Lanthanide-Based Luminescent Probes for Selective Time-Gated Detection of Hydrogen Peroxide in Water and in Living Cells

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I. Synthetic Materials and Methods. All reactions utilizing air- or moisture sensitive reagents were carried out under a dry nitrogen atmosphere. Silica gel P60 (SiliCycle) was used for column chromatography and SiliCycle 60 F254 silica gel (precoated sheets, 0.25 mm thick) was used for analytical thin layer chromatography. 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl) benzaldehyde was purchased from Oakwood Products (West Columbia, SC). Compounds **5**[1] and **[Tb•4]** were synthesized according to literature procedures. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. ^{1}H NMR and ^{13}C NMR were collected in $CDCl_3$ or CD_3OD (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC on a Bruker AVQ-400 spectrometer at the College of Chemistry NMR Facility at the University of California, Berkeley. All chemical shifts are reported in the standard δ notation of parts per million using the peak of residual proton signals of $CDCl_3$ or CD_3OD as an internal reference. 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 at the University of California, Berkeley. Elemental analyses were performed by the Microanalytical Laboratory, University of California, Berkeley, CA. Terbium loading for complexes **[Tb•1]**–**[Tb•4]** were determined using a Perkin Elmer Optima 7000 DV Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) at the Department of Chemistry at the University of California, Berkeley.

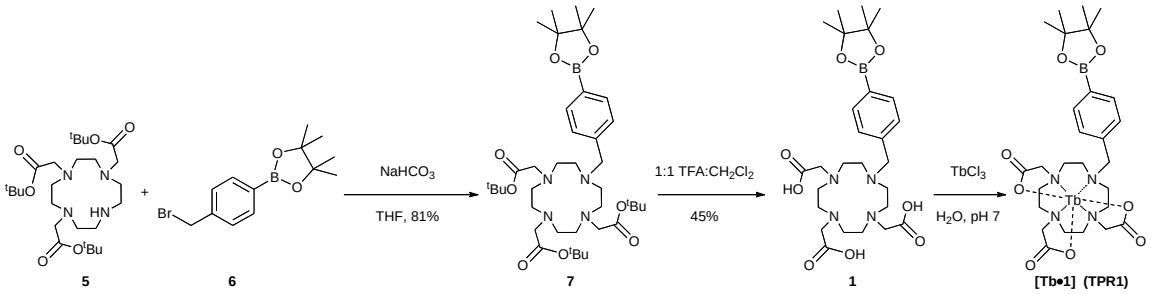

Figure S1. Synthesis of **[Tb•1] (TPR1)**.

***tert*-Butyl 2,2',2''-(10-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan—yl)benzyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate** (**7**). A pressure flask was charged with 2-(4-(bromomethyl)phenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (100 mg, 0.34 mmol, 1.7 equiv), **5** (100 mg, 0.200 mmol, 1.0 equiv), $NaHCO_3$ (70.9 mg, 1.00 mmol, 5.0 equiv), and THF (4 mL) under a N_2 atmosphere. The mixture was heated at 95 ºC

(1) S. Mizukami, K. Tonai, M. Kaneko, and K. Kikuchi. *J. Am. Chem. Soc.* **2008**, *130*, 14376.

overnight, before it was cooled to rt. The reaction mixture was then filtered, washed with MeOH, and concentrated by rotary evaporation to give a yellow solid. Purification by silica column chromatography (45:1 → 4:1 CH_2Cl_2:MeOH) yielded **7** (115 mg, 81%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.59 (d, *J* = 8 Hz, 2H), 7.44 (d, *J* = 8 Hz, 2H), 3.03 (s, 4H), 2.86 (s, 4H), 2.68 (s, 4H), 2.58 (s, 4H), 1.41–1.34 (m, 27H); ^{13}C NMR (400 MHz, CD_3OD) δ 175.21, 174.40, 142.56, 136.25, 130.92, 85.27, 83.97, 83.41, 60.58, 56.83, 28.62, 25.49; LRMS (ESI) calcd for $C_{39}H_{68}BN_4O_{18}$ ($M+H^+$), 731.5130, found 731.5.

