

Supporting Information to Accompany

**Real-Time Monitoring of Vesicle pH in an Endocytic Pathway using an EGF-Conjugated Two-Photon Probe**

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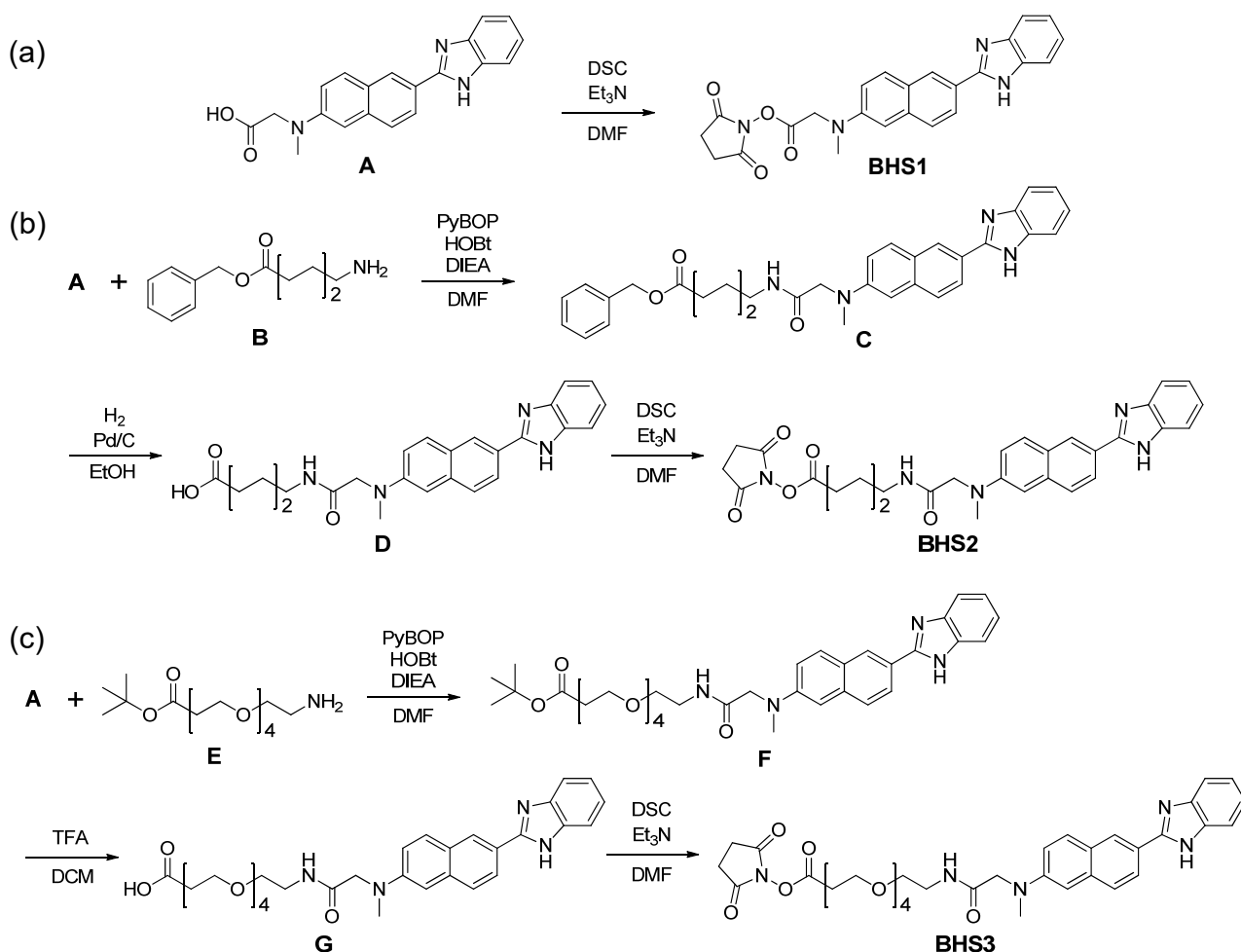
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**Synthesis of BHS1-3.** **A**<sup>1</sup>, **B**<sup>2</sup> and **E**<sup>3</sup> were prepared by the literature methods. Synthesis of **BHS1**, **BHS2**, and **BHS3** is described below.



**Scheme S1.** Synthesis of **BHS1-3**.

**BHS1.** A solution of **A** (0.05 g, 0.15 mmol) in 10 mL DMF with trimethylamine (0.03 ml, 0.23 mmol) was added dropwise and the mixture was stirred at room temperature for 15 min under nitrogen atmosphere. To this mixture, *N,N'*-disuccinimidyl carbonate (DSC, 0.06g, 0.23 mmol) was added and the mixture was stirred at room temperature for 2 h. The mixture was poured into H<sub>2</sub>O and extracted with CH<sub>2</sub>Cl<sub>2</sub>. The organic layer was washed with H<sub>2</sub>O and brine, and then the organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under vacuo to obtain the crude. The crude product was triturate with Hexane:CH<sub>2</sub>Cl<sub>2</sub> (95:5) to get yellow solid. Yield 0.05 g (82 %); M.p. 199-204 °C; <sup>1</sup>H NMR (*d*<sub>6</sub>-DMSO): δ 12.88 (br s, 1H), 8.54 (s, 1H), 8.16 (dd, *J* = 8.8, 1.2 Hz, 1H), 7.88 (d, *J* = 8.8 Hz, 1H), 7.80 (d, *J* = 8.8 Hz, 1H), 7.65 (d, *J* = 8.0 Hz, 1H), 7.52 (d, *J* = 8.0 Hz, 1H), 7.32 (dd, *J* = 8.8, 2.4 Hz, 1H), 7.21-7.17 (m, 2H), 7.08 (d, *J* = 2.4 Hz, 1H), 4.88 (s, 2H), 3.17 (s, 3H), 2.80 (s, 4H). <sup>13</sup>C NMR (100 MHz,

*d*<sub>6</sub>-DMSO):  $\delta$  173.3, 172.4, 152.5, 148.6, 135.9, 129.9, 127.1, 126.3, 124.8, 123.8, 122.5, 116.9, 116.8, 105.9, 54.1, 26.1 ppm. HRMS (FAB+): *m/z* calcd for [C<sub>24</sub>H<sub>20</sub>O<sub>4</sub>N<sub>4</sub>+H<sup>+</sup>]: 429.1559, found: 429.1557

