Electronic Supplementary Information

Design and synthesis of efficient heavy-atom-free photosensitizers for photodynamic therapy of cancer

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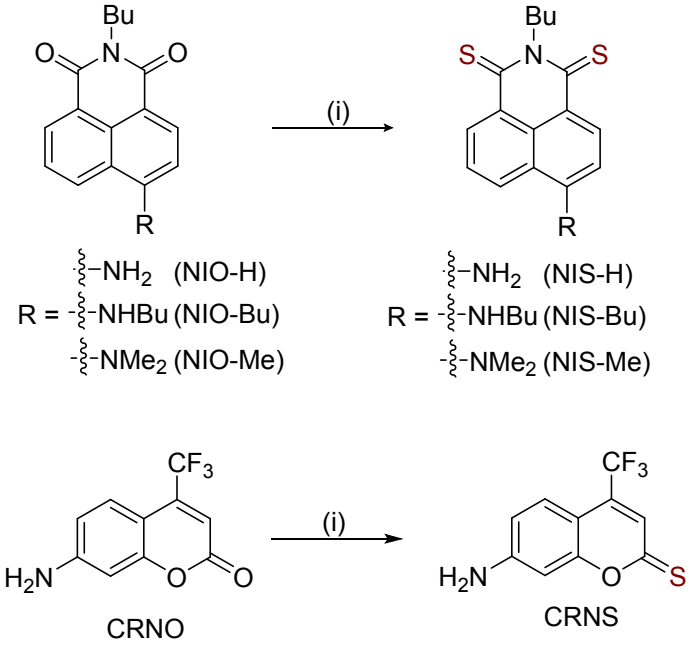
1. Experimental data

1.1. General considerations

All operations were performed under an inert nitrogen atmosphere using standard Schlenk techniques. Anhydrous-grade solvents (Aldrich), spectrophotometric-grade solvents, and all commercial reagents were used as received. Compounds, NIO-H,[1] NIO-Bu,[2] NIS-Bu[2] and NIS-Me,[3] were synthesized according to the modified literature procedures. Deuterated solvents from Cambridge Isotope Laboratories were used. NMR spectra were recorded on a Bruker AM 300 (300.13 MHz for ^{1}H, 75.48 MHz for ^{13}C) spectrometer at ambient temperature. Chemical shifts (ppm) were referenced against external Me_4Si (^{1}H, ^{13}C). Mass spectrometric data were obtained with the Synapt G2-HDMS mass spectrometer.

1.2. Synthesis



(i) Lawesson's reagent, toluene, reflux, 18 h

1.2. Synthesis

NIS-H

NIO-H (0.14 g, 0.52 mmol), Lawesson's reagent (0.84 g, 2.08 mmol) were dissolved in toluene (20 mL). The mixture was refluxed under nitrogen for 18 h. Afterward the reaction mixture was cooled down to room temperature, and the solvent was evaporated under reduced pressure, and the residue was purified by column chromatography on silica gel using *n*-hexane/DCM as eluent. Drying in vacuo afforded a powder of **NIS-H** (yield, 26%).

^{1}H NMR ($CDCl_3$): δ 8.95 (dd, J = 7.7, 1.1 Hz, 1H), 8.79 (d, J = 8.6 Hz, 1H), 7.95 (dd, J = 8.3, 1.0 Hz, 1H), 7.51–7.46 (m, 1H), 6.75 (d, J = 8.6 Hz, 1H), 5.34 (s, 2H), 5.06 (s, 2H), 1.97–1.87 (m, 2H), 1.48 (sextet, J = 7.3 Hz, 2H), 1.00 (t, J = 7.4 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 190.5, 189.2, 149.7, 141.7, 138.9, 129.9, 126.4, 125.7, 124.9, 121.3, 118.9, 111.6, 54.8, 27.4, 20.1, 13.8. EI–MS calculated for $C_{16}H_{16}N_2S_2$ $[M]^+$: 300.0755; found: 300.0753.

NIS-Bu

This compound was prepared according to the modified literature procedures.[2]

^{1}H NMR ($CDCl_3$): δ 8.94 (dd, J = 7.8, 1.0 Hz, 1H), 8.84 (d, J = 8.9 Hz, 1H), 7.92 (dd, J = 8.3, 0.9 Hz, 1H), 7.44 (t, J = 8.0 Hz, 1H), 6.63 (d, J = 9.0 Hz, 1H), 5.47 (s, 2H), 3.40 (t, J = 7.2 Hz, 2H), 1.98–1.88 (m, J = 7.5 Hz, 2H), 1.85–1.76 (m, J = 7.3 Hz, 2H), 1.59–1.42 (m, J = 14.6, 7.3 Hz, 4H), 1.06–0.98 (m, 6H).

CRNS

This compound was prepared in a manner analogous to the synthesis of **NIS-H** using **CRNO** (0.14 g, 0.50 mmol) and Lawesson's reagent (0.61 g, 1.5 mmol) to give a powder of **CRNS** (yield, 78%). ^{1}H NMR ($CDCl_3$): δ 7.52 (dq, J = 8.7, 2.0 Hz, 1H), 7.24 (d, J = 0.9 Hz, 1H), 6.73 (d, J = 2.3 Hz, 1H), 6.67 (dd, J = 8.7, 2.3 Hz, 1H), 4.47 (s, 2H). ^{13}C NMR ($CDCl_3$): δ 195.9, 159.4, 151.4, 126.4, 124.2, 122.4, 120.5, 113.6, 106.9, 100.6. EI–MS calculated for $C_{10}H_6F_3NOS$ $[M]^+$: 245.0122; found: 245.0124.

1.3. Photophysical measurements

UV–vis absorption and photoluminescence (PL) spectra were recorded on a Thermo Scientific Evolution 201 UV–vis spectrometer and FS-2 spectrophotometer (Scinco), respectively. Dilute sample solutions (typically 10 μM) were prepared from stock acetonitrile (ACN) solutions (1.0 mM) at ambient conditions. The UV–vis and PL spectra of solutions (10 μM) were obtained using a 1-cm quartz cuvette. The photoluminescence quantum yield (PLQY) of the coumarin derivatives was determined by:

$$\Phi_s = \Phi_{ref} \times \left[\frac{I_s}{I_{ref}}\right] \times \left[\frac{A_{ref}}{A_s}\right] \times \left[\frac{\eta_s}{\eta_{ref}}\right]^2 \quad (1)$$

where Φ_{ref} is the PLQY of the reference, I is the area under the emission spectra, A is the absorbance at the excitation wavelength, η is the refractive index of the used solvent, and s and ref stand for coumarin derivatives and reference, respectively. Rhodamine 6G (Φ_{ref} = 0.94 in ethanol) was used as a reference for the PLQY.[4]

1.4. Measurement of ROS generation

Following the procedure in the literature,[5] the relative singlet oxygen quantum yields were determined using Rose Bengal as a reference for **NIS** (Φ_Δ (RB) = 0.54 in ACN)5 and $[Ru(bpy)_3]^{2+}$ as a reference for **CRNS** (Φ_Δ = 0.73 in MeOH).[6] 1,3-Diphenylisobenzofuran (DPBF, as singlet oxygen trap, abs ≈ 1.00) and photosensitizers (abs ≈ 0.2) were placed in a cuvette containing air-saturated organic solvents and the solutions were kept in dark until the absorbance reading was stable, followed by continuous light irradiation. The absorption of DPBF at 414 nm was recorded every 2 seconds to obtain the decay rate of the photosensitizing process. The measurements were performed using ~560 nm light that was

wavelength-selected from a 500 W halogen lamp by using an optical bandpass filter or a blue LED light source (454 nm).