2,2‘,2‘‘-(10-4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2yl)benzyl)-1,4,7,10-tetraazacyclo-dodecane-1,4,7-tri-yl)triacetic acid (**1**).Trifluoroacetic acid (5 mL) and 1 drop of triisopropylsilane was added to a solution of **7** (100 mg, 0.14 mmol, 1 equiv) in dry CH_2Cl_2 (5 mL) under a N_2 atmosphere. The resulting mixture was stirred for two days at rt. Et_2O was added and the resulting precipitate was collected by filtration, washed with Et_2O, and dried to yield **1** as a white TFA salt (24 mg, 37%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.71 (d, *J* = 7.4 Hz, 2H), 7.50 (d, *J* = 7.3 Hz, 2H), 4.33 (s, 4H), 3.73 (s, 4H), 3.27 (s, 4H), 3.10 (s, 4H), 1.22 (s, 12H); ^{13}C NMR (400 MHz, CD_3OD) δ 163.24 162.90, 136.86, 131.92, 131.67, 85.58, 75.98, 59.33, 54.34, 52.33, 51.02, 25.32, 25.17; LRMS (ESI) calcd for $C_{27}H_{44}BN_4O_8$ ($M+H^+$), 563.3252, found 563.3.

[Tb•1]. Ligand **1** (90 mg, 0.16 mmol, 1.0 equiv) and $TbCl_3$•$6H_2O$ (60 mg, 0.16 mmol, 1.0 equiv) were dissolved in water (3 mL). The pH was adjusted to 7 by the slow addition of 1M NaOH in portions and the reaction mixture was stirred at rt overnight. The pH was raised to 9–10 with careful addition of 1M NaOH. The cloudy solution was filtered and then brought to pH 7 with careful addition of 1M HCl. The clear solution was concentrated to yield **[Tb•1]** as a white solid. λ_{max} = 226 nm; ε = 6700 M^{-1} cm^{-1}; τ^{Tb} (H_2O, pH = 7.4, 10 μM) = 1.26 ms; Φ = 0.029; HRMS calcd for $C_{27}H_{40}N_4O_8BNaTb$ ($M+Na^+$), 741.2085, found 741.2087. A salt-free sample was obtained by preparative HPLC purification (MeOH/H_2O, C18 Silica Gel) to provide the corresponding boronic acid. Anal. Found: C, 35.40; H, 5.36; N, 7.45; $C_{21}H_{30}N_4O_8BTb$•$4H_2O$ requires: C, 35.61; H, 5.41; N, 7.91.

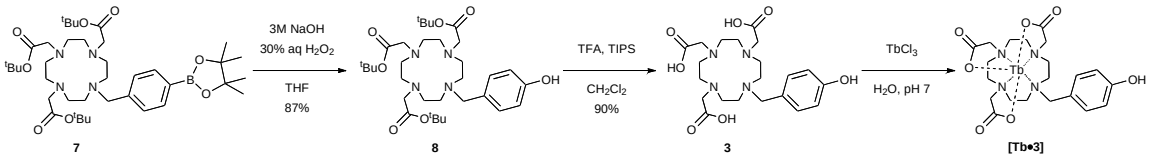

Figure S2. Synthesis of **[Tb•3]**.

