Supporting Information

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SI Text

Synthetic Reagents. Anhydrous methanol (Aldrich) was used as received. Ethyl[(8-amino-2-methylquinolin-6-yloxy)acetate] (1), and 4′,5′-fluoresceindicarboxaldehyde (2), were prepared by previously reported procedures. Sodium borohydride (Sigma), sodium hydroxide (Mallinckrodt Chemicals), and all deuterated solvents (Cambridge Isotope Labratories) were used as received.

Synthetic Materials and Methods. Silica gel (SiliaFlash F60, Silicycle, 230-400 mesh) was used for column chromatography. Thin-layer chromatography (TLC) was performed on EMD Chemicals F254 silica gel-60 plates (1 mm thickness) and viewed by either UV light or ninhydrin staining. ^{1}H and ^{13}C{^{1}H} NMR spectra were obtained on either a Varian 300 MHz or 500 MHz spectrometer and referenced to the residual solvent resonance of the deuterated solvent. High-resolution mass spectra were measured by staff at the MIT Department of Chemistry Instrumentation Facility.

2-{4,5-Bis[(6-(2-ethoxy-2-oxoethoxy)-2-methylquinolin-8-ylamino) methyl]-6-hydroxy-3-oxo-3*H*-xanthen-9-yl}benzoic acid (1, FL2E). 4′,5′-Fluoresceindicarboxaldehyde (95.3 mg, 245 μmol) and ethyl[(2-methyl-8-aminoquinolin-6-yloxy)acetate] (141 mg, 542 μmol) were suspended in methanol (13 mL) and stirred at room temperature for 1 h. The dark red reaction suspension was cooled to 0 °C and sodium borohydride (67.4 mg, 1.78 mmol) was added. The reaction clarified to form a dark red solution which was stirred for 1 h as it warmed to room temperature. The solvent was removed and the crude product was purified by column chromatography on silica (gradient from 100% CH_2Cl_2 to 19:1 CH_2Cl_2:CH_3OH) to afford a dark red solid (99.9 mg, 46%). TLC $R_f = 0.74$ (silica, 9:1 CH_2Cl_2:CH_3OH); mp: 174–175 °C (dec); ^{1}H NMR (DMSO-d_6, 300 MHz) δ 1.19 (6H, t, $J = 7.2$ Hz), 2.56 (6H, s), 4.15 (4H, q, $J = 7.2$ Hz), 4.74 (4H, s), 4.89 (4H, d, $J = 5.7$ Hz), 6.37 (2H, d, $J = 2.4$ Hz), 6.51 (2H, d, $J = 8.7$ Hz), 6.70–6.73 (4H, m), 6.88 (2H, t, $J = 6.3$ Hz), 7.27–7.30 (3H, m), 7.67–7.76 (2H, m), 7.94 (2H, d, $J = 8.4$ Hz), 7.97 (1H, d, $J = 7.2$ Hz), 10.62 (1H, broad s); ^{13}C{^{1}H} NMR (DMSO-d_6, 125 MHz) δ 15.19, 25.56, 37.04, 49.77, 52.95, 61.74, 65.59, 65.73, 94.44, 97.61, 111.00, 113.22, 113.44, 123.94, 125.36, 125.80, 127.48, 128.23, 128.78, 131.29, 135.07, 136.51, 136.69, 145.76, 151.76, 153.15, 154.21, 157.66, 158.61, 169.65, 169.81, 170.33; HRMS (m/z): $[M - H]^-$ calcd 875.2934, found 875.2919; Anal. Calcd for $C_{50}H_{44}N_4O_{11}\cdot H_2O$: C, 67.10; H, 5.18; N: 6.26; Found: C, 67.26; H, 4.94; N, 6.21.

