Supporting Information for:

Carbazole-based Two-photon Fluorescent Probe for Selective imaging of Mitochondrial Hydrogen Peroxide in Living Cells and Tissues

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Materials and apparatus. All reagents and starting materials were purchased from commercial sources. All the solvents were pretreated by standard methods wherever needed. Stock solution (500 μM) of CBZ-H_2O_2 the probe was prepared by dissolving CBZ-H_2O_2 in DMSO. UV-Vis absorption spectra were recorded in 1.0 cm path length quartz cuvettes on a Hitachi U-4100 UV/Vis spectrophotometer (Kyoto, Japan). Fluorescence emission spectra were measured on a PTI QM4 Fluorescence System (Photo Technology International, Birmingham, NJ). Fluorescence images of living cells and tissues were obtained using an Olympus FV1000-MPE multiphoton laser Scanning confocal microscope (Japan). ^{1}H NMR spectra were recorded using a Bruker advanced-Ⅲ 400 NMR spectrometer and referenced to the residual $CHCl_3$ of 7.26 ppm or DMSO-d_6 of 2.5 ppm. ^{13}C NMR spectra were recorded using NMR spectrometer of the same model and referenced to the $CDCl_3$ of 77.23 ppm or DMSO-d_6 of 39.5 ppm. Mass spectrometer (MS) measurements were performed using fast atom bombardment (FAB) on the API ASTAR pulsar Ⅰ Hybrid Mass Spectrometer or matrix-assisted desorption ionization-time-of-flight (MALDI-TOF) technique.

Synthesis of **3-bromocarbazole (1).** To a solution of carbazole (5.0 g, 29.9 mmol) in DMF (50 mL) was added dropwise a solution of NBS (5.32 g, 29.9 mmol) in DMF (50 mL) at 0 °C. After stirred for 6 h at 0 °C, the reaction mixture was poured into 200 mL water, and the white precipitation was filtered, and dried by vacuum oven to afford the desired product (6.8 g, 94%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.50-7.20 (m, 5H), 8.02 (s, 1H), 8.21 (d, J = 7.2 Hz, 1H), 11.70 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 135.4, 131.4, 124.0, 122.2, 121.1, 121.0, 120.1, 118.9, 113.3, 111.1.

Synthesis of **9-(4-Bromobutyl)-3-bromocarbazole (2).** To a mixture of 3-bromocarbazole **(1)** (2.4 g, 9.76 mmol), TBAB (200 mg), and 1,4-dibromobutane (5.8 mL, 48.7 mmol) in benzene (20 mL), aqueous 50% NaOH solution (10 mL) was added at rt. The resulting mixture was stirred for 6 h, then poured into water (50 mL), and extracted with DCM (50 mL×3). The combined organic phase was washed by

brine (50 mL×3), dried over anhydrous Na_2SO_4. After solvent removal, the residual was purified by chromatographic column using hexane/ethyl acetate mixture as the eluent to afford the desired product as a white solid (2.72 g, 72%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.19 (d, J = 1.7 Hz, 1H), 8.03 (d, J = 7.59 Hz, 1H), 7.55-7.47 (m, 2H), 7.38 (d, J = 8.53 Hz, 1H), 7.27-7.23 (m, 2H), 4.31 (t, J = 6.97, 2H), 3.37 (t, J = 6.49, 2H), 2.07-2.00 (m, 2H), 1.92-1.85 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 140.7, 139.1, 128.6, 126.7, 124.8, 123.4, 122.1, 120.8, 119.6, 111.9, 110.2, 109.0, 42.5, 33.2, 30.3, 27.8.