***tert*-Butyl 2,2’,2’’-(10-(4-hydroxybenzyl)-1,4,7,10-tetraaza-cyclododecane-1,4,7-triyl)tri-acetate** (**8**). To a solution of **7** (230 mg, 0.315 mmol, 1.0 equiv) in THF (3 mL) was added 3M NaOH (300 μL, 0.90 mmol, 2.9 equiv) and 10 M H_2O_2 (30 μM, 0.90 mmol, 2.9 equiv). The mixture was stirred for 4 h at rt, extracted with CH_2Cl_2, and the organic layer was separated, dried over Na_2SO_4, and concentrated by rotary evaporation to give a light yellow solid. Purification by silica column chromatography (8:1:1 CH_2Cl_2:MeOH:EtOAc) afforded **8** as a white solid (170 mg, 87%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.11 (d, *J* = 8.4 Hz, 2H), 6.55 (d, *J* = 8.4 Hz, 2H), 2.95 (s, 4H), 2.68 (s, 4H), 2.15 (s, 4H), 1.50–1.21 (m, 27H); ^{13}C NMR (400 MHz, CD_3OD) δ 175.19, 174.41, 158.20, 132.74, 129.57, 116.53, 83.94, 83.43, 59.75, 56.95, 56.88, 28.12; LRMS (ESI) calcd for $C_{33}H_{57}N_4O_7$ ($M+H^+$), 621.4227, found 621.5.

2,2’,2’’-(10-(4-Hydroxybenzyl)-1,4,7,10-tetraazacyclodo-decane-1,4,7-triyl)triaceticacid (**3**). Trifluoroacetic acid (3 mL) and 1 drop of triisopropylsilane was added to **8** (33 mg, 5.32 mmol) in CH_2Cl_2 (1 mL) under a N_2 atmosphere. The resulting mixture was stirred for 2 days at rt under N_2. Et_2O was then added to give a white

precipitate. The solid was collected by filtration and rinsed with Et_2O to give **3** as a white TFA salt (22 mg, 72%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.35 (d, *J* = 8 Hz, 2H), 6.75 (d, *J* = 8 Hz, 2H), 4.31 (s, 4H), 3.88 (s, 4H), 3.40 (s, 4H), 3.01 (s, 4H); ^{13}C NMR (400 MHz, CD_3OD) δ 163.23, 162.88, 160.71, 134.37, 120.24, 117.45, 59.21, 56.62, 53.92, 52.89, 50.76; LRMS (ESI) calcd for $C_{21}H_{33}N_4O_7$ ($M+H^+$), 453.2349, found 453.3.

[Tb•3]. Ligand **3** (90 mg, 0.16 mmol, 1.0 equiv) and $TbCl_3.6H_20$ (60 mg, 0.16 mmol, 1.0 equiv) were dissolved in water (3 mL). The pH was adjusted to 7 by slow addition of 1M NaOH in portions and the reaction mixture was stirred at rt for 2 days. The pH was raised to 9-10 and filtered. After readjusting to pH 7 with 1M HCl, the clear solution was evaporated to yield the complex **[Tb•3]** as a white solid. λ_{max} = 226 nm; ε = 10200 M^{-1}; τ^{Tb} (H_2O, pH = 7.4, 10 μM) = 1.23 ms; Φ = 0.054; HRMS calcd for $C_{21}H_{30}N_4O_7Tb$ ($M+H^+$), 608.1520, found 608.1524; Anal. Found: C, 17.14; H, 2.51; N, 3.44; $C_{21}H_{29}N_4O_7Tb•3H_2O•14NaCl$ requires C, 17.03; H, 2.38; N, 3.78.

