Supporting information for

Development of a Unique Family of Two-photon Full-color-tunable Fluorescent Materials for Imaging in Live Subcellular Organelles, Cells, and Tissues

Hua Chen,[‡] Yonghe Tang,[†] Huiming Shang,[†] Xiuqi Kong,[†]

Rui Guo,[‡] and Weiying Lin*[,†,‡]

[†] Institute of Fluorescent Probes for Biological Imaging, School of Materials Science and Engineering, School of Chemistry and Chemical Engineering, University of Jinan, Jinan, Shandong 250022, P.R. China.

[‡] State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha, Hunan 410082, P. R. China

Email: weiyinglin2013@163.com

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Materials and instruments. Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. Mass spectra were performed using an LCQ Advantage ion trap mass spectrometer from Thermo Finnigan or Agilent 1100 HPLC/MSD spectrometer. NMR spectra were recorded on an INOVA-400 spectrometer, using TMS as an internal standard. Electronic absorption spectra were obtained on a Labtech UV Power PC spectrometer. Photoluminescent spectra were recorded at 37˚C with a HITACHI F4600 fluorescence spectrophotometer. The fluorescence imaging of the cells was performed with OLYMPUS FV1000 (TY1318) confocal microscopy and Nikon A1MP confocal microscopy. TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals.

Determination of the fluorescence quantum yield[1-3]: Fluorescence quantum yields for **TPFC-1~5** were determined by using ICG (Φ_f = 0.13 in DMSO) as a fluorescence standard.[1] The quantum yield was calculated using the following equation:

$$\Phi_{F(X)} = \Phi_{F(S)} (A_S F_X / A_X F_S) (n_X / n_S)^2$$

Where Φ_F is the fluorescence quantum yield, A is the absorbance at the excitation wavelength, F is the area under the corrected emission curve, and n is the refractive index of the solvents used. Subscripts $_S$ and $_X$ refer to the standard and to the unknown, respectively.

References

1. Valeur, B. Molecular Fluorescence: Principles and Applications, Wiley-VCH, 2001.
2. Magde, D.; Rojas, G. E.; Seybold, P. *Photochem. Photobiol*. **1999**, *70*, 737-744.
3. Oushiki, D.; Kojima, H.; Terai, T.; Arita, M.; Hanaoka, K.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc*. **2010**, *132*, 2795-2801.

Measurement of two-photon absorption cross-sections. The two-photon absoprtion cross-section (σ) was determined by using a femtosecond (fs) fluorescence measurement technique. **TPFC-1~5** were dissolved in EtOH, at a concentration of

5.0×10^{-5} M, and then the two-photon fluorescence was excited at 700-900 nm by using fluorescein in pH = 11 aqueous solution (σ = 32 GM in 810 nm) as the standard, whose two-photon property has been well characterized in the literature. The two-photon cross-section was calculated by using $\sigma = \sigma_r(F_t n_t^2 \Phi_r C_r)/(F_r n_r^2 \Phi_t C_s)$, where the subscripts t and r stand for the sample and reference molecules. *F* is the average fluorescence intensity integrated from two-photon emission spectrum, n is the refractive index of the solvent, *C* is the concentration, Φ is the quantum yield, and σ_r is the two-photon cross-section of the reference molecule.

Cell culture and fluorescence imaging

HeLa cells were grown in MEM (modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were plated in 35 mm glass-bottom culture dishes and allowed to adhere for 24 h. Immediately before the experiments, the cells were washed with PBS buffer. The cells were further incubated with **TPFC** or **TPFC-Lyso** dyes (5 μM) for 30 min at 37 °C and imaged with a Olympus FV1000 equipped with a CCD camera.

Preparation of fresh mouse slices and two-photon fluorescence imaging.

Kunming mice were purchased from the Experimental Animal Center of Xiangya School of Medicine Central South University (Changsha, China). All animal procedures for this study were approved by the Animal Ethical Experimentation Committee of the Central South University according to the requirements of the National Act on the use of experimental animals (China). Slices were prepared from the brain or liver of 14-day-old mice. Slices were cut to 400 μm thickness by using a vibrating-blade microtome in 25 mM PBS (pH 7.4). For the full-color imaging

experiments, the slices were incubated with 20 μM **TPFC** in PBS buffer bubbled with 95% O_2 and 5% CO_2 for 0.5 h at 37 ˚C. The slices were then washed three times with PBS, transferred to the glass-bottomed dishes, and observed under a two-photon confocal microscope (OLYMPUS FV1000 (TY1318)). The two-photon fluorescence emission was collected at different fluorescence emission windows upon excitation at 780 nm with a femtosecond pulse. The blue, green, yellow, orange, and red channels correspond to the emission windows of 420-470, 480-530, 530-580, 580-630, and 630-690 nm, respectively.

Synthesis of compound 5.

A mixture of 4-bromo-1,8-naphthalic anhydride (5.54 g, 20.0 mmol) and N-(2-aminoethyl) morpholine (2.4 mL, 24 mmol) in ethanol (200 mL) was refluxed under nitrogen for 12 h. The solution was cooled to room temperature and formed a crystal. The crystal was separated by filtration and washed with cold ethanol to give the compound **5** as a pale yellow crystal (4.9 g, 64.5 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.65 (d, *J* = 7.3 Hz, 1H), 8.57 (d, *J* = 8.5 Hz, 1H), 8.41 (d, *J* = 7.9 Hz, 1H), 8.04 (d, *J* = 7.9 Hz, 1H), 7.88-7.83 (m, 1H), 4.34 (t, *J* = 6.9 Hz, 2H), 3.70-3.66 (m, 4H), 2.71 (t, *J* = 6.8 Hz, 2H), 2.60 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.25, 132.96, 131.69, 130.88, 130.76, 130.31, 129.96, 128.70, 127.74, 122.71, 121.84, 66.67, 55.73, 53.46, 36.96. MS (ESI) m/z = 389.2$[M+H]^+$.

Synthesis of compound 6.

Compound **5** (1.1 g, 3.0 mmol) in 2-methoxy ethanol(20 mL) was heated to 120 ℃ until the mixture became clear. After that, hydrazine hydrate (1 mL, 80 %) was added to the solution dropwisely with stirring in 10 min. Then, the mixture was refluxed for another 3.5 h under nitrogen. After cooling, the crystal was separated by filtration and washed with cold ethanol to afford the compound **6** as a pale yellow crystal (0.61 g, 60.1 %). ^{1}H NMR (400 MHz, d6-DMSO) δ 8.65 (d, *J* = 8.4 Hz, 1H), 8.45 (d, *J* = 7.3 Hz,

1H), 8.32 (d, J = 8.6 Hz, 1H), 7.73-7.64 (m, 1H), 7.28 (d, J = 8.6 Hz, 1H), 4.72 (s, 2H), 4.20 (d, J = 7.0 Hz, 2H), 3.58 (t, J = 4.4 Hz, 4H), 2.57 (dd, J = 11.4, 4.4 Hz, 4H). ^{13}C NMR (100 MHz, d6-DMSO) δ 164.06, 163.16, 153.50, 134.53, 130.89, 129.61, 128.57, 124.41, 121.96, 118.70, 107.55, 104.29, 66.49, 56.10, 55.19, 53.71, 36.65. MS (ESI) m/z = 341.2$[M+H]^+$.

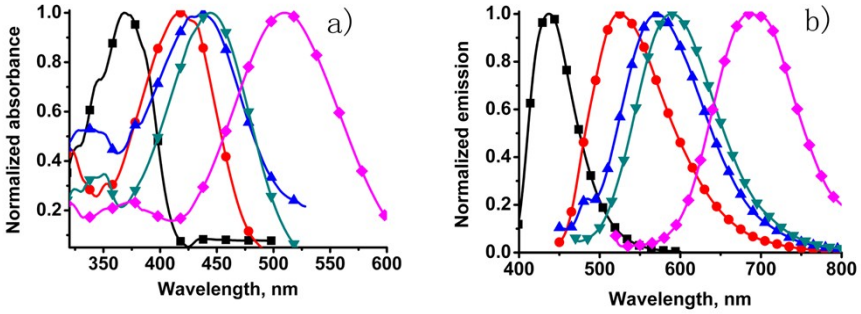

Figure S1. Normalized absorption (a) and fluorescence emission (b) spectra of 5 μM compounds **TPFC-1** (■), **TPFC-2** (●), **TPFC-3**(▲), **TPFC-4** (▼), and **TPFC-5** (◆) in EtOH.

Table S1. Photophysical data of **TPFC** Fluorophores in EtOH.

Compd.	λ_{max} (nm)[a]	ε_{max}	λ_{em} (nm)[b]	Φ	Stokes Shifts (nm)
TPFC-1	368	11120	436	0.277	68
TPFC-2	418	23020	524	0.110	106
TPFC-3	436	16650	571	0.091	135
TPFC-4	446	39040	589	0.114	143
TPFC-5	510	34920	687	0.165	177

[a] The maximal absorption of the dye.
[b] The maximal emission of the dyes.

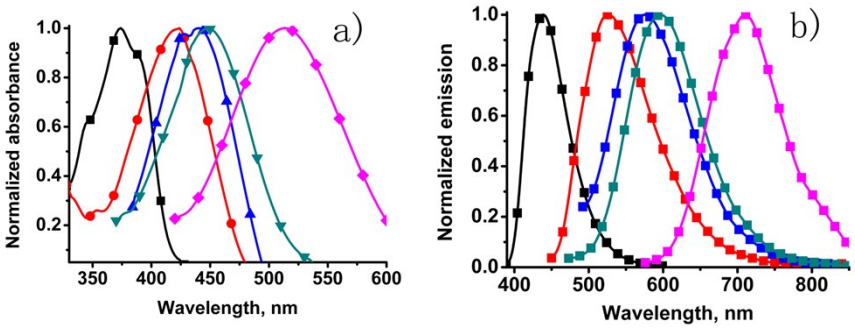

Figure S2. Normalized absorption (a) and fluorescence emission (b) spectra of 5 μM compounds **TPFC-1** (■), **TPFC-2** (●), **TPFC-3**(▲), **TPFC-4** (▼), and **TPFC-5** (◆) in DMSO.

