Supporting Information for

An Azo Dye for Photodynamic Therapy that is Activated Selectively by Two-Photon Excitation

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Emission: 590-640 nm (green, NCC1) and 650-700 nm (red, PI). The laser exposure area $(290 \times 290 \ \mu m^2)$. Scanning laser: 770 nm, 1.6 mW, 1.3 s per scan, 100 scans. All the images share the same scale bar: 60 S14 μm..... Fig. S13 Confocal fluorescence images of HeLa cells incubated with 1 µM ACC1 before and after TP irradiation. Live/dead cell images of HeLa cells showed by using 10 µM PI before and after TP irradiation. After incubation for 15 min, the cells were stained with PI. Note that the cells were pretreated with or without vitamin C (Vit C; 50 µM) and sodium azide (NaN₃; 100 μM). Excitation: 552 nm (for PI). Emission: 650-700 nm (red, PI). Scanning laser: 770 nm, 1.6 mW, 1.3 s per scan. The experiment was run up to 100 scans. All the images share the same scale bar: 60 µm...... S14 Fig. S14 Cytotoxicity of HeLa cells treated with ACC1. 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and 650-700 nm (MTDR), respectively. The excitation wavelengths were 488 (NCC1 and LTG) and 552 nm (ETR and MTDR), respectively. Scale bar, 50 mm. Cells shown are representative images from replicate experiments (n = 5)......

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Scheme S1 Synthetic route to the NCC1, ACC1 and ACC2 dyes

Synthesis of NCC1, ACC1 and ACC2 dyes are described below.

2-hydroxy-4-nitrobenzaldehyde. The compound 2-Methoxy-4-nitrobenzaldehyde (1 g, 5.52 mmol) in CH₂Cl₂ (10 mL) stirred at 0 °C for 10 mins. Now, 1 M BBr₃ in CH₂Cl₂ (1.8 eq, 10 mL) added dropwise to the reaction mass. Then reaction mixture was brought to room temperature slowly and monitored reaction with TLC. After completion, excess BBr₃ quenched by methanol and mixture was diluted with 1N NaOH and extracted diethyl ether. The aqueous layer acidified with 1N HCl and further extracted CH₂Cl₂ (4 times) to obtain 2-hydroxy-4-nitrobenzaldehyde (840 mg) as a orange solid. Yield: 91 %; ¹H NMR (DMSO-d₆, 600 MHz): δ (ppm) 11.59 (s, 1H), 10.34 (s, 1H), 7.82 (d, J = 8.2 Hz, 1H), 7.74 (d, J = 2.7 Hz, 1H), 7.68 (d, J = 7.5 Hz, 1H); ¹³C-NMR (151 MHz, DMSO-d₆) δ (ppm) 189.99, 161.28, 152.06, 130.08, 127.29, 114.25, 112.54.

1'-(2-(1,3-dioxolan-2-yl)ethyl)-3',3'-dimethyl-7-nitrospiro[chromene-2,2'-indoline] (1). A mixture of 2-hydroxy-4-nitrobenzaldehyde (250 mg, 1.49 mmol) and 1-(2-(1,3-dioxolan-2-yl)ethyl)-3,3-dimethyl-2-methyleneindoline (387 mg, 1.49 mmol) in EtOH (4 mL) refluxed (80 °C) overnight. Reaction mass brought to room temperature, and cold EtOH (10 mL) added to reaction mass. The solid filtered and further washed with cold EtOH to obtain 1 (543 mg) as a yellowish orange solid. Yield: 89 %; ¹H NMR (CDCl₃, 600 MHz): δ (ppm) 7.68 (d, J = 6.9 Hz, 1H), 7.54 (s, 1H), 7.18-7.20 (m, 1H), 7.16 (d, J = 8.3 Hz, 1H), 7.08 (d, J = 6.9 Hz, 1H), 6.91 (d, J = 10.3 Hz, 1H), 6.86 (t, J = 6.9 Hz, 1H), 6.66 (d, J = 13.1 Hz, 1H), 5.92 (d, J = 10.3 Hz, 1H), 4.89 (t, J = 3.8 Hz, 1H), 3.90-3.94 (m, 2H), 3.81-3.85 (m, 2H), 3.40-3.45 (m, 1H), 3.28-3.32 (m, 1H), 2.01-2.06 (m, 1H), 1.92-1.96 (m, 1H), 1.29 (s, 3H), 1.16 (s, 3H); ¹³C-NMR (151 MHz, CDCl₃) δ (ppm) 154.57, 148.54, 146.97, 135.97, 128.29, 127.92, 127.13, 124.53, 124.18, 121.77, 119.50, 115.47, 110.70, 106.84, 105.74, 102.88, 65.03, 52.96, 38.66, 33.06, 25.85, 20.12.

