of dry pyridine under an argon atmosphere. The reaction mixture was stirred for 20 hours at 95 °C. Pyridine was removed and the residue was taken up in 10 mL ethyl acetate, washed with 10 mL of 0.2 M NaOH and twice with 10 mL of brine. The aqueous phase was extracted three times with 10 mL of ethyl acetate. After drying the ethyl acetate phase the solvent was removed under reduced pressure and the crude was purified using preparative HPLC (method A) to obtain the pure title compound in 57 % yield. See Figures S27–S28 for NMR spectra.

¹H-NMR (600 MHz, DMSO-d₆): δ [ppm] = 10.08–10.11 (m, 3H), 7.72 (d, J = 8.80 Hz, 2H), 6.99 (d, J = 8.75 Hz, 2H), 3.99 (td, J = 6.52 Hz, J = 7.06 Hz, 2H), 3.85 (dd, J = 6.18 Hz, J = 6.92 Hz, 2H), 3.65 (dd, J = 5.29 Hz, J = 6.92 Hz, 2H), 2.94 (t, J = 7.33 Hz, 2H), 2.83 (t, J = 7.33 Hz, 2H), 2.56 (t, J = 6.99 Hz, 2H), 1.40 (s, 9H), 1.34 (s, 9H). ¹³C-NMR (125 MHz, DMSO-d₆): δ [ppm] = 200.75, 200.55, 196.28, 170.30, 157.78, 135.27, 128.47, 121.88, 80.14, 78.78, 45.52, 44.61, 42.02, 41.95, 41.06, 32.74, 28.53, 27.77. HMRS (ESI, positive) m/z calculated for C₂₄H₃₈N₃O₃S₃ [$M+H$]⁺: 512.2070, found: 512.2068.

Synthesis of 3-(3-(3-(4-hydroxyphenylthioamido)propanethioamido)propanethioamido)propanoic acid (1)

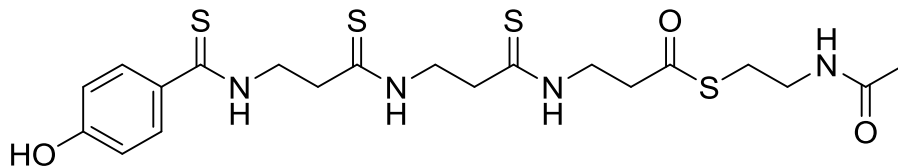


(1)

Compound **10** (32.3 mg, 0.06 mmol) was mixed with NaI (29.2 mg, 0.19 mmol, 3.2 eq.) in 3.5 mL of acetonitrile. To the stirring mixture, CeCl₃ (0.19 mmol, 3.2 eq.) in H₂O (148 μ L, 8.2 mmol, 137 eq.) was added. The reaction was stirred for 28 hours at 75 °C before it was diluted with 15 mL of ethyl acetate. The reaction was then washed with 15 mL of 0.1 M HCl and twice with 15 mL of brine. The aqueous phase was extracted with 30 mL ethyl acetate and the organic layer was dried. The crude was purified using preparative HPLC (method B) to obtain the title product in a 30 % yield. See Figures S29–S30 for NMR spectra.

¹H-NMR (600 MHz, DMSO-d₆): δ [ppm] = 10.09 (m, 2H), 9.92 (t, J = 5.45 Hz, 1H), 7.69 (d, J = 8.86 Hz, 2H), 6.75 (d, J = 8.86 Hz, 2H), 4.03 – 3.96 (m, 2H), 3.83 (dd, J = 6.92 Hz, J = 6.27 Hz, 2H), 3.63 (dd, J = 6.70 Hz, J = 5.40 Hz, 2H), 2.92 (t, J = 7.13 Hz, 2H), 2.82 (t, J = 7.13 Hz, 2H), 2.57 (t, J = 6.92 Hz, 2H). ¹³C-NMR (150 MHz, DMSO-d₆): δ [ppm] = 200.81, 200.46, 196.16, 172.64, 160.17, 131.73, 129.28, 114.45, 45.42, 44.61, 42.12, 41.86, 41.19, 31.66. HMRS (ESI, positive) m/z calculated for C₁₆H₂₂N₃O₃S₃ [$M+H$]⁺: 400.0818, found: 400.0818.

Synthesis of S-(2-acetamidoethyl) 3-(3-(3-(4-hydroxyphenylthioamido)propanethioamido)propanethioamido)propanethioamido)propanethioate (SNAC-1)

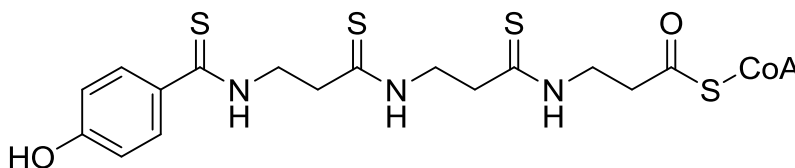


(SNAC-1)

Compound **1** (4.1 mg, 0.01 mmol) was mixed with HOBT (1.7 mg, 0.013 mmol, 1.3 eq.), EDCI (3.2 mg, 0.02 mmol, 2 eq.), *N*-acetyl-cysteamine (1.376 μ L, 0.01 mmol, 1 eq.) and DIPEA (0.498 μ L, 0.003 mmol, 0.3 eq.) in 400 μ L of DCM and 400 μ L of DMF. The reaction was stirred for 5.5 hours and stopped by removing the solvent under reduced pressure. The mixture was taken up in 200 μ L ethylacetate and washed with 200 μ L 0.2 M NaOH and 200 μ L 0.1 M HCl. The title compound was isolated using preparative HPLC (method D) in a yield of 20 %. See **Figures S31–S32 for NMR spectra.**

$^1\text{H-NMR}$ (600 MHz, DMSO- d_6): δ [ppm] = 10.09 (t, J = 5.27 Hz, 1H), 10.02 (t, J = 5.27 Hz, 1H), 9.88 (t, J = 5.27 Hz, 1H), 8.08 (t, J = 5.61 Hz, 1H), 7.65 (d, J = 8.84 Hz, 2H), 6.74 (d, J = 8.84 Hz, 2H), 3.96 (overlapping with H₂O peak), 3.81 (overlapping with H₂O peak), 3.69 (q, J = 6.62 Hz, J = 5.78 Hz, 2H), 3.16 (q, J = 6.46 Hz, J = 6.12 Hz, 2H), 2.92–2.88 (m, 6H), 2.79 (t, J = 7.31 Hz, 2H), 1.78 (s, 3H). $^{13}\text{C-NMR}$ (150 MHz, DMSO- d_6): δ [ppm] = 201.49, 197.59, 196.86, 171.15, 160.66, 132.34, 129.65, 115.19, 45.96, 45.14, 42.73, 42.46, 41.59, 41.46, 38.71, 28.71, 22.96. HMRS (ESI, positive) m/z calculated for C₂₀H₂₉N₄O₃S₄ [$M+H$]⁺: 501.1117, found: 501.1122.

Synthesis of 3-(3-(3-(4-hydroxyphenylthioamido)propanethioamido)propanethioamido)propanoic CoA ester (CoA-1)

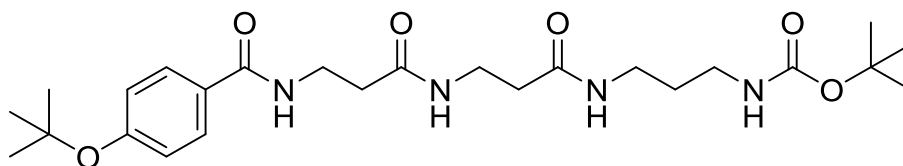


(CoA-1)

Compound **1** (7.7 mg, 0.019 mmol) was mixed with PyBOP (13.3 mg, 0.025 mmol, 1.35 eq.) and K₂CO₃ (9.4 mg, 0.068 mmol, 3.6 eq.) in 500 μ L acetonitrile. To this stirring solution coenzyme A sodium salt was added (17.2 mg, 0.022 mmol, 1.18 eq.). The reaction was stirred for 1 hour and directly purified by preparative HPLC (method C). The pure compound was obtained in a yield of 9 %. See **Figures S33–S34 for NMR spectra.**

$^1\text{H-NMR}$ (600 MHz, DMSO- d_6): δ [ppm] = 9.00 (d, J = 4.74, 1H), 8.78 (d, J = 4.74, 1H), 8.09–8.06 (m, 2H), 7.23–7.21 (m, 2H), 6.52 (dd, J = 5.58 Hz, J = 4.46 Hz, 1H), 5.28 (br, 1H), 5.19 (dd, J = 5.16 Hz, J = 5.09 Hz, 1H), 4.91 (s, 1H), 4.62 (s, 2H), 4.45 (t, J = 6.52 Hz, 2H), 4.36 (d, J = 4.59 Hz, 1H), 4.32–4.26 (m, 3H), 4.15 (t, J = 6.04 Hz, 2H), 3.98 (dd, J = 5.32 Hz, J = 4.59 Hz, 1H), 3.78 (t, J = 5.56 Hz, 2H), 3.68–3.65 (m, 3H), 3.42 (t, J = 6.52 Hz, 2H), 3.35–3.28 (m, 6H), 2.76 (t, J = 6.52 Hz, 2H), 2.39–2.37 (m, 6H). $^{13}\text{C-NMR}$ (125 MHz, DMSO- d_6): δ [ppm] = 201.78, 201.69, 199.41, 197.31, 174.22, 173.18, 144.52, 142.36, 132.35, 129.12, 114.83, 87.61, 82.95, 74.10, 73.77, 73.67, 72.11, 64.76, 45.09, 44.54, 42.68, 42.28, 41.25, 40.67, 38.29, 35.16, 28.07, 20.73, 18.09.

Synthesis of *tert*-butyl (3-(3-(3-(4-(*tert*-butoxy)benzamido)propanamido)propanamido)propyl)carbamate (10)

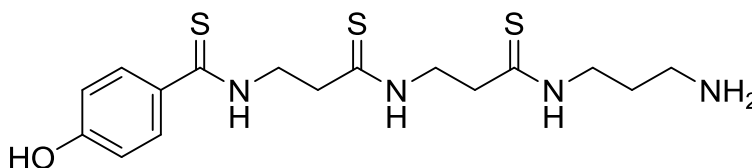


(11)

Compound **8** (36.9 mg, 0.11 mmol) was mixed with HOBt (80.9 mg, 0.60 mmol, 5 eq.), EDCI (26 mg, 0.17 mmol, 1.5 eq.), Boc-1,3-diaminopropane (36.3 mg, 0.21 mmol, 1.89 eq.) and DIPEA (10 μ L, 0.06 mmol, 0.5 eq.) in 10 mL DCM. The reaction was stopped after 24.75 h by removing the solvent under reduced pressure. The residue was taken up in 10 mL of ethyl acetate, washed twice with 10 mL 0.2 M NaOH and 10 mL sat. NaCl solution. The solvent of the organic layer was removed under reduced pressure and the product was isolated from the reaction mixture by preparative HPLC (method D) to obtain the title product in an 83 % yield. See Figures S35–S36 for NMR spectra.

$^1\text{H-NMR}$ (600 MHz, DMSO- d_6): δ [ppm] = 8.36 (t, J = 5.47 Hz, 1H), 7.92 (t, J = 5.47 Hz, 1H), 7.81 (t, J = 5.47 Hz, 1H), 7.75 (d, J = 8.75 Hz, 2H), 7.02 (d, J = 8.75 Hz, 2H), 6.76 (t, J = 6.01 Hz, 1H), 3.42 (dd, J = 7.11 Hz, J = 6.56 Hz, 2H), 3.23 (dd, J = 7.11 Hz, J = 6.01 Hz, 2H), 3.00 (dd, J = 6.56 Hz, J = 6.01 Hz, 2H), 2.89 (dd, J = 6.56 Hz, J = 6.01 Hz, 2H), 2.32 (t, J = 7.24, 2H), 2.21 (t, J = 6.84 Hz, 2H), 1.48 (q, J = 6.84 Hz, 2H), 1.36 (s, 9H), 1.33 (s, 9H). $^{13}\text{C-NMR}$ (125 MHz, DMSO- d_6): δ [ppm] = 170.36, 170.23, 165.71, 157.79, 155.54, 128.99, 128.81, 128.32, 122.31, 78.65, 77.43, 37.56, 36.12, 36.08, 35.46, 35.38, 35.34, 29.51, 28.52, 28.24. HMRS (ESI, positive) m/z calculated for $\text{C}_{25}\text{H}_{41}\text{N}_4\text{O}_6$ [$M+\text{H}$] $^+$: 493.3021, found: 493.3033.

Synthesis of *N*-(3-((3-((3-aminopropyl)amino)-3-thioxopropyl)amino)-3-thioxopropyl)-4-hydroxybenzothioamide (2)

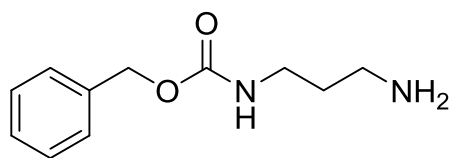


(2)

Compound **11** (4.5 mg, 0.01 mmol) was mixed with Lawesson's reagent (128.4 mg, 0.317 mmol, 35 eq.) in 2 mL of dry pyridine. The reaction was stirred for 19 hours at 85 $^{\circ}\text{C}$ and stopped by removing the solvent under a nitrogen flow. The residue was taken up in 10 mL ethyl acetate and washed with 10 mL 0.2 M NaOH, 10 mL 0.5 M HCl and 10 mL sat. NaCl solution. The solvent was removed and the compound was directly deprotected using 55 % TFA in DCM. The reaction was incubated for 3 minutes, centrifuged for 1 minute at 16,000 \times g and the supernatant was dried down under a N_2 flow. The product was isolated from the crude using preparative HPLC (method D) in a yield of 85 % over two steps. See Figures S37–S38 for NMR spectra.

$^1\text{H-NMR}$ (500 MHz, DMSO- d_6): δ [ppm] = 10.13 (s, 1H), 9.93 (s, 1H), 7.70 (d, J = 8.65 Hz, 2H), 6.76 (d, J = 8.65 Hz, 2H), 3.99 (q, J = 7.01 Hz, J = 5.22 Hz, 2H), 3.86 (t, J = 6.86 Hz, 2H), 3.55 (t, J = 6.94 Hz, 2H), 2.94 (t, J = 7.29 Hz, 2H), 2.87–2.82 (m, 4H), 1.84 (t, J = 7.14 Hz, 2H). $^{13}\text{C-NMR}$ (125 MHz, DMSO- d_6): δ [ppm] = 201.49, 197.59, 196.86, 171.15, 160.66, 132.34, 129.65, 115.19, 45.96, 45.14, 42.73, 42.46, 41.59, 41.46, 38.71, 28.71, 22.96. HMRS (ESI, positive) m/z calculated for $\text{C}_{16}\text{H}_{25}\text{N}_4\text{OS}_3$ [$M+\text{H}$] $^+$: 385.1185, found: 385.1184.

Synthesis of benzyl (3-aminopropyl)carbamate (6)



(6)

To a stirred solution of diaminopropane (DAP, 10 mL, 0.12 mol) in 10 mL chloroform on ice, benzyl chloro formate (1.7 mL, 0.012 mol) in 10 mL chloroform was added drop-wise over 2 hours. The reaction mixture was stirred for 26.5 hours at room temperature and then concentrated. Ethyl acetate (100 mL) was added and the organic phase was washed three times with water (100 mL), once with sat. NaCl solution (100 mL) and three times with 2 M HCl (100 mL). The pH of the combined aqueous layers was adjusted to 12 using NaOH and NaCl was added till saturation. The same volume of ethyl acetate was used to extract the mixture three times. The combined organic layer was dried using Na₂SO₄ and the solvent was removed. The product was isolated from the crude using preparative HPLC method B in a yield of 2 %. See Figures S39–S40 for NMR spectra.

¹H-NMR (500 MHz, CD₃OD): δ [ppm] = 7.34 – 7.28 (m, 5H), 9.93 (s, 1H), 5.08 (s, 2H), 3.21 (t, J = 6.59 Hz, 2H), 2.93 (t, J = 7.26 Hz, 2H), 1.82 (q, J = 7.14 Hz, 2H). ¹³C-NMR (125 MHz, CD₃OD): δ [ppm] = 128.09, 127.67, 127.43, 66.23, 37.02, 36.94. HMRS (ESI, positive) m/z calculated for C₁₁H₁₆N₂O₂ [$M+H$]⁺: 209.1285, found: 209.1288.

Plasmid construction for CRISPR/Cas knockout vector

Suitable target sites containing the necessary PAM (NGG) sequence for generation of the *ctaB* and *ctaK* gene knockouts were identified using the webtool CRISPy-web.^[10] The knockout plasmids were constructed in three steps as previously described.^[2] First, pTargetC^[2] was used as a PCR template to amplify a region between the *EcoRI* and *ScaI* cut-sites using the general primer sgrNA-R and a target-specific primer sgrNA-gene which contains the N20 sequence upstream of the selected PAM sequence (primers listed in Table S3). The resultant amplicon was purified with an innu-PREP PCRpure kit (Analytik Jena) and cloned into the *EcoRI/ScaI*-digested pTargetC plasmid using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs) to afford pTargetCF-*ctaB* and pTargetCF-*ctaK*. In a second step, 1–1.5 kbp of the homologous regions upstream and downstream of the target site were amplified by PCR with primers (listed in Table S3) designed to mutate a portion of the N20 sequence to 5'-gatatccta-3', purified with an innu-PREP PCRpure kit (Analytik Jena) and cloned into the *SalI/StuI*-digested pTargetCF-gene vectors using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs) to generate pTargetCT-*ctaB* and pTargetCT-*ctaK*. For the third step, pTargetCT-gene was used as a template to amplify a DNA fragment with the universal primers TargetCT-F and TargetCT-R (see Table S3 for primer sequences) to create an amplicon including the N20-sgRNA, the P4 synthetic promoter and the homologous regions with the mutated N20 sequence. The resulting DNA fragment was purified with an innu-PREP PCRpure kit (Analytik Jena) and cloned into the *BsaI*-digested pCasC vector using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs) to create pCasC-*ctaB* and pCasC-*ctaK*. Following plasmid assembly, *E. coli* Top10 competent cells were transformed with the resultant plasmids and transformants were selected on LB plates supplemented with 10 $\mu\text{g mL}^{-1}$ gentamicin. Correct clones were identified by colony PCR using OneTaq Quick-load 2X Master Mix (New England Biolabs) and insert-specific primers, and verified by sequencing with the primers listed in Table S3.

Generation of *Ruminiclostridium cellulolyticum* Δ *ctaK* and Δ *ctaB*

R. cellulolyticum Δ *ctaB* and *R. cellulolyticum* Δ *ctaK*, which contain in-frame nonsense mutations in the indicated genes, were generated using pCasC-*ctaB* and pCasC-*ctaK* as previously described.^[2] The design of pCasC-*ctaB/ctaK* vectors was such that successful editing of the target gene would introduce the desired mutation (TAA), as well as an

EcoRV restriction endonuclease site (GATATC) to facilitate the screening of transformants. After electroporation of *R. cellulolyticum*, individual potential edited colonies were randomly picked and subjected to colony PCR using OneTaq DNA Polymerase (New England Biolabs) and the primers indicated in **Table S3**. The presence or absence of the restriction site (corresponding to edited or unedited target gene) was ascertained by digestion of the generated PCR products with restriction endonuclease *EcoRV* (New England Biolabs), followed by agarose gel electrophoresis of the fragments. Pure mutant colonies were identified by the presence of the expected fragments and lack of full-length, undigested PCR product, while the PCR product remained undigested in the case of the wild-type control. Subsequently, genomic DNA (gDNA) was isolated from a putative mutant using a MasterPure Gram Positive DNA Purification Kit (Epicentre). In order to ensure the chosen mutant was free of wild-type contamination, the target gene was amplified using the gDNA as a template and subjected to restriction analysis as described above. The undigested PCR product was purified using a Monarch DNA and PCR DNA cleanup kit (New England Biolabs). DNA sequencing (Eurofins Genomics Germany GmbH) using the primers listed in **Table S3** confirmed the presence of the desired mutation (**Figures S6 and S7**).