Table S2. Photophysical data of **TPFC** Fluorophores in DMSO.

Compd.	λ_{max} (nm)[a]	ε_{max}	λ_{em} (nm)[b]	Φ	Stokes Shifts (nm)
TPFC-1	372	10970	438	0.269	66
TPFC-2	422	20700	525	0.523	103
TPFC-3	440	16890	574	0.317	134
TPFC-4	448	31080	597	0.700	149
TPFC-5	512	32580	710	0.292	198

[a] The maximal absorption of the dye.
[b] The maximal emission of the dyes.

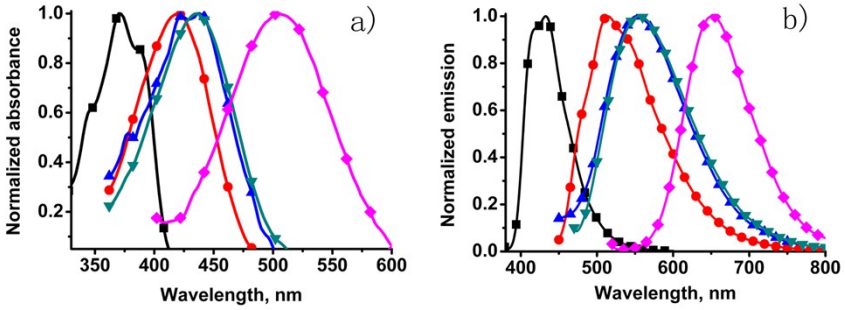

Figure S3. Normalized absorption (a) and fluorescence emission (b) spectra of 5 μM compounds **TPFC-1** (■), **TPFC-2** (●), **TPFC-3**(▲), **TPFC-4** (▼), and **TPFC-5** (◆) in CH_2Cl_2.

Table S3. Photophysical data of **TPFC** Fluorophores in CH_2Cl_2.

Compd.	λ_{max} (nm)[a]	ε_{max}	λ_{em} (nm)[b]	Φ	Stokes Shift (nm)
TPFC-1	370	10710	433	0.381	63
TPFC-2	422	25580	515	0.230	93
TPFC-3	440	17810	555	0.171	115
TPFC-4	436	41780	555	0.101	119
TPFC-5	504	27620	652	0.659	148

[a] The maximal absorption of the dye.
[b] The maximal emission of the dyes.

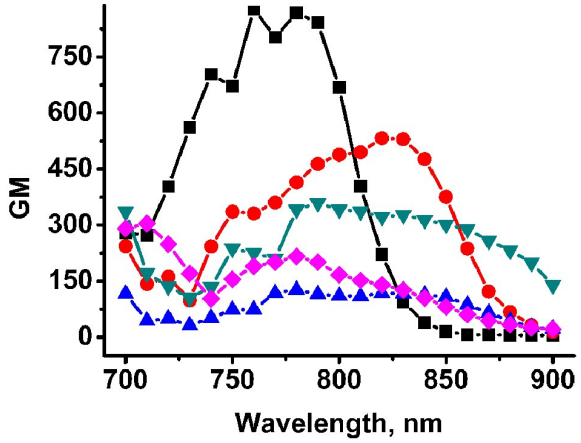

Figure S4. Two-photon cross-sections of **TPFC-1** (■), **TPFC-2** (●), **TPFC-3**(▲), **TPFC-4** (▼), and **TPFC-5** (◆) in EtOH.

Table S4. The maximal two-photon cross-sections and action cross-sections of **TPFC** dyes in EtOH.

	TPFC-1	**TPFC-2**	**TPFC-3**	**TPFC-4**	**TPFC-5**
Two-photon action cross-section (GM)	244	58	12	41	34
Two-photon cross-section (GM)	883	532	125	358	200

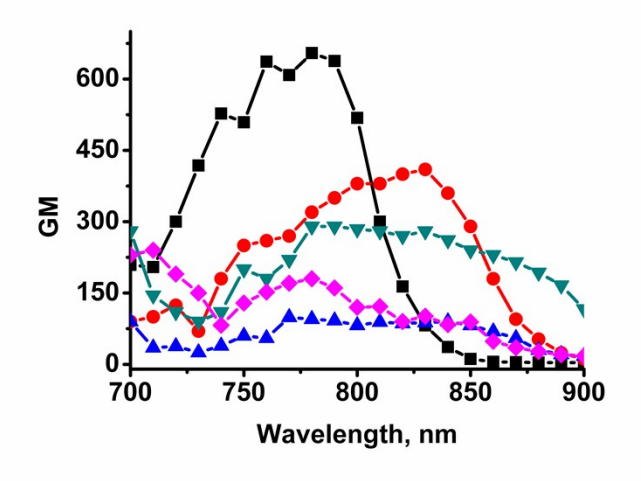

Figure S5. Two-photon cross-sections of **TPFC-1** (■), **TPFC-2** (●), **TPFC-3**(▲), **TPFC-4** (▼), and **TPFC-5** (◆) in PBS.

Table S5. Calculated absorption wavelengths and oscillator strengths of the **TPFC** dyes. All data were obtained from Gaussian 09 programs using the B3LYP exchange functional, together with 6-31+G(d) basis sets.

	TPFC-1	**TPFC-2**	**TPFC-3**	**TPFC-4**	**TPFC-5**
Absorption wavelength (nm)	367	432	450	452	500
Oscillator strength	0.32	0.99	1.11	0.72	1.13

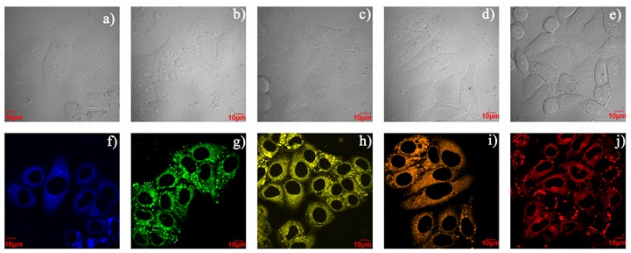

Figure S6. Bright field and one-photon full-color imaging of the living HeLa cells stained with **TPFC-1~5** dyes. a-e) Bright field images of the living HeLa cells treated with 5 μM **TPFC-1**, **TPFC-2**, **TPFC-3**, **TPFC-4**, or **TPFC-5**, respectively, for 0.5 h. f-j) one-photon fluorescence images of the Hela cells stained with **TPFC-1**, **TPFC-2**, **TPFC-3**, **TPFC-4**, **TPFC-5**, respectively (5 μM) for 0.5 h. Scale bar = 10 μm. The blue, green, yellow, orange, and red channels correspond to the emission windows of 420-470, 480-530, 530-580, 580-630, and 630-690 nm, respectively.

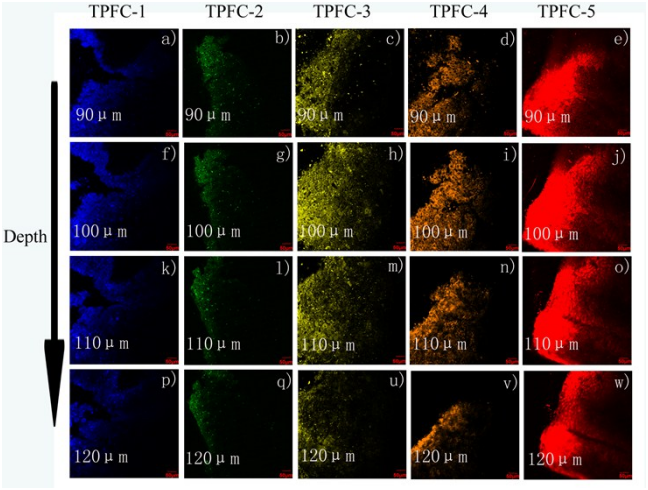

Figure S7. Two-photon full-color imaging of the living liver slices of mice incubated with the **TPFC-1~5** dyes. a, f, k, p) Full-color imaging of the living liver slices of mice incubated with the **TPFC-1** dye; b, g, l, q) Full-color imaging of the living liver slices of mice incubated with the **TPFC-2** dye; c, h, m, u) Full-color imaging of the living liver slices of mice incubated with the **TPFC-3** dye; d, i, n, v) Full-color imaging of the living liver slices of mice incubated with the **TPFC-4** dye; e, j, o, w) Full-color imaging of the living liver slices of mice incubated with the **TPFC-5** dye. Scale bar = 50 μm. The blue, green, yellow, orange, and red channels correspond to the emission windows of 420-470, 480-530, 530-580, 580-630, and 630-690 nm, respectively. Excitation at 780 nm.

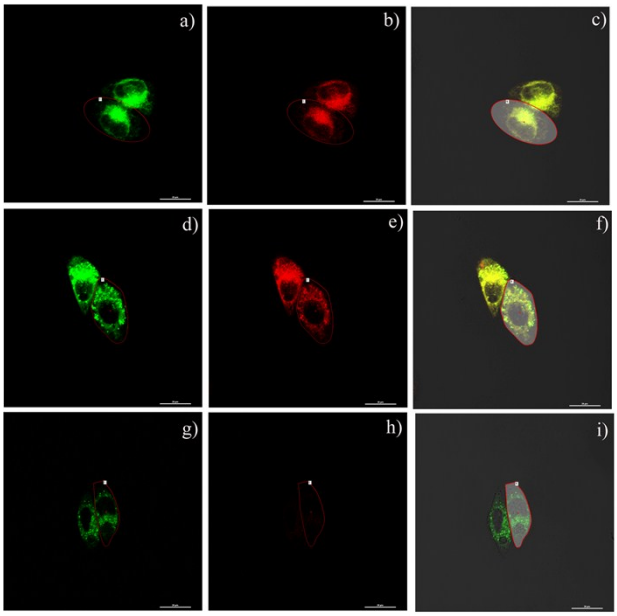

Figure S8. Confocal fluorescence images of **TPFC-2** dye (5 μM) incubated with 1 μM LysoTracker Red (a-c), 1 μM Mitotracker Red (d-f) and 1 μM Golgi-Tracker Red (g-i) in living HeLa cells at pH=7.4. Images were acquired using 488 nm excitation and emission channel of 500-540 nm for **TPFC-2** dye, 561 nm excitation and emission channel of 580−640 nm for organelle Tracker; Scale bar = 20 μm.

