Supporting Information for:

Direct Detection of Nitroxyl in Aqueous Solution using a Tripodal Copper(II) BODIPY Complex

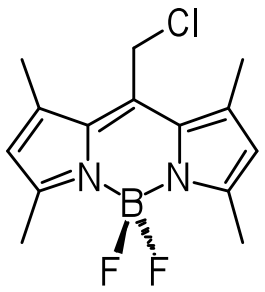
Joel Rosenthal and Stephen J. Lippard*

Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139.

Index *Page*

General Synthetic Materials and Methods. Silica gel 60 (70 - 230 and 230 - 400 mesh, Merck) and Merck 60 F254 silica gel (pre-coated sheets, 0.2 mm thick) were used for column and analytical thin-layer chromatography, respectively. Solvents for synthesis were of reagent grade or better and were dried according to standard methods.[1] All reagents were purchased from commercial sources and used as received.

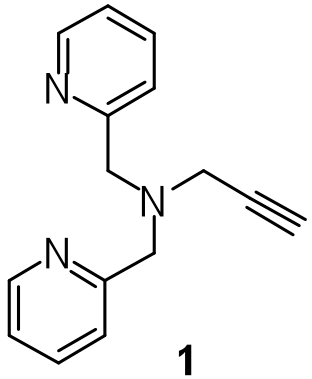
NMR spectra were obtained on a Varian 300, 500 or 501 MHz spectrometer, referencing to the residual proton resonance of the deuterated solvent. Low resolution mass spectra were obtained by using an Agilent 1100 Series LC/MSD mass spectrometer.



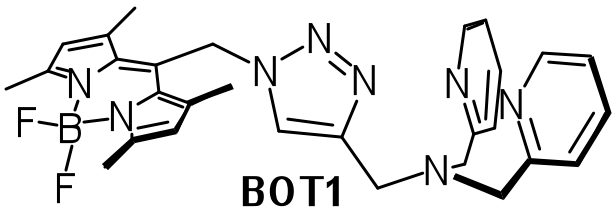
TM-BODIPY–CH_2Cl

8-(Chloromethyl)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene (TM-BODIPY–CH_2Cl). To 100 mL of DCM that had been sparged with N_2 for 30 min were added 0.64 mL (8.1 mmol) of chloroacetyl chloride and 1.67 mL (16.2 mmol) of 2,4-dimethylpyrrole. The resulting solution was stirred at 50 ºC under N_2. After 90 min the solvent was removed under reduced pressure and the residue dissolved in 5% DCM in toluene (200 mL). To the solution was added 5.5 mL of TEA and the mixture was stirred under air for 30 min, after which time 7.0 mL of $BF_3{\cdot}OEt_2$ was added. The solution was then stirred at 50 ºC for 90 min. The solvent was removed under reduced pressure and the residue redissolved in DCM. The organic solution was washed with water and dried over Na_2SO_4. The crude product was purified on silica eluting first with hexanes and toluene (3:1) while slowly moving to a mobile phase of hexanes and toluene (1:1). A second silica column was needed to purify the product completely (eluent = hexanes and DCM (1:1)) to deliver 430 mg of the desired product as a brick red powder (24%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.07 (s, 2H), 4.82 (s, 2H), 2.59 (s, 12H).

N,N-Bis(2-Pyridylmethyl)-N-Propargylamine (1).[2] The hydrochloride salt of 2-picolyl chloride (2.06 g, 12.6 mmol) was dissolved in 30 mL of methanol + water (2:1) and the solution was purged with nitrogen for 20 min. Following the addition of 0.40 mL of propargyl amine (6.2 mmol) the reaction solution was and heated at 65 °C under nitrogen for 12 h. After removing the solvent under reduced pressure, 80 mL of saturated sodium bicarbonate was added to the crude material and the resulting solution was extracted three times with 50 mL of DCM. The organic phases were combined and washed once with 40 mL of 3 M NaOH and then dried over $MgSO_4$. Removal of the solvent afforded 0.47 g of the desired product as a dark yellow oil (32%). The title compound was also prepared as follows. Dipicolylamine (0.90 mL, 5.0 mmol) was dissolved in 10 mL of THF. K_2CO_3 (2.76 g, 20.0 mmol) was added in one portion and the resulting suspension was stirred. To this mixture was added 557 µL (5.00 mmol) of propargyl bromide (80% in toluene) in a dropwise fashion. The resulting suspension was stirred for 24 h under nitrogen at 70 °C. After cooling the yellow mixture to room temperature saturated sodium bicarbonate was added and the solution was extracted with DCM. The organic extract was dried over $MgSO_4$. The title compound was purified on a column of basic alumina by eluting first with DCM to remove nonpolar impurities followed by a solution of 2% methanol in DCM. The alkyne product was isolated in 84% yield as a dark yellow oil. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.56 (ddd, J = 4.90, 1.76, 0.91 Hz, 2H), 7.67 (dt, J = 7.65, 7.61, 1.82 Hz, 2H), 7.52 (d, J = 7.85 Hz, 2H), 7.17 (ddd, J = 7.42, 4.92, 1.22 Hz, 2H), 3.90 (d, J = 18.57 Hz, 4H), 3.43 (d, J = 2.38 Hz, 2H), 2.35-2.25 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 159.00, 149.50, 136.72, 123.40, 122.35, 73.87, 59.70, 42.79. LRMS (ESI) Calcd for $[M + Na]^+$ = 260.1, found 260.1.