Synthesis of **9-(4-Bromobutyl)-2-(4,4,5,5-tetramethyl[1,3,2]dioxaborolan-2-yl) -carbazole (3).** To a solution of 9-(4-Bromobutyl)-3-bromocarbazole **(2)** (1.13 g, 3 mmol), and bis(pinacolato)diboron (1.2 g, 4 mmol) in dioxane (30 mL), was added Pd(dppf)Cl_2 (33 mg, 0.045 mmol), and KOAc (882 mg, 9 mmol). The mixture was then heated to 80 °C and stirred for 6 h under N_2. After removing the solvent by evaporation, DCM (100 mL) was added. The organic phase was washed by brine (50 mL), dried over anhydrous Na_2SO_4. And then the solvent was removed under vacuum, and the residual was purified by column chromatography, eluted by DCM/hexane, the desired product was afforded as colorless oil (640 mg, 50%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.62 (s, 1H), 8.15 (d, J = 8.09 Hz, 1H), 7.94 (dd, J = 1.12, 8.26 Hz, 1H), 7.50-7.46 (m, 1H), 7.41-7.39 (m, 2H), 7.29-7.25 (m, 1H), 4.36 (d, J = 6.9 Hz, 2H), 3.36 (d, J = 6.42 Hz, 1H), 2.10-2.02 (m, 2H), 1.93-1.86 (m, 2H), 1.41 (s, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 142.6, 140.5, 132.4, 128.0, 126.0, 123.4, 122.9, 120.9, 119.6, 108.8, 108.2, 183.8, 42.4, 33.3, 30.4, 27.8, 25.1.

Synthesis of **CBZ-H_2O_2.** To a solution of 9-(4-Bromobutyl)-2-(4,4,5,5-tetramethyl[1,3,2]dioxaborolan-2-yl)-carbazole **(3)** (100 mg, 0.23 mmol) in 20 mL acetonitrile, was added triphenylphosphine (122 mg, 0.49 mmol). The reaction mixture was then heated to 100 °C, and stirred for 3 h. Removing the solvent by evaporation, the residual was purified by column chromatography using ethyl acetate/methanol as the eluent to afforded the desired product (110 mg, 70%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.45 (s, 1H), 8.20 (d, J = 7.76 Hz, 1H), 7.88-7.84 (m, 3H), 7.73-7.58 (m, 15H), 7.44 (d, J = 7.1 Hz, 1H), 7.21 (d, J = 7.38 Hz, 1H), 4.47

(d, J = 6.67 Hz, 2H), 3.62-3.54 (m, 2H), 1.99-1.92 (m, 2H), 1.63-1.52 (m, 2H), 1.35 (s, 12H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 142.0, 140.0, 134.8, 133.5, 133.4, 131.8, 130.2, 130.1, 127.2, 120.4, 119.3, 118.7, 117.8, 109.5, 108.9, 83.3, 73.5, 41.4, 30.9, 29.2, 24.7. ^{31}P NMR (162 MHz, DMSO-d_6) δ: 24.42. HRMS (FAB$^+$): m/z calcd for $[C_{40}H_{42}BNO_2P]^+$: 610.3041, found: 610.3033.

Spectrophotometric measurements. Both the fluorescence and UV/vis absorption measurements were conducted in PBS (20 mM, pH 7.4) containing 20% DMSO. For H_2O_2 assay in aqueous solution, 1.0 mL buffer solution containing 1.0 μM CBZ-H_2O_2 was first introduced to a quartz cell. Following additions of 10 μL different concentration of H_2O_2, the mixture solutions were kept at ambient temperature for 15 min and then the fluorescence intensities were recorded at excitation wavelength 350 nm with emission wavelength range from 360 to 600 nm.

Cell Cytotoxic Assays. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin-streptomycin (10,000 U/mL, 10,000 μg mL-1, Invitrogen), 10% heat-inactivated fetal bovine serum (Invitrogen), 100 U/mL penicillin and 100 U/mL gentamicin at 37°C in an atmosphere containing 5% CO_2 and 95% air at 37 °C. The cytotoxic effects of CBZ-H_2O_2 and the product after reacting with H_2O_2 were assessed using the standard cell viability assay (MTT assay).

