

# Supporting Information

## Genetically Anchored Fluorescent Probes for Subcellular Specific Imaging of Hydrogen Sulfide

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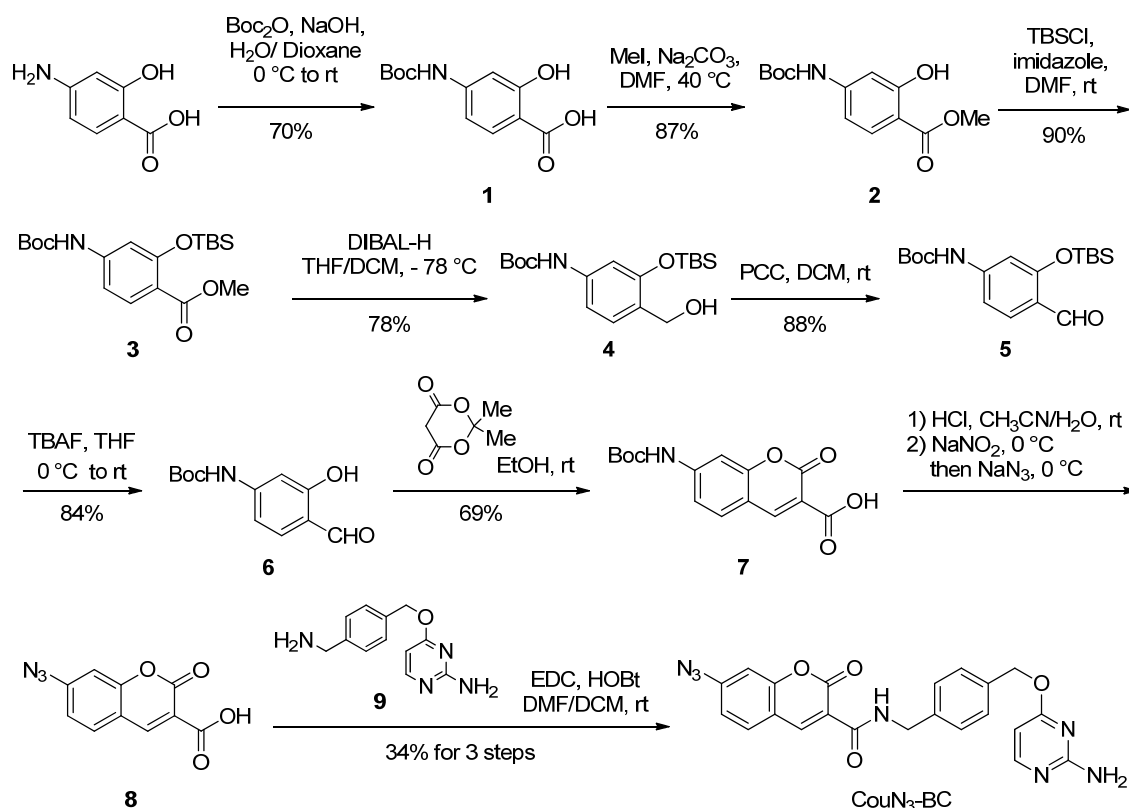
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## Materials and Methods:

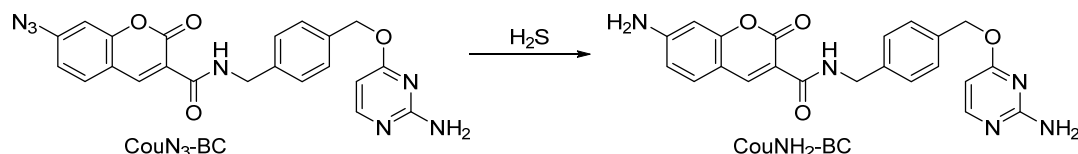
**Materials:** All the chemicals were purchased from Sigma-Aldrich and Alfa Aesar unless otherwise specified. Tetrahydrofuran (THF) was distilled over sodium benzophenone ketyl and  $\text{CH}_2\text{Cl}_2$  was distilled over phosphorus pentoxide. All the other solvents and reagents were used as received without further purification.

The mitochondria specific primary antibody anti-COX IV (Cell Signaling, 1:200) was used. The fluorescent secondary antibody donkey anti-Rabbit (Alexa Fluor® 488, Jackson Laboratory, 1:200) was used. The nuclear stain used was DAPI or NucRed® Live 647 ReadyProbes® Reagent (Life Technologies). Lipofectamine® 3000 was from Life Technologies.

**Instrumentation:** NMR spectra were recorded on a Varian NMR ( $^1\text{H}$  at 400 MHz and  $^{13}\text{C}$  at 101 MHz) spectrometer. Chemical shifts ( $\delta$ ) were given in ppm with reference to solvent signals [ $^1\text{H}$  NMR:  $\text{CDCl}_3$  (7.26),  $\text{CD}_3\text{OD}$  (3.31),  $\text{DMSO}-d_6$  (2.50);  $^{13}\text{C}$  NMR:  $\text{CDCl}_3$  (77.0),  $\text{DMSO}-d_6$  (40.0)]. UV-Vis measurements were performed in 10×10 mm quartz cuvettes with a Cary 60 UV-Vis Spectrometer. Fluorescence measurements were performed in 10×10 mm quartz cuvettes with a Cary Eclipse fluorescence spectrophotometer. Flash chromatography was performed on a Teledyne ISCO CombiFlash Rf 200. ESI mass spectrometry was measured on an Agilent Mass Spectrometer (6130 single quad).



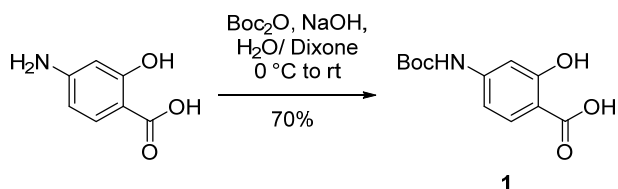
**Scheme S1** Synthetic route for CouN<sub>3</sub>-BC.



**Scheme S2.** Reaction between CouN<sub>3</sub>-BC and  $\text{H}_2\text{S}$ .

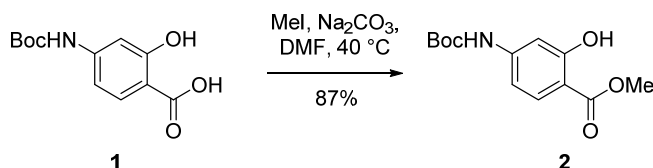
Compounds **1**<sup>S1</sup> and **9**<sup>S2</sup> were synthesized according to literature.

### Synthesis of compound 1



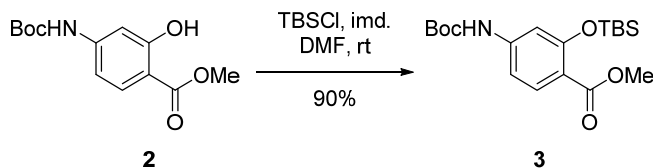
To a solution of 4-amino-2-hydroxybenzoic acid (3.06 g, 20 mmol) in 1 M NaOH (20 mL) was added di-*tert*-butyl dicarbonate (8.73 g, 40 mmol) in dioxane (30 mL) under  $0\text{ }^\circ\text{C}$ . The mixture was stirred at room temperature for 4 h. Then the reaction solution was acidified with a dilute solution of  $\text{KHSO}_4$  to pH 2 – 3 and extracted with ethyl acetate. The organic layer was washed with brine and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure. The residue was recrystallized in dichloromethane (DCM) to afford **1** (3.54 g, 70%).  $^1\text{H}$  NMR (400 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  9.16 (s, 1H), 7.70 (d,  $J$  = 8.4 Hz, 1H), 7.11 (d,  $J$  = 2.0 Hz, 1H), 6.87 (dd,  $J$  = 8.4, 2.0 Hz, 1H), 1.50 (s, 9H).

### Synthesis of compound 2



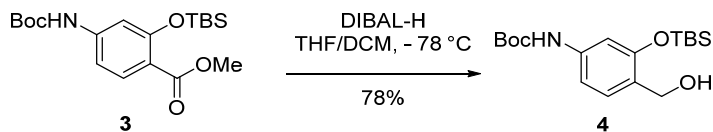
To the solution of compound **1** (1.66 g, 6.55 mmol) in dry dimethylformamide (DMF, 10 mL) was added  $\text{Na}_2\text{CO}_3$  (0.694 g, 6.55 mmol) and stirred at room temperature for 10 minutes. Then methyl iodide (0.62 mL, 9.83 mmol) was added, and the mixture was stirred at  $40\text{ }^\circ\text{C}$  overnight. To the mixture was added water (30 mL) and extracted with ethyl acetate. The organic layer was washed with saturated sodium bicarbonate solution, brine and dried over anhydrous sodium sulfate, then filtered and concentrated under reduced pressure. The residue was purified by flash chromatography using (hexane/ethyl acetate = 4:1 to 3:1) to afford compound **2** (1.52 g, 87%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  10.82 (s, 1H), 7.73 (d,  $J$  = 8.4 Hz, 1H), 6.99 (d,  $J$  = 1.6 Hz, 1H), 6.92 (dd,  $J$  = 8.8, 1.6 Hz, 1H), 6.65 (br s, 1H), 3.91 (s, 3H), 1.51 (s, 9H).

### Synthesis of compound 3



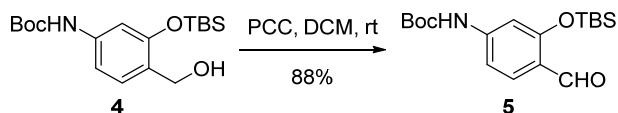
To the solution of compound **2** (500 mg, 1.87 mmol) in dry DMF (9 mL) were added imidazole (191 mg, 2.81 mmol) and TBSCl (366 mg, 2.43 mmol). The mixture was stirred at room temperature for overnight. Then to the mixture was added water (30 mL) and extracted with ethyl acetate. The organic layer was washed with brine and dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography using (hexane/ethyl acetate = 10:1 to 4:1) to afford compound **3** (590 mg, 90%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.75 (d,  $J$  = 8.4 Hz, 1H), 7.03 (d,  $J$  = 2.0 Hz, 1H), 6.92 (dd,  $J$  = 8.4, 2.0 Hz, 1H), 6.56 (br s, 1H), 3.83 (s, 3H), 1.51 (s, 9H), 1.01 (s, 9H), 0.23 (s, 6H). MS (ESI):  $m/z$  382.2  $[\text{M}+\text{H}]^+$

### Synthesis of compound 4



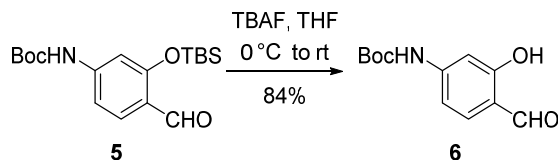
To a solution of compound **3** (590 mg, 1.55 mmol) in DCM (10 mL) were added DIBAL-H (1.0 M solution in THF, 7.8 mL, 7.8 mmol) at  $-78\text{ }^{\circ}\text{C}$  under nitrogen. The reaction was stirred for 4 hours at  $-78\text{ }^{\circ}\text{C}$ . Then saturated potassium sodium tartrate solution (15 mL) was added and stirred overnight. Then ethyl acetate was added, the organic layer was separated and the aqueous layer was extracted with ethyl acetate. The combined organic layer was washed with brine, dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography using (hexane/ethyl acetate = 10:1 to 5:1) to afford compound **4** (430 mg, 78%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.18 (d,  $J = 8.4$  Hz, 1H), 7.01 (s, 1H), 6.85 (dd,  $J = 8.4, 2.0$  Hz, 1H), 6.42 (br s, 1H), 4.60 (s, 2H), 1.95 (br s, 1H), 1.51 (s, 9H), 1.02 (s, 9H), 0.28 (s, 6H).