**Compound C.** Compound **A** (0.15 g, 0.45 mmol), **B** (0.12 g, 0.54 mmol) and hydroxybenzotriazole (HOBt, 0.07 g, 0.54 mmol) were dissolved in dry DMF (15 mL). To this mixture *N,N*-diisopropylethylamine (DIEA, 0.06 g, 0.08 mL, 0.45 mmol) was added and the reaction mass was stirred under nitrogen atmosphere for 10 min. After stirring for 1 h, (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP, 0.28 g, 0.54 mmol) was added to the reaction mass and the reaction mass was allowed to stirring for 12 h under nitrogen atmosphere. The solvent was evaporated and the crude was purified by column chromatography using 30 % hexane in ethyl acetate as an eluent to give **C** as a yellow solid. Yield: 0.14 g (58 %); <sup>1</sup>H NMR (*d*<sub>6</sub>-DMSO):  $\delta$  12.82 (br s, 1H), 8.49 (s, 1H), 8.11 (dd, *J* = 8.8, 1.2 Hz, 1H), 7.93 (t, *J* = 5.6 Hz, 1H, amide-NH), 7.81 (d, *J* = 8.8 Hz, 1H), 7.74 (d, *J* = 8.8 Hz, 1H), 7.76-7.46 (m, 2H), 7.37-7.26 (m, 5H), 7.17-7.12 (m, 3H), 6.92 (s, 1H), 5.03 (s, 2H), 4.02 (s, 2H), 3.11 (s, 3H), 3.05 (q, *J* = 6.4 Hz, 2H), 2.27 (t, *J* = 7.2 Hz, 2H), 1.53-1.45 (m, 2H), 1.41-1.34 (m, 2H), 1.24-1.16 (m, 2H).

**Compound D.** Compound **C** (0.14 g, 0.26 mmol) and 10% Pd/C (0.08g, 0.79 mmol) in EtOH (10mL) were stirred under hydrogen atmosphere for 18 h at room temperature. The reaction mixture was filtered and washed with EtOH, and the solvent was removed in vacuo. Yield: 0.08 g (69 %); <sup>1</sup>H NMR (*d*<sub>6</sub>-DMSO):  $\delta$  12.82 (br s, 1H), 8.51 (s, 1H), 8.12 (dd, *J* = 8.8, 1.2 Hz, 1H), 7.97 (t, *J* = 5.6 Hz, 1H, amide-NH), 7.81 (d, *J* = 8.8 Hz, 1H), 7.73 (d, *J* = 8.8 Hz, 1H), 7.59-7.51 (m, 2H), 7.17-7.12 (m, 3H), 6.92 (s, 1H), 4.03 (s, 2H), 3.11 (s, 3H), 3.05 (q, *J* = 6.4 Hz, 2H), 2.11 (t, *J* = 7.2 Hz, 2H), 1.48-1.42 (m, 2H), 1.40-1.34 (m, 2H), 1.25-1.17 (m, 2H).

**BHS2.** A solution of **D** (0.08 g, 0.18 mmol) in 10 mL DMF with trimethylamine (0.04 ml, 0.27 mmol) was added dropwise and the mixture was stirred at room temperature for 15 min under nitrogen atmosphere. To this mixture, *N,N'*-disuccinimidyl carbonate (DSC, 0.07 g, 0.27 mmol) was added and the mixture was stirred at room temperature for 2 h. The mixture was poured into H<sub>2</sub>O and extracted with CH<sub>2</sub>Cl<sub>2</sub>. The organic layer was washed with H<sub>2</sub>O and brine, and then the organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under vacuo to obtain the crude. The crude product was triturate with Hexane: CH<sub>2</sub>Cl<sub>2</sub> (95:5) to get yellow solid. Yield 0.75 g (77 %); M.p. 142-147 °C; <sup>1</sup>H NMR (*d*<sub>6</sub>-DMSO):  $\delta$  12.82 (br s, 1H), 8.50 (s, 1H), 8.11 (dd, *J* = 8.8, 1.2 Hz, 1H), 7.95 (t, *J* = 5.6 Hz, 1H, amide-NH), 7.82 (d, *J* = 8.8 Hz, 1H), 7.74 (d, *J* = 8.8 Hz, 1H), 7.62-7.48 (m, 2H), 7.17-7.13 (m, 3H), 6.92 (s,

1H), 4.03 (s, 2H), 3.11 (s, 3H), 3.07 (q,  $J = 6.4$  Hz, 2H), 2.78 (s, 4H), 2.59 (t,  $J = 7.2$  Hz, 2H), 1.64-1.54 (m, 2H), 1.46-1.38 (m, 2H), 1.35-1.27 (m, 2H).  $^{13}\text{C}$  NMR (100 MHz,  $d_6$ -DMSO):  $\delta$  174.9, 173.2, 170.7, 169.6, 169.4, 152.4, 148.8, 135.9, 129.8, 127.0, 126.4, 124.8, 123.4, 122.6, 117.0, 105.9, 56.4, 30.9, 29.7, 29.4, 26.7, 26.2, 26.1, 25.1, 24.8. ppm. HRMS (FAB<sup>+</sup>):  $m/z$  calcd for  $[\text{C}_{30}\text{H}_{31}\text{O}_5\text{N}_5+\text{H}^+]$ : 542.2395, found: 542.2398

**Compound F.** Compound **A** (0.15 g, 0.45 mmol), **E** (0.17 g, 0.54 mmol) and hydroxybenzotriazole (HOBt, 0.07 g, 0.54 mmol) were dissolved in dry DMF (15 mL). To this mixture *N,N*-diisopropylethylamine (DIEA, 0.06 g, 0.08 mL, 0.45 mmol) was added and the reaction mass was stirred under nitrogen atmosphere for 10 min. After stirring for 1 h, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 0.28 g, 0.54 mmol) was added to the reaction mass and the reaction mass was allowed to stirring for 12 h under nitrogen atmosphere. The solvent was evaporated and the crude was purified by column chromatography using 5 % methanol in chloroform as an eluent to give **F** as a yellow solid. Yield: 0.16 g (57 %);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  8.49 (s, 1H), 8.16 (dd,  $J = 8.0, 1.6$  Hz, 1H), 7.78 (d,  $J = 9.2$  Hz, 1H), 7.73 (d,  $J = 8.4$  Hz, 1H), 7.66 (br s, 1H, amide-NH), 7.28-7.44 (m, 3H), 7.07 (dd,  $J = 9.2, 2.4$  Hz, 1H), 6.94 (d,  $J = 2.4$  Hz, 1H), 6.83 (s, 1H), 4.88 (br s, 1H), 4.05, (s, 2H), 3.74 (t,  $J = 6.4$  Hz, 2H), 3.61-3.58 (m, 2H), 3.51-3.48 (m, 2H), 3.47-3.42 (m, 4H), 3.36-3.28 (m, 4H), 3.22-3.18 (m, 2H), 3.17 (s, 3H), 3.07 (t,  $J = 5.6, 5.2$  Hz, 2H), 2.52 (t,  $J = 6.4$  Hz, 2H), 1.41 (s, 9H).

