Supporting Information for

Nonenzymatic Carbamoylation and Phosphorylation via Urea Nitrosylation under Mild Aqueous Conditions

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1. Materials and Methods

1.1. Chemicals

Copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$, 99.0%), sodium sulfide pentahydrate ($Na_2S \cdot 5H_2O$, 98.0%), copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, 99.5%), sodium L(+)-aspartate monohydrate (97.0+%), sodium nitrite ($NaNO_2$, 98.5%), 3-(N-morpholino)propanesulfonic acid (MOPS, 99.0%), maleic acid (98.0+%), ammonium acetate (97.0+%), ammonium hydroxide (NH_4OH, 25%), sodium hydrogen carbonate ($NaHCO_3$, 99.5%), acetonitrile (LC-MS grade), p-toluenesulfonic acid monohydrate (99.0%), 2-aminoethanol (99.0%), calcium chloride ($CaCl_2$, 95.0%), 3-(trimethylsilyl)-1-propanesulfonic acid-d_6 sodium salt (DSS-d_6, 99.0+%), and D_2O (99.8%) were purchased from Fujifilm Wako. ^{13}C-labeled urea (99 atom%^{13}C), sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$, >99.0%), sodium phosphate dibasic (Na_2HPO_4, 99.0%), ethylenediaminetetraacetic acid (EDTA, 99.995%), carbamyl phosphate disodium salt (>80%), Chelex® 100 resin (50-100 mesh, sodium form), uridine (≥99%), uridine 5'-monophosphate disodium salt (5'-UMP, ≥99%) were purchased from Sigma-Aldrich. Uridine-3'-monophosphate disodium salt (3'-UMP, 99.8%) was purchased from CHEM-INPEX. Urea (>99.0%), 1-Methylurea (>98.0%) hexamethylphosphoric triamide (HMPA, ≥ 98.0%) were purchased from Tokyo Chemical Industry. 15N-labeled sodium nitrite ($Na^{15}NO_2$, 98%) was purchased from Cambridge Isotope Laboratories (CIL, USA). Nitrous oxide standard gas (N_2O, 99.5%, 0.8MPa) was purchased from GL Science.

1.2. Preparation of CuS

Copper sulfide (CuS) was prepared by adding 100 mM of Na_2S dropwise to 100 mM of $CuCl_2$ under vigorous stirring until the Cu:S mixing ratio reached 1:1. The resulting precipitate was separated from the supernatant solution by centrifugation (8000 rpm, 10 min) and dried under vacuum. To avoid oxidation by atmospheric oxygen (O_2), the CuS precipitation was carried out in an anaerobic chamber filled with argon (Ar) and hydrogen (H_2) gases (the volumetric ratio = 96:4).

1.3. Characterization of CuS

XRD patterns of CuS were measured using a Rigaku Smart Lab-SPI/TISM diffractometer with Cu Kα radiation (λ= 1.5418 angstrom) at a scan rate of $1°min^{-1}$ in steps of 0.02°. The obtained XRD data were analyzed using the Rigaku data analysis software PDXL. Peak identification was performed based on the reference patterns reported in the Power Diffraction File, which was published by the International Centre for Diffraction Data.

Raman spectra of CuS were collected on a Raman microscope system (Nanophoton, Raman touch) using an excitation wavelength of 532 nm from a laser source with a 300 gr mm^{-1} grating and a ×20 objective (Nikon, TU Plan Fluor 20×, NA = 0.45). The laser

intensity was adjusted to 2.79 mW. Twenty spectra were co-added with an integration time of 10 s.

The microscopic morphology and elemental composition of CuS were assessed using a scanning electron microscope (SEM) equipped with energy dispersive X-ray spectroscopy (EDS) (Hitachi High-Tech, S3400-N). An acceleration voltage of 15 kV was applied. Point analysis was conducted at ×3700 magnification.

1.4. Synthesis of carbamoyl phosphate

In a typical experiment, a phosphate-buffered aqueous solution (400 mM, pH 6) dissolving 100 mM of ^{13}C-labeled urea and 400 mM of $NaNO_2$ was prepared. After purging with Ar gas for over 20 min, 3 mL of the sample solution was pipetted into a serum bottle (5 mL) containing 60 mg of CuS. The bottle was then sealed with a butyl rubber cap and an aluminum stopper in an anaerobic chamber filled with Ar and H_2 gases. After gently rotating the bottle using a rotator (VMR-5R, AS ONE) at room temperature (25 °C), the sample suspension was filtered using a polytetrafluoroethylene (PTFE) membrane filter (pore size = 0.22 μm) and analyzed using nuclear magnetic resonance (NMR) spectroscopy.

1.5. NMR measurement of carbamoyl phosphate

^{13}C and ^{31}P spectra were obtained using a Bruker AvanceIII spectrometer at 400.1 MHz and 100.6 MHz, respectively, at a sample temperature of 300.5 K. In ^{13}C measurement, typically, 540 μL of sample solutions was mixed with 60 μL of D_2O containing 50 mM DSS-d_6 and placed in an NMR tube (5 mm outside diameter; Fujifilm Wako). DSS-d_6 was used for the calibration of the 0 ppm position. ^{31}P NMR measurements were conducted following procedure written in O. Maguire et al. 2021.[1] In ^{31}P NMR measurement, 480 μL of sample solutions mixed with 60 μL of D_2O and 60 μL of 500 mM $HMPA_{aq}$ and placed in an NMR tube. HMPA was used for the calibration of the 29.85 ppm position. D1 delay of 30.0 s and acquisition time of 1.5 s were used in order to guarantee the quantitative ^{31}P NMR measurement. The NMR peaks were assigned based on those of standard samples at identical pH.