The singlet oxygen ($^1O_2$) quantum yields of the RNI derivatives were determined by the equation,

$$\Phi_\Delta(PS) = \Phi_\Delta(ref)\ x \left[\frac{m(PS)}{m(ref)}\right] x \left[\frac{F(ref)}{F(PS)}\right] x \left[\frac{PF(ref)}{PF(PS)}\right]$$

Where, PS and ref stand for LSNI derivatives and the reference, respectively. m is the slope of the change in absorbance of DPBF at the absorbance maxima with the irradiation time. F is the absorption correction factor, which is given as $F = 1\text{-}10^{-OD}$, and PF is absorbed photonic flux (μEinstein dm^{-3} s^{-1}).

To manufacture 2 mL of TP PDT PSs and DHR123 in H_2O solution (containing 1% DMSO), 4 μL of 10 mM PSs, 5 μL of 1 mM DHR123 and 11 μL of DMSO were added to 1.98 mL H_2O. After measuring the initial fluorescence intensity of the DHR123, the solution was irradiated with TP excitation source for 30 min. Then, the initial value was removed from the increased fluorescence intensity of DHR123, it was recorded the fluorescence intensity change. Fluorescence intensity of DHR123 was recorded at 500 nm excitation and 525 nm emission wavelength.

1.5. Cell studies

1.5.1. Cell viability

MTT kit (AbCareBio CL) assay was performed to assess the dark cytotoxicity. HeLa cells were cultured in 96-well plates for 24 h. The stock solutions of TP PSs in DMSO (500 μM, 1 mM, 2 mM and 10 mM) were prepared, and 1 μL of each stock solution was added to 96-well plates containing 99 μL of cultured medium. After incubation for 2 h, the cultured medium was replaced with serum-free medium containing 10% MTT, and further incubated for 2 h. MTT containing medium was removed and DMSO was added to dissolve the formed formazan precipitate. Absorbance was measured at 600 nm.

1.5.2. Two-photon microscopy and cell imaging

HeLa cells were incubated for 48 h in minimum essential medium (MEM) which was contained with fetal bovine serum 10% (FBS), penicillin (100 units mL^{-1}), and streptomycin (100 μg mL^{-1}). The cell culture medium was replaced with a serum-free MEM and PSs were stained and incubated before imaging. To sustain optimum imaging conditions, humidity, temperature, and CO_2 were maintained by live-cell chamber (Chamlide IC) during the imaging study. Two-photon fluorescence images were acquired with a multiphoton and spectral confocal microscope (Leica TCS SP8 MP) with ×40 oil objective. TP PDT PSs were excited at 800 nm by a mode-locked fs Ti:Sapphire laser source (Mai Tai HP) with 2.97 W output power, corresponding to ~8.11×10^5 W cm^{-2} in the focal plane. In this condition, the laser power delivered to the sample was measured to be 2.68 mW.

1.5.3. ROS generation study in cells.

The HeLa cells were stained with the TP PSs (20 μM) and DCF-DA (10 μM) for 30 min. After incubation, cells were washed 2 times with serum-free medium and 800 nm of TP light was irradiated each 0, 50, 100 and 200 scans at the same position. After each TP scan, the fluorescence intensity of DCF-DA was

recorded. Image conditions of DCF-DA were 488 nm excitation and 500–550 nm emission windows.

1.5.4. Calcein-AM/PI assays

The HeLa cells were stained with the TP PSs (20 μM). After incubation, cells were washed 2 times with serum-free medium and 800 nm of TP light was irradiated from 0 to 400 scans. Then, the cells were stained with the calcein-AM (1 μM) and PI (10 μM) for 1 h. Image conditions were 488 nm excitation and 505–525 nm emission windows for calcein-AM and 552 nm excitation and 605–625 nm emission windows for PI.

1.5.5. Cell spheroid images

To form HeLa Spheroid, cells were incubated in 3D cell culture dish (MicroFIT) for 24 h. The cell culture medium was replaced with a serum-free MEM and each TP PDT PSs (100 μM) and DHR123 (15 μM) were incubated for 1 h. After incubation, 800 nm of TP light was irradiated to evaluated ROS production efficiency. To obtain 3D-spheroid images, over 100 section images were acquired using *xyz* mode and then combined each section images using LAS-X program. Image conditions of DHR123 was 488 nm excitation and 500–550 nm emission windows.

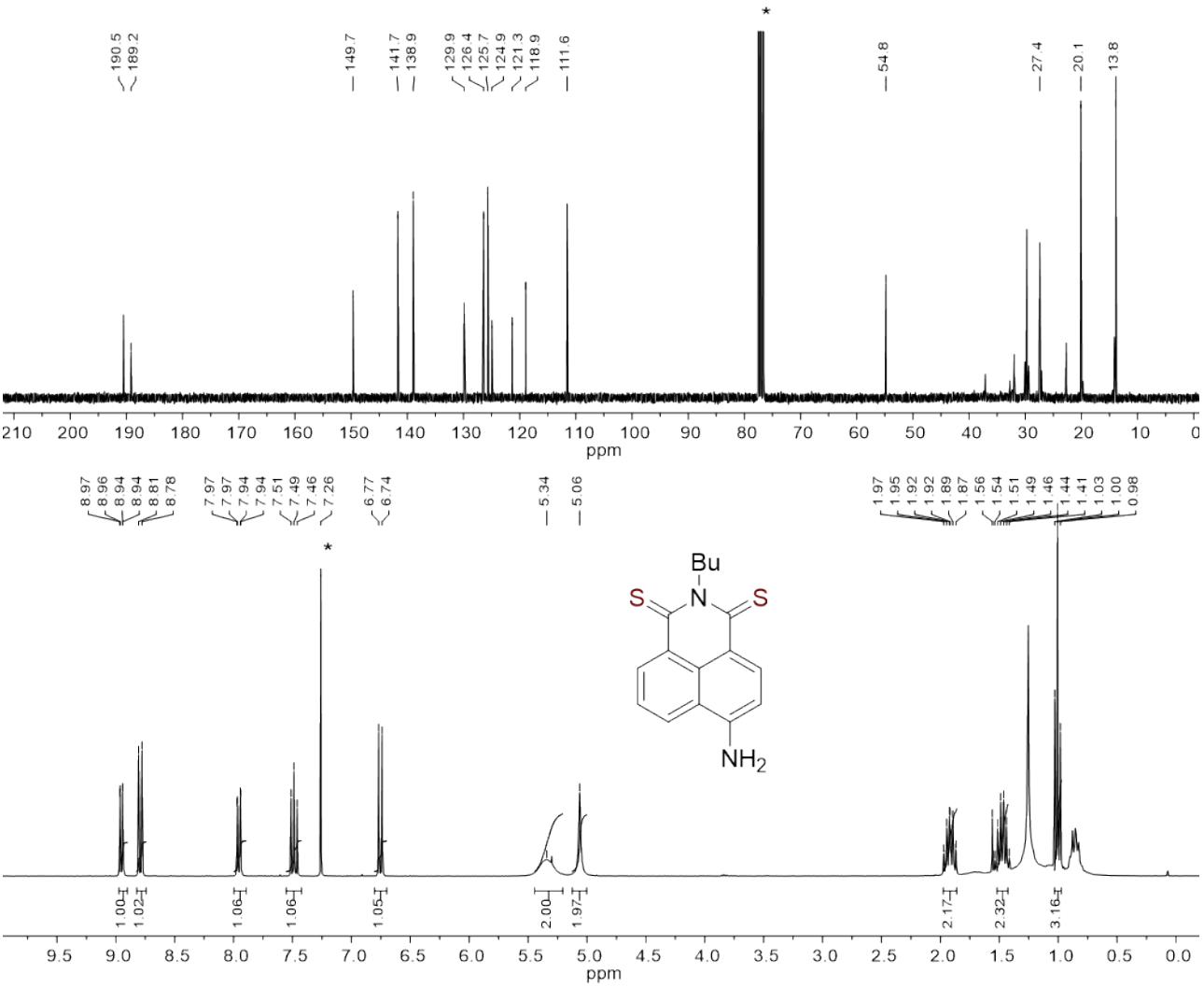


Fig. S1 ^{13}C (top) and ^{1}H (bottom) NMR spectra of NIS-H (∗ from residual solvent).

