Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary Information for

A Selective Reaction-Based Fluorescent Probe for Detecting Cobalt in Living Cells

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1. Synthesis

General. All solvents were of reagent grade. MeCN was dried by passing through activated alumina. All commercially purchased chemicals were used as received. Tokyo Green[1] and 2-(2-pyridylmethyl)aminoethanol **1**[2] were synthesized according to literature procedures. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVB-400 NMR spectrometer at the College of Chemistry NMR Facility, UCB. Signals were internally referenced to methanol residues (^{1}H: 3.31 ppm; ^{13}C: 49.00 ppm). Low-resolution mass spectral analyses were carried out using a LC-MS (Agilent Technology 6130, Quadrupole LC/MS). High-resolution mass spectral analyses (ESI-MS) were carried out at the College of Chemistry Mass Spectrometry Facility, UCB.

Synthesis of Receptor 2. To a mixture of 2,6-dichloromethylpyridine (352 mg, 2 mmol) and $NaHCO_3$ (84 mg, 1 mmol) in 30 ml of dried MeCN heated at 70°C, a solution of compound **2** (152 mg, 1 mmol, 15 ml) in the same solvent was added slowly. The resulting mixture was stirred at 70°C for overnight and cooled to room temperature. Insoluble materials were removed by filtration. The filtrate was concentrated and purified by basic alumina column (100% ethyl acetate → 9:1 ethyl acetate/MeOH). The product was obtained as pale yellow oil. Yield = 106 mg, 36%. ^{1}H NMR (400 MHz, CD_3OD, 298 K) δ (ppm): 8.42 (d, J = 4.8 Hz, 1 H), 7.76–7.72 (m, 2 H), 7.57 (d, J = 7.6 Hz, 1 H), 7.50 (d, J = 8.0 Hz, 1 H), 7.37 (d, J = 7.6 Hz, 1 H), 7.23 (t, J = 6.0 Hz, 1 H), 4.65 (s, 2 H), 3.85 (s, 2 H), 3.84 (s, 2 H), 3.67 (t, J = 5.6 Hz, 2 H), 2.75 (t, J = 5.6 Hz, 2 H). ^{13}C NMR (100.6 MHz, CD_3OD, 298 K) δ (ppm): 160.7, 160.5, 157.2, 149.4, 139.2, 138.5, 125.0, 124.0, 123.7, 122.8, 61.2, 61.1, 60.6, 58.1, 47.1. LRMS (ESI) calcd. for $C_{15}H_{19}NO_3{}^{35}Cl$ $[M+H]^+$ *m/z*: 292.1; found: 292.2.

Synthesis of Cobalt Probe 1 (CP1). A mixture of Tokyo Green (50 mg, 0.15 mmol) and compound receptor **2** (66 mg, 0.23 mmol) was dissolved in dried MeCN/DMF (30 ml/5 ml). K_2CO_3 (250 mg, 1.8 mmol) was added and the resulting mixture was heated at 90°C for overnight. After cooling to room temperature, insoluble material was removed by filtration, and the filtrate was purified by basic alumina column (95:5 EA/MeOH). The product was isolated as yellow oil. Yield = 41 mg, 47%. ^{1}H NMR (400 MHz, CD_3OD, 298 K) δ (ppm): 8.41 (d, J = 4.8 Hz, 1 H), 7.77 (t, J = 7.8 Hz, 1 H), 7.73 (dt, J = 7.8 Hz, 1.6 Hz, 1 H), 7.57 (d, J = 7.6 Hz, 1 H), 7.51 (d, J = 8.0 Hz, 1 H), 7.42 (d, J = 7.6 Hz, 1 H), 7.25–7.22 (m, 2 H), 7.15–7.09 (m, 3 H), 7.04–6.95 (m, 3 H), 6.57 (dd, J = 9.6 Hz, 2.0 Hz, 1 H), 6.40 (d, J = 1.2 Hz, 1 H), 5.31 (s, 2 H), 3.88 (s, 3 H), 3.87 (s, 2 H), 3.85 (s, 2 H), 3.66 (t, J = 5.6 Hz, 2 H), 2.75 (t, J = 5.6 Hz, 2 H), 1.99 (s, 3 H). ^{13}C NMR (100.6 MHz, CD_3OD, 298 K) δ (ppm): 187.3, 165.6, 162.3, 161.3, 160.8, 160.6, 156.4, 156.2, 155.0, 149.4, 139.1, 139.0, 138.6, 132.9, 131.6, 131.4, 129.7, 125.4, 125.0, 124.2, 123.7, 121.8, 119.3, 117.1, 116.4, 116.0, 112.8, 105.6, 102.8, 72.3, 61.3, 61.2, 60.7, 58.0, 55.9, 20.0. HRMS (ESI) calcd. for $C_{36}H_{33}N_3O_5Na$ $[M+Na]^+$ *m/z*: 610.2318; found: 610.2324.