3-amino-13,13-dimethyl-5a,6,7,13-tetrahydrochromeno[3',2':3,4]pyrido[1,2-a]indol-8-ium (NCC1). A mixture of **1** (500 mg, 1.22 mmol) and SnCl₂ (2.4 g, 10 eq) in EtOH (25 mL) stirred at 80 °C for 48 h. The reaction mass passed through celite bed and washed with MeOH (25 mL). The filtrate was concentrated under reduced pressure and purified twice with column chromatography with gradient from (CHCl3/MeOH=9.7:0.3) to (CHCl3/MeOH=8.5:1.5) over 30 min to obtain **NCC1** (138 mg) as a dark solid. Yield: 32 %; ¹H-NMR (600 MHz, Methanol-d₄) δ (ppm) 8.11 (s, 1H), 7.60 (d, J = 7.6 Hz, 1H), 7.50 (q, J = 7.0 Hz, 2H), 7.42-7.44 (m, 1H), 7.30 (d, J = 8.3 Hz, 1H), 6.39 (dd, J = 8.3, 1.4 Hz, 1H), 6.14 (s, 1H), 5.30 (q, J = 5.5 Hz, 1H), 4.54 (dd, J = 14.5, 4.8 Hz, 1H), 4.13 (td, J = 13.8, 4.1 Hz, 1H), 2.78 (dd, J = 11.7, 4.8 Hz, 1H), 2.45 (qd, J = 12.4, 5.5 Hz, 1H), 1.74 (d, J = 26.2 Hz, 6H); ¹³C-NMR (151 MHz, Methanol-d₄) δ (ppm) 175.02, 162.43, 160.74, 144.81, 143.63, 142.52, 136.28, 130.09, 128.67, 123.64, 114.16, 113.47, 112.11, 110.28, 100.31, 72.09, 51.84, 42.98, 27.83, 27.20, 26.32; HRMS (ESI⁺): m/z found for [C₂₁H₂₁N₂O]⁺:317.1633.

3-((4-(dimethylamino)phenyl)diazenyl)-13,13-dimethyl-5a,6,7,13tetrahydrochromeno[3',2':3,4]pyrido[1,2-a]indol-8-ium (ACC1).

The fluorophore **NCC1** (30 mg, 0.085 mmol) in 10 mL of ACN:CH₂Cl₂ (1:1) stirred at 0 °C for 10 mins. Now, 80 µL of trifluoroacetic acid added dropwise and continued stirring for 10 more minutes. To the reaction mass, 2 eq of NaNO₂ was added and stirred until almost fluorescence disappeared (approx. 10 min). Now, added 100 of N,N-dimethylaniline and continued stirring for 10 mins at 0 °C and 1 h at room temperature. The reaction mass was concentrated to dryness under reduced pressure and purified with column chromatography with gradient from (CHCl3/MeOH=9.7:0.3) to (CHCl3/MeOH=8.5:1.5) over 30 min to obtain **ACC1** (20 mg) as a dark solid. Yield: 53 %; ¹H-NMR (600 MHz, Methanol-d₄) δ (ppm) 8.12 (s, 1H), 7.68-7.70 (m, 3H), 7.55-7.59 (m, 2H), 7.49-7.53 (m, 2H), 7.44 (dd, J = 8.3, 2.1 Hz, 1H), 7.26 (d, J = 2.4 Hz, 1H), 6.63 (d, J = 9.6 Hz, 2H), 5.31 (q, J = 5.7 Hz, 1H), 4.59 (dd, J = 15.1, 4.8 Hz, 1H), 4.18 (td, J = 14.1, 3.4 Hz, 1H), 3.07 (s, 6H), 2.82-2.86 (m, 1H), 2.57 (qd, J = 12.6, 5.6 Hz, 1H), 1.79 (d, J = 1.7 Hz, 6H); ¹³C-NMR (151 MHz, Methanol-d₄) δ (ppm) 178.29, 159.41, 159.20, 155.18, 145.08, 144.65, 143.01, 142.00, 133.69, 130.99, 130.46, 127.07, 123.94, 123.53, 119.54, 119.16, 115.48, 112.74, 109.43, 71.61, 53.47, 44.71, 40.38, 26.91, 26.59, 25.92; HRMS (ESI⁺): m/z found for [C₂₉H₂₉N₄O]⁺:449.2316.