2,2′-{8,8′-[9-(2-Carboxyphenyl)-6-hydroxy-3-oxo-3*H*-xanthene-4,5-diyl] bis(methylene)bis(azanediyl)bis(2-methylquinolin-8,6-diyl)}bis(oxy)diacetic acid (2, FL2A). FL2E (**1**, 75.5 mg, 87.0 μmol) was dissolved in aqueous sodium hydroxide (0.5 N, 9 mL) and methanol (3 mL) and heated to 90 °C for 19 h. The solution was cooled to room temperature and the pH was adjusted with concentrated hydrochloric acid until an orange precipitate formed. The solid was filtered, washed with water, and dried to yield the product (65.9 mg, 92%). TLC $R_f = 0.88$ (silica, 100% CH_3OH); mp: 183–184 °C (dec); ^{1}H NMR (CD_3OD + drops of DMSO-d_6, 300 MHz) δ 2.48 (6H, s), 4.67 (4H, s), 4.68 (4H, s), 6.32 (2H, d, $J = 2.4$ Hz), 6.51 (2H, s), 6.63 (2H, d, $J = 8.7$ Hz), 6.69 (2H, d, $J = 9$ Hz), 7.06 (2H, d, $J = 8.7$ Hz), 7.30 (1H, d, $J = 7.5$ Hz), 7.72–7.84 (4H, m), 8.03 (1H, d, $J = 7.5$ Hz); ^{13}C{^{1}H} NMR (DMSO-d_6, 125 MHz) δ 37.41, 56.09, 65.56, 84.51, 94.68, 110.91, 112.71, 113.41, 120.62, 123.89, 125.41, 125.81, 127.30, 128.60, 128.93, 129.95, 131.28, 136.74, 151.79, 153.53, 153.75, 158.06, 158.71, 169.80, 171.20, 171.30; HRMS (m/z): $[M - H]^-$ calcd 819.2308, found 819.2297; Anal. Calcd for $C_{44}H_{36}N_4O_{11}\cdot 3HCl$: C, 59.40; H, 4.23; N: 6.02; Found: C, 59.12; H, 4.29; N, 5.85.

Spectroscopic Materials and Methods. Piperazine-*N*, *N*′-bis(2-ethanesulfonic acid) (PIPES) was purchased from Calbiochem. Potassium chloride (99.999%) was purchased from Aldrich. Buffer solutions (50 mM PIPES, 100 mM KCl, pH 7) were prepared in Millipore water. Nitric oxide was purchased from Airgas and purified as previously described (3). *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP, Cayman Chemical), potassium nitrate (Aldrich), sodium nitrite (Aldrich), sodium peroxynitrite (Cayman Chemical), Angeli's salt ($Na_2N_2O_3$, Cayman Chemical), hydrogen peroxide (Mallinckrodt Chemicals), and sodium hypochlorite (J. T. Baker) were prepared as 50 mM stock solutions in Millipore water. Nitric oxide and other reactive oxygen and nitrogen species (RONS) were introduced into buffered solutions via gas tight syringes. Copper chloride dihydrate (99+ %) was purchased from Alfa Aesar and stock solutions of 10 mM and 1 mM were prepared in Millipore water. Stock solutions of 1 mM ligands were prepared in DMSO and stored in aliquots at −80 °C. UV-visible spectra were acquired on a Cary 50-Bio spectrometer using PMMA cuvettes from Perfector Science (3.5 mL volume, 1 cm path length). Acquisitions were made at 25.00± 0.05 °C. Fluorescence spectra were obtained on a Quanta Master 4 L-format scanning spectrofluorimeter (Photon Technology International) at 37.0 ± 0.1 °C using 1 μM Cu_2(L) (L = FL2E or FL2A) generated in situ by combining stock solutions of $CuCl_2$ and L in a 2:1 ratio, and then introducing either 1.3 mM NO or 167 μM RONS. Fluorescence measurements were made under anaerobic conditions, with cuvette solutions prepared in an inert atmosphere box. Replicate fluorescence measurements were taken at time points between 40 s and 60 min.