1. Y. Urano, M. Kamiya, K. Kanda, T. Ueno, K. Hirose, T. Nagano, *J. Am. Chem. Soc.* **2005**, *127*, 4888-4894.
2. S. Striegler, M. Dittel, *Inorg. Chem.* **2005**, *44*, 2728-2733.

2. Fluorescence spectroscopy

Fluorescence spectra were recorded on a Photon Technology International Quanta Master 4 L-format scanning spectrofluorometer (Lawrenceville, NJ) equipped with an LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with an integrated igniter, switchable 814 photon-counting/analog photomultiplier detection unit, and MD5020 motor driver. Samples for emission measurements were contained in quartz cuvette with path length of 1 cm, 1.5 ml cell volume (Starna, Atascadero, CA).

Millipore water was used to prepare all aqueous solutions. Spectroscopic measurements were performed in 50 mM Tris buffer at pH 7.4. A typical sample for fluorescence measurement of CP1 in the presence of 20 eq. of Co^{2+} was prepared as follow: 1 μl of a stock solution of CP1 (1 mM in DMSO) was added to 50 mM Tris, followed by 1 μl of 20 mM $CoCl_2$ and 500 μl of 10 mM glutathione (GSH) to a final volume of 1000 μl to give a solution of CP1, Co^{2+} and GSH at final concentrations of 1 μM, 20 μM and 5 mM respectively. The solution was mixed well with a plastic disposable pipette after addition of each reagent and at 5 minutes intervals until the emission spectrum was recorded. For fluorescence measurements at 10, 1, 0.5, and 0.1 equivalence of Co^{2+}, 1 μl each of $CoCl_2$ stock solutions at 10 mM, 1 mM, 0.5 mM and 0.1 mM were added. For metal selectivity study, aqueous metal solutions of NaCl, KCl, $MgCl_2{\cdot}6H_2O$, $CaCl_2{\cdot}2H_2O$, $MnCl_2{\cdot}4H_2O$, $FeCl_2$, $Fe(NO_3)_3{\cdot}9H_2O$, $NiCl_2{\cdot}6H_2O$, $CuCl_2{\cdot}2H_2O$ and $ZnCl_2$ and MeCN solution of $Cu(MeCN)_4PF_6$ at 20 mM were used. For fluorescence measurement in the absence of O_2, the Tris buffer was degassed by bubbling N_2 for 30 minutes, and all the solutions were prepared from the degassed buffer. For study of the role of GSH, 10 mM GSH solution was replaced by either 50 mM Tris buffer or 10 mM of glutathione disulfide. Excitation was at 470 nm and emission spectra were collected from 480–700 nm.

3. ESI-MS product analysis

Samples for ESI-MS analysis were prepared as followed: 1 μl of a stock solution of CP1 (1 mM in DMSO) was added to 50 mM Tris at pH 7.4, followed by 1 μl of $CoCl_2$ (20 mM in the same buffer) to a final volume of 1 ml. The solution was mixed well with a plastic disposable pipette at 10 minutes intervals for 2 hours, which was then injected to a LC-MS (Agilent Technology 6130, Quadrupole LC/MS) and analyzed by flow injection analysis.

4. Cell culture and confocal microscopy

Cells were grown in the UCB Tissue Culturing Facility with technical assistance from Ann Fisher and Isabel Ribeiro. A549 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone), and incubated at 37°C in 5% CO_2. One or two days before imaging, the cells were passaged and plated in phenol red-free medium on 12 mm glass coverslips in 24-well plates (Corning, Corning NY), and allowed to grow to 50–70% confluence. Cobalt uptake was performed in the same medium

with supplementation with $CoCl_2$ at 100 μM for 20 hours. Hypoxic conditions were mimicked by incubation with 500 μM DMOG or 50 μM DFO in PBS. Immediately before staining, the cells were washed twice with 200 μM EDTA in PBS. A solution of CP1 in DMSO (1 mM) was diluted into DMEM at 1 μM, added to the cells and incubated for 3 hours. Before imaging, the coverslips were removed from the 24-well plate, rinsed in PBS, and plated on a glass slide. For nuclear staining, cells were incubated with 1 μM Hoechst at 37°C for 30 min prior to imaging.

Confocal fluorescence imaging studies were performed with a Zeiss laser scanning microscope 710 with a 40x water-immersion objective lens, with Zen 2009 software (Carl Zeiss). Tokyo Green was excited at 488 nm with an Ar laser, and emission collected using a META detector between 500 and 625 nm. Hoechst 33342 was excited with a 405 nm diode laser, and emission collected using a META detector between 410 and 490 nm. The cells were imaged at 37°C and 5% CO_2 throughout the course of the experiment. Image analysis was performed using ImageJ (National Institute of Health).

5. ICP-MS analysis of cobalt content in cell lysates

Inductively-coupled plasma mass spectrometry (ICP-MS) was performed in a Perkin Elmer Sciex Eland DRCII ICP-MS in the standard mode at the Earth Sciences Division, Lawrence Berkeley National Laboratory.