Two-Photon Fluorescence Microscopy Imaging in Cells. Before executing the imaging experiments, HeLa cells were washed with Dulbecco's phosphate buffered saline (DPBS), followed by incubating with CBZ-H_2O_2 (5.0 μM). 30 min later, the cells were washed with PBS three times, and then two-photon confocal fluorescence imaging of HeLa cells was observed under an Olympus FV1000-MPE multiphoton laser scanning confocal microscope (Japan), with a mode-locked titanium-sapphire laser source (120 fs, pulse width, 80 MHz repetition rate) set at wavelength 720 nm with laser power of 2.2 mW.. To monitor the concentration change of endogenous H_2O_2, HeLa cells were pretreated the stimulant PMA (1.0 μg/mL) or DMSO (5%) for 30 min, then CBZ-H_2O_2 was incubated with the cells for another 30 min. After washing with PBS for three times, the cells were subjected to imaging analysis. To monitor exogenous H_2O_2 in cells, the cells were pretreated with 50 μM H_2O_2 for 30

min, washed with PBS, and then further incubated with 5.0 μM CBZ-H_2O_2 for 30 min. After washing with PBS for three times, the cells were subjected to imaging analysis.

For the co-localization experiments, HeLa cells were incubated with CBZ-H_2O_2 (10.0 μM) and H_2O_2 (50 μM) for 30 min at 37 °C, and then Mitochondria tracker Red (1.0 μM) were further incubated for 30 min at the same condition. Finally, the cells were washed three times with PBS before imaging. (CBZ-H_2O_2 channel: λ_{ex}=720 nm, λ_{em}=420–460 nm bandpass. Mito Tracker channel: λ_{ex}=590 nm, λ_{em}=620–660 nm bandpass. Pink: colocalization of red and blue fluorescence).

Two-Photon fluorescence microscopy imaging in liver tissues. For deep tissue imaging, two pieces of 1.0 mm-thick liver tissue slice were prepared from a 10-week-old nude mouse. One of them was pretreated with 100 μM H_2O_2, and then incubated with 20 μM CBZ-H_2O_2 in 10% bovine serum-contained PBS for 1.0 h at 37 ^oC. The other slice was incubated with 20 μM CBZ-H_2O_2 only as control. After washing with PBS three times to remove the remaining CBZ-H_2O_2, Z-scan two-photon imaging and the 3D confocal image accumulated along the Z-direction at depth of 0-200 μm of this treated liver tissue were observed under the two-photon confocal microscope upon excitation at 800 nm with a femtosecond pulse.

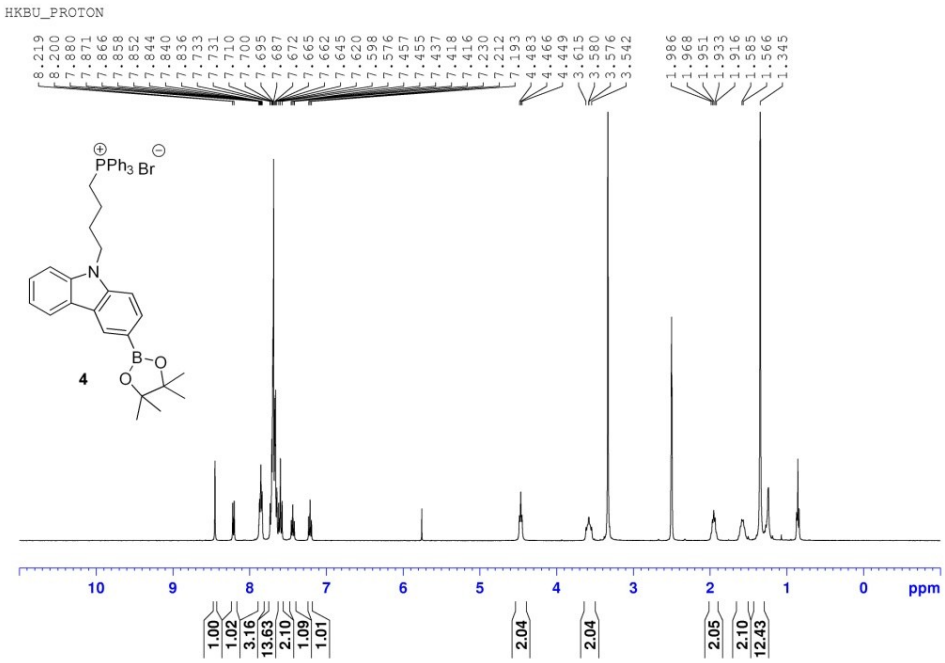

Figure S1. ^{1}H NMR of compound **CBZ-H_2O_2**.