### Synthesis of compound 5



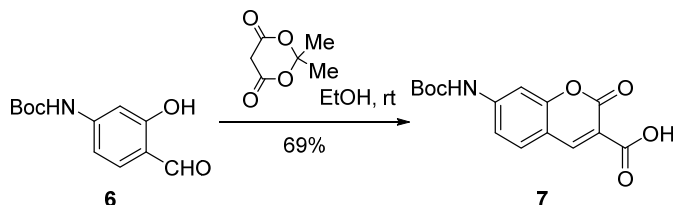
To a solution of compound **4** (400 mg, 1.13 mmol) in DCM (10 mL) was added PCC (244 mg, 1.13 mmol) at room temperature. The mixture was stirred at room temperature for 4 h. Then the mixture was filtered through silicic gel, the filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography using (hexane/ethyl acetate = 10:1 to 5:1) to afford compound **5** (350 mg, 88%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  10.31 (s, 1H), 7.73 (d,  $J = 8.4$  Hz, 1H), 7.23 (d,  $J = 1.6$  Hz, 1H), 6.82 (dd,  $J = 8.4, 2.0$  Hz, 1H), 6.72 (br s, 1H), 1.52 (s, 9H), 1.02 (s, 9H), 0.30 (s, 6H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  188.7, 160.3, 151.8, 145.4, 129.3, 122.3, 111.1, 108.4, 81.3, 28.2 (3C), 25.7 (3C), 18.3, -4.3.

### Synthesis of compound 6



To a solution of compound **5** (180 mg, 0.51 mmol) in THF (5 mL) was added TBAF (1.0 M solution in THF, 0.56 mL, 0.56 mmol) at  $0\text{ }^{\circ}\text{C}$ . The mixture was stirred for 4 h at room temperature. THF was removed under reduced pressure. The residue was suspended in water (10 mL) and the aqueous phase was extracted with ethyl acetate (10 mL). The organic layer was washed with brine, dried over anhydrous sodium sulfate, filtrated and concentrated under reduced pressure. The residue was purified by flash chromatography using (hexane/ethyl acetate = 10:1 to 5:1) as eluent to afford compound **6** (102 mg, 84%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  11.26 (s, 1H), 9.73 (s, 1H), 7.44 (d,  $J = 8.4$  Hz, 1H), 7.05 (dd,  $J = 8.4, 2.0$  Hz, 1H), 6.99 (d,  $J = 2.0$  Hz, 1H), 6.68 (br s, 1H), 1.53 (s, 9H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  194.5, 163.3, 151.7, 146.4, 135.0, 116.4, 109.7, 105.1, 81.8, 28.2 (3C). MS (ESI):  $m/z$  238.1  $[\text{M}+\text{H}]^+$ .

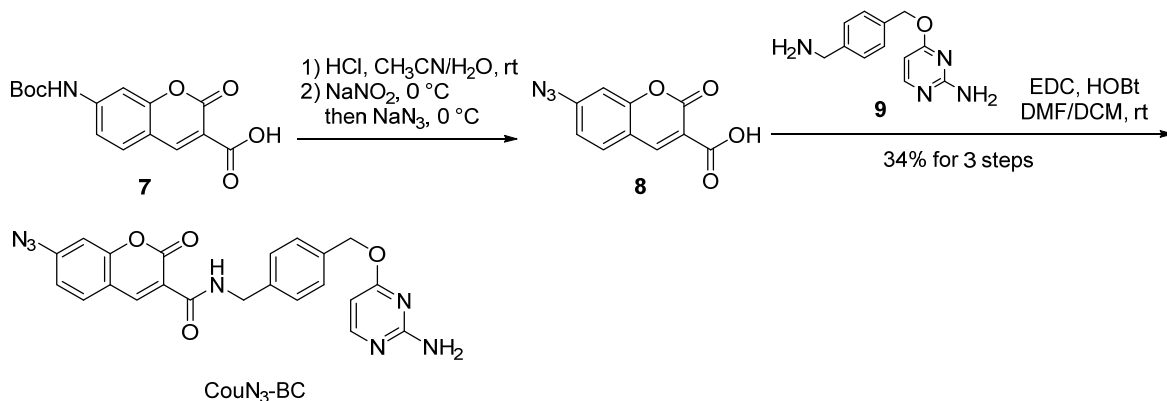
### Synthesis of compound 7



The mixture of compound **6** (73 mg, 0.31 mmol) and meldrum acid (51.4 mg, 0.35 mmol) in absolute ethanol (3 mL) was stirred overnight at room temperature. The solvent was removed under reduced pressure and the

residue was washed with ethyl acetate (1 mL) to remove the excess Meldrum acid to give compound **7** (65 mg, 69%). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>) δ 10.06 (s, 1H), 8.65 (s, 1H), 7.78 (d, *J* = 8.8 Hz, 1H), 7.57 (s, 1H), 7.42 (dd, *J* = 8.4, 1.6 Hz, 1H), 1.50 (s, 9H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>) δ 164.6, 157.7, 156.3, 152.8, 149.1, 146.0, 131.3, 115.2, 114.9, 112.9, 104.0, 80.8, 28.4 (3C). MS (ESI): *m/z* 306.1 [M+H]<sup>+</sup>, 328.1[M+Na]<sup>+</sup>

### Synthesis of CouN<sub>3</sub>-BC



To the solution of compound **7** (10 mg, 0.033 mmol) in acetonitrile (3 mL) was added 3 M HCl (3 mL) at room temperature. The reaction solution was stirred for 10 hours at room temperature to remove *t*-butoxycarbonyl (Boc) group.

To the above solution was added a solution of NaNO<sub>2</sub> (7 mg) in H<sub>2</sub>O (0.2 mL) at 0 °C. The reaction solution was stirred for 10 min at 0 °C and then a solution of NaN<sub>3</sub> (10 mg) in H<sub>2</sub>O (0.2 mL) was added slowly. After the addition was completed, the cooling bath was removed and the reaction mixture was stirred for additional 1 hour at room temperature. Then the mixture was extracted with ethyl acetate, washed with brine, dried over anhydrous sodium sulfate, filtrated and concentrated under reduced pressure. The afforded intermediate **8** was directly used without further purification.

To the solution of intermediate **8** in DCM (1 mL) were added EDC (12.6 mg, 0.066 mmol), HOBT (8.91 mg, 0.066 mmol) and compound **9** (15.2 mg, 0.066 mmol) in DMF (1.5 mL) at room temperature. The reaction solution was stirred overnight. The solvent was removed under reduced pressure. The residual was diluted with water and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous sodium sulfate, filtrated and concentrated under reduced pressure. The residue was purified by flash chromatography using (hexane/ethyl acetate/ethanol = 5:5:1) as eluent to afford CouN<sub>3</sub>-BC (5 mg, 34% for 3 steps). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>) δ 9.07 (t, *J* = 6.0 Hz, 1H), 8.85 (s, 1H), 8.00 (d, *J* = 8.4 Hz, 1H), 7.96 (d, *J* = 5.6 Hz, 1H), 7.40 (d, *J* = 8.4 Hz, 2H), 7.35 (d, *J* = 8.0 Hz, 2H), 7.30 (d, *J* = 2.4 Hz, 1H), 7.21 (dd, *J* = 8.4, 2.4 Hz, 1H), 6.54 (s, 2H), 6.02 (d, *J* = 5.6 Hz, 1H), 5.27 (s, 2H), 4.54 (d, *J* = 6.0 Hz, 2H); <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>) δ 169.5, 163.8, 161.7, 160.6, 159.2, 155.5, 147.5, 146.0, 139.2, 135.8, 132.2, 128.8 (2C), 127.9 (2C), 118.0, 117.1, 116.0, 106.9, 96.7, 66.6, 43.0; MS (ESI): *m/z* 444.0 [M+H]<sup>+</sup>

### References:

- (S1) Wang, C.; Shi, W.; Cai, L.; Lu, L.; Wang, Q.; Zhang, T.; Li, J.; Zhang, Z.; Wang, K.; Xu, L.; Jiang, X.; Jiang, S.; Liu, K. *Journal of Medicinal Chemistry* **2013**, *56*, 2527.  
 (S2) Kindermann, M.; Schwab, M.; (Covalys Biosciences A.-G., Switz.). Patent Application **2006**: WO 2006114409 A1.

### Measurements of reaction kinetics of CouN<sub>3</sub>-BC with H<sub>2</sub>S

To avoid oxidation of NaHS and photodegradation of the probe, all the solutions were prepared under nitrogen atmosphere in a glovebox in dark. CouN<sub>3</sub>-BC (4  $\mu$ L, 5 mM stock solution in DMF) was diluted in 996  $\mu$ L of phosphate buffer saline (PBS, 10 mM, pH 7.4). To the above solution was added 1000  $\mu$ L of PBS containing 200  $\mu$ M of NaHS. The final concentrations of CouN<sub>3</sub>-BC and NaHS were 10  $\mu$ M and 100  $\mu$ M, respectively. In a sealed fluorescence cuvette, the solution was monitored continuously with fluorimeter ( $\lambda_{\text{ex}}$  = 405 nm) with 5 min interval for 1 h.

### Reactivity of CouN<sub>3</sub>-BC towards different redox species

To avoid oxidation of redox species and photodegradation of the probe, all the solutions were prepared under nitrogen atmosphere in a glovebox in dark. CouN<sub>3</sub>-BC (20  $\mu$ L, 1 mM stock solution in DMF) was diluted in 980  $\mu$ L of phosphate buffer saline (PBS, 10 mM, pH 7.4). To the above solution was added an equal volume (1000  $\mu$ L) of PBS solution containing 200  $\mu$ M (unless otherwise specified) of redox species, including H<sub>2</sub>S (dissolution of NaHS), GSH, cysteine, lipoic acid, Na<sub>2</sub>SO<sub>3</sub>, Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, KSCN, NADPH, NaNO<sub>2</sub>, NO (produced by proli-NONOate), H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup>, (produced by KO<sub>2</sub>) and *t*-BuOOH. The final concentrations of CouN<sub>3</sub>-BC and the redox species were 10  $\mu$ M and 100  $\mu$ M (unless otherwise specified), respectively. The final concentrations for GSH and cysteine are 5 mM and 500  $\mu$ M, respectively. The solution was monitored continuously with fluorimeter ( $\lambda_{\text{ex}}$  = 405 nm) every 5 min for 1 h.

### Limit of detection (LOD) for CouN<sub>3</sub>-BC with H<sub>2</sub>S

To 10  $\mu$ M solution of CouN<sub>3</sub>-BC in PBS (600  $\mu$ L) was added 600  $\mu$ L of PBS containing various concentrations of NaHS. The final concentration of CouN<sub>3</sub>-BC was 5  $\mu$ M. The mixture were incubated for 90 min at 20 °C in 1.5 mL Eppendorf tubes under nitrogen atmosphere. To a 96-well plate were added the above samples and the fluorescence intensity was measured using a plate reader ( $\lambda_{\text{ex}}$  = 405 nm,  $\lambda_{\text{em}}$  = 450 nm).

### Evaluation of cell permeability of CouN<sub>3</sub>-BC

HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin–streptomycin at 37 °C under 5% CO<sub>2</sub>. HeLa cells were incubated with 10  $\mu$ M CouN<sub>3</sub>-BC or DMSO (as a negative control) for 30 min (diluted from a 100  $\mu$ M stock solution of CouN<sub>3</sub>-BC in DMSO). Cells treated with CouN<sub>3</sub>-BC were further incubated in culture medium containing NaHS (250  $\mu$ M) or PBS (equal volume as a negative control) for another 30 min and 6 h, respectively. Then, HeLa cells were washed for 15 min twice with full medium. The slides were washed with PBS for another three times prior to confocal imaging.