A549 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone), and incubated at 37°C in 5% CO_2. Two days before imaging, the cells were passaged and plated in phenol red-free medium in 6-well plates (Corning), and allowed to grow to confluence. For the final 20 hours of the incubation period, the medium was supplemented with 100 μM $CoCl_2$. Cells were washed 3 times with PBS and scraped into RIPA buffer, in which they were incubated for 30 min on ice. An aliquot (1 μl) was taken for protein quantification by bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL), and the remainder was heated to 90°C with an equal volume of 40% nitric acid. The samples were diluted in 2% nitric acid and analyzed against a calibration curve of known cobalt concentrations. An internal gallium standard (20 μg/L) was added to each sample and standard.

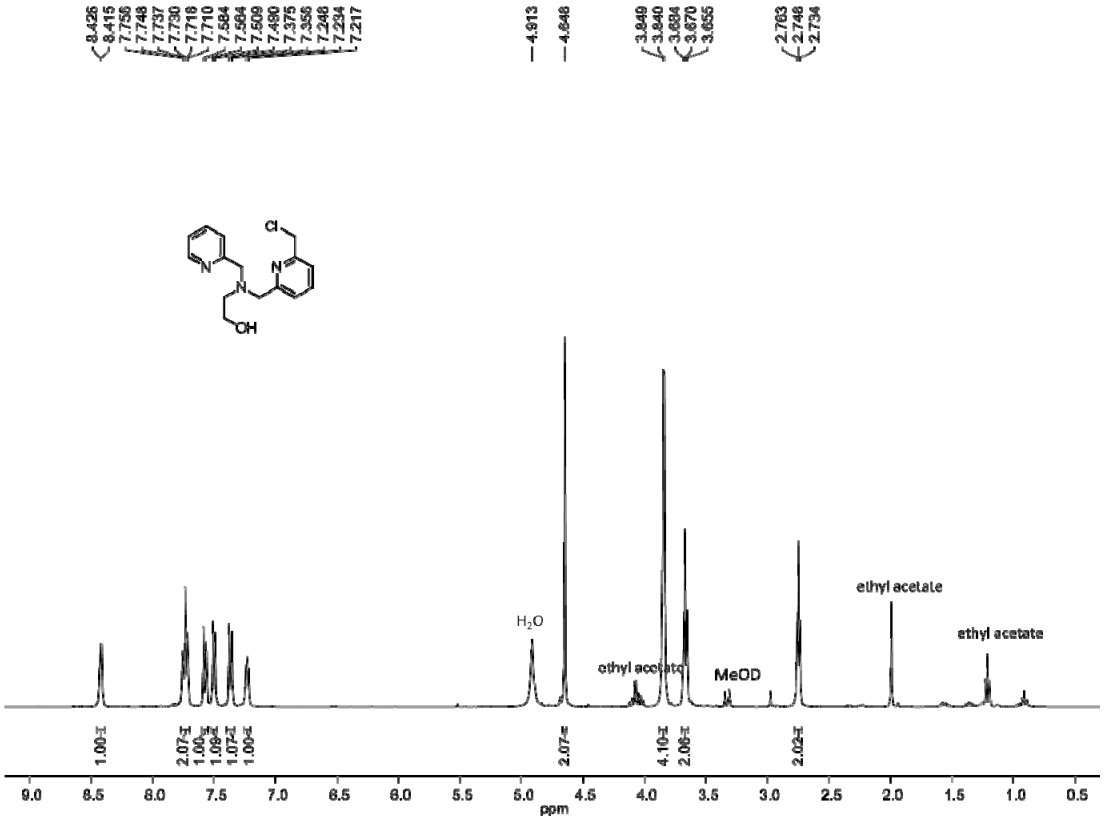


Figure S1. ^{1}H NMR (CD_3OD, 400 MHz, 298 K) of receptor **2**.