3-((4-(dimethylamino)-2-methoxyphenyl)diazenyl)-13,13-dimethyl-5a,6,7,13-tetrahydrochromeno[3',2':3,4]pyrido[1,2-a]indol-8ium (ACC2). To obtain **ACC2** synthetic procedure for **ACC1** has been followed. **ACC2** (15 mg) was obtained from **NCC1** and 3-methoxy-N,N-dimethylaniline as a dark solid. Yield: 37 %; ¹H NMR (Methanol-d₄, 600 MHz): δ (ppm) 7.98 (s, 1H), 7.65 (d, J = 6.9 Hz, 1H), 7.53-7.55 (m, 2H), 7.43 (t, J = 7.2 Hz, 2H), 7.37 (d, J = 8.3 Hz, 1H), 7.32 (dd, J = 8.3, 2.1 Hz, 1H), 7.20 (d, J = 1.7 Hz, 1H), 6.16 (dd, J = 9.3, 2.4 Hz, 1H), 6.01 (d, J = 2.8 Hz, 1H), 5.20 (dd, J = 11.7, 4.1 Hz, 1H), 4.44 (dd, J = 14.8, 5.2 Hz, 1H), 4.07 (td, J = 13.9, 3.9 Hz, 1H), 3.85 (s, 3H), 3.12 (s, 6H), 2.75-2.79 (m, 1H), 2.56 (qd, J = 12.4, 5.5 Hz, 1H), 1.77 (d, J = 8.3 Hz, 6H); ¹³C-NMR (151 MHz, Methanol-d₄) δ (ppm) 177.64, 161.61, 159.59, 158.99, 157.37, 144.81, 143.04, 141.65, 134.24, 133.72, 130.64, 130.23, 123.78, 122.70, 118.80, 118.30, 118.07, 115.16, 110.49, 106.94, 95.14, 71.34, 56.19, 53.34, 44.48, 40.56, 26.54, 26.44, 26.32; HRMS (ESI⁺): m/z found for [C₃₀H₃₁N₄O₂]⁺: 479.2418. **Spectroscopic Measurements.** Absorption spectra and fluorescence spectra were recorded with UV-Vis spectrophotometer (S-3100) and fluorescence spectrophotometer (FS-2), respectively. The fluorescence quantum yield was measured with rhodamine-6G (Φ = 0.98 in MeOH) as the reference. ¹H NMR spectra was recorded using 600 MHz NMR spectrometers (JNM-ECZR). Fluorescence images were obtained with spectral confocal microscopes (Leica TCS SP8).

Measurement of TP Cross Section. The TP cross section (δ) was determined by using a femto-second (fs) fluorescence measurement technique as described.^{1,2} NCC1, ACC1 or ACC2 (5.0 ×10⁻⁶ M) was dissolved in PBS buffer (10 mM, pH = 7.4) and the TP-induced fluorescence intensity were measured at 690–800 nm by using rhodamine 6G as the reference. The TP properties of which have been well characterized in the literature. The intensities of the TP-induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by using the following equation: $\delta = \delta_r (S_s \Phi_r \phi_r c_r)/(S_r \Phi_s \phi_s c_s)$, where the subscripts s and r stand for the sample and reference molecules, respectively. The intensity of the signal collected by a CCD detector was denoted as S. Φ is the fluorescence quantum yield. ϕ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as c. δ_r is the TPA cross section of the reference molecule (Figure S1).



Fig. S1 Two-photon cross section ($\delta\Phi$) values of NCC1, ACC1 and ACC2 under different TP wavelength in PBS buffer.



Fig. S2 (a) and (b) Fluorescence spectra of 20 μ M of ACC1 and ACC2 with 5 μ M DHR 123 after 30 min irradiation at 770 nm (TP) in the presence ROS inhibitors; D-mannitol (for •OH), Ebselen (for ONOO⁻), NaN₃ (for ¹O₂), sodium pyruvate (for H₂O₂) and Tiron (for O₂^{•-}) to detect ROS inhibition in PBS buffer (pH 7.4). (c) and (d) Cellular ROS generation studies by Fluorescence intensity of 10 μ M DHR 123 with 1 μ M of ACC1 and ACC2 in the HeLa cell in the presence of ROS inhibitors.