**Compound G.** A solution of **F** (0.16 g, 0.25 mmol) in 5 mL  $\text{CH}_2\text{Cl}_2$  was added  $\text{CF}_3\text{CO}_2\text{H}$  (2 mL) and the mixture was stirred for 3 h. The solvent was removed under vacuo and the residue co-distilled three times with toluene (10 mL). Yield: 0.12 g (83 %);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  8.19 (s, 1H), 7.61 (d,  $J = 8.0$  Hz, 1H), 7.49 (d,  $J = 9.2$  Hz, 1H), 7.38-7.35 (m, 2H), 7.20 (d,  $J = 8.8$  Hz, 1H), 7.14 (br s, 1H, amide-NH), 7.10-7.07 (m, 2H), 6.83 (dd,  $J = 9.2, 2.4$  Hz, 1H), 6.46 (s, 1H), 4.02, (s, 2H), 3.77 (t,  $J = 5.6$  Hz, 2H), 3.58-3.55 (m, 2H), 3.52-3.49 (m, 4H), 3.48-3.39 (m, 6H), 3.36-3.29 (m, 4H), 3.09 (s, 3H), 2.65 (t,  $J = 5.6$  Hz, 2H).

**BHS3.** A solution of **G** (0.15 g, 0.21 mmol) in 10 mL DMF with trimethylamine (0.04 ml, 0.31 mmol) was added dropwise and the mixture was stirred at room temperature for 15 min under nitrogen atmosphere. To this mixture, *N,N'*-disuccinimidyl carbonate (DSC, 0.08 g, 0.31 mmol) was added and the mixture was stirred at room temperature for 2 h. The mixture was poured into  $\text{H}_2\text{O}$  and extracted with  $\text{CH}_2\text{Cl}_2$ . The organic layer was washed with  $\text{H}_2\text{O}$  and brine, and then the organic layer was dried over

anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under vacuo to obtain the crude. The crude product was triturated with Hexane: CH<sub>2</sub>Cl<sub>2</sub> (95:5) to get yellow solid. Yield 0.91 g (65 %); M.p. 131-136 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 8.45 (s, 1H), 8.12 (dd, *J* = 8.8, 2.0 Hz, 1H), 7.79 (d, *J* = 9.2 Hz, 1H), 7.74 (d, *J* = 8.8 Hz, 1H), 7.66 (br s, 1H, amide-NH), 7.29-7.25 (m, 3H), 7.07 (dd, *J* = 9.2, 2.8 Hz, 1H), 6.95 (d, *J* = 2.4 Hz, 1H), 6.89 (s, 1H), 4.04, (s, 2H), 3.78 (t, *J* = 6.4 Hz, 2H), 3.59-3.55 (m, 2H), 3.53-3.50 (m, 2H), 3.48-3.44 (m, 4H), 3.42-3.39 (m, 2H), 3.37-3.28 (m, 4H), 3.19 (d, *J* = 5.2 Hz, 2H), 3.17 (s, 3H), 2.81 (t, *J* = 6.4 Hz, 2H), 2.78 (s, 4H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub> contained 2-drops of CD<sub>3</sub>OD): δ 174.5, 170.8, 152.3, 152.1, 147.9, 138.3, 135.6, 130.2, 127.1, 126.9, 126.7, 126.4, 124.4, 122.9, 122.8, 116.3, 114.8, 106.7, 70.5, 70.4, 70.3, 69.9, 67.2, 57.9, 40.2, 39.3, 35.7, 33.5, 32.2, 30.0, 23.0, 14.5 ppm. HRMS (FAB<sup>+</sup>): *m/z* calcd for [C<sub>35</sub>H<sub>41</sub>O<sub>9</sub>N<sub>5</sub>+H<sup>+</sup>]: 676.2974, found: 676.2977

**Conjugation of EGF with BHS1-3.** The probe stock solutions were prepared by dissolving 10 mg of each fluorescence dye in 1 mL absolute, amine-free DMF. The EGF stock solution was prepared by dissolving EGF powder (Sigma Aldrich, Cat No. E9644) in 10 mM acetic acid at a concentration of 2 mg/mL. 50 μL of the EGF stock solution was mixed with 450 μL of a HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.8), and then 200 μL of the probe stock solution was added slowly into the diluted EGF solution. The reactions continued for 3 hours at room temperature. The conjugated EGF products were purified by using a size exclusion chromatography of Superdex 75 10/300 GL (GE Healthcare). The purified conjugate was concentrated via ultrafiltration using Centricon (MWCO: 3000, Millipore), and the concentrations of protein and dye were determined using reported formulas.<sup>4</sup>

$$\text{EGF concentration (M)} = \frac{A_{280} - (A_{\text{max}} \times \text{CF})}{\epsilon}$$

$$\text{Ratio of BHS3 to EGF} = \frac{A_{\text{max}} \text{ of the labeled protein}}{\epsilon' \times \text{protein concentration (M)}}$$