1.6. Analysis of CO_2

Total amount of CO_2 in samples was quantified by a Shimadzu HPLC system equipped with an electric conductivity detector and an anion exchange column (Shim-pack SCR-102H, Shimadzu) set at 45°C. The p-toluenesulfonic acid aqueous solution (2 mM) was used as an eluent at a rate of 0.8 ml min^{-1}. The solution containing p-toluensulfonic acid (2 mM), EDTA (0.1 mM), 2-aminoethanol (8 mM) was used as an reaction solution of separated eluent for detecting CO_2 in sample solution.

1.7. Urea nitrosylation experiment

In a typical experiment, a phosphate-buffered aqueous solution (400 mM, pH 7) dissolving 100 mM of urea and 100 mM of ^{15}N-labeled $NaNO_2$ was prepared. After purging with Ar gas for over 30 min, 10 mL of the sample solution was pipetted into a serum bottle (15 mL) containing 100 mg of CuS. The bottle was then sealed with a butyl rubber cap and an aluminum stopper in an anaerobic chamber filled with Ar and H_2 gases. After gently rotating the bottle using a rotator (VMR-5R, AS ONE) at room temperature (25 °C), the headspace gas was analyzed using a gas chromatograph mass spectrometer (GCMS-TQ8030, Shimadzu).

1.8. Analysis of nitrogenous gas products

The identification and quantification of ^{14}NO, ^{15}NO, $^{28}N_2O$, $^{29}N_2O$, $^{30}N_2O$, $^{29}N_2$ and $^{30}N_2$ were performed using a Shimadzu GC-MS spectrometer (GCMS-TQ8030) as following previous methods.[2] Helium (He) gas with a purity of 99.99995% was used as the carrier gas at a pressure of 80 kPa with a total flow rate of 22.4 mL min^{-1}. Split injection mode was applied with a split ratio of 20 and an injection temperature of 100 °C. Restek's Rt-Msieve 5A capillary column (30 m × 0.32 mm inner diameter × 30 μm film thickness) was used at an oven temperature of 180 °C. The ion source and interface temperatures were set to 200 °C. The detector voltage of the mass spectrometer was fixed at 0.8 kV. The obtained data were analyzed using GC–MS post-run analysis software with commercial MS libraries.

1.9. Quantification of Cu^{2+} dissolved during the urea nitrosylation experiments

In a typical experiment, 60 mg of CuS was placed in a serum bottle containing 3 mL of a MOPS buffer (200 mM, pH 6) with 100 mM of urea and 400 mM of $NaNO_2$. After gently rotating the bottle at 25 °C for a given reaction period (0–16 days), the suspension was filtered through a polytetrafluoroethylene (PTFE) membrane syringe filter (0.22 μm). This was followed by the addition of aspartate (final concentration = 10 mM) to form a Cu^{2+}-aspartate complex, and subsequent mixing with Milli-Q water to achieve a tenfold dilution. The resultant complex was analyzed with a UV-Vis spectrometer (Jasco V670 spectrophotometer) to quantify the dissolved concentration of Cu^{2+} released from CuS. Absorption photometric mode was applied with a scan speed of 400 nm min^{-1} within the range of ~200-800 nm. Dissolved Cu^{2+} was quantified as the Cu^{2+}-aspartate complex using a calibration curve constructed from $CuSO_4$ standards (0-10 mM) prepared under the same matrix conditions with 10 mM L-aspartate. The absorbance at a wavelength of 641 nm was used to determine the Cu^{2+} concentration.

1.10. Nucleoside phosphorylation experiment

In a typical experiment, a phosphate-buffered aqueous solution (400 mM, pH 6) dissolving 100 mM of urea, 200 mM of $NaNO_2$ and 100 mM of uridine was prepared.

After purging with Ar gas for over 20 min, 3 mL of the sample solution was pipetted into a serum bottle (5 mL) containing 60 mg of CuS. The bottle was then sealed with a butyl rubber cap and an aluminum stopper in an anaerobic chamber filled with Ar and H_2 gases. After gently rotating the bottle using a rotator (VMR-5R, AS ONE) at room temperature (25 °C), the sample suspension was filtrated using a PTFE membrane filter, mixed with a cation exchange resin (Chelex® 100 Na form; Sigma Aldrich) for removing metal cations, filtered again, and analyzed using electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-QTOF-MS).