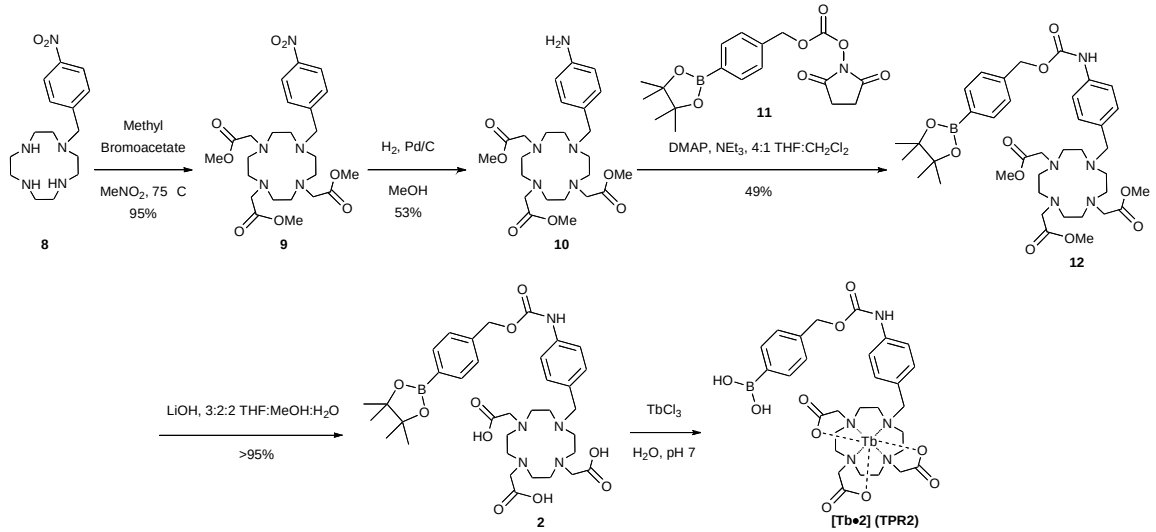

Figure S3. Synthesis of **[Tb•2] (TPR2)**.

Trimethyl 2,2',2''-(10-(4-nitrobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (9). The monosubstituted cyclen **8** (365.8 mg, 1.191 mmol, 1.0 equiv) was dissolved in 10 mL $MeNO_2$ in a dry sealable flask. Sodium carbonate (937.0 mg, 8.841 mmol, 7.4 equiv) and methyl bromoacetate (0.38 mL, 4.1 mmol, 3.5 equiv) were added, the flask was sealed, and the reaction mixture was heated at 100 °C for 16 h. The reaction mixture was filtered and concentrated to yield a crude oil which was purified by silica chromatography (95:5 CH_2Cl_2:MeOH) to yield the tetra-substituted cyclen **9** (549 mg, 88%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19 (d, *J* = 8.0 Hz, 2H), 7.65 (d, *J* = 8.0 Hz, 2H), 3.79 (s, 9H), 3.40–2.31 (m, 24H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.81, 174.04, 147.26, 144.84, 131.36, 123.54, 58.67, 55.40, 52. 73, 52.48, 50.00; LRMS (ESI) calcd for $C_{24}H_{38}N_5O_8$ ($M+H^+$), 524.272, found 524.3.

Trimethyl 2,2',2''-(10-(4-aminobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (10). The cyclen **9** (223.3 mg, 0.4265 mmol, 1 equiv) was dissolved in 2.1 mL MeOH and Pd/C (43.3 mg) was added. The flask was briefly evacuated and backfilled with H_2 from a balloon. The reaction was stirred under a H_2 atmosphere for 6 h. The reaction was filtered, concentrated, and purified by silica column chromatography (10:1 CH_2Cl_2:MeOH) to yield the aniline **10** (109.0 mg, 52%) as a mixture of conformational isomers. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27 (d, *J* = 8.0 Hz, 2H),* 7.16 (d, *J* = 8.0 Hz, 2H), 6.63 (d, *J* = 8.0 Hz),* 6.58 (d, *J* = 8.0 Hz), 3.70 (s, 9H), 3.66 (s, 9H),* (m, 24H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.76, 174.18, 171.78, 171.50, 149.74,

147.12, 132.50, 130.96, 125.75, 116.62, 114.66, 114.60, 78.31, 77.98, 77.65, 58.16, 55.92, 55.11, 54.81, 54.37, 53.99, 53.53, 52.38, 51.75, 51.45, 50.88, 50.76, 50.56, 50.32, 49.92, 49.31; LRMS (ESI) calcd for $C_{24}H_{40}N_5O_6$ ($M+H^+$), 494.298, found 494.3. * Peaks assigned to the minor conformational isomer.