Fig. S3 Fluorescence intensity bar graph at 526 nm of 5 μM DHR 123 with or without 20 μM of NCC1, ACC1, or ACC2 for 30 min at 770 nm TP irradiation to detect generation of ROS in PBS buffer for 30 min at 100 mW incident power (pH 7.4).



Fig. S4 (a) and (b) are absorption of 2 μ M of ACC1 and ACC2 in 10 mM GSH in the PBS buffer (pH 7.4). (c) absorption of 2 μ M of ACC1 and ACC2 for 30 min at 770 nm (TP) at 100 mW power in the PBS buffer (pH 7.4) and (d) Fluorescence spectra of 2 μ M of ACC1, ACC2 and NCC1 in the PBS buffer (pH 7.4).

Quantum mechanical calculation was done to understand electronic structure of the PDT agents.

Oscillator strength indicates the strength of photon absorption to reach the excited state. Triplet oscillator strength is zero for all triplet states because spin multiplicity is conserved during photon absorption. Each electronic transition is described as a sum of promotions of an electron from an occupied orbital to an unoccupied orbital. For example, the S₁ state of Azo-CC1 is mostly characterized as a promotion of an electron from HOMO to LUMO, but (HOMO-1)-LUMO and HOMO-(LUMO+1) promotions are added for a better description. The "orbitals" column indicates major contributing promotions to each transition.

Excited	Excitation	Oscillator	Orbitals	Excited	Excitation	Oscillator	Orbitals
state	energy (eV)	strength		state	energy (eV)	strength	
T ₁	1.2915	0.000	HOMO-LUMO	S ₁	2.3616	1.9139	HOMO-LUMO
T ₂	1.8980	0.000	(HOMO-2)-	S ₂	2.4918	0.0018	(HOMO-2)-
			(LUMO+1)				LUMO
							(HOMO-2)-
							(LUMO+1)
T ₃	1.9153	0.000	(HOMO-1)-	S ₃	3.1105	0.0334	(HOMO-1)-
			lumo				LUMO
							(HOMO-4)-
							LUMO
T_4	2.8584	0.000	(HOMO-4)-	S ₄	3.7111	0.0658	(HOMO-4)-
			lumo				LUMO
T ₅	3.1001	0.000	HOMO-LUMO	S 5	3.9056	0.2118	HOMO-
			(HOMO-5)-				(LUMO+1)
			lumo				
T ₆	3.3110	0.000	(HOMO-1)-	S ₆	4.1313	0.0001	(HOMO-2)-
			(LUMO+1)				LUMO
T ₇	3.6551	0.000	(HOMO-3)-	S ₇	4.1953	0.0016	(HOMO-3)-
			(LUMO+1)				lumo
T ₈	3.7442	0.000	(HOMO-6)-	S ₈	4.4888	0.0078	(HOMO-5)-
			(LUMO+2)				LUMO

Table S1 Excitation energy of ACC1

Excited	Excitation	Oscillator	Orbitals	Excited	Excitation	Oscillator	Orbitals
state	energy	strength		state	energy	strength	
	(eV)				(eV)		
T ₁	1.2237	0.000	HOMO-LUMO	S ₁	2.3022	1.9895	HOMO-LUMO
T ₂	1.8484	0.000	(HOMO-2)-	S ₂	2.4219	0.0009	(HOMO-2)-LUMO
			(LUMO+1)				(HOMO-2)-
							(LUMO+1)
T ₃	1.8987	0.000	HOMO-(LUMO+1)	S ₃	3.1186	0.0432	(HOMO-3)-LUMO
T_4	2.8497	0.000	(HOMO-4)-LUMO	S ₄	3.6879	0.0112	(HOMO-4)-LUMO
T ₅	3.0504	0.000	(HOMO-5)-LUMO	S ₅	3.7644	0.0398	(HOMO-1)-LUMO
			HOMO-LUMO				
T ₆	3.2576	0.000	(HOMO-3)-	S ₆	3.9117	0.1660	HOMO-(LUMO+1)
			(LUMO+1)				
T7	3.3554	0.000	(HOMO-1)-	S ₇	4.0252	0.0001	(HOMO-2)-LUMO
			(LUMO+1)				
T ₈	3.7258	0.000	(HOMO-6)-	S ₈	4.5307	0.0103	(HOMO-5)-LUMO
			(LUMO+2)				

Table S2 Excitation energy of ACC2



Fig. S5 Energy diagram (a and b) and Orbitals and electronic transitions (c and d) of ACC1 and ACC2



Fig. S6 photographic images and PTT efficiency of ACC2 for 10 min under 600 nm and 770 nm.

