Supporting Information

Two-Photon Probes for the Endoplasmic Reticulum: Its Detection in a Live Tissue by Two-Photon Microscopy

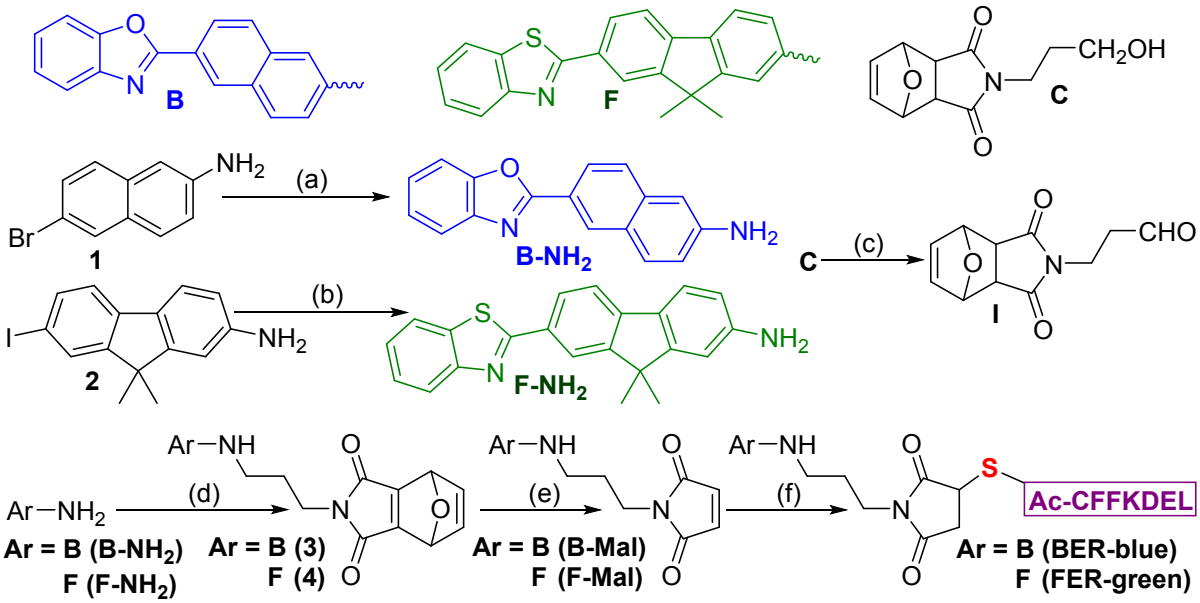
Ji-Woo Choi[a,‡], Seung-Hye Choi[a,‡], Seung Taek Hong[a], Mun Seok Kim[b], Seong Shick Ryu[a], Yeo Uk Yoon[b], Kyu Cheol Paik[b], Man So Han[b], Taebo Sim[a,c], and Bong Rae Cho[a,b,d,*]

[a] KU-KIST Graduate School of Converging Science and Technology, Korea University, 145 Anam-ro, Seongbuk-gu, Seoul 02841, Republic of Korea
[b] Department of Chemistry, Daejin University, 1007 Hoguk-ro, Pocheon-si, Gyeonggi-do 11159, Republic of Korea
[c] Severance Biomedical Science Institute, Yonsei University College of Medicine, 50 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea
[d] Department of Chemistry, Korea University, 145 Anam-ro, Sungbuk-gu, Seoul 02841, Republic of Korea
* Corresponding author E-mail: chobr@korea.ac.kr

TABLE OF CONTENTS **Page**

Synthesis

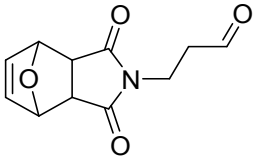
Compounds **1** and **2** were available from earlier works,[1,2] and compound **C** was prepared by



a method from the literature.[3] The synthesis of **BER-blue** and **FER-green** is shown below.

Figure S1. Synthesis of BER-blue and FER-green. (a) i) Benzoxazole, $Pd(OAc)_2$, CuI, PPh_3, Cs_2CO_3, dimethylformamide (DMF), 145°C. (b) Benzothiazole, $Pd(OAc)_2$, CuI, PPh_3, Cs_2CO_3, DMF, 145°C. (c) (2,2,6,6-Tetramethylpiperidin-1-yl)oxyl [TEMPO], NaOCl, CH_2Cl_2. (d) **I**, $NaBH_4$, DMF, room temperature (rt). (e) Toluene, 140°C. (f) Ac-CFFKDEL, $P(CH_2CH_2CO_2H)_3$, Et_3N, dimethyl sulfoxide (DMSO), rt.

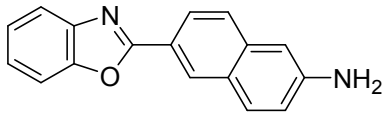
Compound I



A solution of NaOCl (aq) (4%, 20 mL) was added dropwise to a mixture of compound **C** (1.12 g, 5 mmol), $NaHCO_3$ (0.84 g, 10 mmol), KBr (0.71 g, 6 mmol), and TEMPO (0.02 g, 0.13 mmol) in CH_2Cl_2 (10 mL), and the resultant solution was stirred for 2 h at 0°C. The product was extracted with CH_2Cl_2 (3 × 20 mL), washed with 10% $Na_2S_2O_3$ (aq) (3 × 10 mL) and brine (10 mL) and dried over $MgSO_4$, and the solvent was evaporated. The crude product was purified by silica gel chromatography with a 0–10% gradient of MeOH/CH_2Cl_2 as the eluent. Yield: 1.01 g (96%); ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.57 (1 H, t, J = 1.5 Hz), 6.53 (2 H, s),

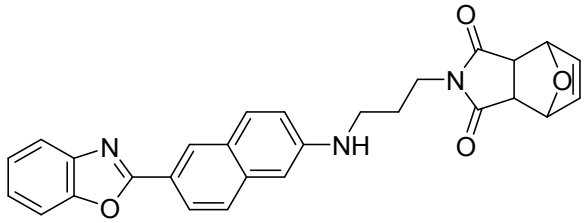
5.10 (2 H, s), 3.61 (2 H, t, J = 6.9 Hz), 2.90 (2 H, s), 2.61 (1 H, td, J = 7.0, 1.5 Hz) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ 201.8, 177.0, 137.2(2C), 81.0(2C), 47.9(2C), 41.6, 32.6 ppm.

Compound B-NH_2



A flame-dried round-bottom flask with a magnetic stirring bar was loaded with CuI (0.09 g, 0.47 mmol), $Pd(OAc)_2$ (0.03 g, 0.11 mmol), PPh_3 (0.31 g, 0.12 mmol), Cs_2CO_3 (1.7 g, 4.7 mmol), benzoxazole (0.56 g, 4.7 mmol), and **1** (0.52 g, 2.3 mmol). Anhydrous DMF (1.0 mL) was then added to the mixture using a syringe in an atmosphere of Ar. The reaction mixture was heated to 145°C for 20 min, cooled to rt, and filtered through a small pad of Celite. The solid residue was washed with CH_2Cl_2 (10 mL), and the combined organic layers were evaporated. The product was purified by silica gel column chromatography with 50% EtOAc in *n*-hexane. Yield: 0.45 g (74%); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.62 (1 H, d, J = 1.7 Hz), 8.19 (1 H, dd, J = 8.5, 1.7 Hz), 7.80−7.77 (2 H, m), 7.69 (1 H, d, J = 8.5 Hz), 7.61−7.58 (1 H, m), 7.38–7.32 (2 H, m), 6.98−7.00 (2 H, m), 4.06 (2 H, br, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 163.7, 150.7, 146.1, 142.3, 136.6, 130.5, 128.2, 127.1, 126.4, 124.7, 124.5, 124.4, 120.7, 119.6, 118.9, 110.4, 108.1 ppm.

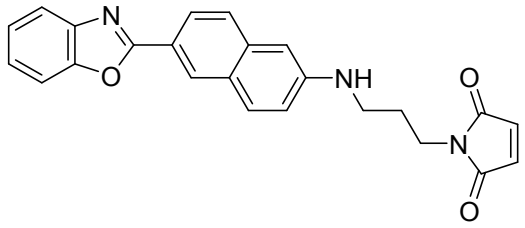
Compound 3



A solution containing **B-NH_2** (0.72 g, 2.8 mmol) and **I** (0.61 g, 2.8 mmol) in anhydrous DMF was purged with argon several times with stirring. $NaBH_4$ (0.58 g, 4.2 mmol) was added to this solution, and the reaction mixture was stirred vigorously for 2 h under argon at rt. The organic layer was separated, washed with saturated brine, dried over $MgSO_4$, and filtered. The solvent was evaporated, and the crude product was purified by silica gel column chromatography with 30–50% ethyl acetate in *n*-hexane. Yield: 0.98 g (76%); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.58 (1 H, d, J = 1.7 Hz), 8.17 (1 H, dd, J = 8.5, 1.7 Hz), 7.78–7.68 (3 H, m), 7.60−7.57 (1 H, m), 7.35−7.32 (2 H, m), 6.95 (1 H, dd, J = 8.8, 2.2 Hz), 6.79 (1 H, d, J = 2.2 Hz), 6.52 (2 H, s), 5.29 (2 H, s), 4.52 (1 H, brs), 3.64 (2 H, t, J = 6.5 Hz), 3.25 (2 H, t, J = 6.3 Hz), 2.86 (2 H, s), 1.95 (2 H, quin, J = 6.3 Hz) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 176.6(2C), 163.9, 150.7,

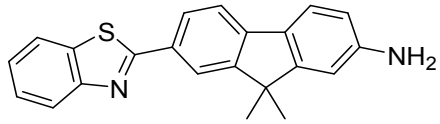
147.2, 142.4, 137.0, 136.5(2C), 130.2, 128.1, 126.5, 126.4, 124.5(2C), 124.4, 120.0, 119.6, 118.8, 110.3, 103.7, 81.0(2C), 47.4(2C), 40.0, 36.3, 26.4 ppm.

Compound B-Mal



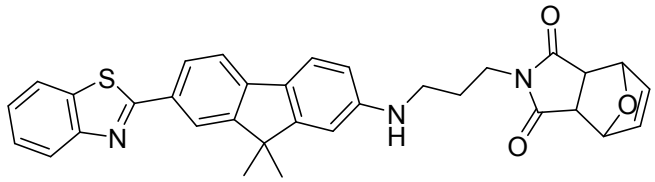
A solution of **3** (0.33 g, 0.71 mmol) in toluene (50 mL) was refluxed overnight. The solvent was evaporated, and the crude product was purified by silica gel column chromatography with 30–50% ethyl acetate in *n*-hexane. Yield: 0.25 g (88%); ^{1}H NMR (800 MHz, DMSO-d_6): δ 8.54 (1 H, d, J = 1.4 Hz), 8.05 (1 H, dd, J = 8.4, 1.4 Hz), 7.84 (1 H, d, J = 8.8 Hz), 7.79–7.77 (2 H, m), 7.73 (1 H, d, J = 8.4 Hz), 7.41–7.40 (2 H, m), 7.07 (1 H, dd, J = 8.8, 1.9 Hz), 7.04 (2 H, s), 6.76 (1 H, d, J = 1.9 Hz), 6.39 (1 H, t, J = 5.6 Hz), 3.57 (2 H, t, J = 6.9 Hz), 3.16 (2 H, td, J = 6.9, 5.6 Hz), 1.88 (2 H, quin, J = 6.9 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.1(2C), 164.0, 150.4, 147.3, 141.8, 137.0, 134.1(2C), 130.1, 128.1, 126.4(2C), 124.6, 124.4, 124.3, 119.6, 119.2, 118.7, 110.3, 103.5, 40.1, 35.2, 27.4 ppm.

Compound F-NH_2



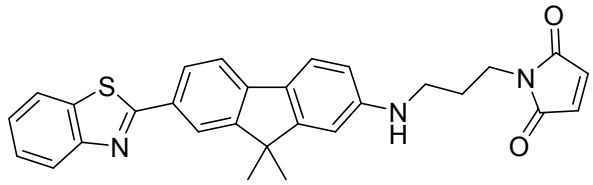
A flame-dried round-bottom flask with a magnetic stirring bar was loaded with CuI (0.05 g, 0.02 mmol), $Pd(OAc)_2$ (0.01 g, 0.006 mmol), PPh_3 (0.16 g, 0.06 mmol), Cs_2CO_3 (0.5 g, 1.4 mmol), benzothiazole (0.13 g, 1.2 mmol), and **2** (0.4 g, 1.2 mmol). Next, anhydrous DMF (1.0 mL) was added to the mixture via a syringe under Ar. The reaction mixture was heated to 145°C for 30 min, cooled to rt, and filtered through a small pad of Celite. The solid residue was washed with CH_2Cl_2 (10 mL), and the combined organic layers were evaporated. The product was purified by silica gel column chromatography with 10−25% EtOAc in *n*-hexane. Yield: 0.18 g (44%); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.26 (1 H, d, J = 1.7 Hz), 8.18 (1 H, d, J = 8.0 Hz), 7.99 (1 H, dd, J = 8.0, 1.7 Hz), 7.88 (1 H, dd, J = 8.0, 1.1 Hz), 7.62 (1 H, d, J = 8.0 Hz), 7.52 (1 H, d, J = 8.2 Hz), 7.52 (1 H, td, J = 7.5, 1.1 Hz), 7.37 (1 H, td, J = 7.5, 1.1 Hz), 6.73 (1 H, d, J = 2.1 Hz), 6.65 (1 H, dd, J = 8.2, 2.1 Hz), 3.97 (2 H, br. s.), 1.53 (6 H, s) ppm ; ^{13}C NMR (75 MHz, $CDCl_3$): δ 168.7, 156.2, 153.9, 153.2, 147.1, 142.7, 134.6, 130.3, 128.5, 127.1, 126.0, 124.6, 122.5, 121.4, 121.3, 120.9, 118.7, 113.9, 108.9, 46.5, 26.9(2C) ppm.