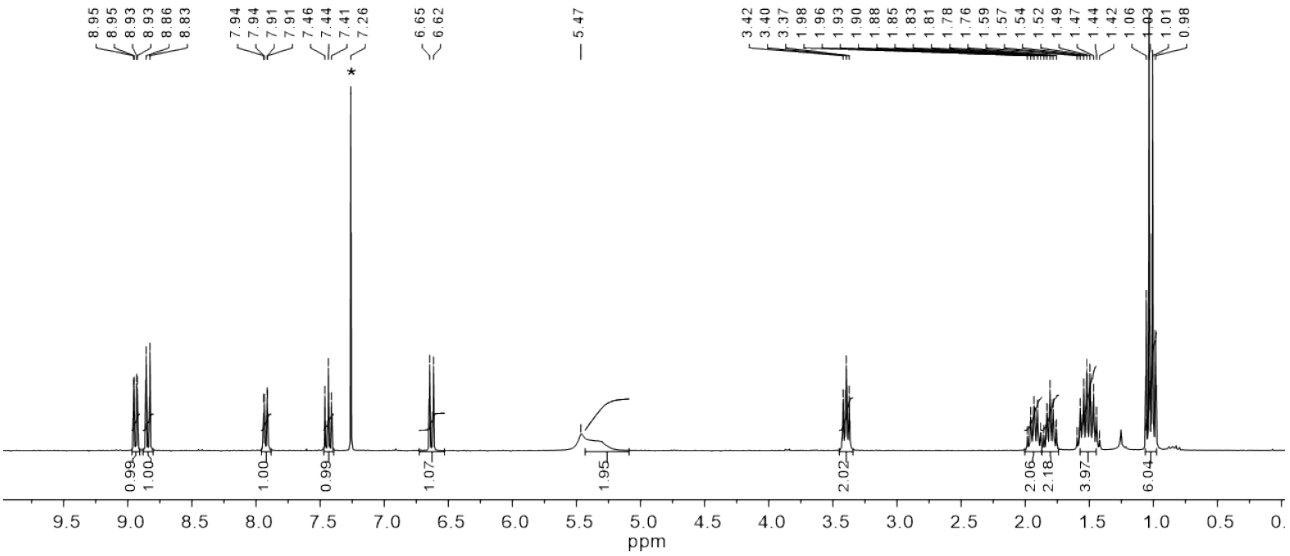


Fig. S2 ^{1}H NMR spectra of **NIS-Bu** (∗ from residual solvent).

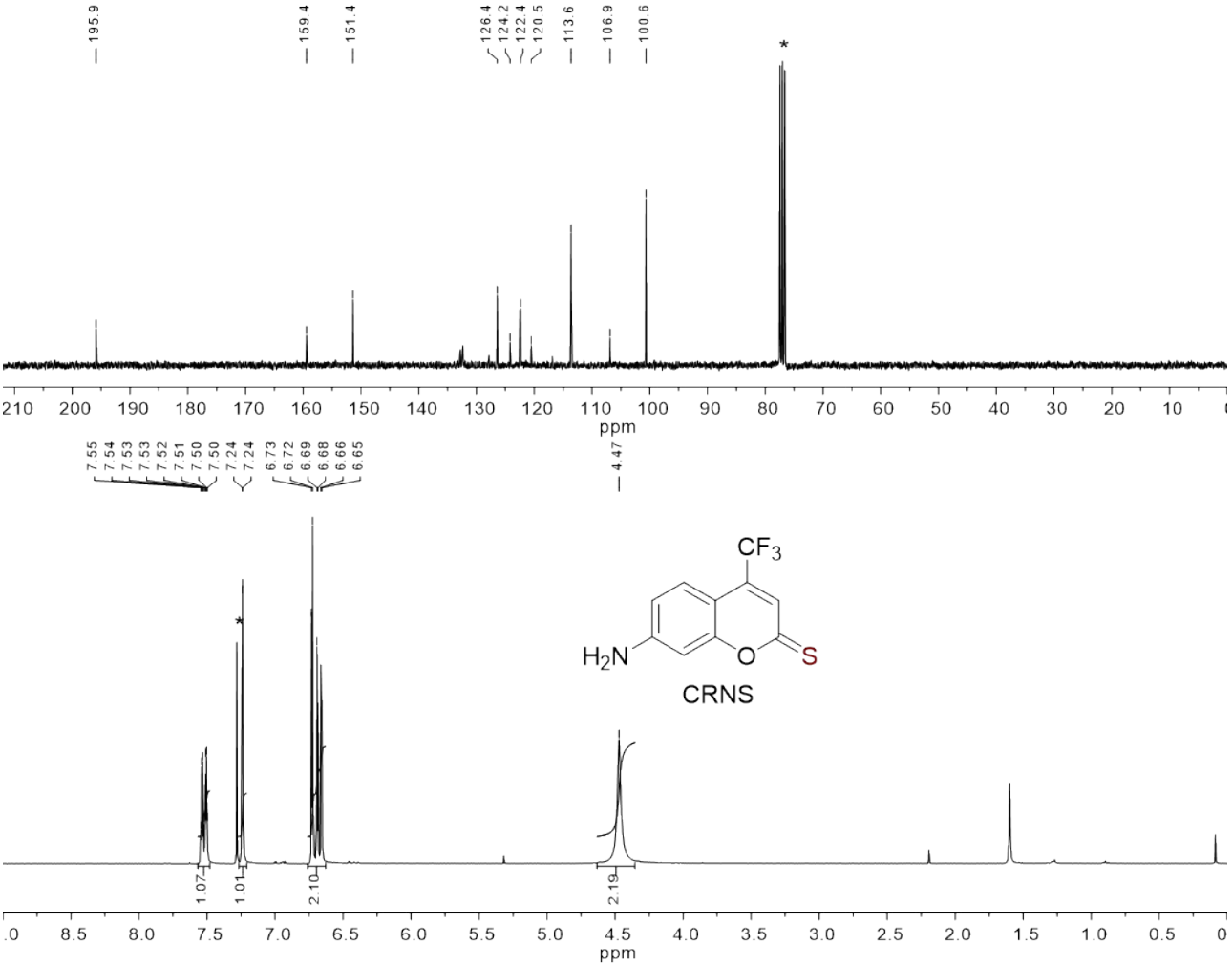


Fig. S3 ^{13}C (top) and ^{1}H (bottom) NMR spectra of **CRNS** (∗ from residual solvent).

Table S1. Photophysical and photosensitizing properties of **NI** and **CRN** compounds.

Compounds	λ_{abs}[a]/nm ($\varepsilon \times 10^{-3}/M^{-1}\ cm^{-1}$)	λ_{PL}/nm[a]	Φ_{PL}[a,b]	Φ_{Δ}[c]
CRNO	358 (16.70)	372	0.99	[e]
NIO-H	402 (12.51)	472	0.99	[e]
NIO-Bu	422 (20.53)	476	0.95	[e]
NIO-Me	405 (17.41)	486	0.98	[e]
CRNS	430 (20.34)	[d]	~0.0	~0.92
NIS-H	524 (16.47)	[d]	~0.0	~0.94
NIS-Bu	566 (33.87)	[d]	~0.0	~0.98
NIS-Me	538 (27.98)	[d]	~0.0	~1.00

[a]10 μM in toluene. [b]Fluorescence quantum yields estimated using rhodamine 6G as a standard (Φ_{PL} = 0.94 in ethanol).[7] [c]The singlet oxygen quantum yields of the **NIS** compounds were determined with respect to RB (Φ_{Δ} = 0.54 in ACN)[8] using ~560 nm light source and that of **CRNS** was determined with respect to $Ru(bpy)_3^{2+}$ (Φ_{Δ} = 0.73 in MeOH) using ~454 nm light source.[9] [d]Not observed. [e]Not determined.

