

Electronic Supplementary Information for

Direct ratiometric detection of nitric oxide with Cu(II)-based fluorescent probes

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TABLE OF CONTENTS

Experimental Section	S2
Table S1. Photophysical parameters of FL3A-P ₉ K-CC and FL3A-Ppz-CC	S12
Table S2. Comparison of the reduction potentials	S12
Figures S1-S11. NMR spectra	S13
Figures S12-S15. HRMS spectra	S19
Figure S16. Analytical HPLC traces	S21
Figure S17. Analytical HPLC traces and ESI-MS detection of the major degradation products of FL3A-P ₉ K-CC and FL3A-Ppz-CC in deionized water	S22
Figure S18. Representative UV-Vis spectra for titration of the ligands with CuCl ₂	S22
Figure S19. Normalized fluorescence emission spectra and ratiometric response of Cu(FL3A-P ₉ K-CC) vs added equiv of the NO donor DEA NONOate	S23
Figure S20. Plots of fluorescence emission over time upon addition of NO	S23
Figure S21. ESI-MS detection of the reaction product of Cu(FL3A-Ppz-CC) with NO	S24
Figure S22. Selectivity of Cu(FL3A-P ₉ K-CC) and Cu(FL3A-Ppz-CC) monitored by the individual fluorescence turn-on of the hydroxycoumarin and fluorescein fluorophores	S25
Figure S23. Fluorescence kinetic traces of Cu(FL3A-P ₉ K-CC) and Cu(FL3A-Ppz-CC) upon addition of cysteine, Angeli's salt, glutathione, and peroxynitrite	S26
Figure S24. Cyclic voltammograms of metal-free and Cu(II)-bound FL3A-Ppz-CC	S26
Figure S25. Analytical HPLC traces and ESI-MS detection of the reaction products of Cu(FL3A-Ppz-CC) with cysteine and glutathione in water	S27
Figure S26. Fluorescence microscopy of HeLa cells incubated with Cu(FL3A-P ₉ K-CC) ...	S28
Figure S27. Fluorescence microscopy of HeLa cells incubated with Cu(FL3A-Ppz-CC) ...	S28
Figure S28. Fluorescence microscopy of A549 cells incubated with Cu(FL3A-Ppz-CC) ...	S29
Figure S29. Fluorescence microscopy of HeLa cells incubated with Cu(FL3A-Ppz-CC) with or without treatment with <i>N</i> -methylmaleimide	S29
References	S30

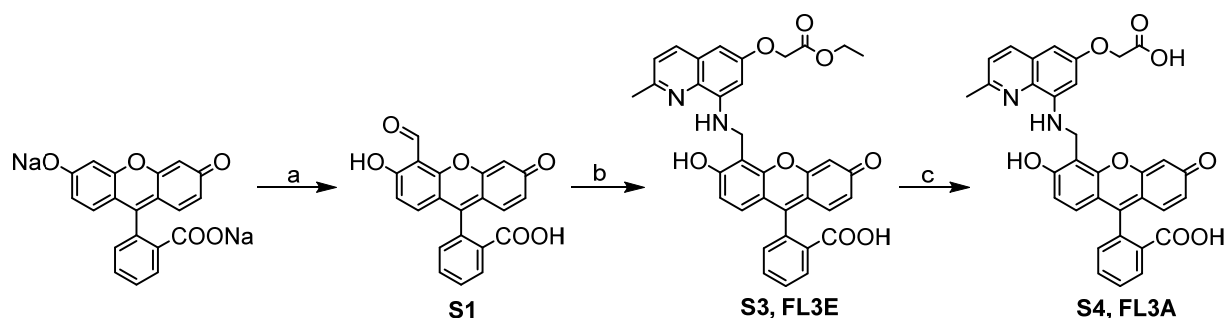
Experimental Section

General Methods. Syntheses involving air-sensitive compounds were performed using a Schlenk line. Dry inert gas was provided by an in-house nitrogen line. Reaction mixtures were monitored by thin-layer chromatography (TLC) on pre-coated, aluminum-backed silica gel 60 F₂₅₄ plates. Chromatographic separations were performed on silica gel 60 (230-400 mesh). HPLC separations were carried out at the semi-preparative scale on an Agilent 1200 Series system equipped with multi-wavelength detector and automated fraction collector, using a reverse stationary phase column (Zorbax SB-C18, 5 μ m, 9.5 mm $\times$ 250 mm). Analytical HPLC was performed on a similar system using a Zorbax SB-C18 reverse stationary phase column (5 μ m, 4.6 mm $\times$ 250 mm). FT-NMR spectra were acquired at ambient temperature on a Bruker Avance III 400 or a Varian Inova 500 instrument. ¹³C NMR spectra were acquired with proton decoupling. ¹H and ¹³C chemical shifts are reported in ppm relative to SiMe₄ (δ = 0.00) and were referenced internally to the residual solvent signals.¹ Low-resolution mass spectra (LRMS) were acquired on an Agilent 1100 Series LC/MSD Trap spectrometer by electrospray ionization (ESI). The MIT Department of Chemistry Instrumentation Facility acquired high-resolution mass spectra (HRMS) in the ESI mode on a Bruker Daltonics APEXIV 4.7 T FT-ICR-MS instrument. The pH measurements were performed with a Mettler Toledo FE20 pH meter. Electronic absorption spectra were collected on a Varian Cary 50 Bio UV-vis spectrophotometer. Fluorescence spectra were acquired on a Quanta Master 4 L-format scanning spectrofluorimeter (Photon Technology International). The samples for UV-visible and fluorescence spectroscopy were prepared as dilute (1-20 μ M) solutions of the analyte in 1.0 cm quartz cuvettes.

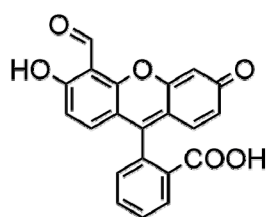
Peptide Syntheses. Solid-phase peptide syntheses were carried out using an Aapptec Focus Xi automated peptide synthesizer. Fmoc groups were removed by treating the resin with a solution of 20% (v/v) piperidine in DMF for 15 min. For coupling reactions, 4 equiv of an Fmoc-protected amino acid were combined as a solid with 4 equiv of solid HBTU. Immediately prior to the coupling reaction, the mixture was dissolved in a freshly prepared solution of 10% (v/v) DIPEA in anhydrous DMF. The resin was allowed to react in the coupling solution for 30 min. Peptides were cleaved from the resin by treatment with a TFA/water/triisopropylsilane cocktail (2 mL, 95:2.5:2.5 v/v/v) for 2 h.

Materials. All solvents employed were ACS reagent grade or higher. Rink Amide AM resin (100-200 mesh) and Fmoc-Lys(Mtt)-OH were obtained from EMD Millipore. Fmoc-Pro-OH and 1-hydroxybenzotriazole (HOBt) were obtained from NovaBiochem. 2-(7-Aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HATU) and *N,N,N',N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) were procured from Oakwood Chemicals. *N,N*-Diisopropylethylamine (DIPEA, Aldrich) was distilled fresh before use and stored in a nitrogen-filled Schlenk flask under 3 Å molecular sieves. Ethyl (2-methyl-8-aminoquinolin-6-yloxy)acetate (**S2**)² and 7-hydroxycoumarinyl-3-carboxylic acid (HCCA, **S5**)³ were produced according to published procedures. All other reagents were purchased from commercial sources and used as received.

Scheme S1. Synthesis of FL3A (**S4**)[†]

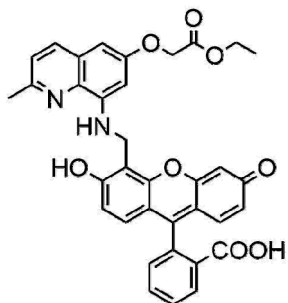


[†]Conditions: (a) CHCl₃, NaOH aq, MeOH, 55 °C, 5 h; (b) 1.1 equiv of **S2**, MeOH, 40 °C, 2 h, then 10 equiv NaBH₄, MeOH, room temperature, 18 h; (c) 0.5 M NaOH aq, MeOH, 90 °C, 2 h.



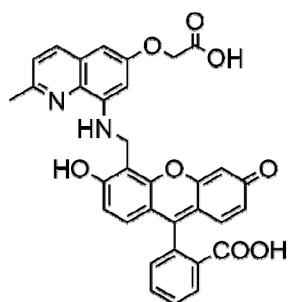
Fluorescein Monoaldehyde (S1). Concentrated aq NaOH (17.8 g, 440 mmol, 40 mL of 33% aq solution) was added with stirring to a suspension of fluorescein disodium salt (11.3 g, 30 mmol) in 10 mL of MeOH. The mixture was brought to 55 °C and CHCl₃ (14.8 g, 124 mmol, 10 mL) was added dropwise over 45 min. The mixture was kept at 55 °C for 5 h, then cooled to room temperature and acidified with 10 M aq H₂SO₄ until a red solid precipitated. The precipitate was filtered, dried under air overnight, and purified by flash chromatography on silica gel (CH₂Cl₂/EtOAc = 85:15 v/v), eluting the compound as the first fraction. Removal of the solvent followed by drying to constant weight in vacuo afforded fluorescein monoaldehyde **S1** as a pale yellow powder in 13% yield (1.36 g). ¹H NMR (500 MHz, (CD₃)₂SO): δ 11.89 (s, 1H, Ar-OH/COOH), 10.63 (s, 1H, Ar-OH/COOH), 10.27 (s, 1H, Ar-CHO), 8.02 (d, *J* = 7.7 Hz, 1H, Ar-

H), 7.81 (t, *J* = 7.7 Hz, 1H, Ar-*H*), 7.73 (t, *J* = 7.5 Hz, 1H, Ar-*H*), 7.32 (d, *J* = 7.5 Hz, 1H, Ar-*H*), 6.95 (d, *J* = 9.0 Hz, 1H, Ar-*H*), 6.85 (d, *J* = 1.7 Hz, 1H, Ar-*H*), 6.71 (d, *J* = 9.0 Hz, 1H, Ar-*H*), 6.62 (m, 2H, Ar-*H*); ^{13}C { ^1H } NMR (125 MHz, $(\text{CD}_3)_2\text{SO}$): δ 192.85, 168.6, 162.9, 159.6, 152.4, 152.2, 150.85, 136.55, 135.8, 130.3, 129.0, 125.9, 124.8, 124.0, 113.5, 113.4, 109.7, 109.2, 109.1, 102.6, 81.8; MS (ESI⁺): *m/z* calcd for $[\text{M} + \text{H}]^+$ ($\text{C}_{21}\text{H}_{13}\text{O}_6$)⁺ 361.1, found 361.1.



2-(6-Hydroxy-5-(((6-(2-ethoxy-2-oxoethoxy)-2-methylquinolin-8-yl)amino)methyl)-3-oxo-3*H*-xanthen-9-yl)benzoic Acid (FL3E, S3).

Ethyl (2-methyl-8-aminoquinolin-6-yloxy)acetate (**S2**, 0.3 g, 1.15 mmol), fluorescein monoaldehyde (**S1**, 0.36 g, 1.00 mmol), and 1 g of anhydrous Na_2SO_4 were suspended in 30 mL of dry MeOH. The mixture was kept at 40 °C under nitrogen with stirring for 2 h. After cooling to room temperature, sodium borohydride (0.4 g, 10.6 mmol) was added in portions over 10 min under nitrogen stream, and the cloudy red solution was kept at room temperature under nitrogen with stirring for 18 h. The insoluble materials were filtered, the filtrate was concentrated to dryness under reduced pressure, and the crude solid was purified by flash chromatography on silica gel ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ = 10:0 to 8:2 v/v), affording FL3E (**S3**) as a dark red powder in 58% yield (0.35 g), a ~1:1.8 mol mixture of the methyl and ethyl ester isomers (by integration of the ^1H NMR spectrum). Partial trans-esterification with MeOH occurs during chromatography. ^1H NMR (400 MHz, $\text{CD}_2\text{Cl}_2/\text{CD}_3\text{OD}$ = 9:1): δ 7.96 (dt, *J* = 7.4, 1.4 Hz, 1H, Ar-*H*), 7.83 (d, *J* = 8.4 Hz, 1H, Ar-*H*), 7.65 (td, *J* = 7.4, 1.4 Hz, 1H, Ar-*H*), 7.62 (td, *J* = 7.4, 1.4 Hz, 1H, Ar-*H*), 7.22 (d, *J* = 8.4 Hz, 1H, Ar-*H*), 7.16 (dt, *J* = 7.4, 1.4 Hz, 1H, Ar-*H*), 6.91 (m, 2H, Ar-*H*), 6.52-6.59 (m, 4H, Ar-*H*), 6.32 (d, *J* = 2.6 Hz, 1H, Ar-*H*), 4.83 (d, *J* = 1.7 Hz, 2H, Ar- CH_2), 4.71 (s, 2H, Ar- OCH_2), 4.27 (q, *J* = 7.1 Hz, 2H, $\text{CO}_2\text{CH}_2\text{CH}_3$), 2.62 (s, 3H, Ar- CH_3), 1.30 (t, *J* = 7.1 Hz, 3H, $\text{CO}_2\text{CH}_2\text{CH}_3$); MS (ESI[−]): *m/z* calcd for $[\text{M} - \text{H}]^-$ ($\text{C}_{34}\text{H}_{25}\text{N}_2\text{O}_8$)[−] 589.2, found 589.2.

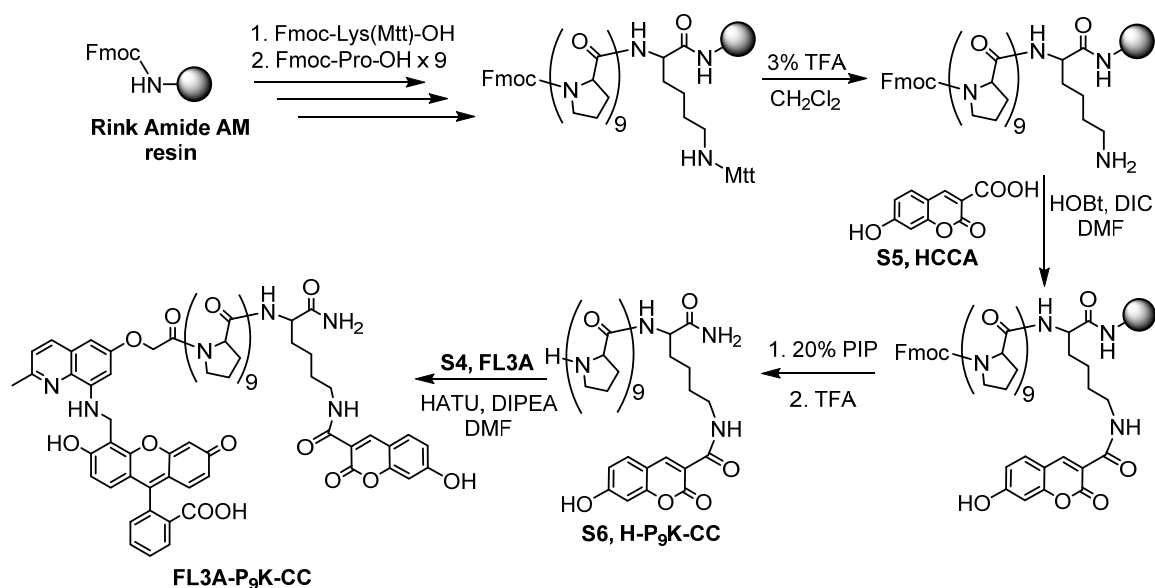


2-(6-Hydroxy-5-(((6-(carboxymethoxy)-2-methylquinolin-8-yl)amino)methyl)-3-oxo-3*H*-xanthen-9-yl)benzoic Acid (FL3A, S4).