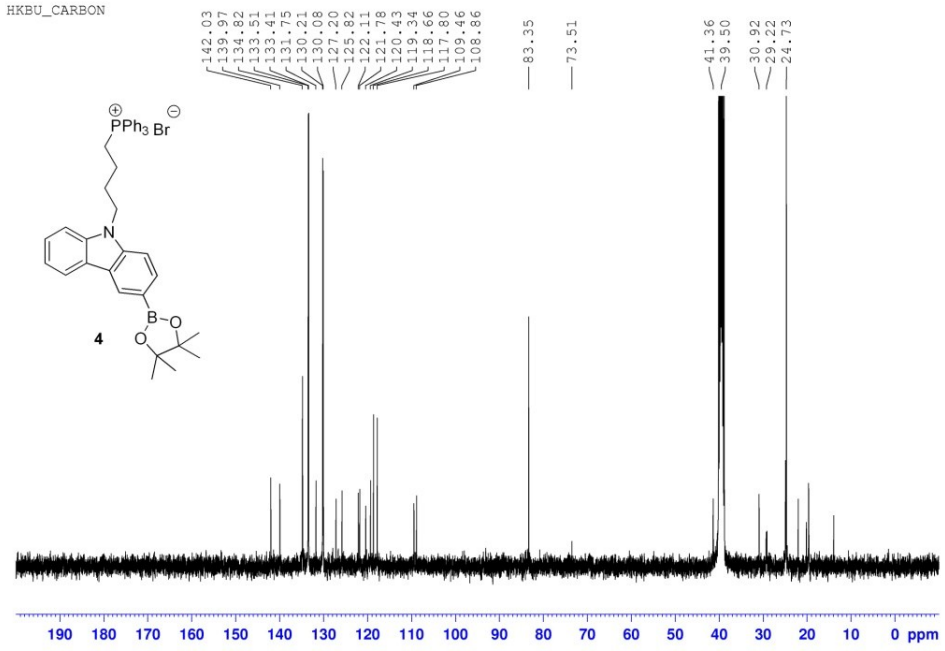

Figure S2. ^{13}C NMR of compound **CBZ-H_2O_2**.

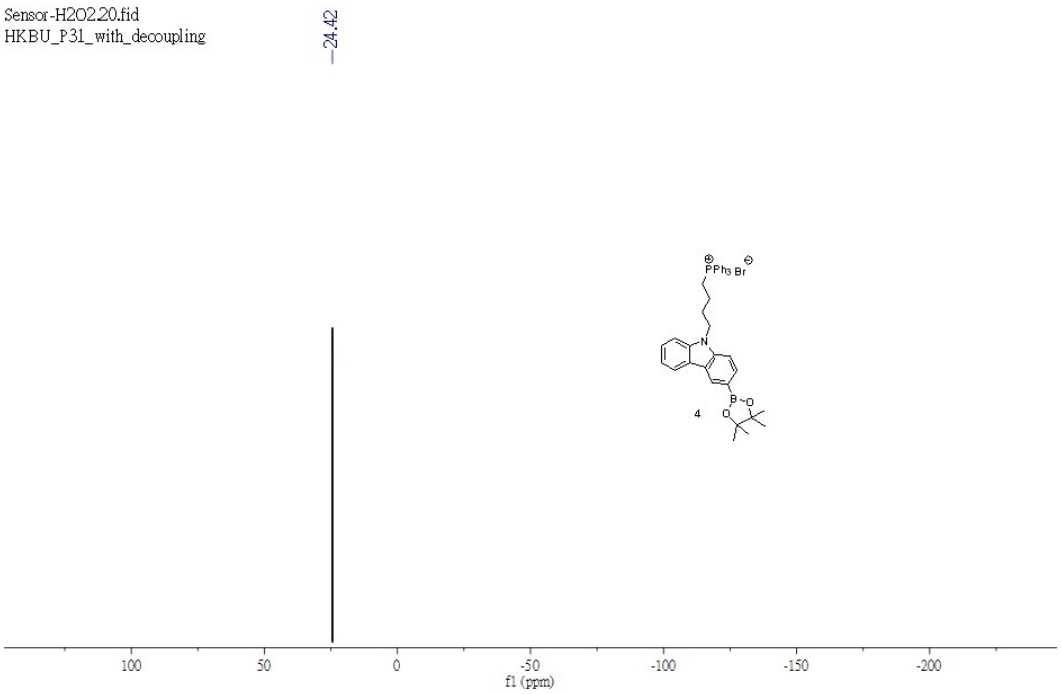

Figure S3. ^{31}P NMR of compound **CBZ-H_2O_2**.