1.11. Analysis of UMP

The identification and quantification of UMP were performed based on a protocol developed by Tagawa et al. 2024.[3] Briefly, 30 µL of the sample solution was mixed with 1.0 mL of an aqueous solution of $CaCl_2$ (21.6 mM), 1.93 mL of MQ water, and 40 µL of NH_4OH_{aq} for removing phosphate. After leaving mixed sample for overnight under 4 °C, the resulting white precipitate was separated from the supernatant solution by centrifugation (3000 rpm, 10 min) and 3 µL of filtered supernatant solution was injected into a mass spectrometer (Xevo-Q-TOF-MS, Waters) attached to a Waters ACQUITY UPLC H-Class PLUS system (Waters Corp., MA, USA) via direct infusion at a flow rate of 0.30 ml min^{-1}. All chromatographic separations were conducted on an Atlantis Premier BEH C18 AX Column (2.1 × 100 mm, 1.7 µm). Running buffers included A (10 mM ammonium acetate in H_2O at pH9.2) and B (H_2O/Acetonitrile (10/90)). The gradient was 0 min, 100% of A; 0 → 4 min, 90% of A; 4 → 6 min, 0% of A; 6 → 7 min, 0% of A; 7 → 8 min, 100% of A; 8 → 10 min, 100% of A. The TOF analyzer was set to the sensitivity mode with a resolving power of 22,000, and the *m/z* range of 50–1200 was calibrated using leucine-enkephalin. The following conditions were applied: ionization mode (ESI negative), source temperature (150 °C), desolvation temperature (400 °C), cone gas flow (1.7 L min^{-1}), flow rate of desolvation gas (11.7 L min^{-1}), and capillary voltage (3.0 kV). The obtained data were analyzed using MestReNova software, version 1.50.1621.

1.12. Synthesis of N-carbamoyl aspartate (NCA) from L-aspartate (Asp) and urea

In a typical experiment, a MOPS-buffered aqueous solution (200 mM, pH 7) dissolving 200 mM of urea and 100 mM of $NaNO_2$ was prepared. After purging with Ar gas for 30 min, 5 mL of the sample solution was pipetted into a serum bottle (10 mL) containing 100 mg of CuS. The bottle was then sealed with a butyl rubber cap and an aluminum stopper in an anaerobic chamber filled with Ar and H_2 gases and rotated at 60 rpm using a rotator (VMR-5R, AS ONE) at room temperature (25 °C) for 8 days. The sample suspension was filtrated using a PTFE membrane filter, mixed with a cation exchange resin (Chelex® 100 Na form; Sigma Aldrich) for removing metal cations, filtered again, adjusted to a pH of 8 by adding $NaHCO_3$, and analyzed using ESI-QTOF-MS.

1.13. Analysis of NCA

The identification and quantification of NCA were performed based on a protocol developed by Yi et al.[4]. Briefly, 1.6 mL of the sample solution was mixed with 0.4 mL of an aqueous solution of maleic acid (10 mM). 5 µL of this mixture was injected into a mass spectrometer (Xevo-Q-TOF-MS, Waters) via direct infusion at a flow rate of 0.35 ml min^{-1}. All chromatographic separations were conducted on an ACQUITY UPLC® BEH Amide Column (2.1 × 150 mm, 1.7 µm), with a flow rate of 0.35 ml/min. Running buffer included A (10 mM ammonium acetate in H_2O at pH9.2) and B (10 mM ammonium acetate in H_2O/Acetonitrile (10/90) at pH9.2) and pH of both phases were adjusted by adding NH_4OH solution. The gradient was 0 min, 5% of A; 0 → 2 min, 13% of A; 2 → 14.5 min, 13% of A; 14.5 → 16.5 min, 95% of A; 16.5 → 18 min, 5% of A; 18 → 20 min, 5% of A. The TOF analyzer was set to the sensitivity mode with a resolving power of 22,000, and the *m*/*z* range of 50–1200 was calibrated using leucine-enkephalin. The following conditions were applied: ionization mode (ESI negative), source temperature (150 °C), desolvation temperature (400 °C), cone gas flow (1.7 L min^{-1}), flow rate of desolvation gas (11.7 L min^{-1}), and capillary voltage (3.0 kV). The obtained data were analyzed using MestReNova software, version 1.50.1621.

1.14. NMR measurement of NCA

^{1}H and ^{13}C spectra were obtained using a Bruker AvanceIII spectrometer at 400.1 MHz and 100.6 MHz, respectively, at a sample temperature of 300.5 K. In ^{1}H and ^{13}C NMR measurement, typically, 540 µL of sample solutions was mixed with 60 µL of D_2O containing 50 mM DSS-d_6 and placed in an NMR tube (5 mm outside diameter; Fujifilm Wako). DSS-d_6 was used for the calibration of the 0 ppm position. During the ^{1}H-NMR measurement, a solvent suppression run was performed to minimize the HOD signal derived from D_2O. The NMR peaks were assigned based on those of standard samples at identical pH.