Screen for production of CTA and intermediates by *Ruminiclostridium cellulolyticum* strains

Strains (*R. cellulolyticum* wild type, $\Delta ctaB$, $\Delta ctaJ$, and $\Delta ctaK$) were cultivated and extracted with ethyl acetate as previously described.^[2] Organic extracts were analyzed by LC-HRMS (Exactive).

Detection of polyamines from *Ruminiclostridium cellulolyticum* cultures

Strains were cultivated under CTA-producing conditions as previously described^[2] and the cellular polyamines were isolated according to an established method.^[11] Specifically, cells from a 7 mL culture (OD₆₀₀ 0.2–0.3) were pelleted by centrifugation at 8,000 × *g* for 10 min. The spent medium was removed and the cell pellets were washed three times with phosphate-buffered saline (10 mM Na₂PO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl, pH 7.5) to remove any residual spent medium. Next, the cell pellets were resuspended in 75 µL of lysis buffer (20 mM MOPS, 10 mM NaCl, 4 mM MgCl₂, pH 8.0) supplemented with 2 µL of Ready Lyse Lysozyme (Lucigen) and incubated at 37 °C for 2 h. The samples were then lysed by 5 freeze-thaw cycles in liquid nitrogen (freeze for 5 min, thaw at 55 °C for 5 min). Following lysis, the cellular debris was removed by centrifugation at 16,000 × *g* for 10 min and the cellular polyamines were labeled by the addition of diisopropylethylamine (DIPEA) and Sanger's reagent (10 mM dissolved in ACN) to a final concentration of 0.5 mM and 5 mM, respectively. Reactions were allowed to proceed at 50 °C for 1 h before the samples were dried under reduced pressure. The resultant solid was dissolved in MeOH and analyzed by LC-HRMS (Exactive).

To aid in the identification of the polyamines, labeled standards for diaminopropane and spermidine were generated by the incubation of 1 mM of the polyamine (pH 7.0) with 1 mM DIPEA and 5 mM Sanger's reagent (10 mM dissolved in ACN) for 1 h at 55 °C.

Plasmid construction for *E. coli* expression vectors

The target genes (*ctaB*, *ctaJ*, and *ctaK*) were amplified by PCR from the gDNA of *R. cellulolyticum* DSM 5812 using the oligonucleotide primers listed in **Table S3**. PCRs were performed with Phusion High-Fidelity DNA Polymerase (New England Biolabs) and amplicons were purified using an innuPREP gel extraction kit (Analytik Jena). Following purification, the DNA fragments were digested with *NheI* and *BamHI*. The digested inserts were purified using an innuPREP PCRpure kit (Analytik Jena) and ligated with an appropriately digested pET28a vector using T4 DNA ligase (New England Biolabs). *E. coli* Top10 cells were transformed with the ligation reactions and transformants were selected on LB agar supplemented with 50 µg mL⁻¹ kanamycin. Plasmids were isolated from the transformants and the sequence of the construct was confirmed by sequencing using the oligonucleotide primers listed in **Table S3**.

Mutagenesis of pET28-*ctaJ*

The plasmid was amplified by PCR using the mutagenesis primers listed in **Table S3** and Phusion High-Fidelity DNA Polymerase (New England Biolabs). Template DNA was removed by digestion with DpnI (1 U; New England Biolabs) for 4 h at 37 °C. Digested samples were used to transform *E. coli* Top10 cells and transformants were selected on LB agar supplemented with 50 µg mL⁻¹ kanamycin. Plasmids were isolated from transformants and verified by sequencing using the primers in **Table S3**.

Heterologous Protein Production

E. coli Rosetta (DE3) cells were transformed with pET28a derived expression vectors and transformants were selected on LB agar plates supplemented with 50 µg mL⁻¹ kanamycin/25 µg mL⁻¹ chloramphenicol. A single colony was used to inoculate 4 mL of LB supplemented with the appropriate antibiotic and cultures were grown for 18–20 h at 37 °C and 160 rpm. The entire overnight culture was used to inoculate 400 mL of fresh LB medium supplemented with the appropriate antibiotics. Cultures were grown at 37 °C and 160 rpm until an optical density at 600 nm (OD₆₀₀) of approximately 0.6 was reached. Cultures were then iced for 10 min before protein expression was induced with the addition of IPTG to a final concentration of 0.4 mM. Following induction, cultures were grown for an additional 16–18 h at 18 °C before the cells were harvested by centrifugation at 3,220 × *g* for 15 min. Cell pellets were washed with Tris-buffered saline (10 mM Tris pH 7.5, 150 mM NaCl) and stored at –20 °C for up to one month before use.

Purification of N-terminal His₆ fusion proteins

Cell pellets were resuspended in 30 mL of lysis buffer [50 mM Tris pH 8.0, 300 mM NaCl, 25 mM imidazole, 5 % glycerol (v/v)] supplemented with 1 mg mL⁻¹ lysozyme (Roth). After incubation on ice for 30 min, cells were disrupted by sonication at 4 °C using a SONOPLUS ultrasonic homogenizer with an MS73 microtip (Bandelin) and the following parameters: 30 % power, three 2 min cycles, with a 50 % pulse frequency and 5–10 min breaks between cycles. The insoluble debris was removed from the lysate by centrifugation at 17,000 × *g* for 30 min and the cleared lysate was loaded onto 2 mL of TALON Superflow resin (GE Healthcare) equilibrated with lysis buffer. The resin was washed with 100 mL of lysis buffer supplemented with 1 mM tris(2-carboxyethyl)phosphine (TCEP), and His₆-tagged proteins were eluted using 15 mL of elution buffer [50 mM Tris pH 8.0, 300 mM NaCl, 250 mM imidazole, 1 mM TCEP, 5 % glycerol (v/v)]. The eluent was concentrated in an appropriate molecular weight cutoff Amicon Ultra 15 mL centrifugal filter (Merck Millipore) to approximately 1.5 mL and diluted 10-fold with storage buffer [50 mM HEPES pH 7.5, 300 mM NaCl, 1 mM TCEP, 20 % glycerol (v/v)]. Following a second round of concentration and dilution with storage buffer, the His-tagged proteins concentrated to a final time and stored at –80 °C. Protein concentration was determined by absorbance at 280 nm and purity was assessed by SDS-PAGE (**Figure S8**).

UV-visible Spectroscopy

Spectra were measured using a Varioskan Lux microplate reader (Thermo Fisher Scientific) and a 384-well microtiter plate with a 50 µL sample volume. All protein solutions were analyzed at a concentration of 20 µM in protein storage buffer. Where indicated, 20 µM pyridoxal phosphate (PLP) was added to the protein solution and the sample was incubated at room temperature for 30 min before the UV-vis spectrum was measured. Protein storage buffer (with PLP supplementation when appropriate) was used as a blank.

Reconstitution of CtaB activity

Initial CtaB activity assays were conducted with 1 µM His-tagged CtaB, 25 µM PLP, 0.5 mM 3-[(carbobenzyloxy)-amino]propionaldehyde (AH-1 surrogate; Cbz-propionaldehyde; dissolved in DMSO) and 2 mM of the indicated amine donor in CtaB reaction buffer [50 mM Na₂HPO₄ pH 7.5, 150 mM NaCl, 1 mM DTT]. Following a 24 h reaction at 25 °C, the assays were quenched with the addition of one volume of methanol and processing of Cbz-

propionaldehyde was monitored by LC-HRMS (Exactive). Control assays were performed with heat-inactivated CtaB (incubation at 99 °C for 10 min) or reactions lacking an amine donor.

To determine the preferred substrate for CtaB, assays were set up as indicated above and processing of Cbz-propionaldehyde was monitored after 2 h and 5 h.

CtaB kinetic assay

Assays were carried out with 1 μ M His-tagged CtaB, 25 μ M PLP, 2 mM Cbz-propionaldehyde (dissolved in DMSO) and variable concentrations of the indicated amine donor in CtaB reaction buffer [50 mM Na₂HPO₄ pH 7.5, 150 mM NaCl, 1 mM DTT] supplemented with 0.5 mg mL⁻¹ caffeine. Reactions were initiated by the addition of the amine donor and incubated at 25 °C. At the indicated time points, 2 μ L of the reaction mixture was analyzed by LC-HRMS (QExactive HF-X). The concentration of **6** in the reactions was determined by comparing the peak area of the extracted ion chromatogram (EIC) for **6** ($[M+H]^+$ = 209.1285; 5 ppm window) to a standard curve generated with synthetic **6**. The peak areas of the EICs in both the reactions and the standard curve were corrected by the peak area of caffeine (EIC; $[M+H]^+$ = 195.0877; 5 ppm window).

To generate the standard curve, a dilution series of **6** ranging from 1 mM to 0.001 mM was performed in the reaction buffer supplemented with 2 % DMSO and 0.05 mg mL⁻¹ caffeine. The samples were analysed by LC-HRMS (QExactive HF-X).

CtaJ reconstitution assays with **2**

Assays were performed with 1 μ M His-tagged CtaJ and 100 μ M **2** in CtaJ reaction buffer [50 mM HEPES pH 7.5, 125 mM NaCl, 1 mM TCEP]. Reactions were allowed to proceed for 18 h at 25 °C before being quenched by the addition of one volume of acetonitrile and analyzed by LC-HRMS (QExactive). Control reactions were performed with heat-inactivated CtaJ (incubation at 99 °C for 10 min).

Screens for the effect of additives on CtaJ activity were conducted as above in CtaJ reaction buffer supplemented with 1 mM CaCl₂ or 1 mM GTP. For the GTP-containing reactions, 20 mM MgCl₂ was also included in the reaction buffer.

CtaJ reconstitution assays with SNAC-1 and **1**

Assays contained 1 μ M His-tagged CtaJ, 500 μ M substrate (SNAC-1 or **1**), 100 μ M **2**, 50 mM HEPES (pH 7.5), 125 mM NaCl, 20 mM MgCl₂, and 1 mM TCEP. Assays were incubated at 25 °C for 18 h, quenched by the addition of one volume of acetonitrile, and analyzed by LC-HRMS (QExactive). Control reactions were either conducted with heat-inactivated CtaJ (incubation at 99 °C for 10 min) or by the omission of **2**.

***In vitro* phosphopantetheinylation of PCPs**

His-tagged CtaE was isolated from *E. coli* in the apo form as previously described.^[12] To generate crypto-CtaE, 30 μ M apo-CtaE was mixed with 60 μ M synthetic coenzyme A ester (**1**-CoA) and 0.5 μ M Sfp PPTase (New England Biolabs) in PCP loading buffer [50 mM HEPES pH 6.5, 125 mM NaCl, 20 mM MgCl₂, 1 mM TCEP]. Reactions were allowed to proceed for 60 min at 25 °C and CtaE-**1** formation was verified by MALDI-TOF-MS. Following PCP loading, His-CtaE-**1** was directly used in CtaJ reactions without further purification.

CtaJ reconstitution assays with His-CtaE-1

Assays contained 50 mM HEPES (pH 6.5), 125 mM NaCl, 20 mM MgCl₂, 1 mM TCEP, 1 μ M His-tagged CtaJ (wild type, C114A, H148A, or D165A), 30 μ M His-tagged CtaE-**1** and either 100 μ M **2** or 1 mM diaminopropane. Assays were incubated at 25 °C for variable times and progress was monitored by LC-HRMS and MALDI-TOF-MS. Control reactions were conducted with heat-inactivated CtaJ (incubation at 99 °C for 10 min).

For LC-HRMS analysis, reactions containing **2** and diaminopropane proceeded for 4 h and 18 h, respectively, before being quenched with the addition of one volume of acetonitrile. Samples were then analyzed by LC-HRMS (QExactive). For MALDI-TOF-MS analysis, reactions proceeded for 18 h before being quenched with the addition of TFA to a final concentration of 0.2 %. Samples were then analyzed by MALDI-TOF-MS.

CtaJ inhibition by CTA

Inhibition assays were performed with 1 μM His-tagged CtaJ, 30 μM His-tagged CtaE-1, 100 μM **2**, and variable concentrations of CTA (0, 1.9, 3.8, 7.5, 15, and 30 μM) in PCP loading buffer [50 mM HEPES pH 6.5, 125 mM NaCl, 20 mM MgCl_2 , 1 mM TCEP]. The reaction mixtures were incubated at 25 $^\circ\text{C}$ and CtaE-1 processing by CtaJ was monitored by MALDI-TOF-MS after 4 and 18 h.

Figure S1. CtaB is homologous to class-III pyridoxal phosphate-dependent aminotransferases. Neighbor-joining phylogenetic tree of diverse aminotransferase class-III protein family members (PF00202) with 1,000 bootstrap replicates. Where known, the amine donor accepted by the aminotransferase is indicated. Homologs of CtaB encoded in CTA-like biosynthetic gene clusters are denoted by an asterisk.

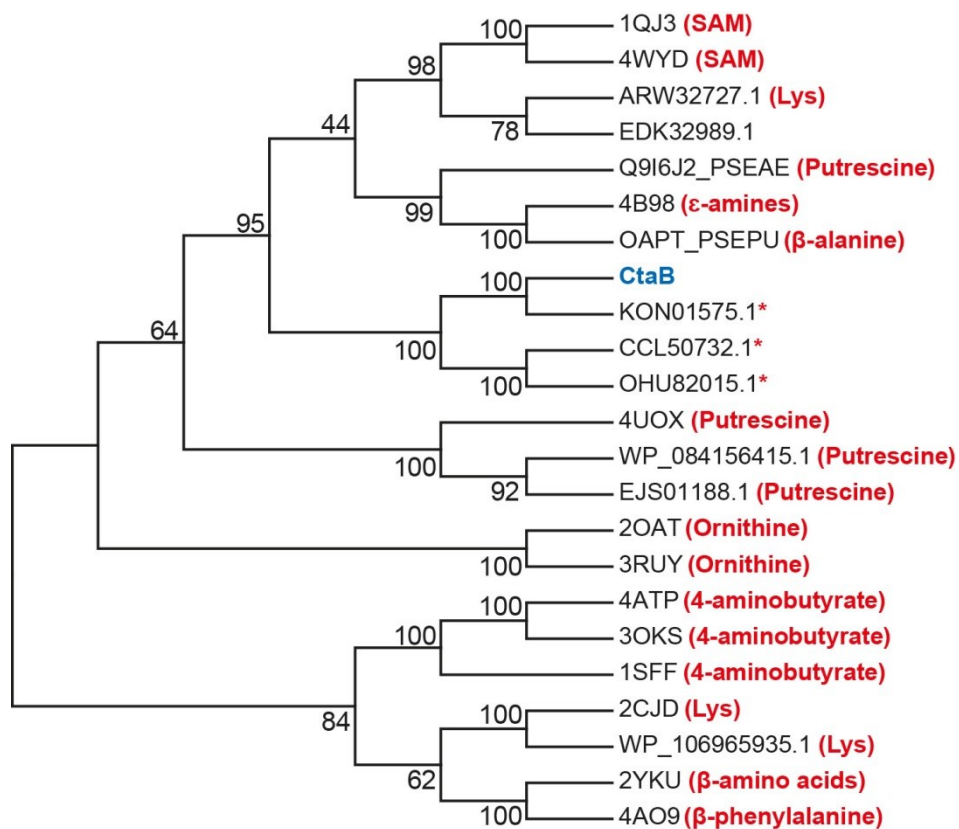


Figure S2. CtaK is a member of the aldo-keto reductase protein family. A) HHPred output for CtaK. Two domains were detected: an N-terminal aldo-keto reductase (AKR) domain and a 4Fe-4S cluster binding domain. B) A multiple sequence alignment of CtaK and diverse members of the AKR protein family. The AKRs are classified by general function. The residues comprising the catalytic tetrad (Asp-Tyr-Lys-His) of canonical ARKs are colored red. CtaK contains an atypical Asp-Tyr-Lys-Trp catalytic tetrad. C) A structural model of CtaK is displayed along with a zoom-in of the active site. The modeled CtaK catalytic tetrad (red) closely aligns to the catalytic tetrad of a canonical aldo-keto reductase (PDB: 4XK2; yellow) in a structural overlay. The Trp residue of CtaK's putative catalytic tetrad adopts a conformation reminiscent of the canonical His-utilizing homologs.

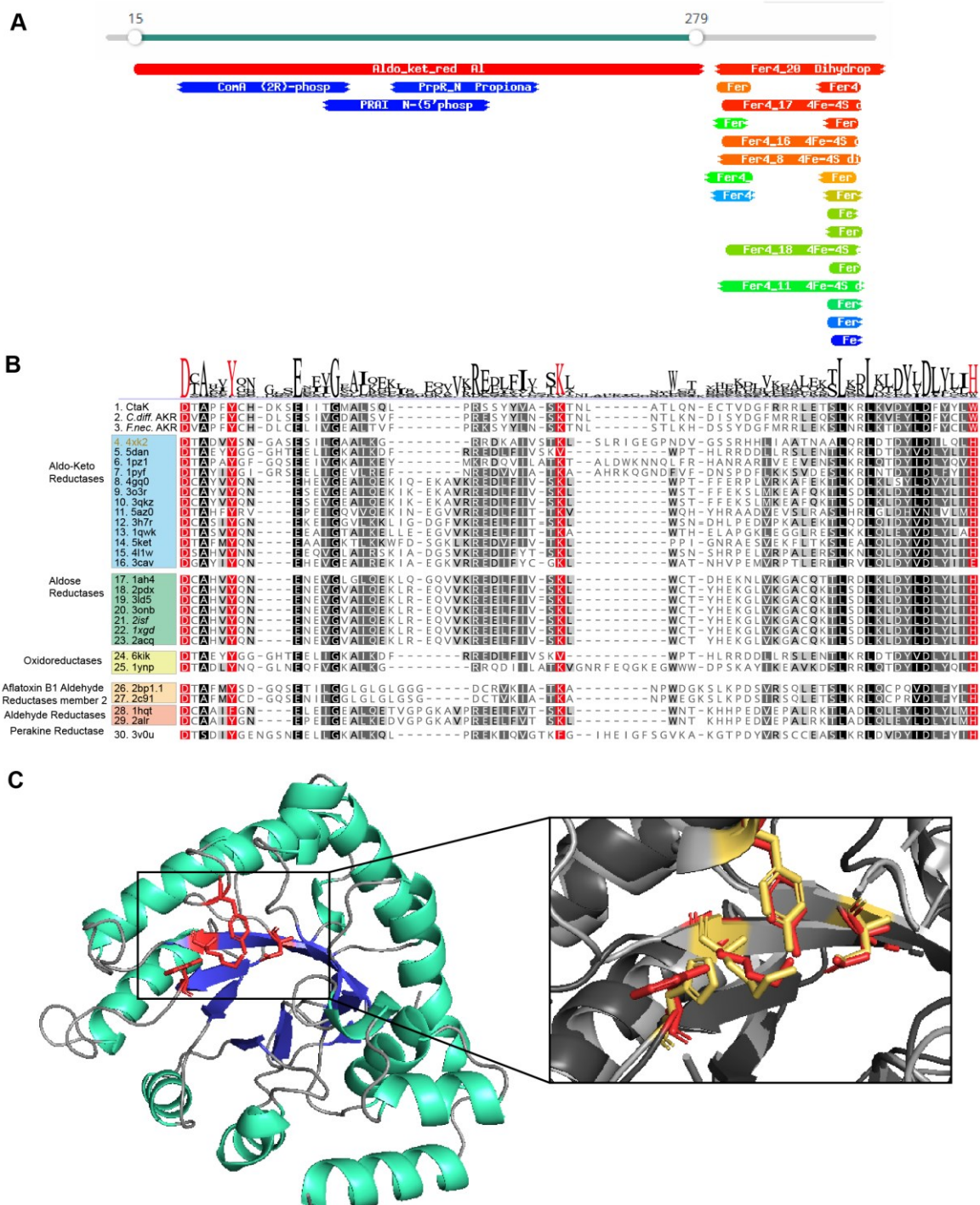


Figure S3. Production of 2 by *Ruminiclostridium cellulolyticum* strains. Extracted ion chromatograms (EICs) corresponding to the $[M+H]^+$ ionic species for 2 are displayed. All EICs are displayed with m/z values ± 5 ppm from the calculated exact mass for the compound. *R. cell* WT, wild-type *R. cellulolyticum*.