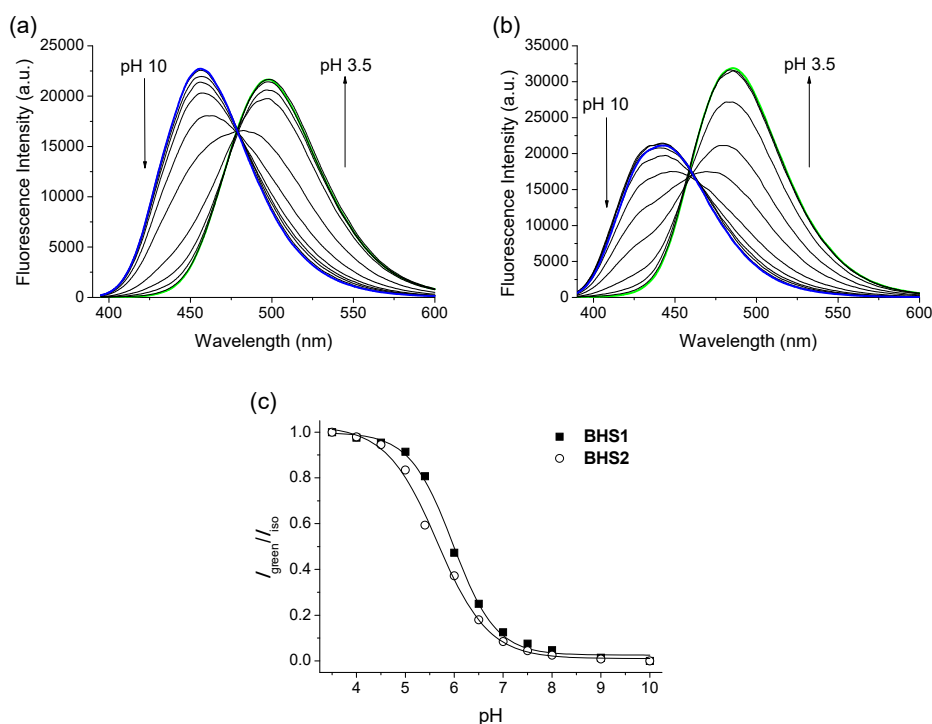
$\epsilon$  (=18835 M<sup>-1</sup>cm<sup>-1</sup>) and  $\epsilon'$  (=23700 M<sup>-1</sup>cm<sup>-1</sup> at pH 7.2) are the molar extinction coefficient of EGF and BHS3 respectively;  $A_{280}$  is absorbance of the BHS3-EGF solution at 280 nm;  $A_{\text{max}}$  is absorbance of the BHS3-EGF solution at  $\lambda_{\text{max}}$  (= 343nm at pH 7.2) for BHS3; and CF (correction factor) equals the  $A_{280}$  of BHS3 divided by the  $A_{\text{max}}$  of BHS3. Similar methods were used for synthesis of BHS1-EGF and BHS2-EGF.

**Spectroscopic Measurements.** Absorption spectra were recorded on a S-3100 UV-Vis spectrophotometer and fluorescence spectra were obtained with FluoroMate FS-2 fluorescence spectrophotometer with a 1 cm standard quartz cell. The fluorescence quantum yield was determined by using 9,10-diphenylanthracene ( $\Phi = 0.93$  in cyclohexane) as the reference by the literature method.<sup>5</sup>

**pK<sub>a</sub> Value.** A 3.0  $\mu$ L of the stock solution of probe in DMSO ( $1.0 \times 10^{-3}$  M) was added to a cuvette containing 3.0 mL of universal buffer solution by using a micro syringe to prepare 1.0  $\mu$ M of probe solution and the spectral changes in the fluorescence were measured as a function of the pH (3.5-10.0). pK<sub>a</sub> values were calculated by linear regression analysis of the fluorescence data to fit Eq (1).

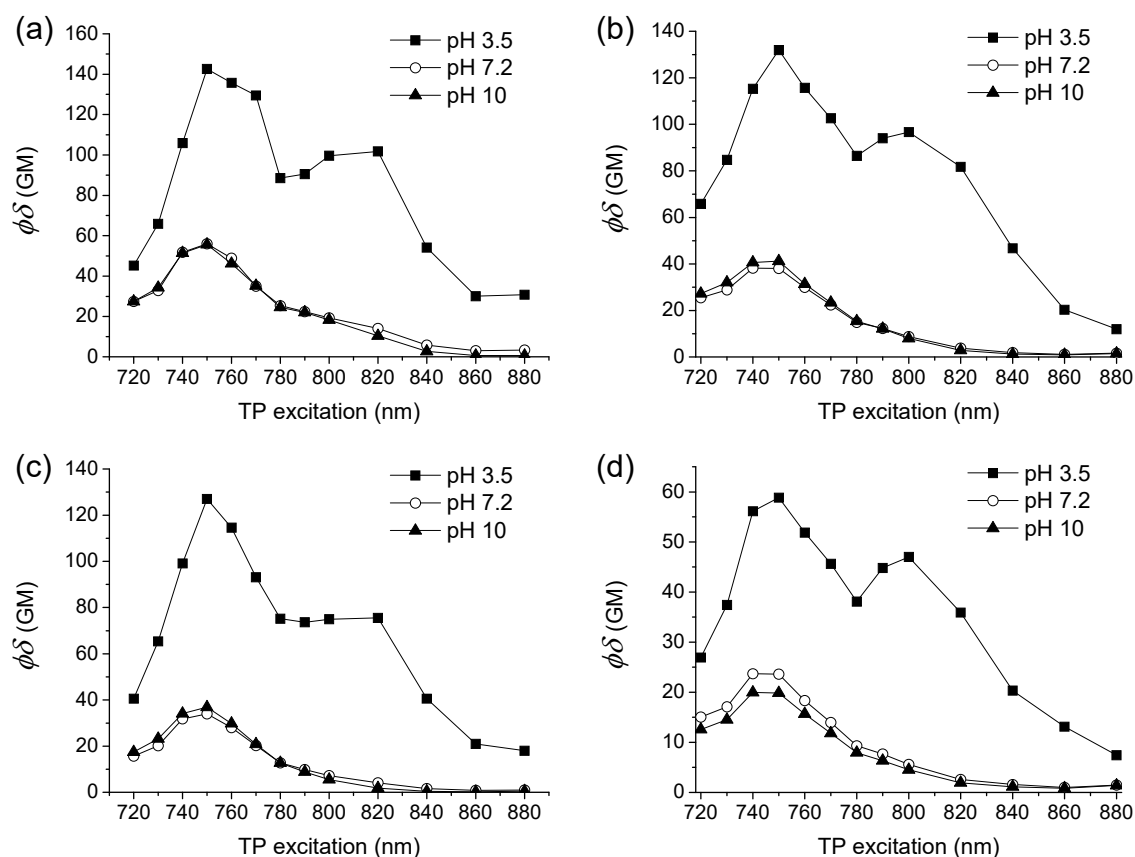
$$\text{pH} = \text{pK}_a + c \left[ \log \frac{R - R_{\min}}{R_{\max} - R} \right] + \log \frac{I^a}{I^b} \quad (1)$$

where R is the observed ratios ( $I_{\text{green}}/I_{\text{iso}}$ ) at isoemission point ( $I_{\text{iso}}$ ) and 500-550 nm ( $I_{\text{green}}$ ) at a given pH.  $R_{\max}$  and  $R_{\min}$  are maximum and minimum limiting value of R, respectively, and c is the slope.<sup>6</sup>  $I^a/I^b$  is the ratio of the fluorescent intensity in acid (pH 3.5) to the intensity in base (pH 10.0) at the wavelength chosen for the denominator of R. In this case, this correction vanishes by using the sharp isoemission point.