### Evaluation of organelle specificity of H<sub>2</sub>S probe CouN<sub>3</sub>-BC

HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin–streptomycin at 37 °C under 5% CO<sub>2</sub>. Cells were transfected with pCLIP-H2B (New England Biolabs® Inc.), pCLIP-COX8A (gift from Dr. C. Provost at New England Biolabs), and a control plasmid (5  $\mu$ g of plasmid per well) in 6-well plates using Lipofectamine® 3000 (3.75  $\mu$ L per well) following the standard protocol from Life Technologies. The transfection medium was removed after 24 h incubation. Cells were further cultured for another 48 h in full medium. The cells were then transferred to glass coverslips. Once the cells grew to 60% confluence on the coverslips, they were incubated with 10  $\mu$ M of CouN<sub>3</sub>-BC (diluted from a 1 mM stock solution of CouN<sub>3</sub>-BC in DMSO) or DMSO (as a negative control) for 30 min. The cells treated with CouN<sub>3</sub>-BC were further incubated in culture medium containing NaHS (250  $\mu$ M) or PBS (equal volume as a negative control) for another 30 min. After treatment, the cells were washed twice with PBS, and fixed with pre-chilled methanol at room temperature for 10 min. Antigen accessibility was increased by treatment with 0.2% Triton X-100. The cells were then incubated with the primary antibodies for 1 h, and, after washing with PBS, stained with a secondary antibody for another 1 h, and then with NucRed® Live 647 ReadyProbes® (Life Technologies) for another 30 min. Cell images were captured with an Olympus FluoView 1200 Confocal Microscope with a 60X objective (Alexa Fluor® 405 nm, 488 nm and 647 nm channels).

### **Evaluation of endogenous H<sub>2</sub>S level in Nucleus via Organelle Specific CouN<sub>3</sub>-BC**

HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin–streptomycin at 37 °C under 5% CO<sub>2</sub>. Cells were transfected with pCLIP-H2B (New England Biolabs® Inc.), and a control plasmid (5 µg of plasmid per well) in 6-well plates using Lipofectamine® 3000 (3.75 µL per well) following the standard protocol from Life Technologies. The transfection medium was removed after 24 h incubation. Cells were further cultured for another 48 h in full medium. The cells were then transferred to glass coverslips. Once the cells grew to 60% confluence on the coverslips, they were incubated in culture medium containing 10 µM of CouN<sub>3</sub>-BC (diluted from a 1 mM stock solution of CouN<sub>3</sub>-BC in DMSO) or DMSO (as a negative control) for 30 min. Cells were washed to remove excess CouN<sub>3</sub>-BC, then treated with 100 µM of SNP or PBS (as a negative control) for 45 min. After treatment, the cells were washed twice with PBS, and fixed with pre-chilled methanol at room temperature for 10 min. The cells were then incubated with the primary antibodies for 1 h at room temperature, and, after washing with PBS, stained with a secondary antibody for another 1 h at room temperature, and then with NucRed® Live 647 ReadyProbes® (Life Technologies) for another 30 min. Cell images were captured with an Olympus FluoView 1200 Confocal Microscope with a 60X objective (Alexa Fluor® 405 nm, 488 nm and 647 nm channels).

### **Evaluation of cytotoxicity of CouN<sub>3</sub>-BC**

HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin–streptomycin at 37 °C under 5% CO<sub>2</sub>. Cells were seeded in 96-well plate with 5000 cells per well, allowed to adhere and grow for overnight. Cells were incubated in culture medium containing 10 µM of CouN<sub>3</sub>-BC (diluted from a 1 mM stock solution of CouN<sub>3</sub>-BC in DMSO) or DMSO (as a negative control). After 2 h, 4 h and 24 h, the cellular viability were measure by alamar blue assay following the standard protocol from Life Technologies. The fluorescence were measured with BioTek® Synergy H1 Multi-Mode Reader (Excitation: 544 nm, Emission: 590 nm). Four replicates of all experimental and no-cell control samples were measured at every time point to minimize experimental errors. Data were plotted with Graph Prism® 5 (showing Mean ± SD).



**Table S1.** Quantitative Analyses of Co-localization between H<sub>2</sub>S Probes and Organelles of Interest

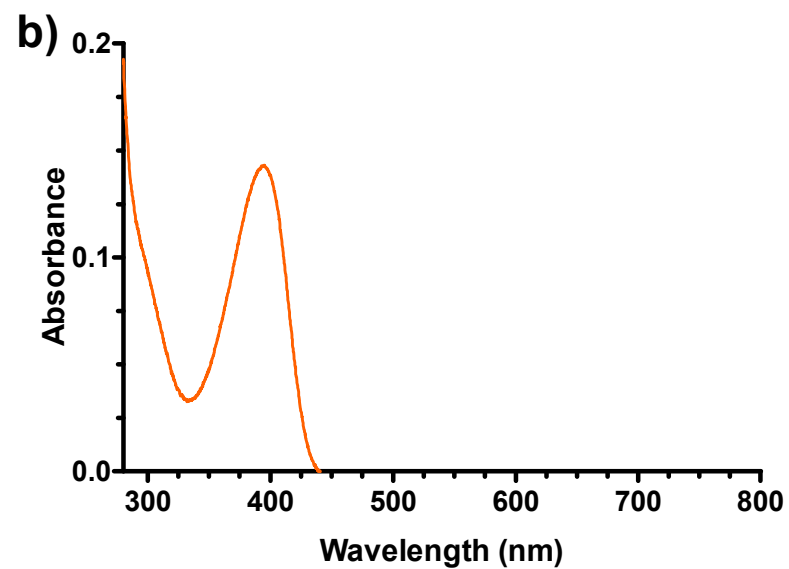
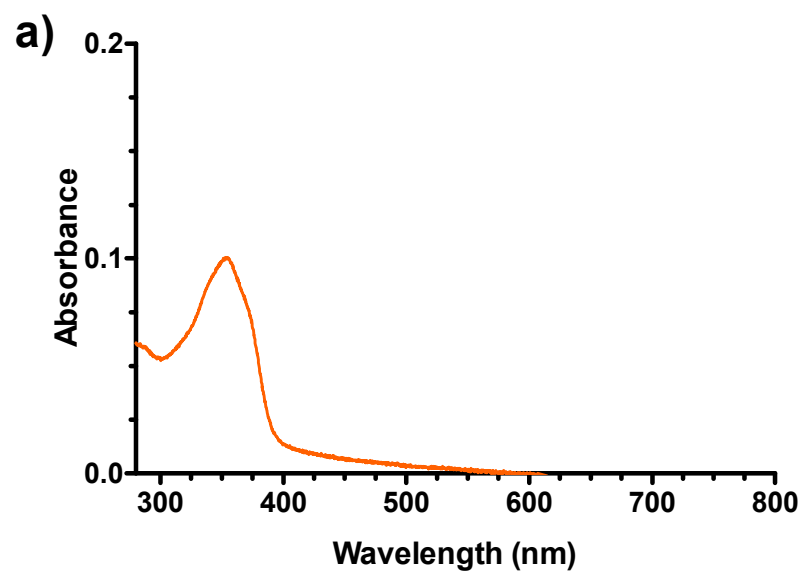
Probes	Plasmids	Pearson's Coefficient	Manders' Overlap Coefficient			Manders' Coefficients	
			Overlap Coefficient	k1	k2	M1	M2
CouN <sub>3</sub> -BC	CLIP-COX8A	0.721	0.928	0.853	1.009	0.458	0.88
CouN <sub>3</sub> -BC	CLIP-H2B	0.695	0.927	0.776	1.107	0.684	0.563

Note:

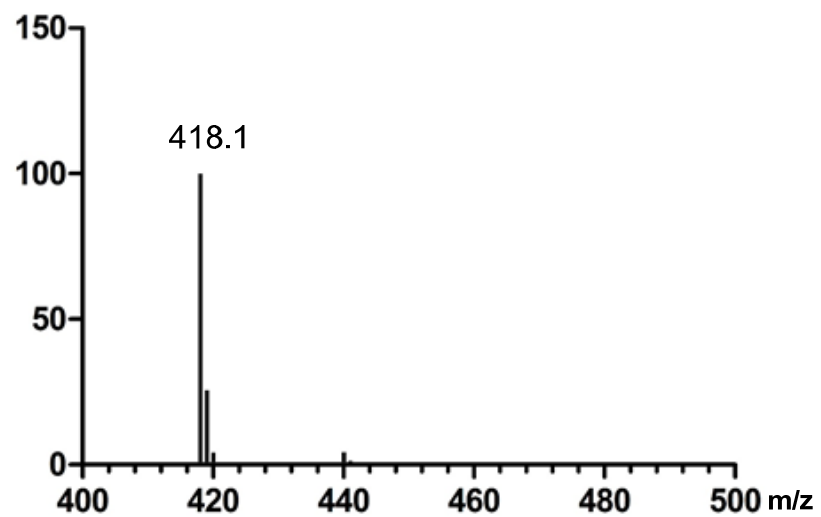
a. The dual-color correlations in confocal images were quantitatively measured by ImageJ with JACoP plugin. (Bolte, S. & Cordelières, F. P. (2006) A guided tour into subcellular colocalization analysis in light microscopy, *Journal of Microscopy*, 224, 213-232.) Co-localization methods used in these tests were Pearsons' coefficient, Manders' overlap coefficient with k1 & k2, and Manders' coefficients with M1 & M2. (Manders, E. M. M., Verbeek, F. J., and Aten, J. A. (1993) Measurement of co-localization of objects in dual-color confocal images, *Journal of Microscopy*, 169, 375-382).

b. In the pCLIP-COX8A group, CouN<sub>3</sub>-BC (405 nm, defined as component A in Manders' coefficients) and Alexa 488 labeled anti-COX IV antibody (488 nm, defined as component B in Manders' coefficients) were evaluated. In the pCLIP-H2B group, CouN<sub>3</sub>-BC (405 nm, defined as component A in Manders' coefficients) and NucRed® Live 647 ReadyProbes® Reagent (647 nm, defined as component B in Manders' coefficients) were evaluated.

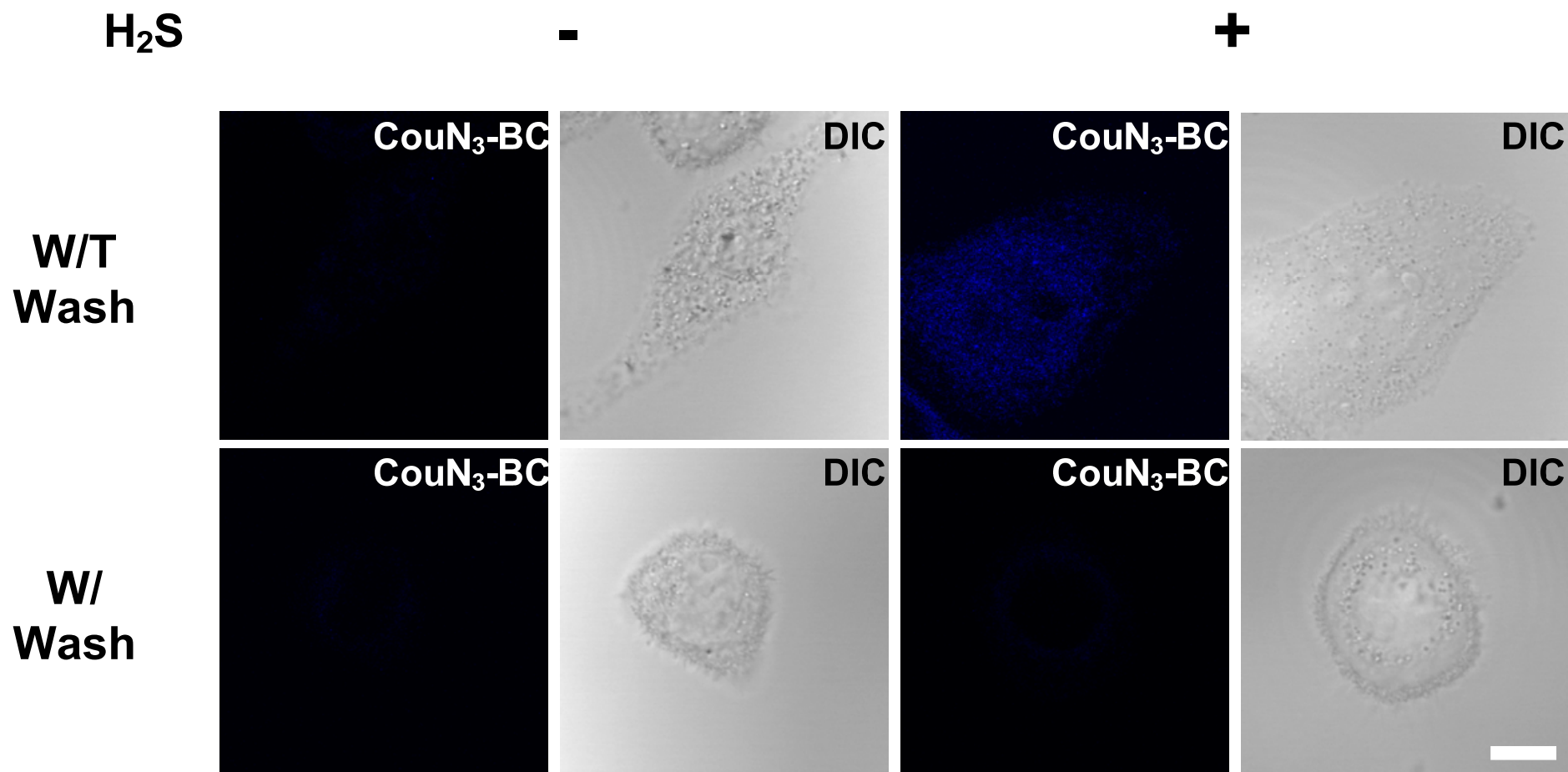
c. The results showed positive correlations between our probes and the targeted organelles: high positive correlation for the pCLIP-COX8A group, and moderate positive correlation for the pCLIP-H2B and pHalo-H2B groups. (Mukaka, M.M., (2012) A guide to appropriate use of correlation coefficient in medical research, *Malawi Med J.*, 24, 69–71).