2,5-Dioxopyrrolidin-1-yl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl carbonate (11). N,N'-Disuccinimidyl carbonate (120.3 mg, 0.4696 mmol, 1.5 equiv) was added to a solution of (4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)methanol[2] (71.3 mg, 0.305 mmol, 1.0 equiv) in CH_2Cl_2. NEt_3 (0.13 mL, 0.93 mmol, 3.1 equiv) was added and the reaction was stirred for 2.5 h at rt. The solvent was removd in vacuo and the residue was redissolved in 10 mL EtOAc, washed with 3 mL sat. $NaHCO_3$ and 3 mL brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated to yield **11** (109.9 mg, 96%) as a clear oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (d, *J* = 8.0 Hz, 2H), 7.40 (d, *J* = 8.0 Hz, 2H), 5.33 (s, 2H), 2.83 (s, 4H), 1.35 (s, 12H).

Trimethyl-2,2',2''-(10-(4-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyloxy)carbonylamino)benzyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (12). The tetra-substituted cyclen **10** (59.8 mg, 0.121 mmol, 1.0 equiv) was dissolved in 1.25 mL 4:1 THF:CH_2Cl_2. The activated ester **11** (109.9 mg, 0.293 mmol, 2.4 equiv) was added as a solution in 3 x 0.33 mL THF. DMAP (27.3 mg, 0.223 mmol, 1.8 equiv) and NEt_3 (53 μL, 0.38 mmol, 3.1 equiv) were added and the reaction was stirred for 14 h at rt. The reaction mixture was concentrated and purified by silica column chromatography (96:4 CH_2Cl_2:MeOH → 90:10 CH_2Cl_2:MeOH) to yield **12** (43.3 mg, 47%) as a clear oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (d, *J* = 8.0 Hz, 2H), 7.52 (d, *J* = 8.0 Hz, 2H), 7.40 (d, *J* = 8.0 Hz), 7.28 (d, *J* = 8.0 Hz), 5.18 (s, 2H), 3.75 (s, 6H), 3.66 (s, 3H), 3.60–2.40 (m, 24H), 1.32 (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.51, 174.35, 173.76, 173.25, 153.39, 139.34, 137.94, 134.94, 131.64, 130.72, 127.17, 118.47, 83.83, 66.54, 58.77, 55.23, 54.75, 52.84, 52.44, 50.61, 25.42, 24.81; LRMS (ESI) calcd for $C_{38}H_{57}BN_5O_{10}$ ($M+H^+$), 754.4198, found 754.4.

2,2',2''-(10-(4-((4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzyloxy)carbonylamino)benzyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (2). 12 (43.3 mg, 0.0575 mmol, 1.0 equiv) was dissolved in 0.64 mL 3:2:2 THF:MeOH:H_2O. 1M LiOH in H_2O (60 μL, 0.06 mmol, 1.0 equiv) was added and the reaction was monitored by LC/MS. Additional 1M LiOH was added after 3.5 h (120 μL, 0.12 mmol, 2.1 equiv) and after 7 h (180 μL, 0.18 mmol, 3.1 equiv). After a total of 20 h, the reaction mixture was concentrated to yield **2** (45.2 mg) as a white solid. ^{1}H NMR (400 MHz, MeOD) δ 7.59–7.48 (m, 2H), 7.35 (br s, 4H), 7.22–7.15 (m, 2H), 7.28 (d, *J* = 8.0 Hz), 5.09 (s, 2H), 3.60–2.40 (m, 24H), 1.32 (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.05, 180.07, 179.58, 178.32, 175.68, 169.99,156.17,156.68, 134.29, 133.92, 133.09, 132.06, 127.62, 127.47, 119.66, 75.84, 68.56, 60.83, 60.17, 53.11, 52.00, 49.85, 35.88, 26.07, 25.04; LRMS (ESI) calcd for $C_{35}H_{51}BN_5O_{10}$ ($M+H^+$), 712.3729, found 712.4.