**Figure S1.** The changes in one-photon fluorescence spectra of (a) **BHS1** and (b) **BHS2** with pH in universal buffer. The excitation wavelengths were (a) 365 and (b) 368 nm, respectively. (c) Plots of  $I_{\text{green}}/I_{\text{iso}}$  versus pH for **BHS1** and **BHS2** determined by one-photon mode.

**Measurement of Two-Photon Cross Section.** The two-photon cross section ( $\delta$ ) was determined by using femto second (fs) fluorescence measurement technique as described.<sup>7</sup> Probe ( $1.0 \times 10^{-6}$  M) was dissolved in universal buffer solutions at pH = 3.5, 7.2 and 10, respectively, and the two-photon induced fluorescence intensity was measured at 720–880 nm by using rhodamine 6G as the reference, whose two-photon property has been well characterized in the literature.<sup>8</sup> The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by using  $\delta = \delta_r(S_s\Phi_r\phi_r c_r)/(S_r\Phi_s\phi_s c_s)$ : where the subscripts  $s$  and  $r$  stand for the sample and reference molecules. The intensity of the signal collected by a CCD detector was denoted as  $S$ .  $\Phi$  is the fluorescence quantum yield.  $\phi$  is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as  $c$ .  $\delta_r$  is the TPA cross section of the reference molecule.



**Figure S2.** Two-photon action spectra of (a) BHS1, (b) BHS2, (c) BHS3, and (d) BHS3-EGF in universal buffer solutions at pH 3.5, 7.2, and 10, respectively. The estimated uncertainties for the two-photon action cross section values ( $\delta\Phi$ ) are  $\pm 15\%$ .



**Table S1.** Photophysical data for **BH1**, **BHS1**, **BHS2**, **BHS1-EGF** and **BHS2-EGF**.<sup>a</sup>

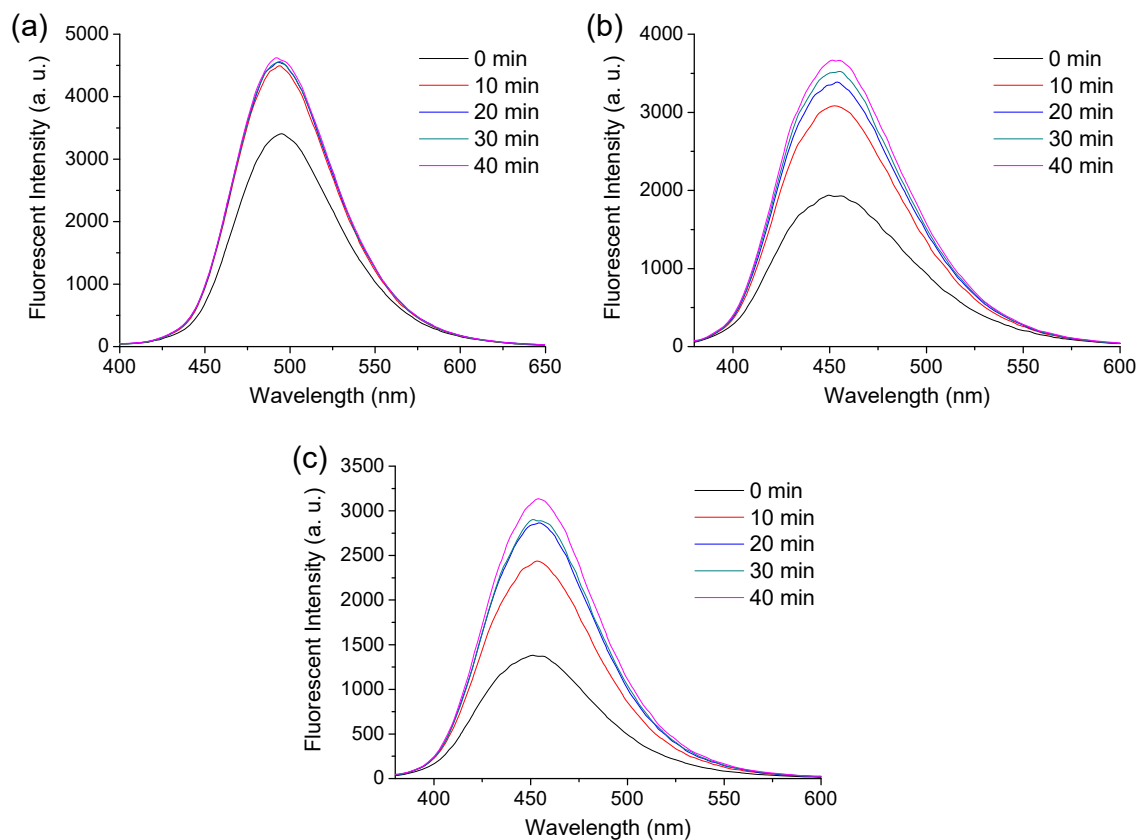
Probe	pH	$\lambda_{max}^{(1)}$ ( $10^{-4} \epsilon$ ) <sup>b</sup>	$\lambda_{max}^{fl}$ <sup>c</sup>	$\Phi$ <sup>d</sup>	pK <sub>a</sub> <sup>e</sup>	$\lambda_{max}^{(2)}$ <sup>f</sup>	$\delta$ <sup>g</sup>	$\Phi\delta$ <sup>h</sup>
<b>BH1</b>	3.5	368 (1.94)	494	0.76		750	185	140
	7.2	337 (2.53)	455	1.00	5.91	740	40	40
	10.0	337 (2.76)	453	1.00		740	35	35
<b>BHS1</b>	3.5	381 (2.07)	493	0.93		750	154	143
	7.2	345 (2.67)	458	1.00	5.97	750	56	56
	10.0	345 (2.91)	456	1.00		750	55	55
<b>BHS1-EGF</b>	3.5	375	494	n.d. <sup>i</sup>		n.d. <sup>i</sup>	n.d. <sup>i</sup>	n.d. <sup>i</sup>
	7.2	346	450	n.d. <sup>i</sup>	n.d. <sup>i</sup>	n.d. <sup>i</sup>	n.d. <sup>i</sup>	n.d. <sup>i</sup>
	10.0	347	450	n.d. <sup>i</sup>		n.d. <sup>i</sup>	n.d. <sup>i</sup>	n.d. <sup>i</sup>
<b>BHS2</b>	3.5	388 (2.18)	487	1.00		750	132	132
	7.2	342 (2.29)	442	1.00	5.65	740	38	38
	10.0	342 (2.42)	442	1.00		750	41	41
<b>BHS2-EGF</b>	3.5	386	488	n.d. <sup>i</sup>		n.d. <sup>i</sup>	n.d. <sup>i</sup>	n.d. <sup>i</sup>
	7.2	342	448	n.d. <sup>i</sup>	n.d. <sup>i</sup>	n.d. <sup>i</sup>	n.d. <sup>i</sup>	n.d. <sup>i</sup>
	10.0	341	445	n.d. <sup>i</sup>		n.d. <sup>i</sup>	n.d. <sup>i</sup>	n.d. <sup>i</sup>