**Figure S1.** UV-Vis spectra of H<sub>2</sub>S probes and their corresponding products. (a) CouN<sub>3</sub>-BC (10 μM) and (b) CouNH<sub>2</sub>-BC (10 μM) in PBS containing 20% (v/v) of acetonitrile.

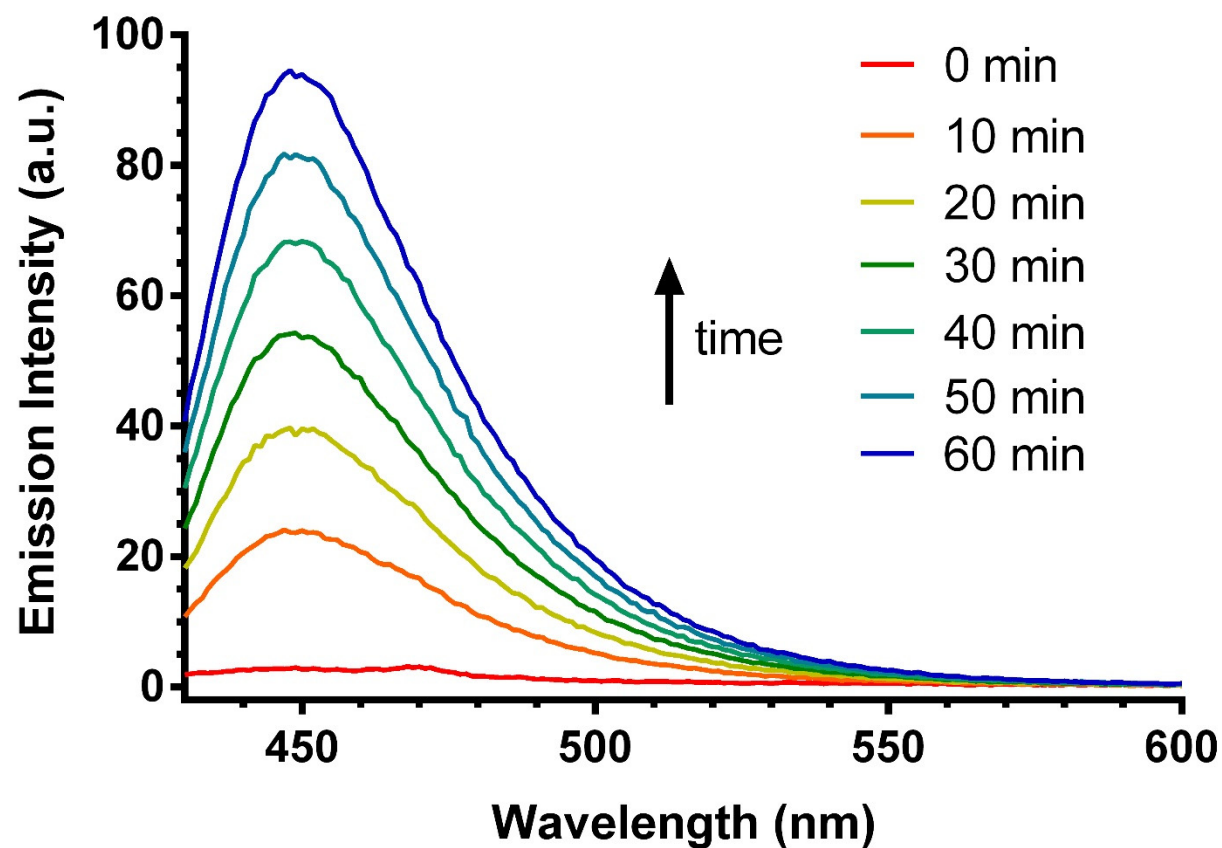


**Figure S2.** LC-MS (ESI) spectra of CouNH<sub>2</sub>-BC.

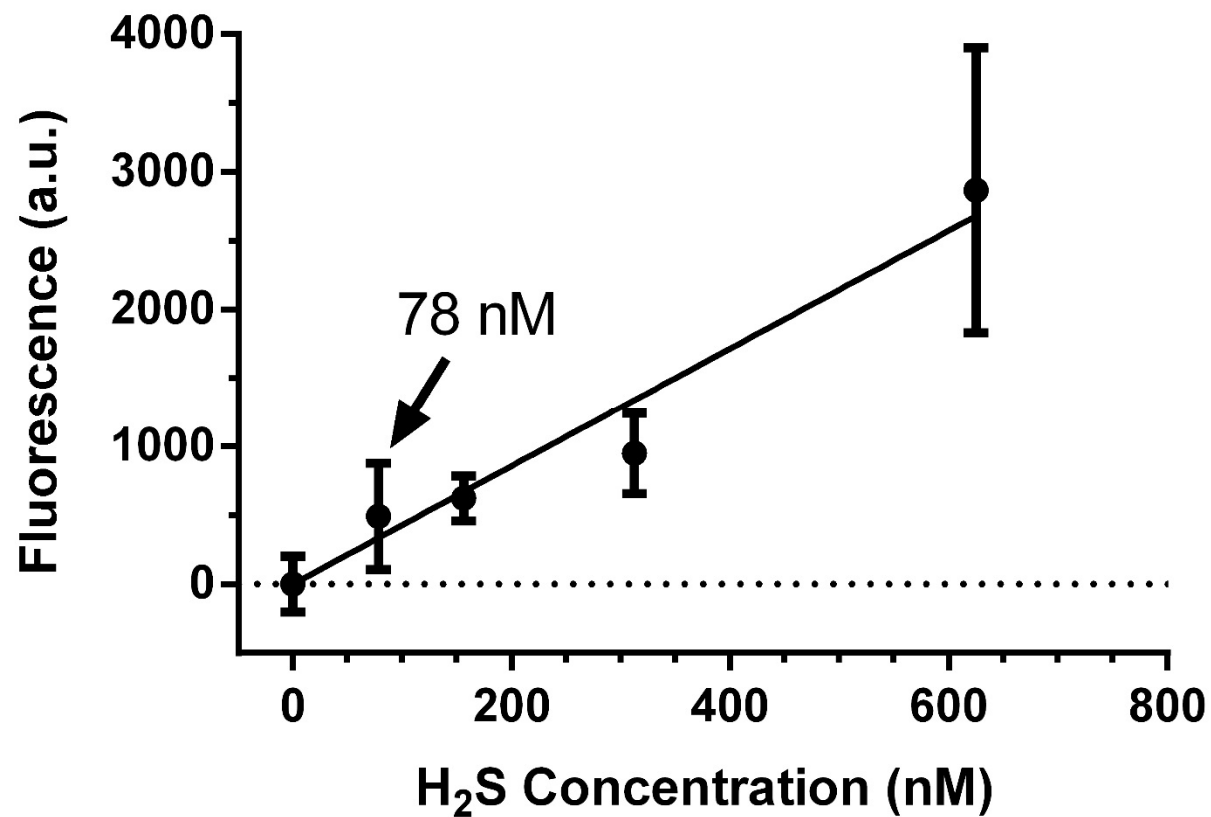


**Figure S3.** Cellular permeability of CouN<sub>3</sub>-BC.

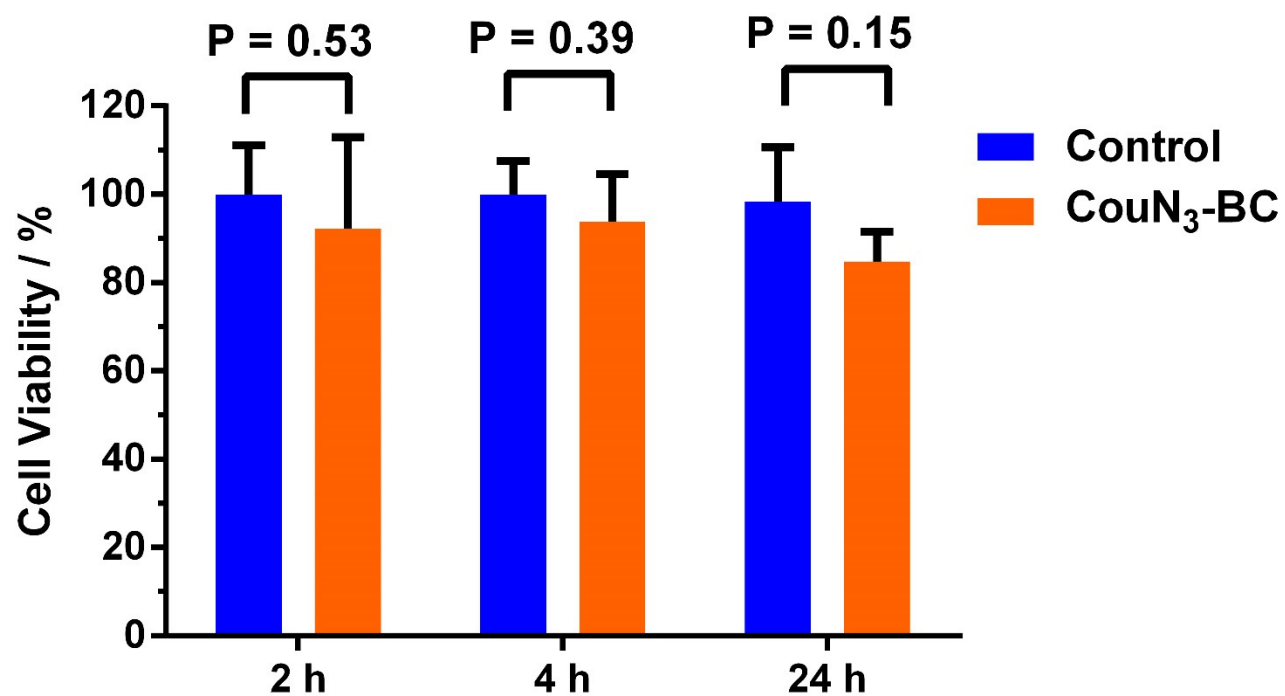
HeLa cells were firstly incubated with 10  $\mu$ M of CouN<sub>3</sub>-BC for 30 min, and then incubated for another 30 min in full medium with 250  $\mu$ M of H<sub>2</sub>S or in full medium without H<sub>2</sub>S. The cells were imaged by confocal microscope with or without washing to remove CouN<sub>3</sub>-BC. The result showed positive response for H<sub>2</sub>S of CouN<sub>3</sub>-BC, but the signal of CouN<sub>3</sub>-BC can be effectively removed by double PBS wash steps without CLIP-anchored (described in Methods). Scale bar is equal to 10  $\mu$ m.