Compound 4



Compound **4** was prepared from **F-NH$_2$** (0.38 g, 1.11 mmol) and **I** (0.25 g, 1.11 mmol) as described for **3**. Yield: 0.31 g (51%); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.14 (1 H, d, *J* = 1.7 Hz), 8.07 (1 H, d, *J* = 8.0 Hz), 7.94 (1 H, dd, *J* = 8.0, 1.7 Hz), 7.86 (1 H, dd, *J* = 8.0 Hz), 7.59 (1 H, d, *J* = 8.0 Hz), 7.53 (1 H, d, *J* = 8.2 Hz), 7.47 (1 H, td, *J* = 7.6, 1.1 Hz), 7.34 (1 H, td, *J* = 7.6, 1.1 Hz), 6.68 (1 H, d, *J* = 2.1 Hz), 6.59 (1 H, dd, *J* = 8.2, 2.1 Hz), 6.45 (2 H, s), 5.25 (2 H, s), 3.62 (2 H, t, *J* = 6.6 Hz), 3.18 (2 H, t, *J* = 6.5 Hz), 2.80 (2 H, s), 1.89 (2 H, quin, *J* = 6.4 Hz), 1.52 (6 H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 176.4(2C), 168.8, 156.3, 154.0, 153.2, 148.3, 143.0, 136.3(2C), 134.7, 130.1, 127.7, 127.1, 126.0, 124.6, 122.6, 121.6, 121.4, 121.0, 118.6, 111.8, 106.7, 80.8(2C), 47.3(2C), 46.6, 40.4, 36.2, 27.1(2C), 26.5 ppm.

Compound F-Mal



F-Mal was synthesized from compound **4** (0.31 g, 0.57 mmol) as described for **B-Mal**. Yield: 0.22 g (81%); ^{1}H NMR (800 MHz, $CDCl_3$): δ 8.31 (1 H, s), 8.13 (1 H, d, *J* = 1.4 Hz), 8.10 (1 H, d, *J* = 7.8 Hz), 8.04 (1 H, d, *J* = 7.8 Hz), 7.94 (1 H, dd, *J* = 7.8, 1.4 Hz), 7.70 (1 H, d, *J* = 7.8 Hz), 7.59 (1 H, d, *J* = 8.3 Hz), 7.52 (1 H, t, *J* = 7.8 Hz), 7.43 (1 H, t, *J* = 7.8 Hz), 7.02 (2 H, s), 6.72 (1 H, d, *J* = 1. Hz), 6.58 (1 H, dd, *J* = 8.3, 1.2 Hz), 3.54 (2 H, t, *J* = 7.0 Hz), 3.10 (2 H, t, *J* = 7.0 Hz), 1.82 (2 H, quin, *J* = 7.0 Hz), 1.43 (6 H, s) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.6(2C), 168.7, 156.2, 153.9, 153.1, 148.3, 142.8, 134.5, 133.7(2C), 130.0, 127.6, 127.1, 125.9, 124.5, 122.5, 121.5, 121.3, 120.9, 118.5, 111.7, 106.5, 46.5, 40.5, 35.0, 27.5, 27.0(2C) ppm.

BER-blue

A mixture of **B-Mal** (0.05 g, 0.13 mmol), Et_3N (0.02 g, 0.17 mmol), $(HO_2CCH_2CH_2)P$ (0.0003 g, 0.001 mmol), and an endoplasmic-reticulum–targeting peptide (Ac-CFFKDEL, InterPharm, Gyeonggi-do, Korea) (0.12 g, 0.13 mmol) in DMSO was stirred at rt for 2 h. The crude product was purified by semipreparative reverse-phase high-performance liquid chromatography (RP-HPLC, SunFire Preparative Column C18 OBD, 5 μm, 19 × 50 mm) on a Waters HPLC system (Waters Corporation, Milford, MA) using step gradient elution with 30–90% MeOH/H_2O at flow rate 25 mL/min during 10 min. Yield: 0.09 g (51%); ^{1}H NMR (800 MHz, DMSO-d_6): δ 8.54 (1 H, s), 8.26−8.18 (4 H, m), 8.05 (1 H, d, J = 8.8 Hz), 8.00 (2 H, br. s.), 7.86−7.83 (2 H, m), 7.78−7.77 (2 H, m), 7.73 (1 H, d, J = 8.3 Hz), 7.41−7.40 (2 H, m), 7.25 (4 H, s.), 7.20−7.16 (6 H, m), 7.08 (1 H, d, J = 8.8 Hz), 6.77 (1 H, s), 6.39 (1 H, s), 4.57−4.55 (2 H, m), 4.49−4.45 (2 H, m), 4.27 (2 H, m), 4.16−4.15 (2 H, m), 4.03−4.02 (1 H, m), 3.96−3.95 (1 H, m), 3.60−3.54 (4 H, m), 7.86−7.83 (2 H, m), 3.40 (1 H, m), 3.19−3.13 (5 H, m), 3.07−3.04 (3 H, m), 2.99−2.97 (2 H, m), 2.94−2.87 (2 H, m), 2.84 (2 H, br. s.), 2.75−2.67 (6 H, m), 2.55−2.47 (4 H, m), 2.27−2.21 (2 H, m), 1.95 (1 H, m), 1.86−1.82 (4 H, m), 1.76 (1 H, m), 1.69 (1 H, m), 1.64−1.63 (1 H, m), 1.54−1.50 (5 H, m), 1.36−1.33 (2 H, m), 0.88 (3 H, d, J = 6.4 Hz), 0.83 (3 H, d, J = 6.4 Hz) ppm; high-resolution mass spectrometry (HRMS) (electrospray ionization): *m/z* calcd. for $[C_{68}H_{81}N_{11}O_{16}S+H^+]$: 1339.5583, found: 1340.5662.

The partial ^{1}H NMR spectrum of BER-blue in DMSO in the region δ 8.6–6.3 revealed all the proton peaks of B-Mal [except for the vinylic protons at δ 7.03 (h)] and new peaks due to the aromatic protons of the phenylalanine moiety at δ 7.3–7.1 (10 H) and N-H protons (*) of the CFFKDEL moiety at δ 8.28–8.13 (4 H), 8.03–7.97 (2 H), and 7.88–7.87 (1 H; Figure S2a,b). In addition, the 2D ^{1}H-^{1}H TOCSY spectrum and an overlay of TOCSY and NOESY spectra are consistent with the amide-to-side chain and amino acid residue connectivity in BER-blue (Figure S2c,d and Table S1). The chemical shifts of each proton in the fingerprint region of the TOCSY spectrum are summarized in Table S1. Furthermore, high-resolution mass spectrometry (HRMS) result on BER-blue molecular-weight ion peak at m/e = 1340.5662 (calcd. 1339.5583) (Figures S27). These results confirm that the fluorophores are linked to HS-Ac-CFFKDEL via a sulfide bond and that BER-blue represents a 1:1 mixture of two diastereomers produced after the addition of a thiol group to the maleimide moiety of B-Mal.

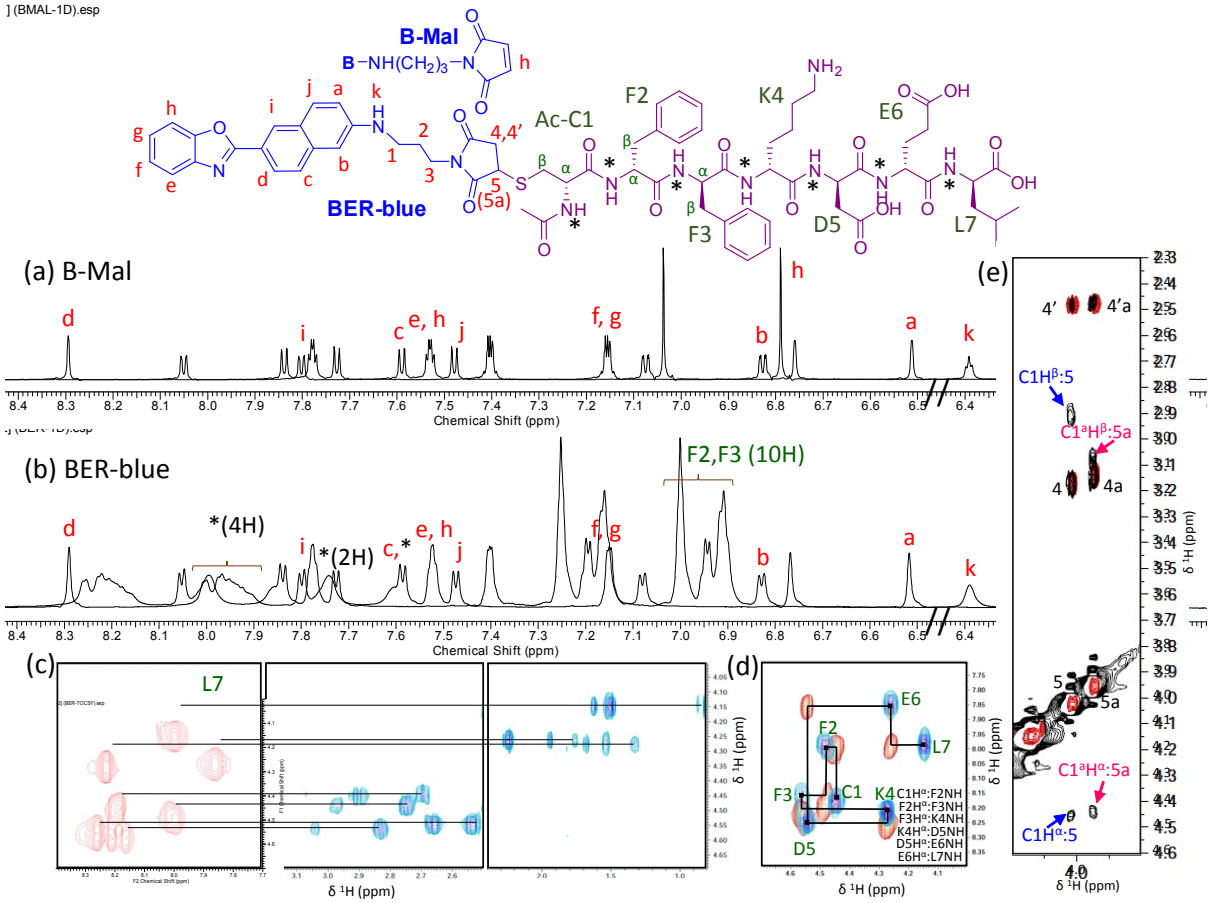


Figure S2. (a,b) Partial ^{1}H NMR spectra of B-Mal (a) and BER-blue (b) in DMSO in the region δ 8.6–6.3. The protons in the B-Mal and Ac-CFFKDEL moieties are designated as a–k, *, F2, and F3, respectively. (c) 2D ^{1}H-^{1}H TOCSY spectrum of BER-blue in the NH-H^{a} fingerprint region. Each line indicates amide-to-side chain connectivity. (d) Overlay of TOCSY (blue) and NOESY (red) spectra. The lines trace the sequential backbone assignment via intramolecular NH–H$^{\alpha}$ NOE connections. (e) An overlay of ^{1}H-^{1}H NOESY (black) and TOCSY (red) spectra showing NOE peaks between protons on succinimide and Cys moieties.

Table S1. Chemical shifts of each proton in the fingerprint region of the TOCSY spectrum of BER-blue in DMSO-d_6.

BER-blue							
Residue	NH	Hα	Hβ, Hβ'	Hγ, Hγ'	Hδ, Hδ'	Hε, Hε'	Hζ
Cys1	8.18	4.47	3.07, 2.84				
Cys1a	8.18	4.45	3.05, 2.90				
Phe2	7.99	4.47	2.98, 2.76		7.25	7.16	7.17
Phe2a	8.01	4.48	2.98, 2.76				
Phe3	8.20	4.55	2.90, 2.70		7.25	7.15	7.17
Phe3a	8.15	4.56	2.90, 2.70				
Lys4	8.22	4.27	1.68, 1.54	1.33	1.53	2.75	
Asp5	8.25	4.55	2.66,2.54		6.30		
Glu6	7.85	4.27	1.94, 1.76	2.24		2.82	
Leu7	7.99	4.15	1.51	1.62	0.87, 0.82		

FER-green

Compound **FER-green** was synthesized from **F-Mal** and the endoplasmic-reticulum peptide as described above. Yield: 0.07 g (64%); ^{1}H NMR (800 MHz, DMSO-d_6): δ 8.35−8.33 (2 H, m), 8.27−8.11 (6 H, m), 8.05 (1 H, d, J = 7.8 Hz), 7.99−7.96 (2 H, m), 7.81 (1 H, d, J = 7.8 Hz), 7.73 (1 H, d, J = 7.8 Hz), 7.61 (1 H, d, J = 8.8 Hz), 7.54 (1 H, t, J = 7.8 Hz), 7.45 (1 H, t, J = 7.8 Hz), 7.26 (4 H, m), 7.20−7.17 (6 H, m), 6.73 (1 H, d, J = 1.0 Hz), 6.58 (1 H, dd, J = 8.3, 1.2 Hz), 4.57−4.55 (2 H, m), 4.49−4.45 (2 H, m), 4.27 (2 H, m), 4.16−4.15 (1 H, m), 4.03 (1 H, m), 3.95 (1 H, m), 3.53−3.51 (3 H, m), 3.20−3.04 (7 H, m), 2.99−2.97 (1 H, m), 2.92−2.90 (1 H, m), 2.83 (1 H, m), 2.75−2.70 (3 H, m), 2.56−2.46 (6 H, m), 2.56−2.46 (2 H, m), 2.26−2.25 (3 H, m), 1.98−1.92 (2 H, m), 1.83−1.80 (8 H, m), 1.65−1.62 (3 H, m), 1.56−1.54 (3 H, m), 1.52−1.43 (9 H, m), 1.27 (1 H, m), 0.89 (3 H, d, J = 6.8 Hz), 0.83 (3 H, d, J = 6.8 Hz) ppm; HRMS (electrospray ionization): *m/z* calcd. for [$C_{73}H_{87}N_{11}O_{16}S_2$+$H^+$]: 1421.5825, found: 1422.5903.