Cell culture

HeLa human cervical carcinoma cells (KCLB, Seoul, Korea) cultured in a glass-bottomed dishes (NEST, Rahway, NJ, USA) supplemented with minimum essential medium eagle (MEM, WelGene Inc, Seoul, Korea) containing fetal bovine serum (10% FBS, WelGene), of 10 mM, whereas the working concentration of probes 1 μ M (unless otherwise stated) incubated with cells, washed three times with PBS (Gibco, Gaithersburg, MD, USA), and used in imaging guided PDT.



Fig. S7 Confocal fluorescence images of HeLa cells incubated with 1 μ M of (a) ACC1, ACC2, and NCC1 for 30 mins to 4h and (b) xy λ cell spectra obtained from CSLM. Scale bar 60 μ m.

Cell Viability. CCK-8 kit assay was performed to assess the cytotoxicity. HeLa cells were cultured in 96-well plate for 24 h, and then each different concentration of probes was added. After incubation for 24 h or 2 h, the cultured medium was replaced with serum free medium containing 10% CCK-8, and further incubated for 2 h. CCK-8 containing medium was removed and DMSO was added to dissolve the formed formazan precipitate. Absorbance was measured at 600 nm.



Fig. S8 Viability of HeLa cells in the presence of (a) ACC1 and ACC2 for 24 h and (b) NCC1 for 2 h as measured by using CCK-8 kit assays. A total of six independent experiments were performed.



Fig. S9 Confocal fluorescence images of HeLa cells incubated with DHR 123 and 1 μM ACC2 before and after TP irradiation. For Images OP Excitation: 488 nm, Emission: 490-540 nm (green, DHR 123) and 590-640 nm (red, ACC2). scanning laser: 488 nm or 770 nm, 1.6 mW, 1.3 s per scan. All the images share the same scale bar: 60 μm.



Fig. S10 ACC2 and PI before and after TP irradiation. Fluorescence Intensity of DHR 123 with 1 μ M ACC2 according to ROS generation upon TP irradiation. After TP Excitation, the cells were stained with 10 μ M PI. Excitation: 488 nm (for ACC2) and 552 nm (for PI). Emission: 590-640 nm (green, ACC2) and 650-700 nm (red, PI). Two-photon scanning laser: 770 nm, 1.6 mW, 1.3 s per scan. All the images share the same scale bar: 60 μ m.



Fig. S11 (a) Confocal fluorescence images of HeLa cells incubated with 10 μM DHR 123 and 1 μM ACC1 before and after TP irradiation. OP Excitation: 488 nm, Emission: 490-540 nm (green, DHR 123) and 590-640 nm (red, ACC1). (b) ACC1 and PI before and after TP irradiation. After incubation for 15 min, the cells were stained with 10 μM PI. Excitation: 488 nm (for ACC1). 552 nm (for PI). Emission: 590-640 nm (green, ACC1) 650-700 nm (red, PI). scanning laser: 770 nm, 1.6 mW, 1.3 s per scan. All the images share the same scale bar: 60 μm.



NCC1



Fig. S12 Confocal fluorescence images of HeLa cells incubated with 1 μ M NCC1 before and after TP irradiation. Confocal live/dead cell images of HeLa cells incubated with NCC1, and 10 μ M PI before and after TP irradiation. Excitation: 488 nm (for NCC1) and 552 nm (for PI). Emission: 590-640 nm (green, NCC1) and 650-700 nm (red, PI). The laser exposure area (290 × 290 μ m²). Scanning laser: 770 nm, 1.6 mW, 1.3 s per scan, 100 scans. All the images share the same scale bar: 60 μ m.



Fig. S13 Confocal fluorescence images of HeLa cells incubated with 1 μ M ACC1 before and after TP irradiation. Live/dead cell images of HeLa cells showed by using 10 μ M PI before and after TP irradiation. After incubation for 15 min, the cells were stained with PI. Note that the cells were pretreated with or without vitamin C (Vit C; 50 μ M) and sodium azide (NaN₃; 100 μ M). Excitation: 552 nm (for PI). Emission: 650-700 nm (red, PI). Scanning laser: 770 nm, 1.6 mW, 1.3 s per scan. The experiment was run up to 100 scans. All the images share the same scale bar: 60 μ m.