1



BODIPY-triazole 1 (BOT1). Sodium azide (8.0 mg, 0.12 mmol) was combined with 30 mg (0.10 mmol) TM-BODIPY–CH_2Cl, 15 mg (0.060 mmol) of $CuSO_4 \cdot 5H_2O$, 25 mg (0.10 mmol) of L-ascorbic acid and 80 mg (0.34 mmol) of alkyne **13**. The solid mixture was suspended in 4 mL of DMF and 1 mL of water under an atmosphere of argon. After 8 h the resulting solution was added to a solution of saturated sodium bicarbonate with a minimal amount of ammonium hydroxide. The aqueous solution was then extracted with ethyl acetate and washed with brine. After drying the organic solution over $MgSO_4$ the crude material was purified on basic alumina eluting first with DCM and then with 2% methanol in DCM to isolate an orange band. The final product was purified further by preparatory TLC on silica using 10% methanol in DCM as the mobile phase to deliver 3.5 mg of the desired product as a red powder (7%); mp = 131 – 133 °C (dec). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.46 (ddd, J = 4.90, 1.74, 0.84 Hz, 2H), 7.59 (dt, J = 7.63, 7.60, 1.81 Hz, 2H), 7.51 (s, 1H), 7.45 (d, J = 7.84 Hz, 2H), 7.10 (ddd, J = 7.47, 4.92, 1.23 Hz, 2H), 6.09 (s, 2H), 5.79 (s, 2H), 3.84 (s, 2H), 3.79 (s, 4H), 2.57 (s, 6H), 2.19 (m, 6H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 159.17, 159.00, 157.81, 149.51, 149.28, 145.48, 136.74, 136.64, 123.40, 122.37, 122.25, 122.06, 78.56, 73.91, 59.80, 42.81, 15.95. LRMS (ESI) Calcd for $[M + Na + H_2O]^+$ = 563.3, found 563.3. HRMS (ESI) calcd 541.2821 ($[M + H]^+$), found 541.2840.

The **Cu^{II}[BOT1]Cl_2** complex was prepared and isolated by adding a solution of $CuCl_2 \cdot 2H_2O$ (2.8 mg, 0.016 mmol) dissolved in 0.5 mL of acetone to a solution of 10 mg (0.016 mmol) **BOT1** in 1.0 mL acetone. The metal complex was precipitated from solution by addition of diethyl ether to deliver 9.6 mg of the desired product as a dark red solid (87%); mp = 137 – 140 °C (dec). HRMS (ESI) calcd 638.1717 ($[M - Cl]^+$), found 638.1638. The isolated product analyzed as the acetone solvate. Anal. Calcd for $C_{32}H_{37}BCl_2CuF_2N_8O$: C, 52.44; H, 5.09; N, 15.29. Found: C, 52.22; H, 4.69; N, 15.03.

General Spectroscopic Materials and Methods. PIPES, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid), purchased from Calbiochem, potassium chloride (99.999%), purchased from Aldrich, and copper chloride dihydrate (99+%), purchased from Alfa Aesar, were used as received. Buffer solutions (50 mM PIPES, 100 mM KCl, pH 7) were prepared with Millipore water. Stock solutions of 1.0 mM of BOT1 were prepared in DMSO and stored at -80 ^{o}C. Stock solutions of 1.0 mM $CuCl_2$ were prepared in Millipore water. UV/visible spectra were acquired on a Cary 50 spectrometer using PMMA cuvettes from Perfector Science (3.5 mL volume, 1 cm path length) or screw cap quartz cuvettes (7q) from Starna. Acquisitions were made at 25.0 ± 0.05 ^{o}C. NO gas was purchased from Airgas and purified as previously described.[3] Solutions of *S*-Nitroso-L-cysteine (SNOC) (100 mM) were prepared as described.[4] Nitric oxide gas and other reactive nitrogen and oxygen species were introduced into buffered solutions via gas tight syringes. Fluorescence spectra were obtained on a Photon Technology International (PTI) fluorimeter at 25.0 ± 0.5 ^{o}C following previously described procedures.[5] X-band EPR spectra were recorded on a Bruker EMX EPR spectrometer. Temperature control was maintained with a quartz finger dewar (77 K). Spectra were recorded in 4 mm o.d. quartz EPR tubes capped with a tight-fitting rubber septum.

The excitation source for luminescence lifetime measurements was a chirped-pulse amplified Ti:Sapphire laser system described elsewhere.[6] Samples were irradiated at λ_{exc} = 400 nm. The excitation was vertically polarized and attenuated to 100 - 200 nJ/pulse. Luminescence lifetime kinetics were measured on a Hamamatsu C4334 Streak Scope streak camera. The emission was collected at the magic angle (θ_m = 54.7^{o}) over a 140-nm window centered on the emission peak.