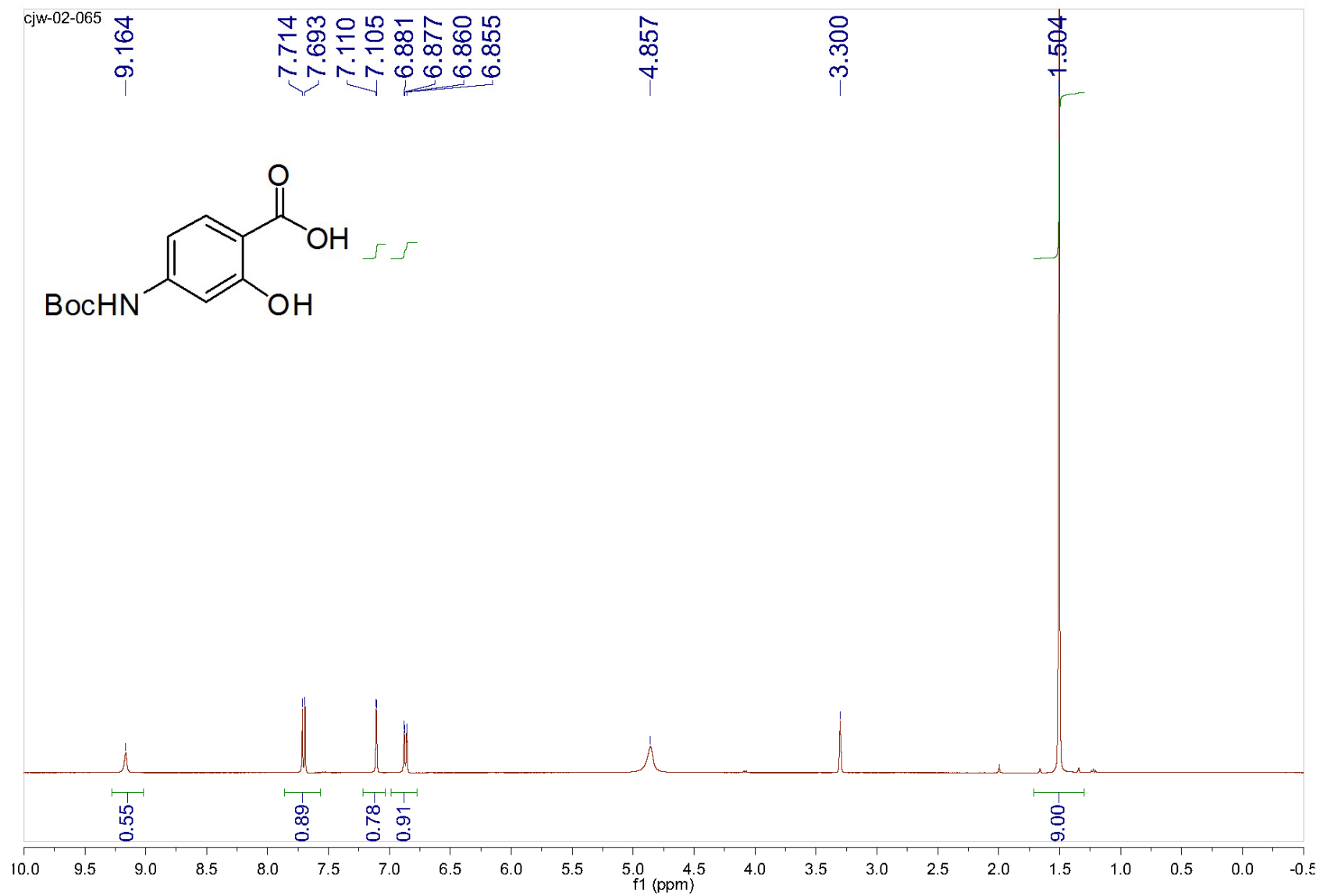
**Figure S4.** Time dependent fluorescence emission spectra of CouN<sub>3</sub>-BC (10 μM,  $\lambda_{\text{ex}}$  = 405 nm) upon reacting with 100 μM of H<sub>2</sub>S in PBS (pH 7.4). Note: this is the data shown in Figure 2B before normalization.



**Figure S5.** Limit of detection (LOD) of CouN<sub>3</sub>-BC (10  $\mu$ M,  $\lambda_{\text{ex}}$  = 405 nm) upon reacting with different concentrations of H<sub>2</sub>S in PBS (pH 7.4) for 90 min. Note: the LOD depends on the sensitivity of the fluorimeter and incubation time. This fluorescence measurement was performed on a BioTek Synergy H1 plate reader. This LOD value cannot be extrapolated as the LOD for confocal imaging.

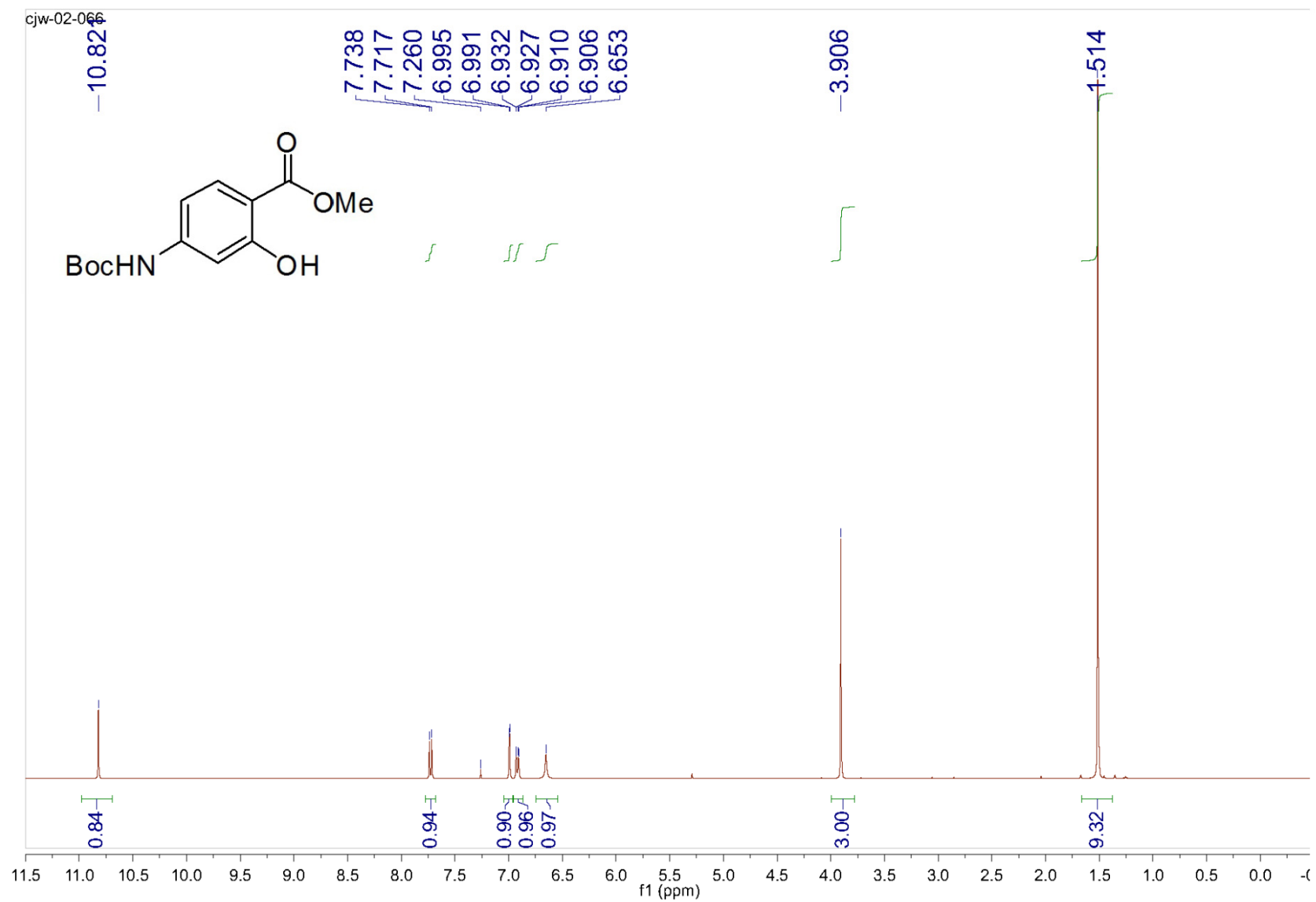


**Figure S6.** Toxicity of CouN<sub>3</sub>-BC (10  $\mu$ M) in HeLa cells. The cell viability was measured using an Alamar Blue assay at different time points. The statistics was analyzed using student t test. As shown, CouN<sub>3</sub>-BC (10  $\mu$ M) does not have statistically significant toxicity to HeLa cells within the experimental time frame, which is usually within 2 h.



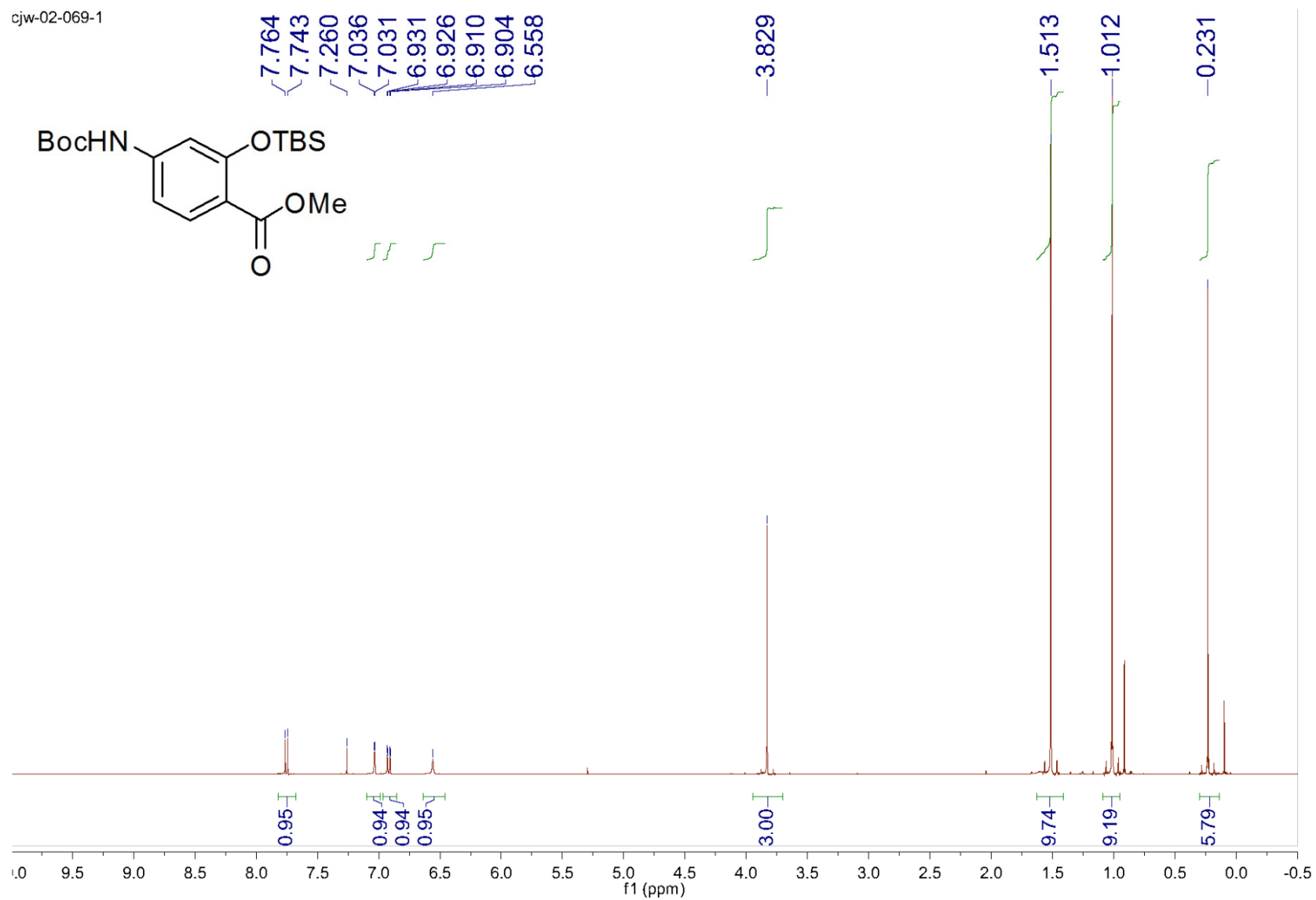
**Figure S7.** <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD) spectrum of **1**