Cell Culture and Imaging Materials and Methods. HeLa, RAW 264.7 murine macrophages, and human SK-N-SH neuroblastoma cells were obtained from American Type Cell Collection (ATCC). HeLa and RAW 264.7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Cellgro, MediaTek, Inc.) supplemented with 10% fetal bovine serum (FBS, HyClone), 1% sodium pyruvate (Cellgro, MediaTek, Inc.), and 1% MEM nonessential amino acids (Sigma). SK-N-SH cells were cultured in Eagle's Minimal Essential Medium (EMEM, Cellgro, MediaTek, Inc.) supplemented with 10% FBS and 1% nonessential amino acids. For imaging studies, cells were passed and plated into poly-d-lysine coated plates (MatTek) containing 2 mL of complete DMEM, and incubated overnight at 37 °C with 5% CO_2. For all cell studies, the sensors were generated in situ by combining stock solutions of $CuCl_2$ (1 mM) and L (1 mM) in a 2:1 ratio (L = FL2E, FL2A).

For CuFL1 diffusion studies, HeLa cells were incubated with 10 μM CuFL1 for 30 min. Cells were washed three times with 1 mL of phosphate buffered saline (PBS, Cellgro, MediaTek, Inc.) prior to imaging and then bathed in 2 mL of PBS during imaging. To mimic media flow, the PBS was removed, and the cells were washed three times with 1 mL of fresh PBS and then bathed in 2 mL of fresh PBS. This process was repeated every 10 min during imaging. For Cu_2(FL2E) diffusion studies, RAW 264.7 macrophages were preincubated with 500 ng/mL of lipopolysaccharide (LPS, Sigma) and 250–1,000 U/mL of interferon-gamma (IFN-γ, BD Biosciences) for 4 h and then incubated

with 1 μM Cu_2(FL2E) for 12 h. Washing and imaging were performed in the same manner as in the CuFL1 diffusion studies.

For nitric oxide detection studies, iNOS was induced in RAW 264.7 macrophages with 500 ng/mL of LPS and 250–1,000 U/mL of IFN-γ for 4 h and the cells were then incubated with either 1 μM Cu_2(FL2E) or 1 μM Cu_2(FL2A) for 2–8 h. In SK-N-SH neuroblastoma cells, nNOS was induced with 100 nM β-estradiol (Sigma) and were coincubated with 1 μM Cu_2(FL2E), 4.5 μM Hoechst 33258 (Sigma), and 0.2 μM Mitotracker Red (Invitrogen) for 30 min. Cells were washed three times with 1 mL of PBS prior to imaging and then bathed in 2 mL of PBS during imaging.

Images were acquired on a Zeiss Axiovert 200M inverted epifluorescence microscope equipped with an EM-CCD digital camera (Hamamatsu) and a MS200 XY Piezo Z stage (Applied Scientific Instruments), lighted by an X-Cite 120 metal-halide lamp (EXFO). Plates were maintained on the microscope stage in an INC-2000 incubator kept at 37 °C with 5% CO_2. Differential interference contrast (DIC) and red, blue, and green fluorescent images were obtained using an oil immersion 63x objective lens, with 2.00 s exposure times for green fluorescent images. The microscope was operated with Volocity software (Improvision). All fluorescent images were corrected for background.

Cytotoxicity Assays. SK-N-SH cells were seeded into 96-well plates (100 μL total volume/well, 3,000 cells/well) in complete DMEM and incubated at 37 °C with 5% CO_2 for 24 h. The medium was replaced and the cells incubated with Cu_2(FL2E) (1 μM–1 mM, with an untreated control lane) for another 72 h. The medium was removed and replaced with 200 μL of a 20:1 mixture of FBS-free DMEM and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL stock in PBS). The cells were incubated for 4 h and then the medium was removed and replaced with DMSO (100 μL/well). The absorbance of each well was recorded at 555 nm on a microplate reader (BioTek, Synergy HT) and the percent cell survival values are reported relative to those of untreated control cells.