A partial ^{1}H NMR spectrum of FER-blue in DMSO in the region δ 8.4–6.5 manifested all the proton peaks of F-Mal [except for the vinylic protons at δ 7.03 (h)] and new peaks due to the aromatic protons of the phenylalanine moiety at δ 7.28–7.13 (10 H) and N-H protons (*) of the CFFKDEL moiety at δ 8.30–8.07 (4 H), 8.03–7.93 (2 H), and 7.84–7.78 (1 H; Figure S3a,b). In addition, a 2D ^{1}H-^{1}H TOCSY spectrum and an overlay of TOCSY and NOESY spectra are consistent with the amide-to-side chain and amino acid residue connectivity in FER-green (Figure S3c,d and Table S2). The chemical shifts of each proton in the fingerprint region of the TOCSY spectrum are summarized in Table S2. Furthermore, high-resolution mass spectrometry (HRMS) result on FER-green revealed molecular-weight ion peak at m/e = 1422.5903 (calcd. 1421.5825) (Figure S28). These results confirm that the fluorophore is linked to HS-Ac-CFFKDEL via a sulfide bond and that FER-green represent a 1:1 mixture of two diastereomers produced after the addition of a thiol group to the maleimide moiety of F-Mal.

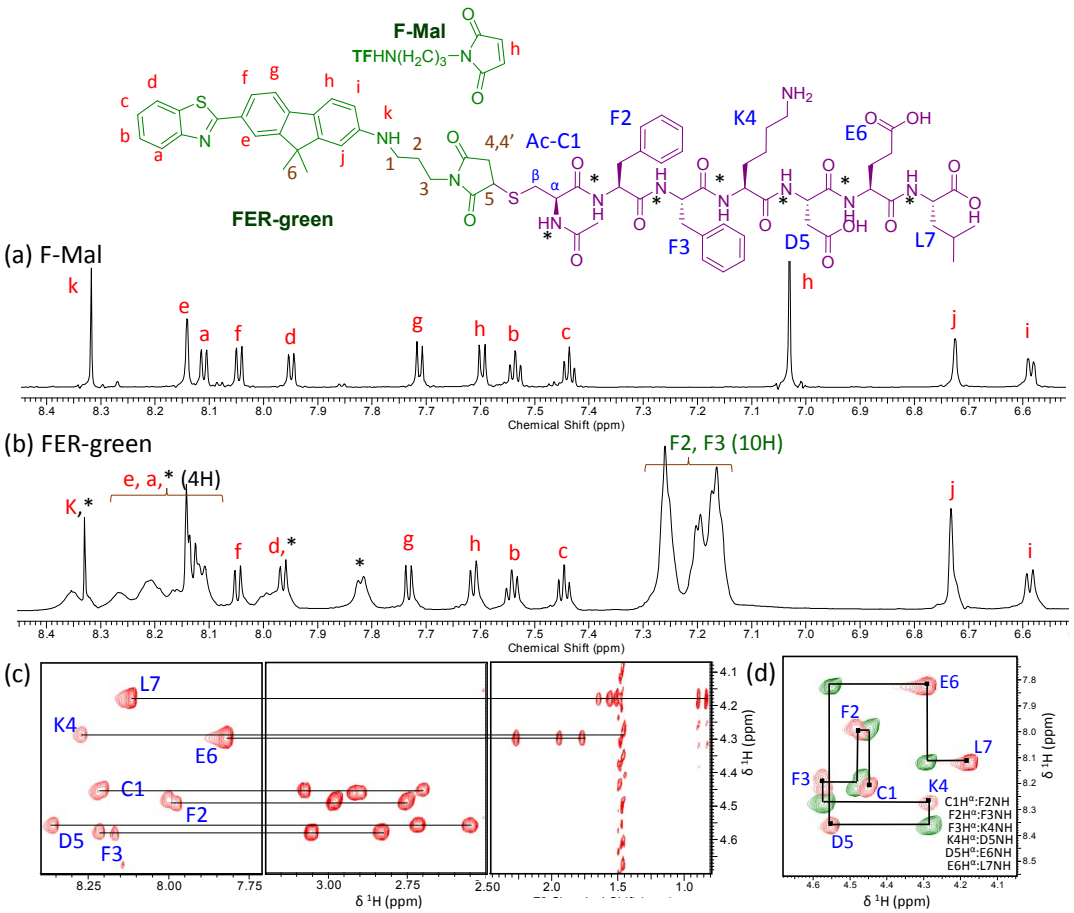


Figure S3. (a,b) Partial ^{1}H NMR spectra of F-Mal (a) and FER-green (b) in DMSO in the region δ 8.4–6.5. The protons in the F-Mal and Ac-CFFKDEL moieties are designated as a–k, *, F2, and F3, respectively. (c) 2D ^{1}H-^{1}H TOCSY spectrum of FER-green in the NH-H^{a} fingerprint region. Each line indicates amide-to-side chain connectivity. (d) Overlay of TOCSY (red) and NOESY (green) spectra. The lines trace the sequential backbone assignment via intramolecular NH–H$^{\alpha}$ NOE connections.

Table S2. Chemical shifts of each proton in the fingerprint region of the TOCSY spectrum of FER-green in DMSO-d_6.

FER-green							
Residue	NH	H$^{\alpha}$	H$^{\beta}$, H$^{\beta'}$	H$^{\gamma}$, H$^{\gamma'}$	H$^{\delta}$, H$^{\delta'}$	H$^{\varepsilon}$, H$^{\varepsilon'}$	H$^{\zeta}$
Cys1	8.20	4.44	3.06, 2.68				
Cys1a	8.18	4.44	3.06, 2.90				
Phe2	7.98	4.47	2.96, 2.74		7.25	7.16	7.17
Phe2a	8.01	4.48	2.98, 2.76				
Phe3	8.18	4.58	3.08, 2.83		7.25	7.15	7.17
Phe3a	8.15	4.58	3.05, 2.83				
Lys4	8.26	4.26	1.76, 1.68	1.53, 1.46	1.61, 1.60		
Asp5	8.36	4.55	2.72, 2.55				
Glu6	7.81	4.28	1.76, 1.93	2.26			
Leu7	8.10	4.17	1.63, 1.55	1.50	0.89, 0.83		

Spectroscopic Properties

The spectral data were obtained as reported elsewhere, and the fluorescence quantum yield (Φ) was determined by a method from the literature.[4,5] The spectral data obtained under various conditions are summarized in Table S3.

Table S3. Spectroscopic properties.

Cpd	Solvent (E_T^N)[a]	$\lambda_{max}/\lambda_{fl}$[b]	Φ[c]	Φδ[d]
BER-blue	EtOH (0.654)	358/445	1.0	71
	PBS (1.00)	354[e]/465	1.0	61
	HeLa cell	–/460[f] (460)[g]	–	1580[h]
FER-green	EtOH (0.654)	385/513	0.93	202
	PBS (1.00)	373[i]/553	0.24	70
	HeLa cell	–/480[f] (497)[g]	–	3030[h]

[a]The numbers in parentheses are normalized empirical parameters of solvent polarity.[6] [b]One-photon absorption and emission maxima unless stated otherwise. [c]Fluorescence quantum yield. [d]TP action cross-section in GM ± 15%. [e]Molar extinction coefficients (ε) = 25,560 $cm^{-1}M^{-1}$. [f]λ_{fl} of emission spectra. [g]λ_{fl} of TPEF spectra. [h]Effective TP action cross-section ($\Phi\delta_{eff}$) values measured in HeLa cells. [i]ε = 32,440 $cm^{-1}M^{-1}$

Solubility

The solubility of BER-blue and FER-green in phosphate-buffered saline (PBS) was determined by a fluorescence method, as reported before.[4]

Two-Photon Microscopy

TPM images of probe-labeled cells and tissues were obtained by spectral confocal and multiphoton microscopes (Leica TCS SP2; Leica Camera, Solms, Germany), using the 100× oil objective with a numerical aperture (NA) of 1.30, and a 10× dry objective with a numerical aperture (NA) of 0.30. The excitation was provided using a mode-locked titanium-sapphire laser (Chameleon, 90 MHz, 200 fs; Coherent Inc., Santa Clara, CA, USA) set at 750 nm and output power of 1305 mW, which corresponded to a power of 4×10^9 mW/cm^2 (100×) at the focal plane. Images were obtained by collecting the emissions at the indicated channels (see Table S4) with PMTs in an 8-bit unsigned 512 × 512 pixel format at a scan speed of 400 Hz.

Measurements of TP Action Cross-Section ($\Phi\delta_{max}$) and Effective TP Action Cross-Section ($\Phi\delta_{eff}$)

$\Phi\delta_{max}$ and $\Phi\delta_{eff}$ of BER-blue and FER-green were determined via a comparison of TP excited fluorescence (TPEF) intensities of the probes with that of Rhodamine 6G in methanol in a cuvette or through a comparison of TPEF intensities from the regions of interest in probe-labeled cells with TPEF intensity of 5.0 μM Rhodamine 6G in methanol in the TPM setup, respectively.[4]

Cell Preparation

Human cervical carcinoma HeLa cells were acquired from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured as described before and seeded 24 h prior to imaging at a density of 5.0×10^4 cells/mL on glass coverslips coated with 0.1 mg/mL poly-L-lysine for high-resolution OPM with Airyscan (LSM 800, Carl Zeiss) or in glass-bottomed dishes (SPL Life Sciences, Gyeonggi-do, Korea) for OPM and TPM live imaging.[4]

Tissue Preparation

All animal procedures were approved by the Korea Institute of Science and Technology Laboratory Animal Facilities and Use Committee and carried out at Association for Assessment and Accreditation of Laboratory Animal Care–accredited facilities. *Ex vivo* brain slices were obtained from the hippocampus of Sprague–Dawley rats aged 2 weeks (Orient Bio Inc., Gyeonggi-do, Korea). Hippocampal coronal slices were cut into 400-μm-thick slices on a vibrating-blade microtome in artificial cerebrospinal fluid and were used for TPM imaging as described before.[7]

Detection Windows

The detection windows of the probes were determined ensuring good separation of the emission bands and similar emission intensities from the two probes under comparison. For the co-localization experiments with the probe-labeled cells, the detection windows were selected via a comparison of TPEF spectra of BER-blue, FER-green, ABI-Nu, and PLT-yellow (a TP probe for lysosomes), and a one-photon fluorescence spectrum of ETR in HeLa cells (Figure S6a and Table S4).[4,8] The detection windows determined for the colocalization experiments in the HeLa cells colabeled with a pair of probes are summarized in Table S4. In each channel, the fluorescence of one probe contributed to that of another by 0–35% (Table S4).

Table S4. Detection windows for the OPM and TPM imaging.

OPM			**TPM**		
BER[a] /ETR[c]	FER[b] /ETR[c]	BER[a] /FER[b]	BER[a] /FER[b]	BER[a] /PLT[d]	BER[a] /ABI[e]
Ch1 (0)[f] (400–550)[g]	Ch1 (0)[f] (400-550)[g]	Ch2 (30)[f] (410–460)[g]	Ch3 (20)[f] (385–450)[g]	Ch4 (1)[f] (385–500)[g]	Ch5 (35)[f] (385–435)[g]
Ch8 (1)[f] (580–700)[g]	Ch8 (15)[f] (580–700)[g]	Ch6 (20)[f] (535–655)[g]	Ch6 (17)[f] (535–655)[g]	Ch7 (0)[f] (600–660)[g]	Ch6 (35)[f] (535–655)[g]

[a]BER-blue. [b]FER-green. [c]ER-tracker Red. [d]PLT-yellow. [e]ABI-Nu. [f]The degree (%) to which the emission intensity of one probe contributes to that of the other probe in a given channel. [g]Wavelength ranges for each channel.

Cell Imaging by High-resolution OPM

High-resolution OPM images of HeLa cells colabeled with BER-blue (5 μM) and ER-tracker Red (ETR; 1 μM) as well as with FER-green (3 μM) and ETR (1 μM) were captured by detection of the emission at 400–550 nm (channel 1 [Ch1], BER-blue and FER-green) and 580–700 nm (Ch7, ETR) upon excitation at 405 nm for BER-blue and FER-green and at 561 nm for ETR during the OPM in Airyscan mode (LSM 800, Carl Zeiss) with an alpha Plan-Apochromat 63×/ 1.40 oil immersion objective lens. The obtained raw images were processed using the Zen software (Carl Zeiss). Pearson's coefficient was calculated in the ImageJ software (NIH).

Cell Imaging by OPM and TPM

OPM images of HeLa cells colabeled with BER-blue (3 μM) and FER-green (1.5 μM) were captured by the emission acquired at 410–460 nm (Ch2, BER-blue) and 535–655 (Ch6, FER-green) after excitation at 405 nm under a confocal fluorescence microscope (LSM 700, Carl Zeiss) with a 63× oil objective lens. TPM images of HeLa cells colabeled with BER-blue (3 μM) and FER-green (1.5 μM) were obtained via recording of the emission at 385–450 nm (Ch3, BER-blue) and 535–655 nm (Ch6, FER-green) upon excitation at 750 nm under a multiphoton microscopy with a 100× oil objective lens as described elsewhere.[4]

Tissue Imaging by TPM

TPM images of *ex vivo* brain slices colabeled with BER-blue (10 μM) and ABI-Nu (a TP probe for nucleus, 3 μM) and those colabeled with BER-blue (10 μM) and FER-green (6 μM) were obtained across wavelength ranges 380–435 nm (Ch5, BER-blue) and 535–655 nm (Ch6, ABI-Nu) as well as 385–450 nm (Ch3, BER-blue) and 535–655 nm (Ch6, FER-green), respectively, after excitation at 750 nm. In all cases, 50–100 sectional TPM images were captured as reported before.[9] Enlarged cellular TPM images of the brain slices were captured by means of a 100× oil objective lens.