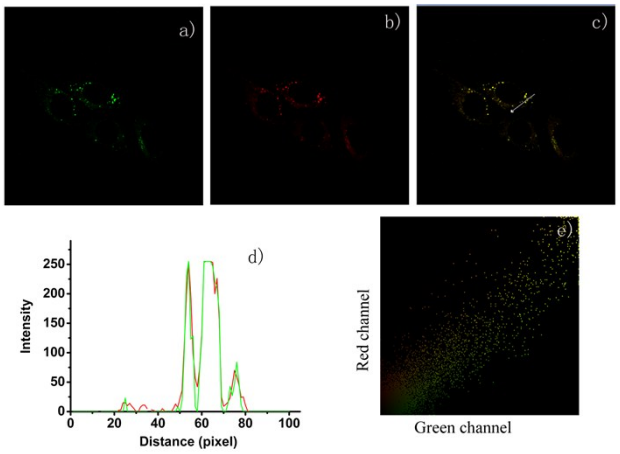

Figure S9. Fluorescent imaging of the living Hela cells co-stained with **TPFC-Lyso-2** and LysoTracker Red. a) Fluorescence image of the cells from the green channel; b) Fluorescence images of the cells from the red channel; c) Overlay of a and b; (d)

Intensity profile of the region of interest (indicated by the white color line in c). Green line for a and red line for b. (e) Correlation plot of **TPFC-Lyso-2** and LysoTracker Red intensities.

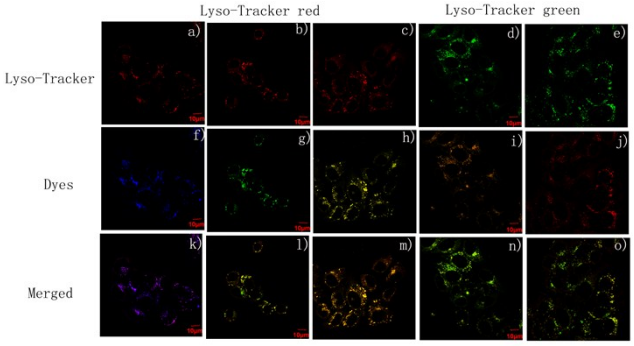

Figure S10. Fluorescence imaging of the living Hela cells co-stained with **TPFC-Lyso** and LysoTracker. a, f, k)**TPFC-Lyso-1** co-stained with LysoTracker Red; b, g, l)**TPFC-Lyso-2** co-stained with LysoTracker Red; c, h, m)**TPFC-Lyso-3** co-stained with LysoTracker Red; d, i, n)**TPFC-Lyso-4** co-stained with LysoTracker Green; e, j, o)**TPFC-Lyso-5** co-stained with LysoTracker Green; Scale bar = 10 μm. The blue, green, yellow, orange, and red channels correspond to the emission windows of 420-470, 480-530, 530-580, 580-630, and 630-690 nm, respectively.

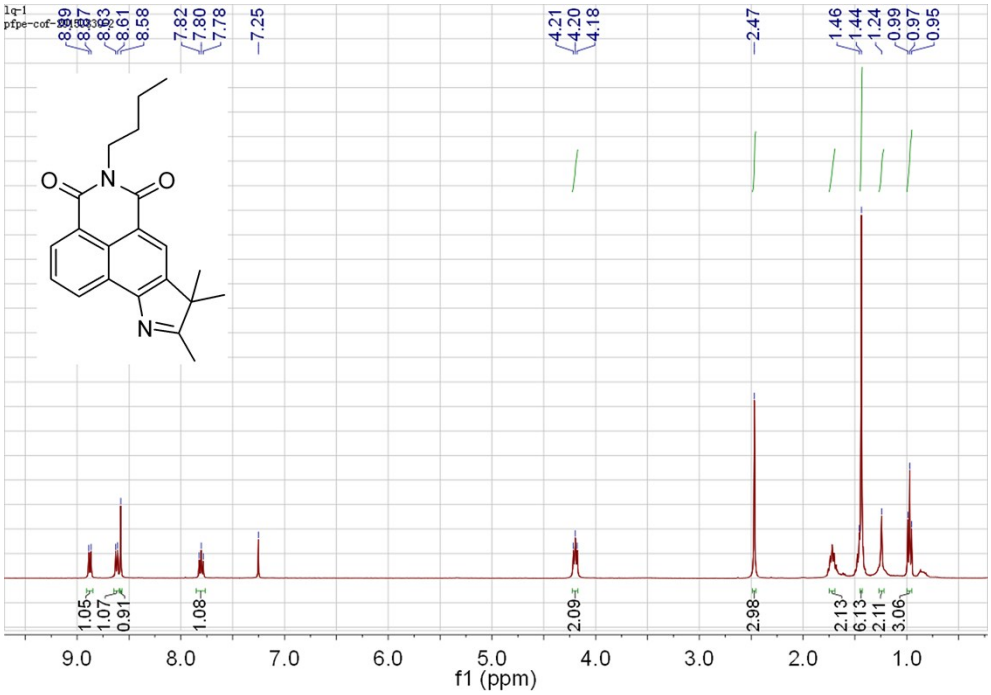

Figure S11. ^{1}H NMR spectrum of **TPFC-1** ($CDCl_3$).

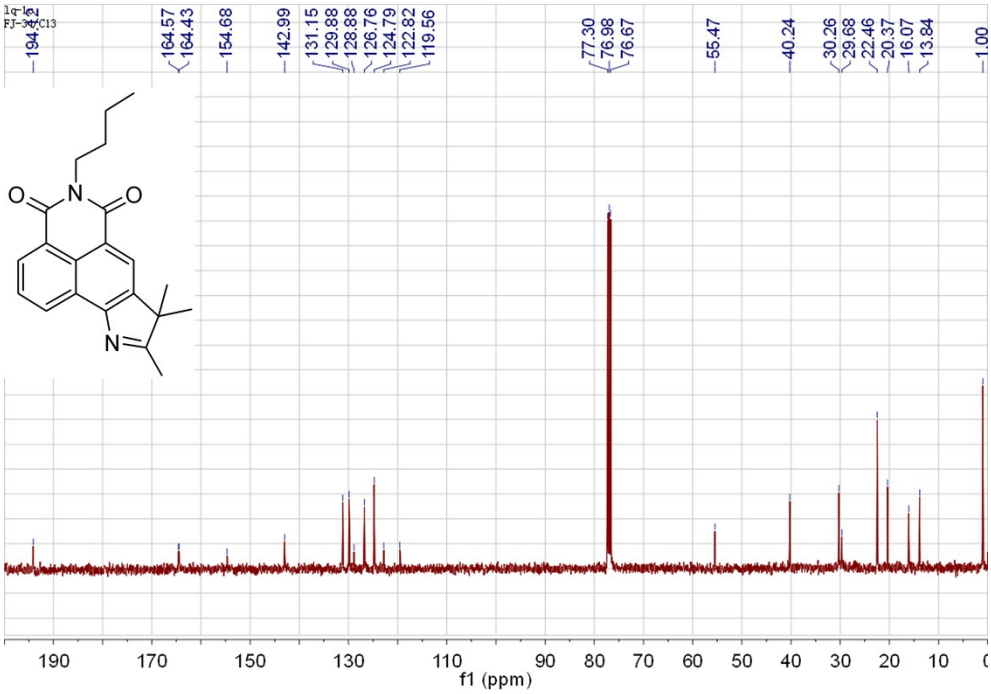

Figure S12. ^{13}C NMR spectrum of **TPFC-1** ($CDCl_3$).

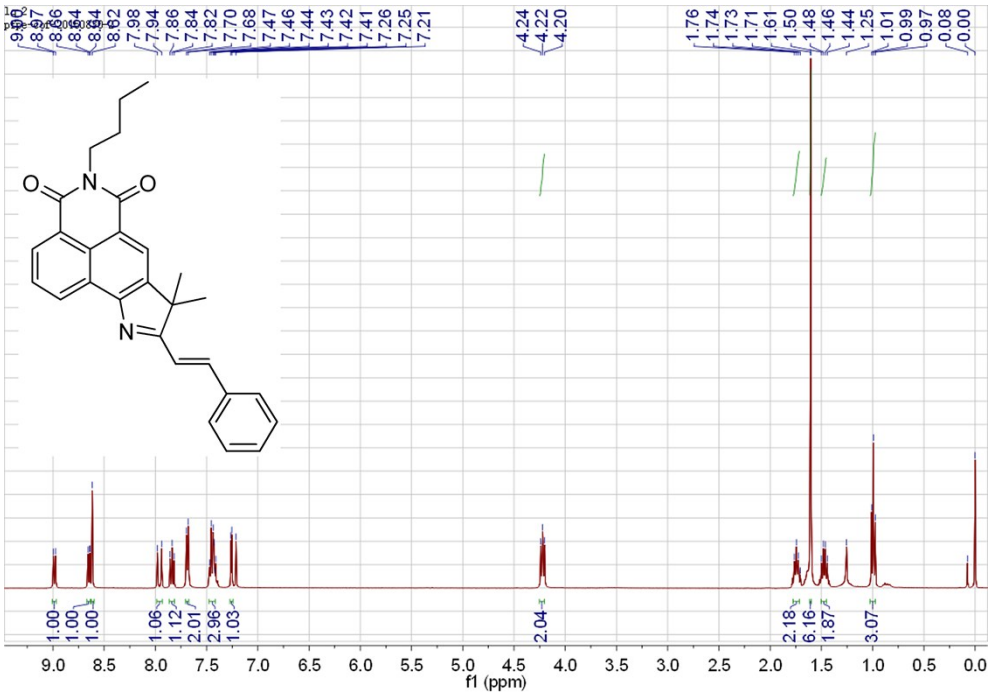

Figure S13. ^{1}H NMR spectrum of **TPFC-2** ($CDCl_3$).