[Tb•2]. The triacid **2** (22.6 mg, 0.0318 mmol, 1.0 equiv) was dissolved in H_2O. $TbCl_3$ (12.6 mg, 0.0390 mmol, 1.2 equiv) was added and the pH was adjusted to pH 7 and stirred for 24 h. The reaction mixture was filtered and concentrated. The complex was resuspended in water, the pH was raised to 9–10 and filtered. After readjusting to pH 7 with 1M HCl, the clear solution was evaporated to yield the complex **[Tb•2]** as a white solid. λ_{max} = 240 nm; ε = 15000 M^{-1} cm^{-1}; τ^{Tb} (H_2O, pH = 7.4, 10 μM) = 1.36 ms; Φ = 0.0060. LRMS calcd for $C_{29}H_{38}O_{10}N_5BTb$ ($M+H^+$), 786.1887, found 786.3; Anal. Found: C, 7.07; H, 0.83; N, 1.45; $C_{29}H_{37}BN_5O_{10}Tb•2H_2O•70NaCl$ requires: C, 7.09; H, 0.84; N, 1.43.

(2) A. de Filippis, C. Morin, C. Thimon, *Synth. Commun.* **2002**, *32*, 2669.

II. Spectroscopic Materials and Methods. Millipore water was used to prepare all aqueous solutions. Spectroscopic measurements were performed in 20 mM HEPES, pH 7.4. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer (Walnut Creek, CA). Fluorescence spectra were recorded using 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 integrated igniter, switchable 814 photon-counting/analog photomultiplier detection unit, and MD5020 motor driver. Time-gated spectra were recorded on a Varian Cary Eclipse fluorescence spectrophotometer (Palo Alto, CA) equipped with a xenon flash lamp. Samples for absorption and emission measurements were contained in 1-cm • 1-cm quartz cuvettes (1.4 and 3.5-mL volume, Starna, Atascadero, CA). Concentrations of Terbium complexes were measured using ICP-OES and lifetimes were measured using a 100 μs delay time and a 10 ms gate time.

Selectivity Studies. Various reactive oxygen species (ROS) were administered to probes **TPR1** and **TPR2** as follows. Superoxide (O_2^-) was generated enzymatically using 2 mM hypoxanthine, 40 U/L xanthine oxidase, and 6.1 x 10^6 U/L catalase. The rate of production of O_2^- was determined using a colorimetric assay.[3] Hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (TBHP), and hypochlorite (OCl^-) were delivered from 30%, 70%, and 5% aqueous solutions, respectively, to give a final concentration of 200 μM. Hydroxyl radical (•OH) and *tert*-butoxy radical (•O^{t}Bu) were generated by reaction of 1 mM Fe^{2+} with 200 μM H_2O_2 or 200 μM TBHP, respectively. Nitric oxide (NO) was added using PROLI NONOate (Cayman Chemical, Ann Arbor, MI) at a final concentration of 100 μM.

Quantum Yields. Quantum yields were determined using aniline **[Tb•4]** as a standard according to a classic method.[4] For each complex, the absorbance spectra was measured at five concentrations so that the absorbance range was between 0.01 and 0.1. These absorbance values were plotted against the integrated emission intensities for each solution and the quantum yield was determined by comparing to the absorbance versus emission plot of the known **[Tb•4]** (Φ = 0.0051) according to the equation: $\Phi_{sample} = \Phi_{standard} (Grad_{sample}/Grad_{standard})(\eta_{sample}/\eta_{standard})^2$; where Φ is the quantum yield, Grad is the slope of the plot of absorbance versus integrated emission intensity, and η is the refractive index of the solvent.

Absorbance Spectra for [Tb•1], [Tb•2], and [Tb•3].