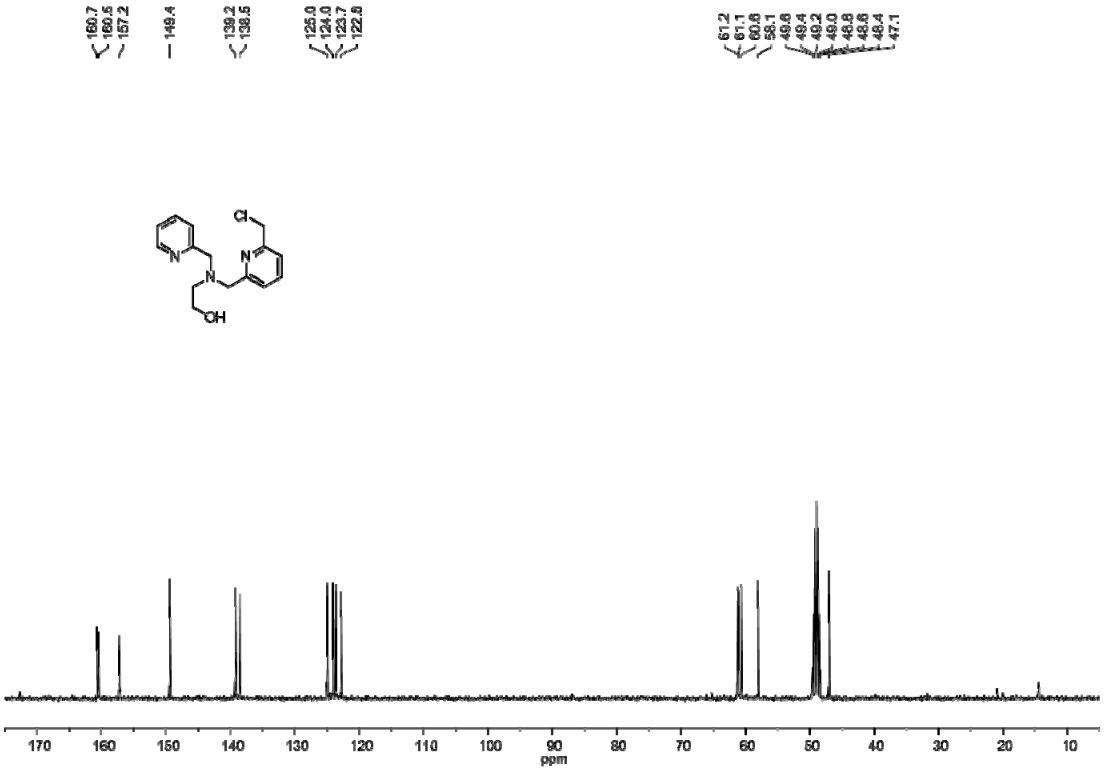


Figure S2. ^{13}C NMR (CD_3OD, 100.6 MHz, 298 K) of receptor **2**.

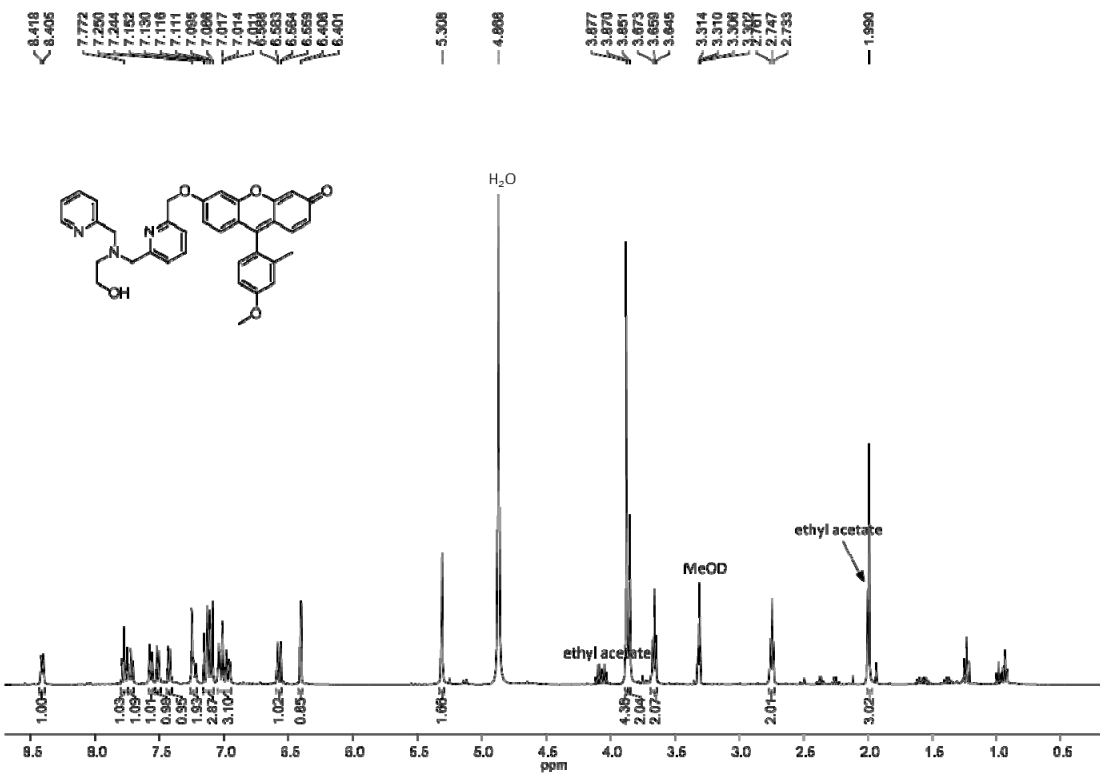


Figure S3. ^{1}H NMR (CD_3OD, 400 MHz, 298 K) of Cobalt Probe 1 (CP1).