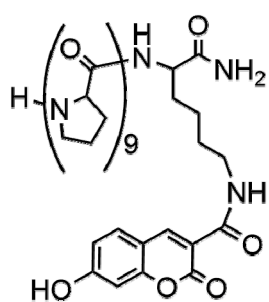
Solid FL3E (**S3**, 0.35 g, 0.58 mmol) was dissolved in a mixture of 4 mL of MeOH and 6 mL of 1.0 M aq NaOH, and heated to 90 °C with stirring for 2 h. The solution was cooled to room temperature and acidified by dropwise addition of conc. aq HCl until an orange

precipitate formed, which was filtered, washed with 50 mL of cold water, and dried under air suction overnight to afford FL3A (**S4**) as an orange powder in 96% yield (0.32 g). ^1H NMR (500 MHz, $(\text{CD}_3)_2\text{SO}$): δ 13.00 (br, 2H, CO_2H), 10.52 (s, 1H, Ar-OH), 7.96 (m, 2H, Ar-H), 7.76 (t, $J = 7.6$ Hz, 1H, Ar-H), 7.70 (t, $J = 7.6$ Hz, 1H, Ar-H), 7.28 (m, 2H, Ar-H), 6.89 (d, $J = 2.2$ Hz, 1H, Ar-H), 6.84 (br, 1H, Ar-NH), 6.69 (m, 2H, Ar-H), 6.53 (m, 3H, Ar-H), 6.38 (d, $J = 2.2$ Hz, 1H, Ar-H), 4.71 (s, 4H, Ar- CH_2/OCH_2), 2.58 (s, 3H, Ar- CH_3); ^{13}C $\{^1\text{H}\}$ NMR (125 MHz, $(\text{CD}_3)_2\text{SO}$): δ 170.2, 168.6, 159.6, 157.5, 156.7, 152.9, 152.1, 151.9, 150.6, 144.7, 135.55, 135.3, 134.0, 130.1, 128.8, 127.4, 127.1, 126.3, 124.65, 124.1, 122.7, 112.75, 112.2, 111.9, 110.1, 109.6, 102.6, 96.6, 92.8, 83.3, 64.4, 35.4, 24.5; HRMS (ESI $^-$): m/z calcd for $[\text{M} - \text{H}]^-$ ($\text{C}_{33}\text{H}_{23}\text{N}_2\text{O}_8$) $^-$ 575.1460, found 575.1468.

Scheme S2. Solid-phase synthesis of the ratiometric peptide ligand FL3A- $\text{P}_9\text{K-CC}^\dagger$

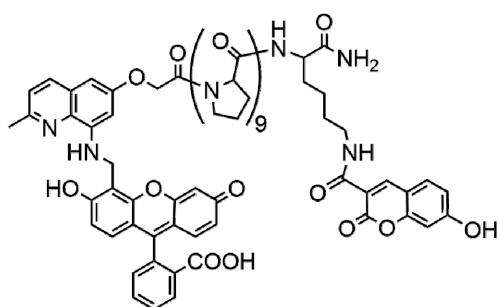


† Abbreviations: Mtt = 4-methyltrityl, HCCA = 7-hydroxycoumarinyl-3-carboxylic acid, HOBt = 1-hydroxybenzotriazole, DIC = N,N' -diisopropylcarbodiimide, PIP = piperidine, TFA = trifluoroacetic acid.



H- $\text{P}_9\text{K-CC}$ (S6**).** The H- $\text{P}_9\text{K-CC}$ peptide was synthesized on the 100 μmol scale using an automated peptide synthesizer, starting from Rink Amide AM resin (130 mg, 0.77 mmol/g loading) and employing 4 equiv of each Fmoc-protected amino acid under HBTU standard coupling conditions. Before the coupling of the coumarin fluorophore, the resin was transferred to a 20 mL peptide synthesis vessel and a solution of

HCCA (**S5**, 62 mg, 300 μmol), HOBt (45 mg, 330 μmol), and DIC (44 mg, 350 μmol , 55 μL) in 4 mL of DMF was added, and the reaction was allowed to complete with stirring at room temperature overnight. The construct was cleaved from the resin by treatment with 2 mL of a TFA/water/triisopropylsilane 95:2.5:2.5 (v/v/v) solution for 90 min, followed by washing the resin with 2×2 mL of acetonitrile and concentrating the combined filtrates to dryness. The crude yellow solid was purified by semi-preparative reverse phase HPLC using the (A) water (0.1% v/v TFA) / (B) CH_3CN (0.1% v/v TFA) solvent system, according to the following protocol: constant flow rate 3 mL min^{-1} ; 5 min, isocratic flow 20% B; 10 min, linear gradient 20-40% B; product retention time: 11.7 min. The equivalent fractions from independent runs were combined and lyophilized to afford the TFA salt of H-P₉K-CC (**S6**) as a white powder in 27% yield (35.0 mg). The purity was judged to be >95% by analytical HPLC, employing the solvent system above according to the following protocol: constant flow rate 1 mL min^{-1} ; 5 min, isocratic flow 10% B; 25 min, linear gradient 10-65% B; retention time: 16.0 min. HRMS (ESI⁺): m/z calcd for $[\text{M} + \text{H}]^+$ ($\text{C}_{61}\text{H}_{83}\text{O}_{12}\text{N}_{14}$)⁺ 1207.6146, found 1207.6121; calcd for $[\text{M} + 2\text{H}]^{2+}$ ($\text{C}_{61}\text{H}_{84}\text{O}_{12}\text{N}_{14}$)²⁺ 604.3109, found 604.3097.

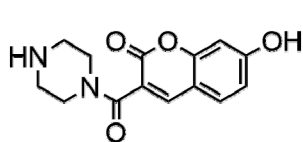
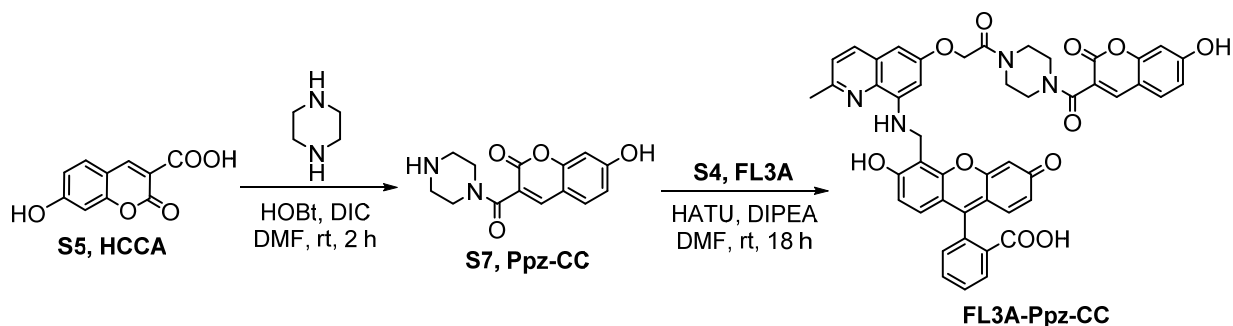


FL3A-P₉K-CC. FL3A (**S4**, 11.5 mg, 20 μmol) and HATU (8.0 mg, 20 μmol) were dissolved in 1.5 mL of a 10% (v/v) solution of DIPEA in anhydrous DMF and mixed for 10 min at room temperature. This solution was added to solid H-P₉K-CC (**S6**, 12.1 mg, 10 μmol) and the mixture was kept at room temperature with

stirring for 2 h. The reaction was quenched with 20 mL of deionized water and the mixture was lyophilized to give a crude brown solid, which was purified by semi-preparative reverse phase HPLC using the (A) water (0.1% v/v TFA) / (B) CH_3CN (0.1% v/v TFA) solvent system, according to the following protocol: constant flow rate 3 mL min^{-1} ; 5 min, isocratic flow 22% B; 20 min, linear gradient 22-50% B; product retention time: 20.0 min. The equivalent fractions from independent runs were combined and lyophilized to afford the TFA salt of the FL3A-P₉K-CC peptide as an orange solid in 11% yield (4.2 mg). The purity was judged to be ~87% by analytical HPLC, employing the solvent system above according to the following protocol: constant flow rate 1 mL min^{-1} ; 5 min, isocratic flow 10% B; 30 min, linear gradient 10-50% B;

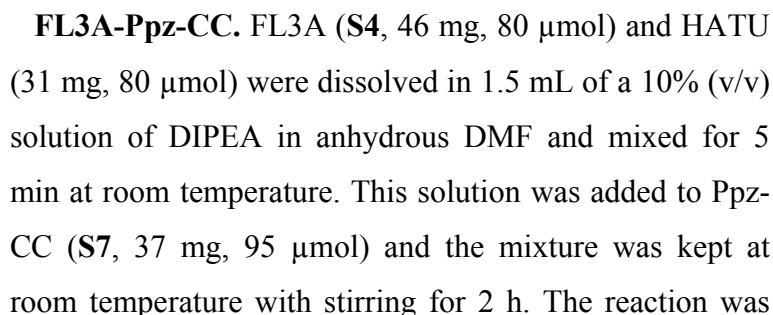
retention time: 25.2 min. HRMS (ESI⁺): m/z calcd for $[M + H]^+$ ($C_{94}H_{105}N_{14}O_{21}$)⁺ 1765.7573, found 1765.7517; calcd for $[M + 2H]^{2+}$ ($C_{94}H_{106}N_{14}O_{21}$)²⁺ 883.3823, found 883.3806.

Scheme S3. Synthesis of the piperazine-based ratiometric ligand FL3A-Ppz-CC



7-Hydroxy-3-(piperazine-1-carbonyl)-2H-chromen-2-one (Ppz-CC, S7). HCCA (S5, 53 mg, 0.25 mmol), HOBt (38 mg, 0.28 mmol), and DIC (35 mg, 0.28 mmol, 45 μ L) were dissolved in 1 mL of

anhydrous DMF and mixed for 3 min at room temperature. This solution was added dropwise to excess piperazine (170 mg, 2.0 mmol) suspended in 1 mL of dry DMF and the mixture was kept at room temperature with stirring overnight. The reaction was quenched with 20 mL of deionized water and the mixture was lyophilized to give a crude dark yellow solid, which was purified by semi-preparative reverse phase HPLC using the (A) water (0.1% v/v TFA) / (B) CH₃CN (0.1% v/v TFA) solvent system, according to the following protocol: constant flow rate 3 mL min⁻¹; 5 min, isocratic flow 10% B; 8 min, linear gradient 10-18% B; product retention time: 10.8 min. The equivalent fractions from independent runs were combined and lyophilized to afford the TFA salt of Ppz-CC (S7) as a pale yellow solid in 38% yield (37 mg). The purity was judged to be ca. 94% by analytical HPLC, employing the solvent system above according to the following protocol: constant flow rate 1 mL min⁻¹; 5 min, isocratic flow 10% B; 25 min, linear gradient 10-65% B; retention time: 7.9 min. ¹H NMR (400 MHz, (CD₃)₂CO with 5% v/v CD₃COOD): δ 8.07 (s, 1H, Ar-*H*), 7.59 (d, J = 8.5 Hz, 1H, Ar-*H*), 6.91 (dd, J = 8.6, 1.9 Hz, 1H, Ar-*H*), 6.81 (d, J = 1.8 Hz, 1H, Ar-*H*), 3.97 (br, 2H, Ppz-CH₂), 3.78 (br, 2H, Ppz-CH₂), 3.32 (br, 4H, Ppz-CH₂); MS (ESI⁺): m/z calcd for $[M + H]^+$ ($C_{14}H_{15}N_2O_4$)⁺ 275.1, found 275.2.



Spectroscopic Methods. All aqueous solutions were prepared using deionized water with a resistivity of 18.2 MΩ·cm, obtained with a Milli-Q water purification system (Millipore). Phosphate-buffered saline (PBS, 1×) solution was obtained from Corning. Nitric oxide gas (Airgas) was purified by passing through an Ascarite (NaOH fused on silica gel) column and a 6 ft silica gel-filled coil cooled at −78 °C, and was collected in glass Schlenk storage bulbs which were subsequently sealed and stored under inert atmosphere in a glove box. Sodium α -oxyhyponitrite (Na₂N₂O₃, Angeli's salt) was prepared according to a previously published procedure.⁴ *S*-Nitroso-*N*-acetyl-DL-penicillamine (SNAP), *S*-nitrosoglutathione (GSNO), diethylammonium (Z)-1-(*N,N*-diethylamino)diazene-1,1,2,2-tetrolate (DEA NONOate), and sodium peroxyxynitrite (NaONOO, ~30 mM solution in 0.3 M NaOH aq) were purchased from

Cayman Chemical. All other reagents for the spectroscopic studies were purchased from Sigma-Aldrich. Spectroscopic measurements were conducted in aqueous PBS buffer (10 mM PO_4^{3-} , 137 mM NaCl, 2.7 mM KCl, pH 7.4). Ultra-dry, high-purity 99.995% CuCl_2 (Alfa Aesar) was used to prepare 0.1 M volumetric solutions anaerobically in deionized water. CuCl_2 solutions of lower concentrations were prepared by dilution of 0.1 M volumetric solutions. Stock solutions (2.0 or 5.0 mM) of the metal-free probes were prepared in spectroscopic grade DMSO, stored at -40°C in 50 or 100 μL aliquots, and thawed immediately before use. For all spectroscopic and cell imaging studies, stock solutions of the Cu(II) complexes were prepared in situ by combining a DMSO solution of the fluorescent ligand with 20 mM CuCl_2 aq in a 1:1.2 molar ratio 30 min prior to the start of the measurements. All measurements were conducted at 25.0 or 37.0°C , maintained by a circulating water bath. Extinction coefficients (ϵ) and fluorescence quantum yields (Φ) were determined in the $0.5\text{--}5\ \mu\text{M}$ range in aqueous buffer solutions at pH 7.4. In determining the fluorescence quantum yields of the metal-free ligands and Cu(II) -bound probes, excitation was provided at 490 nm, the emission spectra were integrated from 495 to 650 nm, and the calculations were standardized to fluorescein ($\lambda_{\text{em}} = 521\ \text{nm}$, $\lambda_{\text{ex}} = 490\ \text{nm}$, and $\Phi = 0.95$ in 0.1 M aq NaOH).⁵ All measurements were repeated in triplicate.