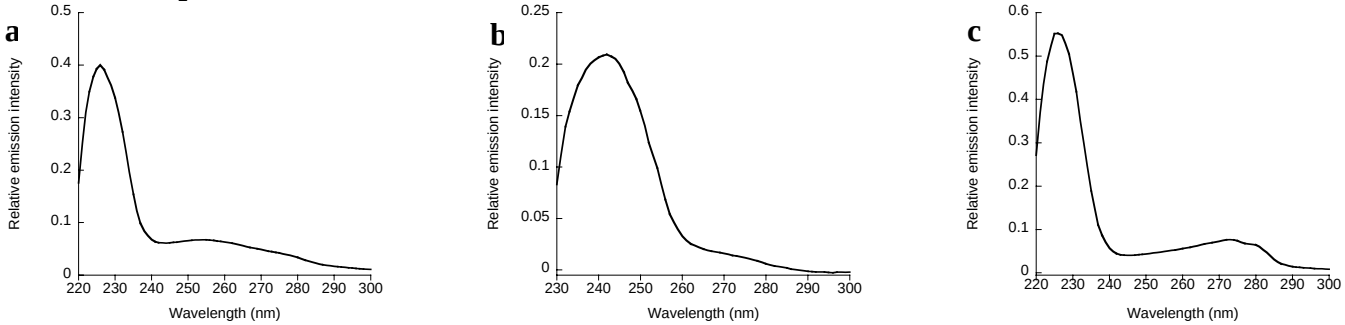

Figure S4. Absorbance spectra of (a) 54 μM **[Tb•1]** (b) 15 μM **[Tb•2]** (c) 60 μM **[Tb•3]** in 20 mM HEPES at pH 7.4.

(3) K. R. Messner, J. A. Imlay, *Methods Enzymol.* **2002**, *349*, 354–361.
(4) A. T. R. Williams, S. A. Winfield, J. N. Miller, *Analyst* **1983**, *108*, 1067–1071.

Excitation Spectra of [Tb•1], [Tb•2], and [Tb•3].

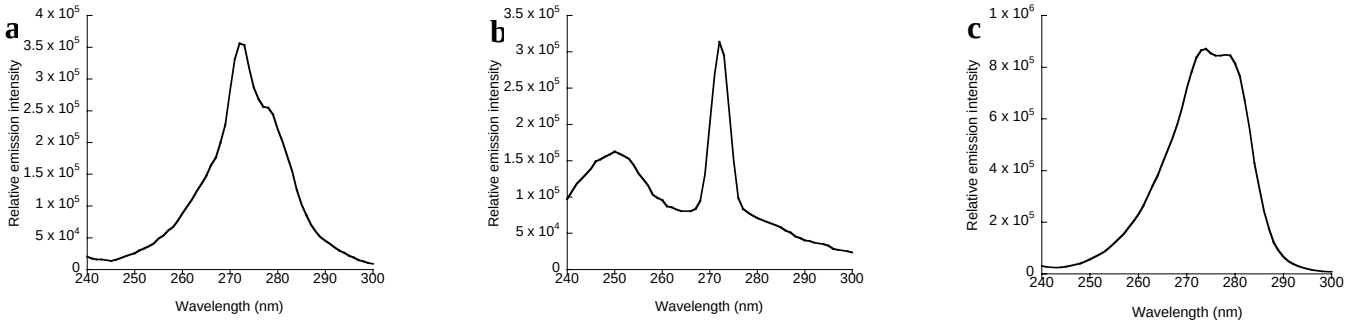

Figure S5. Excitation spectra of (a) 54 μM **[Tb•1]** (b) 15 μM **[Tb•2]** (c) 60 μM **[Tb•3]** in 20 mM HEPES at pH 7.4. λ_{em} = 545 nm, ex slits = 4 nm, em slits = 2 nm.

Emission Spectra of [Tb•1], [Tb•2], and [Tb•3].