Fig. S14 Cytotoxicity of HeLa cells treated with ACC1. (a) Confocal fluorescence microscopic images of HeLa cells treated with 1 μ M ACC1 after TP irradiation (770 nm) for 10-100 scans as a function of irradiation times. Dead/live cells are red/ blue (PI/Hoechst 33342), respectively. Excitation: OP 552 nm (for PI), and TP 750 nm (for Hoechst 33342). Emission: 380-480 nm (Hoechst 33342) and 650-700 nm (red, PI). Two-photon scanning laser: 770 nm, 3.6 mW, 1.3 s per scan. All the images share the same scale bar: 130 μ m. (b) Dead cell ratio (cytotoxicity) with 1 μ M PS is defined as the number of PI positive cells to the total number of cells. (c) The HeLa cells were incubated with 1 μ M ACC1 for 30min. After irradiated with TP laser scans, the dead cell ratio was estimated by using Hoechst 33342 and PI. In vitro dead cell ratio curves produced using TP wavelength of 770 nm and 1.3 s/scan using 5 discrete laser powers (1.1, 1.6, 2.1, 2.5, 3.2 mW). (d) log (LD₅₀) is plotted against log (laser power⁻¹). Scale bar, 120 μ m.



Fig. S15 Confocal fluorescence microscopic images of HeLa cells treated with PBS, after TP irradiation (770 nm) for 200 scans as a function of irradiation times. Dead/live cells are red/ blue (PI/Hoechst 33342), respectively.



Fig.S16 Confocal fluorecence images of HeLa cells co-labeled with (a,b) NCC1 and ETR, (e, f) LTG and NCC1, and (i, j) NCC1 and MTDR (c, g, k) Merged images. The images were collected at 560–620 (NCC1), 490–540 nm (LTG), 650-710 nm (ETR), and 650-700 nm (MTDR), respectively. The excitation wavelengths were 488 (NCC1 and LTG) and 552 nm (ETR and MTDR), respectively. Scale bar, 50 mm. Cells shown are representative images from replicate experiments (n = 5).



Fig. S17 Confocal fluorecence images of HeLa cells co-labeled with (a,b) ACC1 and ETR, (e, f) LTG and ACC1, and (i, j) ACC1 and MTDR (c, g, k) Merged images. The images were collected at 560–620 (ACC1), 490–540 nm (LTG), 650-710 nm (ETR), and 650-700 nm (MTDR), respectively. The excitation wavelengths were 488 (ACC1 and LTG) and 552 nm (ETR and MTDR), respectively. Scale bar, 50 mm. Cells shown are representative images from replicate experiments (n = 5).



Fig. S18 Confocal fluorecence images of HeLa cells co-labeled with (a,b) ACC2 and ETR, (e, f) LTG and ACC2, and (i, j) ACC2 and MTDR (c, g, k) Merged images. The images were collected at 560–620 (ACC2), 490–540 nm (LTG), 650-710 nm (ETR), and 650-700 nm (MTDR), respectively. The excitation wavelengths were 488 (ACC2 and LTG) and 552 nm (ETR and MTDR), respectively. Scale bar, 50 mm. Cells shown are representative images from replicate experiments (n = 5).



Fig. S19¹H-NMR and ¹³C-NMR spectra of **2-hydroxy-4-nitrobenzaldehyde** in DMSO-d₆.



Fig. S20 1 H-NMR and 13 C-NMR spectra of 1 in CDCl₃



Fig. S21 ¹H-NMR and ¹³C-NMR spectra of NCC1 dye in Methanol-d₄



Fig. S22 ¹H-NMR and ¹³C-NMR spectra of ACC1 dye in Methanol-d₄



Fig. S23 1 H-NMR and 13 C-NMR spectra of ACC2 dye in Methanol-d₄



Fig. S25 HRMS data of ACC1 dye

[3-2] MS spectrum (RT : 13.46 min)



rig. 520 millions data of Acc2 dy

References

- (1) D. E. Kang, C. S. Lim, J. Y. Kim, E. S. Kim, H. J. Chun and B. R. Cho, Anal. Chem., 2014, 86, 5353-5359.
- (2) N. S. Makarov, M. Drobizhev and A. Rebane, Opt. Express, 2008, 16, 4029-4047.