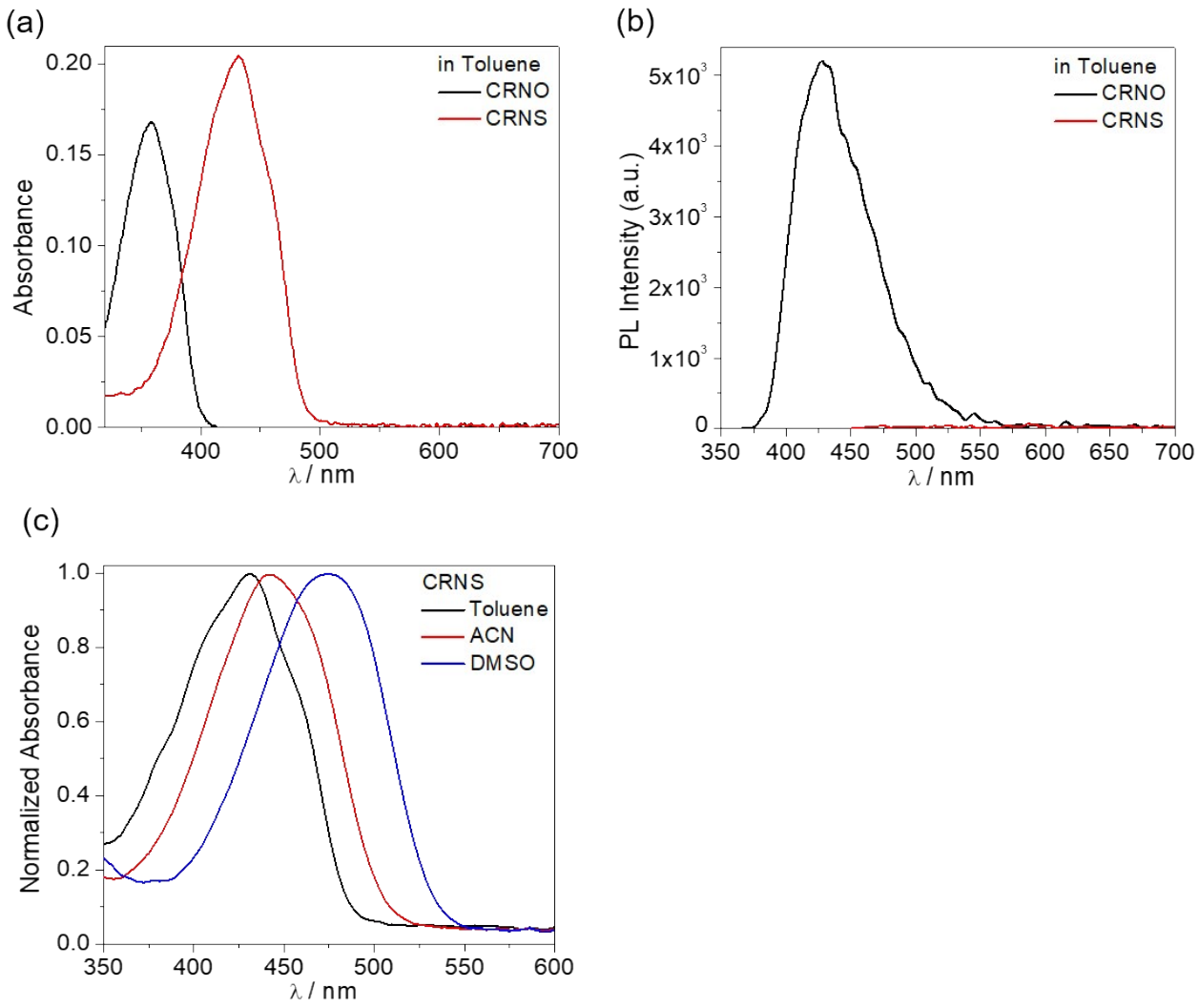


Fig. S4 (a) UV-Vis absorption and (b) PL spectra of CRNO and CRNS in toluene. (c) UV-Vis absorption spectra of CRNS in various solvents (~10 μM).

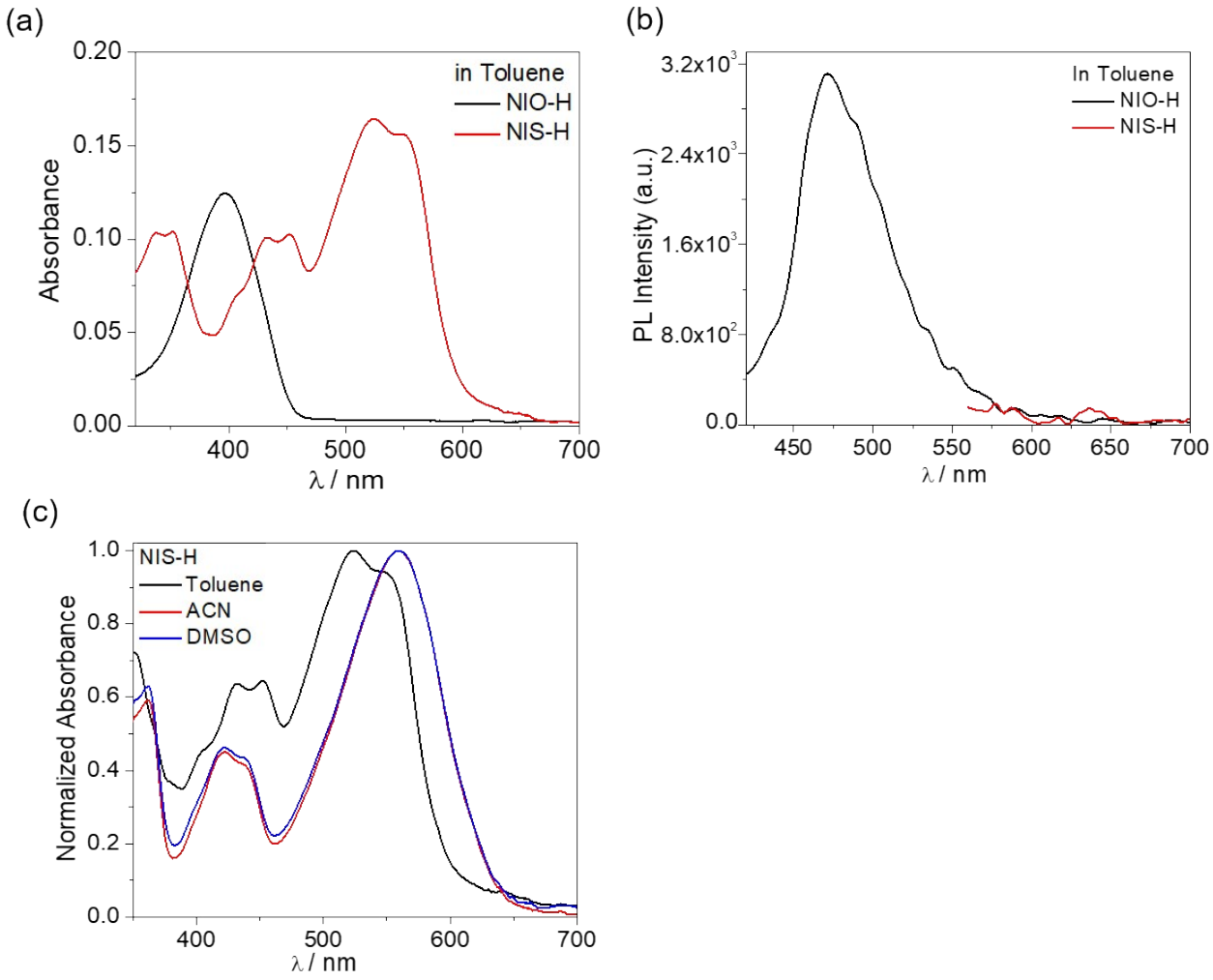


Fig. S5 (a) UV-Vis absorption and (b) PL spectra of NIO-H and NIS-H in toluene. (c) UV-Vis absorption spectra of NIS-H in various solvents (~10 μM).