Mass Spectrometry of NO gas. An Agilent Technologies 5975C Mass Selective Detector operating in electron impact ionization mode was used to collect mass spectrometric data. The experiment was performed in a custom built gas-tight reaction vessel with gas inlet and outlet ports. The cell was connected to the He carrier gas and mass spectrometer and purged for several hours before data collection. The mass

spectrometer was operated in selective ion mode with detection of 30 (NO), 32 (O_2), and 44 (CO_2) amu ions. The 32 and 44 amu signal was used to determine the residual air background. This signal remained at baseline level throughout the experiment.

Cell Culture. HeLa cells were cultured at 37 °C under a 5% CO_2 humidified atmosphere in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, HyClone), glutamine (2 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL). For live cell imaging, cells were plated in 35 mm poly-D-lysine coated glass-bottom culture dishes (MatTek Corporation) in 2 mL of DMEM.

After 1 – 2 d, the growth medium was removed and replaced with a solution containing 1 μM **Cu^{II}[BOT1]**, which was added directly or formed in situ via addition of 1 μM **BOT1** and 2 μM $CuCl_2$ in complete DMEM. After an incubation time of 1 h (37 °C, 5% CO_2), the cells were rinsed with warm PBS (2 × 2 mL) and imaged in 2 mL of PBS. HNO was introduced to the live cells by addition of a freshly prepared stock solution of Angeli's salt in 0.01 N NaOH directly to the cell culture dishes on the microscope stage to reach a 200 μM final concentration.

Fluorescence Microscopy. The imaging experiments were performed using 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). The light source was an X-Cite 120 metal-halide lamp (EXFO) and the fluorescence images were obtained using an oil-immersion objective at 63× magnification. The microscope stage was outfitted with an INC-2000 incubator, which maintained the samples at 37°C under a 5% CO_2 humidified atmosphere. Microscope operation and image processing were conducted using the Volocity software (Improvision). Exposure times for the fluorescence images ranged from 200 to 1000 ms and were kept constant for each image series.

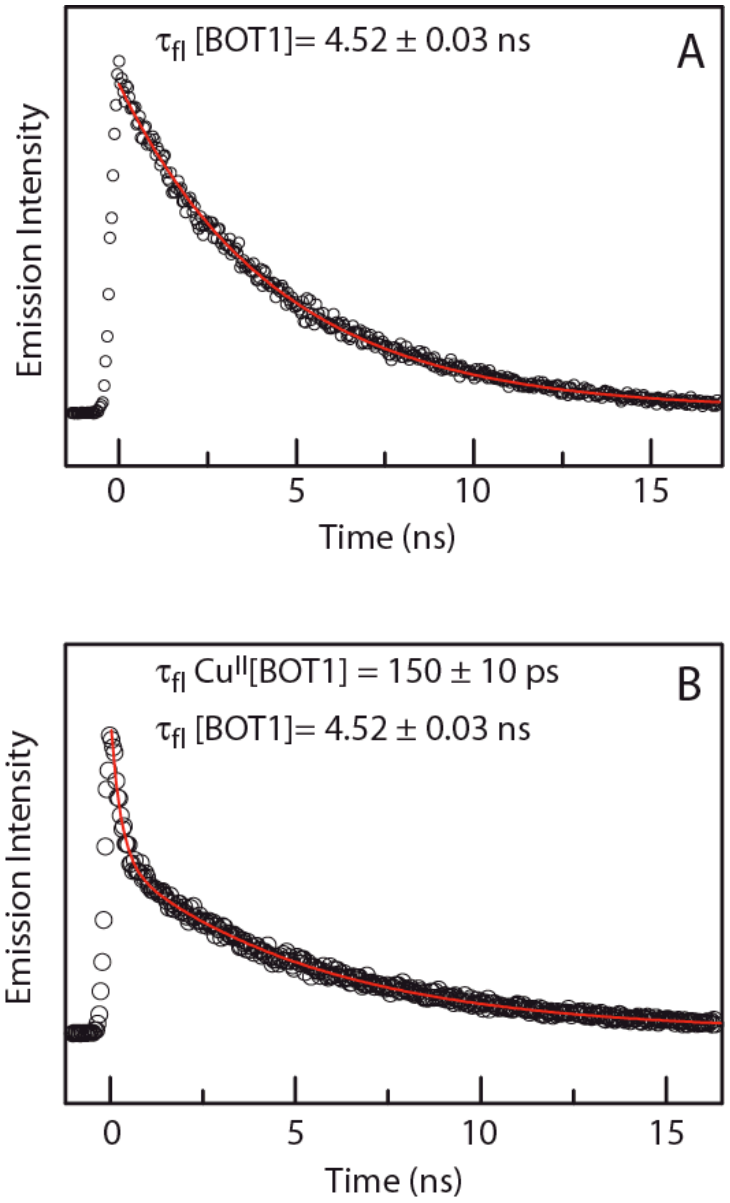


Figure S1. Time resolved fluorescence decay profiles recorded in 50 mM PIPES buffer and 100 mM KCl (pH = 7, T = 25°C, l_{exc} = 400 nm) for (A) 3 μM BOT1 and (B) 3.0 μM BOT1 and 6.0 μM $CuCl_2$. The trace for BOT1 and $CuCl_2$ is biexponential due to the presence of uncomplexed BOT1 (long component) and the Cu[BOT1] complex (short component).