Analyte Selectivity Studies. The analyte selectivity profiles of the Cu(II) -bound probes were determined by comparing the fluorescence emission spectra of $2\ \mu\text{M}$ solutions of the sensors in PBS aqueous buffer at pH 7.4, before and after treatment with 250 equiv of aq ZnSO_4 , NaNO_2 , KNO_3 , H_2O_2 , NaClO , sodium L-ascorbate, sodium glutamate, L-methionine, L-cysteine, glutathione, Na_2S , NaONOO , and Angeli's salt. Reactivity towards HO^- was studied in aq 10 mM NaOH. Stock solutions of Angeli's salt and DEA NONOate in aq 10 mM NaOH were prepared anaerobically ($\text{O}_2 < 2\ \text{ppm}$) under nitrogen atmosphere in a glove box dedicated to work with aqueous solutions ("wet box"), and brought out of the wet box in gastight syringes. For the NO, HNO, and peroxynitrite selectivity studies, the samples were prepared anaerobically in the wet box and brought out in sealed airtight quartz cuvettes. NO gas, ~ 1000 equiv, was removed from the side arm of a storage bulb inside the wet box with a gastight syringe and injected into the headspace of each airtight cuvette before measuring the fluorescence response. The fluorescence emission spectra were recorded before and 15 min after addition of the analyte. The integrated fluorescence ratio was normalized in each case to the initial ratio determined

before addition of the analyte. Excitation was provided at 400 nm and the emission intensity used in the calculation of the fluorescence response ratio was integrated between 410-480 nm (for the hydroxycoumarin moiety, F_{HC}) and 481-650 nm (for the fluorescein moiety, F_{FL}). For measurements of the intensity-based response of the fluorescein chromophore alone, excitation was provided at 490 nm, and the fluorescence emission was integrated between 495-650 nm. All measurements were repeated in triplicate.

Apparent Cu(II) Dissociation Constants. The apparent Cu(II) dissociation constants (K_d) for the binding sites of FL3A-P9K-CC and FL3A-Ppz-CC were determined by titration of aqueous 2 mM CuCl₂ into 2.0 mL of pH 7.4-buffered 5 or 7 μM ligand solutions, respectively, up to a final Cu²⁺ concentration of 70 μM . The formation of the Cu(II) complexes was monitored by the absorbance changes (ΔA) at 495 and 499 nm, respectively. The absorbance change was plotted against the total Cu²⁺ concentration and fitted to the one-step binding equilibrium equation:⁶

$$\Delta A = \frac{\Delta A_{\infty} \times ([\text{Cu}^{2+}]_{\text{total}} + [\text{L}]_{\text{total}} + K_d - \sqrt{([\text{Cu}^{2+}]_{\text{total}} + [\text{L}]_{\text{total}} + K_d)^2 - 4 \times [\text{L}]_{\text{total}} \times [\text{Cu}^{2+}]_{\text{total}}})}{2 \times [\text{L}]_{\text{total}}}$$

where ΔA_{∞} is the maximum absorbance change, $[\text{L}]_{\text{total}}$ is the total ligand concentration, and $[\text{Cu}^{2+}]_{\text{total}}$ is the total concentration of Cu²⁺ titrated in the solution. Under these conditions, the fit parameters obtained were: $K_d = 0.18 \pm 0.04$ and 0.37 ± 0.08 μM , with $R^2 = 0.9960$ and 0.9938 for FL3A-P9K-CC and FL3A-Ppz-CC, respectively.

Cyclic Voltammetry. Cyclic voltammograms were measured using a three-electrode setup with a 2.0 mm diameter glassy carbon working electrode, a platinum auxiliary electrode, and a Ag⁺/Ag pseudoreference electrode in acetonitrile. The solvent contained 0.1 M *n*-Bu₄NPF₆ as the supporting electrolyte. The measurements were performed at ambient temperature with a VersaSTAT 4 potentiostat (Princeton Applied Research). Measurements were carried out at scan rates of 50 to 1000 mV s⁻¹ on ~1 mM quiescent solutions of the analytes sparged with dry nitrogen for 5 min. Data were referenced internally to the Fc⁺/Fc couple, added to the solution at the end of the measurements.

Cell Culture and Staining Procedures. HeLa and A549 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Corning cellgro), supplemented with 10% heat-deactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin, at 37 °C in a humidified atmosphere with 5% CO₂. Cells were plated in 35 mm glass-bottom, poly-D-lysine coated culture dishes with

14 mm opening (MatTek) 24–48 h before imaging. All cells used were at a passage number from 5 to 15, and the experiments were repeated on cells from at least two separate frozen stocks at different passages. Cells were 40–60% confluent at the time of imaging and were incubated with the sensors at 37 °C for 30 min before mounting to the microscope. For all cell imaging studies, stock solutions of the Cu(II) complexes of the probes were prepared in situ by combining a DMSO stock solution of the fluorescent ligand with aq 20 mM CuCl₂ in a 1:1.2 molar ratio 30 min prior to incubation. The growth medium was replaced with 2 mL of fresh DMEM containing the sensor before incubation. In order to block intracellular free thiols, cells were pre-incubated with *N*-methylmaleimide (TCI, final concentration 1 mM) in PBS buffer for 30 min, followed by rinsing with 2 mL of PBS prior to sensor incubation in PBS. Cells were rinsed with sterile PBS buffer (2 × 2 mL) to remove excess unbound dyes. For all imaging experiments, cells were bathed in 2 mL of PBS buffer (pH 7.4) before mounting on the microscope stage. After the initial sets of images were acquired, intracellular fluorescence changes were measured following addition to the PBS buffer of concentrated aqueous stock solutions of selected NO donors (GSNO, DEA NONOate, or SNAP) to reach a final concentration of 1.0–1.5 mM in the imaging dish. Stock solutions of DEA NONOate (in aq 10 mM NaOH), GSNO (in PBS buffer), and SNAP (in PBS buffer) were prepared in degassed solvents immediately prior to addition on the microscope stage.

Fluorescence Microscopy. Fluorescence imaging experiments were carried out on a Zeiss Axiovert 200M inverted epifluorescence microscope equipped with a Hamamatsu EM-CCD C9100 or a Hamamatsu ImageEM X2 digital camera and an MS200 XY Piezo Z stage (Applied Scientific Instruments). An XCite 120 metal halide lamp (EXFO) was used as the light source. Zeiss standard filter sets 49 and 38 HE were employed for acquiring images in the blue and green channels, respectively. The microscope was operated with the aid of the Volocity software (version 6.01, Perkin Elmer). The exposure time and sensitivity were kept constant for each channel upon acquiring images of a given cell culture dish. The ImageJ software (version 1.47, NIH) was employed for quantification of intracellular fluorescence intensity. For each measurement, the whole cell was selected as region of interest and the integrated fluorescence from the background region was subtracted from the integrated fluorescence intensity of the cell body region.

Table S1. Photophysical properties of FL3A-P₉K-CC and FL3A-Ppz-CC in PBS aqueous buffer, pH = 7.4

probe	Metal-free					Cu ²⁺ -bound				
	absorption		emission			absorption		emission		
	λ_{max} (nm)	$\varepsilon \times 10^{-4}$ (M ⁻¹ cm ⁻¹)	λ_{ex} (nm)	λ_{max} (nm)	Φ (%)	λ_{max} (nm)	$\varepsilon \times 10^{-4}$ (M ⁻¹ cm ⁻¹)	λ_{ex} (nm)	λ_{max} (nm)	Φ (%)
FL3A-P₉K-CC	495	3.64 ± 0.07	490	517	59.8 ± 2.8	494	2.80 ± 0.05	490	516	57.6 ± 1.2
FL3A-Ppz-CC	498	6.09 ± 0.04	490	519	6.8 ± 0.5	497	2.79 ± 0.16	490	516	12.1 ± 0.6

Table S2. Comparison of the reduction potentials of Cu(FL3A-Ppz-CC), NO, HNO, and biological thiols*

Redox couple	$E_{1/2}$ or E_{pc} , V vs NHE	Comments	Ref.
$\text{NO}^+ + \text{e}^- \rightarrow \text{NO}^\bullet$	+1.52 ($E_{1/2}$)	in CH ₃ CN	7, 8
$\text{GSSG} + 2\text{H}^+ + 2\text{e}^- \rightarrow 2 \text{GSH}$	-0.29 ($E_{1/2}$)	pH 7	9
$\text{CysSSCys} + 2\text{H}^+ + 2\text{e}^- \rightarrow 2 \text{CysSH}$	-0.36 ($E_{1/2}$)	pH 7	9
$\text{NO}^\bullet + \text{e}^- \rightarrow \text{NO}^-$	-1.01 (E_{pc})	in CH ₃ CN	10
$\text{NO}^\bullet + \text{H}^+ + \text{e}^- \rightarrow \text{HNO}$	-0.55 (E_{pc})	pH 7	11
$\text{Cu}^{\text{II}}(\text{FL3A-Ppz-CC})^+ + \text{e}^- \rightarrow \text{Cu}^{\text{I}}(\text{FL3A-Ppz-CC})$	+0.46 ($E_{1/2}$)	in CH ₃ CN	this work

*Abbreviations: $E_{1/2}$ = half-wave reduction potential, E_{pc} = cathodic peak potential, GSSG = glutathione disulfide, GSH = glutathione, CysSSCys = cystine, CysSH = cysteine.

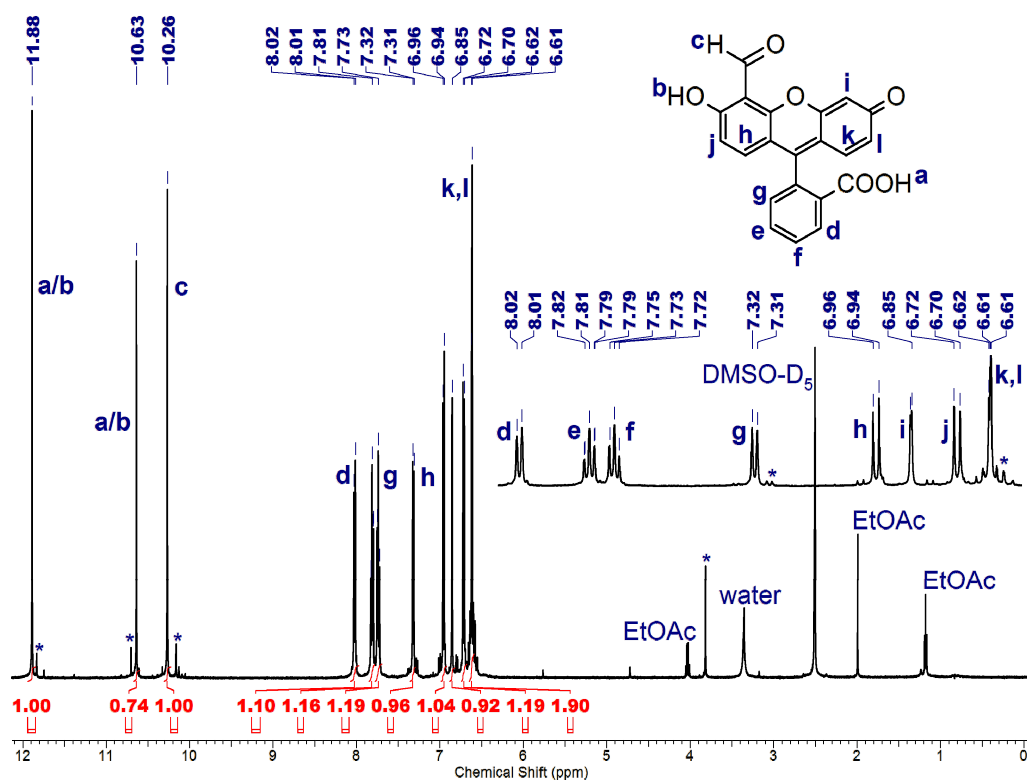


Figure S1. ^1H NMR spectrum of compound **S1** (500 MHz, $(\text{CD}_3)_2\text{SO}$).

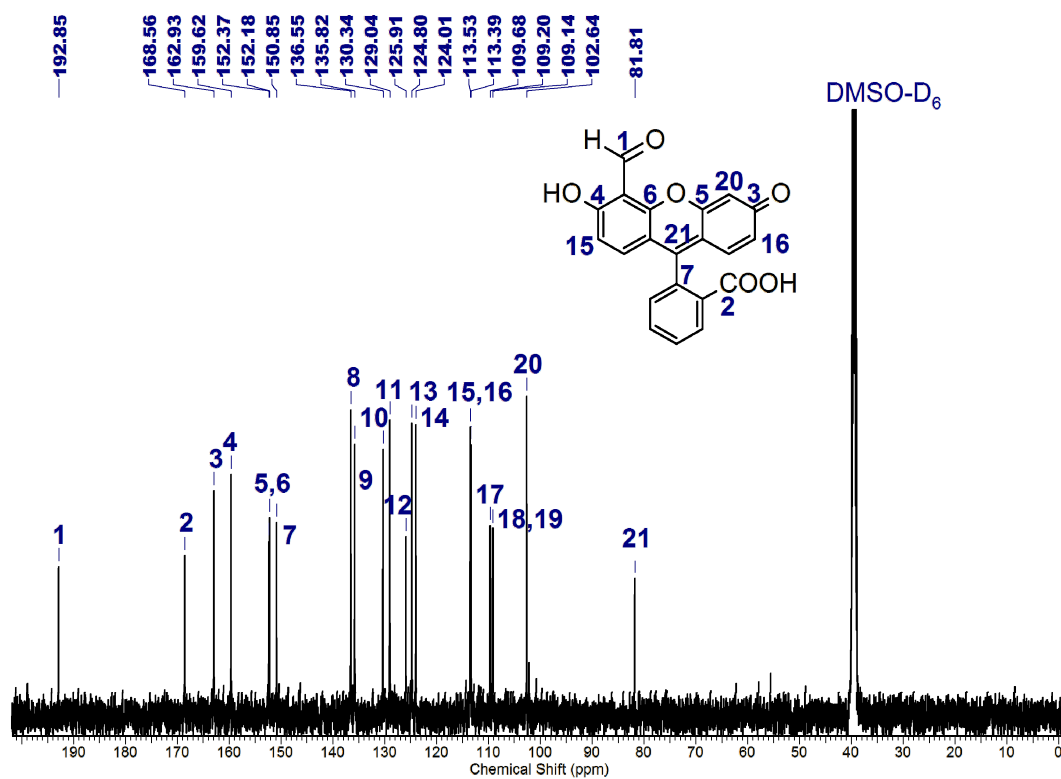


Figure S2. ^{13}C $\{^1\text{H}\}$ NMR spectrum of compound **S1** (125 MHz, $(\text{CD}_3)_2\text{SO}$).