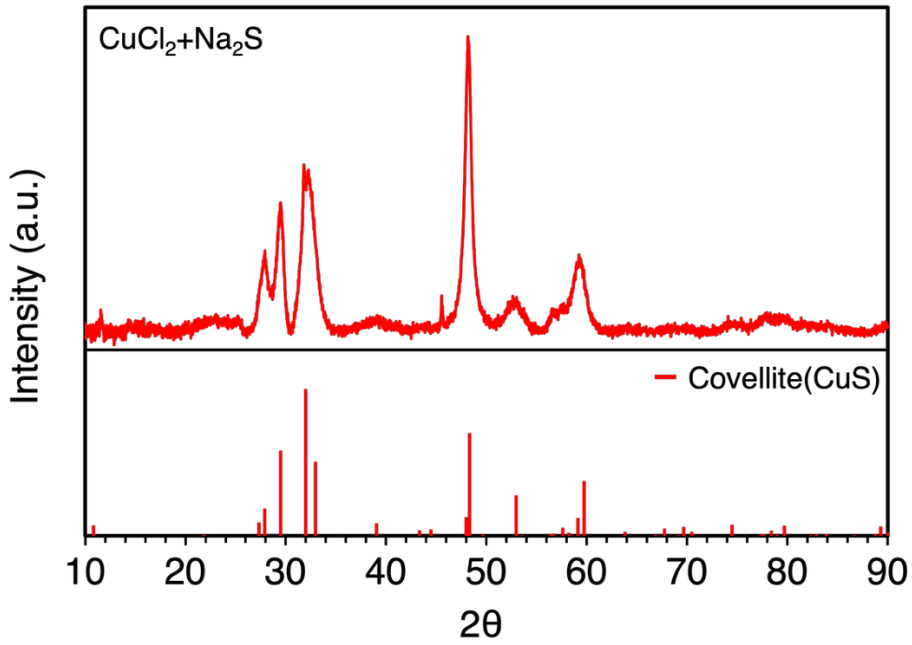

Figure. S1. XRD pattern of CuS prepared in this study. This sample was identified as a hexagonal phase CuS based on the reference pattern for synthetic covellite (JCPDS No. 79-2321) with a space group of $P6_3/mmc$ ($a = b = 3.792$ Å, $c = 16.344$ Å; α = β = 90°, γ = 120°).

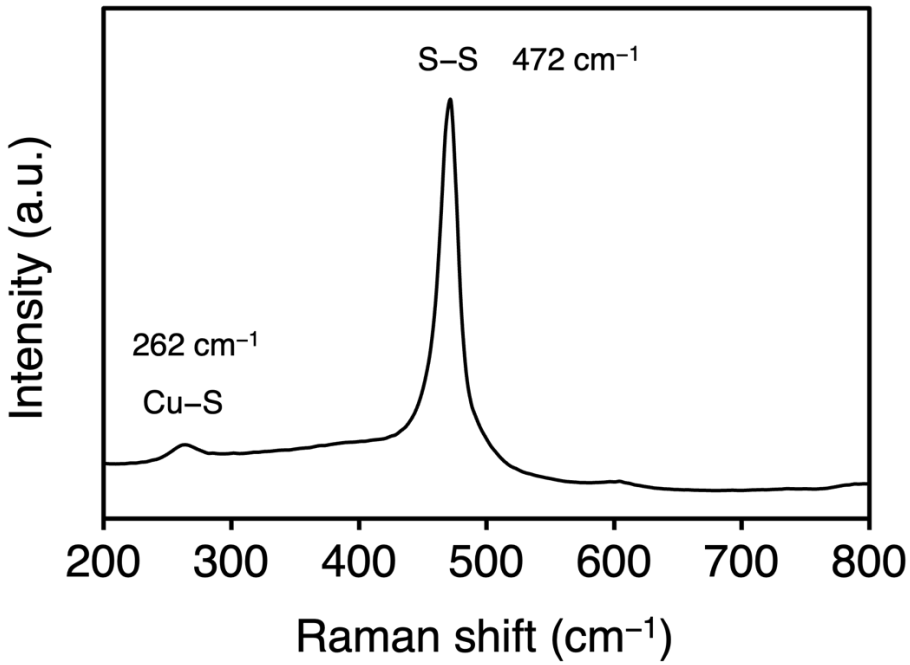

Figure. S2. Raman spectrum of CuS prepared in this study. The signals centered at 262 and 472 cm^{-1} were attributed to the Cu-S and S-S phonon modes, respectively.

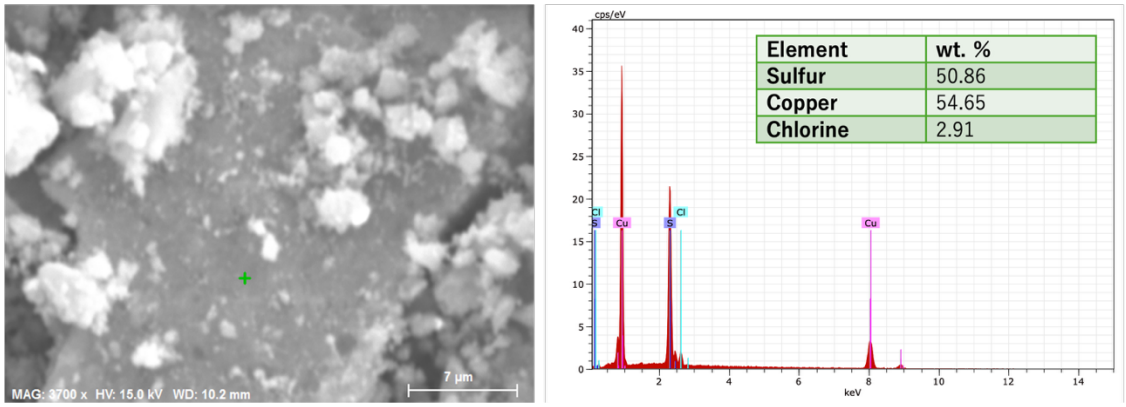

Figure. S3. SEM image (left) and the EDS spectrum (right) of CuS prepared in this study. The green dot in the SEM image shows where the EDS spectrum was measured.