Olfactory Bulb Imaging. Cells in slices were visualized with differential interference contrast (DIC) optics on an Olympus BX50-WI microscope equipped with a 60 × NA 0.90 water immersion objective and viewed with a V-1070 CCD video camera (Marshall Electronics, El Segundo, CA). For dynamic imaging of local fluorescence in slices, we used Hg arc lamp illumination with 425DF45 excitation, 475DCLPO2 dichroic, and 535DF55 emission filters (Omega Optical). Excitation was controlled by a Uniblitz electronic shutter (Vincent Associates) with 8 ms frame exposure to reduce bleaching. Single frames were acquired at 30 s intervals using a NeuroCCD-SM camera controlled by Neuroplex 6.5 software (Red Shirt Imaging, CT). Wider area static images of slices were acquired with a Nikon DXM1200C digital camera attached to a Nikon Eclipse 80i microscope, using Hg arc lamp excitation and FITC filter set (EX480/40 excitation, DM495 dichroic, BA535/50 emission).

Olfactory Bulb Slice Preparation. Olfactory bulb slices were prepared from male mice (P21-28, CD-1, Charles River Laboratories, Wilmington, MA) as described previously (4, 5). Animals were sacrificed under halothane anesthesia, and olfactory bulbs were removed and transferred into ice-cold slicing solution containing 240 mM sucrose, 2.5 mM KCl, 10 mM NaHEPES, 10 mM glucose, 1 mM $CaCl_2$, 4 mM $MgCl_2$, and 0.2 mM ascorbic acid at pH 7.2. Horizontal slices (200–300 μm thickness) were cut and incubated for 45 min (32 °C–23 °C) in an interface chamber containing 124 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 10 mM glucose, 1 mM $CaCl_2$, 3 mM $MgCl_2$, and 2 mM N^{ω}-nitro-L-arginine at pH 7.3 (aerated with 95% O_2, 5% CO_2).

Nitric Oxide Probe Loading. NO probe loading solution was prepared by mixing 5 mL of 2 mM FL2E in DMSO + 20% Pluronic® F-127 (Invitrogen) with an equal volume of 4 mM $CuCl_2$ in water to make Cu_2(FL2E), including 76 μM MK-571 to block multidrug resistant transporters (6). Slices were incubated in loading solution for 2 h in a small shallow dish in the interface chamber and subsequently transferred to an open chamber and perfused (2 mL/min) with ACSF containing 124 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 25 mM glucose, 2 mM $CaCl_2$, and 1.3 mM $MgCl_2$ at pH 7.3 (aerated with 95% O_2, 5% CO_2, 23 °C). N^{ω}-nitro-L-arginine (2 mM) was used initially to inhibit NO synthesis and then replaced with 400 μM L-arginine to promote NO synthesis. During repeated stimulation of slices by step additions of 20 mM KCl, there was a 350–590 s delay between trials.

Animal Care. All experimental procedures adhered strictly to guidelines for animal care, handling, and euthanasia set by the U.S. Public Health Service. All protocols were reviewed and approved by the Monell Institutional Animal Care and Use Committee.