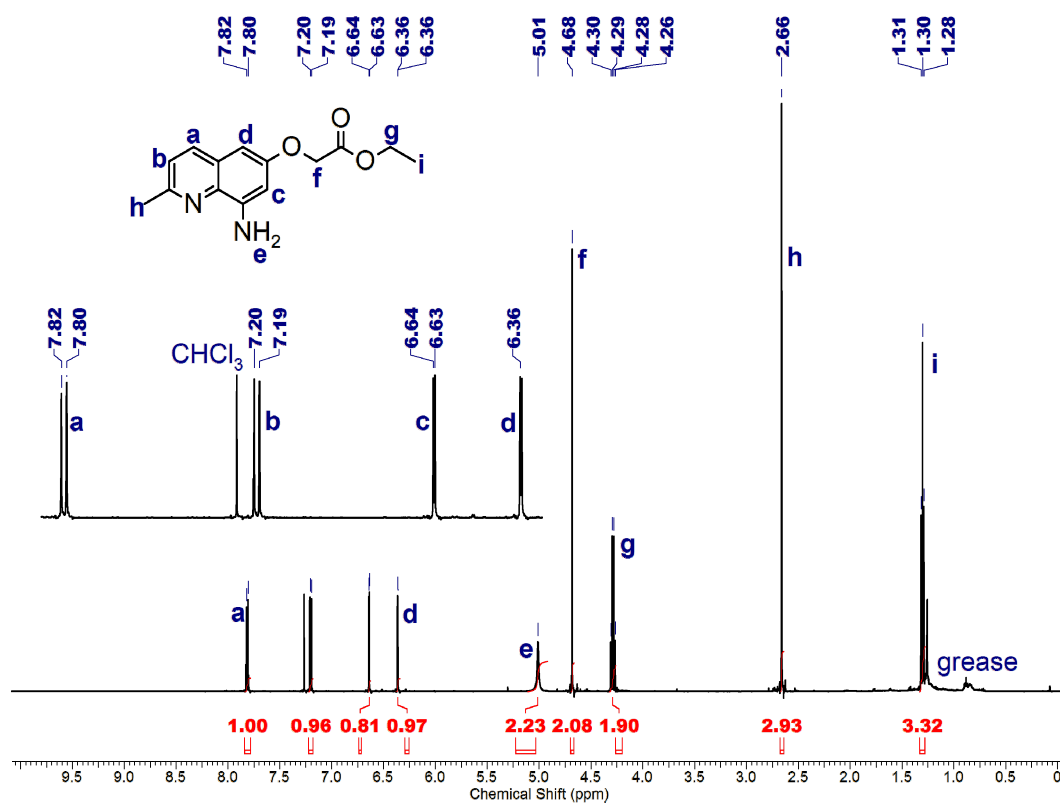


Figure S3. ¹H NMR spectrum of compound S2 (500 MHz, CDCl₃).

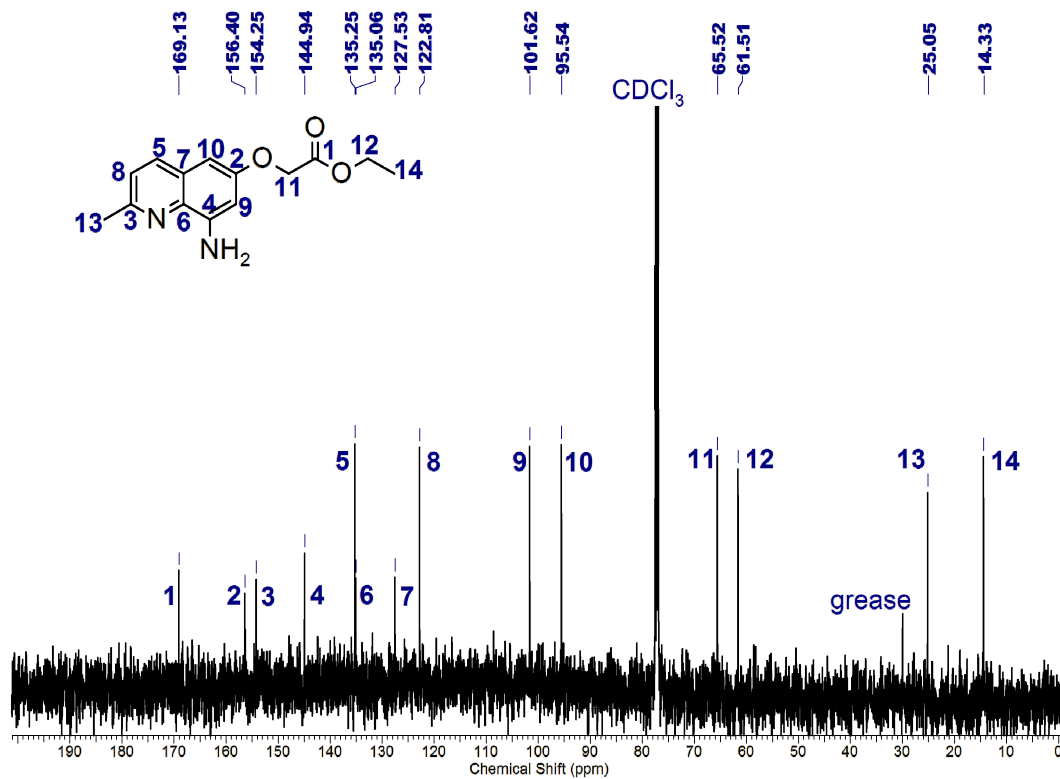


Figure S4. ¹³C {¹H} NMR spectrum of compound S2 (125 MHz, CDCl₃).

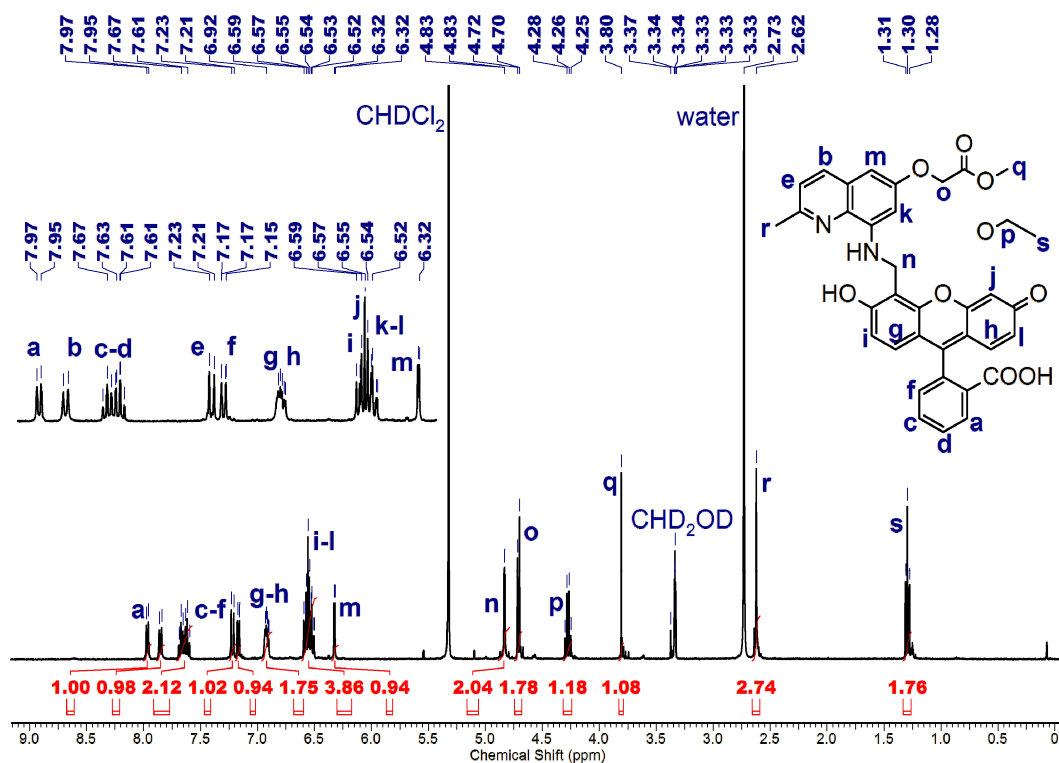


Figure S5. ¹H NMR spectrum of compound S3 (400 MHz, CD₂Cl₂:CD₃OD = 9:1 v/v).

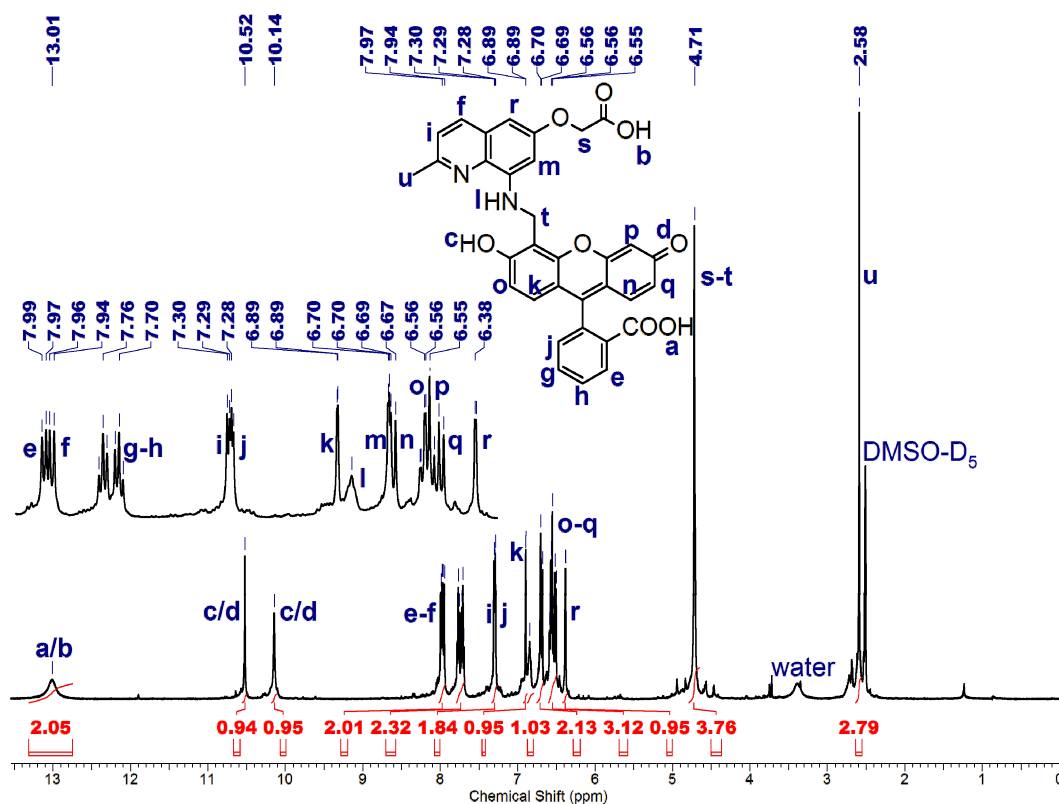


Figure S6. ¹H NMR spectrum of compound S4 (500 MHz, (CD₃)₂SO).

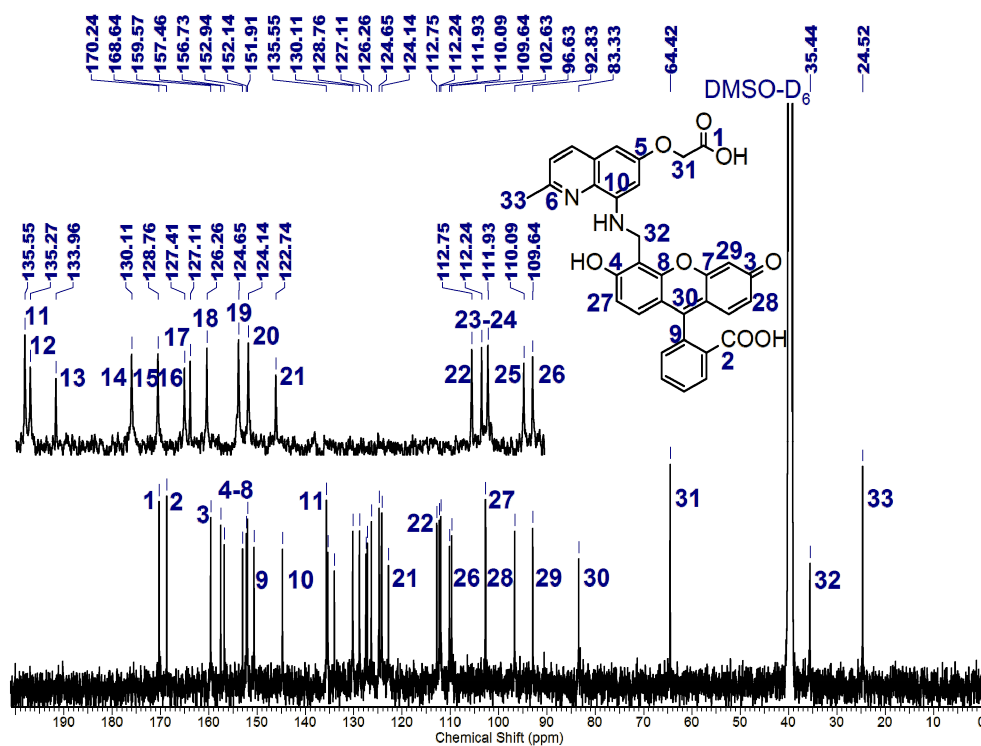


Figure S7. ^{13}C $\{^1\text{H}\}$ NMR spectrum of compound **S4** (125 MHz, $(\text{CD}_3)_2\text{SO}$).