Cytotoxicity

This property of BER-blue and FER-green was determined by the CellTiter-Glo Luminescent Cell Viability Assay (G7572, Promega, USA).

Photostability

This characteristic was determined as reported before.[4]

A Western Blot Assay

HeLa cells were treated with 5 μg/mL tunicamycin for 0−16 h and washed twice with ice-cold PBS. The cells were lysed in NP40 buffer (50 mM Tris-HCl pH 7.5, 1% of NP40, 1 mM EDTA, 150 mM NaCl, 5 mM Na_3VO_4, and 2.5 mM NaF) containing a 1× protease inhibitor cocktail (Roche). The mixture was centrifuged for 20 min at 15,871 × *g* and 4°C, and the supernatants were collected. The protein concentrations were determined with the Bradford Protein Assay Kit (Bio-Rad, Inc., USA).

Samples of the cell lysates containing equal amounts of total protein (25 μg) were separated by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (0.45 μm pore size) in a Wet/Tank Blotting System. The membranes were blocked for 1 h in Tris-buffered saline containing 0.1% of Tween 20 (TBST) and 5% skim milk at room temperature.

The membrane was hybridized at 4°C for 18 h with one of the following primary antibodies: anti-BIP (catalog No. 3183S, Cell Signaling Technology; dilution, 1:1000), anti-CHOP (catalog No. 5554S, Cell Signaling Technology; dilution, 1:1000), anti-cleaved caspase-3 (catalog No. 9661L; dilution, 1:1000), anti-PARP 1/2 (catalog No. 5625L; dilution, 1:3000), and anti-β-actin (catalog No. sc-47798, Santa Cruz Biotechnology; dilution, 1:10000). The membranes were washed three times with TBST (15 min each time) and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000, an anti-mouse immunoglobulin G [IgG] antibody and anti-rabbit IgG antibody, GenDEPOT) in TBST with 1% skim milk at room temperature for 2 h. The protein bands were detected using the Enhanced Chemiluminescence Kit (Pierce™ ECL Western Blotting Substrate and Super-Signal™ West Femto Maximum Sensitivity Substrate, Thermo Fisher Scientific, Inc.).

Statistical Analysis

Numerical data are presented as mean ± standard deviation. The significance of data after a comparison was evaluated by one-way ANOVA in GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

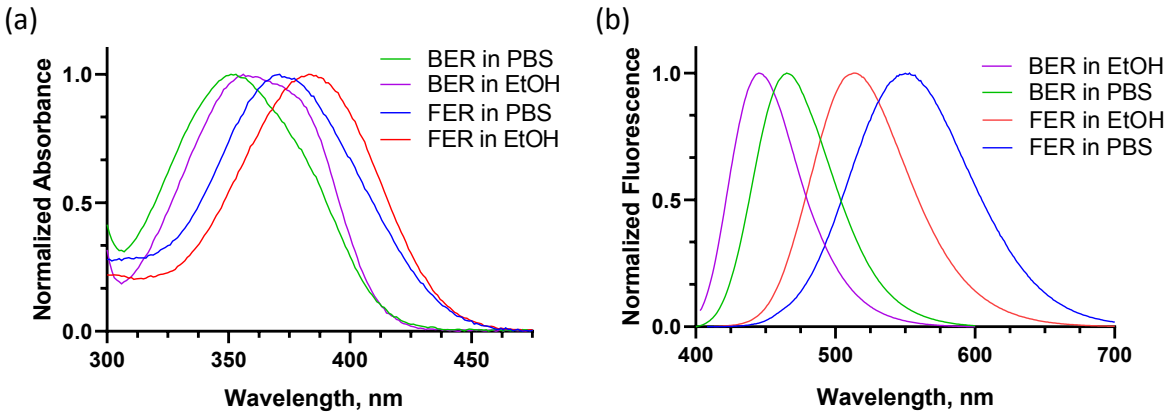


Figure S4. Normalized absorption (a) and emission (b) spectra of BER-blue and FER-green in phosphate-buffered saline (PBS) and EtOH.

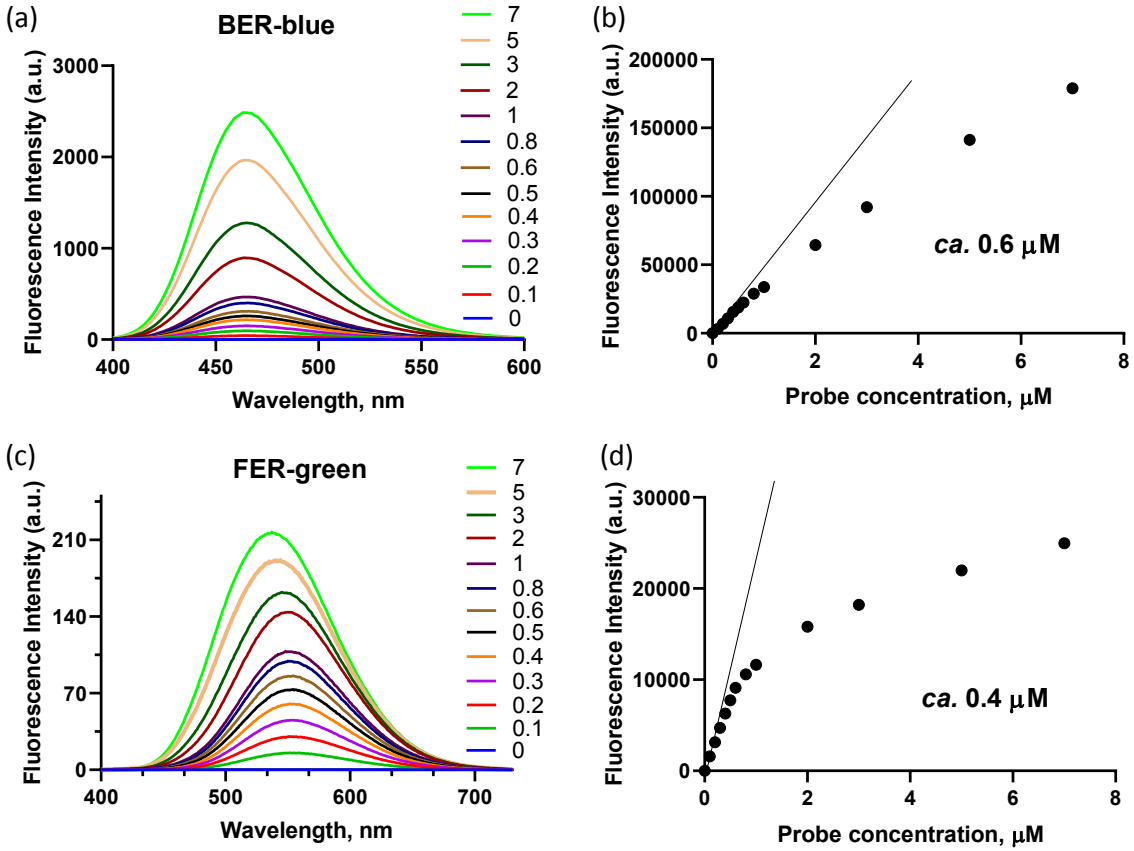


Figure S5. (a,c) Fluorescence spectra of BER-blue (a) and FER-green (c), and plots (b, d) of the fluorescence intensity against probe concentration for BER-blue (b) and FER-green (d) in PBS. The excitation wavelengths for BER-blue and FER-green were 354 and 373 nm,

respectively.

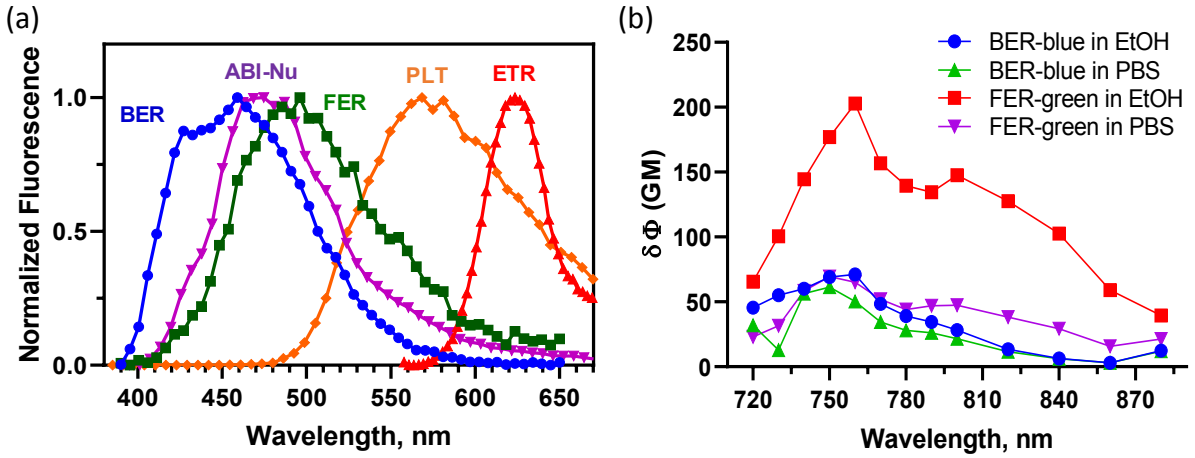


Figure S6. (a) Normalized TPEF spectra of BER-blue (blue), FER-green (green), PLT-yellow (orange), and ABI-Nu (purple) and a normalized fluorescence spectrum of ETR (red) in HeLa cells. The wavelengths for the one- and two-photon excitation were 405 and 750 nm, respectively. (b) TP excitation spectra of BER-blue and FER-green in EtOH and PBS, respectively.

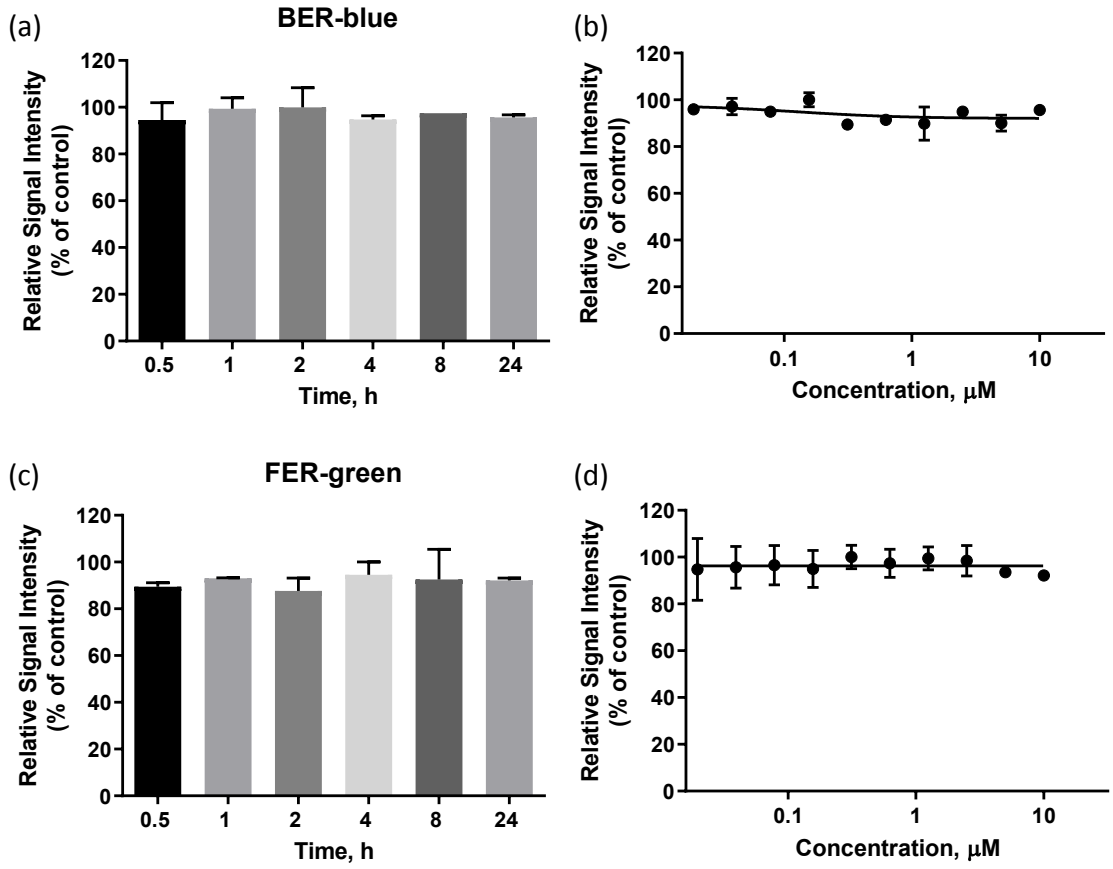


Figure S7. Viability of HeLa cells in the presence of BER-blue (a,b) or FER-green (c,d), as

measured by a 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were incubated with the probes (0−10 μM) for 0–24 h.