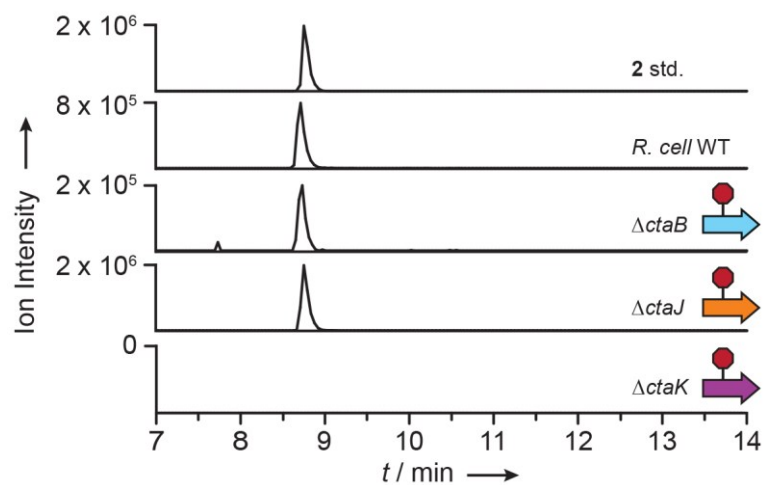


Figure S4. HRMS spectrum of **2** produced by *R. cellulolyticum*.

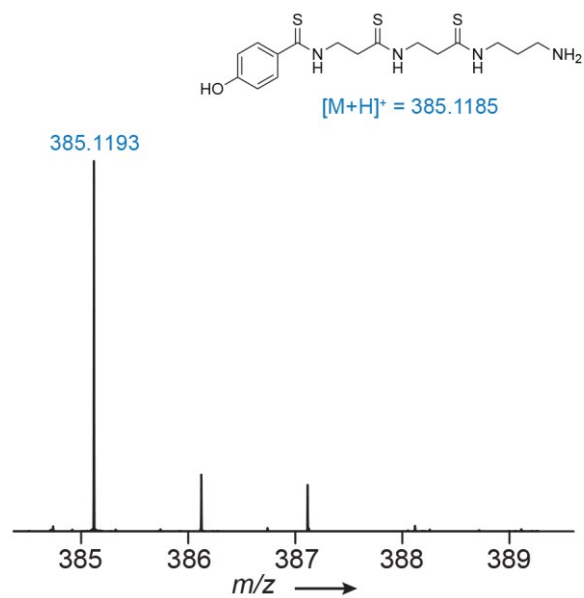


Figure S5. MS² spectra of **2 produced synthetically and by *R. cellulolyticum*.** The structure of **2** is displayed along with the MS² spectra of A) the synthetic reference compound and B) the compound detected in *R. cellulolyticum* culture extracts. The expected masses of assignable fragments are indicated. Ions that were detected in the MS² spectrum are colored blue. Asterisks denote ions formed from the neutral loss of H₂, quotations denote ions formed from the neutral loss of NH₃, and parentheses denote ions formed from the neutral loss of H₂S.

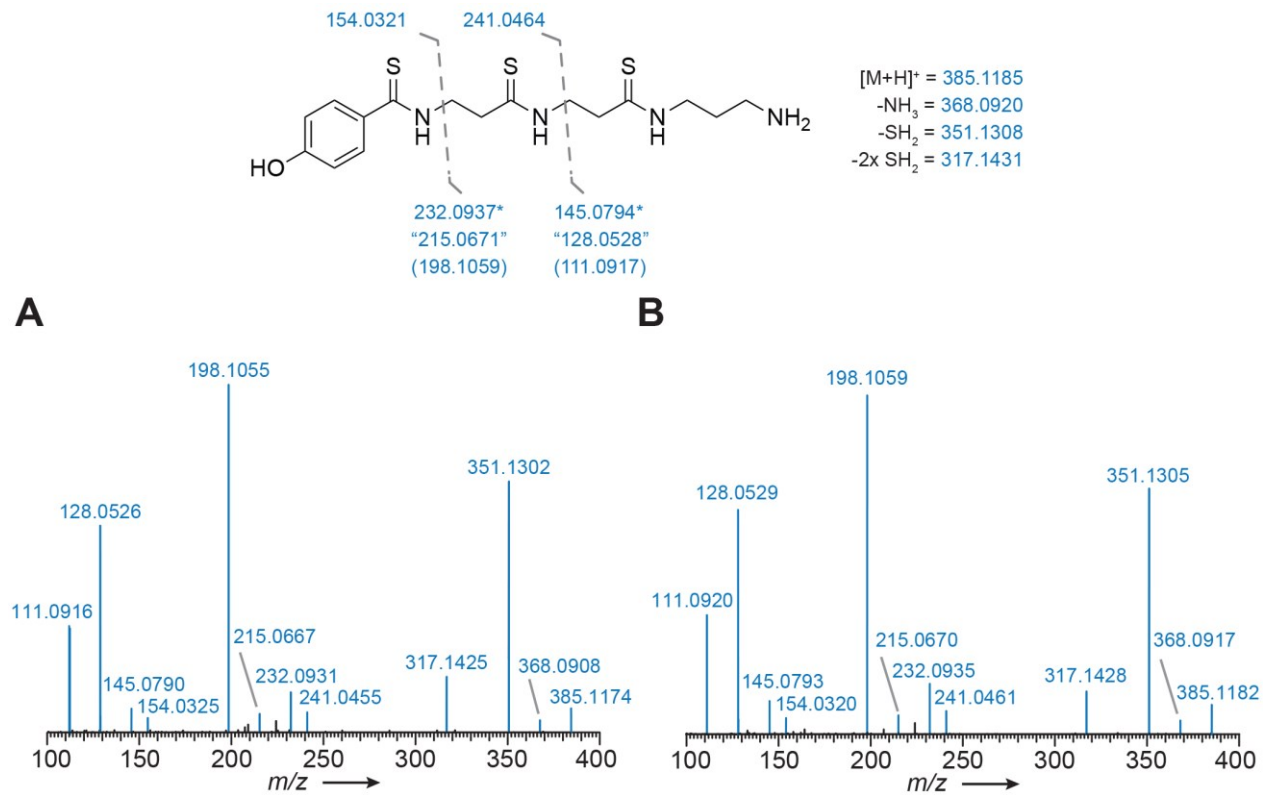


Figure S6. Generation of *R. cellulolyticum* Δ *ctaB* by CRISPR-Cas9 genome editing. A) Scheme depicting the CRISPR-Cas9 genome editing method used for the incorporation of a nonsense mutation and an additional *EcoRV* recognition site into *ctaB*. B) Agarose gel (0.75 %) of *EcoRV*-digested *ctaB*-containing PCR products amplified from gDNA isolated from the indicated strains. The PCR amplicon from the wild-type control (2943 bp) contains one *EcoRV* recognition site, yielding products of 2868 bp and 75 bp (not visible) upon digestion by *EcoRV*. The PCR amplicon from the Δ *ctaB* strain (2943 bp) contains two *EcoRV* recognition sites and is digested to the expected 2141 bp, 727 bp and 75 bp (not visible) by *EcoRV*. L, GeneRuler 1 kb DNA ladder (Thermo Scientific). C) The sequence of Δ *ctaB* in the region of the targeted mutation (stop codon, red; *EcoRV* site, underlined) is displayed beneath the corresponding sequence of *ctaB*. The point of translation termination is indicated by an asterisk. D) DNA sequence chromatogram verifying the precise insertion of the mutated sequence (highlighted) in *R. cellulolyticum* Δ *ctaB*.

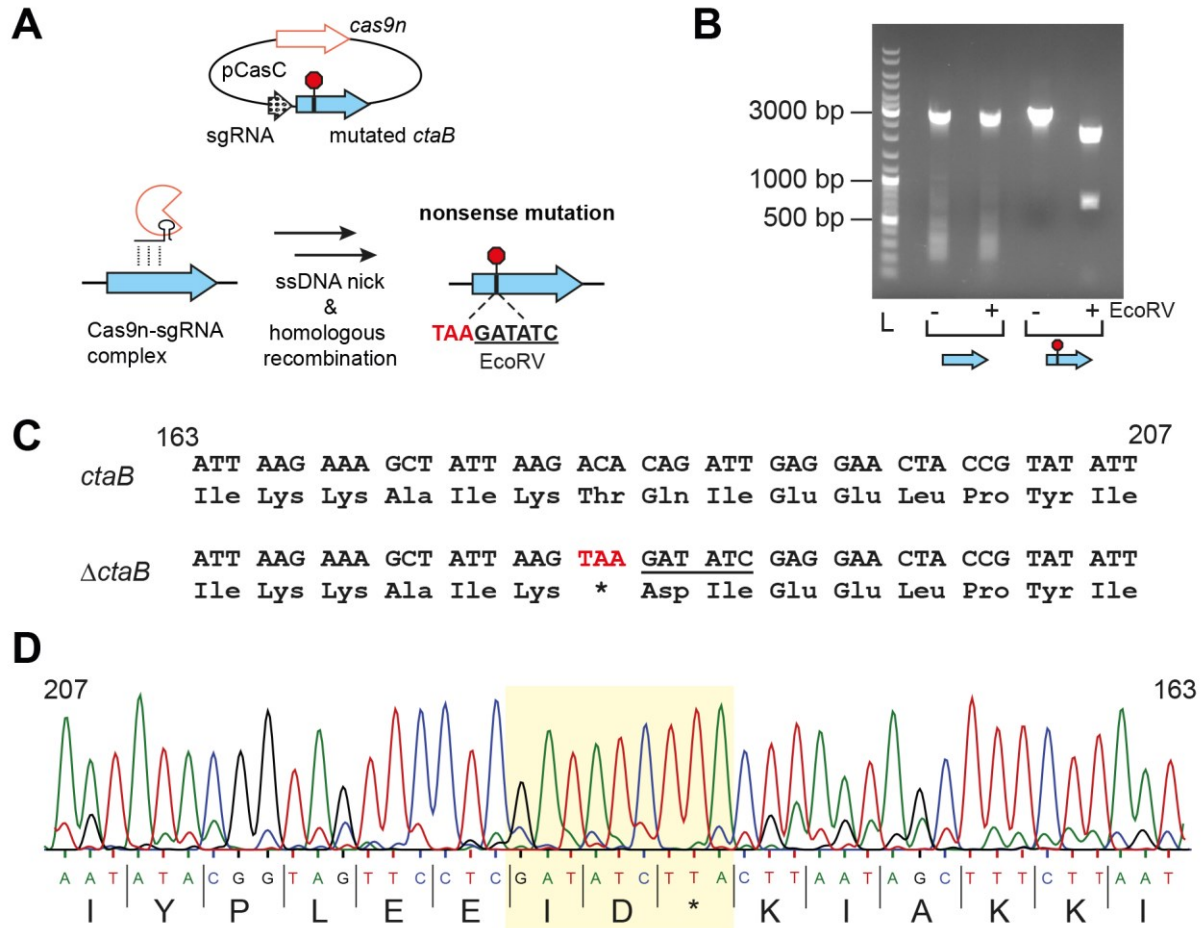


Figure S7. Generation of *R. cellulolyticum* Δ *ctaK* by CRISPR-Cas9 genome editing. A) Scheme depicting the CRISPR-Cas9 genome editing method used for the incorporation of a nonsense mutation and an *EcoRV* recognition site into *ctaK*. B) Agarose gel (1 %) of *EcoRV*-digested *ctaK*-containing PCR products amplified from gDNA isolated from the indicated strains. The PCR amplicon from the wild-type control (2124 bp) does not contain an *EcoRV* recognition site and is not digested by *EcoRV*. The PCR amplicon (2124 bp) from the Δ *ctaK* strain contains one *EcoRV* recognition site and is digested to the expected 1521 bp and 603 bp products by *EcoRV*. L, GeneRuler 1 kb DNA ladder (Thermo Scientific). C) The sequence of Δ *ctaK* in the region of the targeted mutation (stop codon, red; *EcoRV* site, underlined) is displayed beneath the corresponding sequence of *ctaK*. The point of translation termination is indicated by an asterisk. D) DNA sequence chromatogram verifying the precise insertion of the mutated sequence (highlighted) in *R. cellulolyticum* Δ *ctaK*.

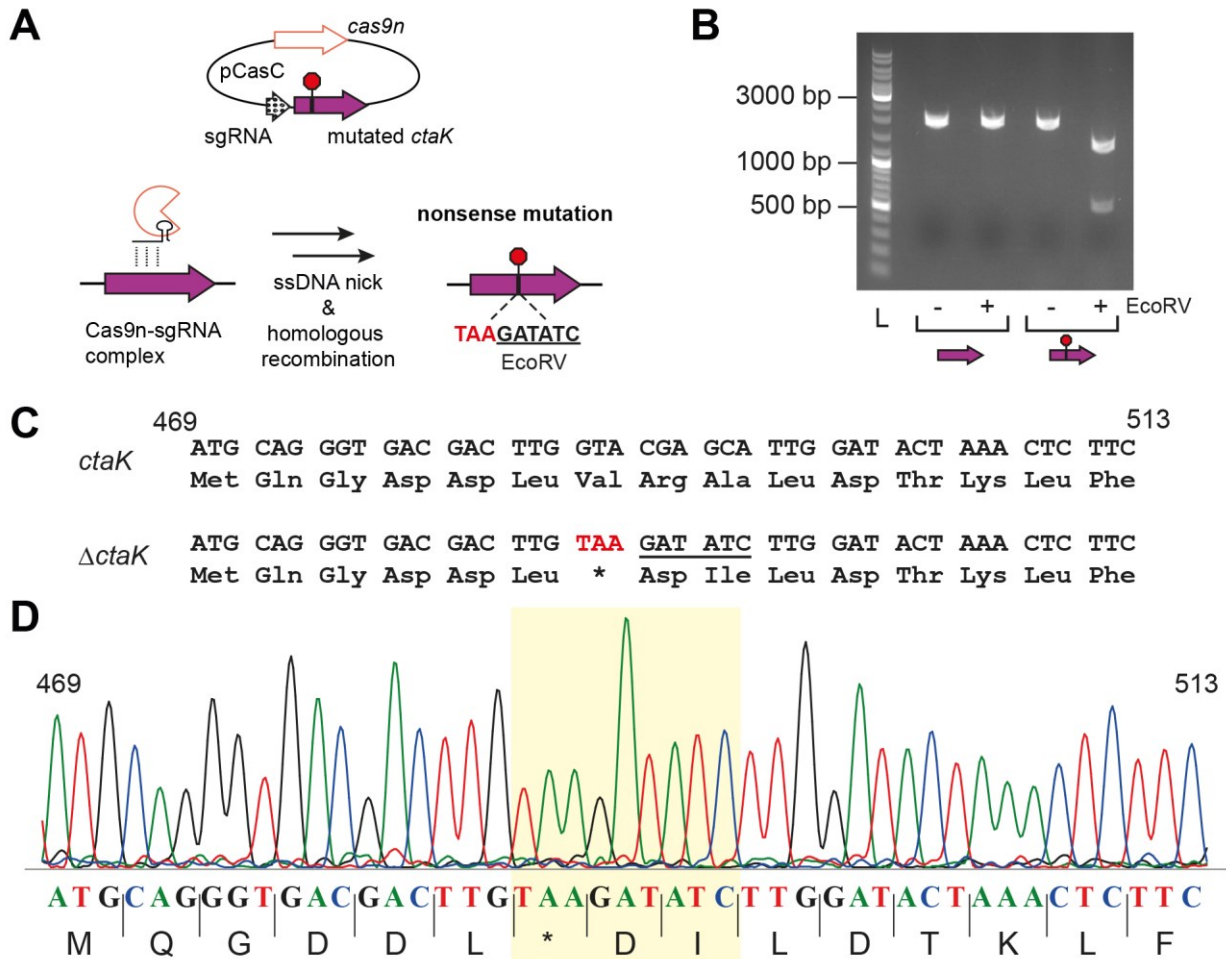


Figure S8. SDS-PAGE analysis of purified proteins. Coomassie-stained SDS-protein gel of purified proteins used in this study displayed. The expected size of each protein is indicated.

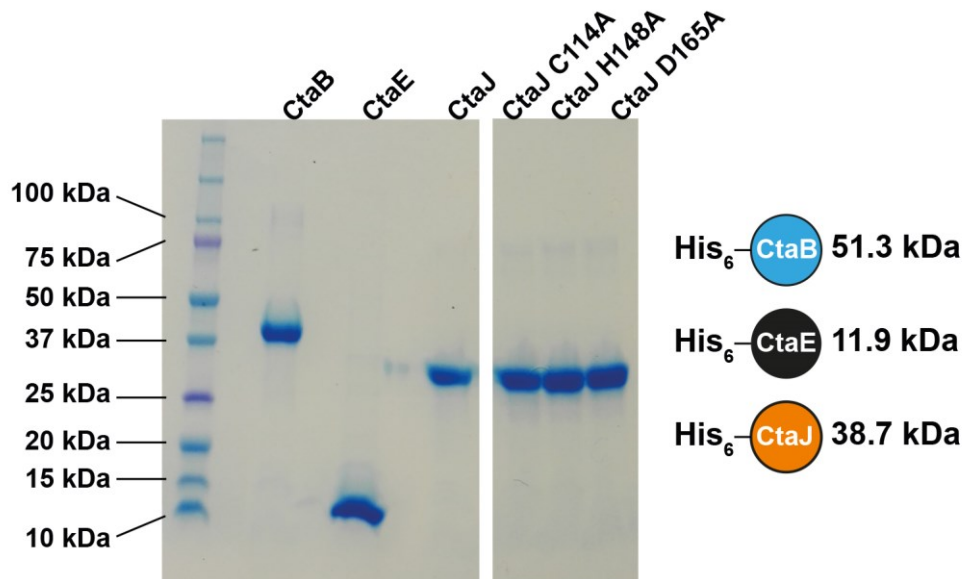


Figure S9. UV-vis spectrum of His-CtaB isolated from *Escherichia coli*. UV-visible absorbance spectra of His-CtaB in the presence or absence of PLP.