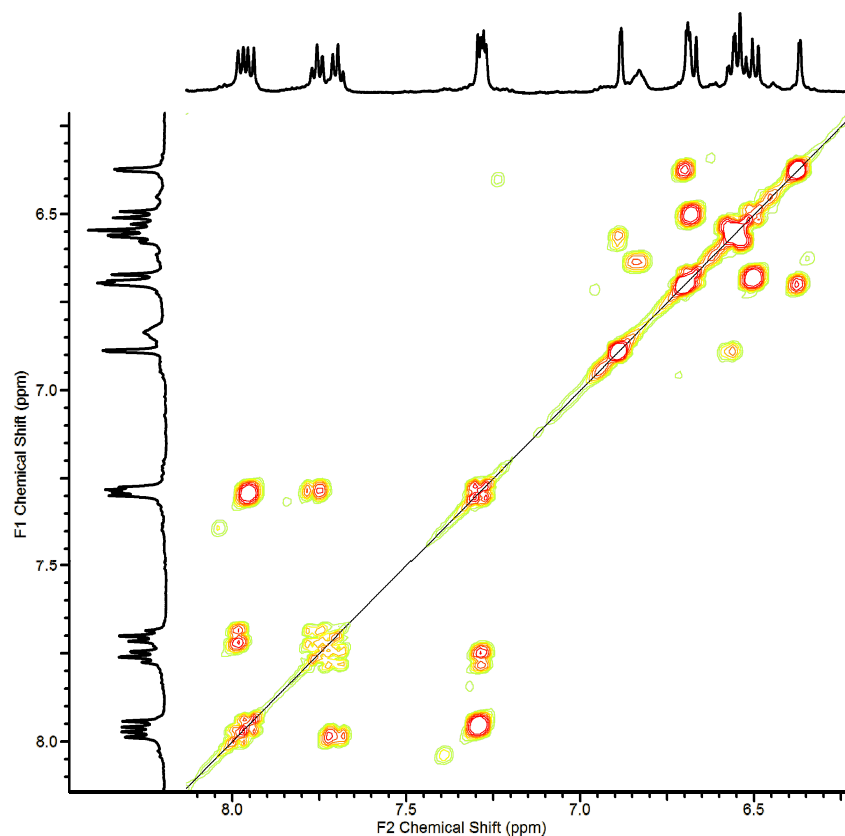


Figure S8. ^1H 2D gCOSY NMR spectrum of compound **S4** (500 MHz, $(\text{CD}_3)_2\text{SO}$).

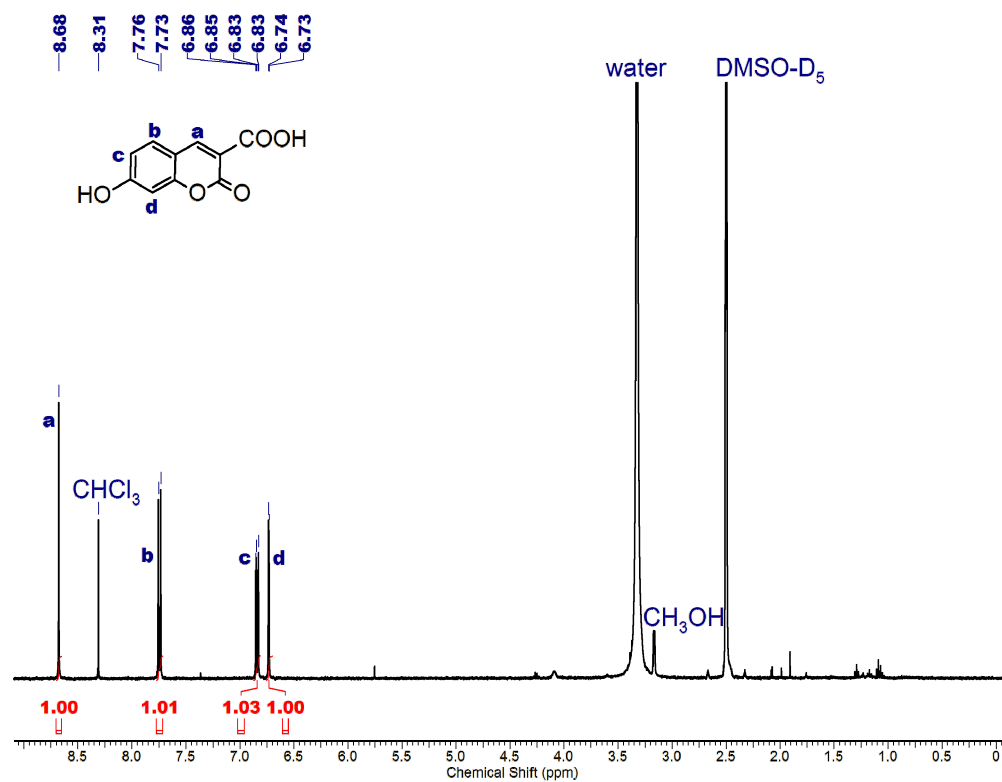


Figure S9. ¹H NMR spectrum of compound **S5** (400 MHz, (CD₃)₂SO).

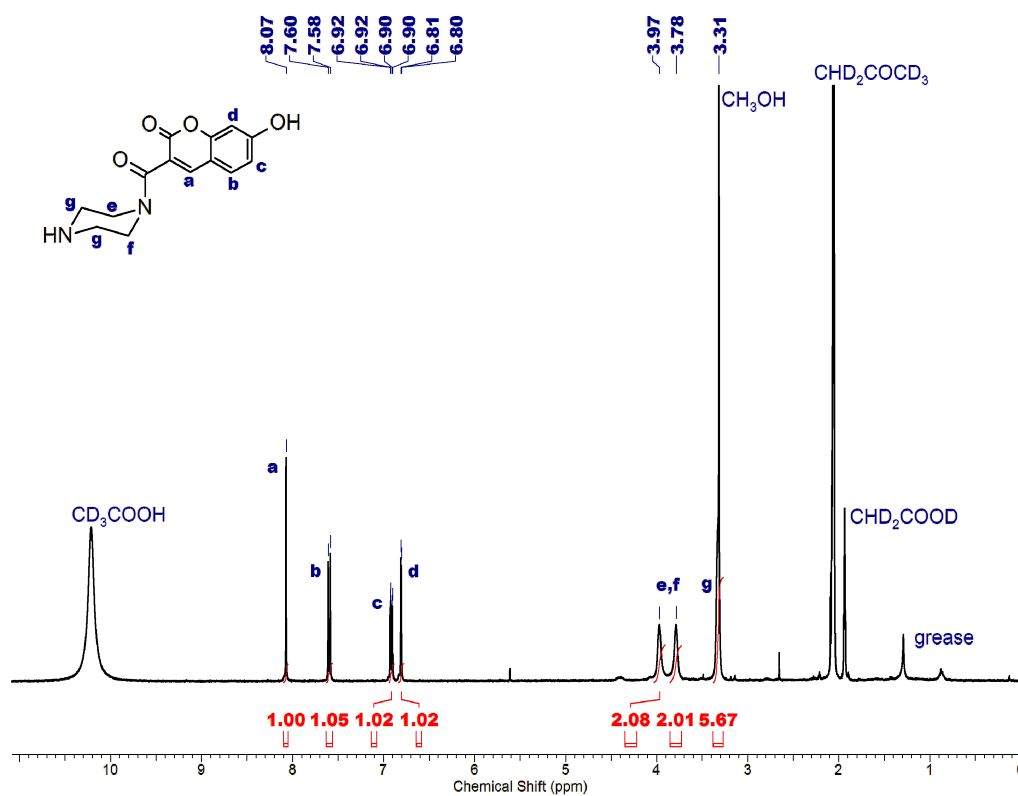


Figure S10. ¹H NMR spectrum of compound **S7** (400 MHz, (CD₃)₂CO:CD₃COOD = 95:5 v/v).

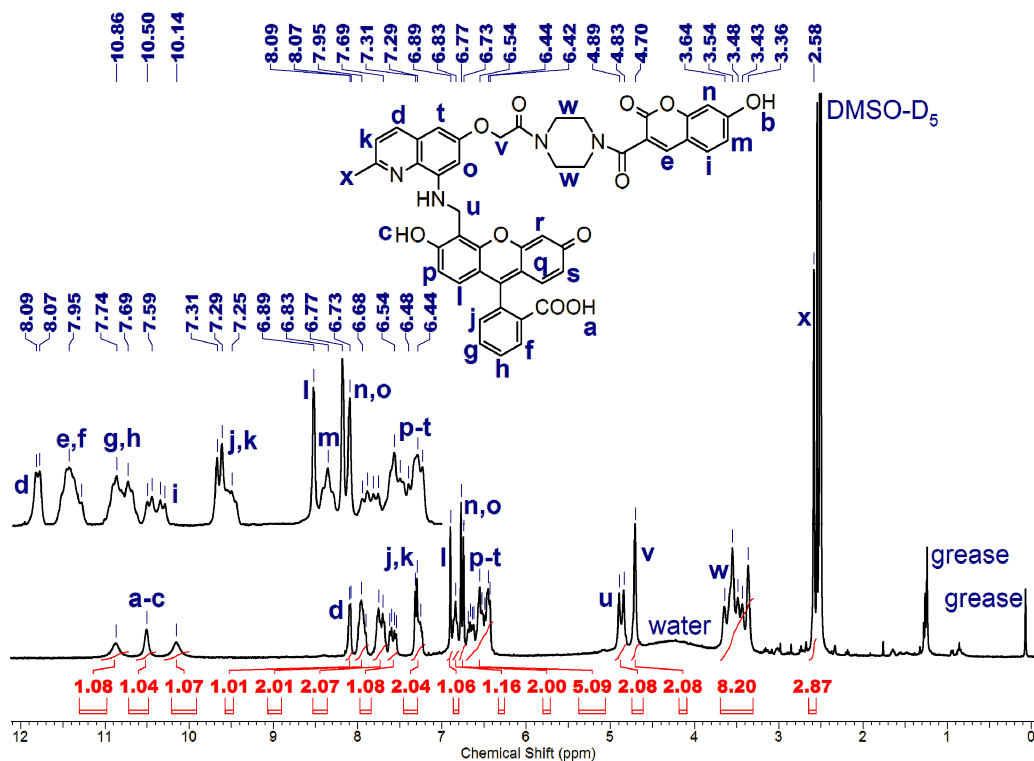


Figure S11. ¹H NMR spectrum of FL3A-Ppz-CC (400 MHz, (CD₃)₂SO).

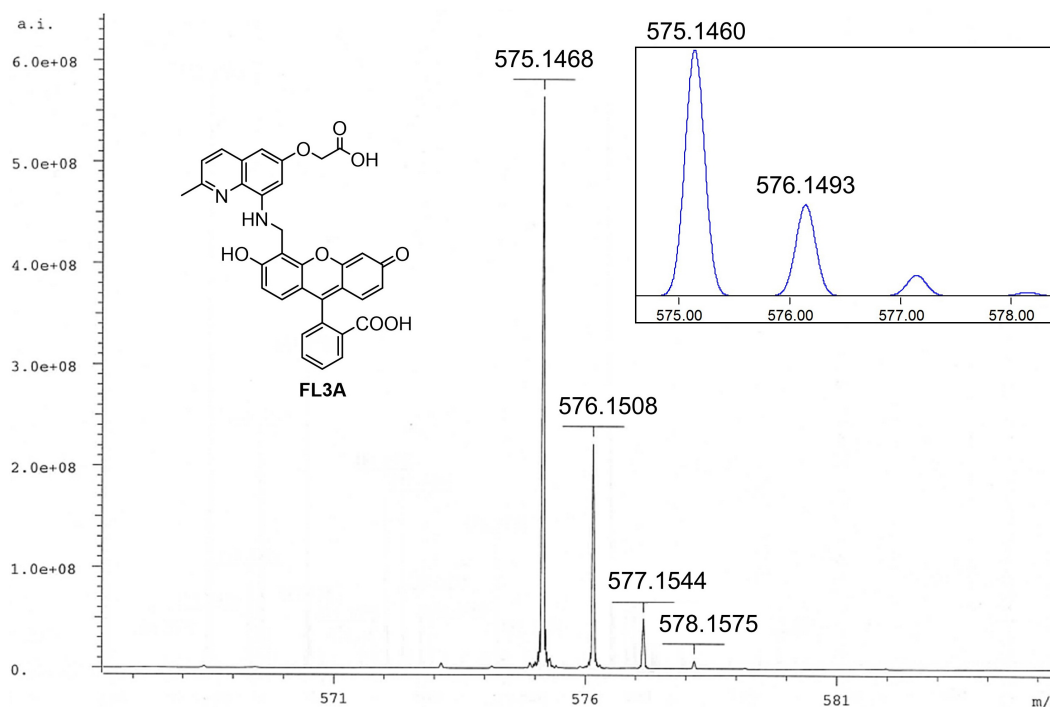


Figure S12. High-resolution mass spectrum (ESI⁻) of compound **S4**, FL3A. Inset: calculated isotope pattern and exact mass.

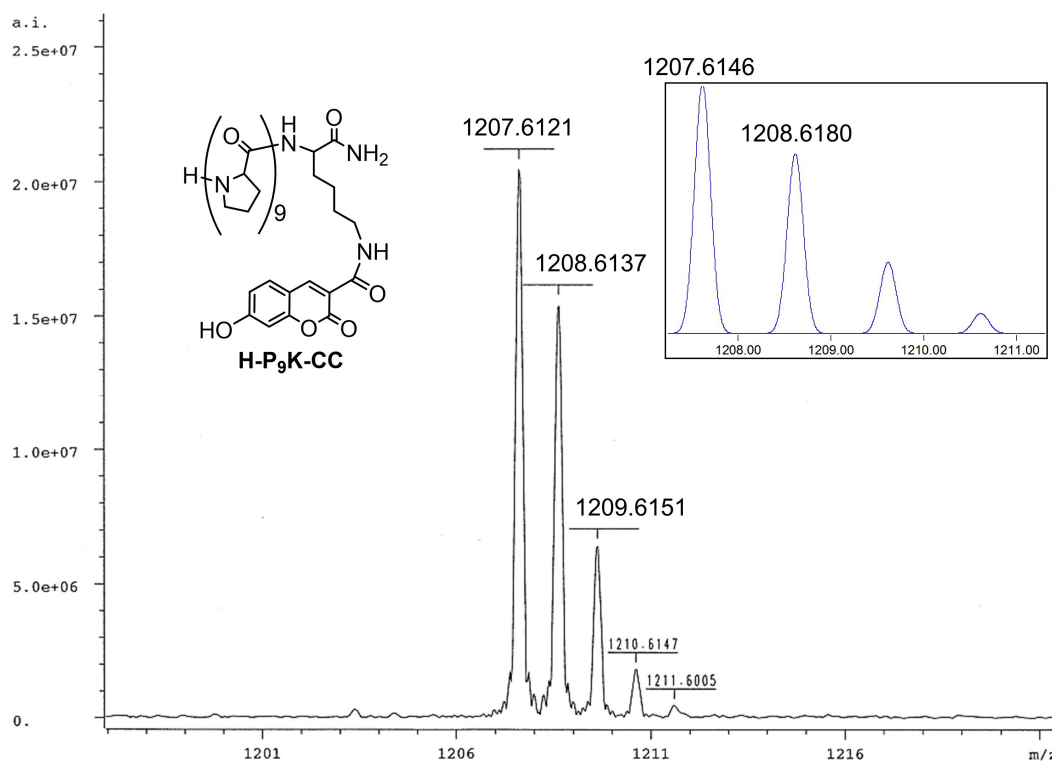


Figure S13. High-resolution mass spectrum (ESI⁺) of compound **S6**, H-P₉K-CC. Inset: calculated isotope pattern and exact mass.