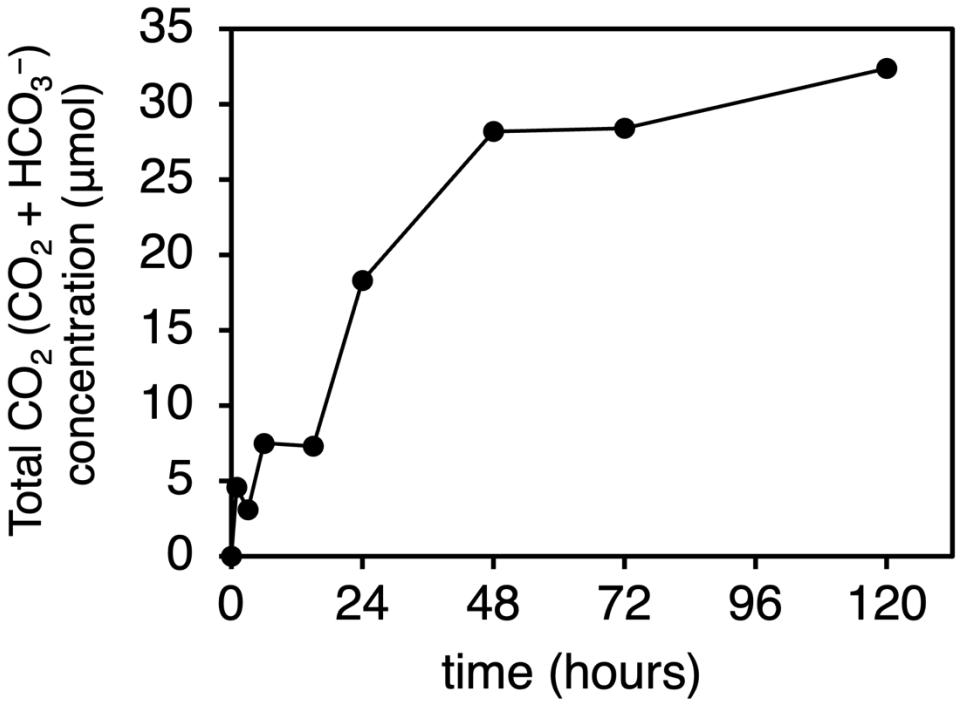

Figure S4. Concentration of CO_2 formed through the CP synthesis experiment as determined by a base-treatment of the sample products (see "**1.6. Analysis of CO_2**").

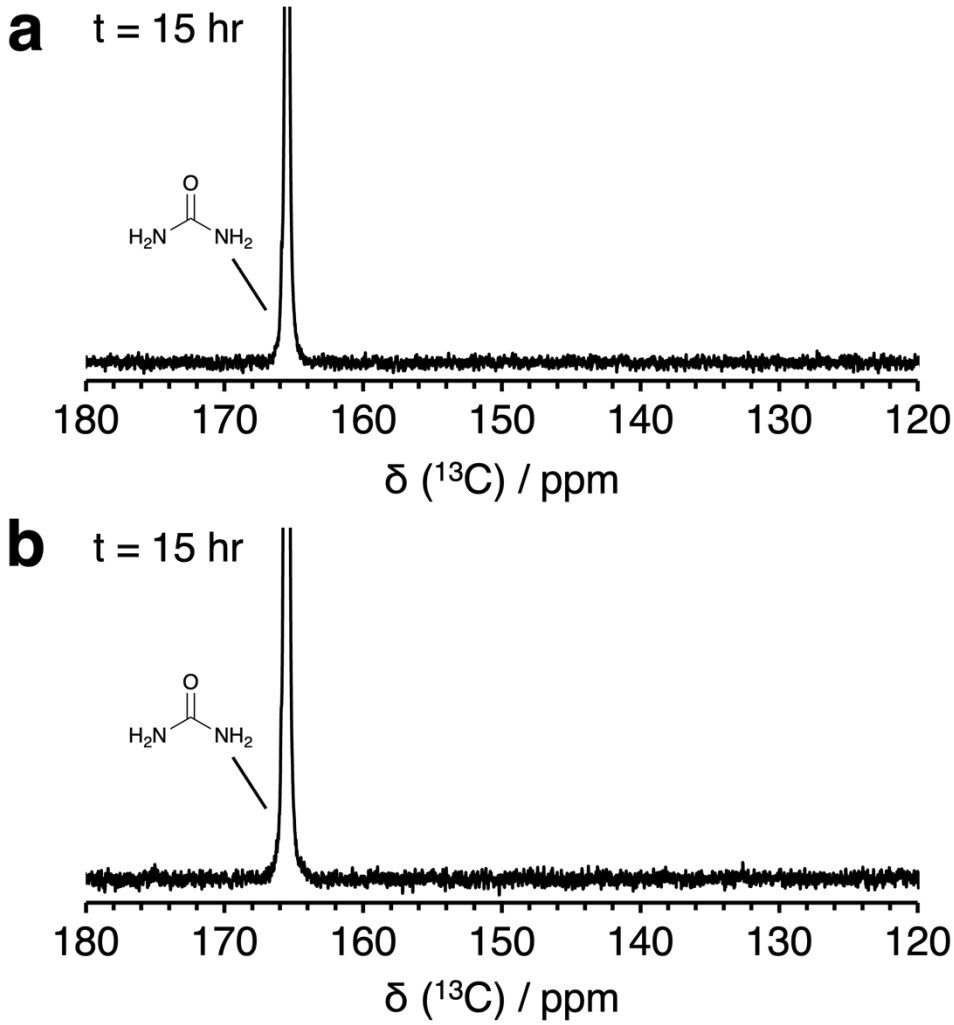

Figure S5. ^{13}C NMR spectra of sample aqueous solutions after the CP synthesis experiment without (a) CuS or (b) NO_2^-. These experiments were performed as described in "**1.4. Synthesis of carbamoyl phosphate"** with either CuS or NO_2^- removed from the starting materials.