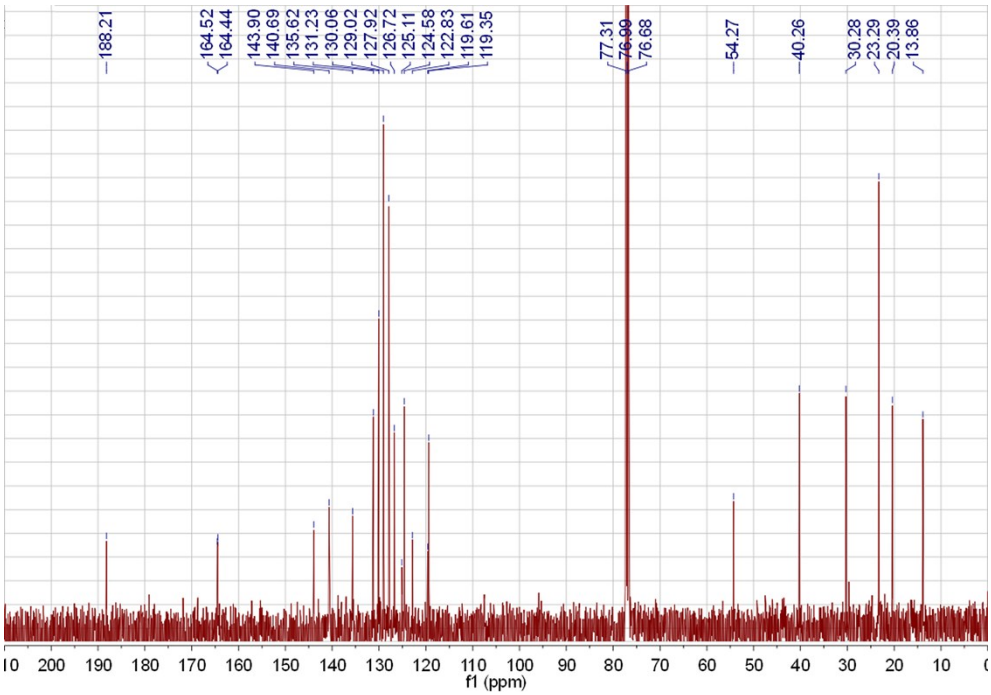

Figure S14. ^{13}C NMR spectrum of **TPFC-2** ($CDCl_3$).

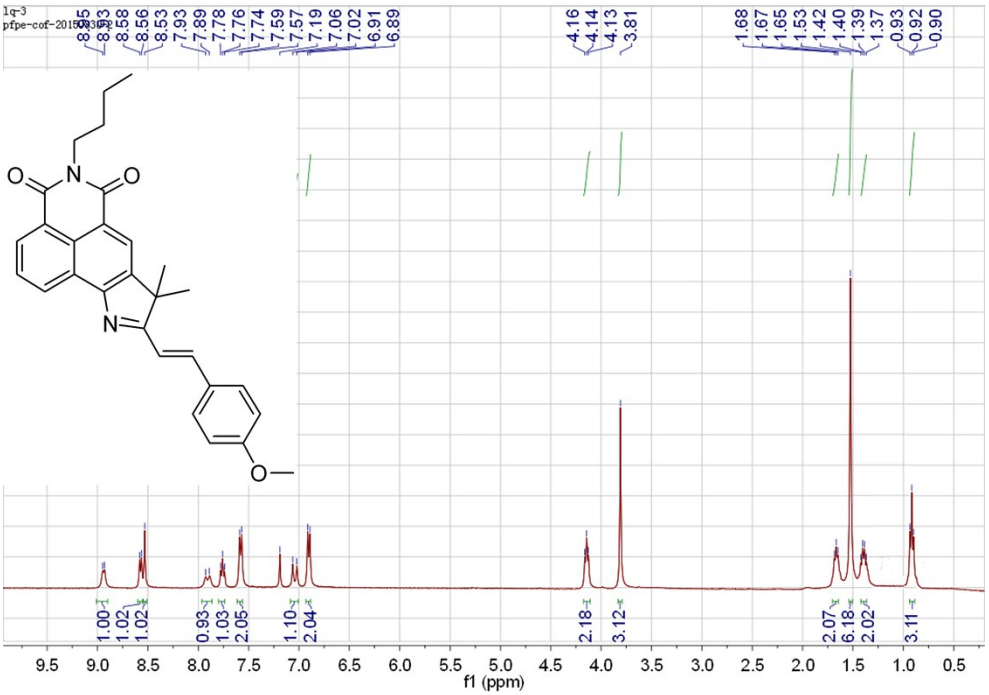

Figure S15. ^{1}H NMR spectrum of **TPFC-3** ($CDCl_3$).

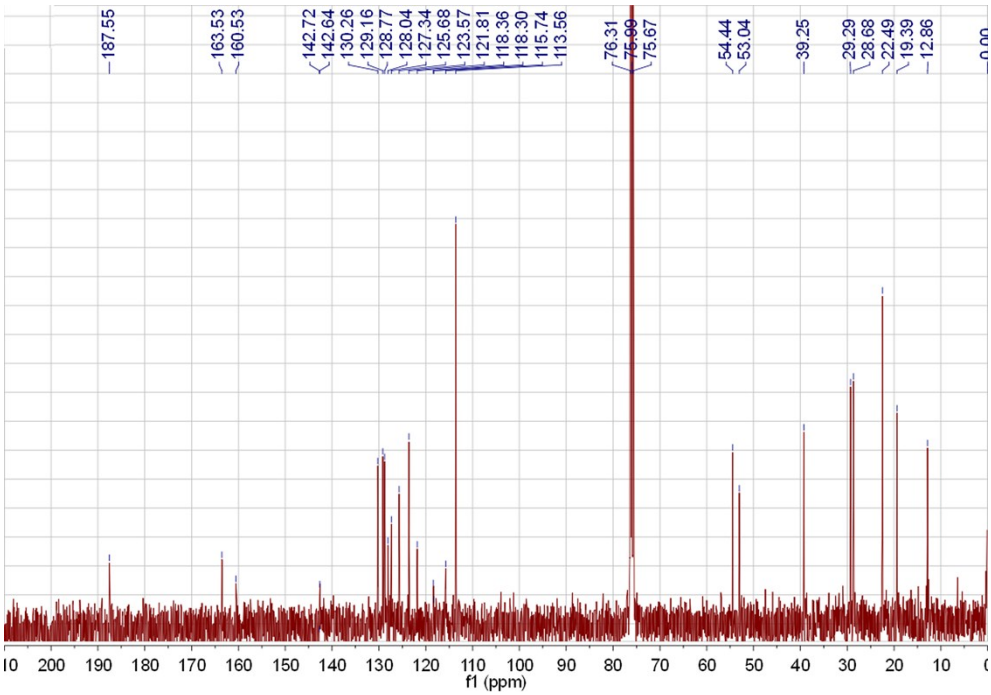

Figure S16. ^{13}C NMR spectrum of **TPFC-3** ($CDCl_3$).

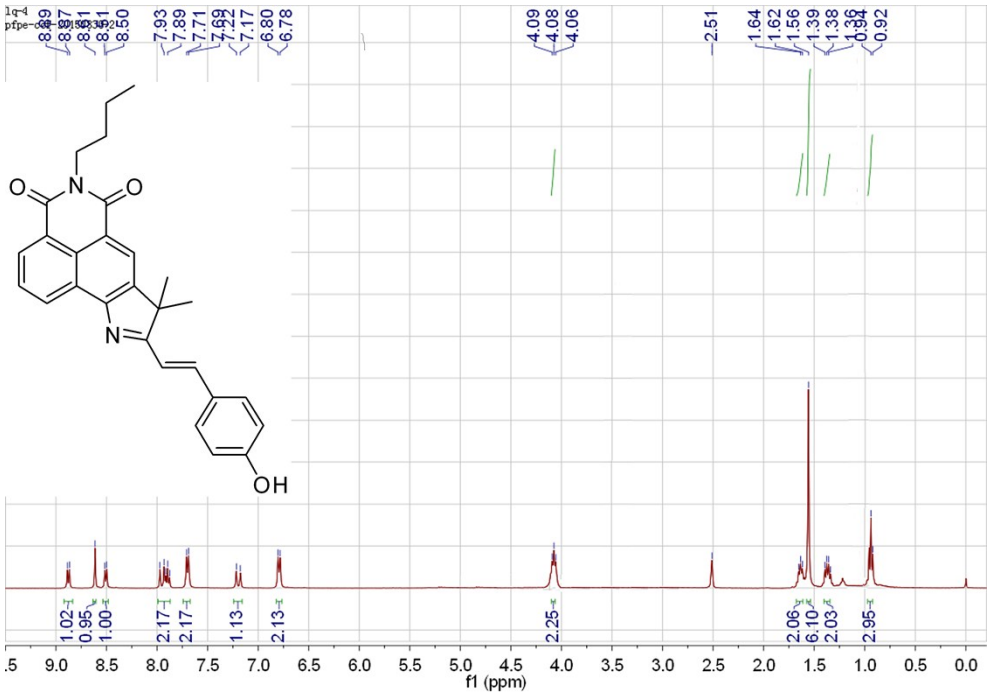

Figure S17. [1]H NMR spectrum of **TPFC-4** (d6-DMSO).