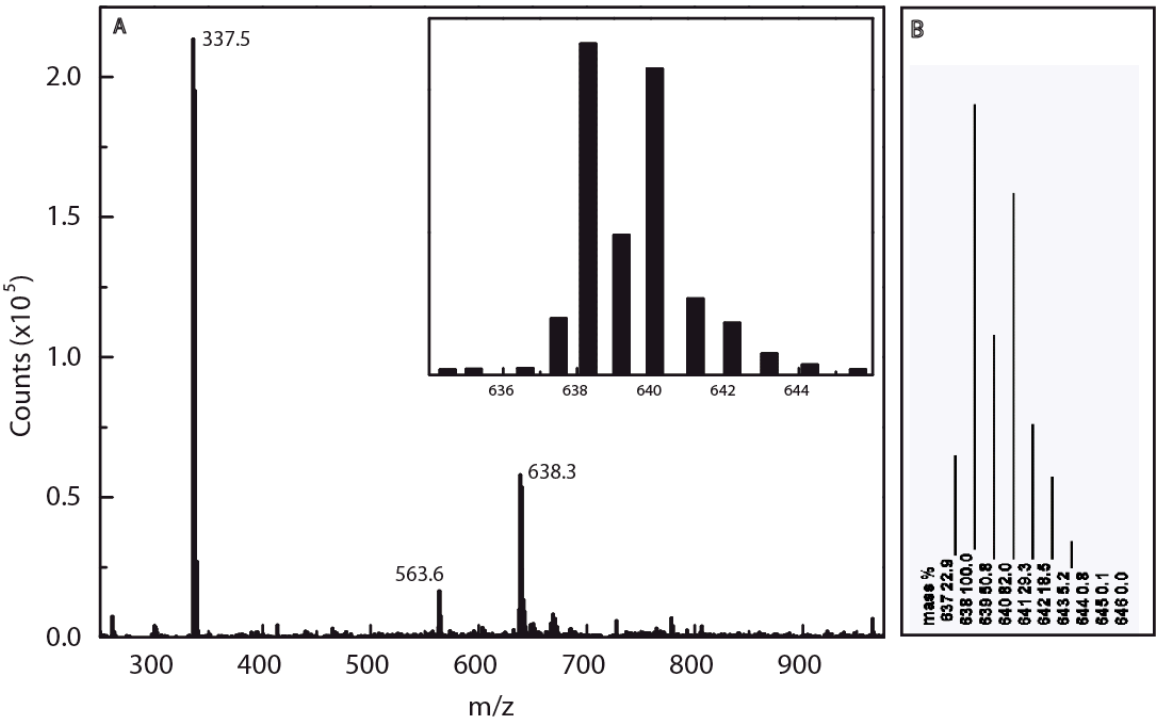


Figure S2. ESI(+)MS spectrum of the Cu[BOT1]Cl^+ species. The peak at m/z = 638.3 corresponds to $[Cu[BOT1]Cl]^+$ (calcd. m/z = 638.17) Peaks m/z = 337.5 and m/z = 563.6 correspond to $[CuCl[BOT1]Cl + 3H]^{2+}$ (calcd. m/z = 338.0) and uncomplexed $[BOT1 + Na]^+$ (calcd. m/z = 563.3), respectively. (A) Experimental, (B) calculated ion distribution for Cu[BOT1]Cl^+ species.