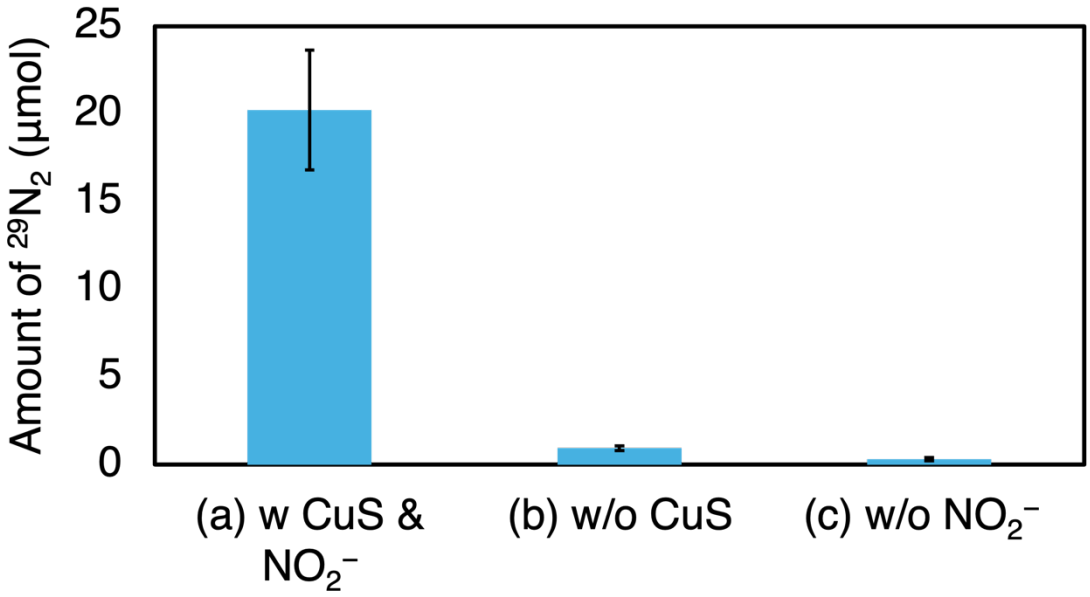

Figure S6. Amount of $^{29}N_2$ formed through the 8-day urea nitrosylation experiment (a) with CuS & NO_2^-, in the absence of (b) CuS or (c) NO_2^-. The error bars were determined based on three independent runs under identical reaction conditions.