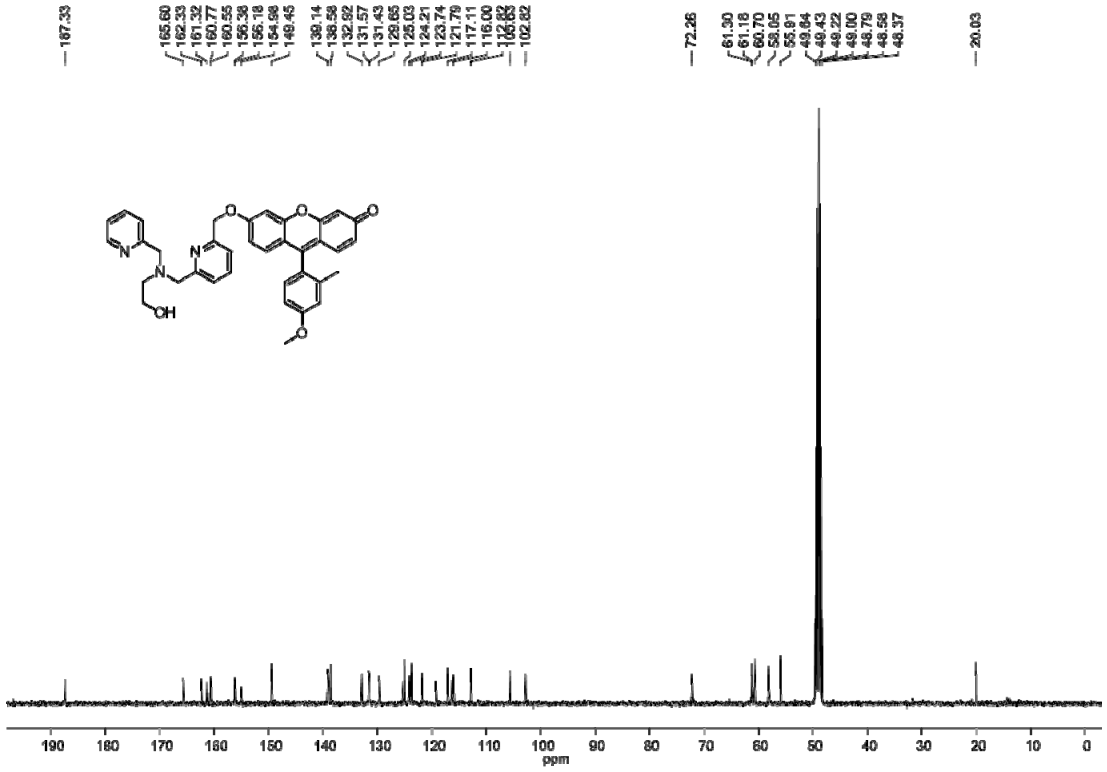


Figure S4. ^{13}C NMR (CD_3OD, 100.6 MHz, 298 K) of Cobalt Probe 1 (CP1).

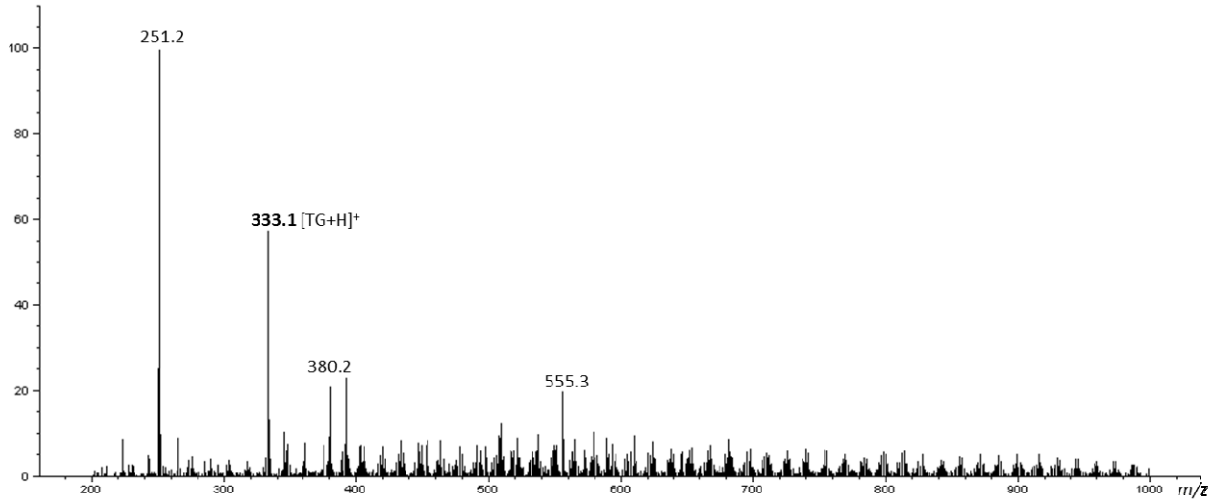
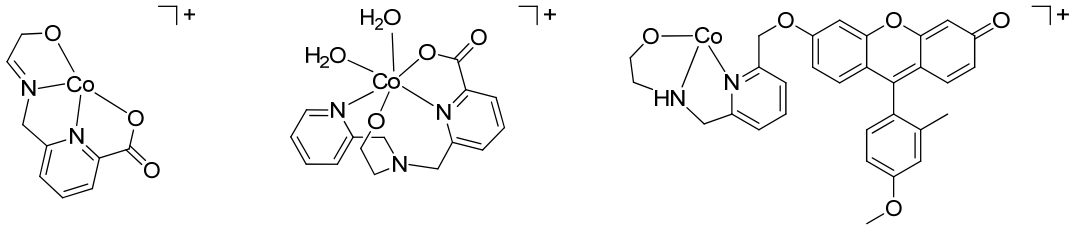