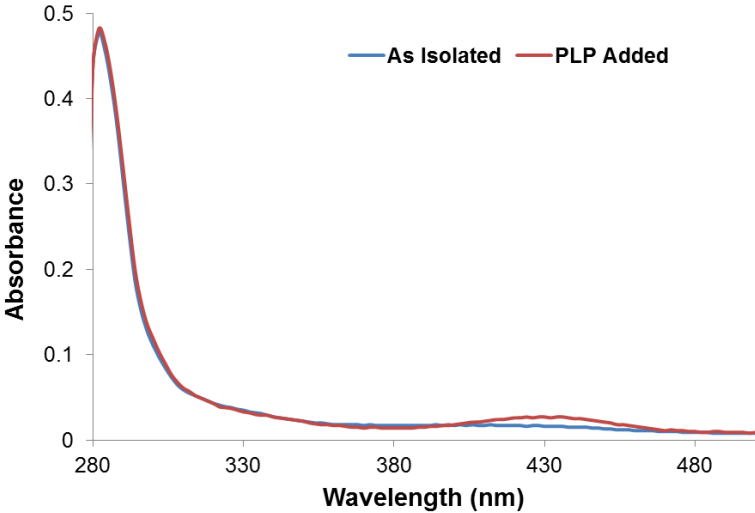


Figure S10. Expanded amine donor specificity profile of CtaB. HPLC profile (255 nm absorbance) of CtaB activity assays performed with Cbz-propionaldehyde and various amine donors. Asp, L-aspartate; Glu, L-glutamate; Gln, L-glutamine; His, L-histidine; β Ala, β -alanine; Ala, L-alanine; Orn, L-ornithine; Put, putrescine; Spm, spermine.

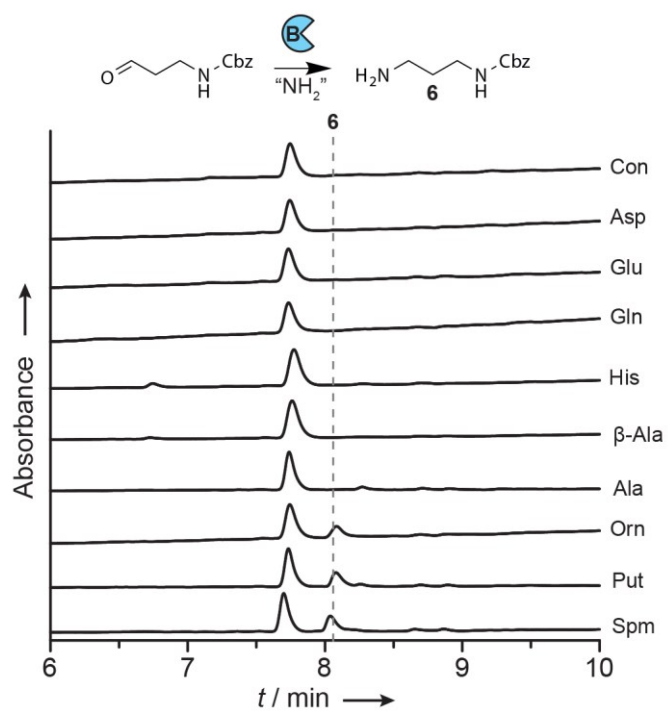


Figure S11. HRMS spectrum of 6 produced in CtaB assays.

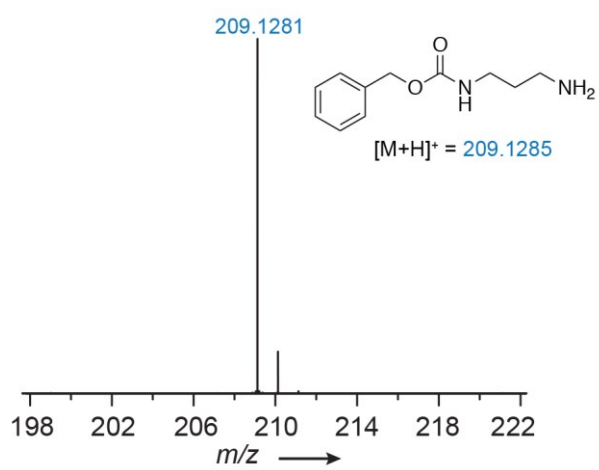


Figure S12. MS² spectra of 6 produced synthetically and in CtaB assays. The structure of 6 is displayed along with the MS² spectra of A) the synthetic standard and B) compound detected in CtaB assays. The expected masses of assignable fragments are indicated. Ions that were detected in the MS² spectrum are colored blue. Asterisks denote ions formed from the neutral loss of H₂ and quotations denote ions formed from the neutral loss of NH₃.

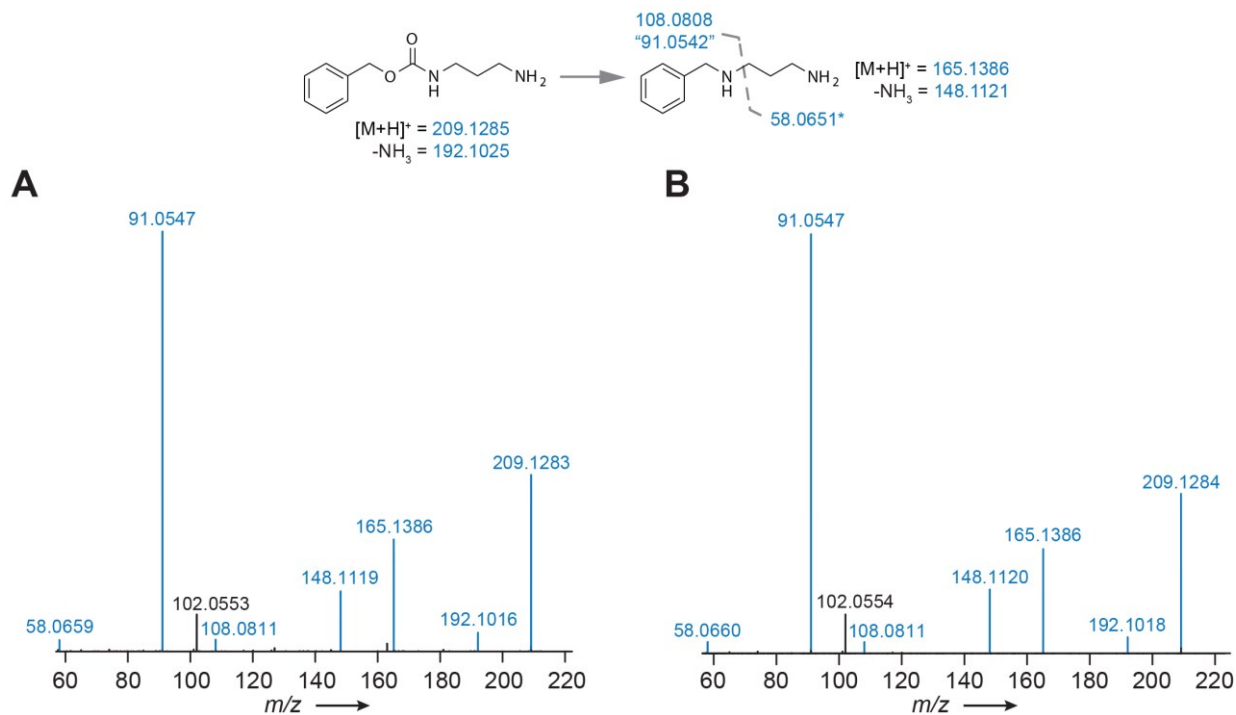


Figure S13. Reaction time course to determine the amine donor specificity of CtaB. HPLC profile (255 nm absorbance) of CtaB assays conducted with Cbz- β Ala aldehyde and various amine donors. A 2 h and 5 h point are shown for each reaction. The fastest processing was observed with Lys, Put and Cad. Lys, L-lysine; Orn, L-ornithine; Put, putrescine; Cad, cadaverine; Spm, spermine.

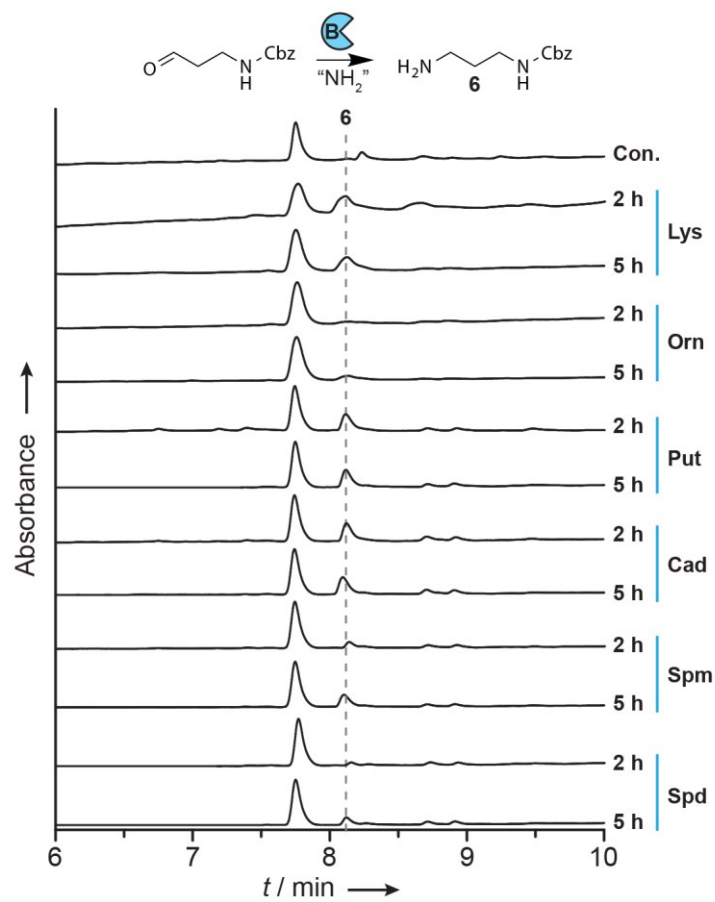


Figure S14. Kinetic assay to determine amine donor specificity of CtaB. A) Standard curve for **6** quantification by LC-HRMS (QExactive HF-X). Values represent the ratio of the EIC peak areas of **6** ($[M+H]^+ = 209.1285$; 5 ppm window) and the internal standard (caffeine; $[M+H]^+ = 195.0877$; 5 ppm window). B–D) Kinetic curves for CtaB assays performed with the indicated amine donor. Regression analysis to obtain the Michaelis-Menten kinetic parameters was performed with Graphpad. The error on the kinetic parameters represents the standard error from the curve fitting. A–D) Error bars on the data points represent the standard deviation from the mean from 3 independent experiments.

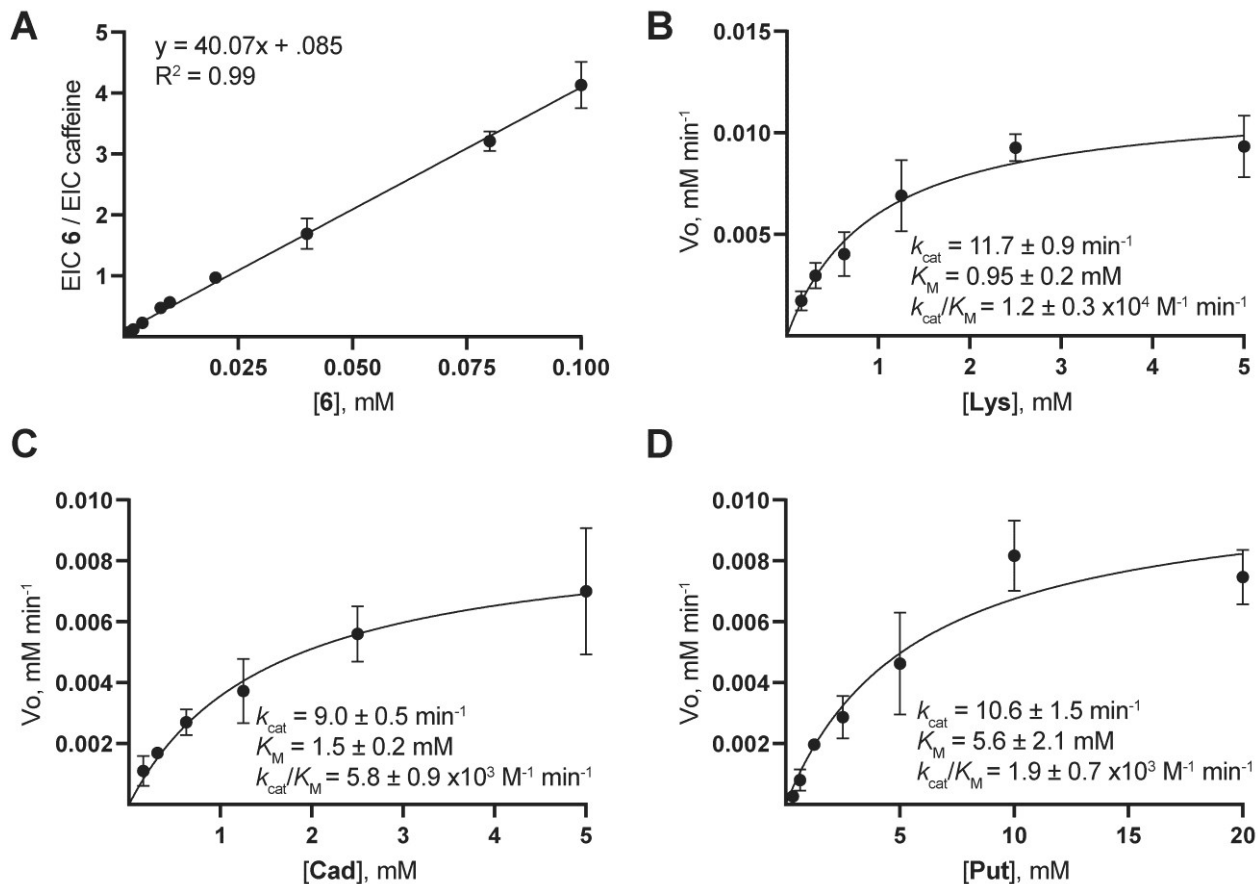


Figure S15. Detection of polyamines produced by *R. cellulolyticum*. A) HPLC profile (350 nm absorbance) of polyamine extracts from *R. cellulolyticum* cells following derivatization with Sanger's reagent. Standards of diaminopropane (DAP) and spermidine (Spd) are displayed. B) Extracted ion chromatograms (EICs) for diamines present in the *R. cellulolyticum* culture are displayed along with the structures of the labeled diamines with the expected m/z for the $[M-H]^-$ ionic species indicated. All EICs are displayed with m/z values ± 5 ppm from the calculated exact mass for the compound. *R. cell* WT, wild type *R. cellulolyticum*; Put, putrescine; Cad, cadaverine; Spd, spermidine; Spm, spermine.

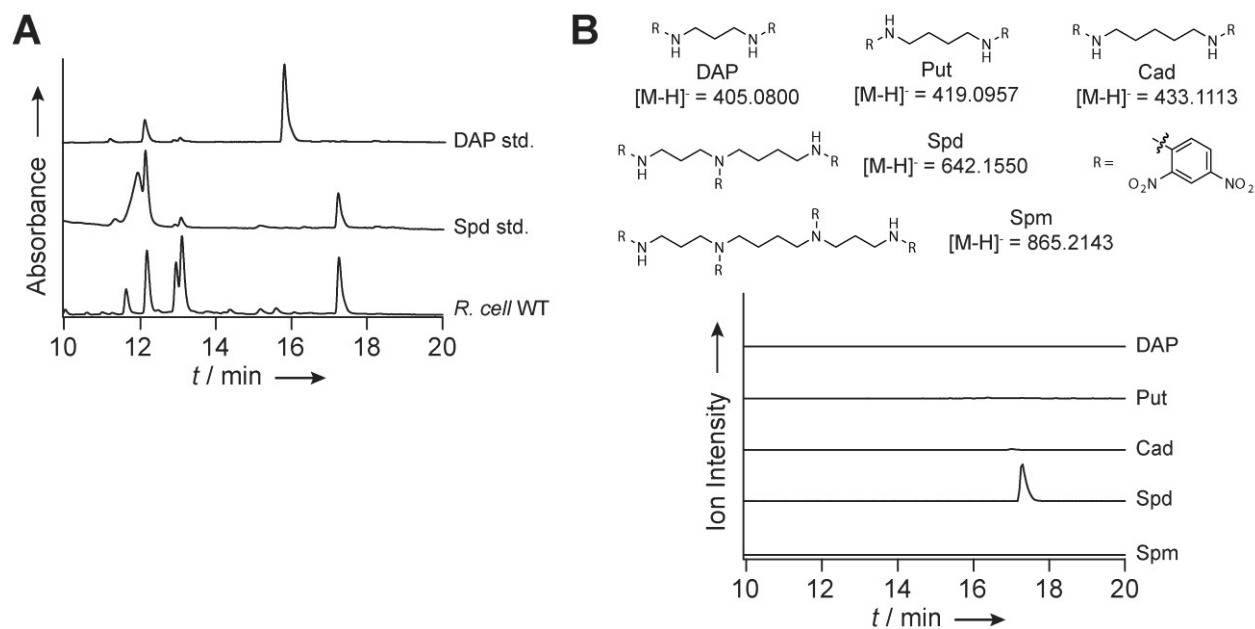


Figure S16. CtaJ activity assays containing only 2. HPLC profile (270 nm absorbance) of CtaJ assays performed with 2. Where indicated reactions were supplemented with CaCl_2 or GTP. Red strikethrough denotes heat-inactivated enzyme.

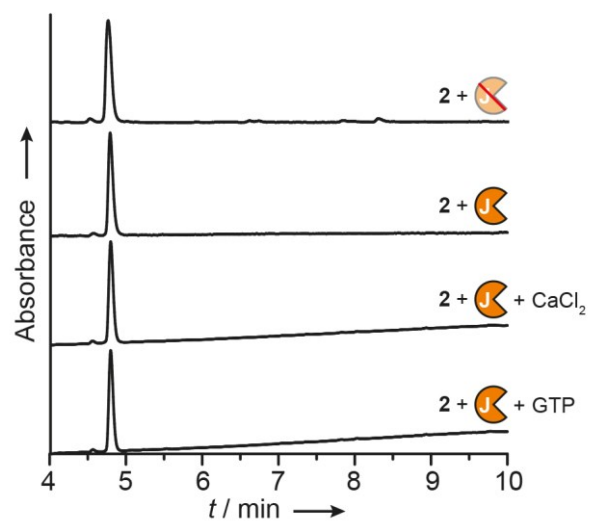


Figure S17. SNAC-1 and 1 processing by CtaJ. A) HPLC profile (270 nm absorbance) of CtaJ assays containing SNAC-1. B) HPLC profile (270 nm absorbance) of CtaJ activity assays containing **1**. A 10-fold vertical zoom of the boxed area is provided to better visualize product formation. The red pound sign denotes a CTA congener bearing an additional β Ala residue formed in the SNAC-1 assays (see **Figure S18–S19**). Red strikethrough denotes heat-inactivated enzyme.