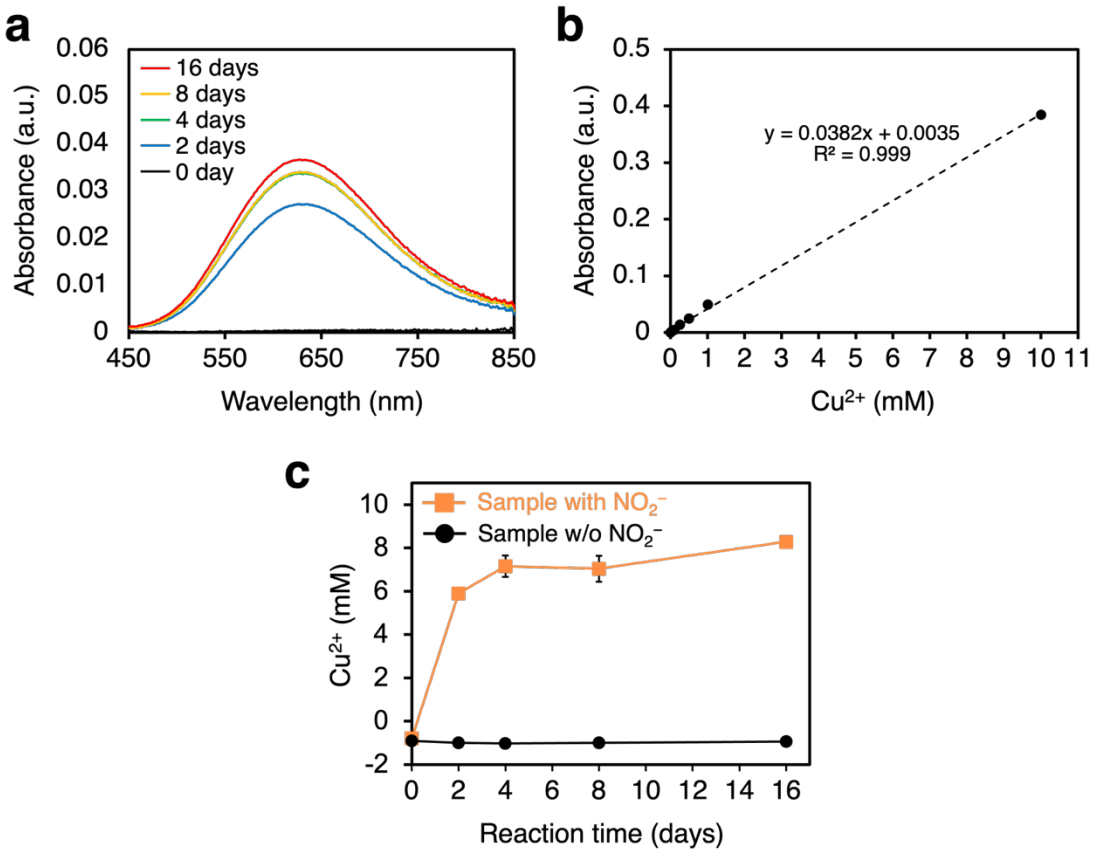

Figure S7. UV–vis assay for soluble Cu(II) released from CuS in the presence of nitrite. **(a)** UV–vis spectra of the filtrates after chelation with aspartate (final 10 mM), showing a broad Cu(II)–aspartate absorption band at 641 nm. **(b)** Calibration curve of A_{641} versus Cu(II) concentration for Cu(II)–aspartate standards (0-10 mM $CuSO_4$) prepared in the identical matrix (MOPS buffer containing urea and $NaNO_2$). **(c)** Time course of dissolved Cu(II) quantified as the Cu(II)–aspartate complex using A_{641}. Orange squares: with $NaNO_2$ (400 mM); black circles: without $NaNO_2$. Reaction conditions: CuS (60 mg) in MOPS buffer (200 mM, pH 6.0) containing urea (100 mM) at 25 °C. The error bars were determined based on two independent runs under identical reaction conditions.

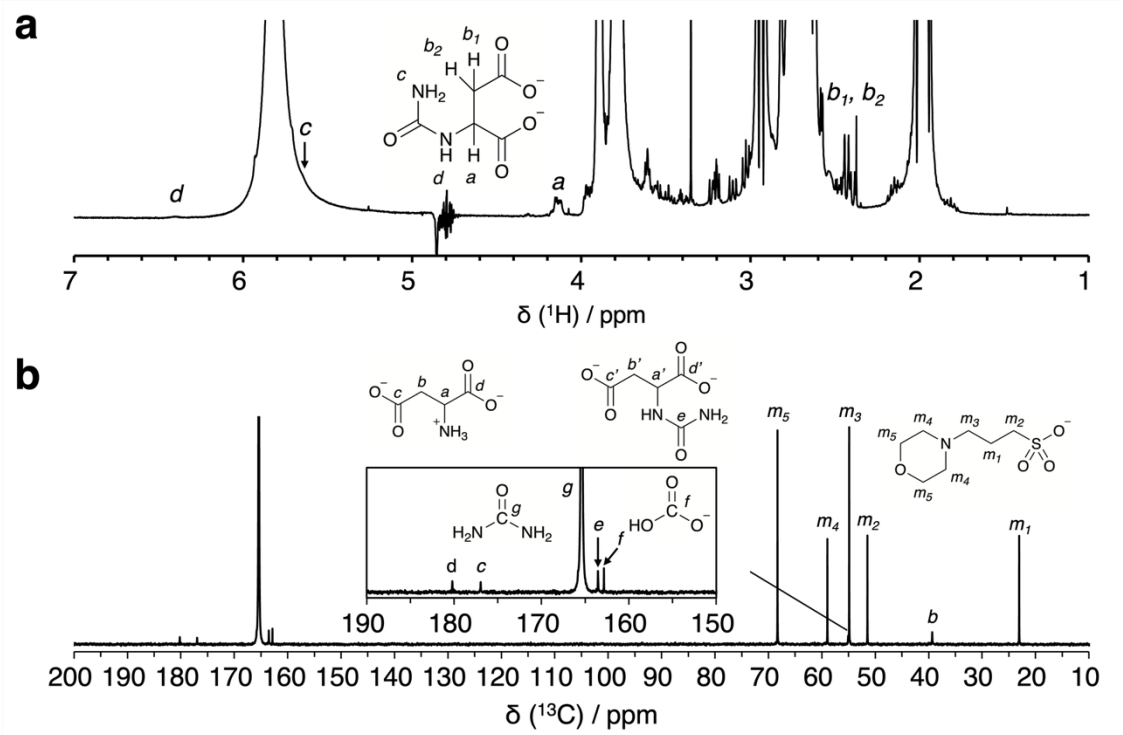

Figure S8. (a) ^{1}H- and (b) ^{13}C-NMR spectra showing Asp carbamoylation in the urea nitrosylation system at 25 °C and pH 7. A 32-day experiment was performed for the ^{1}H-NMR measurement, whereas the ^{13}C-NMR spectra characterize the NCA synthesis through the 16-day experiment using ^{13}C-Urea.

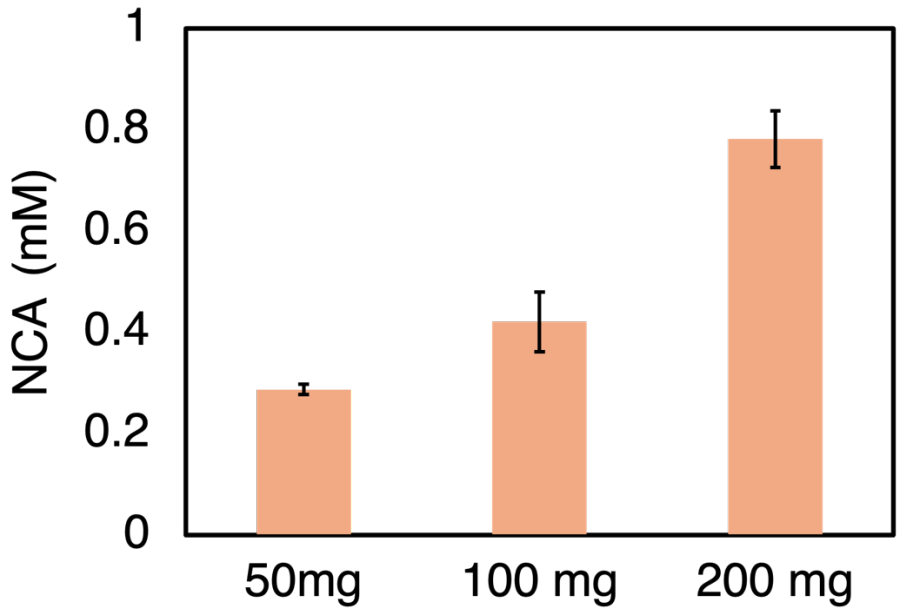

Figure S9. Concentration of NCA after a 4-day Asp carbamoylation experiment in the presence of 50, 100, and 200 mg CuS. See **Section 1.12** for experimental details. The error bars were determined based on three independent runs under identical reaction conditions.