1. Fahrni CJ, O'Halloran TV (1999) Aqueous coordination chemistry of quinoline-based fluorescence probes for the biological chemistry of zinc. *J Am Chem Soc* 121:11448–11458.
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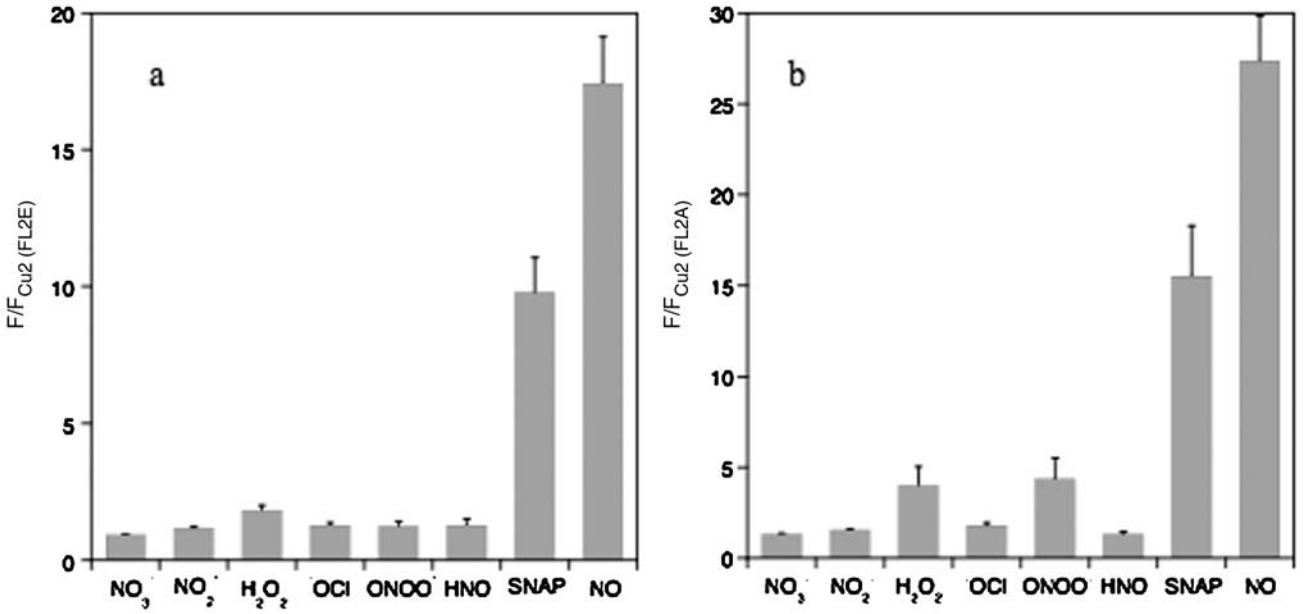

Fig. S1. Selectivity of *A*) 1 μM Cu_2(FL2E) and *B*) 1 μM Cu_2(FL2A) for NO over other RONS. Normalized fluorescence response after 1 h relative to the emission of the sensor in 50 mM PIPES, 100 mM KCl, pH 7, $T = 37$ °C.