<sup>a</sup>All the measurements were performed in the universal buffer solution (0.1 M citric acid, 0.1 M KH<sub>2</sub>PO<sub>4</sub>, 0.1 M Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, 0.1 M Tris, 0.1 M KCl). Data for **BH1** was taken from Ref. 1. <sup>b</sup> $\lambda_{max}$  of the one-photon absorption spectra in nm. The numbers in parentheses are molar extinction coefficients in M<sup>-1</sup>cm<sup>-1</sup>. <sup>c</sup> $\lambda_{max}$  of the one-photon emission spectra in nm. <sup>d</sup>Fluorescence quantum yield. <sup>e</sup>pK<sub>a</sub> values measured by one-photon mode. The values in parentheses are measured by two-photon mode. <sup>f</sup> $\lambda_{max}$  of the two-photon excitation spectra in nm. <sup>g</sup>The peak two-photon absorption cross-section in 10<sup>-50</sup> cm<sup>4</sup>/s/photon (GM). <sup>h</sup>The peak two-photon action cross-section in 10<sup>-50</sup> cm<sup>4</sup>/s/photon. <sup>i</sup>Not determined.

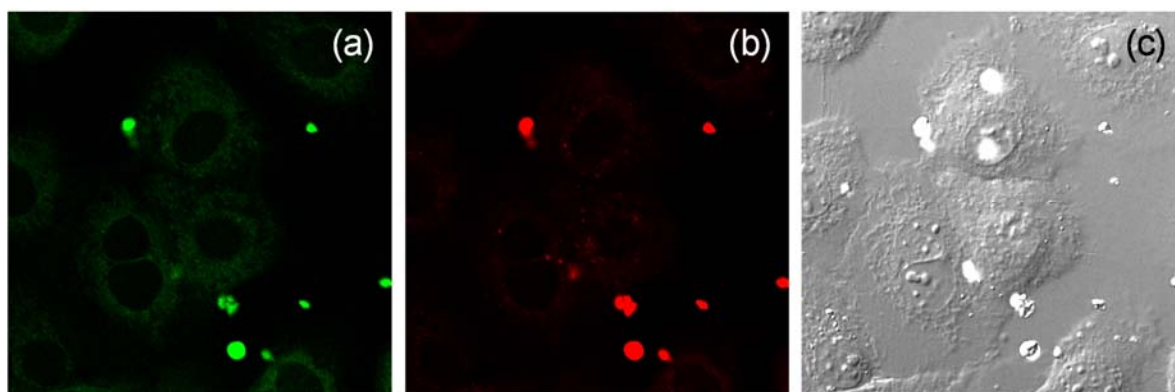
**Cell Culture.** A431 cells (ATCC, Manassas, VA, USA) were cultured in RPMI1640 (WelGene Inc, Seoul, Korea) supplemented with 10 % FBS (WelGene), penicillin (100 units/ml), and streptomycin (100  $\mu\text{g}/\text{mL}$ ). Cells were passed and plated on glass-bottomed dishes (NEST) before imaging for two days. All the cells were maintained in a humidified atmosphere of 5/95 (v/v) of  $\text{CO}_2/\text{air}$  at 37 °C.

**Western Blot.** A431 cells were washed with cold PBS and lysed with RIPAa buffer (1 % NP-40, 150 mL NaCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.2, 0.5 % sodium deoxycholate) containing protease inhibitors (2 mM PMSF, 10  $\mu\text{g}/\text{L}$  leupeptin, 10  $\mu\text{g}/\text{L}$  pepstatin, and 0.5 mM  $\text{NaVO}_3$ ). Proteins were separated by 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membrane was incubated with 5 % non-fat milk to block nonspecific antibody binding and then was incubated with phosphor-EGFR rabbit antibody, or anti-actin antibodies overnight at 4 °C. An anti-rabbit HRP was used as the secondary antibody, and visualized using an enhanced chemiluminescence (ECL) system.

**Two-Photon Fluorescence Microscopy.** Two-photon fluorescence microscopy images of probe-labeled cells and tissues were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP8 MP) with  $\times 10$  dry,  $\times 40$  oil and  $\times 100$  oil objectives, numerical aperture (NA) = 0.30, 1.30 and 1.30, respectively. The two-photon fluorescence microscopy images were obtained with a DMI6000B Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Mai Tai HP; Spectra Physics, 80 MHz pulse frequency, 100 fs pulse width) set at wavelength 750 nm and output power 2510 mW, which corresponded to approximately 2.1 mW average power in the focal plane. To obtain images at 440-460 nm (IR) and 500-550 nm (green) range, internal PMTs were used to collect the signals in an 8 bit unsigned  $512 \times 512$  and  $1024 \times 1024$  pixels at 400 and 200 Hz scan speed, respectively. Ratiometric image processing and analysis was carried out using MetaMorph software.