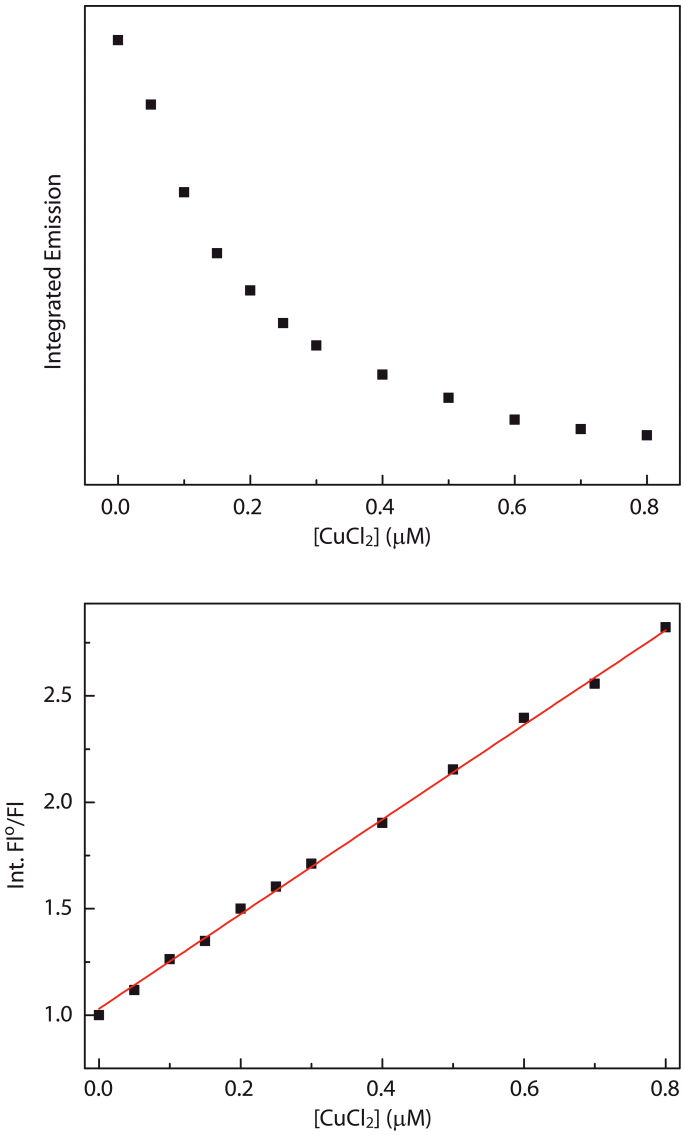


Figure S3. Measurement of the dissociation constant (K_d) of Cu[BOT1]. $CuCl_2$ was titrated into a 3 μM solution of BOT1 (50 mM PIPES, 100 mM KCl, pH 7.0). (A) The formation of Cu[BOT1] was followed by the observing change in integrated emission intensity as a function of added Cu^{2+}. (B) A Benesei-Hildebrand fit of the intensity of the BODIPY luminescence with added $CuCl_2$ yields $K_d = 1/K_a = 3.0 \pm 0.1$ μM.

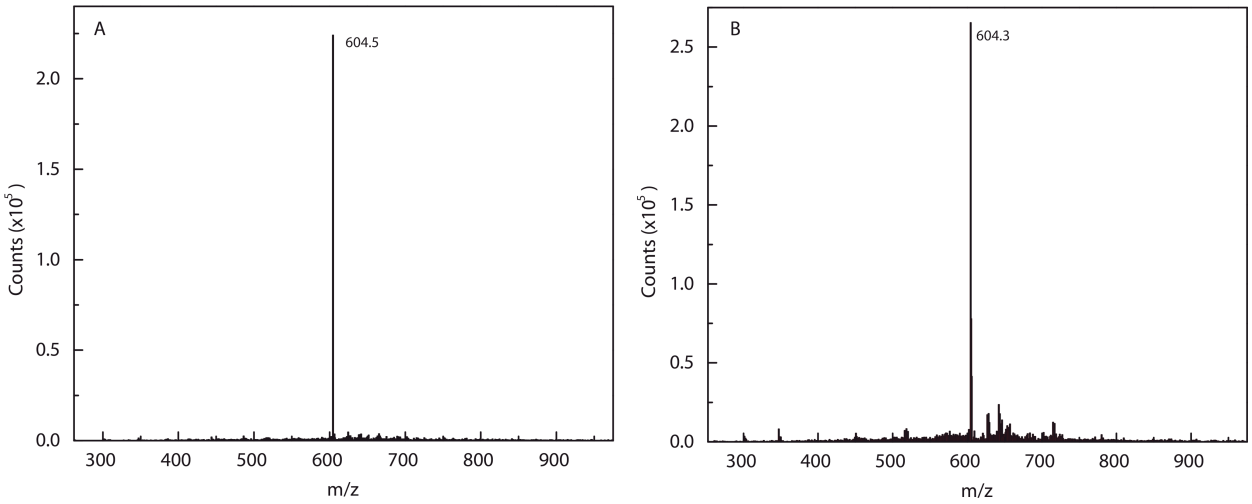


Figure S4. ESI(+)MS spectrum of the $[Cu[BOT1]]^+$ species (calcd. m/z = 604.0) formed upon reduction of **Cu^{II}[BOT1]** with (A) cysteine, (B) Angeli's salt.