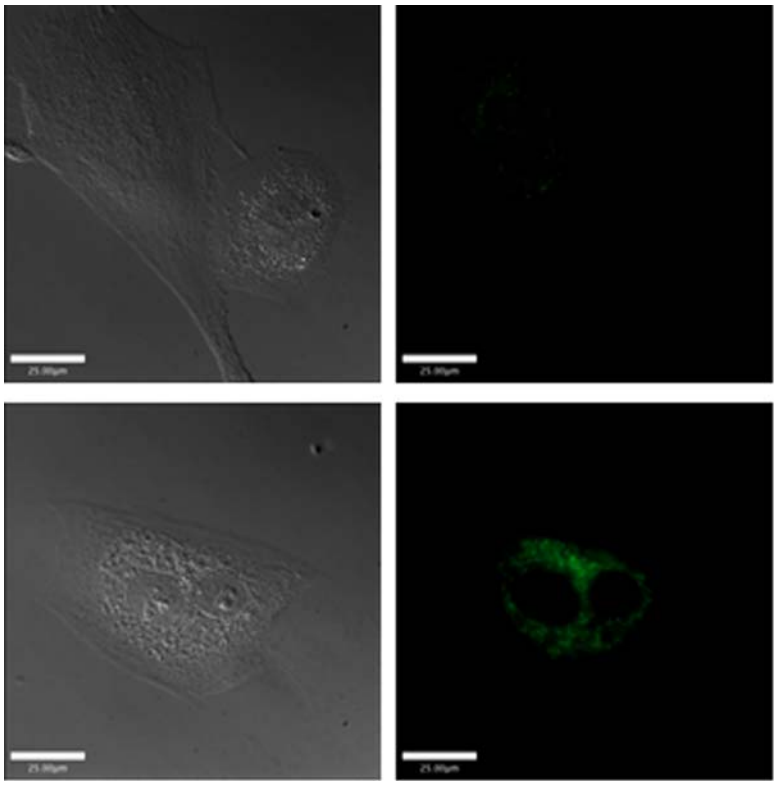

Fig. S2. SK-N-SH cells coincubated with (*top*) 5 μM Cu_2(FL2E) and (*bottom*) 5 μM Cu_2(FL2E) and 100 nM 17-β-estradiol. (*Left*) DIC images; (*Right*) emission from the sensor in the green channel. Scale bars 25 μm.

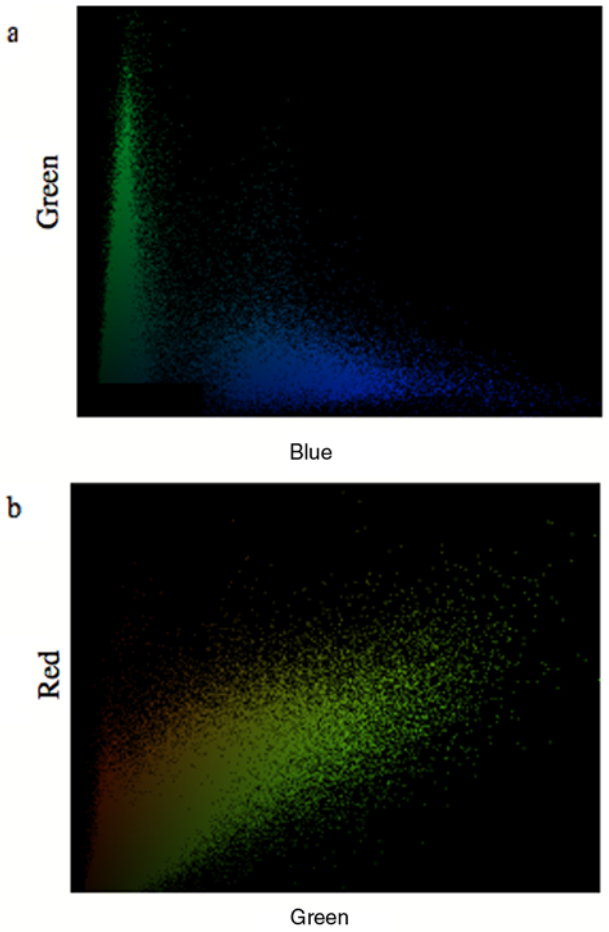

Fig. S3. SK-N-SH cells coincubated with 5 μM Cu_2(FL2E), 100 nM 17-β-estradiol, 0.2 μM Mitotracker Red and 4.5 μM Hoechst 33258 for 30 min. *A*) Colocalization scatter plot of the signals from the blue and green channels and *B*) colocalization scatter plot of the signals from the red and green channels.