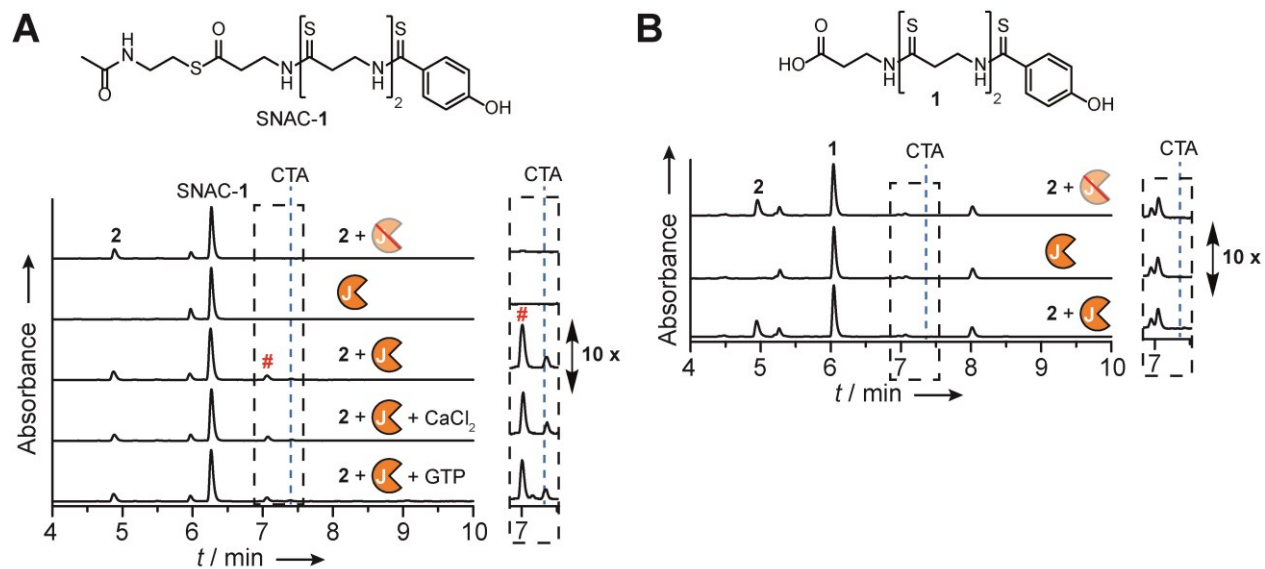


Figure S18. Production of a CTA congener with additional β Ala in CtaJ assays containing SNAC-1. A) The predicted structure of the CTA congener produced in CtaJ assays performed with SNAC-1 and **2** is displayed alongside the HRMS spectrum for the compound. B) Extracted ion chromatograms (EIC) for the new CTA congener in CtaJ assays containing SNAC-1. The EICs are displayed with m/z values ± 5 ppm from the calculated exact mass for the compound. Red strikethrough denotes heat-inactivated enzyme.

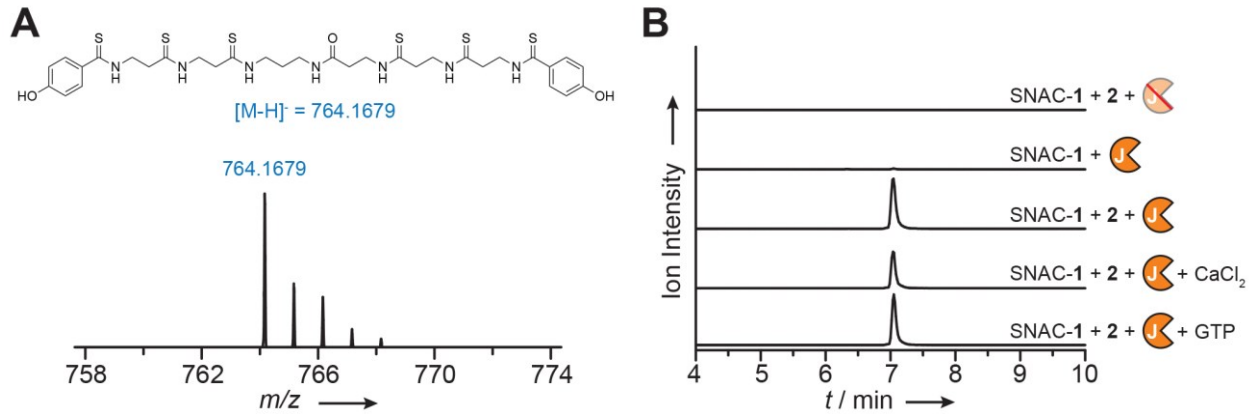


Figure S19. MS² spectrum of CTA congener with additional βAla. The predicted structure of the CTA congener produced in CtaJ assays performed with SNAC-1 is displayed along with the MS² spectrum of the compound produced in the reactions. The expected masses of assignable fragments are indicated. Ions that were detected in the MS² spectrum are colored blue. Asterisks denote ions formed from the neutral loss of H₂ while parentheses denote ions formed from the neutral loss of H₂S.

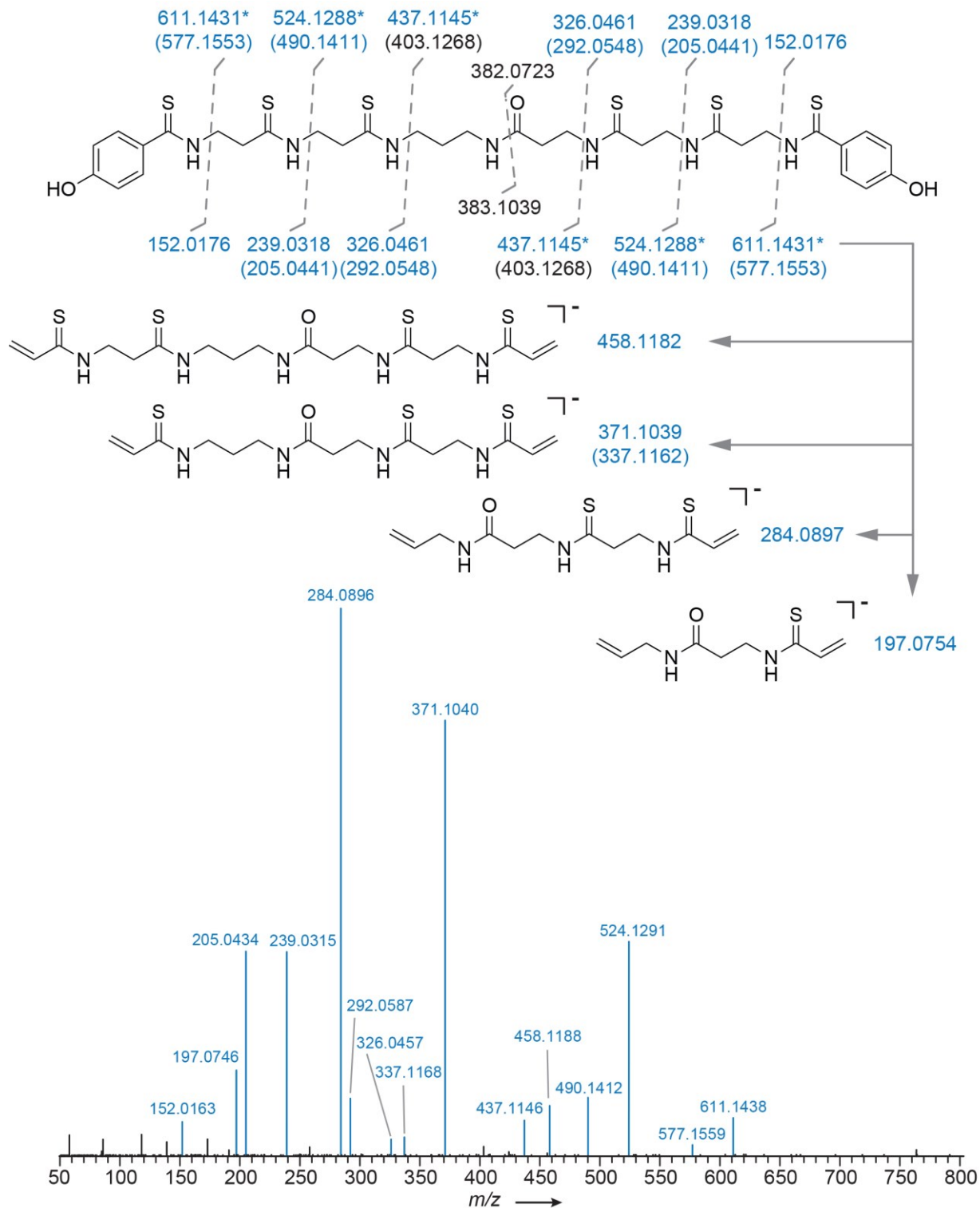


Figure S20. CtaJ activity assay with CtaE-1 and diaminopropane. HPLC profile (270 nm absorbance) of CtaJ assays containing CtaE-1 and diaminopropane (DAP). Red strikethrough denotes heat-inactivated enzyme.

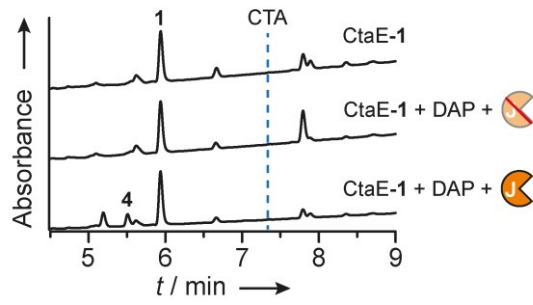


Figure S21. Production of 7 in CtaJ assays containing CtaE-1. A) The predicted structure of the thioacid congener of 4 (7) produced in CtaJ containing only CtaE-1 is displayed alongside the HRMS spectrum for the compound. B) Extracted ion chromatograms (EIC) for 7 produced in CtaJ assays performed with CtaE-1. The EICs are displayed with m/z values ± 5 ppm from the calculated exact mass for the compound. Red strikethrough denotes heat-inactivated enzyme.

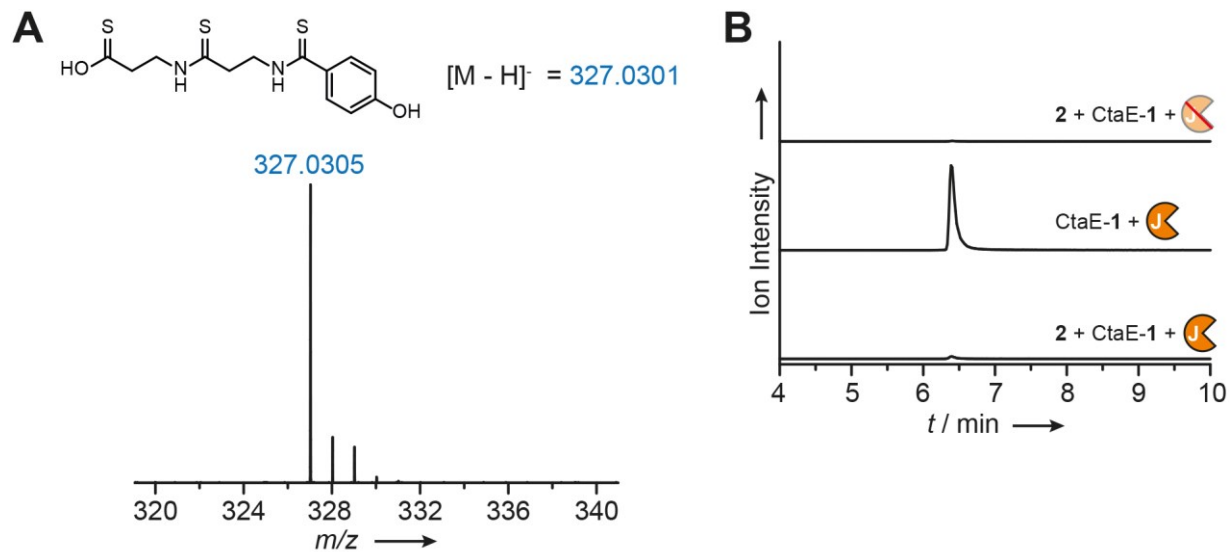


Figure S22. MS² spectrum of 7. The predicted structure of **7** produced in CtaJ assays containing CtaE-1 is displayed along with the MS² spectrum of the compound produced in the reactions. The expected masses of assignable fragments are indicated. Ions that were detected in the MS² spectrum are colored blue. Asterisks denote ions formed from the neutral loss of H₂ while parentheses denote ions formed from the neutral loss of H₂S.

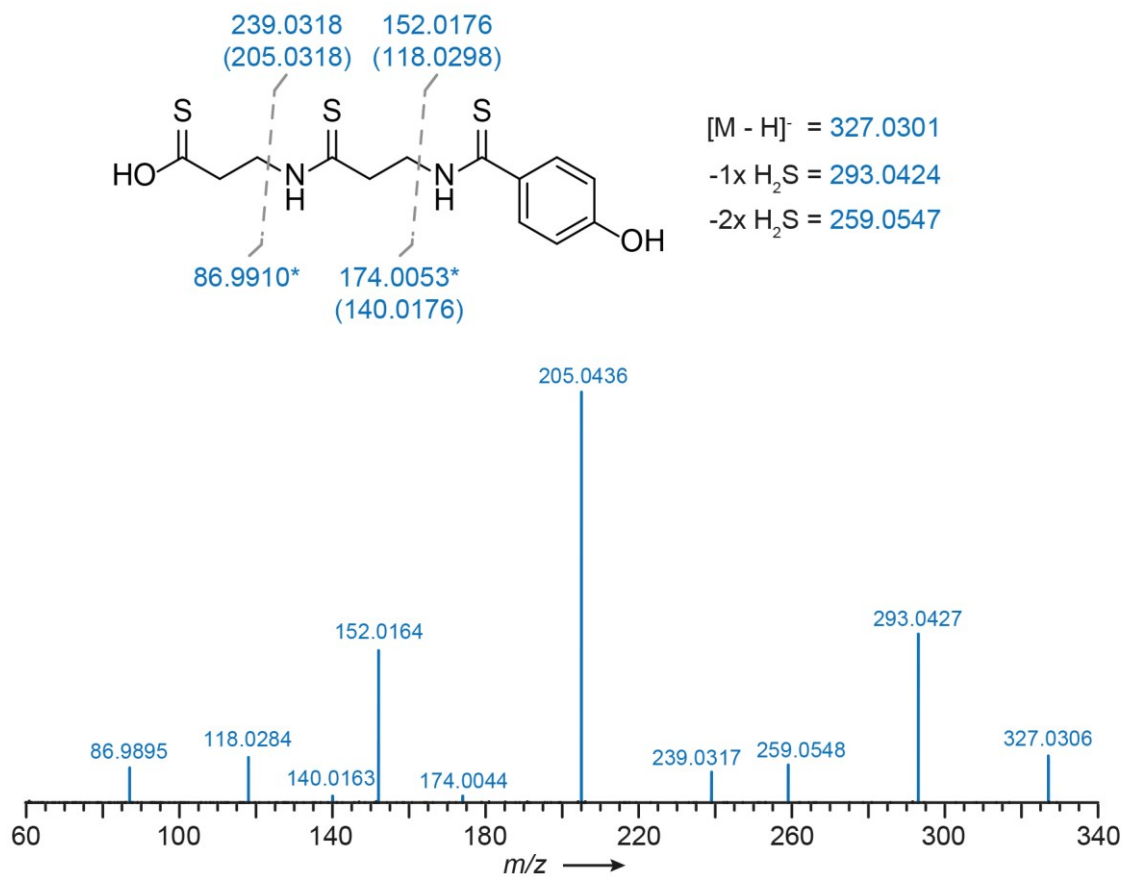


Figure S23. Product inhibition of CtaJ activity. MALDI-TOF-MS spectral overlay of CtaJ assays performed in the presence of varying concentrations of CTA after A) 4 h and B) 18 h. The expected average masses of CtaE-1 and CtaE- β Ala are indicated. The red asterisk denotes holo-CtaE formed from thioester hydrolysis. Red strikethrough denotes heat-inactivated enzyme.

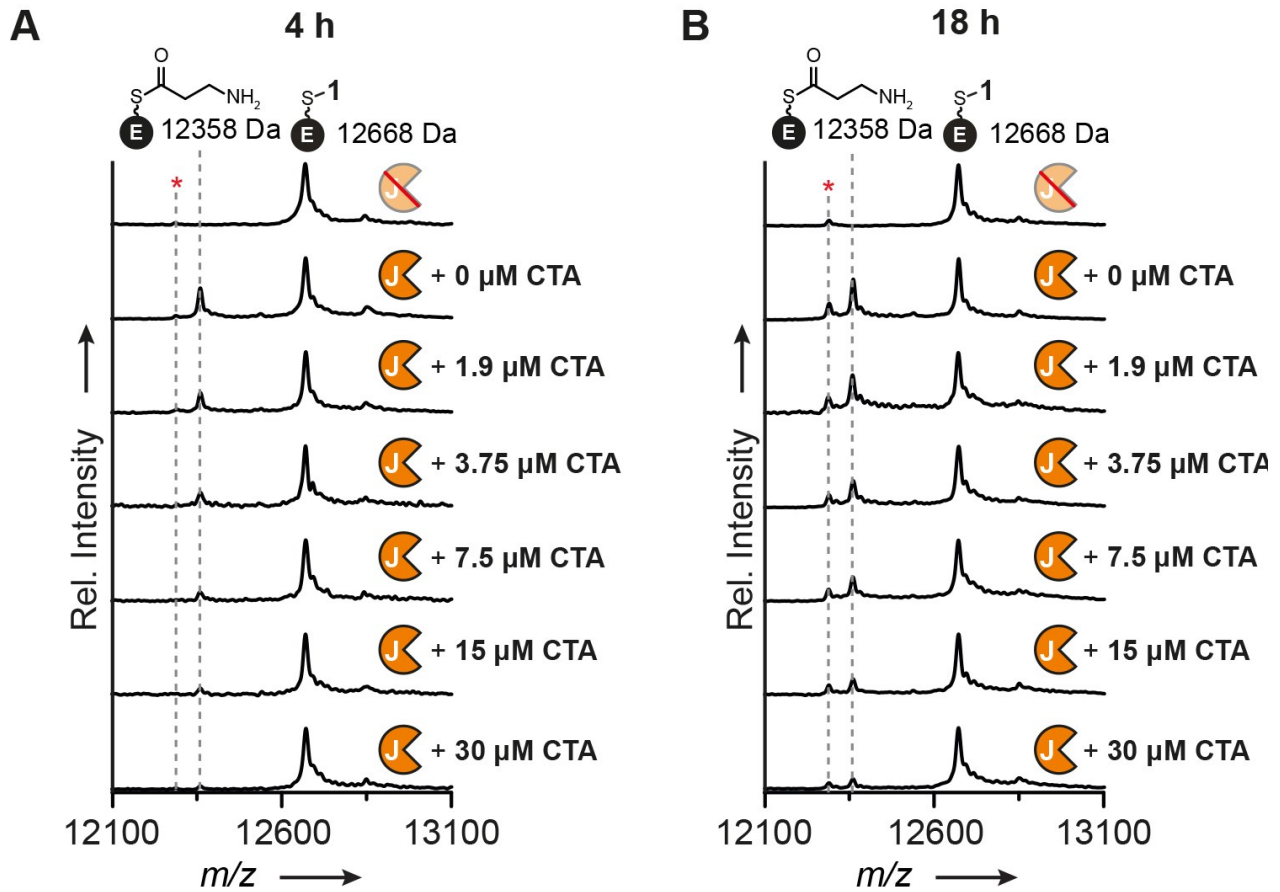


Figure S24. Multiple sequence alignment of CtaJ and characterized transglutaminases. Alignment of diverse transglutaminase (TGase) proteins with the catalytic triad highlighted. TGase 1, BAA34203; TGase 2, 3LY6; TGase 3, 1NUD; TGase 4, AAH07003; TGase 5, AA122860; Factor XIII, 1EVU; AdmF, AAO39100; AdmS, AAO39113.