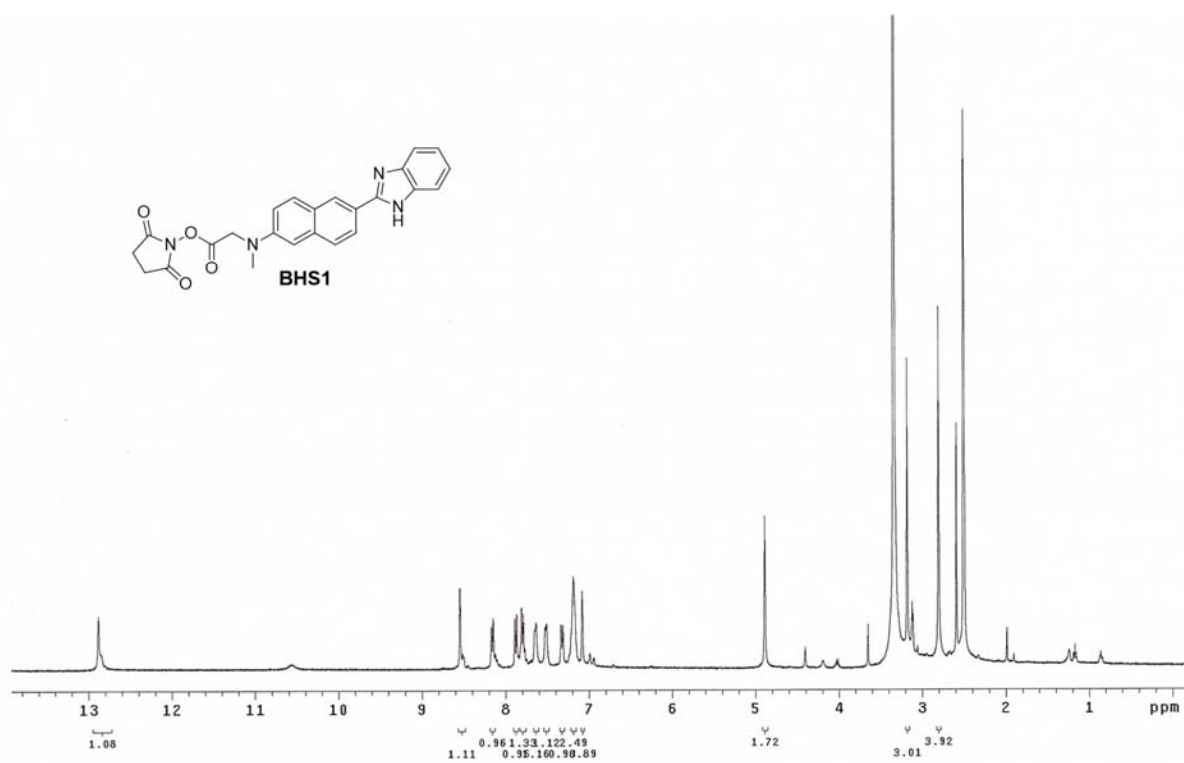


**Figure S3.** The fluorescence spectra of **BHS1-EGF** in pH (a) 3.5, (b) 7.2 and (c) 10. The excitation wavelengths were (a) 375, (b) 346 and (b) 347 nm, respectively.

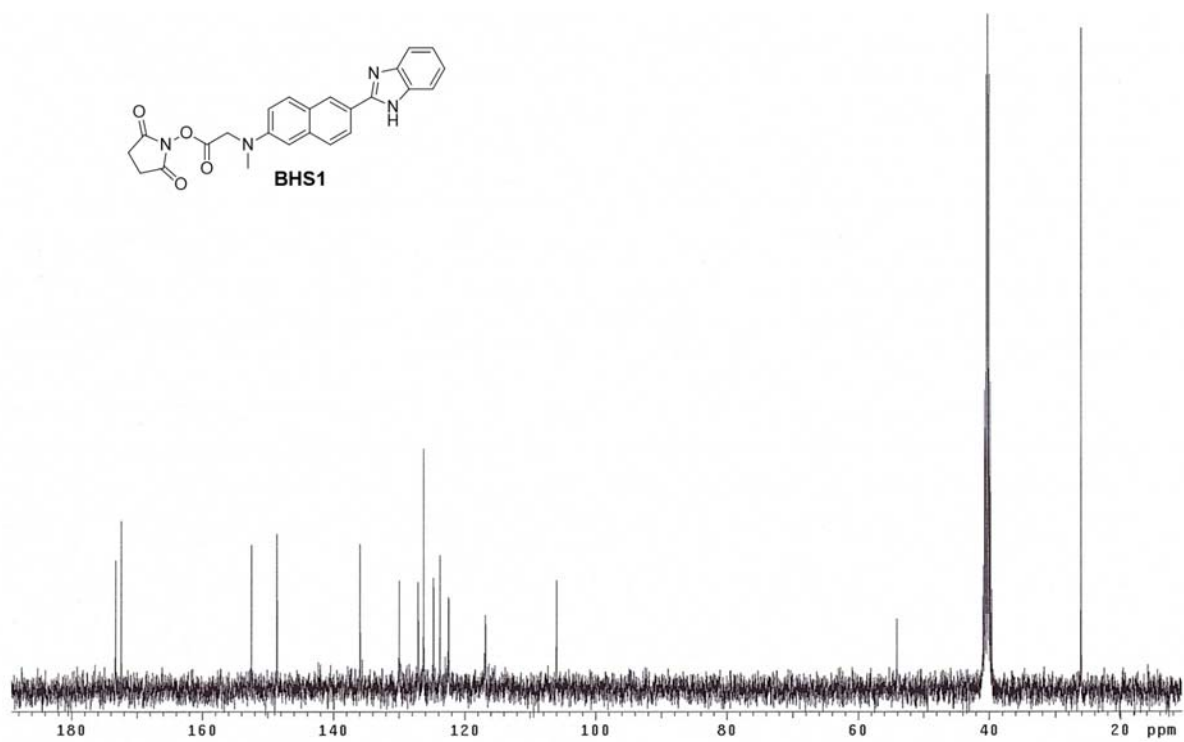


**Figure S4.** TPM images of A431 cells stained with  $0.5 \mu\text{M}$  **BHS2-EGF** ( $[\text{EGF}] = 470 \text{ nM}$ ). The TPEF were collected at (a) 440-460 ( $I_{\text{IR}}$ ) and (b) 500-550 nm ( $I_{\text{green}}$ ) upon excitation at 750 nm with fs pulses. (c) DIC image.