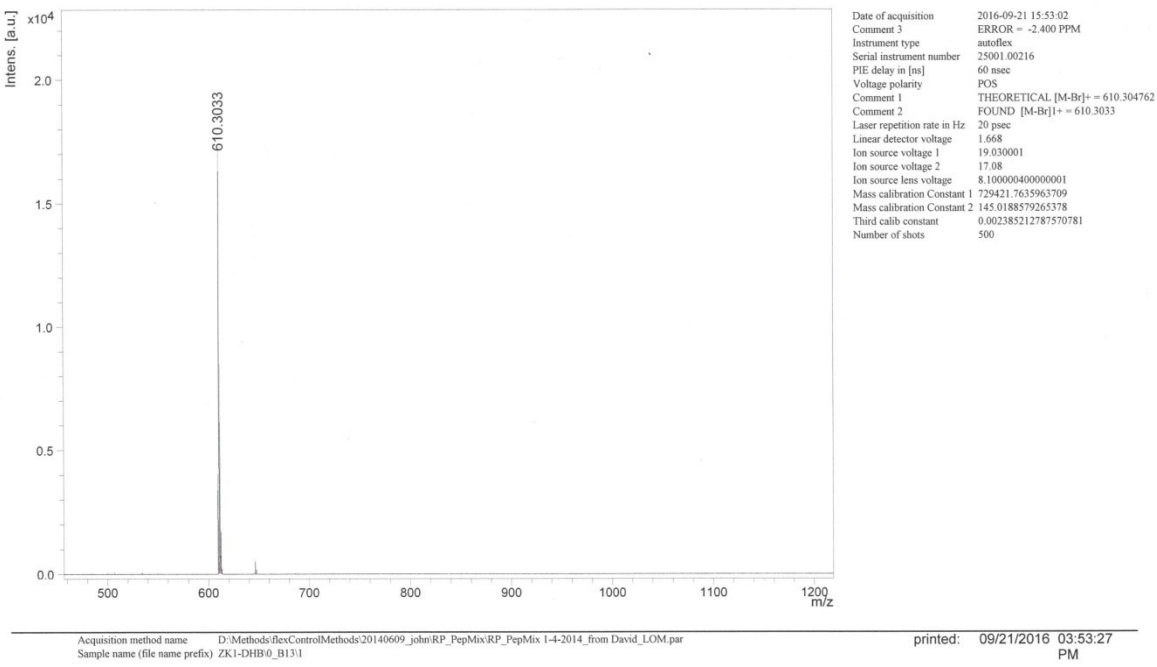

Figure S4. HRMS of compound **CBZ-H_2O_2**.