Figure S5. ESI-MS (+ve) analysis of the reaction mixture of 1 μM CP1 and 20 μM Co^{2+} under aerobic conditions. Uncaged Tokyo Green (TG) dye was observed with *m/z* 333.1; peaks at *m/z* 251.2, 380.2 and 555.3 could be attributed to other oxidation products and their cobalt complexes.

Speculative structures of other observed ions:



calculated *m/z*: 251.0 380.1 555.1

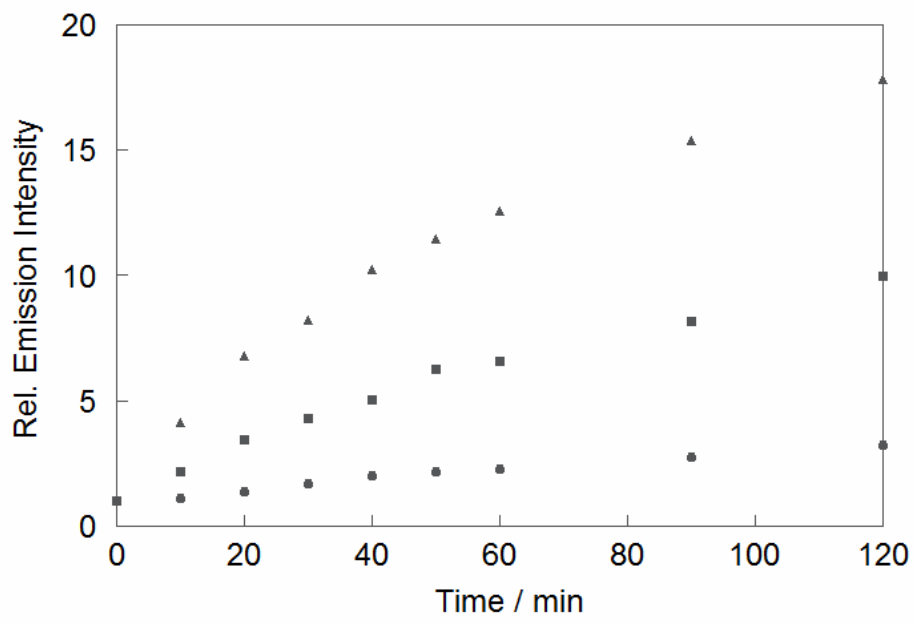


Figure S6. Time-course plots of relative emission intensities at 508 nm of 1 μM CP1 incubated with 20 μM of Co^{2+} in Tris buffered to pH 7.4 under aerobic conditions with (▲) and without (■) 5 mM GSH, and with 5 mM GSH under anaerobic conditions (●). Excitation was provided at 470 nm.

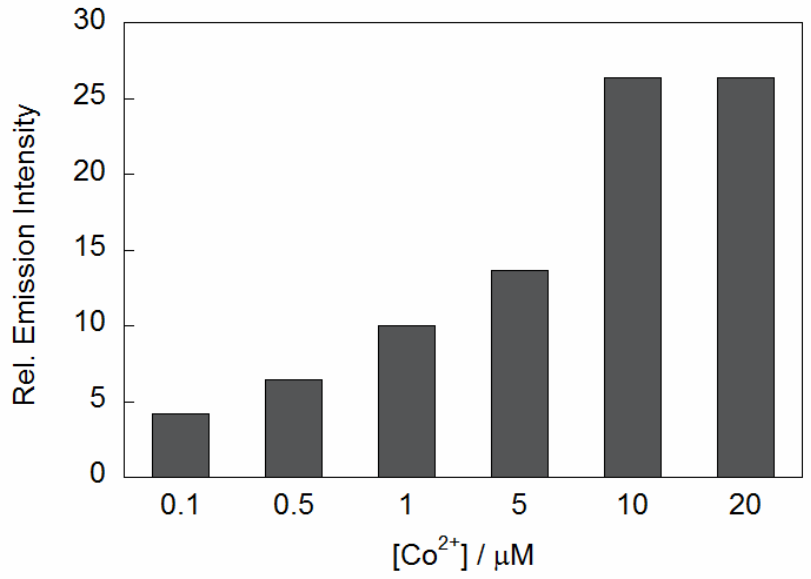


Figure S7. Relative emission intensities at 508 nm of 1 μM CP1 in Tris buffered to pH 7.4 with 5 mM GSH, incubated with different concentrations of Co^{2+} for 2 hours at room temperature. Excitation was provided at 470 nm.