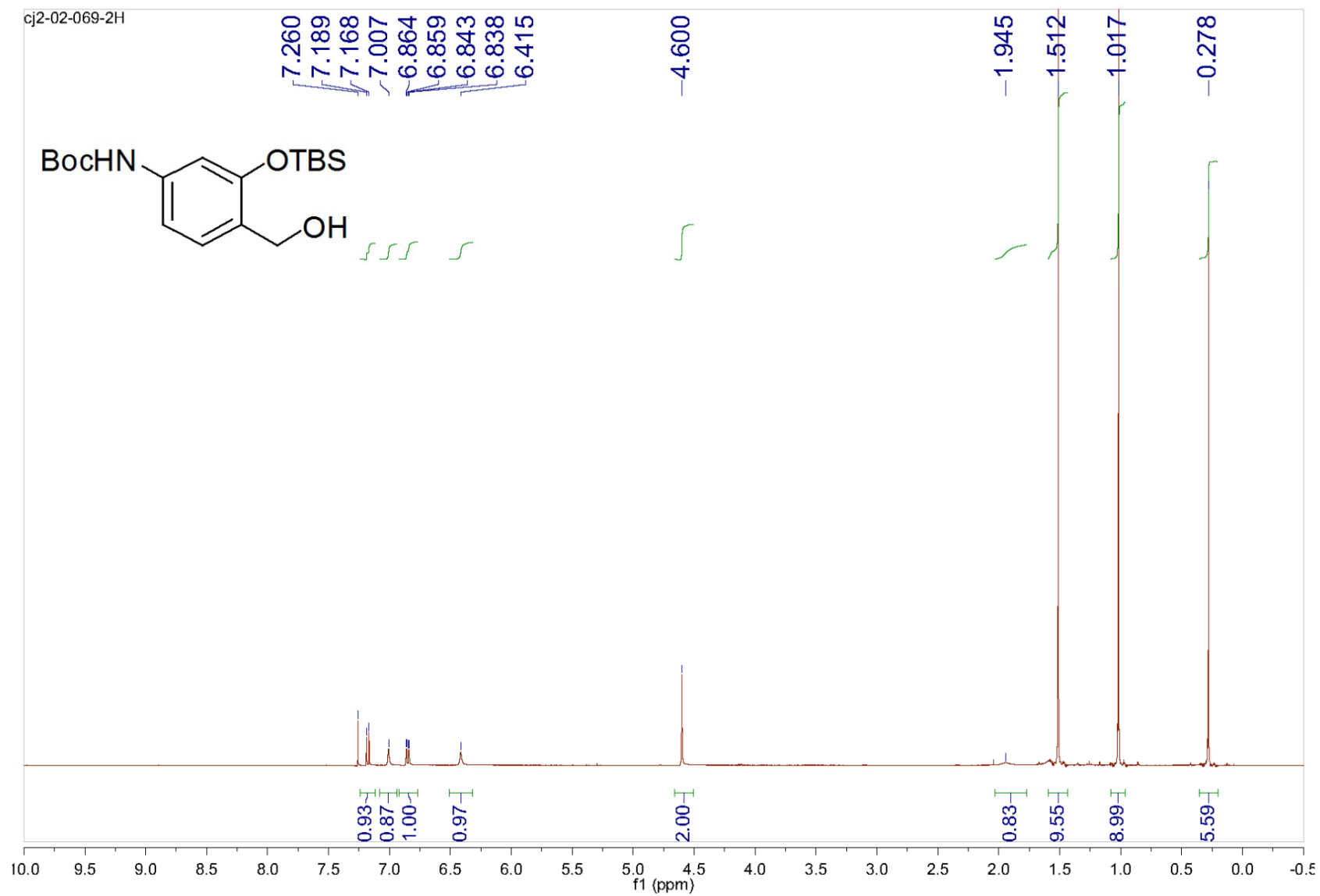


**Figure S8.** <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) spectrum of **2**

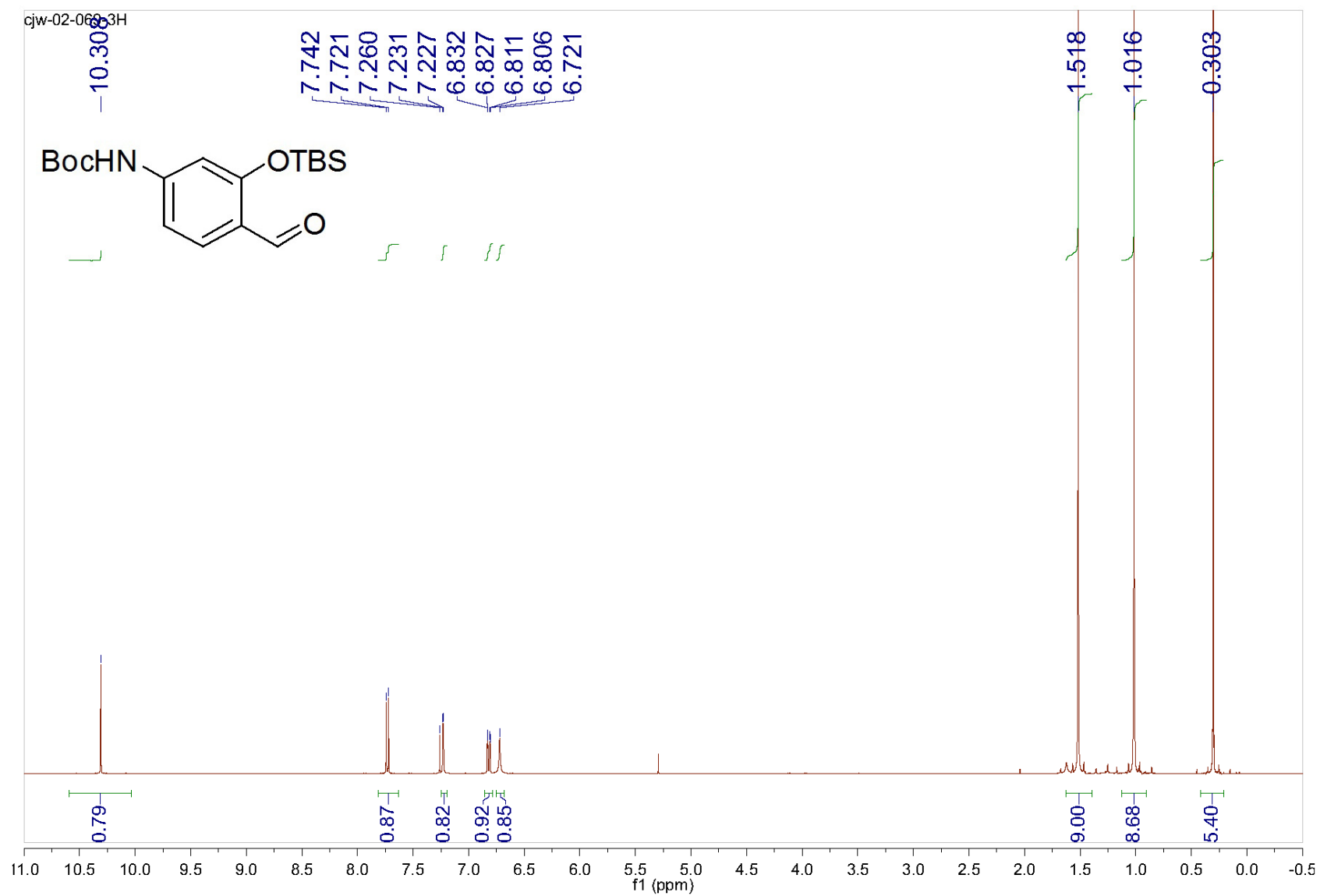
cjw-02-069-1



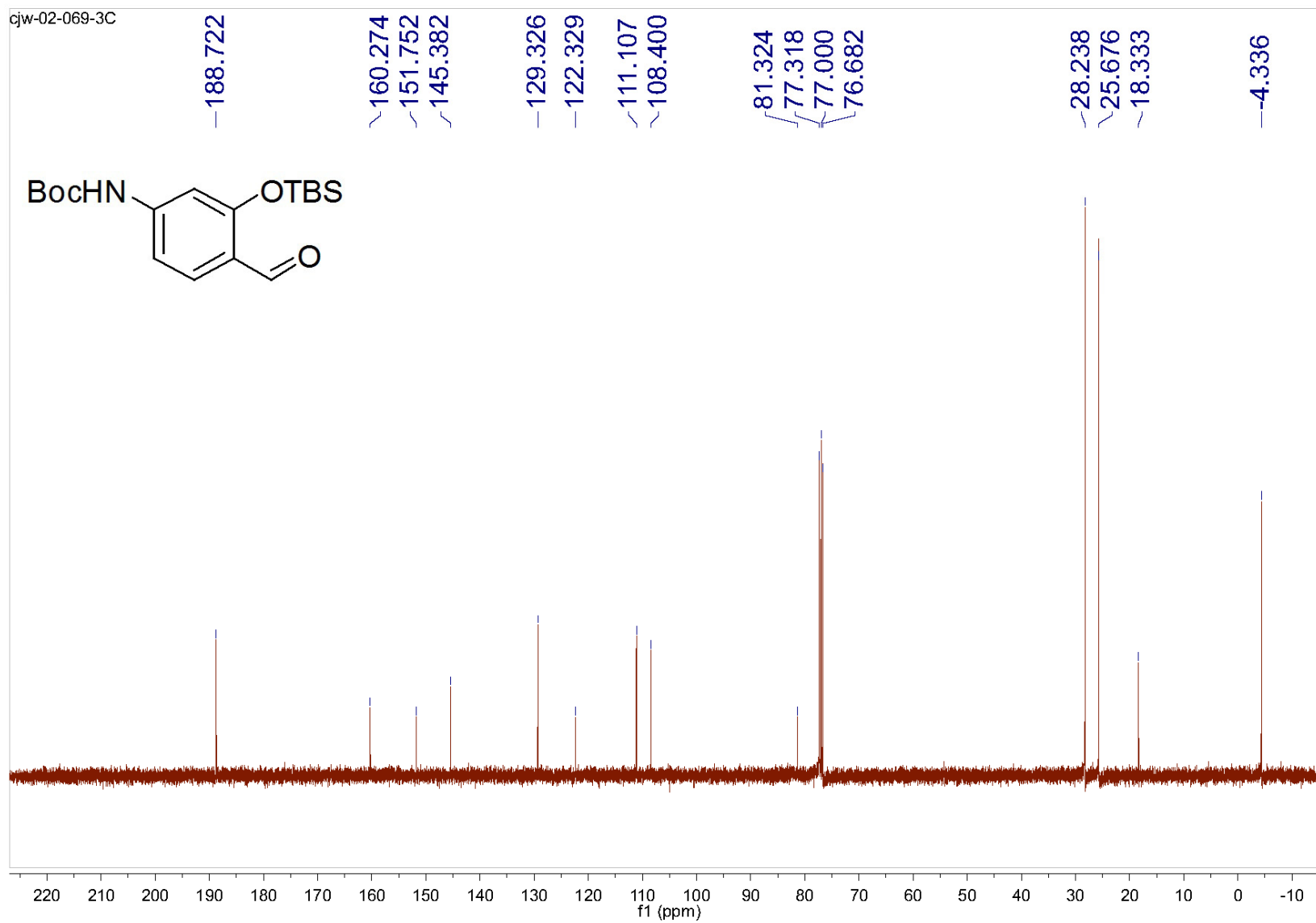
**Figure S9.** <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) spectrum of **3**