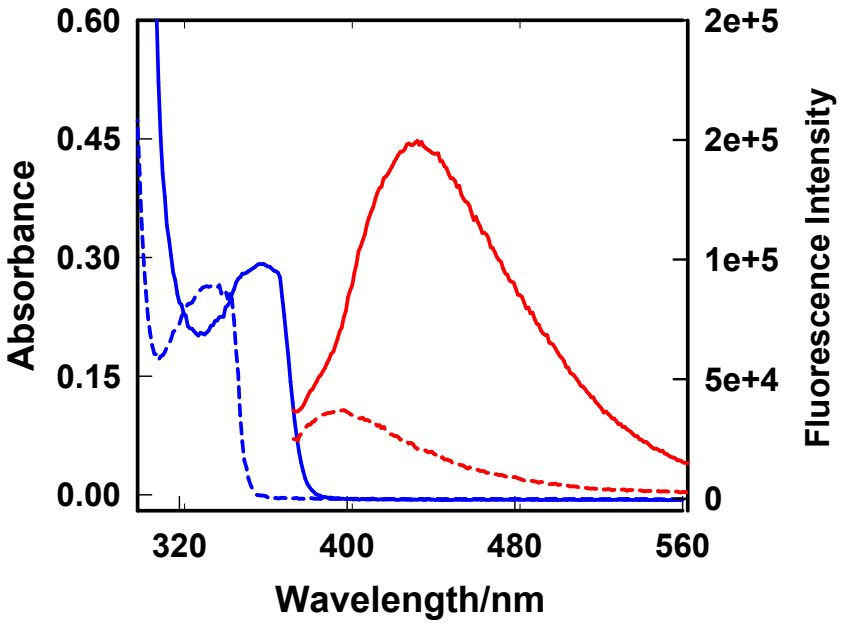

Figure S5. Absorption spectra (blue line) and Fluorescence emission spectra (red line) of CBZ-H_2O_2 (1.0 μM) in the absence (dotted line) and presence (solid line) of H_2O_2 in PBS (20 mM, pH 7.4) containing 20% DMSO.

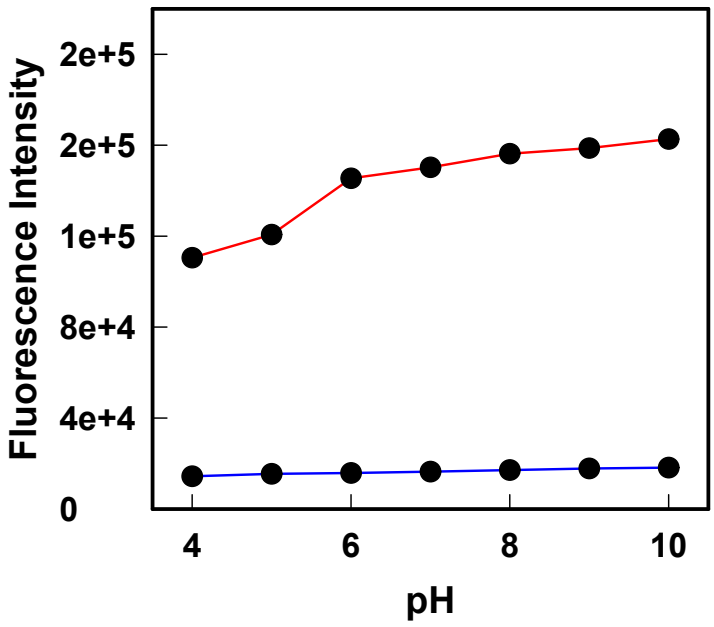

Figure S6. The corresponding fluorescence intensity of CBZ-H_2O_2 (1.0 μM) treated with H_2O_2 as a function of pH, where the blue line and the pink line are the

fluorescence intensities in the absence and the presence of H_2O_2, respectively.