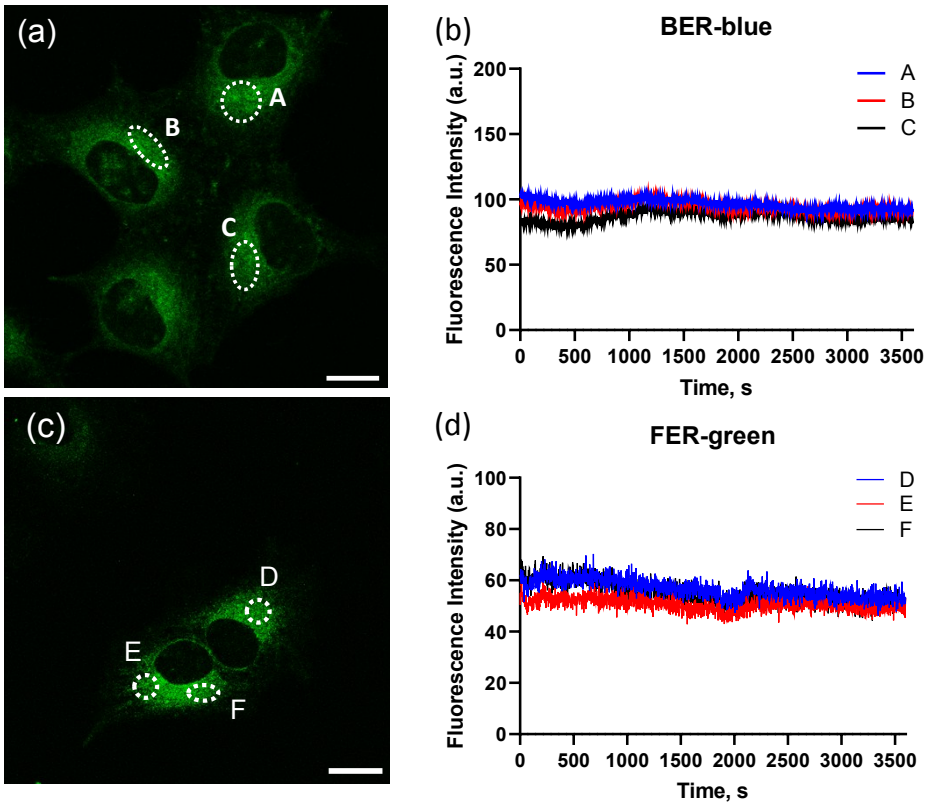


Figure S8. (a,c) Two-photon microscopy images of HeLa cells labeled with BER-blue (a) or FER-green (c). (b,d) Relative TPEF intensity as a function of time. The digitized intensity was recorded with 2 s intervals during 1 h in *xyt* mode. The TPEF intensities at positions A–F were recorded at 380–660 nm upon excitation at 750 nm. Scale bar: 30 μm.

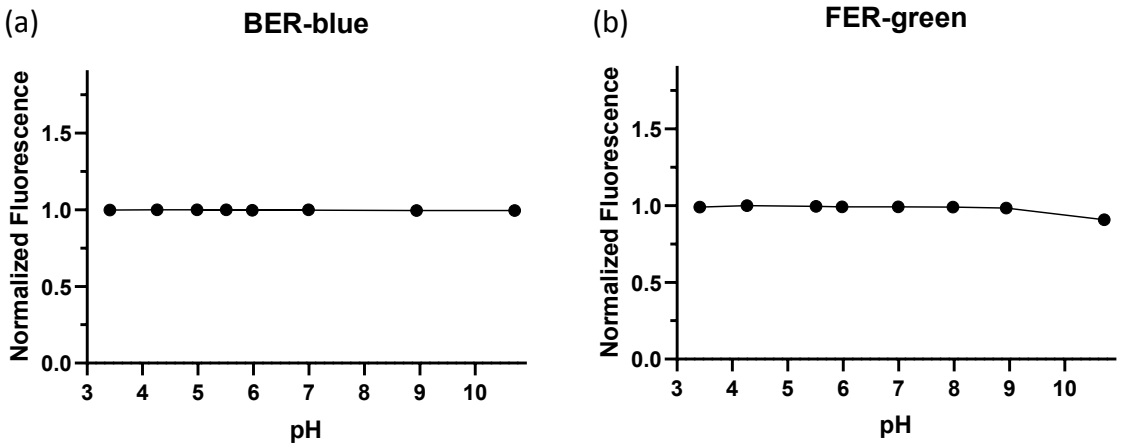


Figure S9. Effects of pH on the one-photon fluorescence intensity of BER-blue and FER-green (2 μM each in universal buffer: 0.1 M citric acid, 0.1 M KH_2PO_4, 0.1 M $Na_2B_4O_7$, 0.1 M Tris, and 0.1 M KCl).

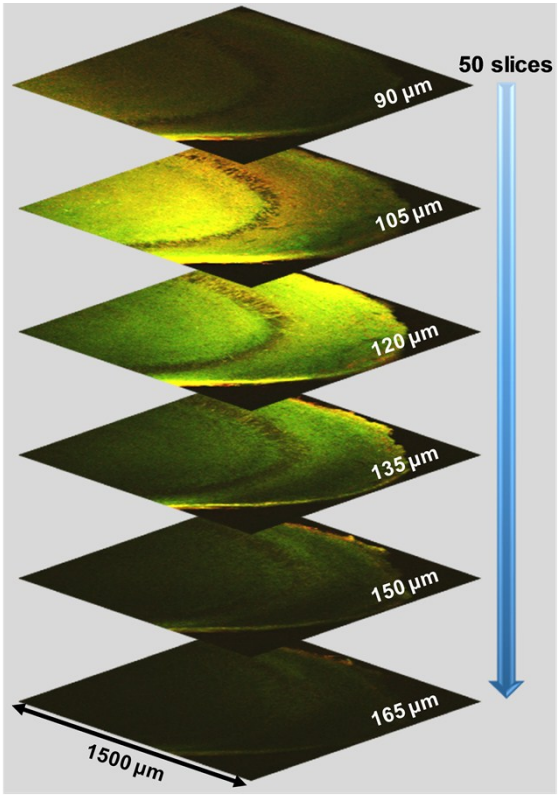


Figure S10. Sectional TPM images of a rat hippocampal slice colabeled with BER-blue (10 µM) and FER-green (6 µM). The images were captured in channel 3 (Ch3: BER-blue, 385–450 nm) and Ch6 (FER-green, 535–655 nm) at a depth of 90–165 µm and 10× magnification after excitation at 750 nm.

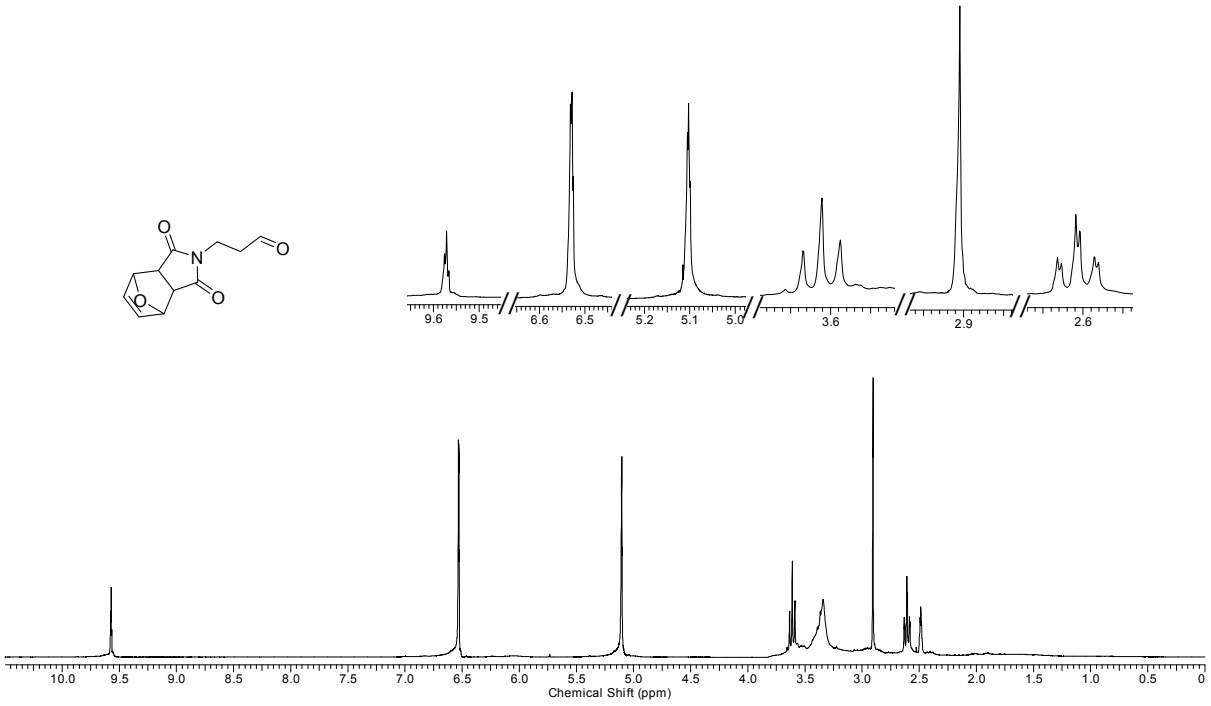


Figure S11. ^{1}H NMR spectrum (300 MHz) of compound **I** in DMSO-d_6.

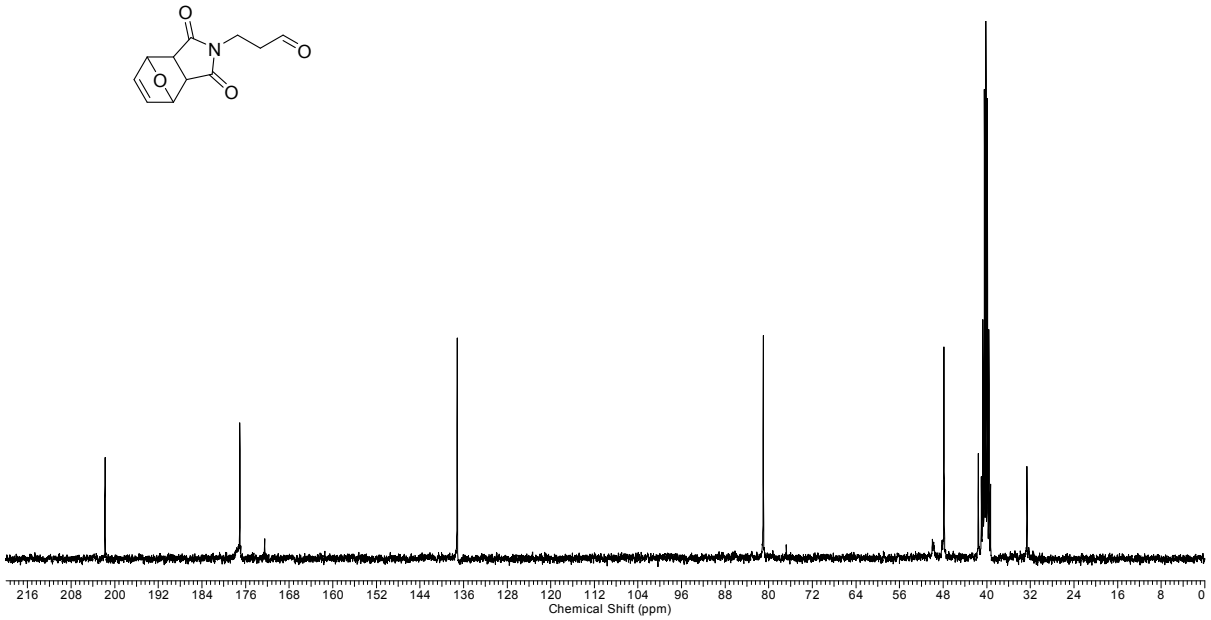


Figure S12. ^{13}C NMR spectrum (75 MHz) of compound **I** in DMSO-d_6.

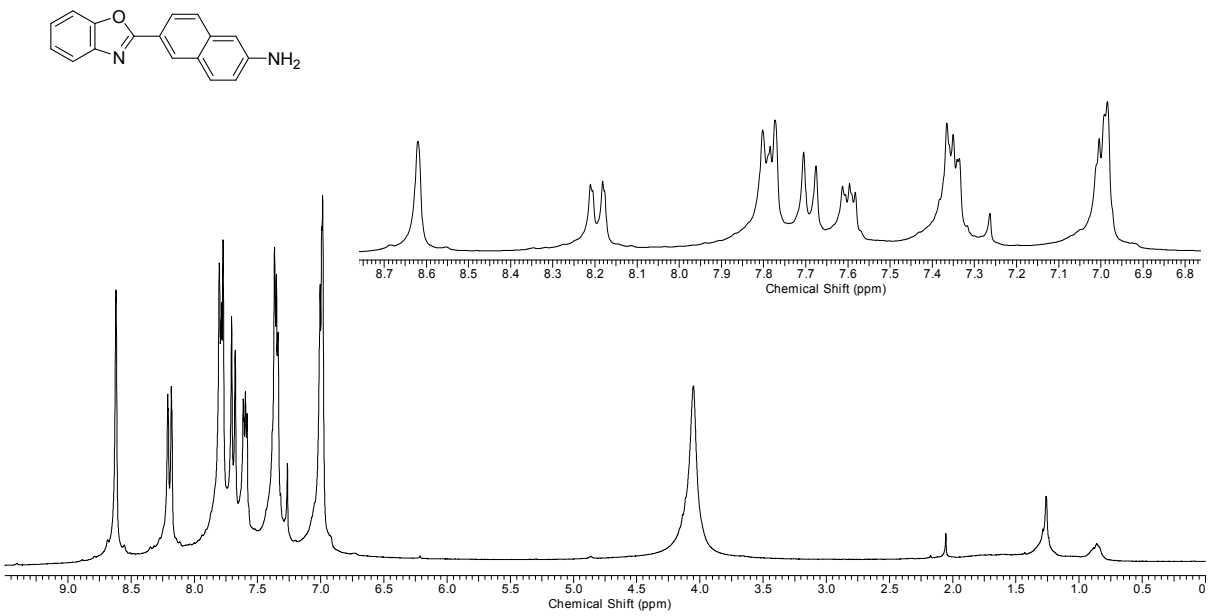


Figure S13. ^{1}H NMR spectrum (300 MHz) of compound **B-NH$_2$** in $CDCl_3$.