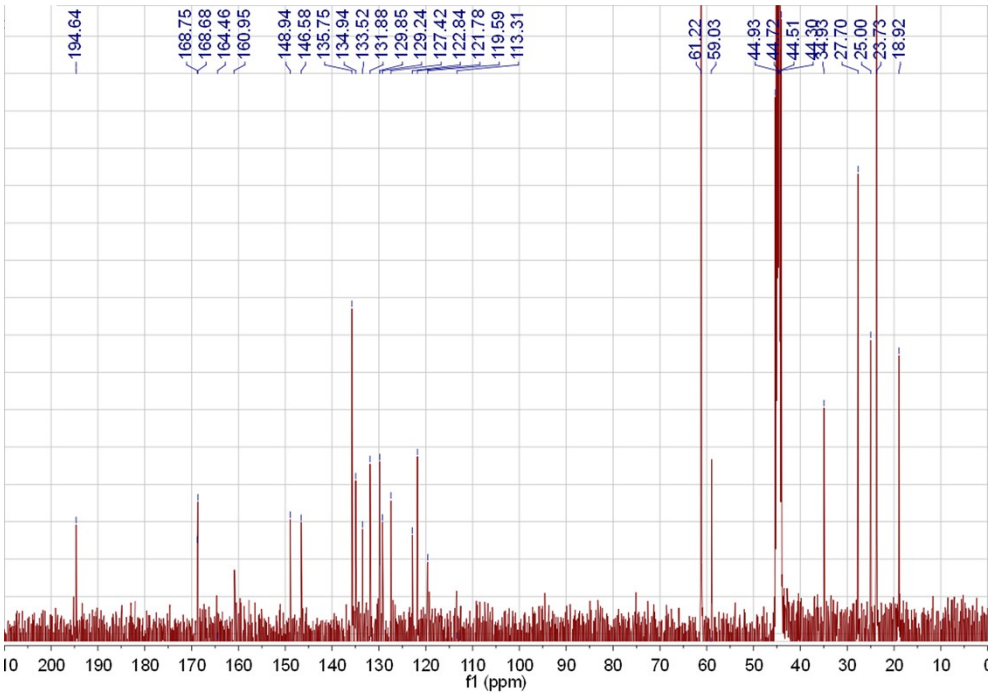

Figure S18. [13]C NMR spectrum of **TPFC-4** (d6-DMSO).

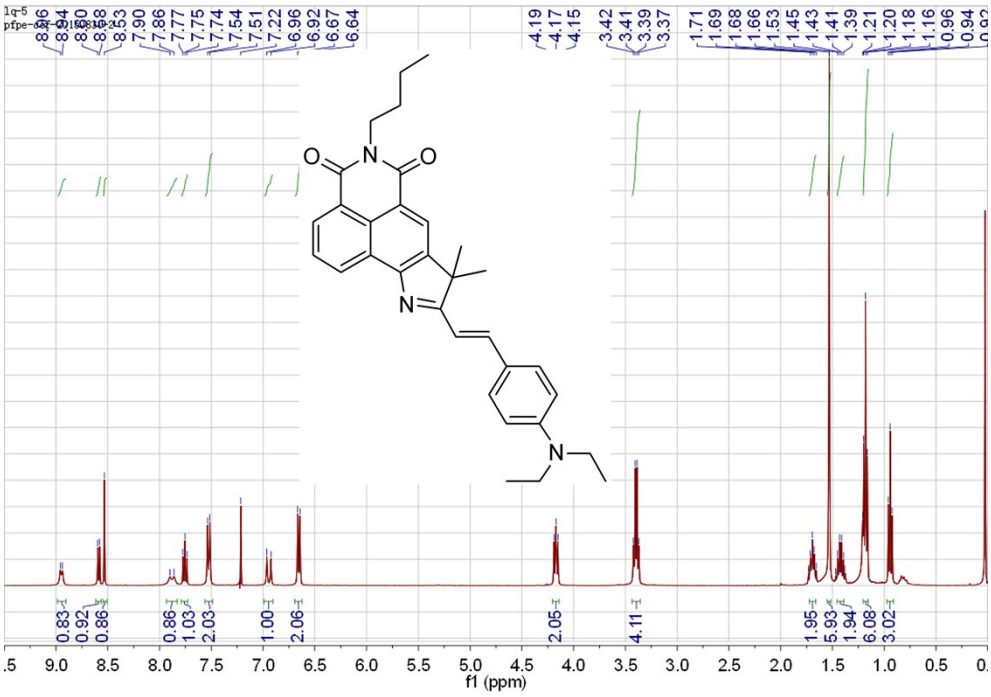

Figure S19. ^{1}H NMR spectrum of **TPFC-5** ($CDCl_3$).

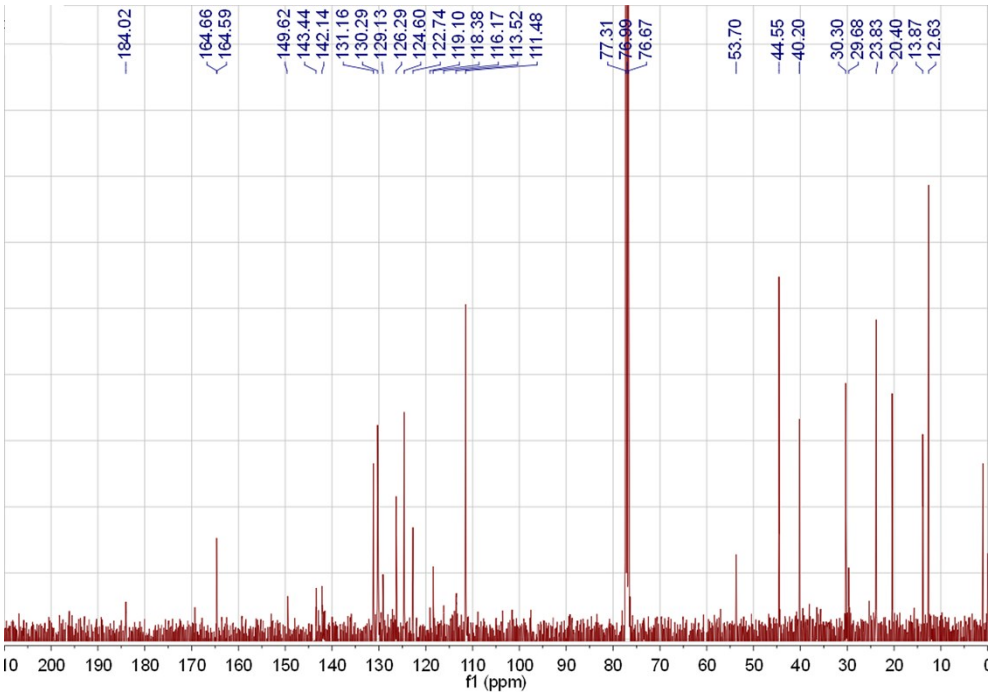

Figure S20. ^{13}C NMR spectrum of **TPFC-5** ($CDCl_3$).

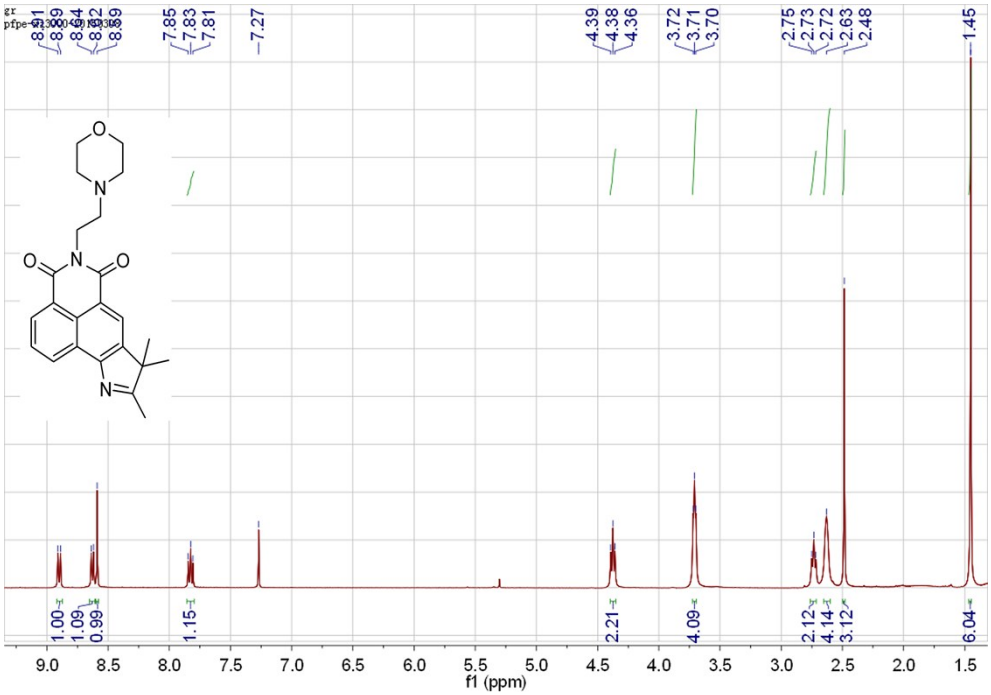

Figure S21. ^{1}H NMR spectrum of **TPFC-Lyso-1** ($CDCl_3$).