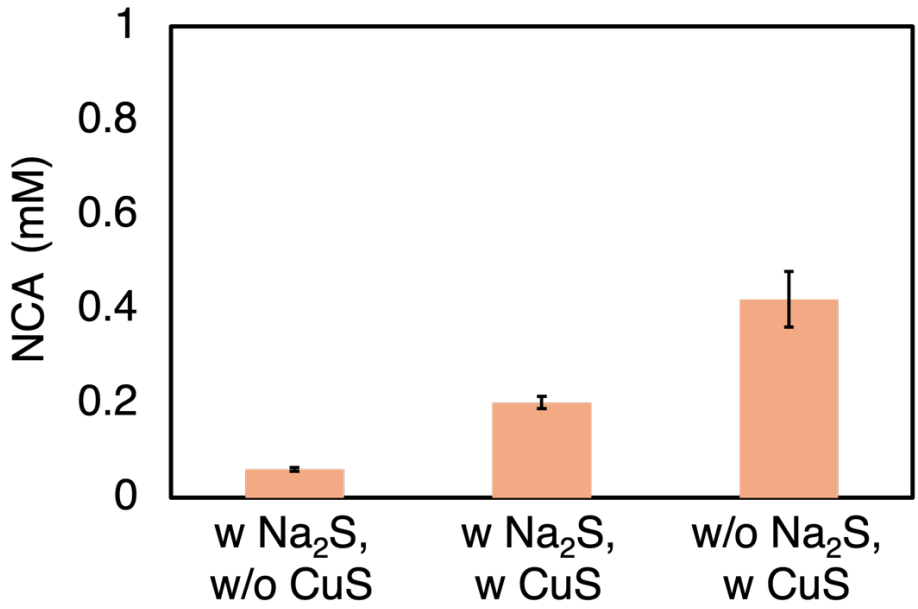

Figure S10. Concentration of NCA formed through a 4-day Asp carbamoylation experiment in the presence of either 10 mM Na_2S or 100 mg CuS, or both. See **Section 1.12** for experimental details. The error bars were determined based on three independent runs under identical reaction conditions.

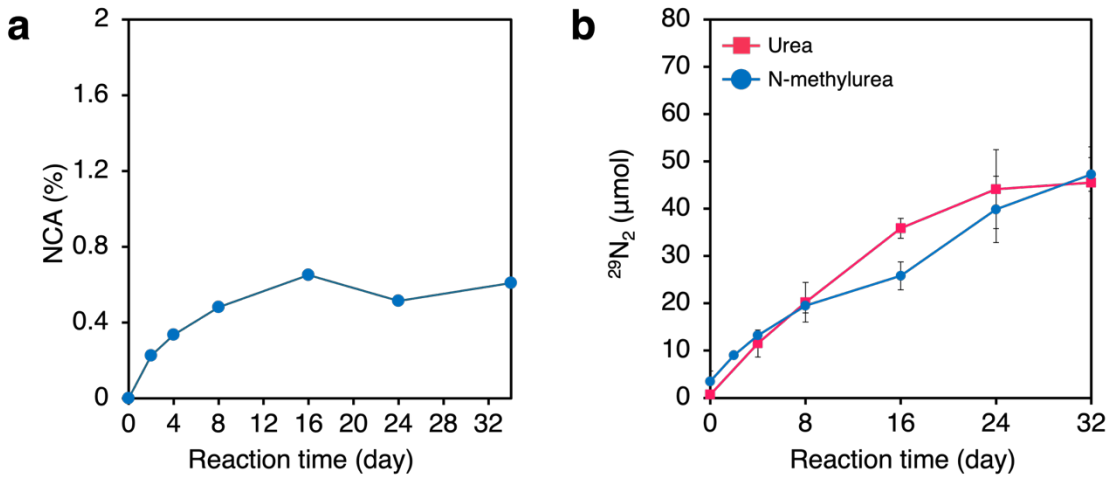

Figure S11. Yields of (a) NCA formed through the Asp carbamoylation experiment. Amount of (b) $^{29}N_2$ in nitrosylation experiment. In (a), 100 mM N-methylurea was used instead of urea as the carbamoylating agent under the otherwise identical condition to that described in **Section 1.12**. (b) compares the amount of $^{29}N_2$ formed in the presence of N-methylurea or urea. In (b), 100 mM N-methylurea was used instead of urea under the otherwise identical condition to that described in **Section 1.7**.

References

1. O. R. Maguire, I. Smokers and W. Huck, *Nat. Commun.*, 2021, ***12***.
2. D. He, K. Adachi, D. Hashizume and R. Nakamura, *Nat. Chem.*, 2024, ***16***, 1605-1611.
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4. J. Yi, H. Kaur, W. Kazöne, S. Rauscher, L. Gravillier, K. Muchowska and J. Moran, *Angew. Chem. Int. Ed.*, 2022. ***61***, e202117211.