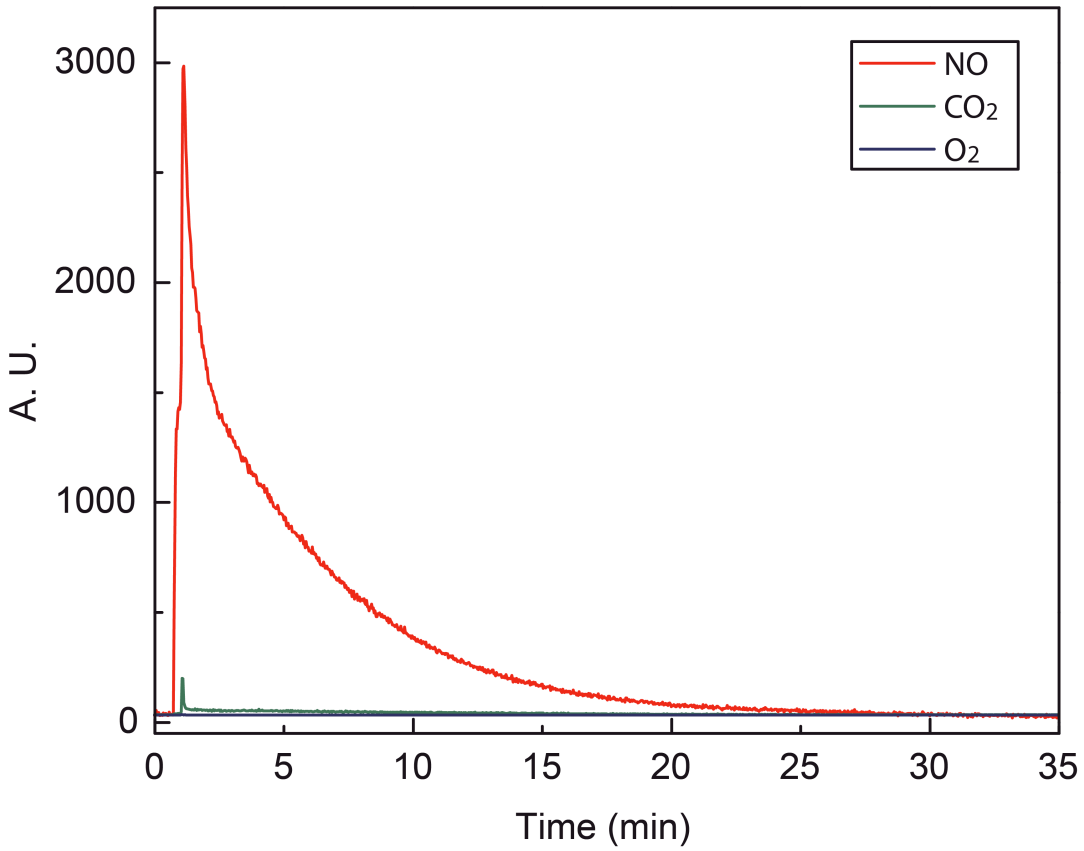


Figure S5. EI-MS detection of NO formed upon reaction of **Cu[BOT1]** with Angeli's salt. Background levels of CO_2 and O_2 remain at baseline levels throughout the course of the experiment.

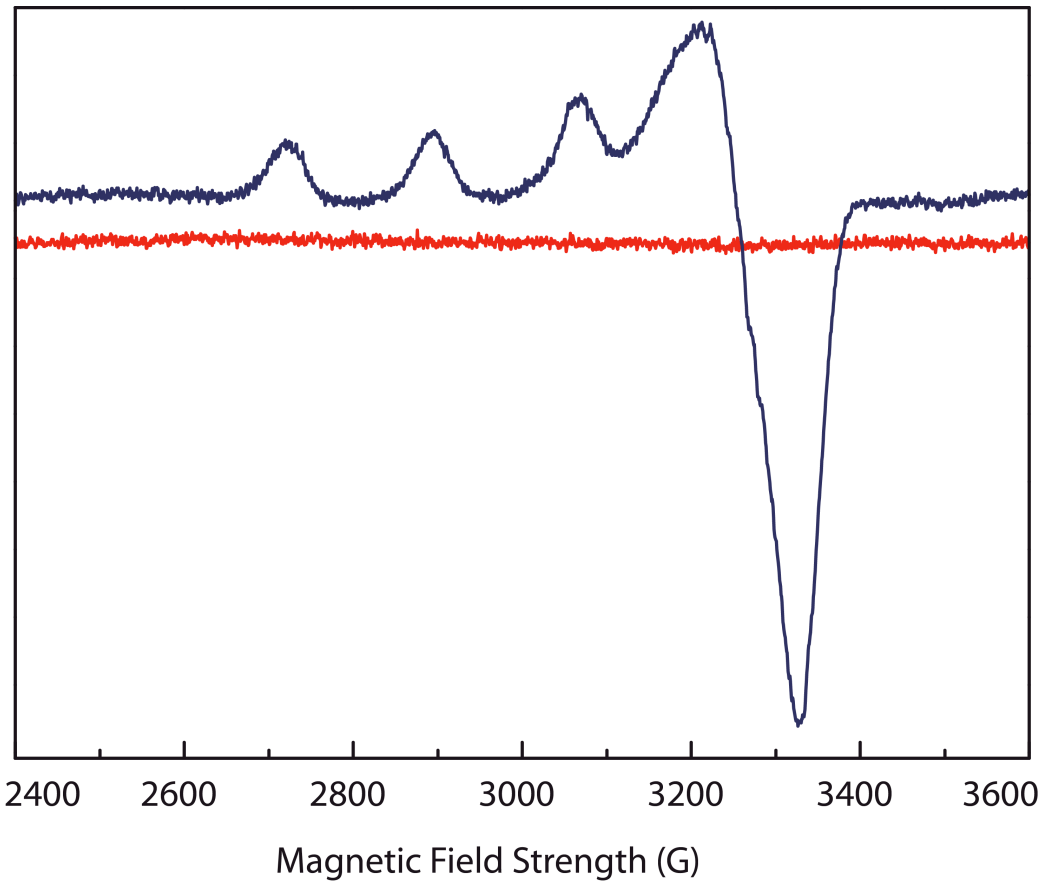


Figure S6. X-band EPR spectra of 0.1 mM **Cu^{II}[BOT1]** in EtOH (blue) in which 5 mg of Angeli's salt was suspended (red). Spectra were recorded at 77 K.