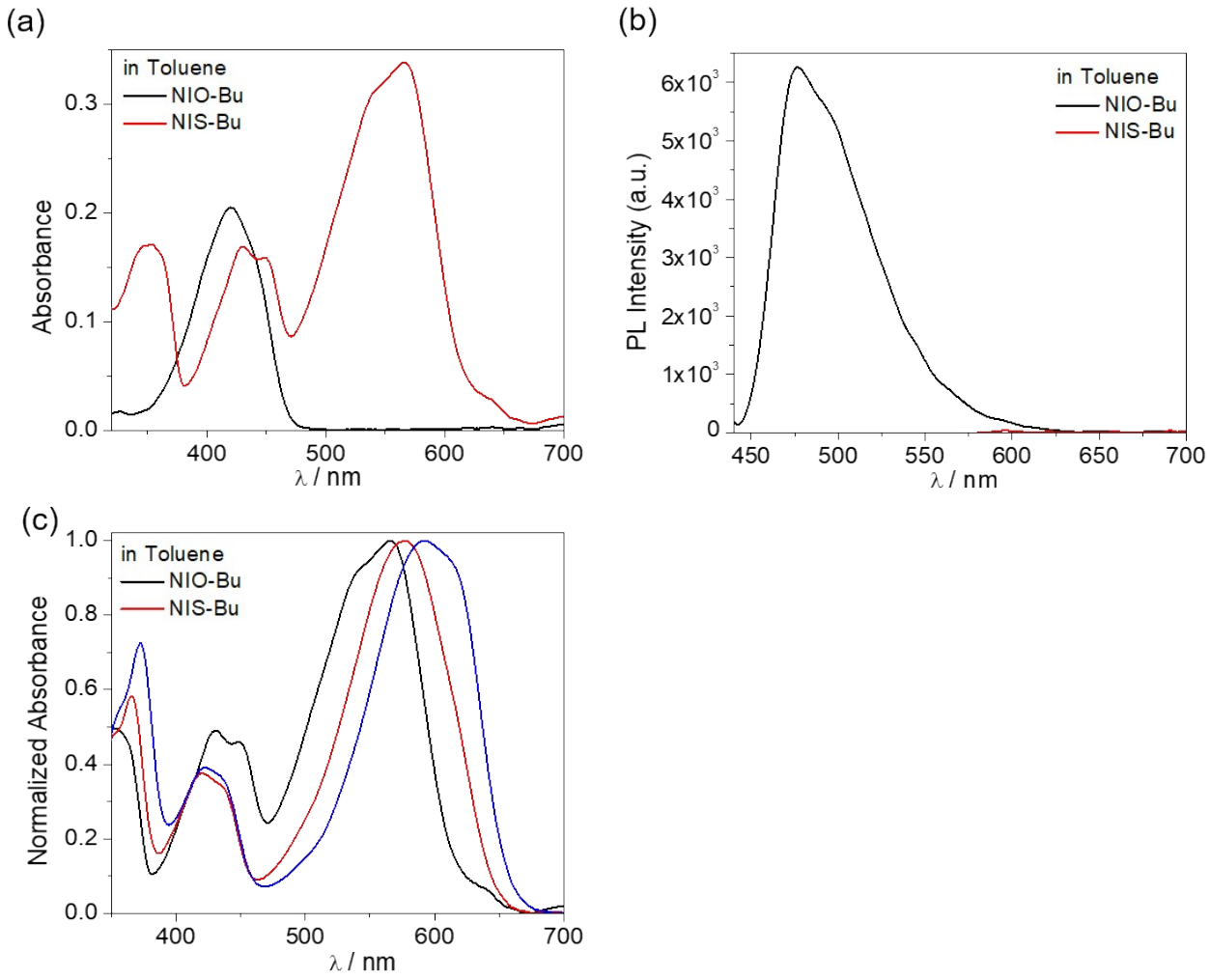


Fig. S6 (a) UV-Vis absorption and (b) PL spectra of NIO-Bu and NIS-Bu in toluene. (c) UV-Vis absorption spectra of NIS-Bu in various solvents (~10 μM).

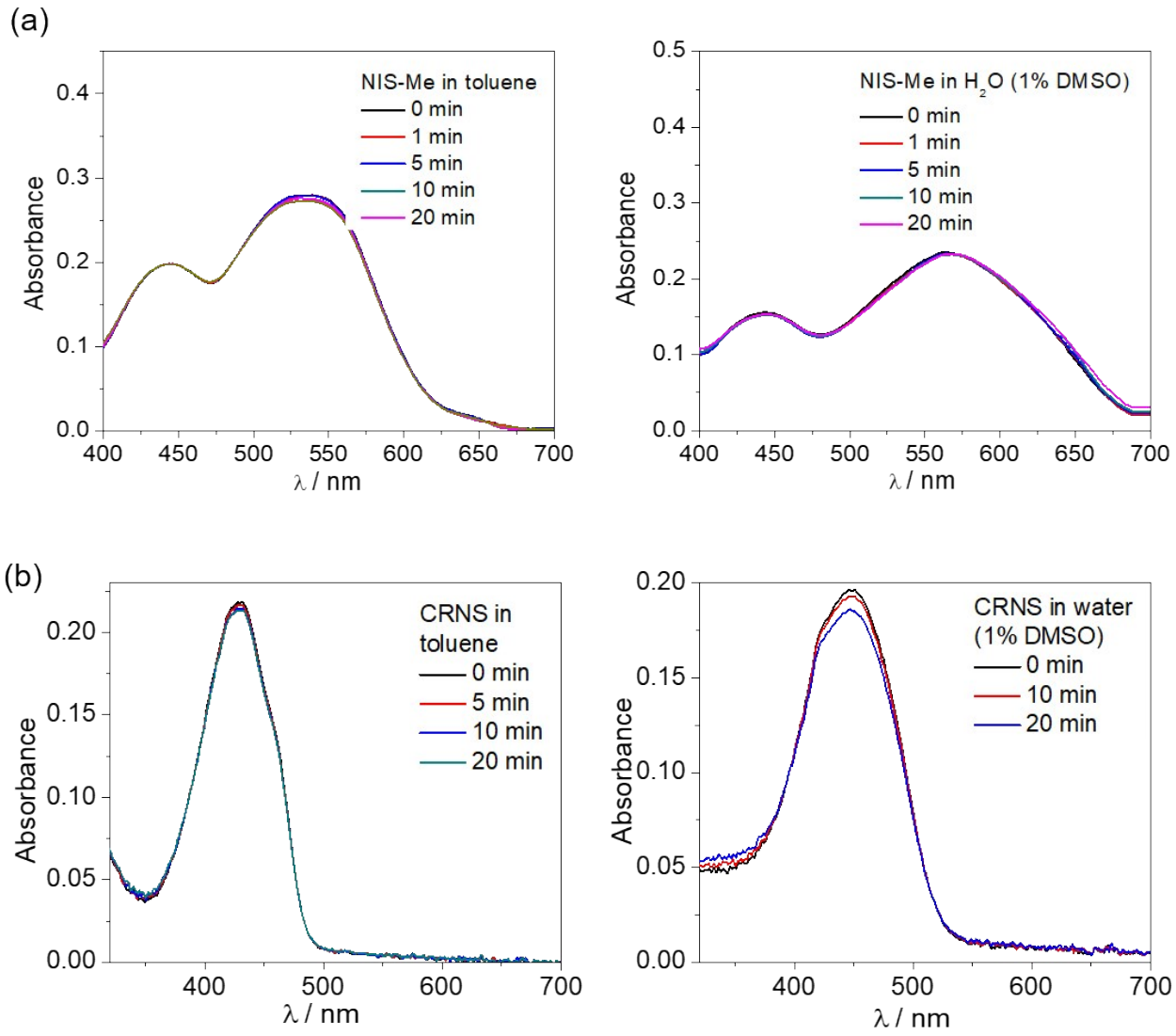


Fig. S7 UV-visible absorption spectra of (a) **NIS-Me** (10 μM) and (b) **CRNS** in toluene and water, before irradiation ($t = 0$) and after irradiation with 560 nm light and blue LED light source (454 nm), respectively.