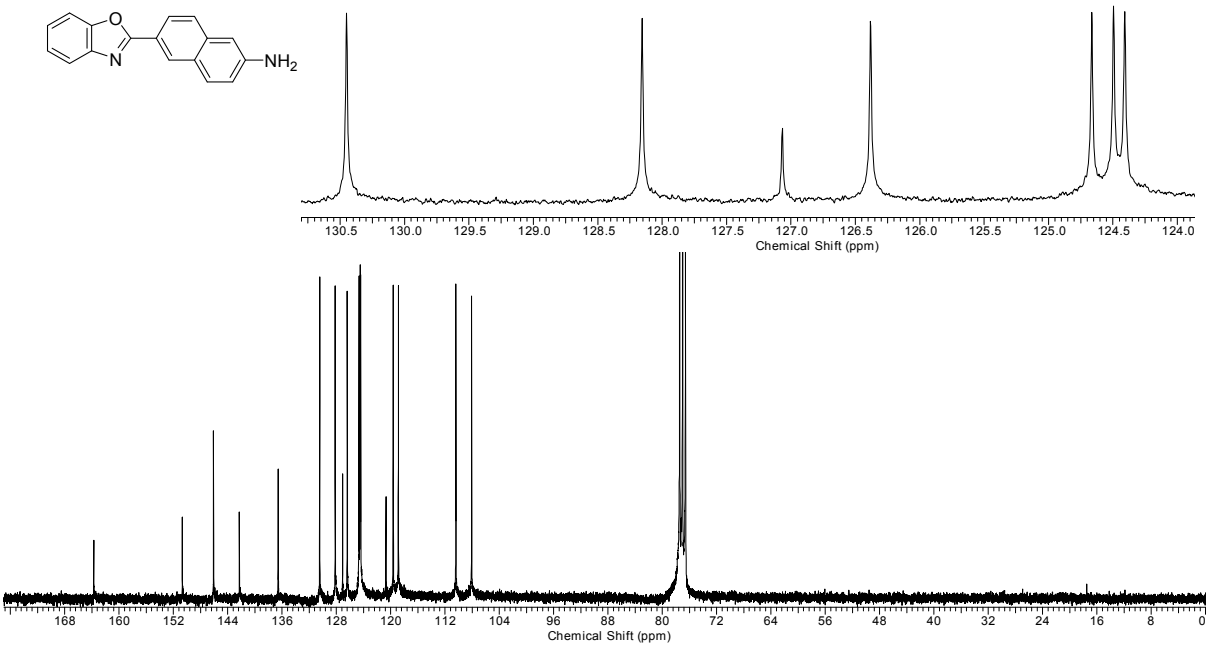


Figure S14. ^{13}C NMR spectrum (75 MHz) of compound **B-NH$_2$** in $CDCl_3$.

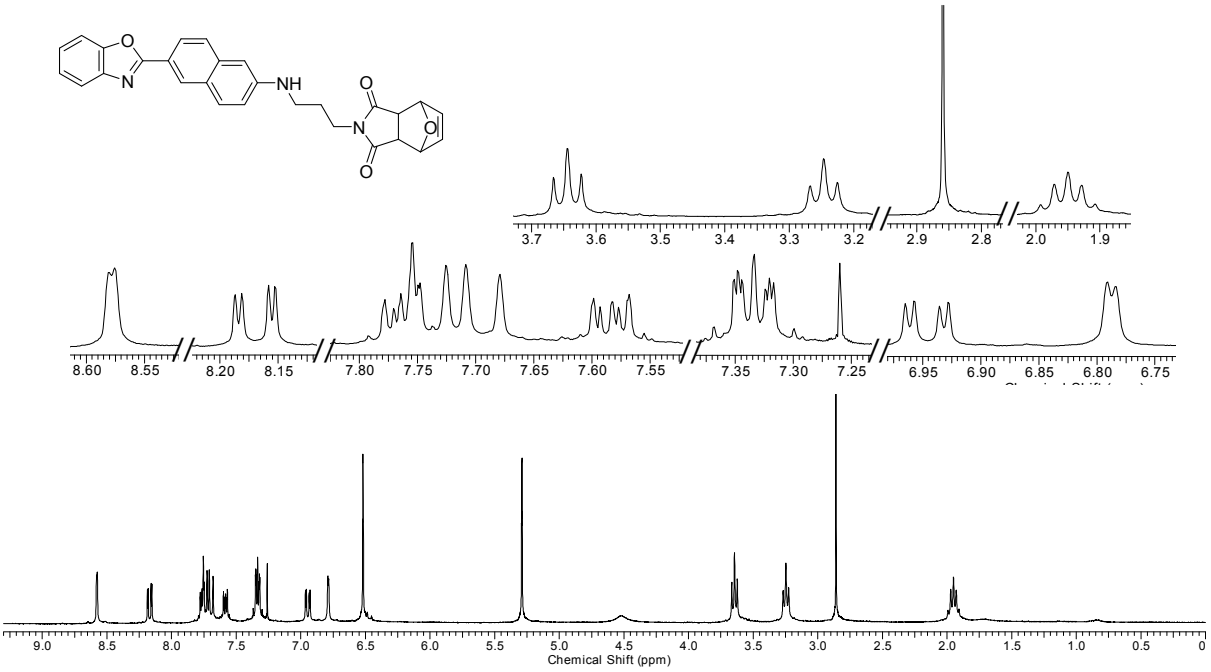


Figure S15. ^{1}H NMR spectrum (300 MHz) of compound **3** in $CDCl_3$.

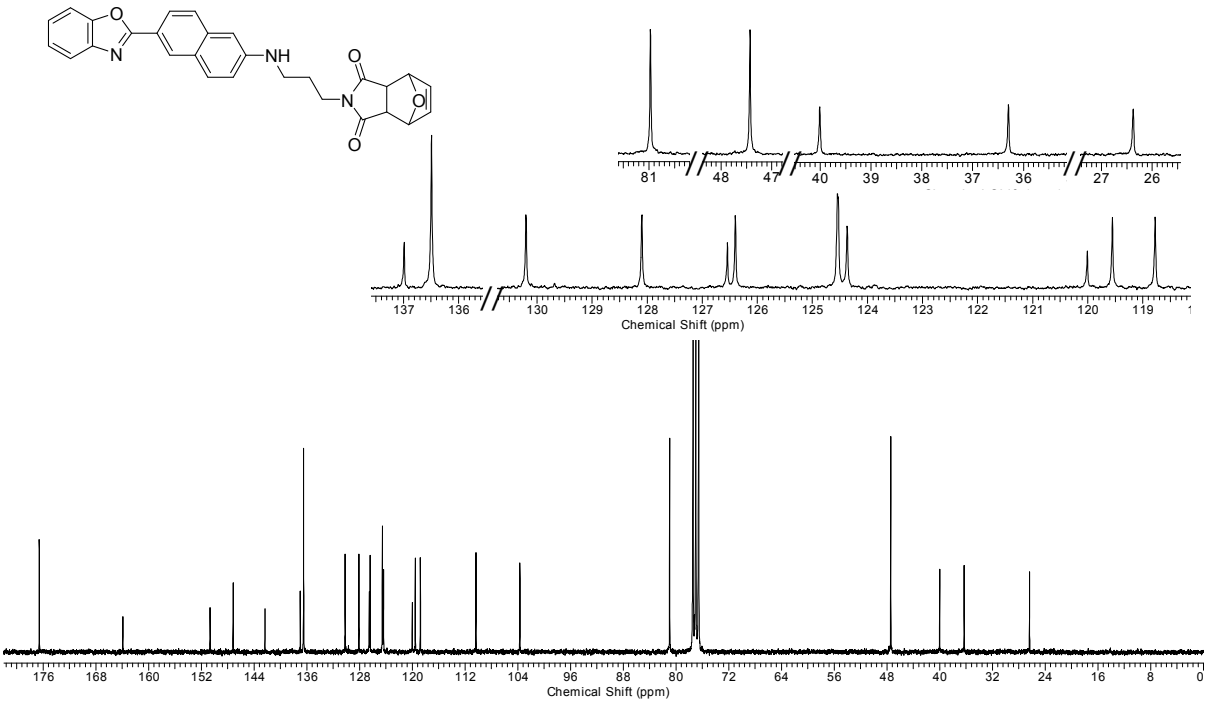


Figure S16. ^{13}C NMR spectrum (75 MHz) of compound **3** in $CDCl_3$.

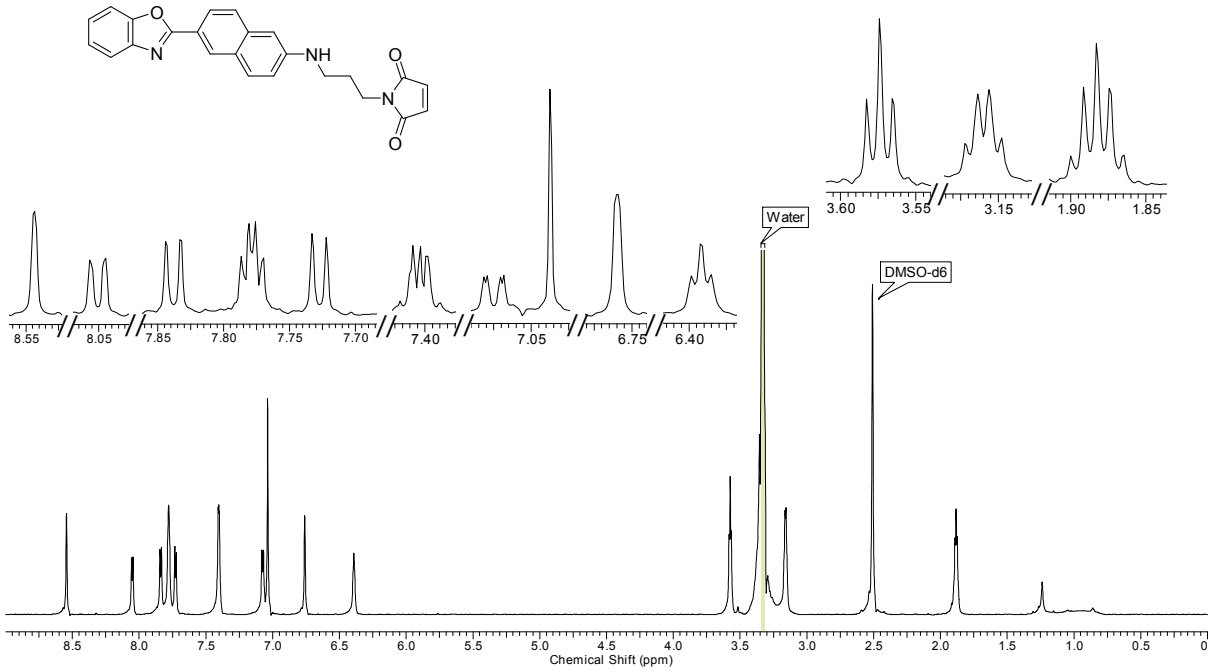


Figure S17. ^{1}H NMR spectrum (800 MHz) of **B-Mal** in DMSO-d_6.

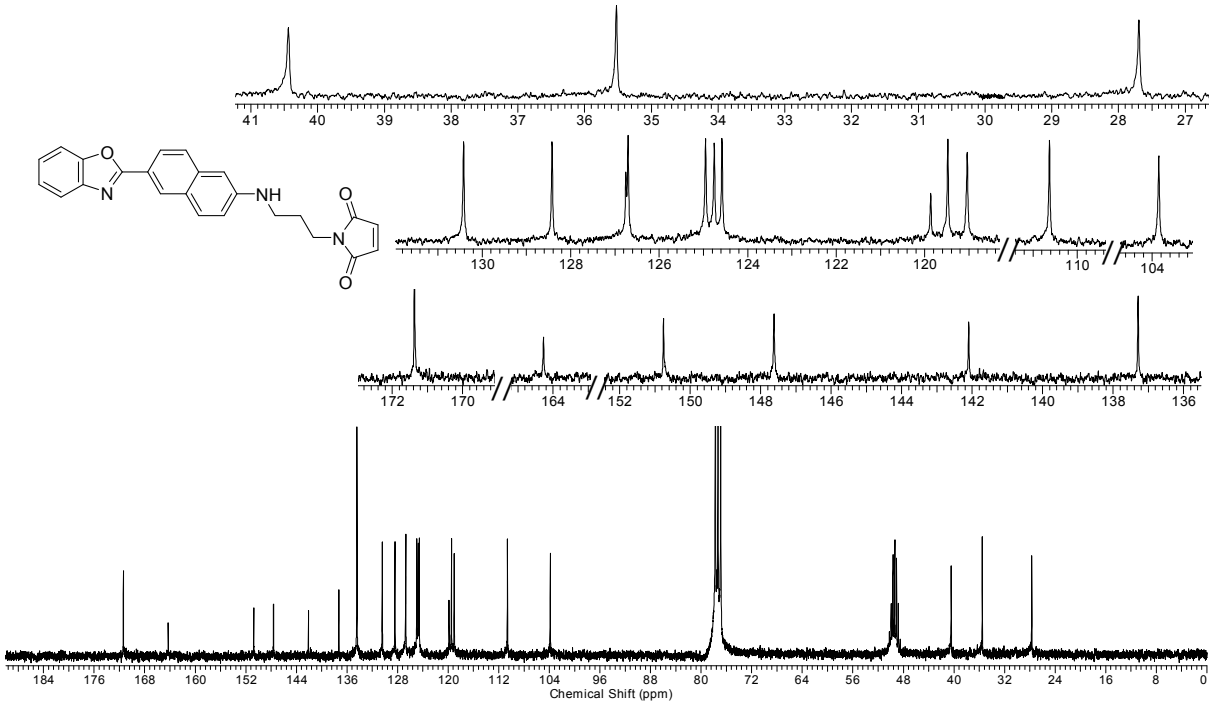


Figure S18. ^{13}C NMR spectrum (75 MHz) of **B-Mal** in $CDCl_3/CD_3OD$ (10/1).