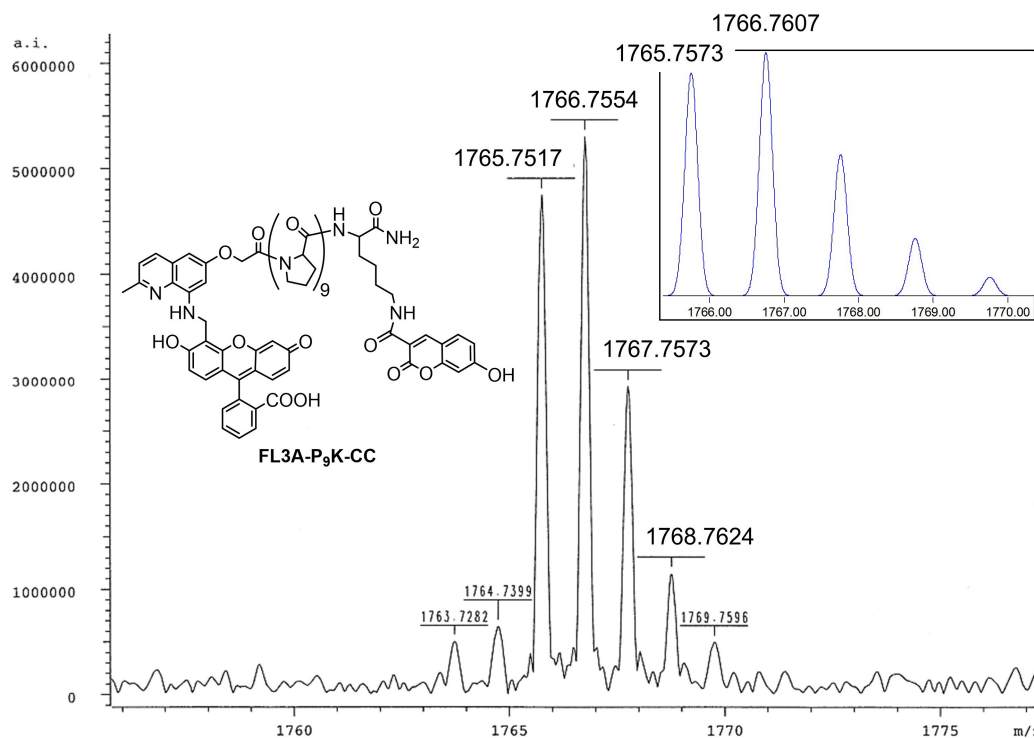


Figure S14. High-resolution mass spectrum (ESI+) of FL3A-P₉K-CC. Inset: calculated isotope pattern and exact mass.

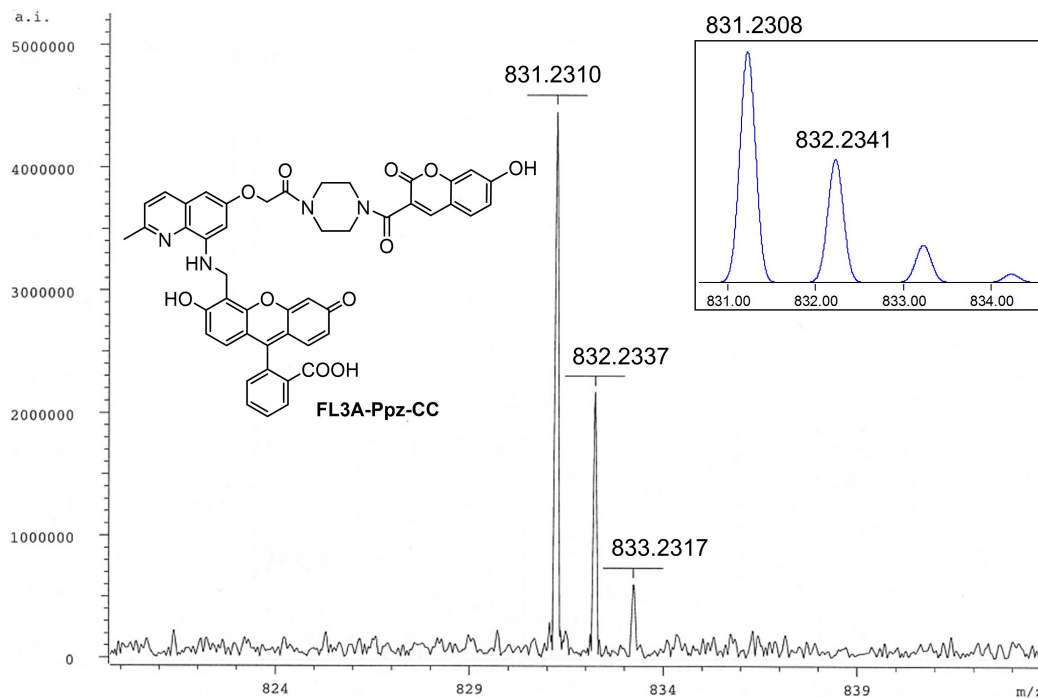


Figure S15. High-resolution mass spectrum (ESI-) of FL3A-Ppz-CC. Inset: calculated isotope pattern and exact mass.

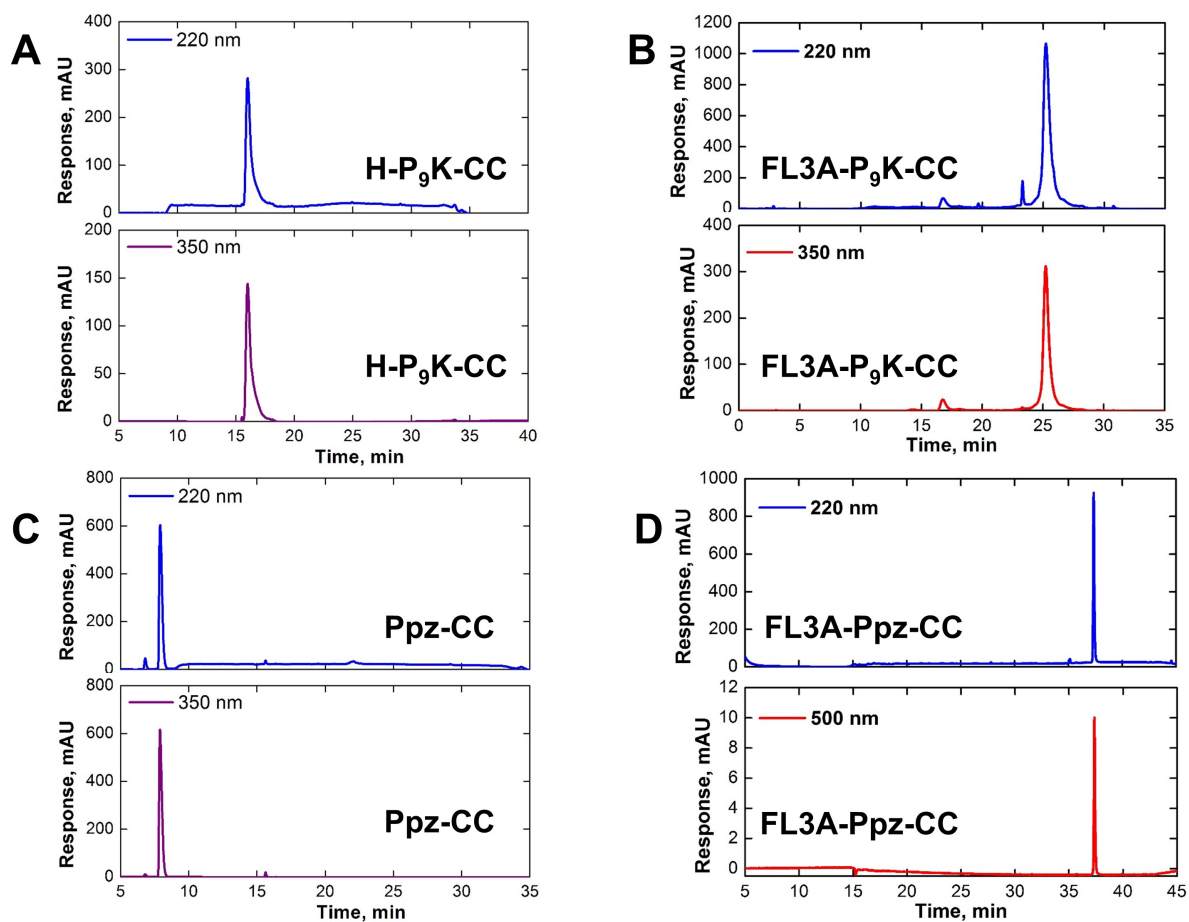


Figure S16. Analytical HPLC traces of (A) H-P₉K-CC (S6), (B) FL3A-P₉K-CC, (C) Ppz-CC (S7), and (D) FL3A-Ppz-CC, detected by the signals at indicated wavelengths.

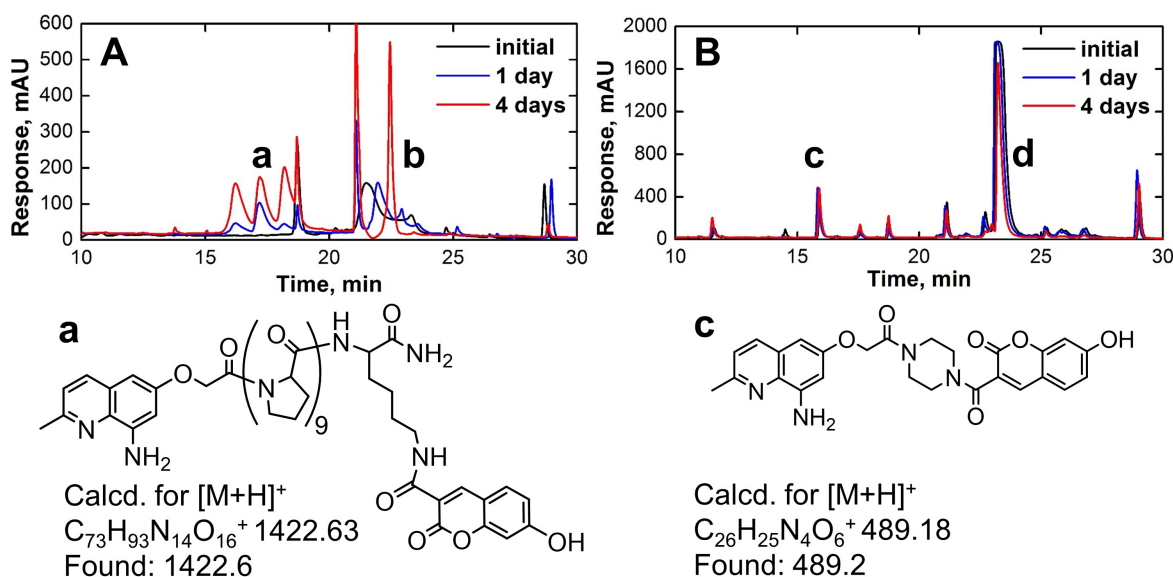


Figure S17. Analytical HPLC traces and ESI-MS detection of the major degradation products of (A) 300 μ M of FL3A-P₉K-CC and (B) 500 μ M of FL3A-Ppz-CC in deionized water (pH $\sim$ 9.0) with the elution region of interest between 10 and 30 min expanded for clarity. The chromatographic traces are plotted based on the signal at 220 nm. The aqueous solutions were kept at ambient temperature in sealed vials and monitored up to 4 days. Low-resolution mass spectra of the isolated fractions **a-d** shown above were then acquired in positive and negative mode. Fractions labeled **b** and **d** correspond to the FL3A-P₉K-CC and FL3A-Ppz-CC ligands, respectively.

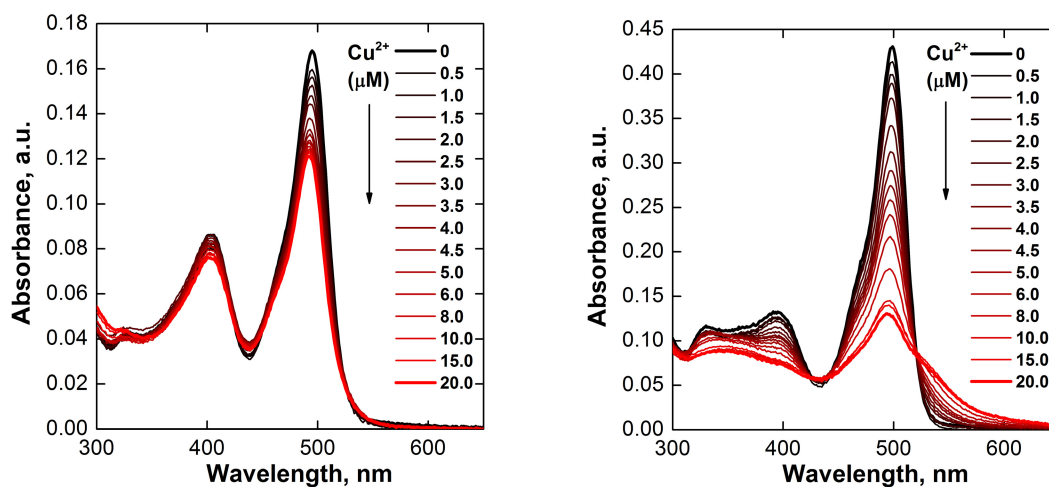


Figure S18. Representative UV-Vis spectra of (left) 4.4 μ M of Cu(FL3A-P₉K-CC) and (right) 7.3 μ M of Cu(FL3A-Ppz-CC) in PBS (25 $^{\circ}$ C, pH 7.4), recorded upon titration with aq. $CuCl_2$. The formation of the Cu(II) complexes was monitored by the absorbance changes (ΔA) at 495 and 499 nm, respectively.