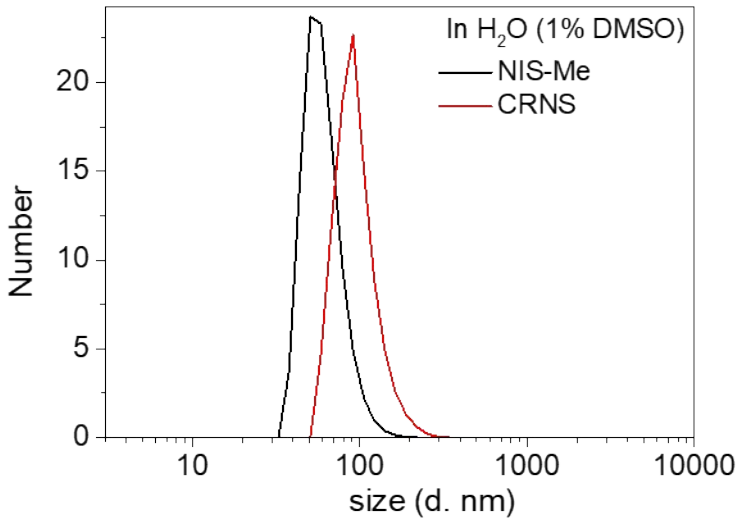


Fig. S8 Size distribution of **NIS-Me** and **CRNS** (10 μM) in water (1%DMSO) detected by DLS. PSs were dissolved in DMSO first and then diluted into water.

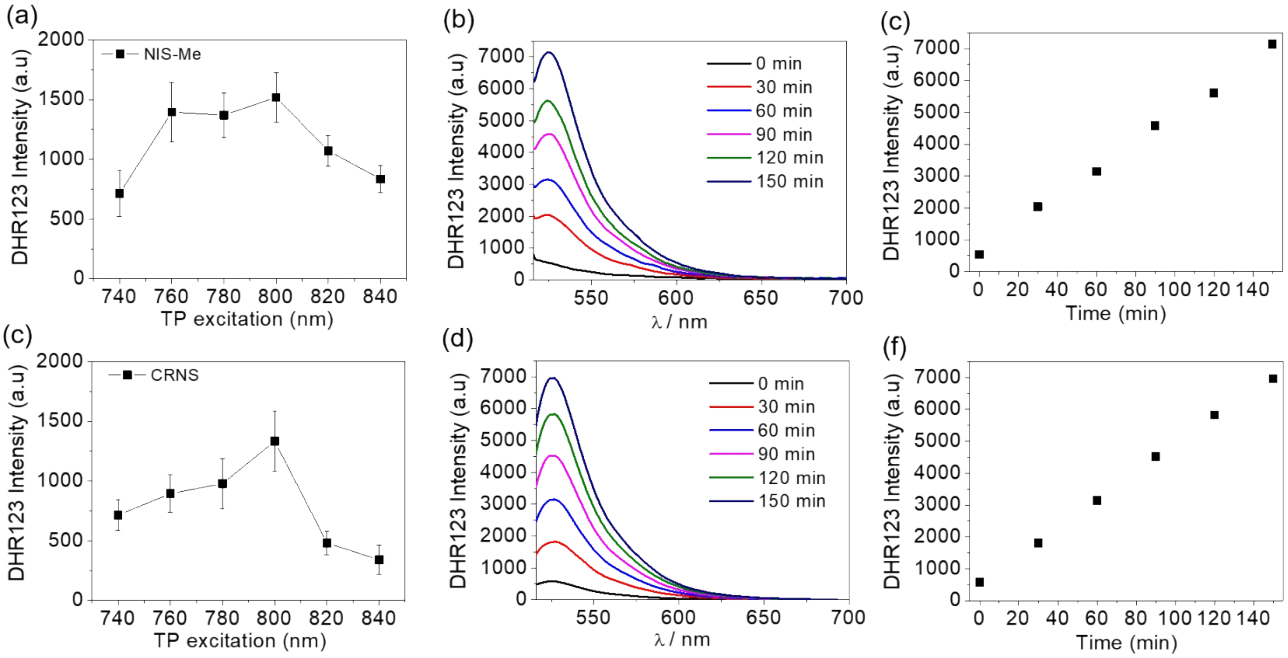


Fig. S9 Two-photon ROS generation efficiency of (a–c) **NIS-Me** (20 μM) and (d–f) **CRNS** (20 μM). Plot of fluorescence intensity of DHR123 (2.5 μM) with (a) **NIS-Me** and (d) **CRNS** according to two-photon excitation wavelength. (b,e) Fluorescence spectra and (c,f) plot of fluorescence intensity for DHR123 with (b,c) **NIS-Me** and (e,f) **CRNS** against TP irradiation time at 800 nm. The excitation wavelengths for DHR123 was 500 nm and fluorescence intensities were acquired at 525 nm.

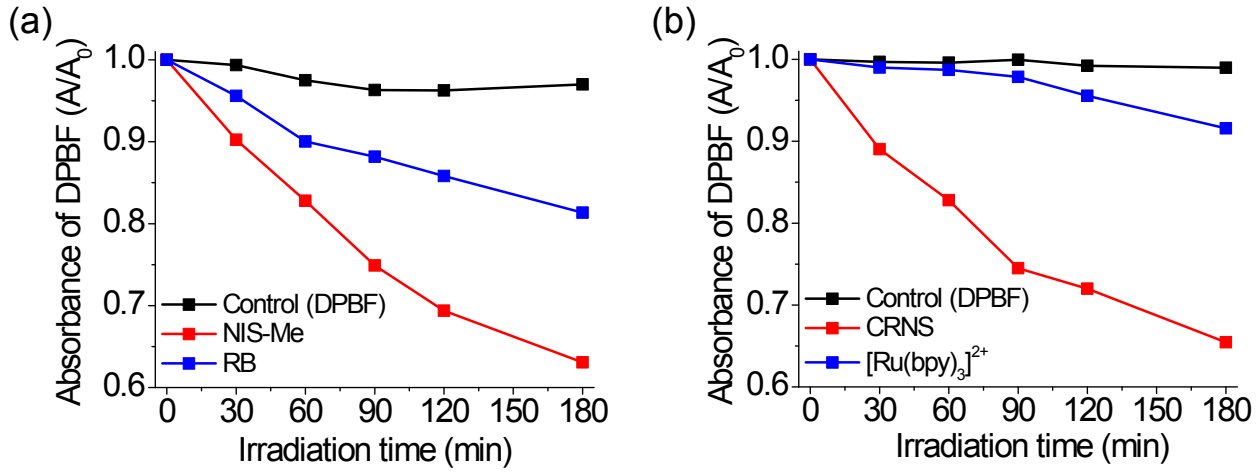


Fig. S10 Comparison of ROS generation of (b) **NIS-Me** and Rose Bengal (RB) in ACN and (b) **CRNS** and $[Ru(bpy)_3]^{2+}$ in MeOH. The concentrations of samples were 20 μM and irradiated by 800 nm Ti:sapphire laser. The absorbance of DPBF at 414 nm was recorded every 30 min.