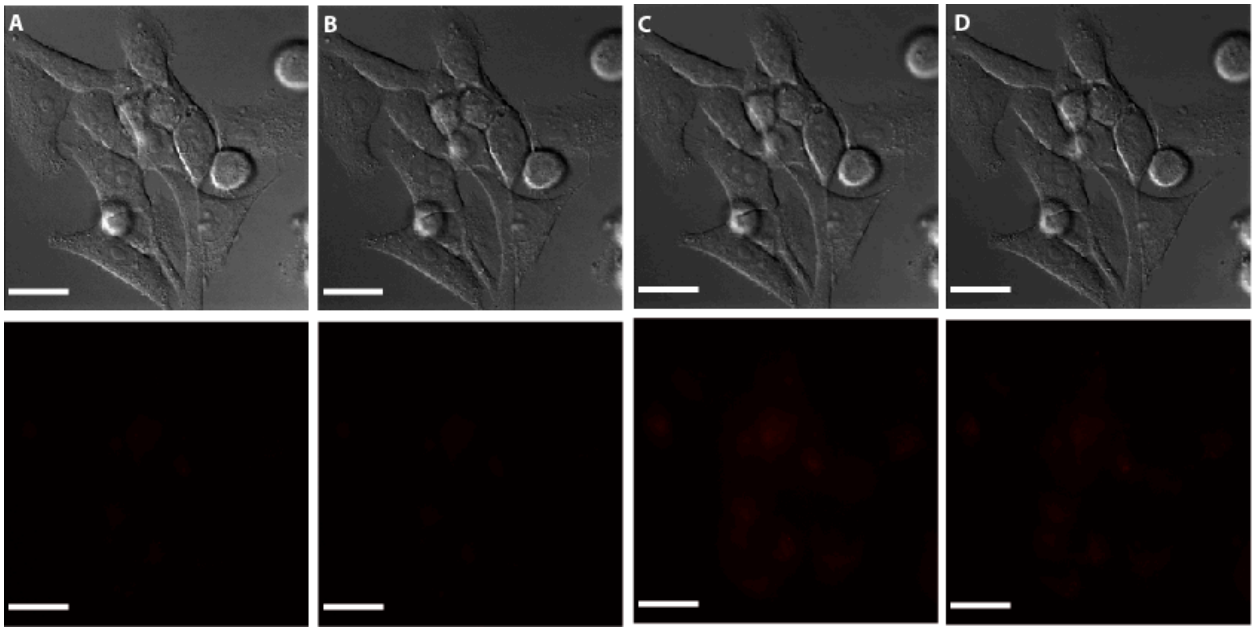


Figure S7. Fluorescence response in HeLa cells (A) stained with 1 μM **Cu^{II}[BOT1]** over the course of (B) 5 min, (C) 10 min and (D) 15 min. (Top) DIC image, (bottom) fluorescence image. Scale bar: 25 μm.

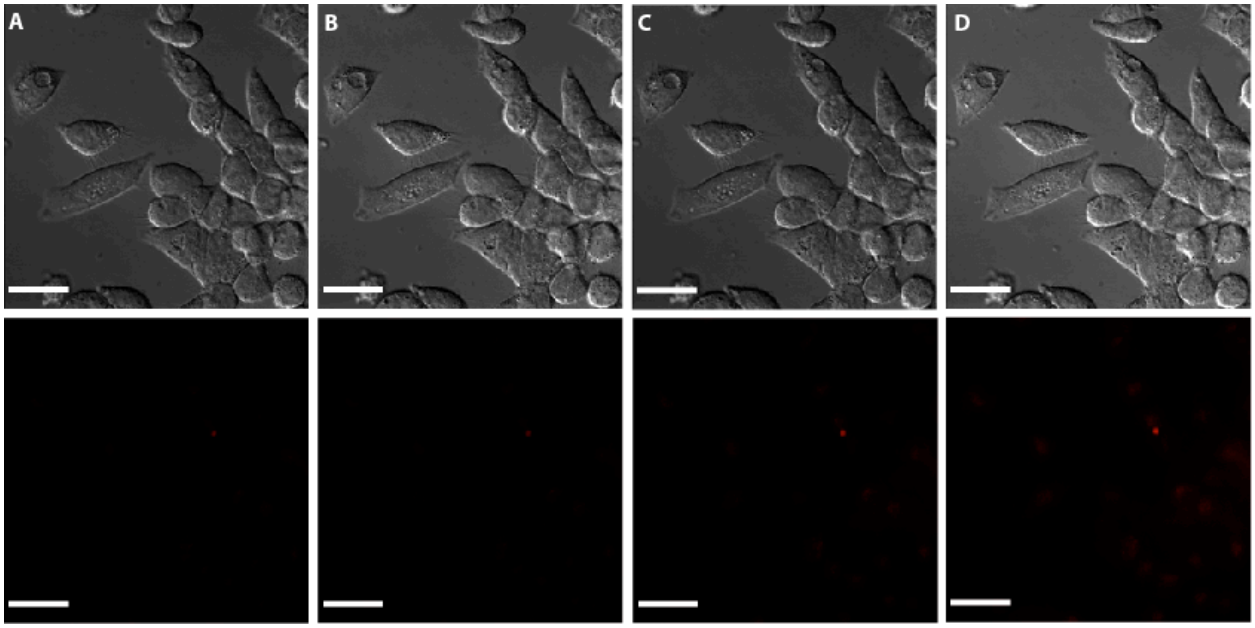


Figure S8. Nitric oxide induced fluorescence response in HeLa cells (A) stained with 1 μM **Cu^{II}[BOT1]** and (B) 5 min, (C) 10 min and (D) 15 min after treatment with diethylamine NONOate (200 μM). (Top) DIC image, (bottom) fluorescence image. Scale bar: 25 μm.