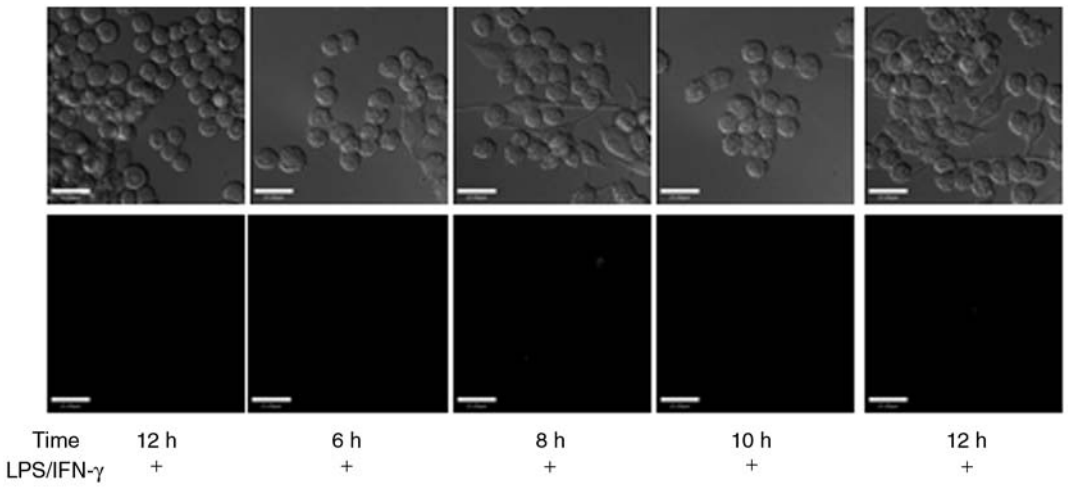

Fig. S4. RAW 264.7 macrophages coincubated with 1 μM Cu_2(FL2A), 500 ng/mL LPS and 250–1,000 U/mL IFN-γ for 6–12 h. Top, DIC images; (*Bottom*) emission from the sensor in the green channel. Scale bars, 25 μm.