TGase 2	DGVSPMSWIG-----SVDILRRWKNHGC-----QRVKYGCCWVFAAVACT	286
TGase 3	GGRDPRSWDG-----SVEILKNWKKSGL-----SPVRYGQCWVFAGTLNT	281
TGase 5	DGANPAEWTG-----SVAILKQWNATGC-----QPVRYGQCWVFAAVMCT	287
TGase 4	GGTAPYKWTG-----SAPILQQYYNTK-----QAVCFGCCWVFAGILTT	277
TGase 1	RGTNPNSAWVG-----SVEILLSYLRTG-----YSVPYGCWVVFAGVTTT	386
Factor XIII	YGVPPSAWTG-----SVDILLEYSSE-----NPVRYGQCWVVFAGVFTT	322
CtaJ	GMDRFPWYRMCCEDDILLTAV-AMTAEIIFRLNEKGFPPGROVEERIVISCRHITVILGA	123
AdmF	SGVNAHKIPL-SRHKDFELRTVTEVLQEAATRRGL-DLTTEPNPFKKIIGNCQTISLLCLG	99
AdmS	KGINKYNINP-ERYNDIDITQPEIILDRLS DAGVTSIINPIPFHKLVGHC LTLAIVALD	104
	- : * :	
TGase 2	VLRCLGIPTRVVTNYSAHDQNSNLLIEYFRNEFGEIQG-DKSEMIWNFEWVESWMTRP	345
TGase 3	AIRSLGIPSRVITNFNSAHDTRNLSVDVYDPMGNPLD-KGSDSVWNFEVWNEGWFVRS	340
TGase 5	VMRCLGIPTRVITNFDSGHDTDGNLIIDEYYDNTGRILGNKKKDTIWNFEVWNECWARK	347
TGase 4	VLRALGIPARSVTGFDSAHDTERNLTVDTYVNEGEKITSMTHDSVWNFEVWTDAMMKRP	337
TGase 1	VLRCLGLATRITVITNFNSAHDITDTSMTMDIYFDENMKPLEHLNHSVWNFEVWVNDCAWTKRP	446
Factor XIII	FIRCLGIPARIVTNYFSAHNDANLQMDIFLEEDGNVNSKLTKDSVWNFEVWNEAWMTRP	382
CtaJ	IIKAKGIPCRSRAGIRAYHGKP-----VDEWINQYWSEK-	157
AdmF	IIRELSIPARYRFLCEYFEPNS-----YAEHIVLEIYWCSS-	135
AdmS	MMRYKNIPSRRLRYAYCTYFSQDI-----YPEQILIEYWCED-	140
	:: -: * :	
TGase 2	DLQPGYEGWQALDPTPQEKSEGTGCCGVPVRAIKEGDLSTKYDAPFVFAEVNADVVDWI	405
TGase 3	DLGPSYGGWQVIDATPQERSQGVFCCGPASVIGVREGDVQINFDMPFFFAEVNADRIITWL	400
TGase 5	DLPPAYGGWQVIDATPQEMSNQVYCCGPASVRAIKEGEVDLNYDTPFVFSMVNADCMHWL	407
TGase 4	DLPKGYDGWQAVDATSQERSQGVFCCGPSPLTAIRKGDIFIVYDTRFVSEVNGDRLIWL	397
TGase 1	DLPSGFDGWQVVDATPQETSSTGIFCCGPCSVESIKNGLVYMKYDTPFFIFAEVNSDKVYWQ	506
Factor XIII	DLPVGFGGWQAVDSTPQENS DGM YRCGPASVQA I K H G H V C F Q F D A P F V F A E V N S D L I Y I T	442
CtaJ	----ENRWVNEDEGEG--IMY-----RECEGLF---TTYDIPDDQFYWI	191
AdmF	----DKKWKIIDPTVTHEIIE-----KNQTSFIDFEFDNIPRIKSNLF	175
AdmS	----KGAWLLGDP SMNQEVL D-----HNQISVNV--DFCNVDNITLSQPI	178
	* * :	

Figure S25. ^1H -NMR spectrum of 9.

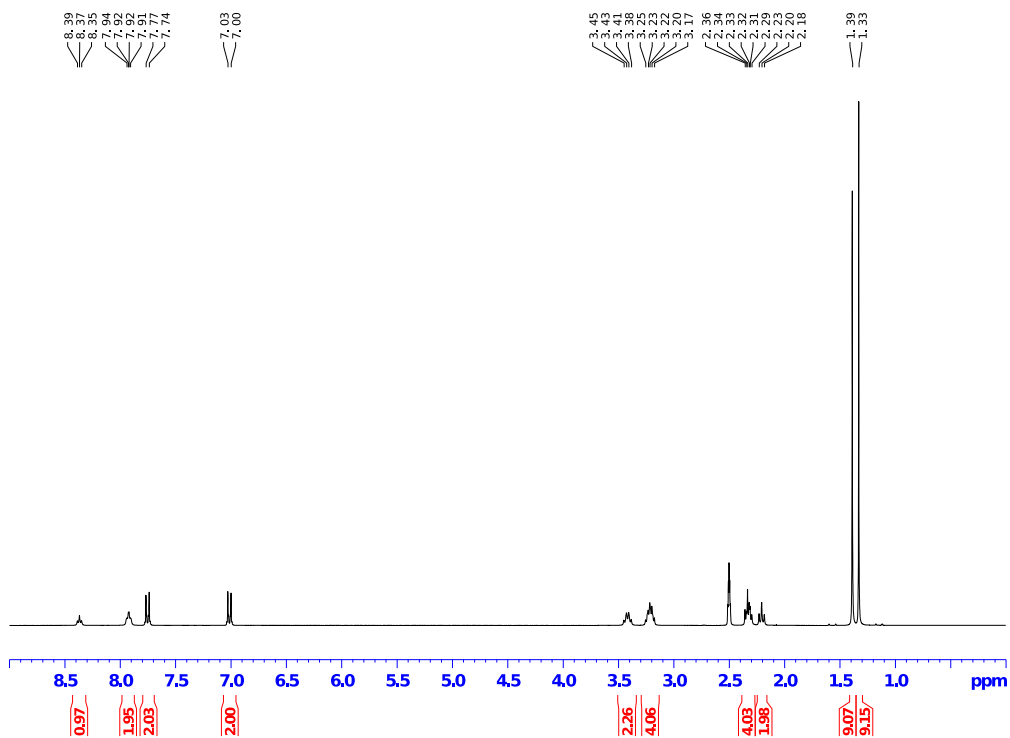


Figure S26. ^{13}C -NMR spectrum of 9.

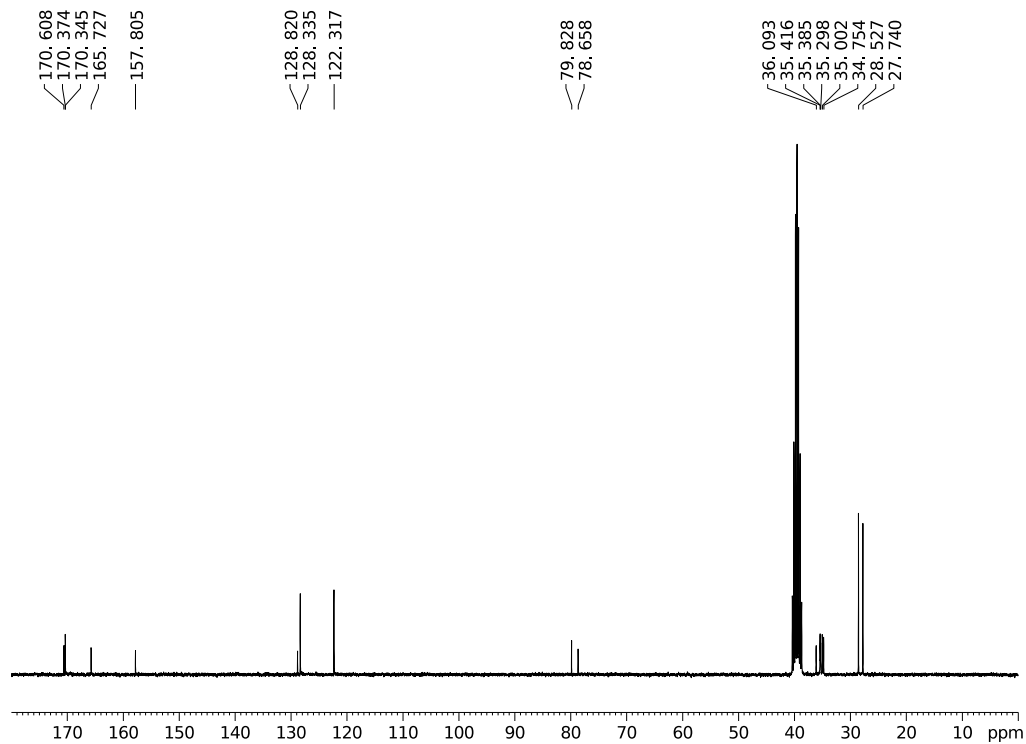


Figure S27. ¹H-NMR spectrum of 10.

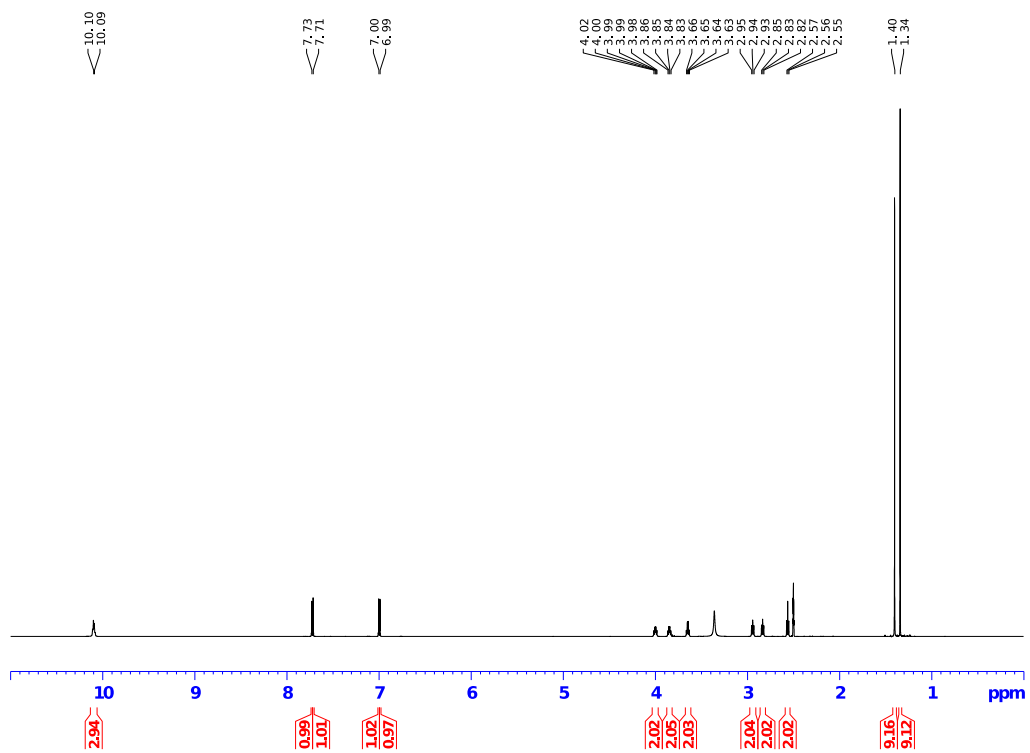


Figure S28. ¹³C-NMR spectrum of 10.

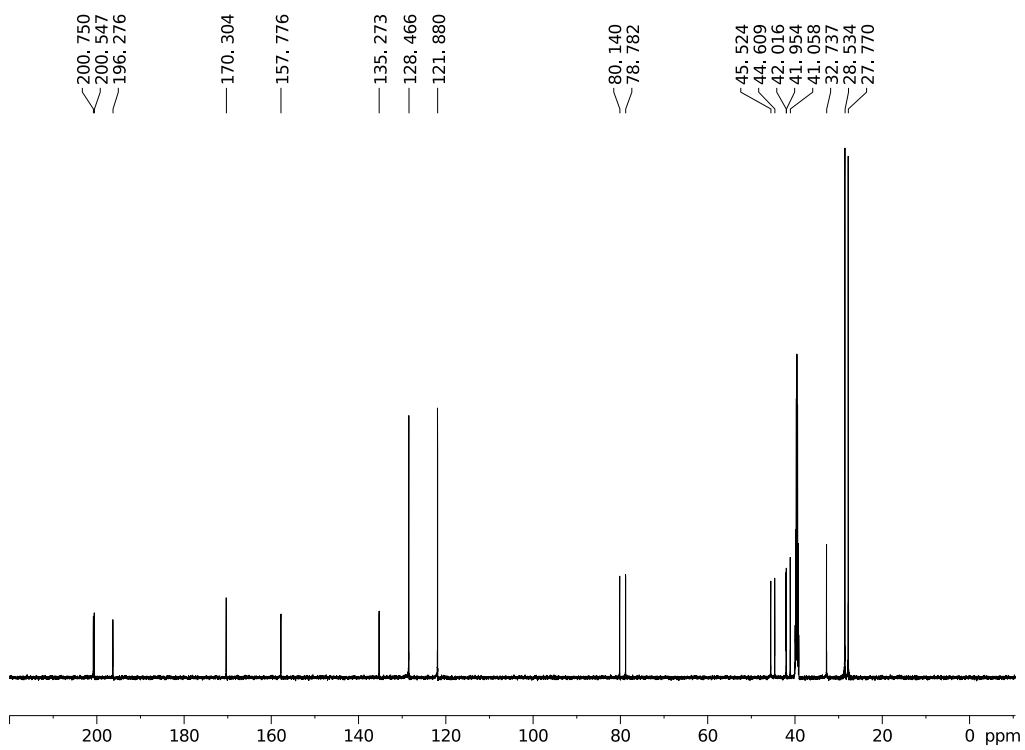


Figure S29. ^1H -NMR spectrum of 1.

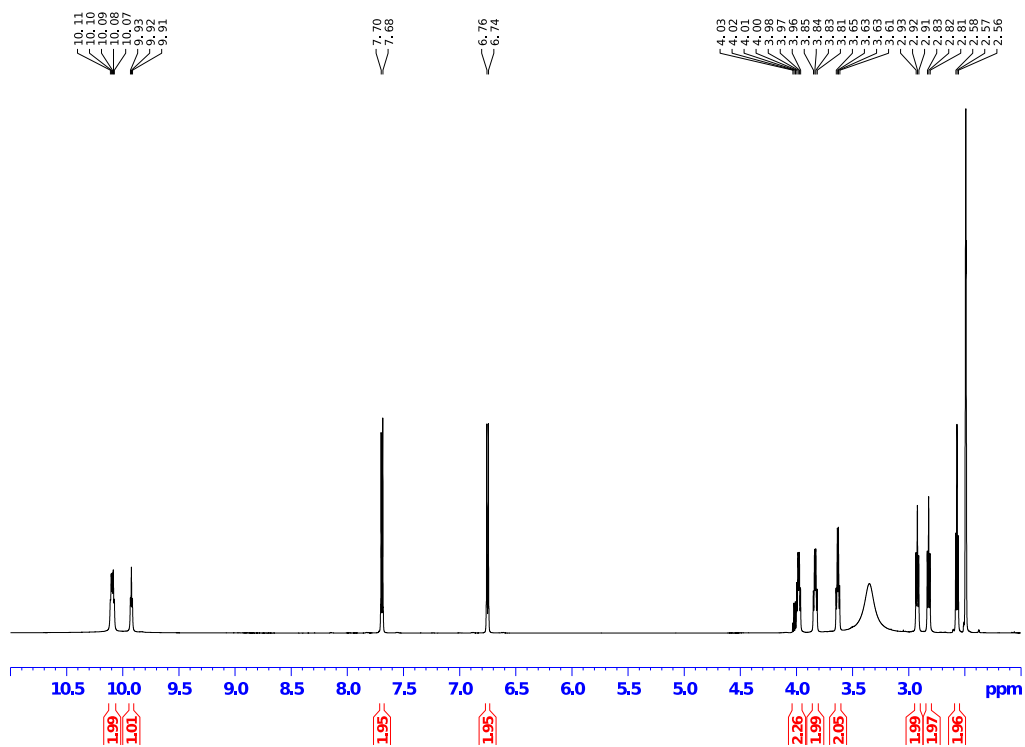


Figure S30. ^{13}C -NMR spectrum of 1.

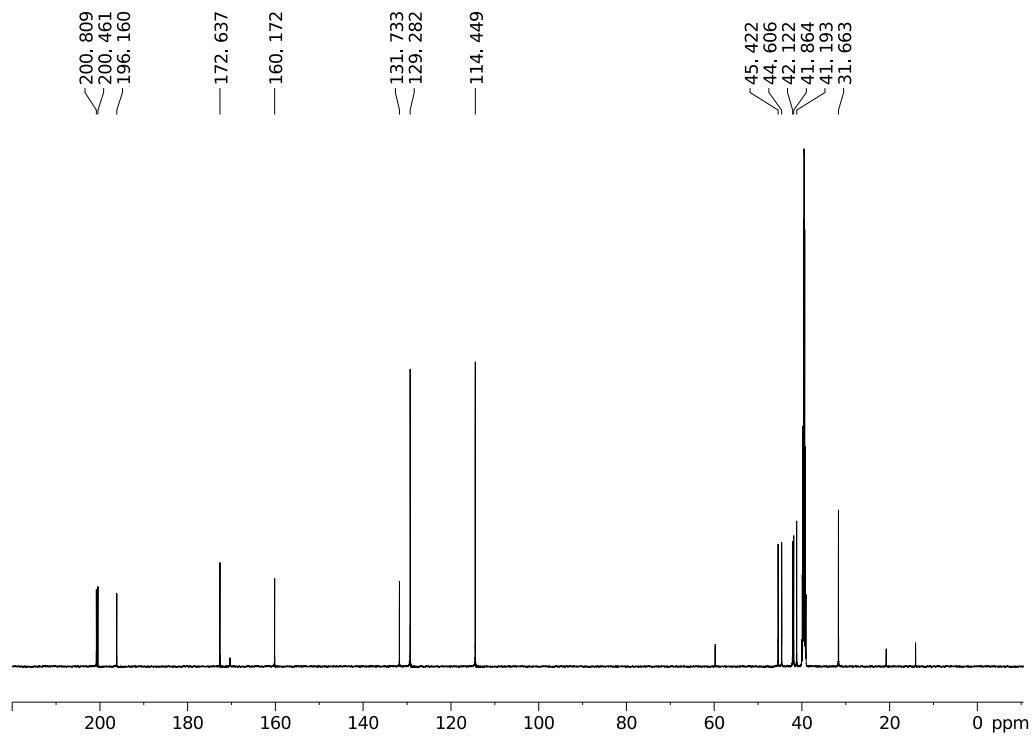


Figure S31. ^1H -NMR spectrum of SNAC-1.