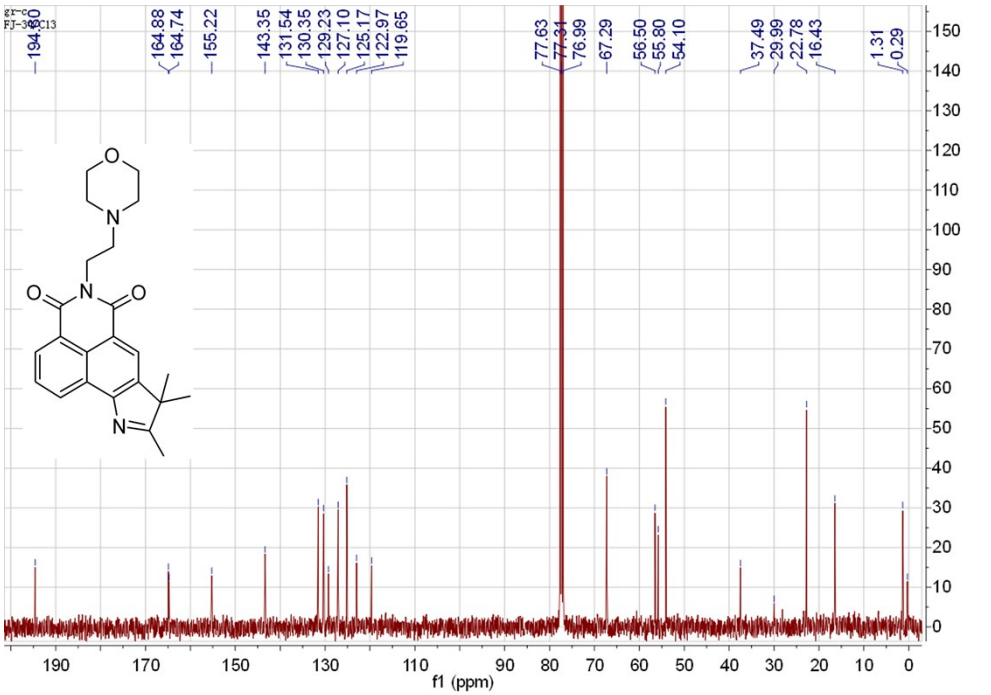

Figure S22. ^{13}C NMR spectrum of **TPFC-Lyso-1** ($CDCl_3$).

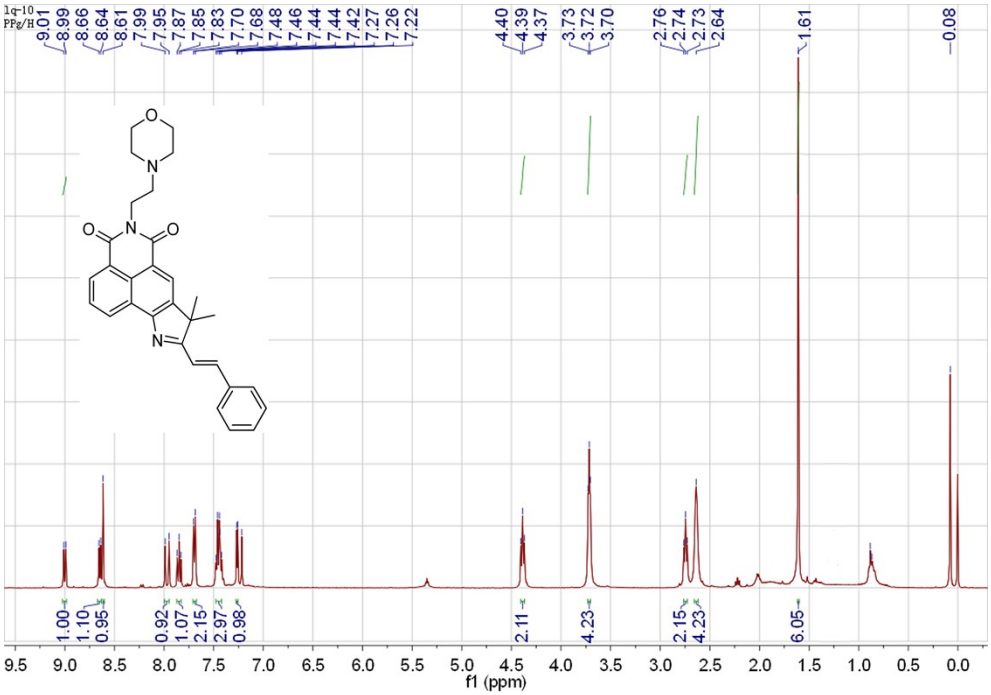

Figure S23. ^{1}H NMR spectrum of **TPFC-Lyso-2** ($CDCl_3$).

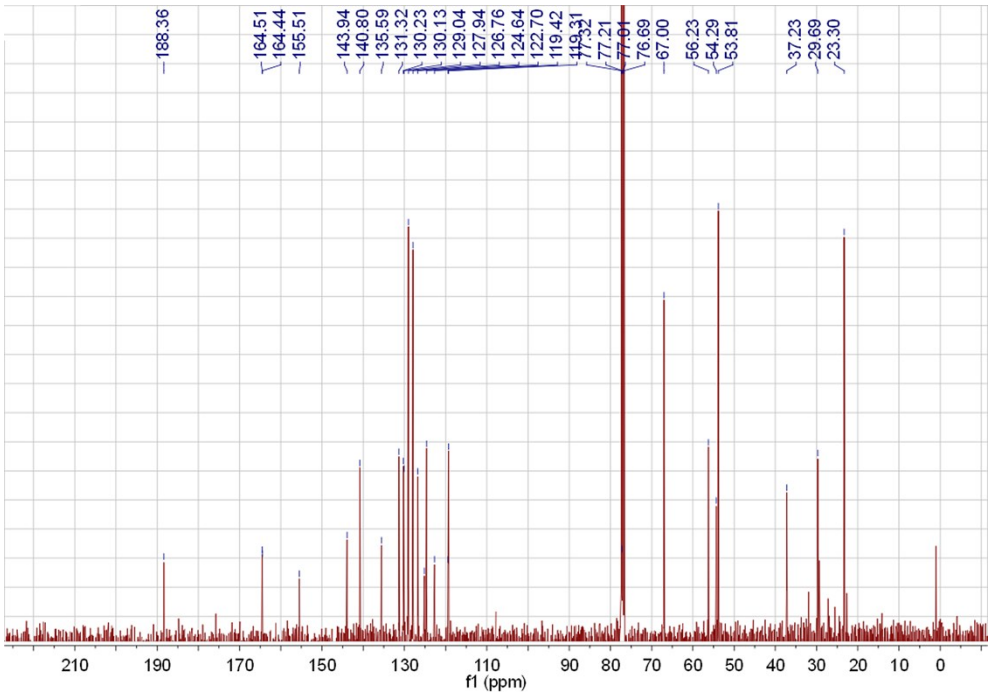

Figure S24. ^{13}C NMR spectrum of **TPFC-Lyso-2** ($CDCl_3$).

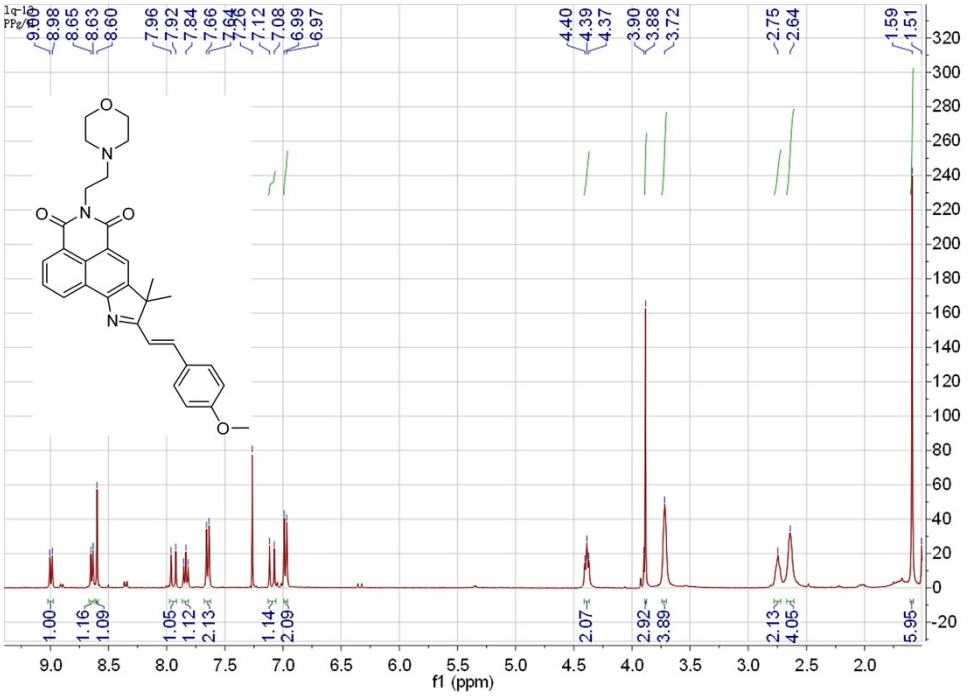

Figure S25. ^{1}H NMR spectrum of **TPFC-Lyso-3** ($CDCl_3$).