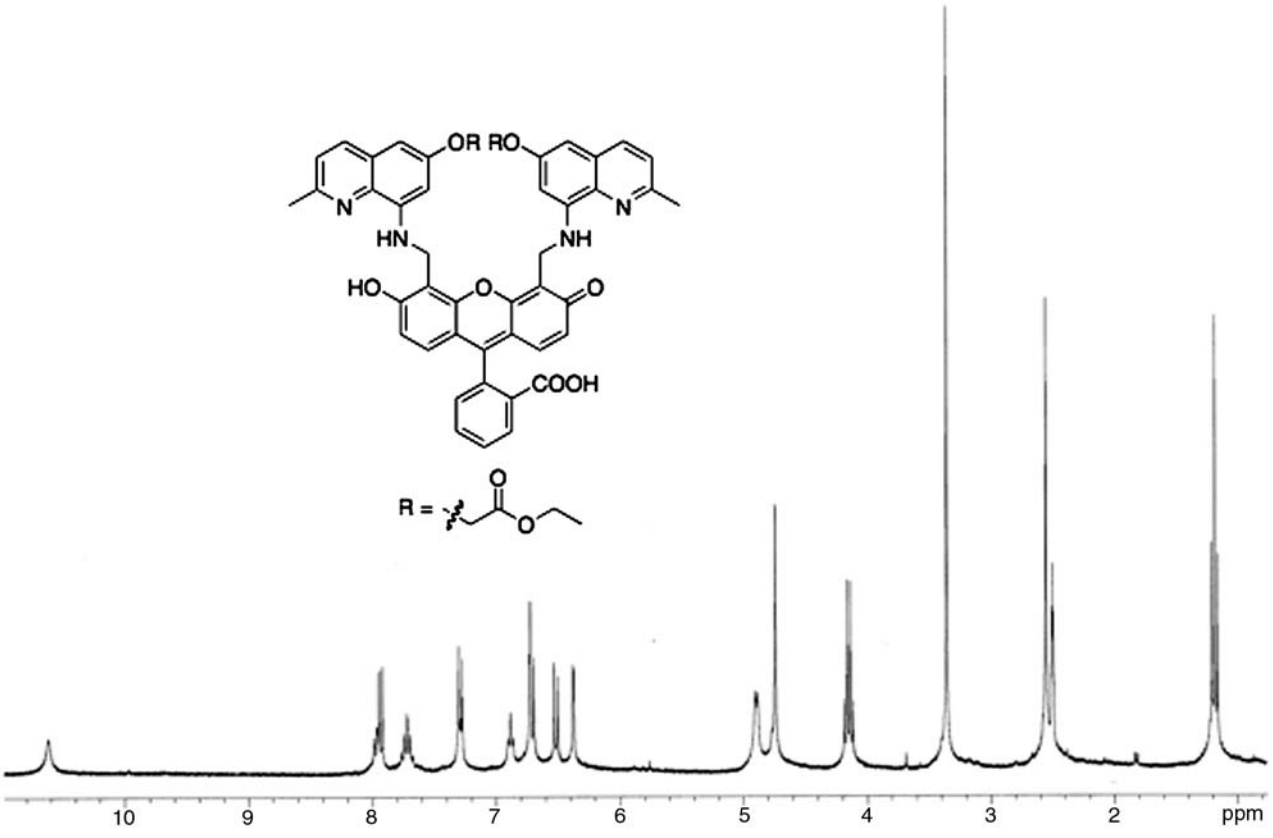

Fig. S5. ^{1}H NMR spectrum of FL2E (300 MHz, d_6-DMSO).

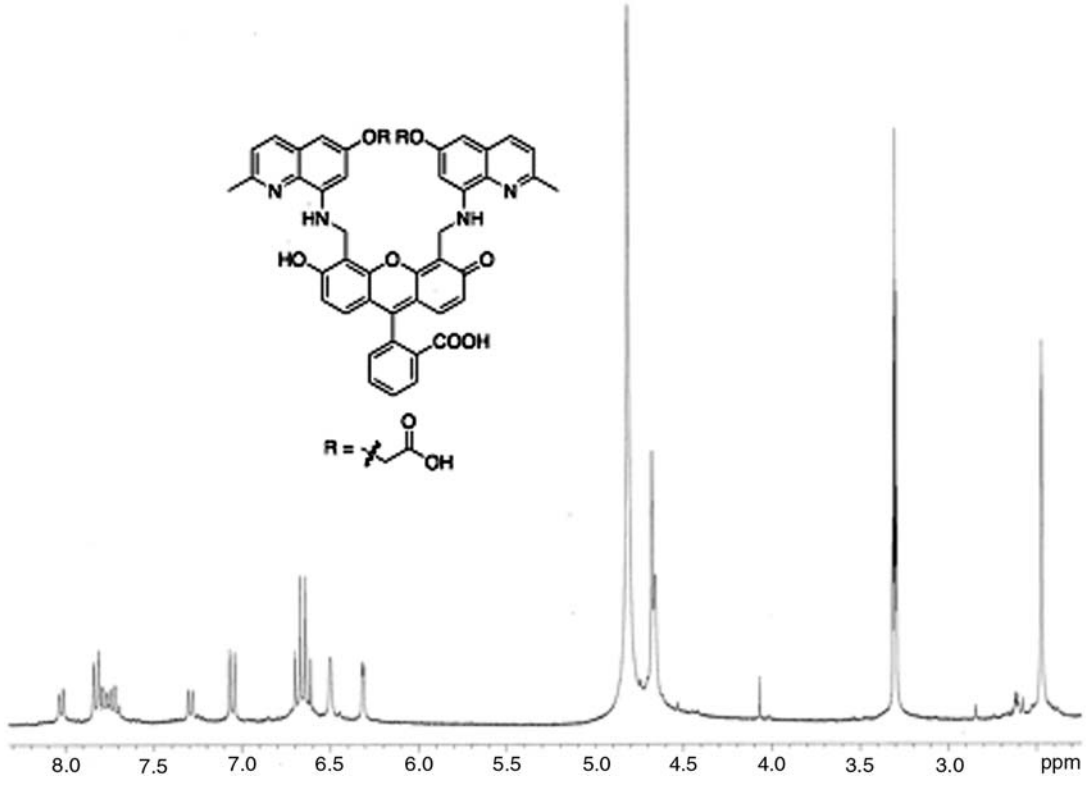

Fig. S6. ^{1}H NMR spectrum of FL2A (300 MHz, CD_3OD with drops of d_6-DMSO).