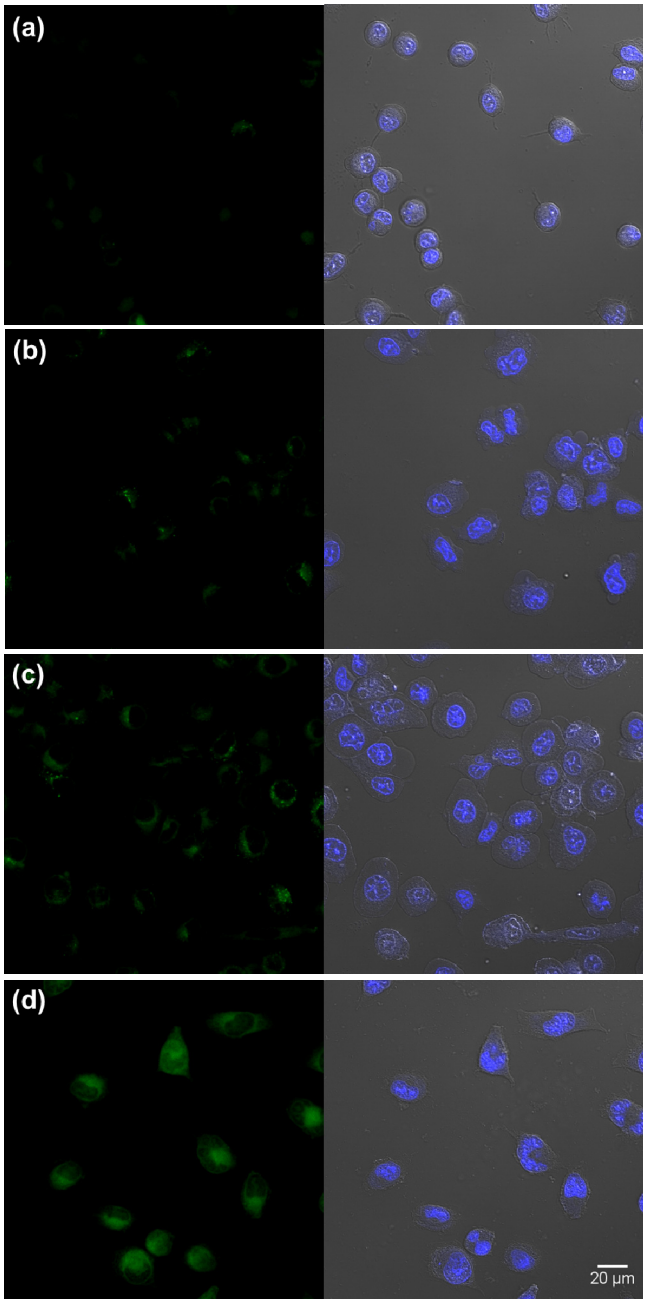


Figure S8. Confocal microscopy images of A549 cells supplemented with (a) 0; (b) 5; (c) 25; and (d) 100 µM $CoCl_2$ for 20 hours. Before imaging, the cells were incubated with 10 µM CP1 for 3 hours and Hoechst 33342 for 30 min at 37 °C. Fluorescence is shown in the left panels. Overlaid brightfield images with Hoescht 33342 staining are shown in the right panels. Excitation and emission wavelengths were as indicated in the methods section.