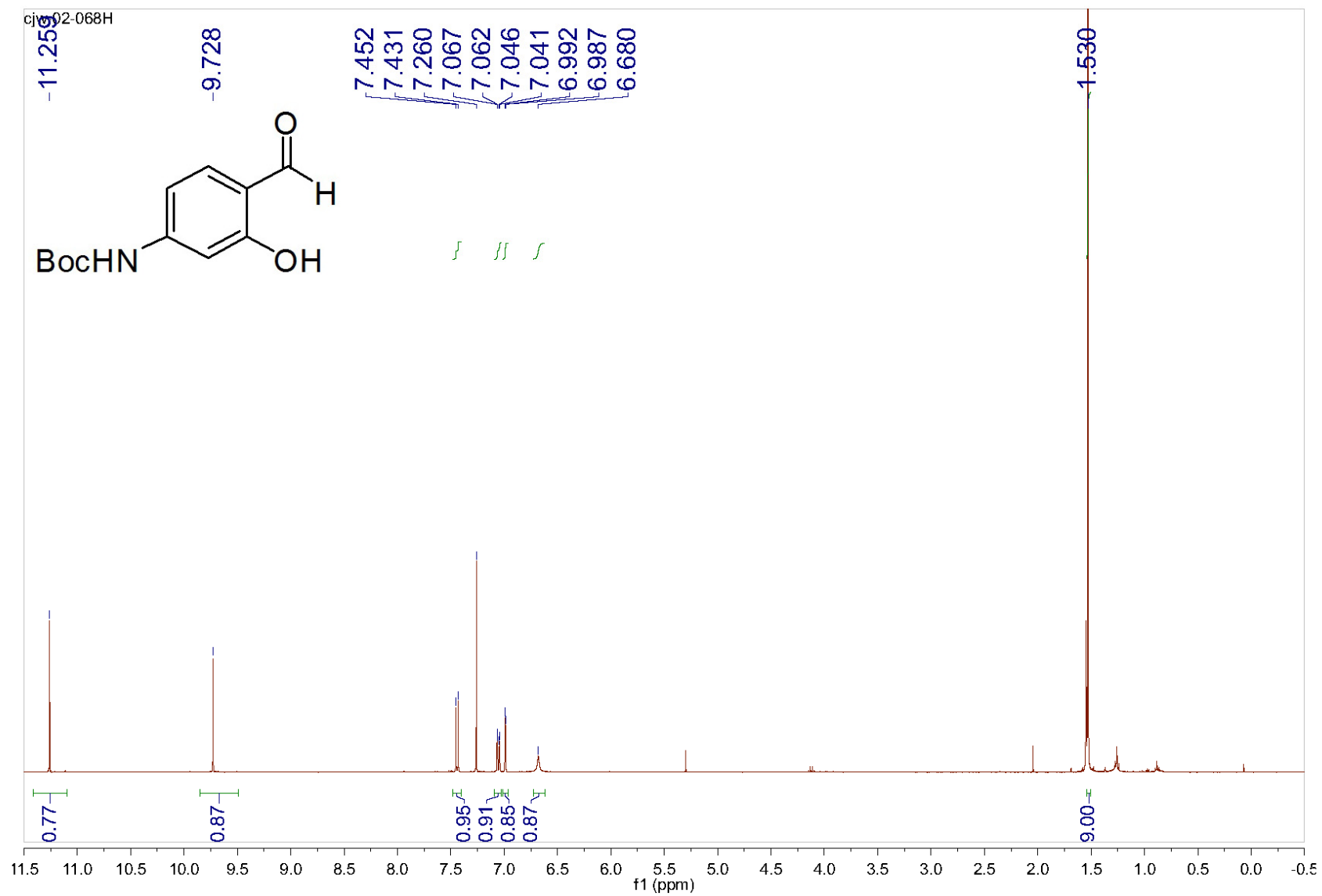
**Figure S10.** <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) spectrum of **4**



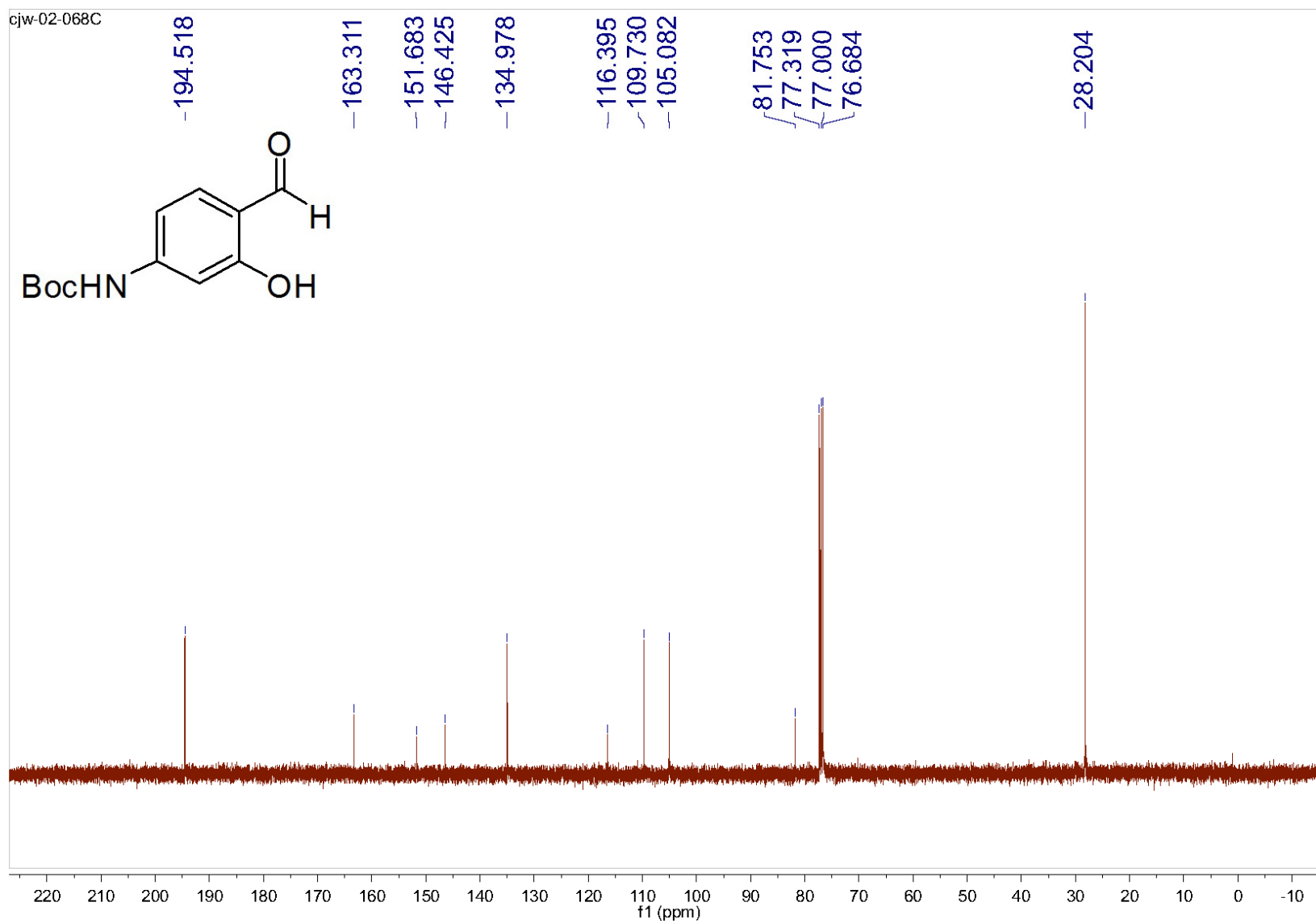
**Figure S11.** <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) spectrum of **5**



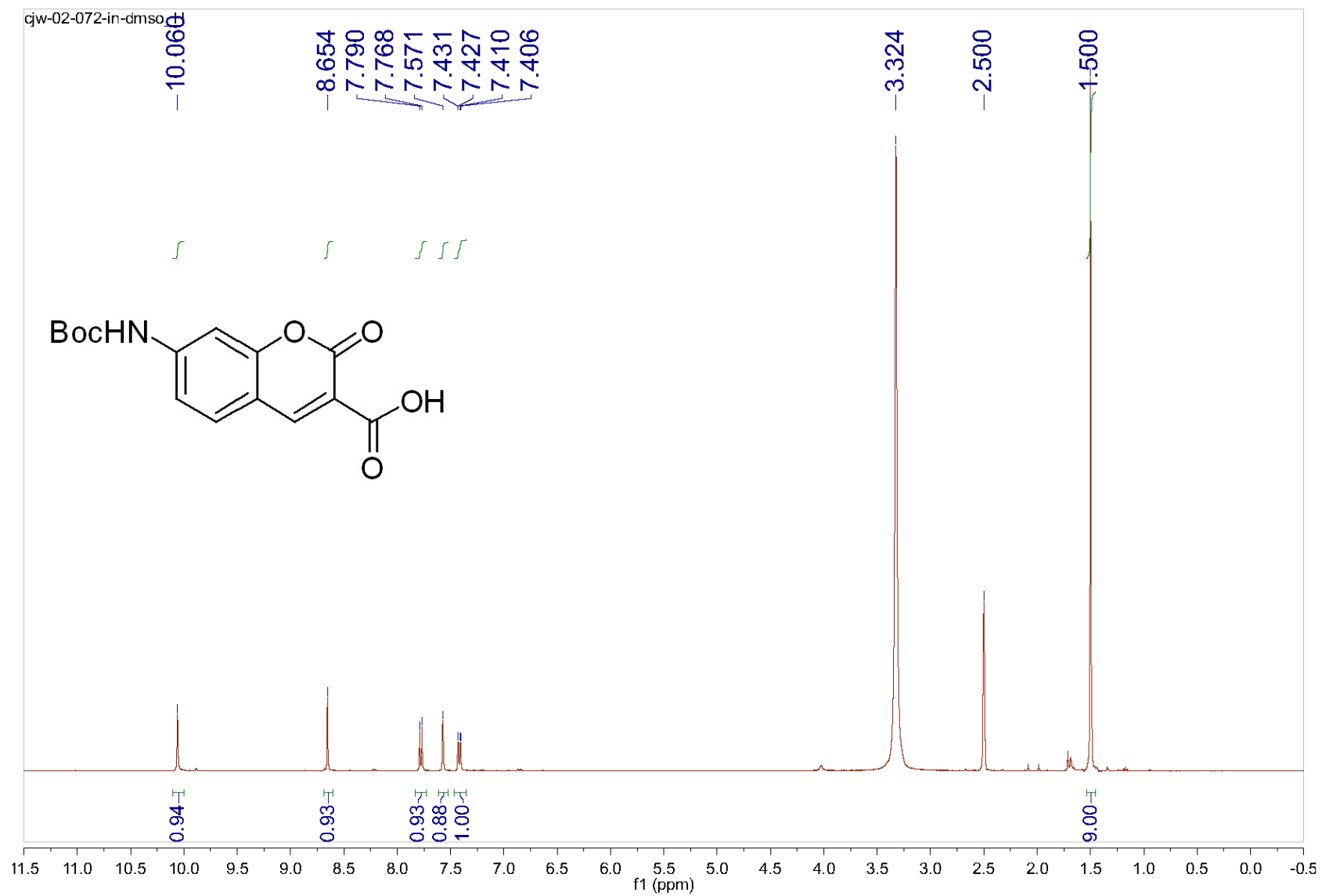
**Figure S12.** <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) spectrum of **5**