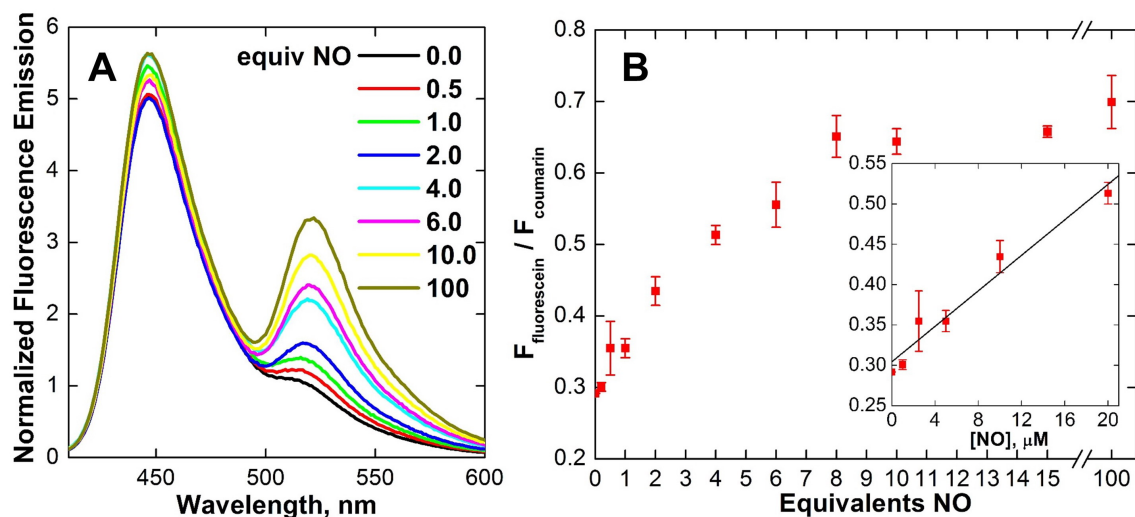


Figure S19. (A) Normalized fluorescence emission spectra and (B) ratiometric fluorescence response of 4 μM Cu(FL3A-P₉K-CC) vs added equiv of the NO donor DEA NONOate in PBS buffer under anaerobic conditions (25 °C, pH 7.4, λ_{ex} = 400 nm). The inset shows the linearity of the fluorescence ratio in the 0-20 μM NO range, as well as the linear fit used in the calculation of the detection limit for NO. The emission intensity in determining the ratio was integrated between 410-480 nm (F_{coumarin}) and 481-650 nm ($F_{\text{fluorescein}}$).

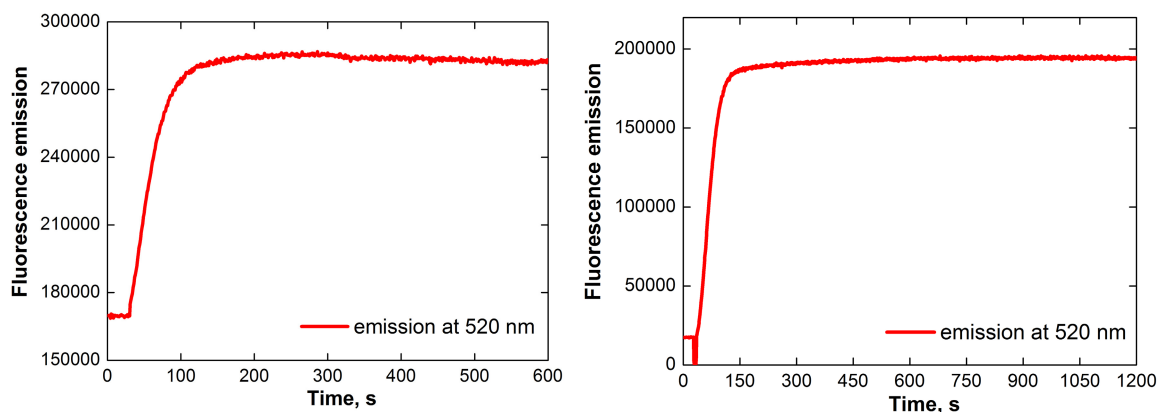


Figure S20. Plots of fluorescence emission at 520 nm over time of anaerobic 5 μM solutions of (left) Cu(FL3A-P₉K-CC) and (right) Cu(FL3A-Ppz-CC) in PBS (25 °C, pH 7.4, λ_{ex} = 400 nm), recorded upon addition of 500 equiv of NO (g).

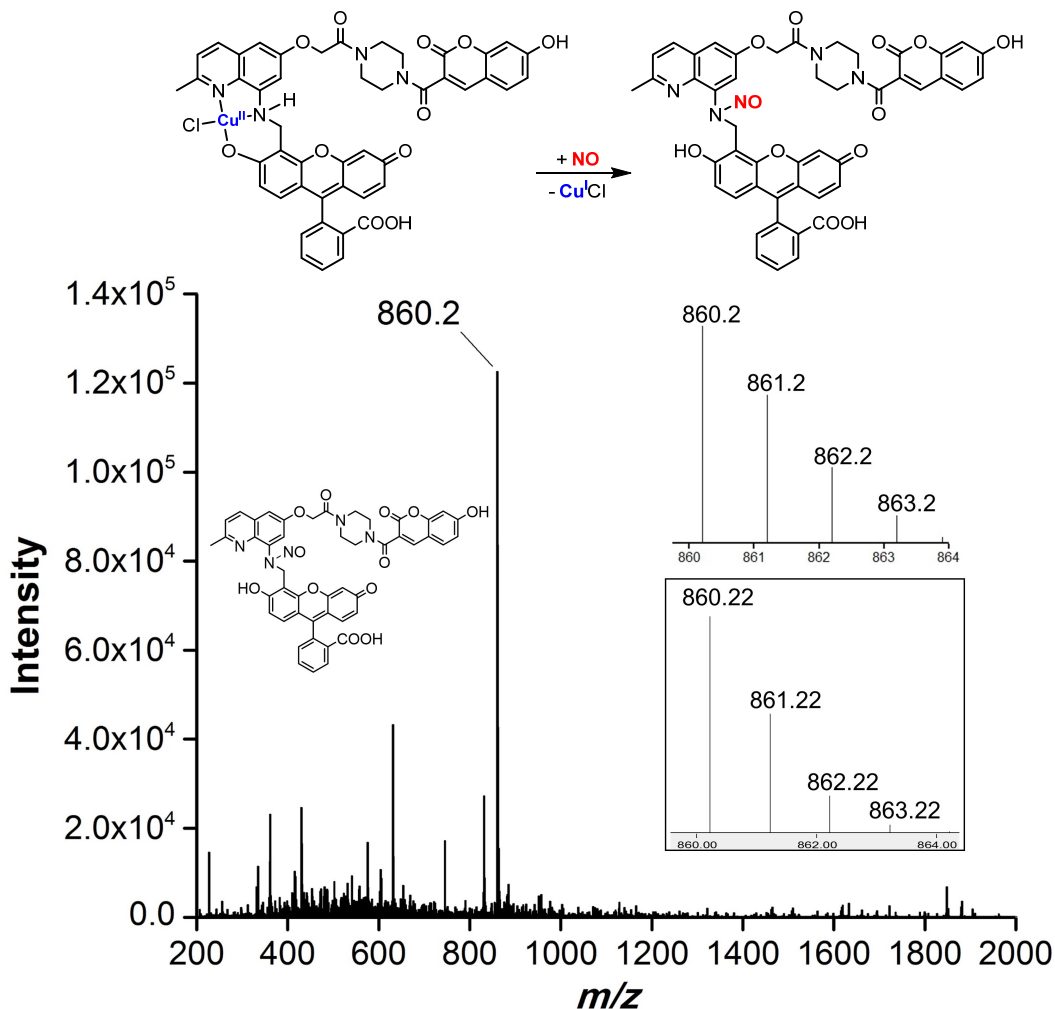


Figure S21. ESI-MS detection in the negative mode of the N-nitrosated reaction product of Cu(FL3A-Ppz-CC) with NO. Inset: calculated isotope pattern for the $[M - H]^-$ species. 240 μL of a $\sim 400 \mu\text{M}$ anaerobic solution of Cu(FL3A-Ppz-CC) in dilute PBS buffer ($\sim 1 \text{ mM PO}_4^{3-}$, pH 7.4) was placed in a septum-sealed vial and treated with 1 mL NO(g) ($\sim 40 \mu\text{mol}$, 250 equiv) by injecting the gas directly into the solution from a gastight syringe in the glove box. After 5 min, the sealed vial was removed from the glove box and the reaction solution was analyzed by direct injection on the LC-MS system.

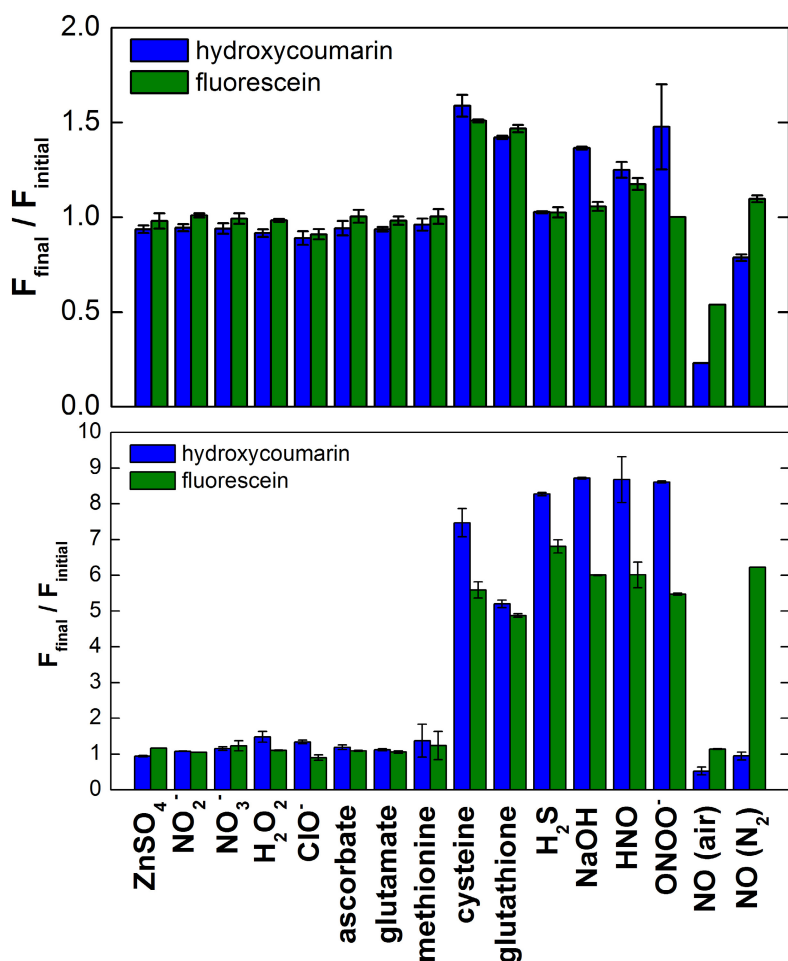


Figure S22. Selectivity of 2 μ M of (top) Cu(FL3A-PpK-CC) and (bottom) Cu(FL3A-Ppz-CC) toward reactive nitrogen and oxygen species, and other biological analytes in PBS buffer (37 $^{\circ}$ C, pH 7.4). The response of the probes was monitored by the individual fluorescence turn-on of the 7-hydroxycoumarin and fluorescein fluorophores, respectively, upon addition of each analyte. For each sample, the fluorescence emission was recorded before and 15 min after addition of excess analyte (1000 equiv NO gas, 250 equiv all other). The measurements for OH⁻ were conducted in 10 mM aq NaOH. The integrated fluorescence intensity arising from each fluorescent moiety was normalized to the initial value recorded for the metal-bound complex before addition of the analyte. For measurements of 7-hydroxycoumarin emission, excitation was provided at 400 nm and the fluorescence intensity was integrated between 410-480 nm. For measurements of fluorescein emission, excitation was provided at 490 nm and the fluorescence intensity was integrated between 495-650 nm. All measurements were repeated in triplicate.

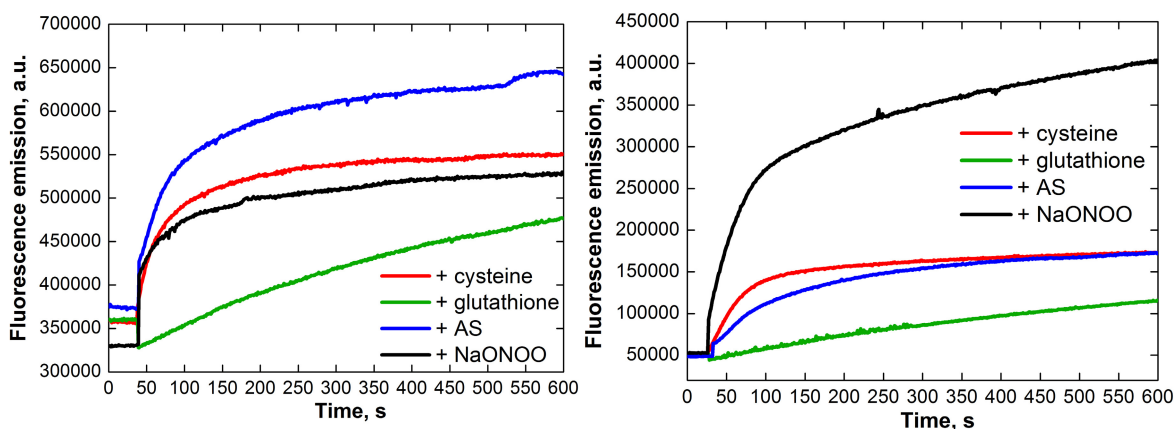


Figure S23. Plots of fluorescence emission over time at 450 nm of anaerobic 2 μM solutions of (left) Cu(FL3A-P₉K-CC) and (right) Cu(FL3A-Ppz-CC) in PBS (25 $^{\circ}\text{C}$, pH 7.4, λ_{ex} = 400 nm), recorded upon addition of 250 equiv of cysteine (Cys), glutathione, Angeli's salt (AS), and sodium peroxynitrite.