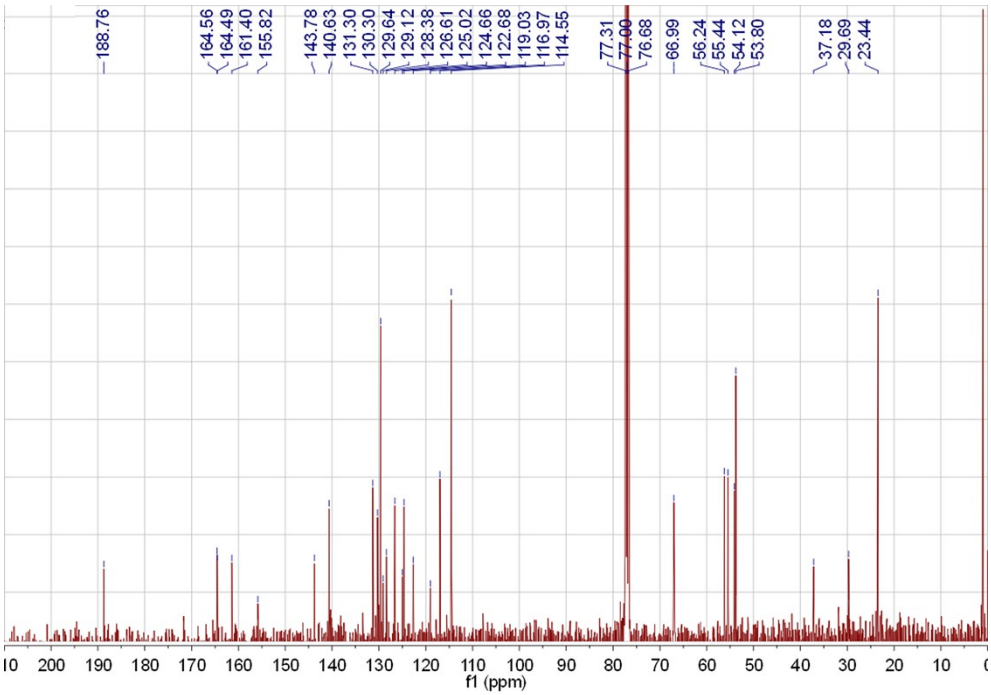

Figure S26. ^{13}C NMR spectrum of **TPFC-Lyso-3** ($CDCl_3$).

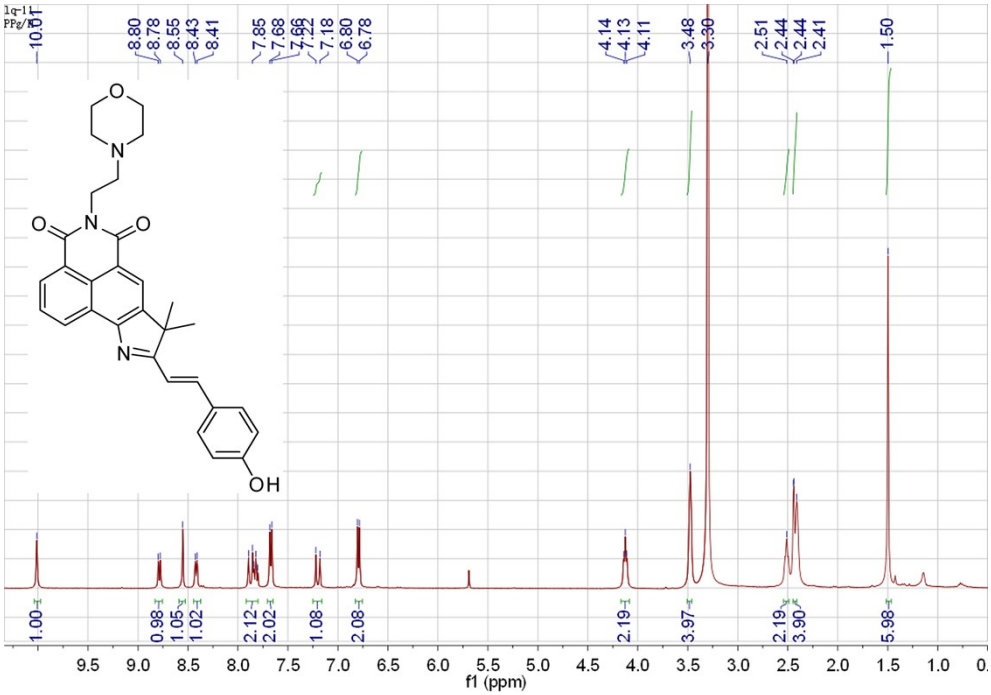

Figure S27. [1]H NMR spectrum of **TPFC-Lyso-4** (d6-DMSO).

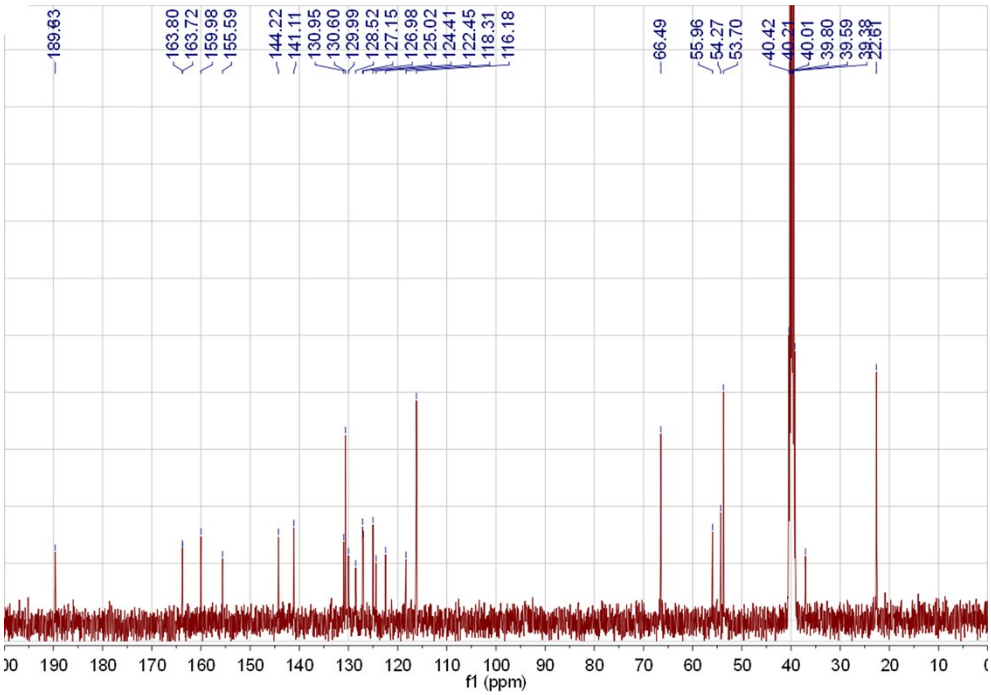

Figure S28. [13]C NMR spectrum of **TPFC-Lyso-4** (d6-DMSO).

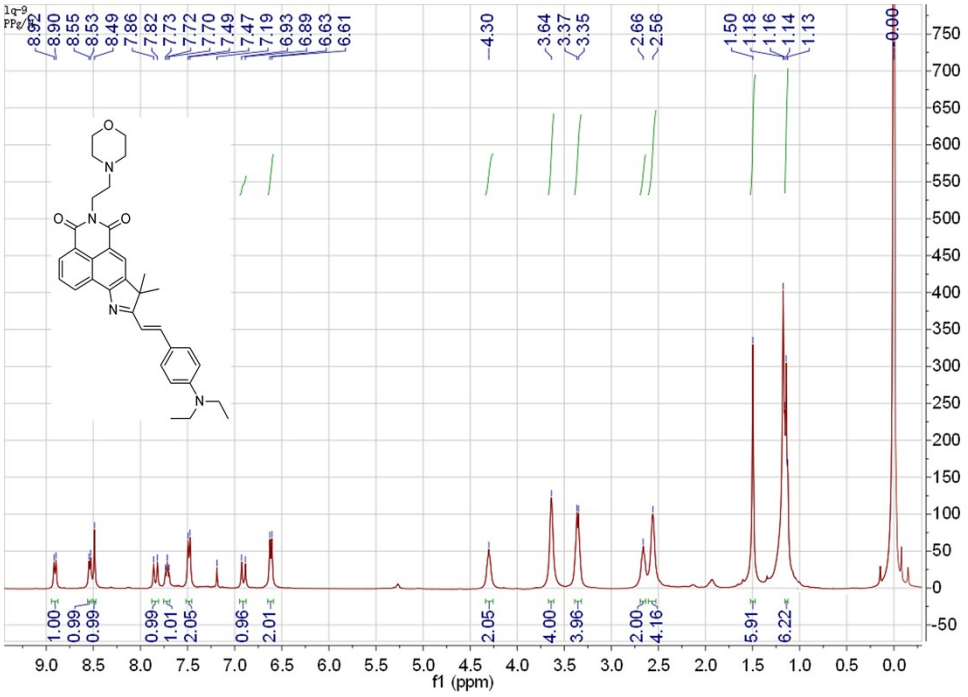

Figure S29. ^{1}H NMR spectrum of **TPFC-Lyso-5** ($CDCl_3$).

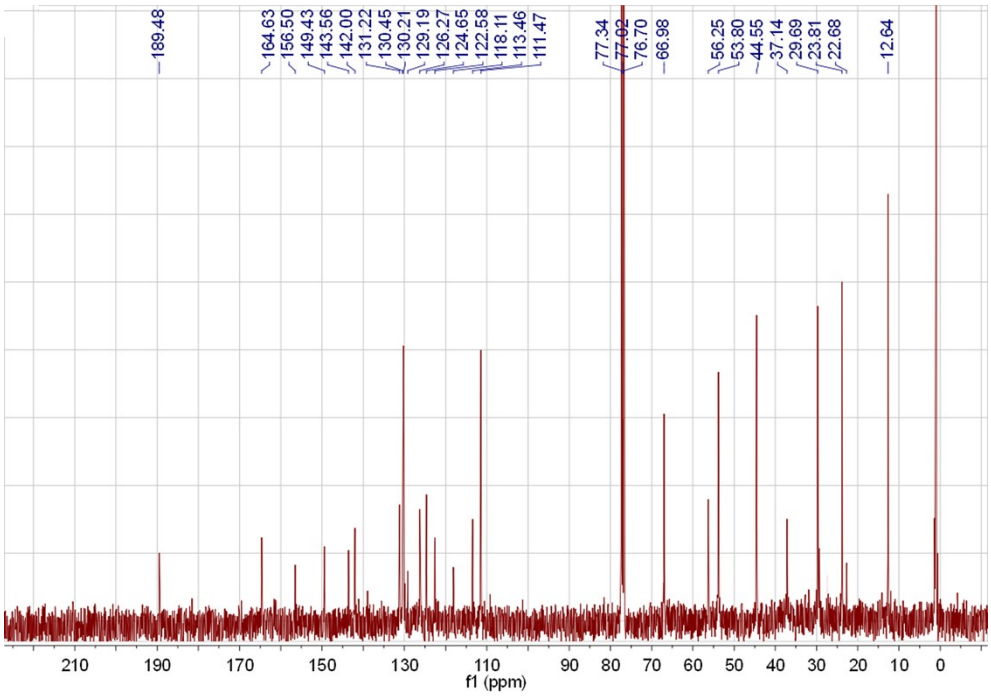

Figure S30. ^{13}C NMR spectrum of **TPFC-Lyso-5** ($CDCl_3$).