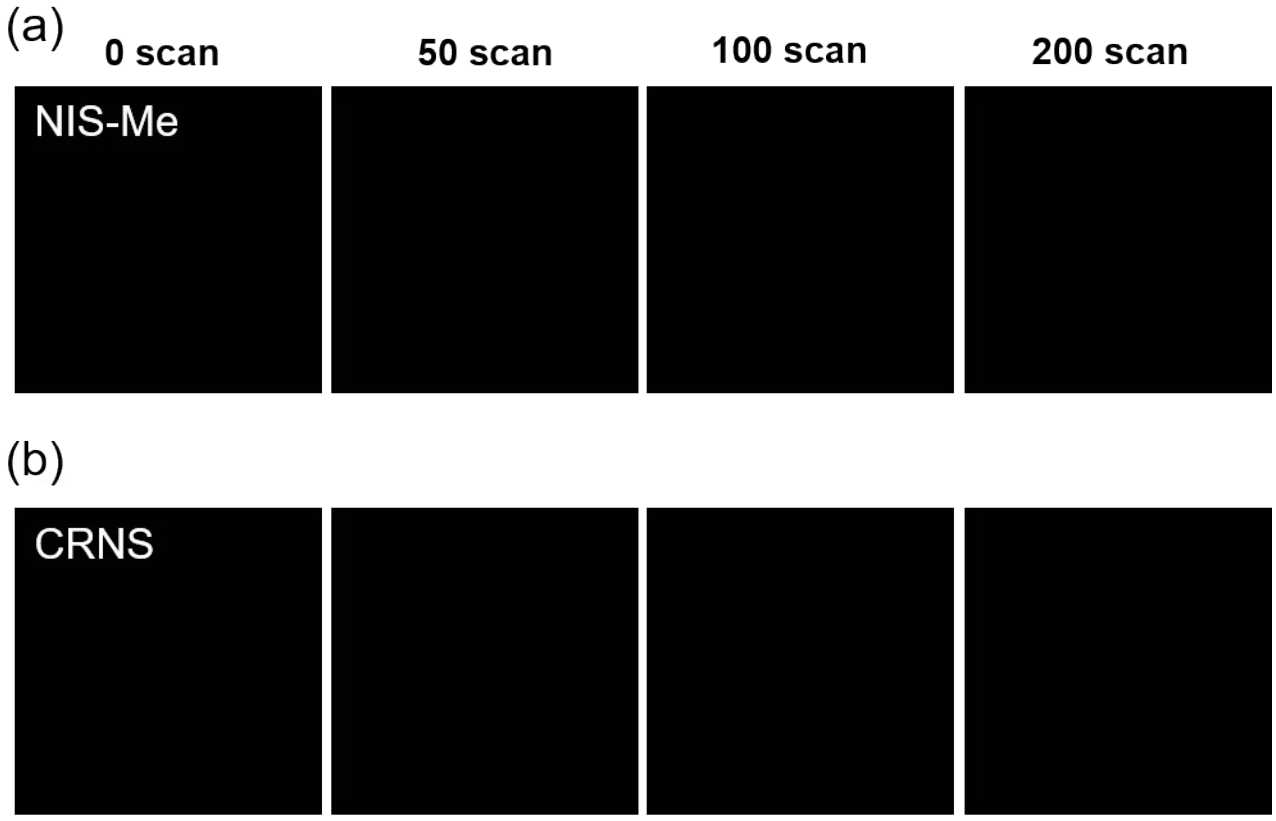


Fig. S11 Fluorescence images of HeLa cells were stained with (a) **NIS-Me** (20 μM, 30 min) and (b) **CRNS** (20 μM, 30 min) followed by different TP scans. Images were obtained from 488 nm excitation and 500–550 nm emission windows. Scale bars = 50 μm.

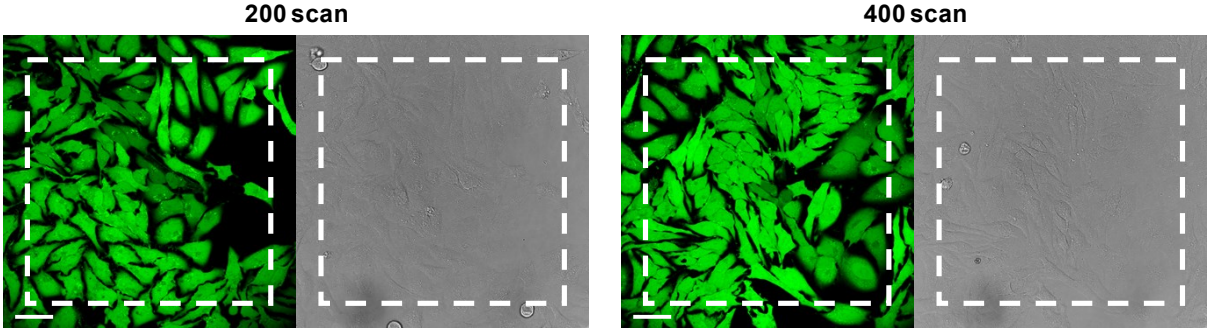


Fig. S12 Overlaid images and brightfield images of calcein-AM and PI. HeLa cells were irradiated for different TP scans. Then calcein-AM (1 μM, 1 h) and PI (10 μM, 1 h) were stained into the cells. Images were obtained from 488 nm excitation and 505–525 nm for calcein-AM and 552 nm excitation and 605–625 nm emission windows for PI. Scale bars = 50 μm.