**Figure S13.** <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) spectrum of 6

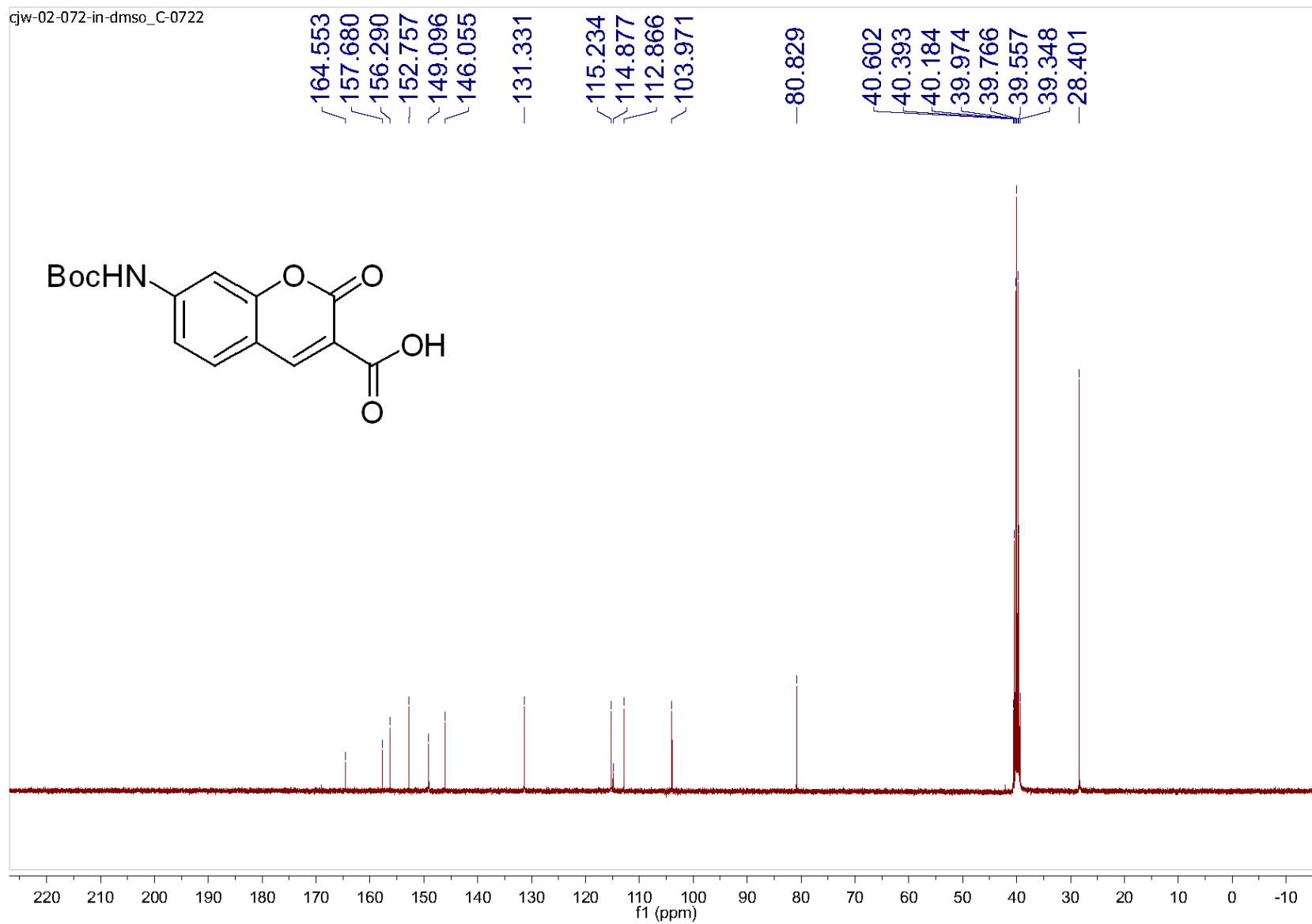


**Figure S14.**  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ ) spectrum of **6**

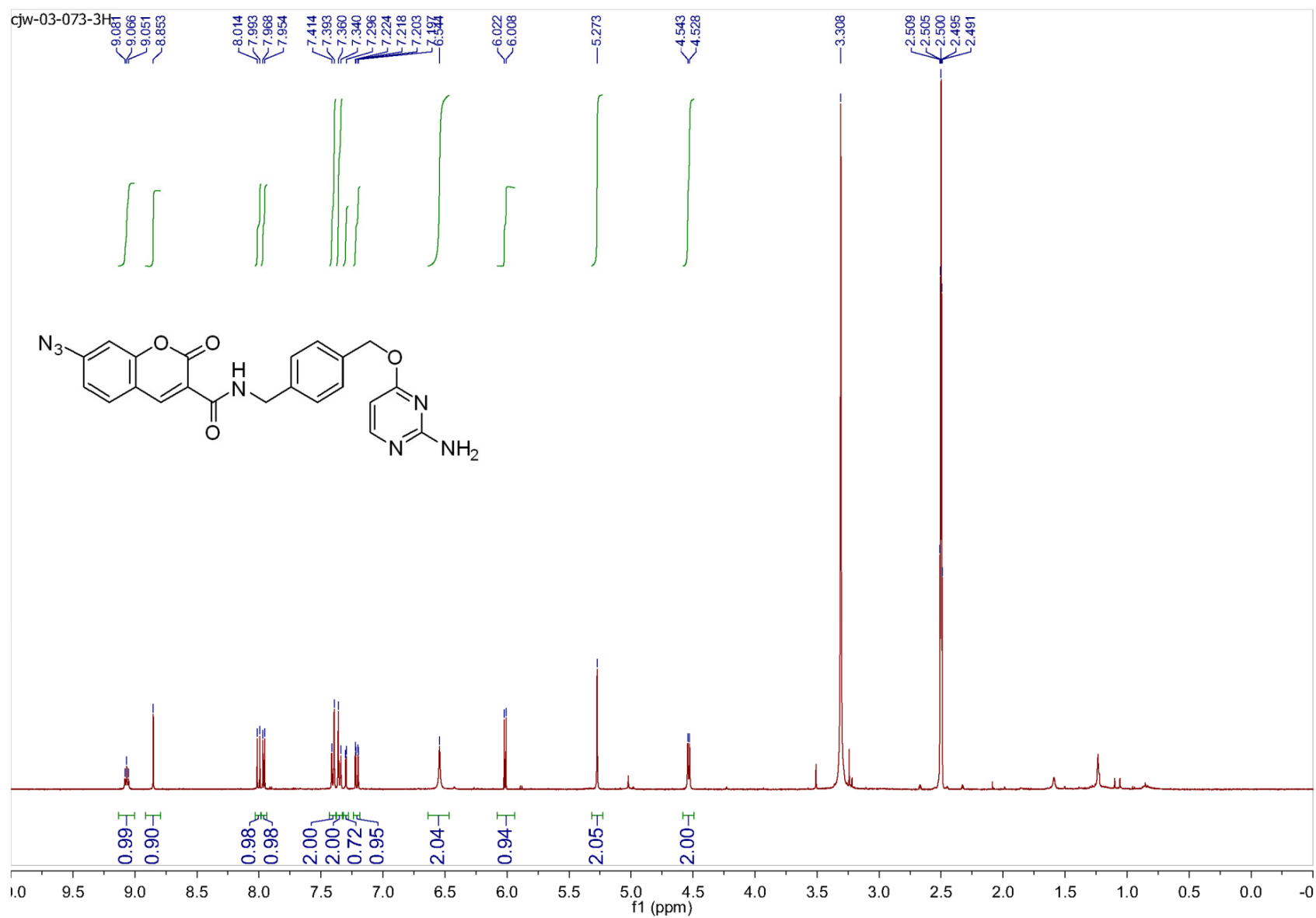


**Figure S15.** <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) spectrum of **7**

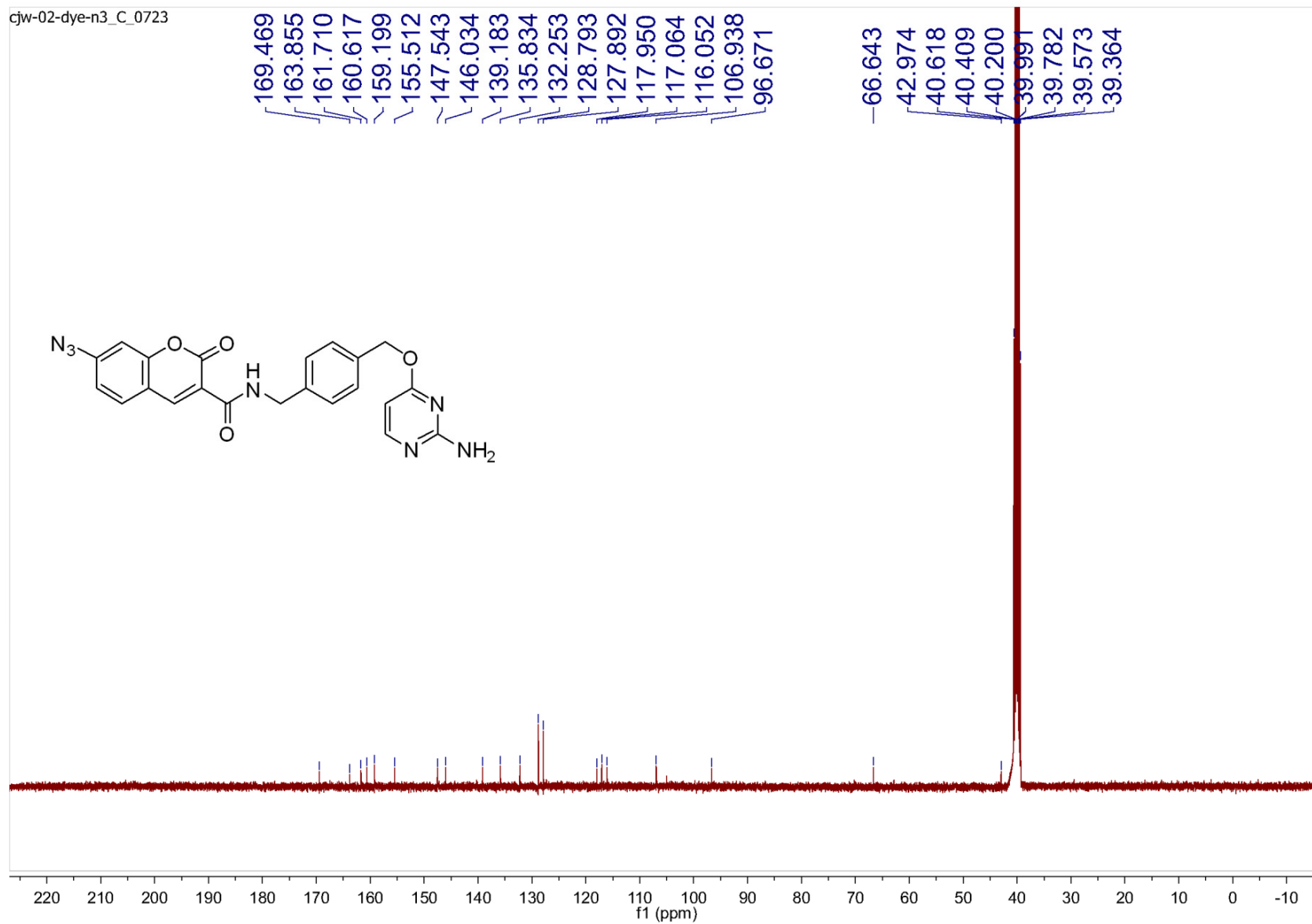




**Figure S16.**  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO}-d_6$ ) spectrum of **7**



**Figure S17.** <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) spectrum of CouN<sub>3</sub>-BC



**Figure S18.** <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) spectrum of **CouN<sub>3</sub>-BC**