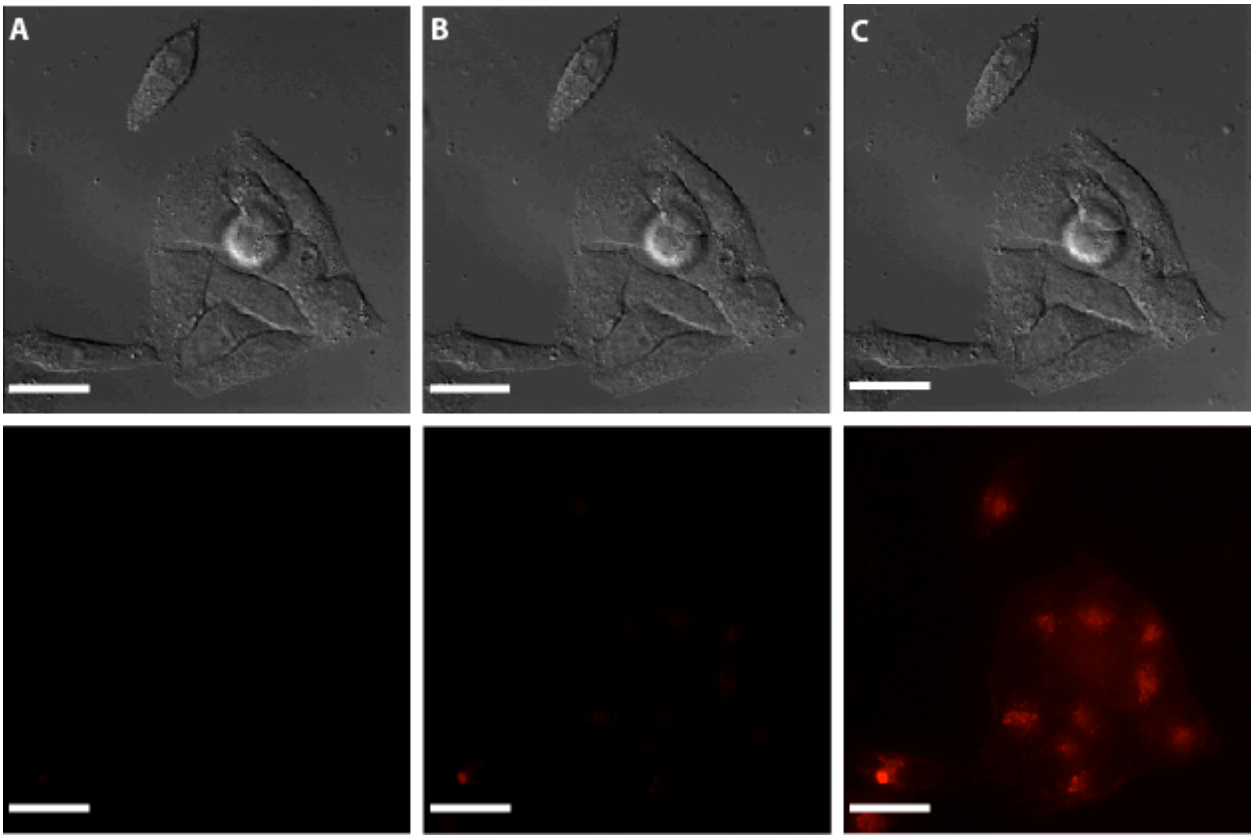


Figure S9. Cysteine induced fluorescence response in HeLa cells (A) stained with 1 μM **Cu^{II}[BOT1]** and (B) 1 min, (C) 5 min after treatment with cysteine (200 μM). (Top) DIC image, (bottom) fluorescence image. Scale bar: 25 μm.

Table S1. Photophysical properties of BOT1 and Cu^{II}BOT1

	Absorbance		Fluorescence		
	λ_{abs}	ε ($M^{-1}cm^{-1}$)	λ_{em}	Φ_{Fl}	τ_{Fl}
BOT1	518 nm	30900 ± 960	526 nm	0.12	4.53 (3) ns
Cu^{II}[BOT1]	518 nm	30150 ± 875	526 nm	0.01	150 (10) ps

(1) Armarego, W. L. F.; Perrin, D. D. *Purification of Laboratory Chemicals*; 4th ed.; Butterworth-Heinmann: Oxford, 1996.
(2) Bergbreiter, D. E.; Hamilton, P. N.; Koshti, N. M.; *J. Am. Chem. Soc.* **2007**, *129*, 10666-10667.
(3) Lorkovic, I. M.; Ford, P. C.; *Inorg. Chem.* **1999**, *38*, 1467-1473.
(4) Kröncke, K. D.; Kolb-Bachofen, V.; Lester, P. In *Methods Enzymol.*; Academic Press: 1999; Vol. Volume 301, p 126-135.
(5) Lim, M. H.; Xu, D.; Lippard, S. J.; *Nat. Chem. Biol.* **2006**, *2*, 375-380.
(6) Damrauer, N. H.; Hodgkiss, J. M.; Rosenthal, J.; Nocera, D. G.; *J. Phys. Chem. B* **2004**, *108*, 6315-6321.