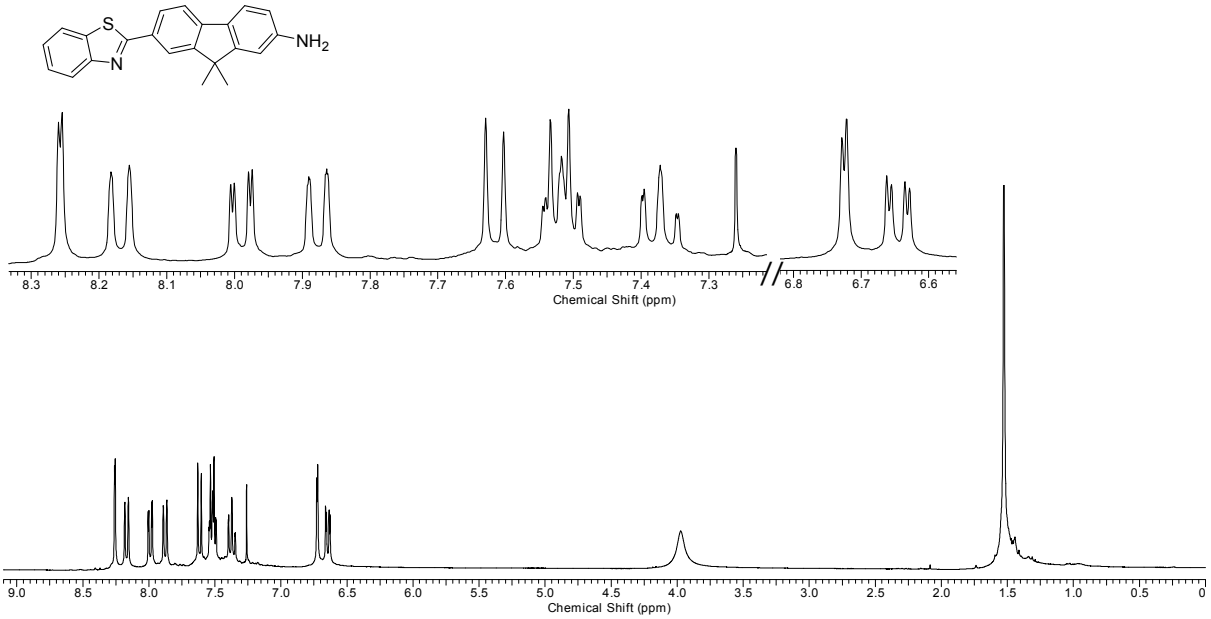


Figure S19. ^{1}H NMR spectrum (300 MHz) of **F-NH$_2$** in $CDCl_3$.

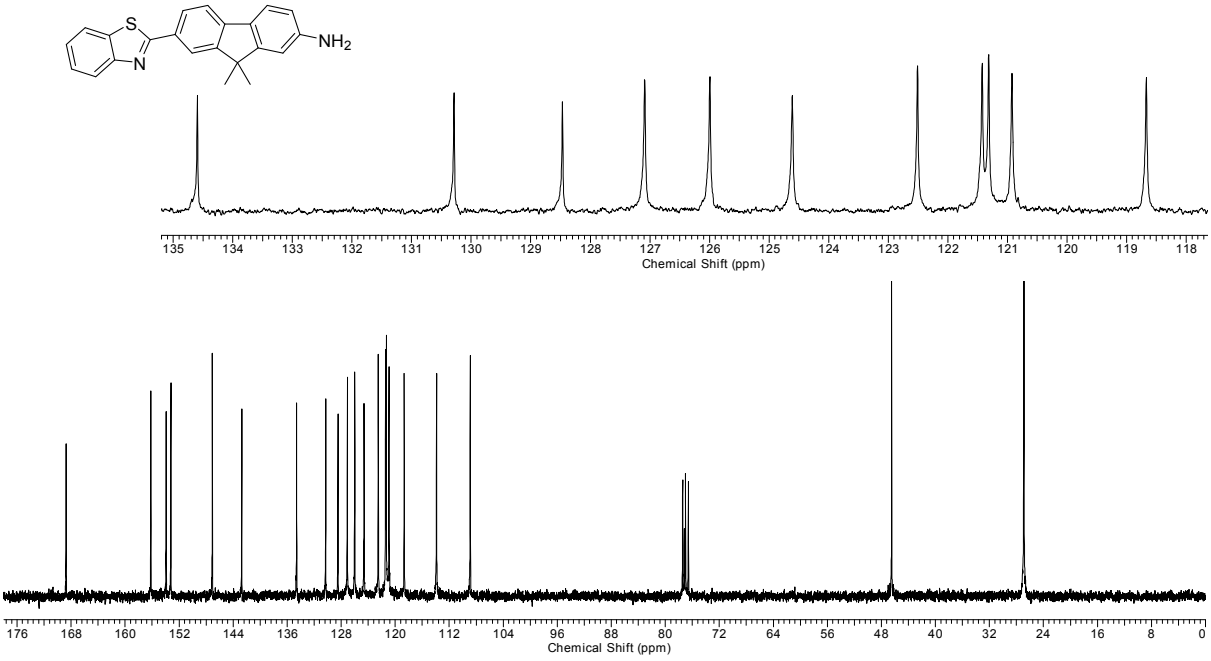


Figure S20. ^{13}C NMR spectrum (75 MHz) of **F-NH$_2$** in $CDCl_3$.

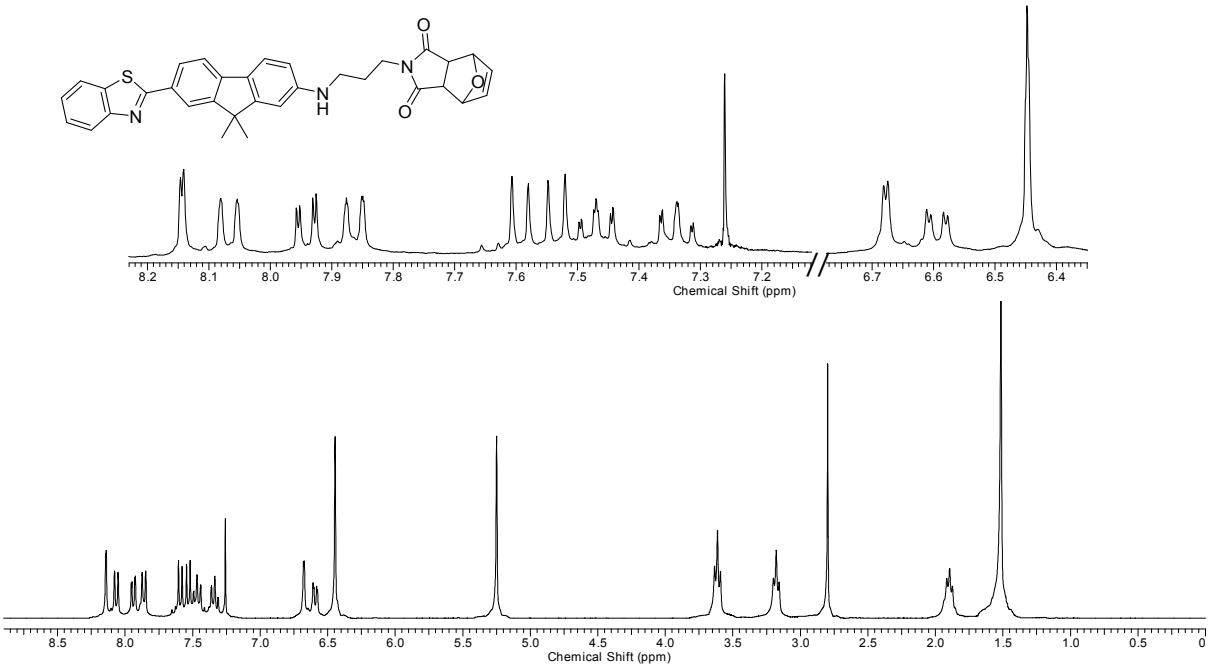


Figure S21. ^{1}H NMR spectrum (300 MHz) of compound **4** in $CDCl_3$.

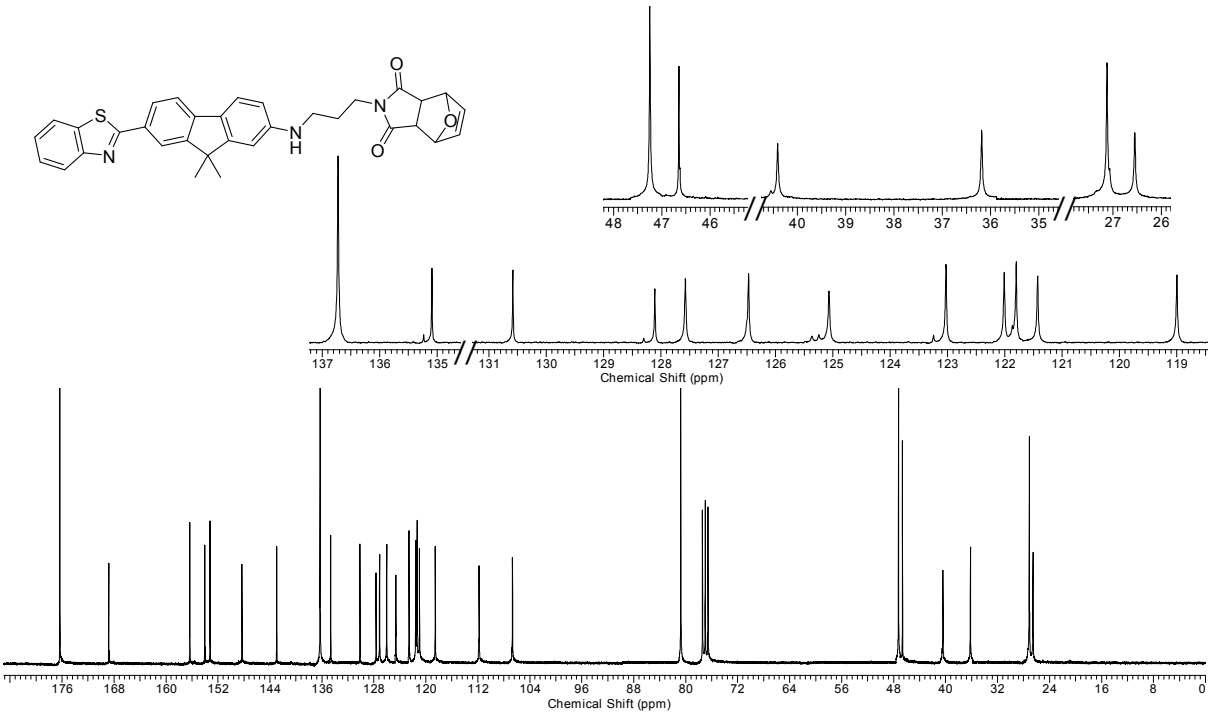


Figure S22. ^{13}C NMR spectrum (75 MHz) of compound **4** in $CDCl_3$.

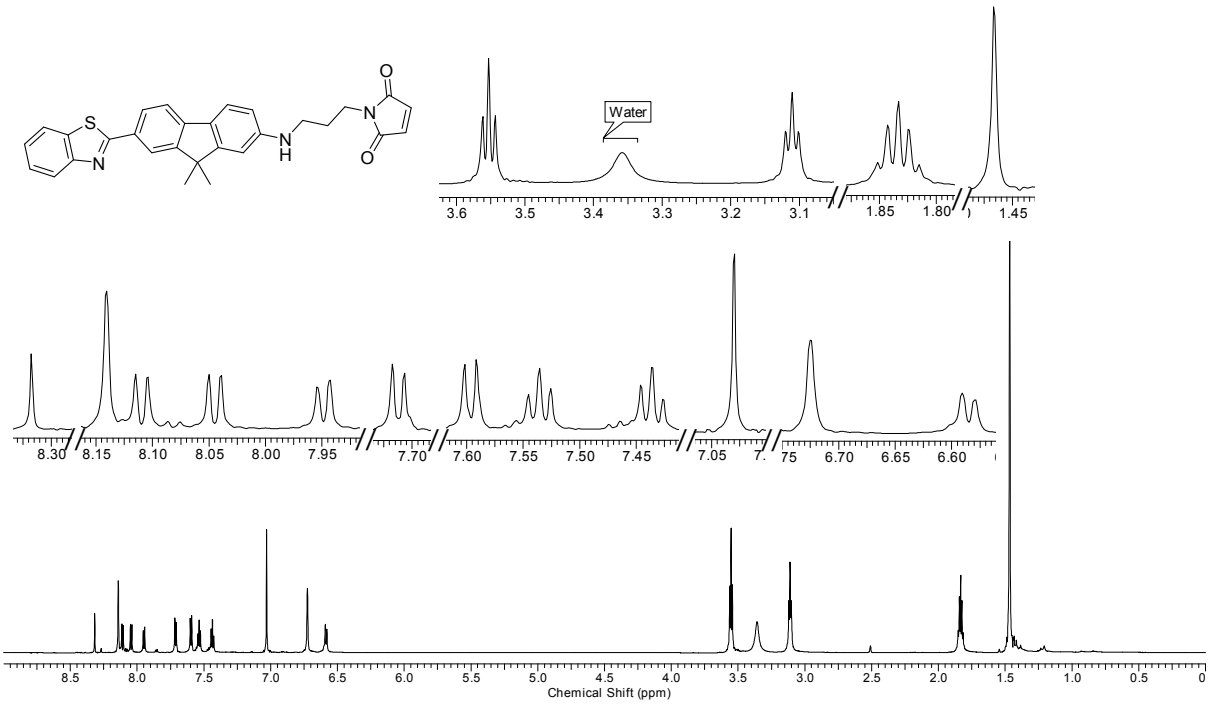


Figure S23. ^{1}H NMR spectrum (800 MHz) of **F-Mal** in DMSO-d_6.