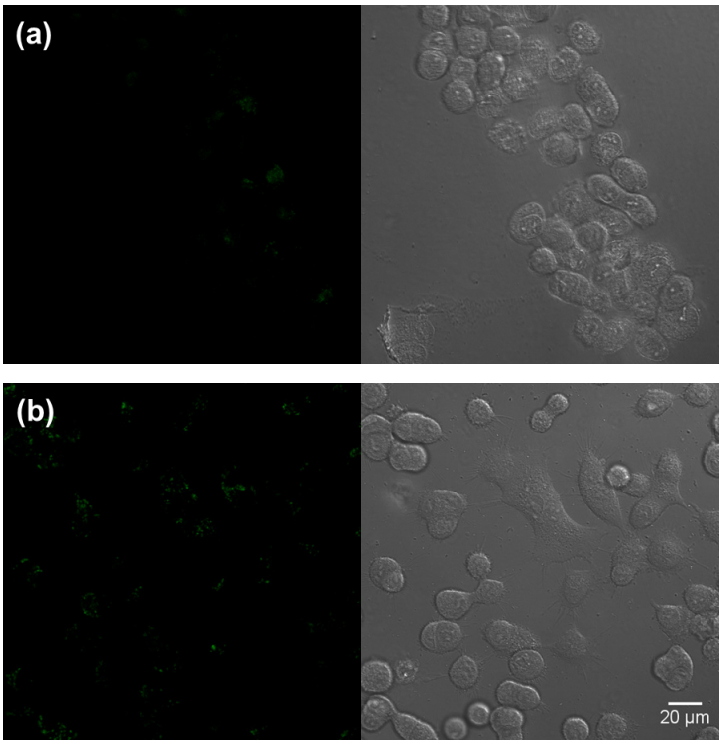


Figure S9. Confocal microscopy images of A549 cells incubated with (a) 50 µM DFO; and (b) 0.5 mM DMOG 20 hours and stained with 10 µM CP1 for 3 hours at 37 °C. Fluorescent and brightfield images are shown in the left and right panels respectively. Excitation and emission wavelengths were as indicated in the methods section.

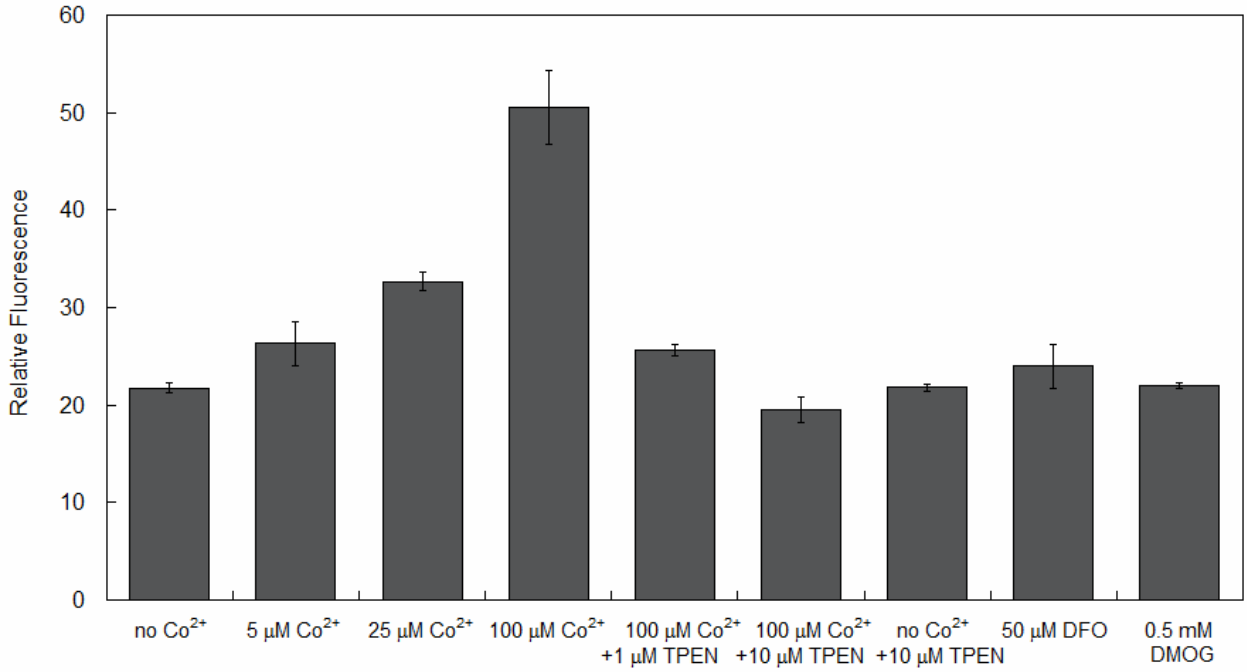


Figure S10. Relative fluorescence values of confocal microscopy images of A549 cells treated under different conditions for 20 hours and stained with 10 μM CP1 for 3 hours at 37 °C. Error bars represent standard deviations (n = 3).

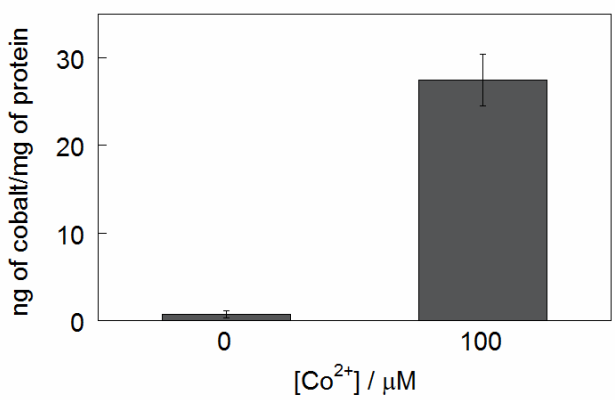


Figure S11. ICP-MS analysis of cobalt content in cell lysates of A549 cells grown in a basal medium and supplemented with 100 µM $CoCl_2$ for 20 hours at 37 °C. Error bars represent standard deviations (n = 3).