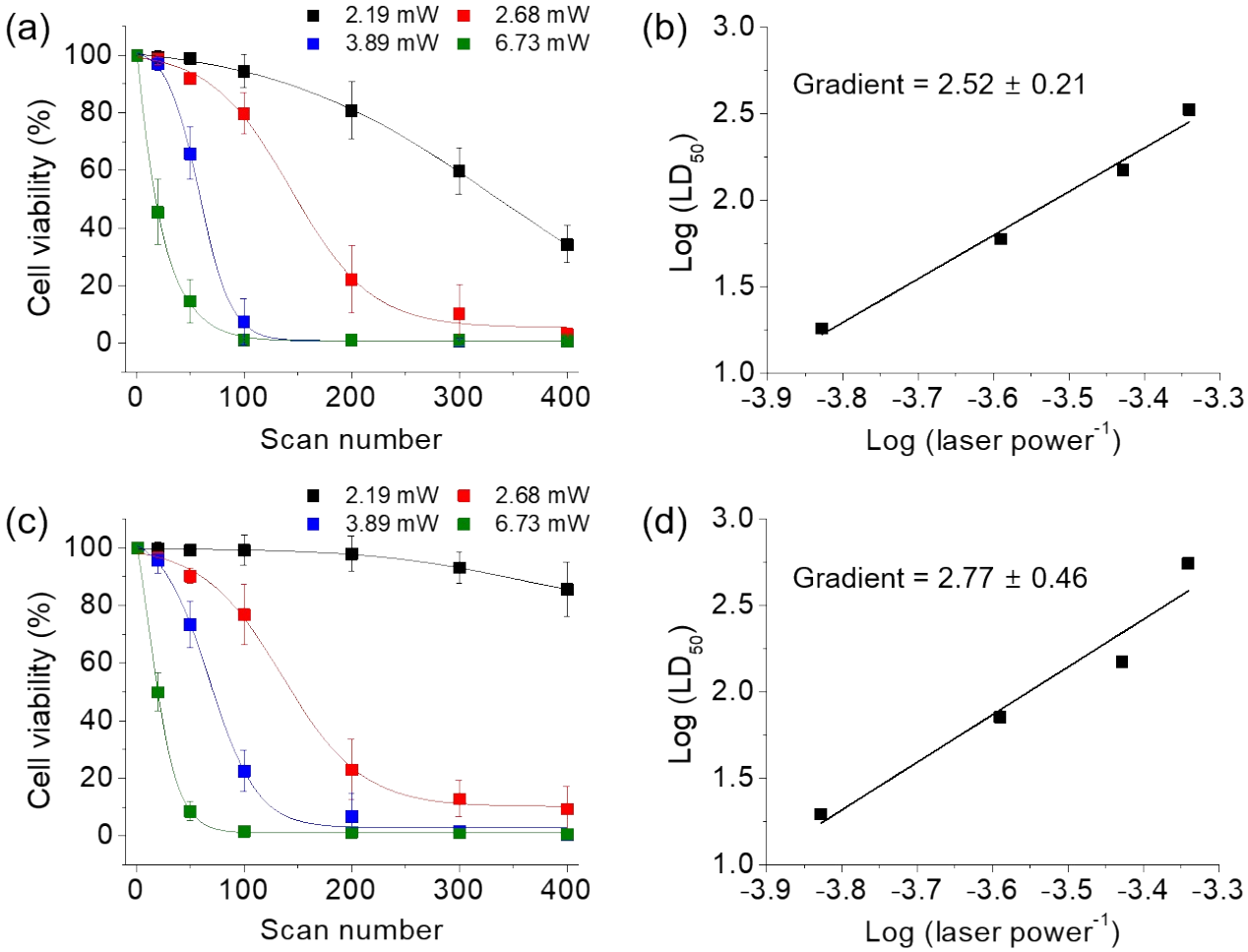


Fig. S13 Two-photon photodynamic efficiency as a function of laser power. Hela cells were incubated for 30 min with **NIS-Me** (20 μM) or **CRNS** (20 μM). After irradiation with a full spectrum laser, cell viability was estimated using calcein-AM and PI fluorescent probes. Viability of cells incubated with (a) **NIS-Me** or (c) **CRNS** and irradiated at 800 nm at discrete laser powers of 2.2, 2.7, 3.9, and 6.7 mW. Log(LD_{50}) of (b) **NIS-Me** and (d) **CRNS** vs. log(laser $power^{-1}$).

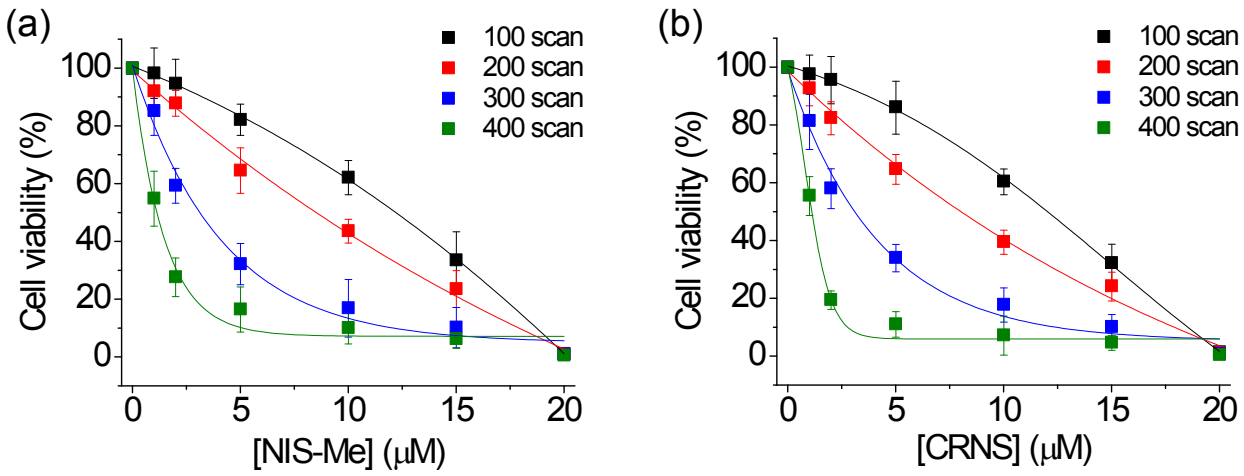


Fig. S14 Two-photon photodynamic efficiency as a function of PS concentration. Hela cells were incubated for 30 min with (a) **NIS-Me** (20 μM) or (b) **CRNS** (20 μM). After 800 nm of TP irradiation (6.73 mW), cell viability was estimated using calcein-AM and PI fluorescent probes. After 800 nm of TP irradiation (6.7 mW), the IC_{50} for 100, 200, 300, and 400 scans are 12.2, 8.7, 3.1, and 1.9 μM in **NIS-Me** and 12.0, 7.9, 3.1, and 1.1 μM in **CRNS**, respectively.

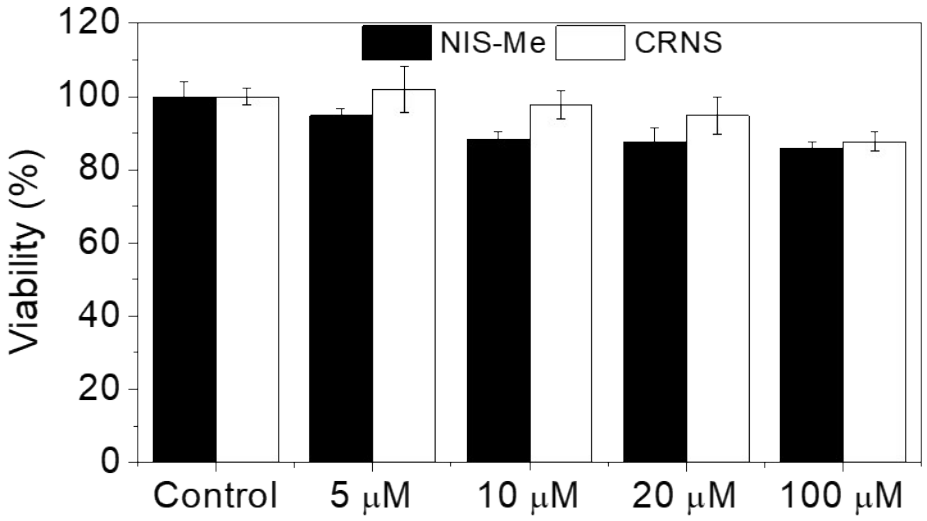


Fig. S15 Dark toxicity of HeLa cells in the presence of **NIS-Me** and **CRNS** as measured by using MTT assays. The cells were incubated with 0–100 μM of PSs for 2 h.

4. References

1. M. Quintana, A. Bilbao, J. Comas-Barcelo, J. Bujons, G. Triola, Eur. J. Med. Chem., 2019, 78, 648-666.

2. J. O. Moon, M. G. Choi, T. Sun, J. I. Choe and S. K. Chang, Dyes Pigm., 2013, 96, 170-175.

3. V. N. Nguyen, S. Qi, S. Kim, N. Kwon, G. Kim, Y. Yim, S. Park and J. Yoon, J. Am. Chem. Soc., 2019, 141, 16243-16248.

4. A. M. Brouwer, Pure Appl. Chem., 2011, 83, 2213-2228.

5. A. M. Durantini, L. E. Greene, R. Lincoln, S. R. Martinez and G. Cosa, J. Am. Chem. Soc., 2016, 138, 1215-1225.

6. Z. Lv, H. J. Wei, Q. Li, X. L. Su, S. J. Liu, K. Y. Zhang, W. Lv, Q. Zhao, X. H. Li and W. Huang, *Chem. Sci.*, 2018, **9**, 502-512.

7. A. M. Brouwer, Pure Appl. Chem., 2011, 83, 2213-2228.

8. A. M. Durantini, L. E. Greene, R. Lincoln, S. R. Martinez, and G. Cosa, J. Am. Chem. Soc., 2016, 138, 1215-1225.

9. Z. Lv, H. J. Wei, Q. Li, X. L. Su, S. J. Liu, K. Y. Zhang, W. Lv, Q. Zhao, X. H. Li and W. Huang, Chem. Sci., 2018, 9, 502-512.

5. Author Contributions

Dr. Van-Nghia Nguyen: Conceptualization, synthesis, data interpretation, and writing of an original draft.
Sang Jun Park: Cell experiments and writing of cell results.
Sujie Qi: Synthesis.
Jeongsun Ha: Photophysical measurements.
Seonye Heo: Photophysical measurements.
Yubin Yim: Synthesis.
Gain Baek: Photochemical measurements.
Chang Su Lim: Cell experiments.
Dong Joon Lee: Cell experiments.
Prof. Hwan Myung Kim: Interpretation and writing of cell results.
Prof. Juyoung Yoon: Project management, reviewing and editing.