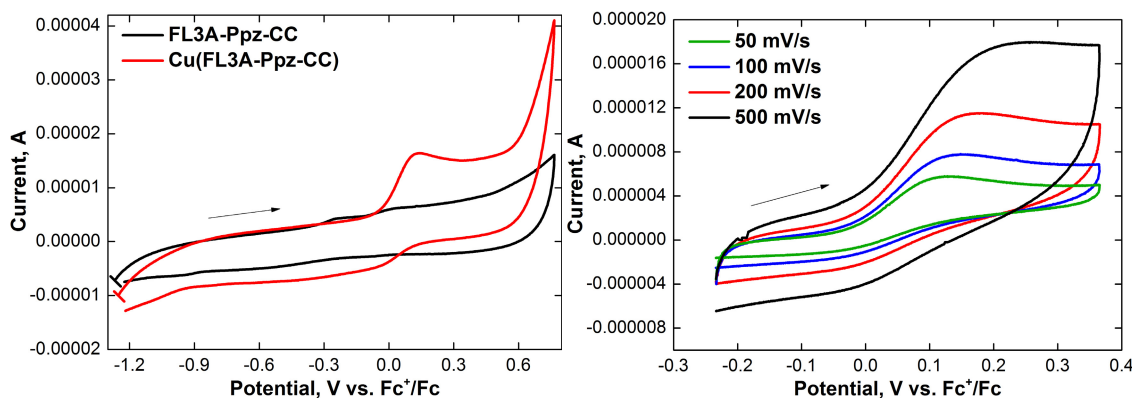


Figure S24. Left: cyclic voltammograms of ~ 1 mM solutions of (black) metal-free and (red) Cu(II)-bound FL3A-Ppz-CC measured at a scan rate of 200 mV/s in acetonitrile containing 0.1 M $n\text{-Bu}_4\text{NPF}_6$ as the supporting electrolyte. Right: cyclic voltammograms showing the Cu(II)-centered reduction of a ~ 1 mM solution of Cu(FL3A-Ppz-CC), measured at gradually increasing scan rates in acetonitrile containing 0.1 M $n\text{-Bu}_4\text{NPF}_6$ as the supporting electrolyte. In all cases, a three-electrode setup was used, consisting of a 2.0 mm glassy carbon working electrode, Pt counter electrode, and Ag^+/Ag pseudo reference electrode. The potentials were referenced to the Fc^+/Fc internal standard, added to the solution at the end of the measurements.

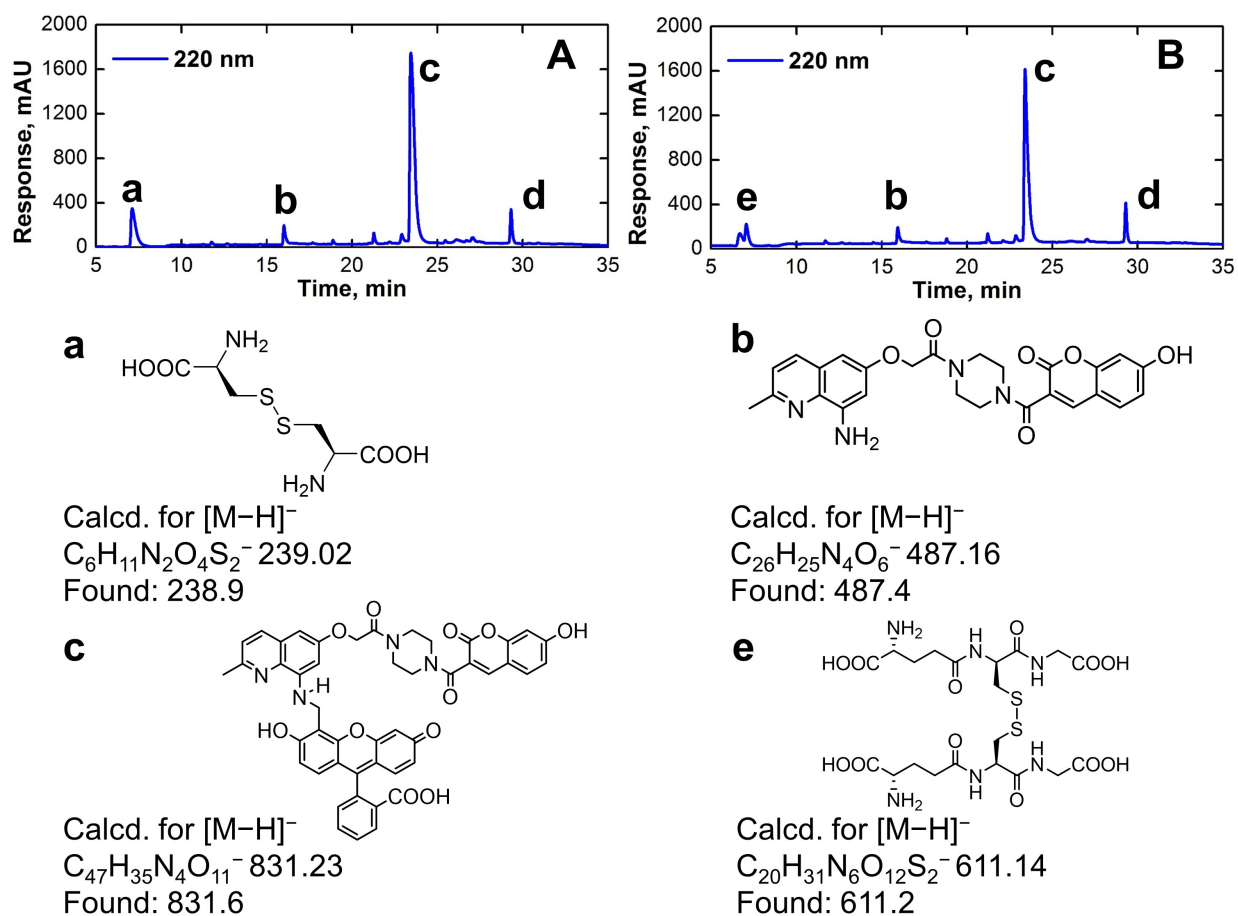


Figure S25. Analytical HPLC traces and ESI-MS detection of the reaction products of 450 μ M of Cu(FL3A-Ppz-CC) with (A) 10 equiv of cysteine and (B) 15 equiv of glutathione in dilute PBS buffer (~ 1 mM PO_4^{3-} , pH 7.4). A 240 μ L aliquot of the solution of Cu(FL3A-Ppz-CC) was placed in a sealed vial and treated with excess thiol. After 20 min at room temperature, the reaction solutions were analyzed by direct injection on the LC-MS system and analytical HPLC. The chromatographic traces are plotted based on the signal at 220 nm. Low-resolution mass spectra of the isolated fractions **a-e** were then acquired in positive and negative mode. Fractions labeled **a** and **e** correspond to the thiol oxidation product (disulfide). The major fraction **c** corresponds to the metal-free FL3A-Ppz-CC ligand. Fractions labeled **b** and **d** correspond to the hydrolysis side products of FL3A-Ppz-CC and are unaffected by addition of thiols.

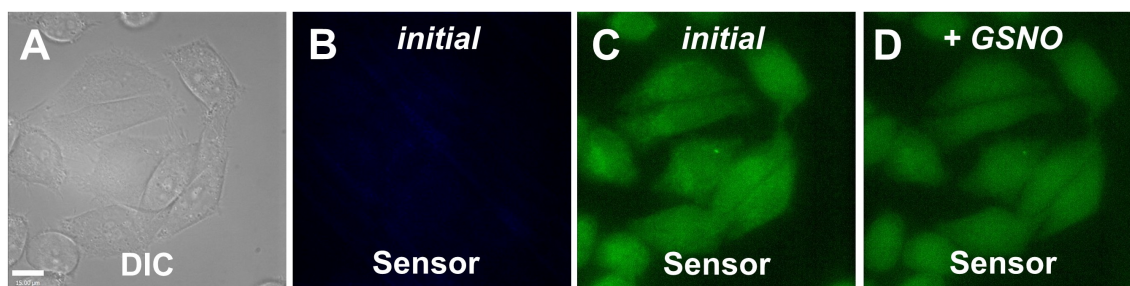


Figure S26. Fluorescence microscopy images of live HeLa cells incubated with 5 μ M of Cu(FL3A-P η K-CC) at 37 °C for 30 min in PBS, illustrating cellular impermeability of the sensor. (A) Differential interference contrast (DIC) image. (B) Signal in the blue channel. (C) Signal in the green channel. (D) Signal in the green channel 15 min after treatment with 1.25 mM of the NO donor GSNO on the microscope stage. Scale bar = 15 μ m.

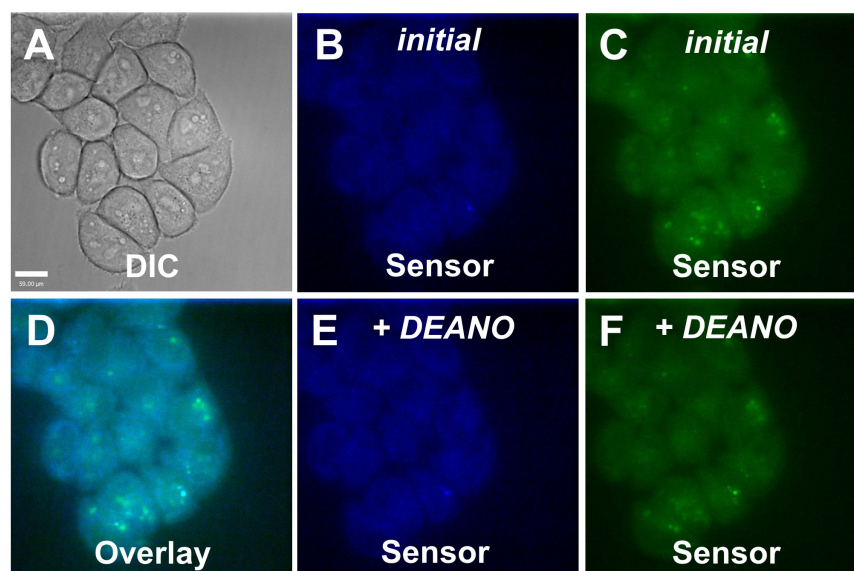


Figure S27. Fluorescence microscopy images of live HeLa cells incubated with 2 μ M of Cu(FL3A-Ppz-CC) at 37 °C for 30 min in DMEM. (A) DIC image. (B) Signal in the blue channel. (C) Signal in the green channel. (D) Overlay of (B) and (C). (E, F) Signal in the blue and green channels, respectively, recorded 5 min after treatment with 1.5 mM of the NO donor DEA NONOate (DEANO) on the microscope stage. Scale bar = 15 μ m.

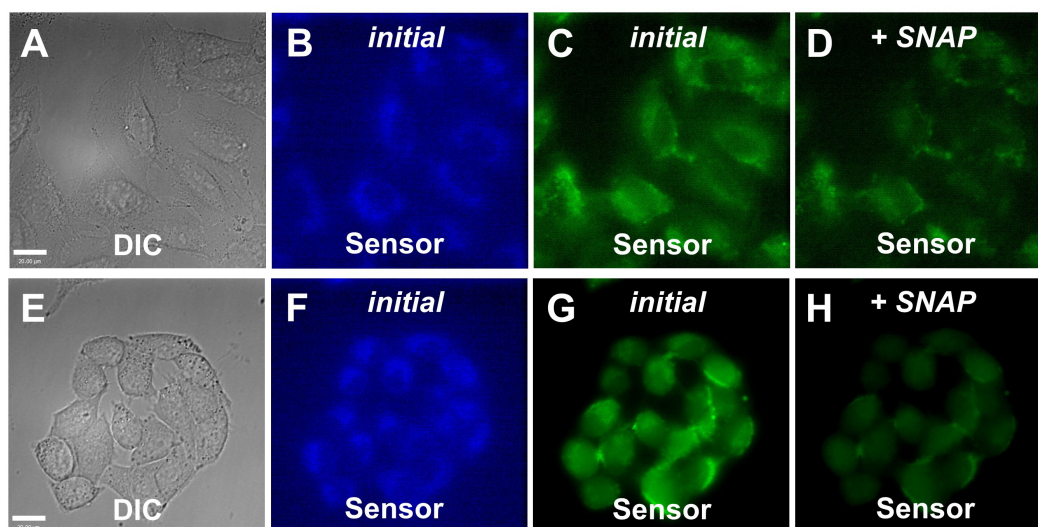


Figure S28. Fluorescence microscopy images of live A549 cells incubated with 2 μM of Cu(FL3A-Ppz-CC) at 37 $^{\circ}\text{C}$ for 30 min in DMEM (top row) or PBS (bottom row). (A, E) DIC images. (B, F) Initial signal in the blue channel. (C, G) Initial signal in the green channel. (D, H) Signal in the green channel recorded 20 min after treatment with 0.5 mM of the NO donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) on the microscope stage. Scale bar = 20 μm .

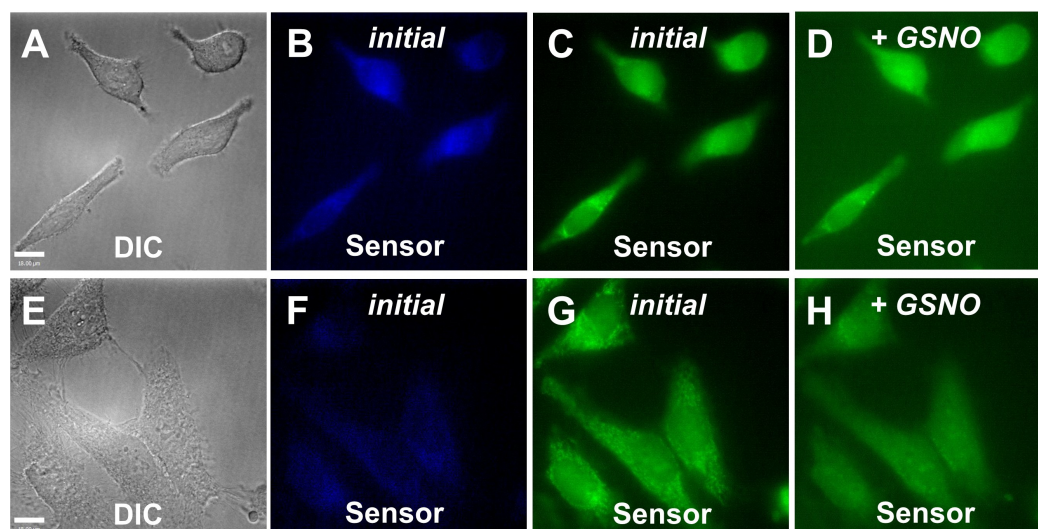


Figure S29. Fluorescence microscopy images of live HeLa cells incubated with 2 μM of Cu(FL3A-Ppz-CC) at 37 $^{\circ}\text{C}$ for 30 min in PBS. Top row: control cells. (A) DIC image. (B) Signal in the blue channel. (C) Signal in the green channel. (D) Signal in the green channel recorded 5 min after treatment with 1 mM of the NO donor GSNO on the microscope stage. Bottom row: cells pre-incubated with 1 mM *N*-methylmaleimide in PBS for 30 min at 37 $^{\circ}\text{C}$. (E) DIC image. (F) Signal in the blue channel. (G) Signal in the green channel. (H) Signal in the green channel recorded 5 min after treatment with 1 mM of the NO donor GSNO on the microscope stage. Scale bar = 18 μm .

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