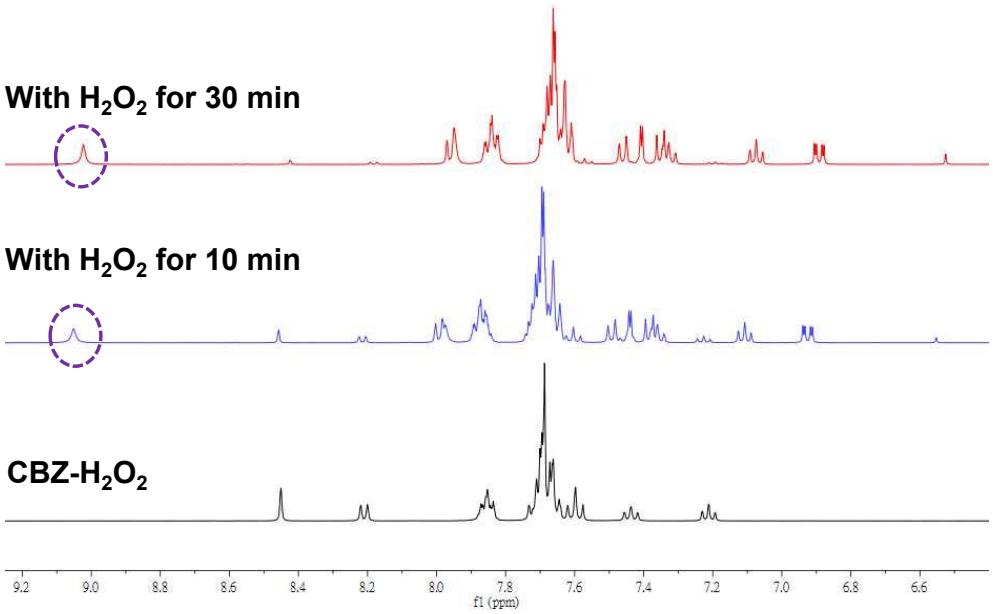

Figure S7. ^{1}H NMR spectra (from d 9.2–6.3) of CBZ-H_2O_2 before and after addition of H_2O_2 (50 eq) in 50% DMSO-d_6/D_2O.

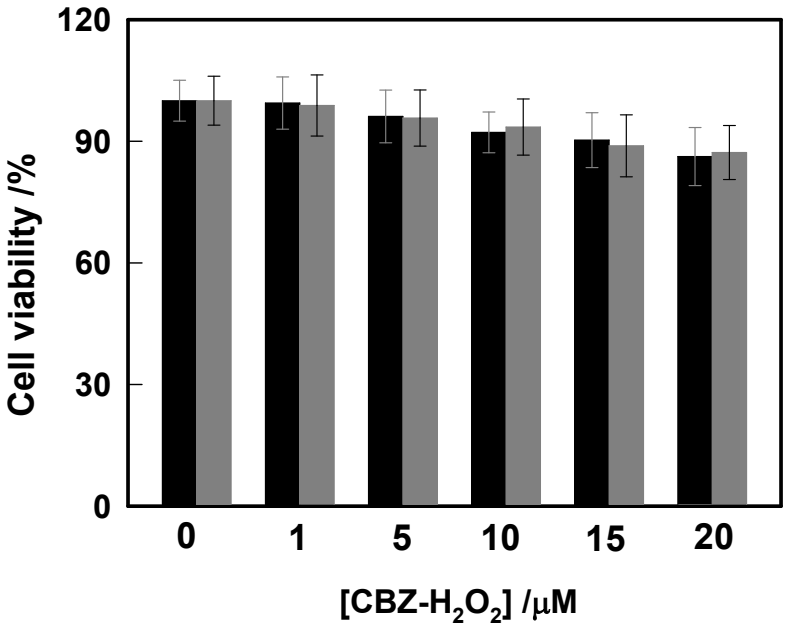

Figure S8. Cell viability values (%) estimated by MTT proliferation. HeLa cells were incubated with different concentrations of CBZ-H_2O_2 in the absence and presence of H_2O_2 at 37 ^{o}C for 24 h, respectively. Cells without addition of probes were taken as

the control experiment, and the viability was set as 100%.

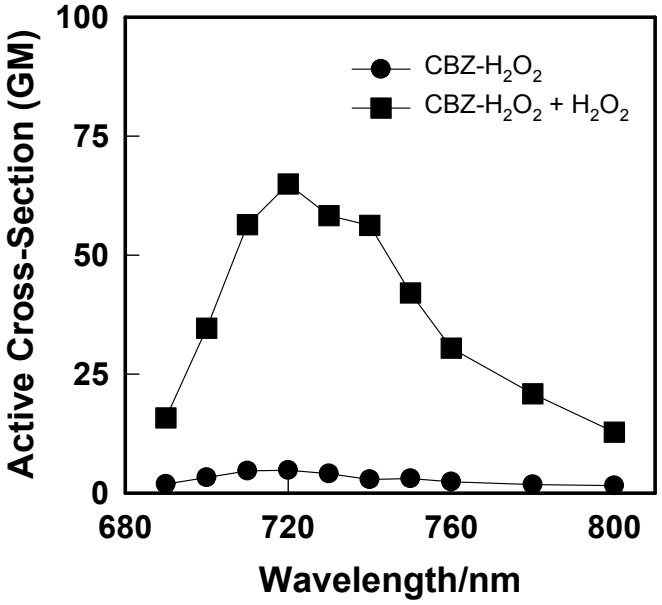

Figure S9. Two-photon action spectra of CBZ-H_2O_2 before and after addition of H_2O_2 in PBS (20 mM, pH 7.4) containing 20% DMSO.