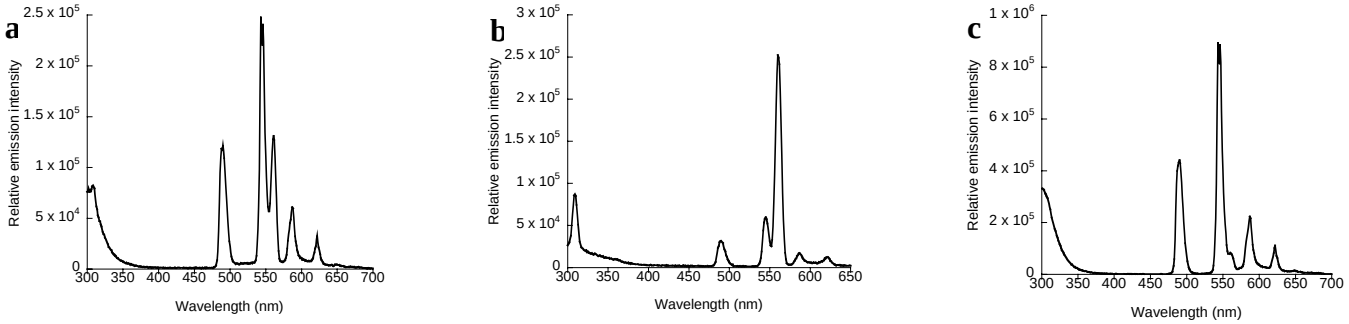

Figure S6. Emission spectra of (a) 54 μM **[Tb•1]** (b) 15 μM **[Tb•2]** (c) 60 μM **[Tb•3]** in 20 mM HEPES at pH 7.4. λ_{max} = 280 nm, ex slits = 4 nm, em slits = 2 nm.

Carbohydrate Compatibility.

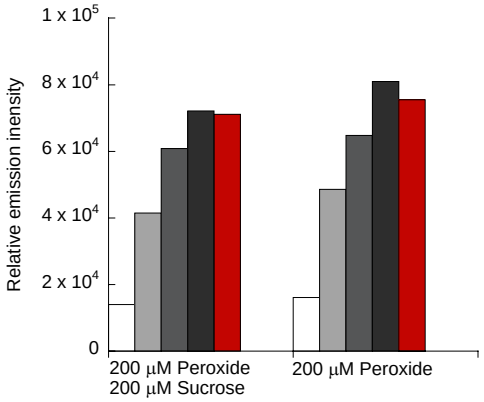

Figure S7. 10 μM **TPR2** was treated with 200 μM H_2O_2 in the presence and absence of 200 μM sucrose in 20 mM HEPES at pH 7.4. Bars are the emission intensity at 545 nm taken at 0, 15, 30, 45, and 60 min. λ_{em} = 280 nm, ex slits = 4 nm, em slits = 2 nm.

III. Cellular Experiments

Preparation of Cell Cultures. RAW264.7 macrophages were seeded at a density of 1.8×10^6 cells/well in a 6-well plate without coverslips at the Cell Culture Facility at the University of California, Berkeley in Dulbecco's Modified Eagle Medium (DMEM 1X, without phenol red, Gibco/Invitrogen, Carlsbad, CA) containing high D-glucose, GlutaMAX (Invitrogen), and 10% Fetal Bovine Serum (HyClone, Logan, UT).

Measurement of Endogenous Hydrogen Peroxide Production. For all cellular experiments, solutions of probes (from 10 mM stocks in DMSO) were made in DMEM with a final concentration of 10 μM. PMA was added from a stock solution in water to a solution of the dye in DMEM to give a final concentration of 1 μg/mL PMA. The cell growth media was aspirated under sterile conditions and either 10 μM **TPR2** in DMEM or 10 μM **TPR2** and 1 μg/mL PMA in DMEM were added. Cells were then incubated at 37 °C and 5% CO_2 for 6 hours. The DMEM was aspirated and each well of cells was washed with 500 μL DMEM. 400 μL of 0.5% Trypsin containing no phenol red was added to each well and the cells were incubated for 5 min at 37 °C and 5% CO_2. 500 μL DMEM was added to each well, the cells were gently scraped, placed as a suspension into cuvettes, and the luminescence spectra was immediately measured. Delay time = 100 μs, gate time = 10 ms, averaging time = 100 ms, ex slits = 20 nm, em slits = 20 nm.