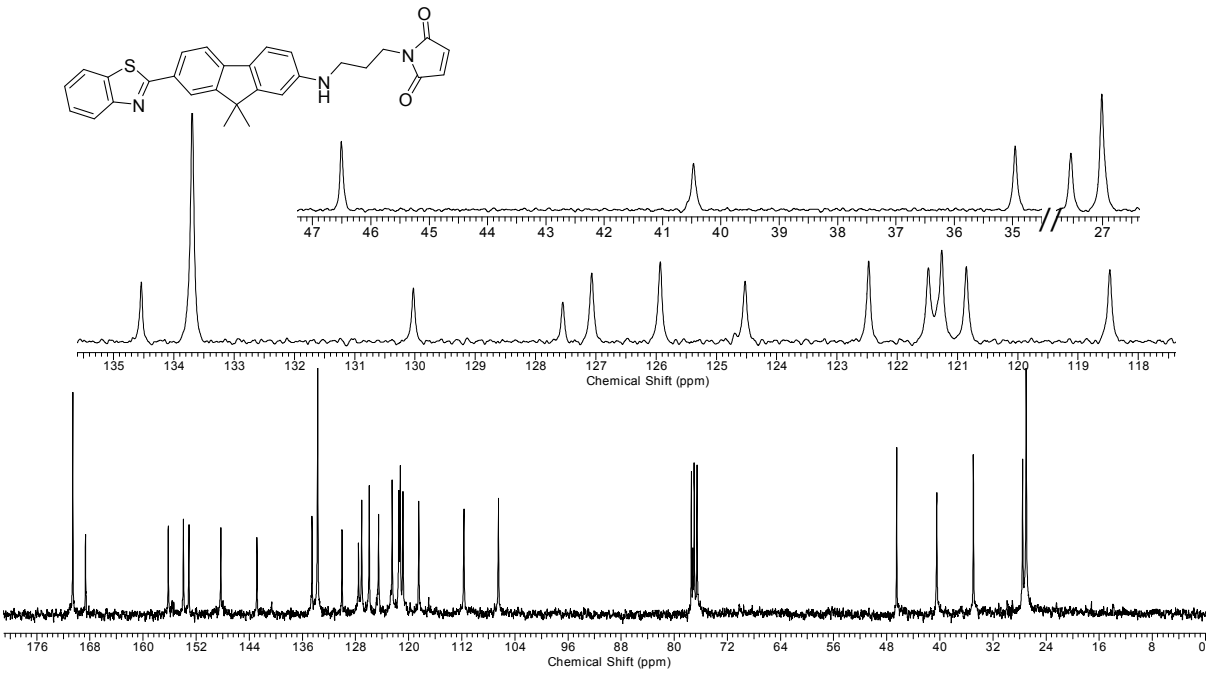


Figure S24. ^{13}C NMR spectrum (75 MHz) of **F-Mal** in $CDCl_3$.

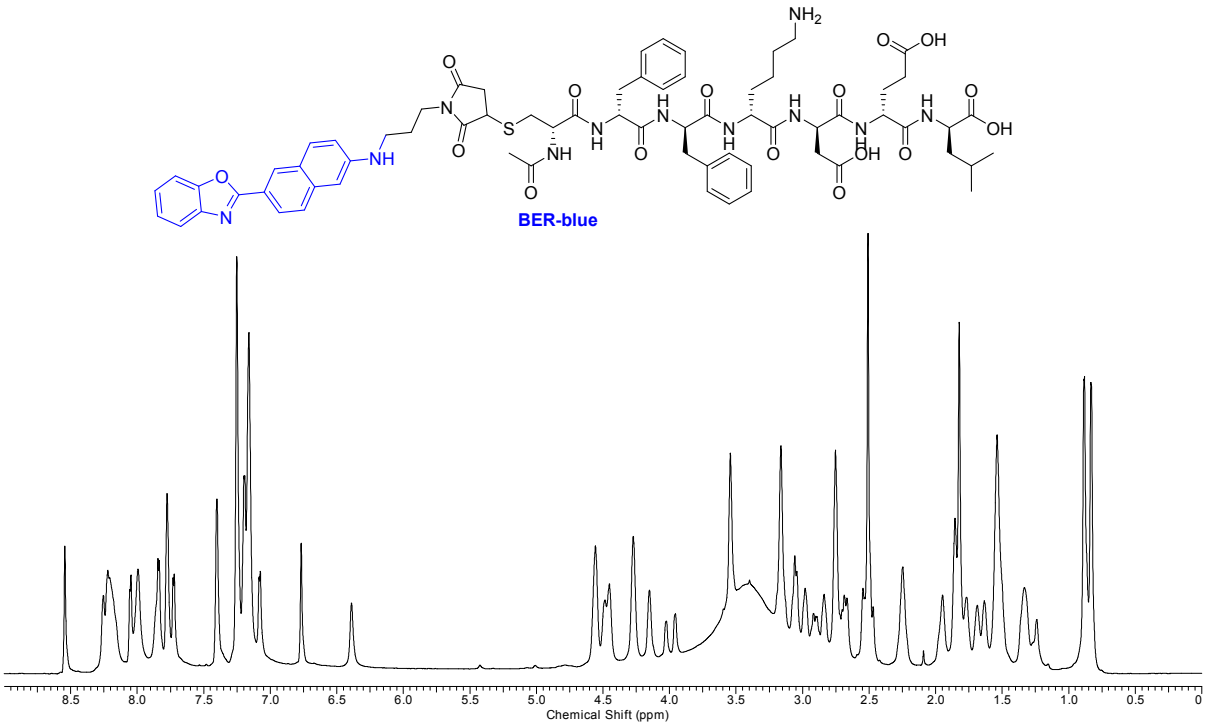


Figure S25. ^{1}H NMR spectrum (800 MHz) of **BER-blue** in DMSO-d_6.

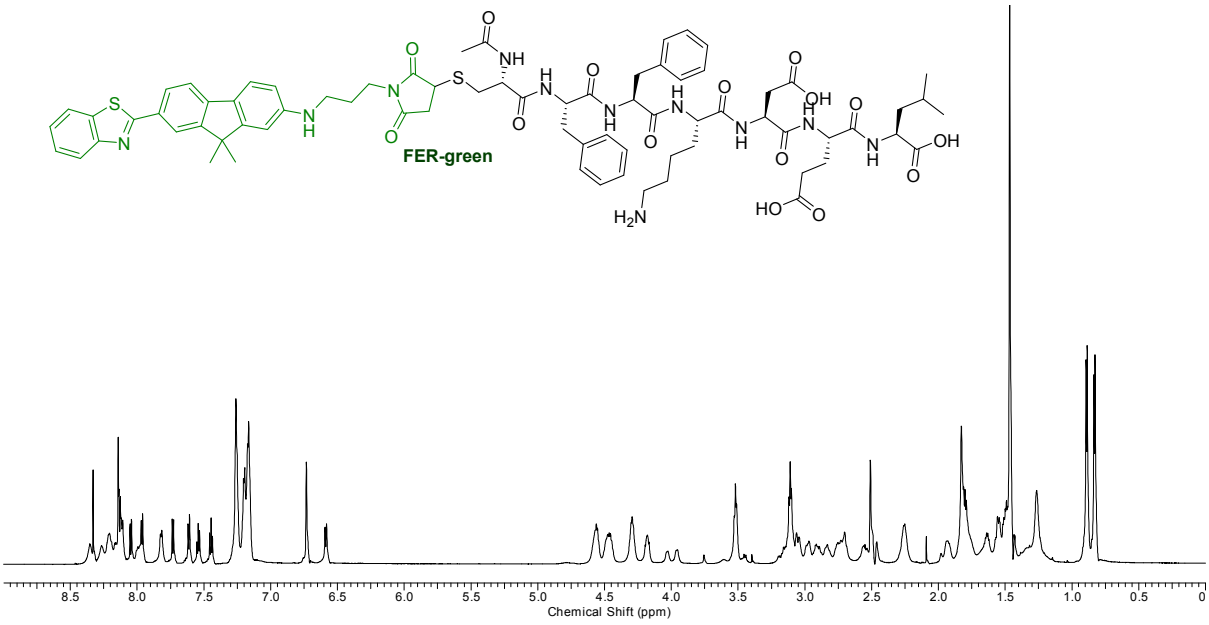


Figure S26. ^{1}H NMR spectrum (800 MHz) of **FER-green** in DMSO-d_6.

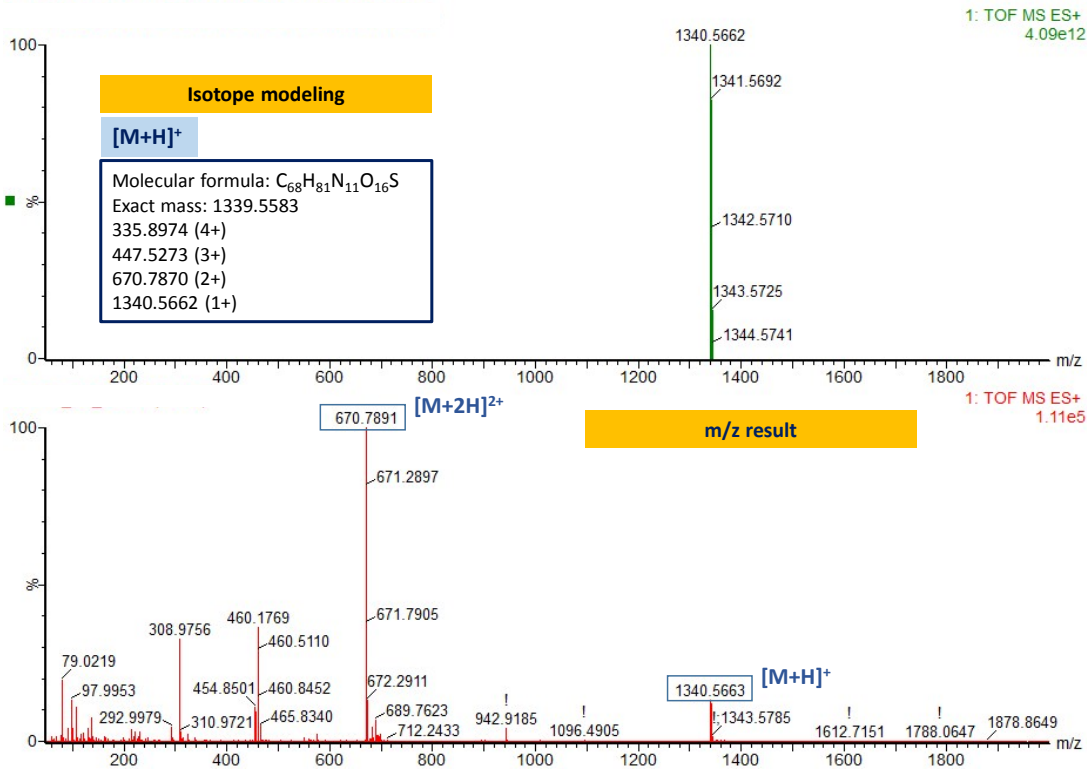


Figure S27. HRMS of **BER-blue**. TOF MS: time-of-flight mass spectrometry.

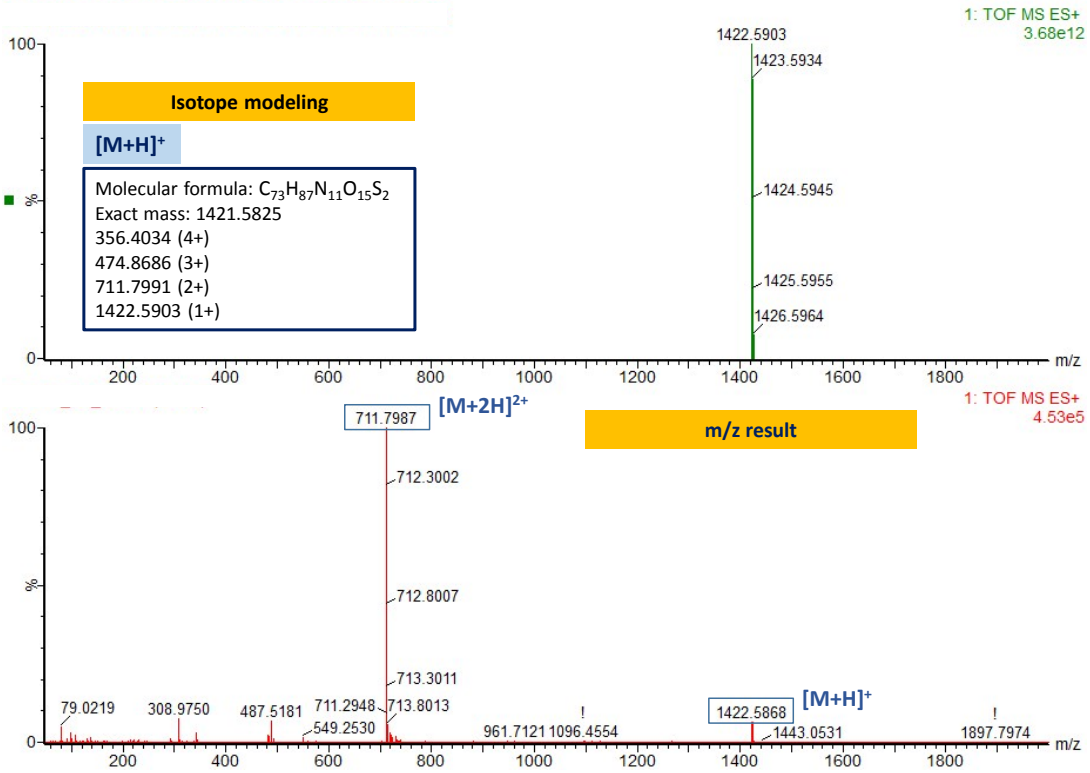


Figure S28. HRMS of **FER-green**. TOF MS: time-of-flight mass spectrometry.

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