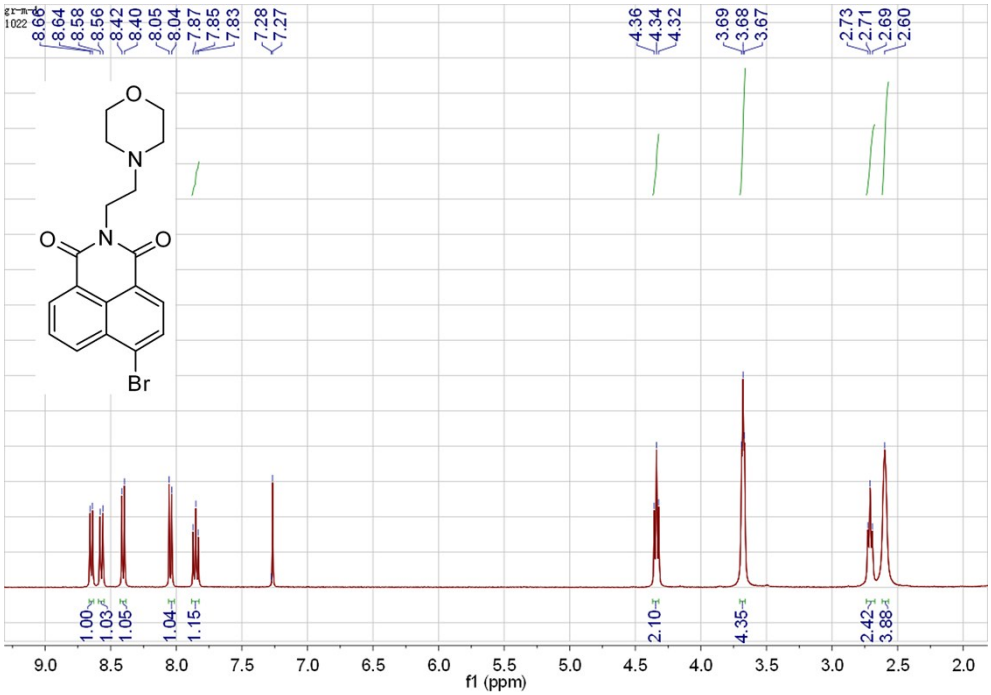

Figure S31. ^{1}H NMR spectrum of Compound **5** ($CDCl_3$).

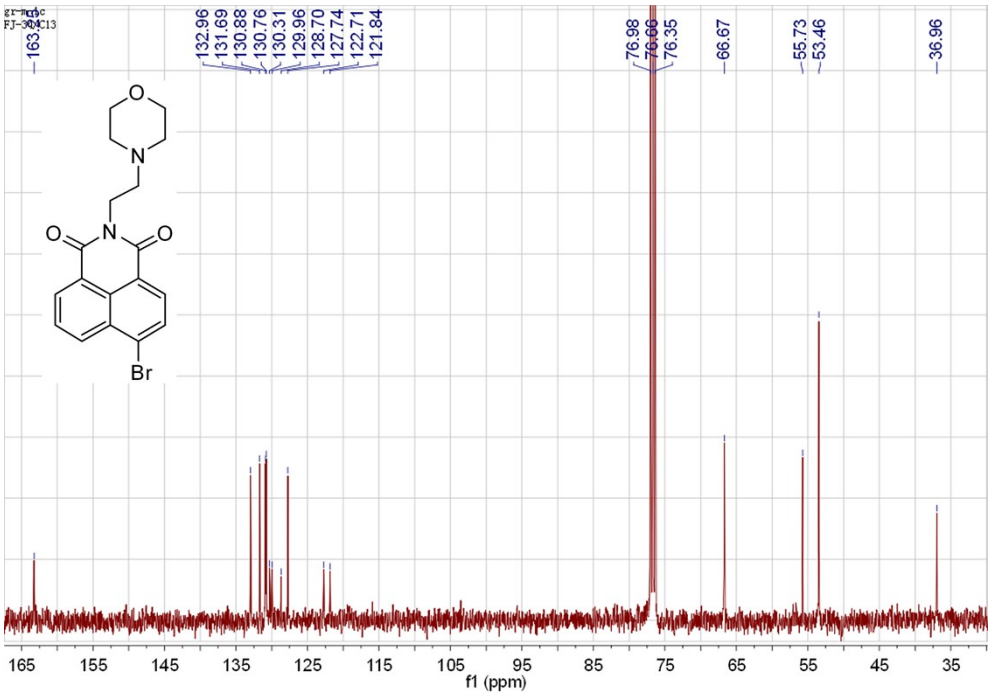

Figure S32. ^{13}C NMR spectrum of Compound **5** ($CDCl_3$).

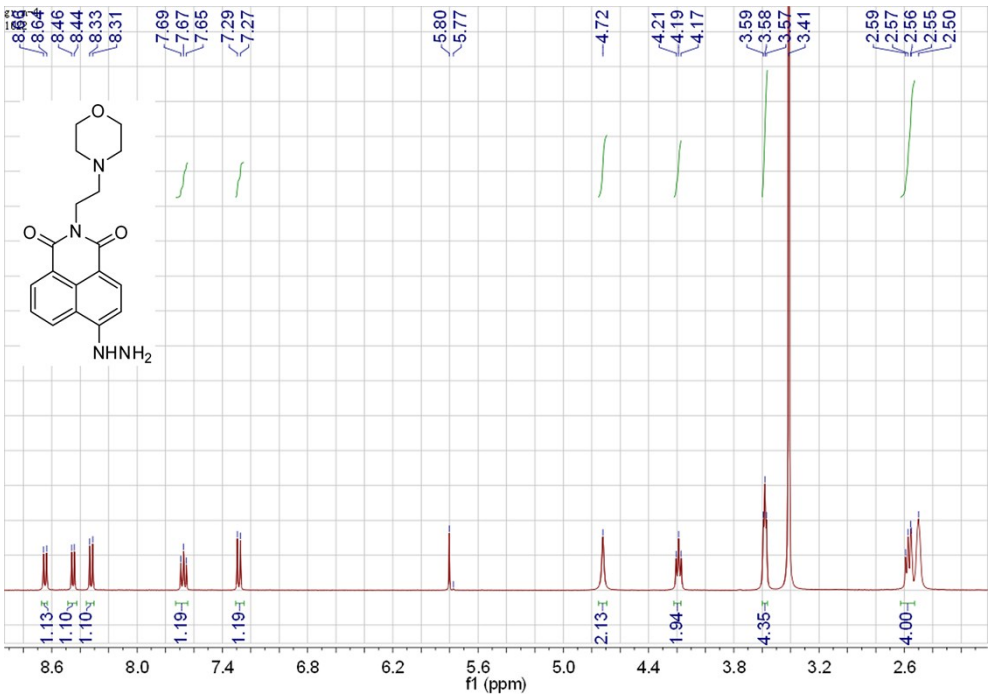

Figure S33. [1]H NMR spectrum of Compound **6** (d6-DMSO).

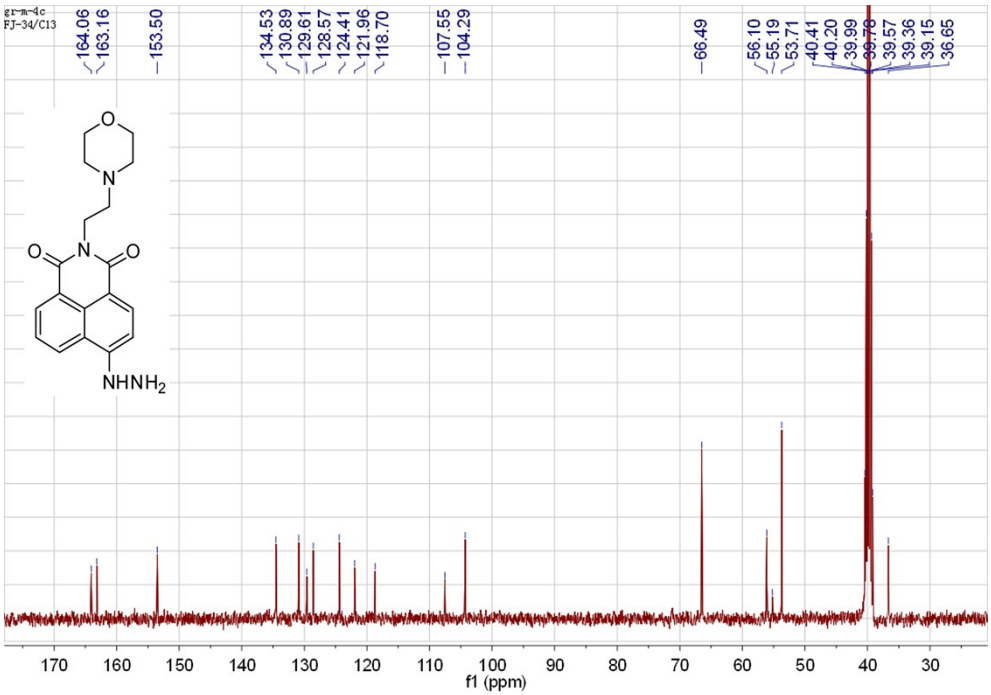

Figure S34. [13]C NMR spectrum of Compound **6** (d6-DMSO).