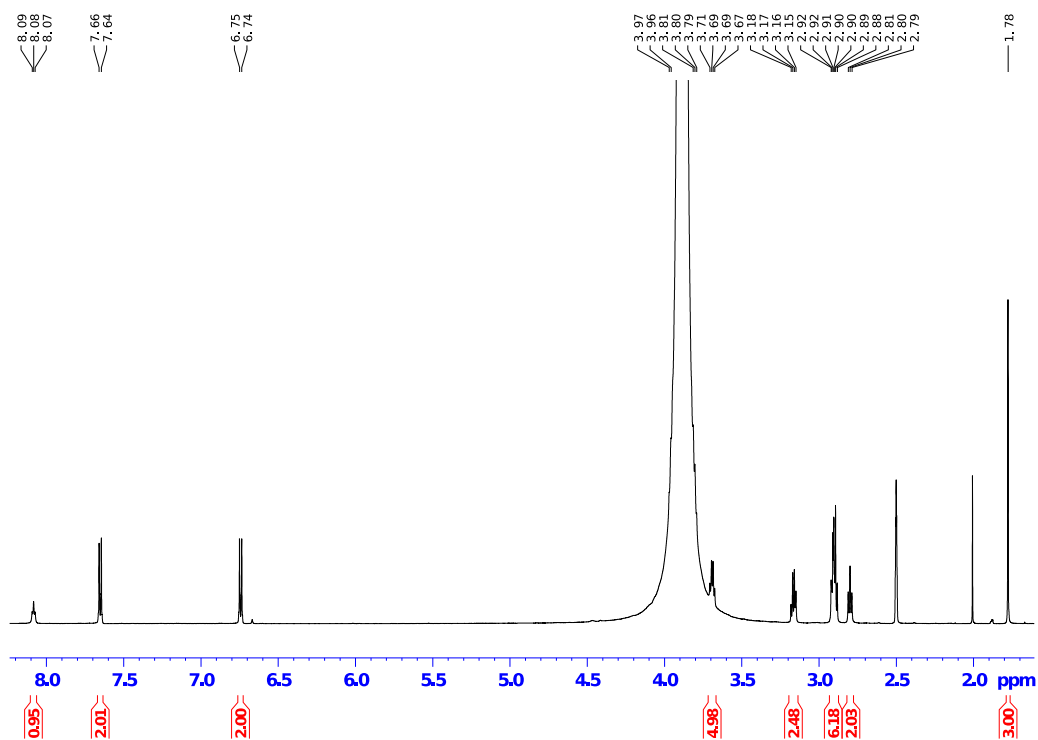


Figure S32. ^{13}C -NMR spectrum of SNAC-1.

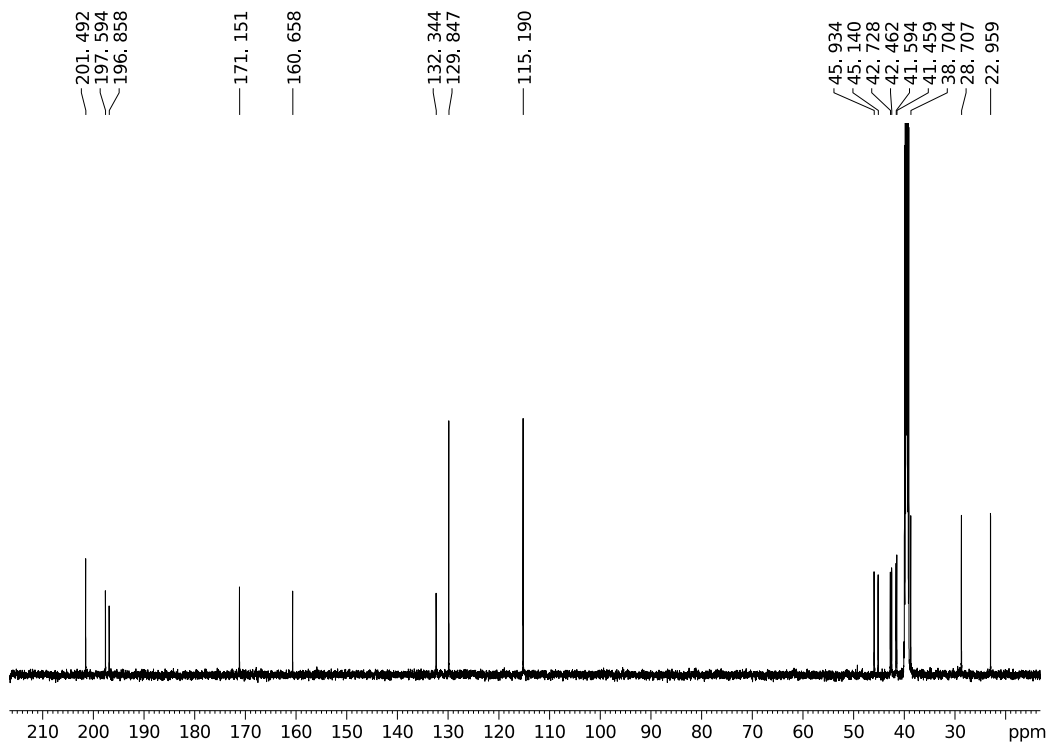


Figure S33. ¹H-NMR spectrum of CoA-1.

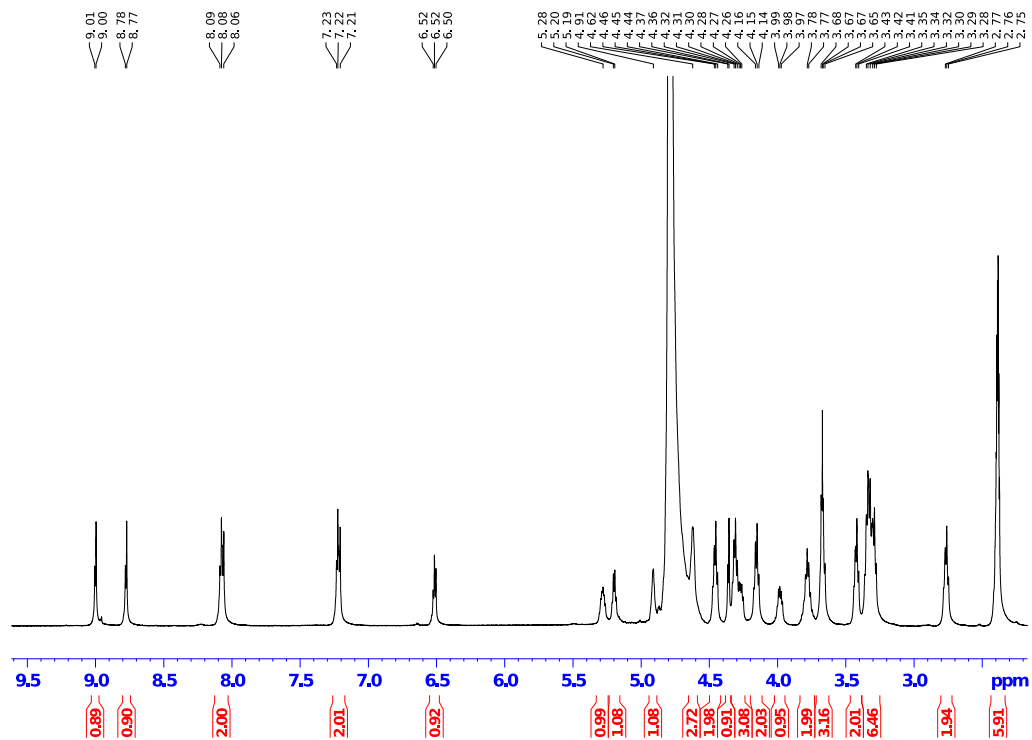


Figure S34. ¹³C-NMR spectrum of CoA-1.

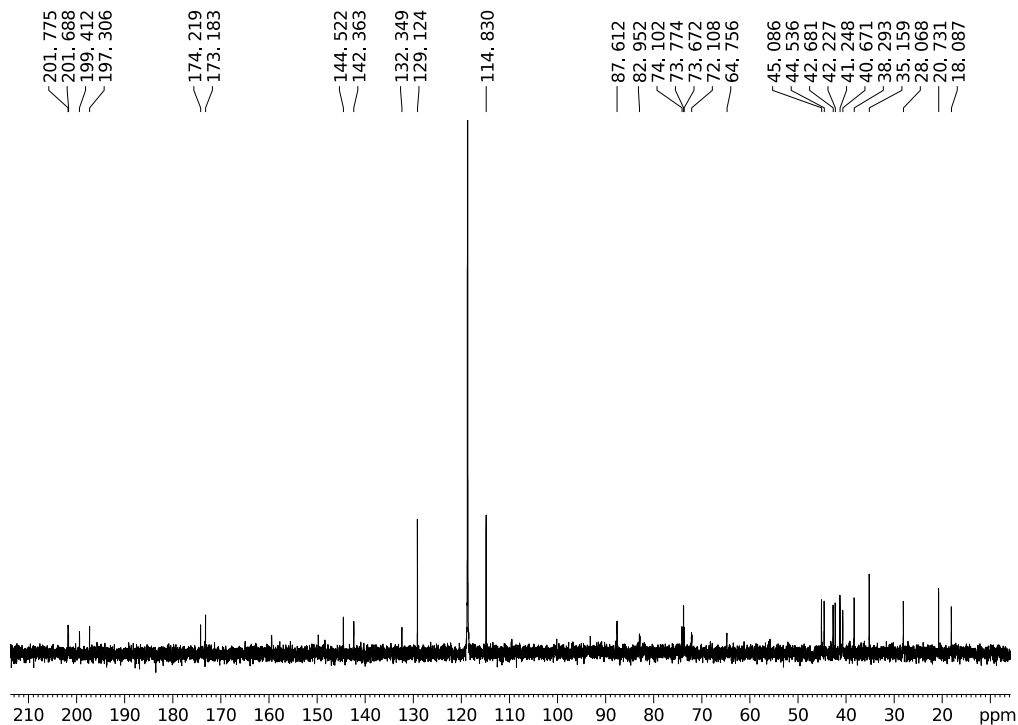


Figure S35. ^1H -NMR spectrum of 11.

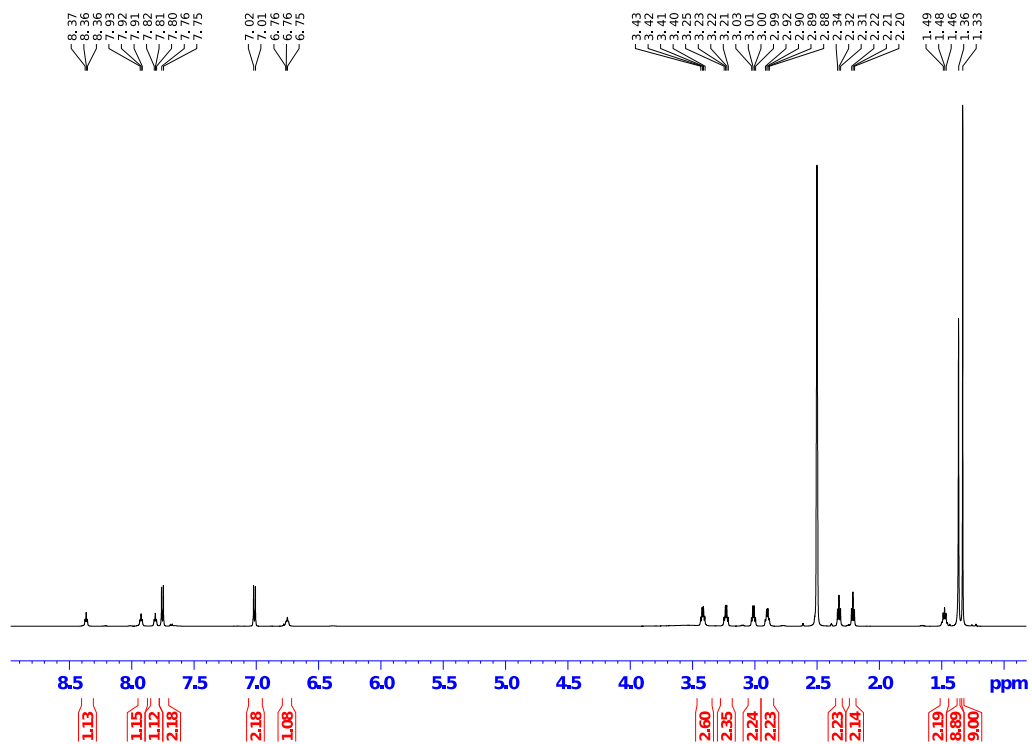


Figure S36. ^{13}C -NMR spectrum of 11.

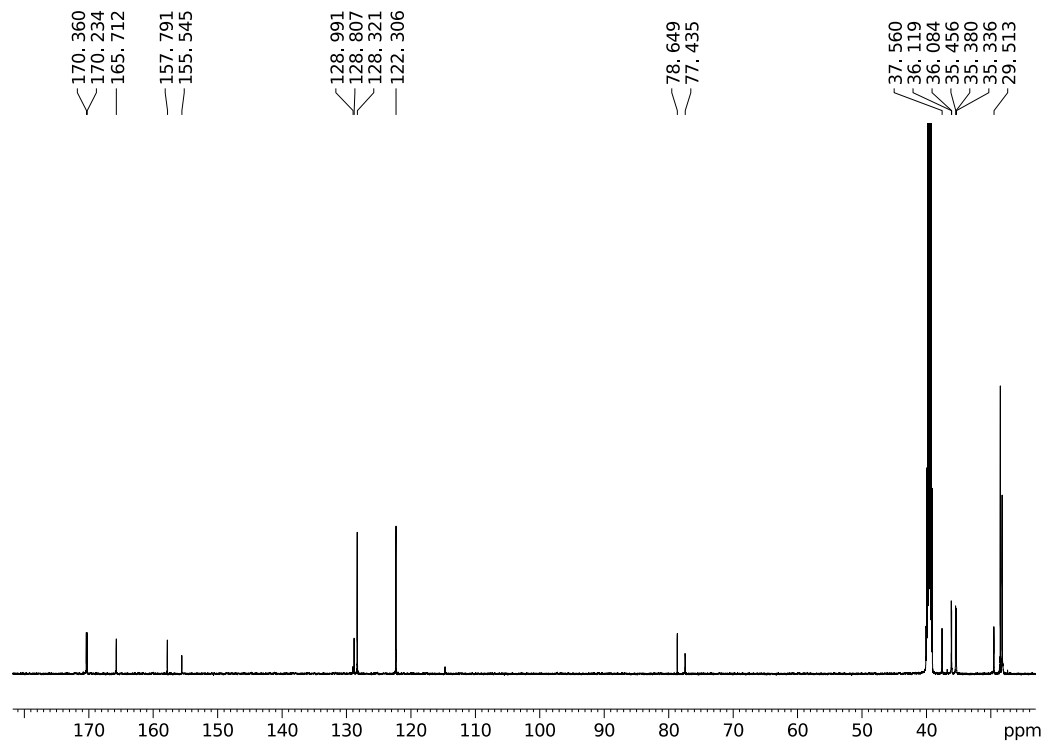


Figure S37. ^1H -NMR spectrum of 2.

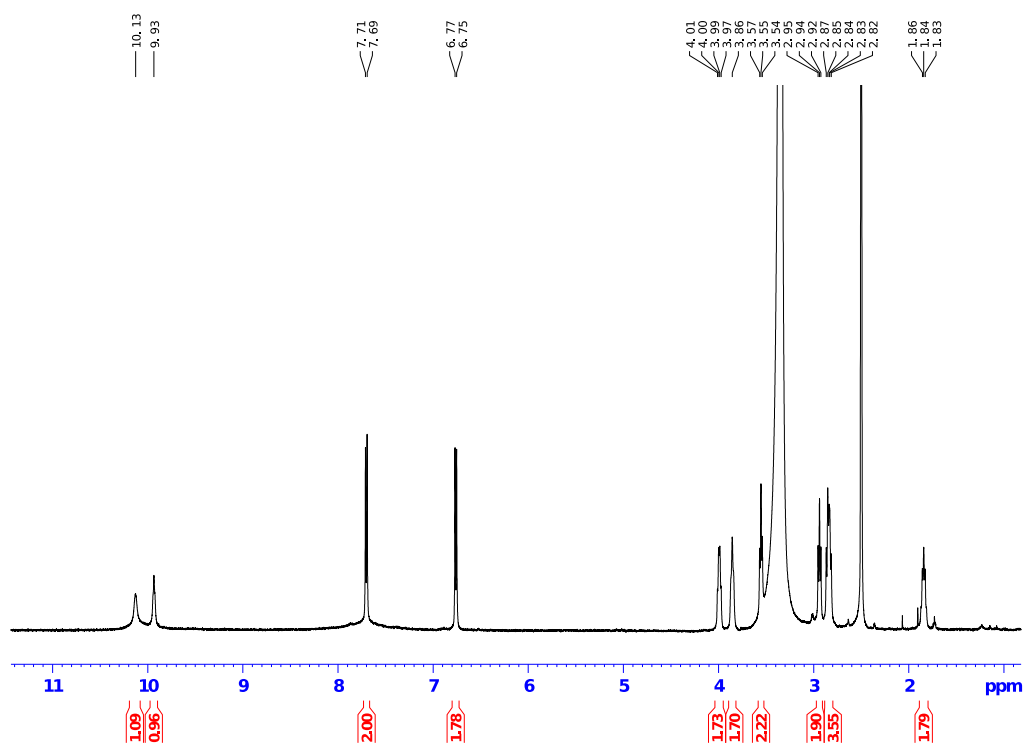


Figure S38. ^{13}C -NMR spectrum of 2.

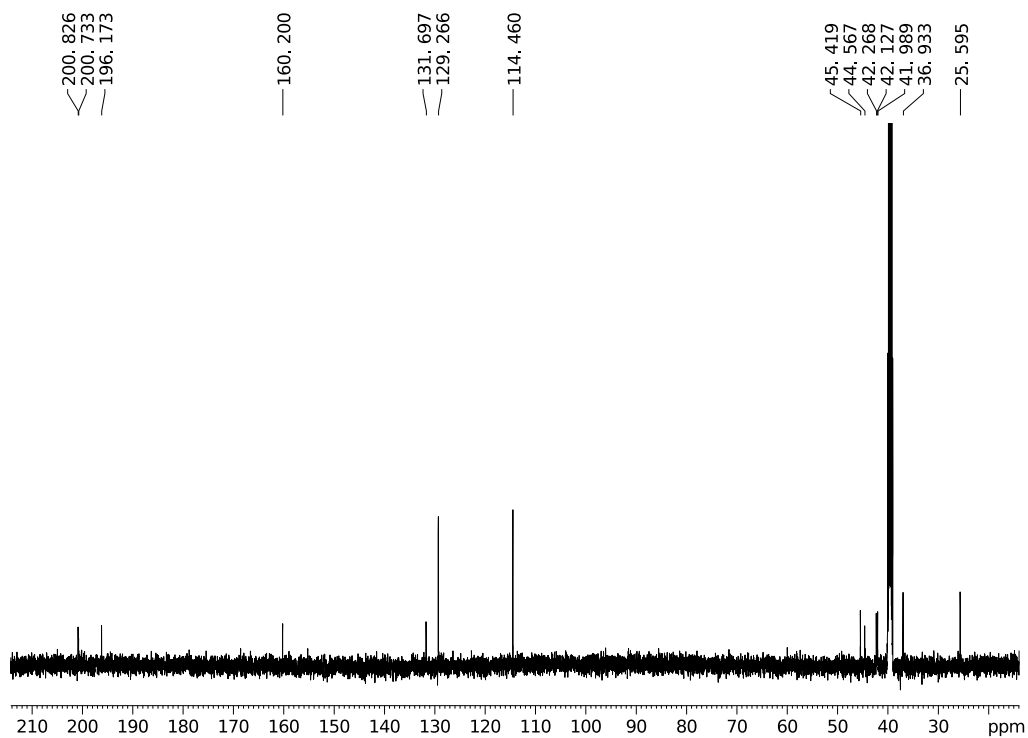


Figure S39. ^1H -NMR spectrum of 6.

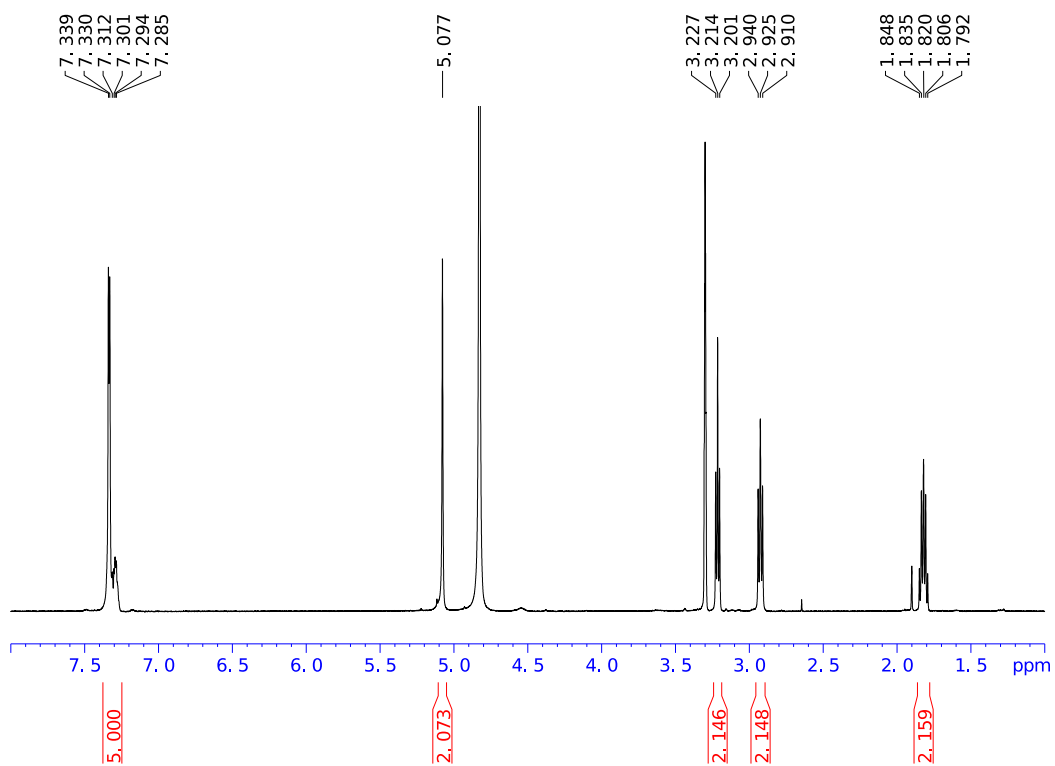
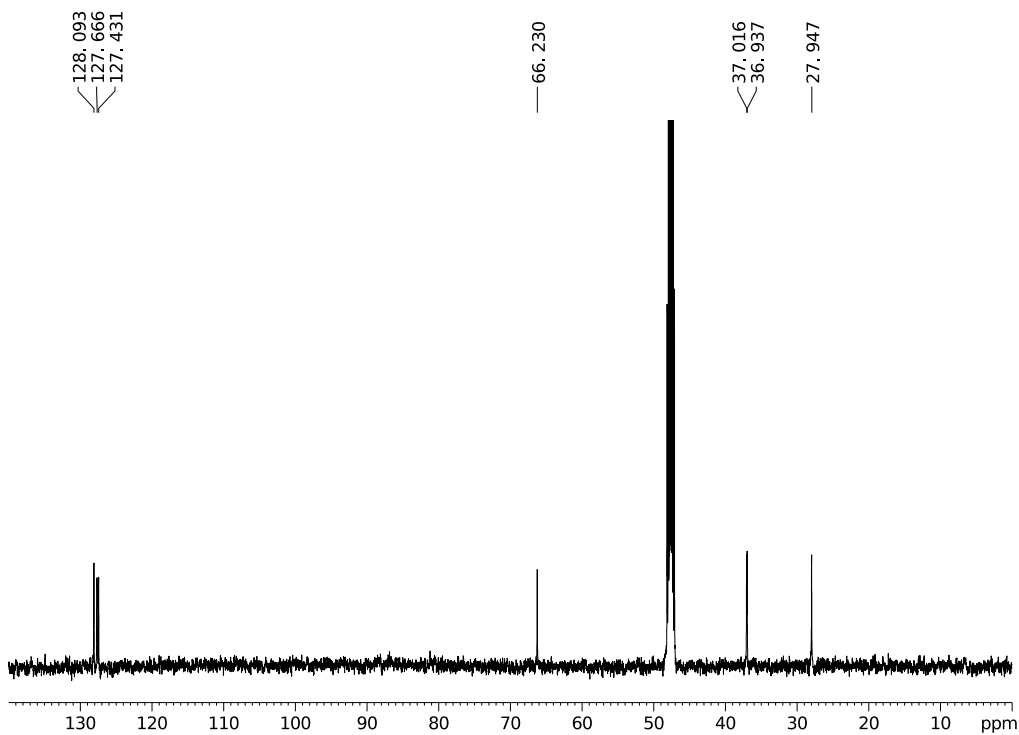


Figure S40. ^{13}C -NMR spectrum of 6.



Scheme S1. Model for the origin of all known CTA biosynthetic intermediates and shunt products. A) CTA derivatives formed by the breakdown of CTA. B) CTA congeners formed from biosynthetic intermediates. Compound numbers are provided for CTA congeners that were discussed in this manuscript. Grey box, compound not identified from *R. cellulolyticum* extracts; Blue box, compound previously detected^[13] but not produced under the cultivation conditions used in this study; Acet., acetylation.

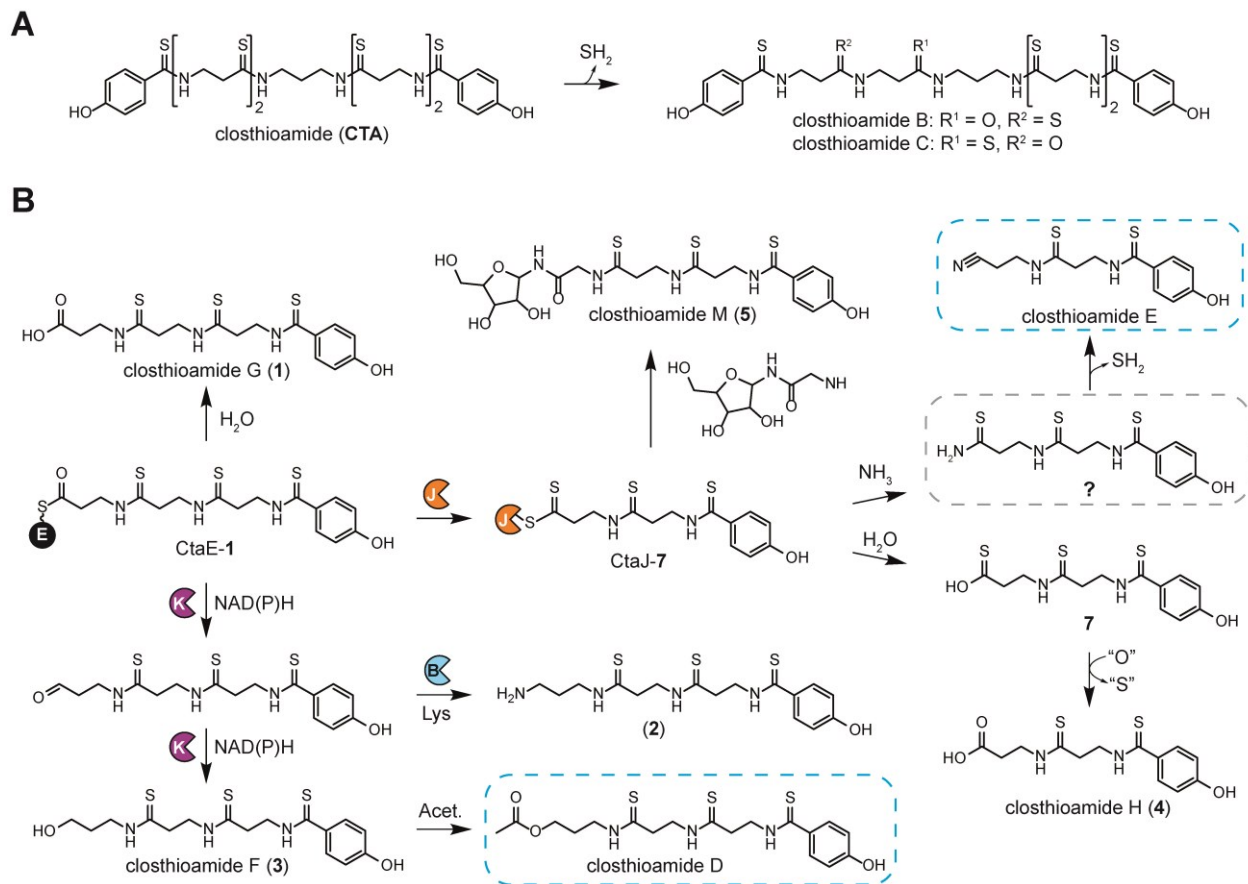


Table S1. Strains used in this study.

Strains	Relevant genotype and descriptions	Source
<i>Ruminiclostridium cellulolyticum</i>	Wild type (DSM 5812)	DSMZ
<i>Ruminiclostridium cellulolyticum</i> Δ <i>ctaB</i>	CRISPR/Cas inactivated Ccel_3259	This study
<i>Ruminiclostridium cellulolyticum</i> Δ <i>ctaJ</i>	CRISPR/Cas inactivated Ccel_3251	[2]
<i>Ruminiclostridium cellulolyticum</i> Δ <i>ctaK</i>	CRISPR/Cas inactivated Ccel_3250	This study
<i>Escherichia coli</i> TOP10	General cloning strain	Laboratory strain
<i>Escherichia coli</i> Rosetta (DE3)	Protein expression strain	NEB
<i>E. coli</i> Rosetta (DE3) pET28- <i>ctaB</i>	Strain contains pET28- <i>ctaB</i>	This study
<i>E. coli</i> Rosetta (DE3) pET28- <i>ctaE</i>	Strain contains pET28- <i>ctaE</i>	[12]
<i>E. coli</i> Rosetta (DE3) pET28- <i>ctaJ</i>	Strain contains pET28- <i>ctaJ</i>	This study
<i>E. coli</i> Rosetta (DE3) pET28- <i>ctaJ</i> _{C114A}	Strain contains pET28- <i>ctaJ</i> _{C114A}	This study
<i>E. coli</i> Rosetta (DE3) pET28- <i>ctaJ</i> _{H148A}	Strain contains pET28- <i>ctaJ</i> _{H148A}	This study
<i>E. coli</i> Rosetta (DE3) pET28- <i>ctaJ</i> _{D165A}	Strain contains pET28- <i>ctaJ</i> _{D165A}	This study
<i>E. coli</i> Rosetta (DE3) pET28- <i>ctaK</i>	Strain contains pET28- <i>ctaK</i>	This study

Table S2. Plasmids used in this study.

Plasmid	Description	Source
pTargetC	Clostridia-adapted sgRNA plasmid used as a staging vector for construction of appropriate sgRNA and homology arms	[2]
pCasC	Clostridia-adapted vector for CRISPR/Cas9 editing in <i>R. cellulolyticum</i> , the Cas9 protein has a D10A mutation to convert it into a nickase	[2]
pTargetCF- <i>ctaB</i>	pTargetC vector with N20 sequence for Ccel_3259 (<i>ctaB</i>)	This study
pTargetCF- <i>ctaK</i>	pTargetC vector with N20 sequence for Ccel_3250 (<i>ctaK</i>)	This study
pTargetCT- <i>ctaB</i>	pTargetCF vector with mutated homology arms for Ccel_3259 (<i>ctaB</i>)	This study
pTargetCT- <i>ctaK</i>	pTargetCF vector with mutated homology arms for Ccel_3250 (<i>ctaK</i>)	This study
pCasC- <i>ctaB</i>	CRISPR/Cas vector for Ccel_3259 (<i>ctaB</i>) inactivation	This study
pCasC- <i>ctaK</i>	CRISPR/Cas vector for Ccel_3250 (<i>ctaK</i>) inactivation	This study
pET28a	Expression vector for N-terminally His ₆ -tagged proteins	Novagen
pET28- <i>ctaB</i>	N-terminal His ₆ -tag on Ccel_3259	This study
pET28- <i>ctaE</i>	N-terminal His ₆ -tag on Ccel_3256	[12]
pET28- <i>ctaJ</i>	N-terminal His ₆ -tag on Ccel_3251	This study
pET28- <i>ctaJ</i> _{C114A}	C114A mutant of CtaJ	This study
pET28- <i>ctaJ</i> _{H148A}	H148A mutant of CtaJ	This study
pET28- <i>ctaJ</i> _{D165A}	D165A mutant of CtaJ	This study
pET28- <i>ctaK</i>	N-terminal His ₆ -tag on Ccel_3250	This study

Table S3. Oligonucleotide primers used in this study. Base pairs changed for gene mutagenesis are bolded.

Name	Sequence	Use
T7 Seq F	TAATACGACTCACTATAGGG	pET28a Sequencing
T7 Seq R	GCTAGTTATTGCTCAGCGG	pET28a Sequencing
pTargetC-Seq-F	CTTGCCAAGGTCATGATG	pTargetCF- <i>ctaB/K</i> sequencing
M13-R	CAGGAAACAGCTATGACC	pTargetCT- <i>ctaB/K</i> sequencing
M13-F	AGGGTTTTCCAGTCACGACGTT	pTargetCT- <i>ctaB/K</i> sequencing
pCasC-seq	AACAAGCCATGAAAACCG	pCasC- <i>ctaB/K</i> sequencing
pUC-ori-seq-R	CTACGGGGTCTGACGCTCAG	pCasC- <i>ctaB/K</i> sequencing
pCasC- <i>ctaB</i> -seq-F	AATAAAGCCATCAACCATTGGTCC	pCasC- <i>ctaB</i> sequencing
pCasC- <i>ctaB</i> -seq-R	TGGGGAGCACATACTGGAGA	pCasC- <i>ctaB</i> sequencing
pCasC- <i>ctaK</i> -seq-F	GGCAGTTAGTAGAGAATG	pCasC- <i>ctaK</i> sequencing
pCasC- <i>ctaK</i> -seq-R	AGGATTGTAATGCTTTATAAGCGCTATGG	pCasC- <i>ctaK</i> sequencing
pET28- <i>ctaK/B</i> -seq	CGTTTACGAAGGCATTGGC	pET28- <i>ctaK-ctaB</i> sequencing
Cas-3259-F	TTTGATAATGAGCCGCGTCG	Amplification of <i>ctaB</i> for CRISPR/Cas mutant verification
Cas-3259-R	TGGGGAGCACATACTGGAGA	Amplification of <i>ctaB</i> for CRISPR
Cas-3259-R2	ATGATGCAGATGAATAATTCCAC	Amplification of <i>ctaB</i> Cas mutant verification
Cas-3259-seq	AATAAAGCCATCAACCATTGGTCC	<i>ctaB</i> CRISPR/Cas mutant sequencing
Cas-3250-F	ATGAATATTGATAAAGCCGTTTTG	Amplification of <i>ctaK</i> for CRISPR/Cas mutant verification
Cas-3250-R	TTACAAAGAAGCAATTTCTTGATTC	Amplification of <i>ctaK</i> for CRISPR/Cas mutant verification
Cas-3250-seq	AGGATTGTAATGCTTTATAAGCGCTATGG	<i>ctaK</i> CRISPR/Cas mutant sequencing
sgRNA-R	GACGTCGACTCTAGAGAA	pTargetCF- <i>ctaB/K</i> cloning
sgRNA- <i>ctaB</i>	TTTAAAGTTAAATTAAGTTGAGCTATTAAGACACAGATTGGTTTTAGAGCTAGAAATAGC	pTargetCF- <i>ctaB</i> cloning
sgRNA- <i>ctaK</i>	TTTAAAGTTAAATTAAGTTGTGACGACTTGGTACGAGCATGTTTTAGAGCTAGAAATAGC	pTargetCF- <i>ctaK</i> cloning
Cc-3259-Front-F	TTTTGAATTCTCTAGAGTCGACTTTTGATAGTATGTAAGC	pTargetCT- <i>ctaB</i> cloning
Cc-3259-Front-R	TAAAGTAAGATATCGAGGAACTACCGTATATTAATC	pTargetCT- <i>ctaB</i> cloning
Cc-3259-Back-F	TTCCTCGATATCTTACTTAATAGCTTTCTTAATATC	pTargetCT- <i>ctaB</i> cloning
Cc-3259-Back-R	CAAGCTTGCATGTCTGCAGGCCTGTTAAAAGAAAAGATGTA	pTargetCT- <i>ctaB</i> cloning
Cc-3250-Front-F	TTTTGAATTCTCTAGAGTCGACATTCACTGAACAATCTTC	pTargetCT- <i>ctaK</i> cloning
Cc-3250-Front-R	CGACTTGTAAGATATCTTGGATACTAACTCTTCG	pTargetCT- <i>ctaK</i> cloning
Cc-3250-Back-F	CCAAGATATCTTACAAGTCGTCACCCTGCATATG	pTargetCT- <i>ctaK</i> cloning
Cc-3250-Back-R	CAAGCTTGCATGTCTGCAGGCCTGTCAACAAGTACATATACA	pTargetCT- <i>ctaK</i> cloning
TargetCT-F	GCAATGATACCGCGAGACCATATGCTGGATCCTTGAC	pCasC- <i>ctaB/K</i> cloning
TargetCT-R	GGTTTCGGTCTCCACGCAGCTTGCATGTCTGCAGGCC	pCasC- <i>ctaB/K</i> cloning
Cc-3259-F	AAAGCTAGCATGGGGAGCACATACTGGAG	pET28- <i>ctaB</i> cloning
Cc-3259-R	TTTGATCCTTAGATAACCACTACTATTGCTTATAGCTGTTTTAAC	pET28- <i>ctaB</i> cloning
Cc-3251-F	AAAGCTAGCATGAATATTGATAAAGCCGTTTTG	pET28- <i>ctaJ</i> cloning
Cc-3251-R	TTTGATCCTTATCCGTTTGTAGATTCAAATAG	pET28- <i>ctaJ</i> cloning
CtaJ-C114A-F	GTAATATCC G CTAGACACATAACTGTTATATTAGGGG	pET28- <i>ctaJ</i> mutagenesis

Name	Sequence	Use
CtaJ-C114A-R	GTGTCTAG CG GGATATTACAATCCTCTCCTCAAC	pET28- <i>ctaJ</i> mutagenesis
CtaJ-H148A-F	GTAGAT GCT TTGGATTAAACCAATACTGGAGTGAAAAAG	pET28- <i>ctaJ</i> mutagenesis
CtaJ-H148A-R	GTTAATCCA AGC ATCTACAGGCTTACCATGATATGC	pET28- <i>ctaJ</i> mutagenesis
CtaJ-D165A-F	GTTAATTTTG CT GGGGAAGGAATAATGTATCG	pET28- <i>ctaJ</i> mutagenesis
CtaJ-D165A-R	CTTCCC CAG CAAATAACCCATCTGTTTTTC	pET28- <i>ctaJ</i> mutagenesis
Cc-3250-F	AAAGCTAGCATGCTTTATAAGCGCTATGG	pET28- <i>ctaK</i> cloning
Cc-3250-R	TTTGGATCCTTACAAAGAAGCAATTTCTTGATTC	pET28- <i>ctaK</i> cloning

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