$^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$  and HRMS of **BHS1**, **BHS2**, and **BHS3**



**Figure S5.**  $^1\text{H-NMR}$  spectrum (400 MHz) of **BHS1** in  $d_6$ -DMSO.



**Figure S6.**  $^{13}\text{C-NMR}$  spectrum (100 MHz) of **BHS1** in  $d_6$ -DMSO.

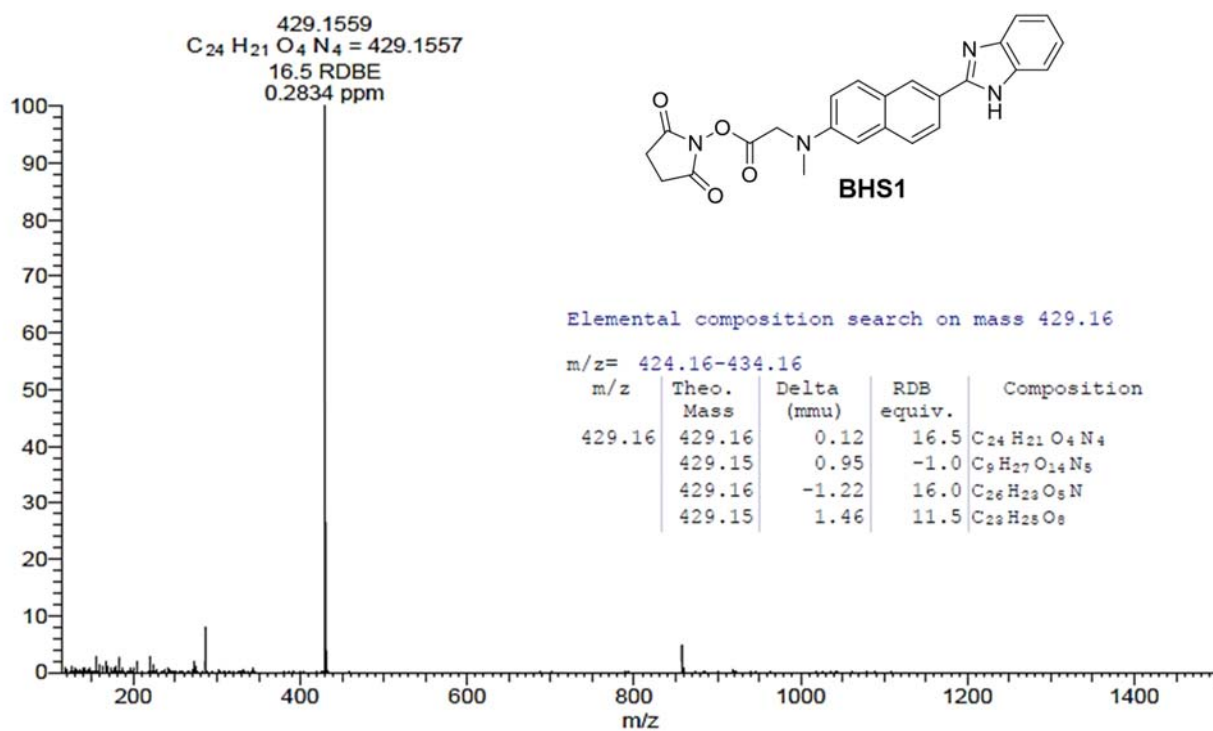


Figure S7. HRMS spectrum of **BHS1**.

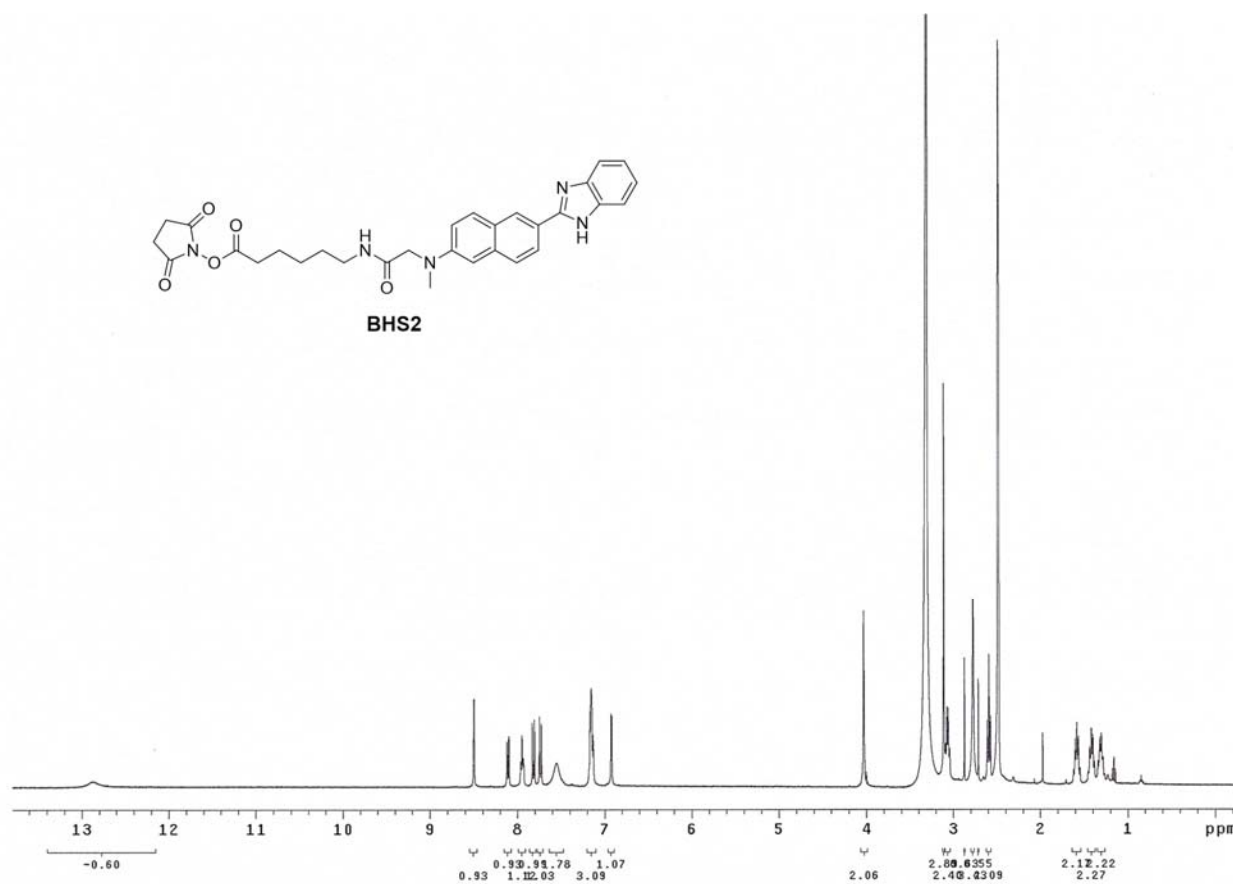
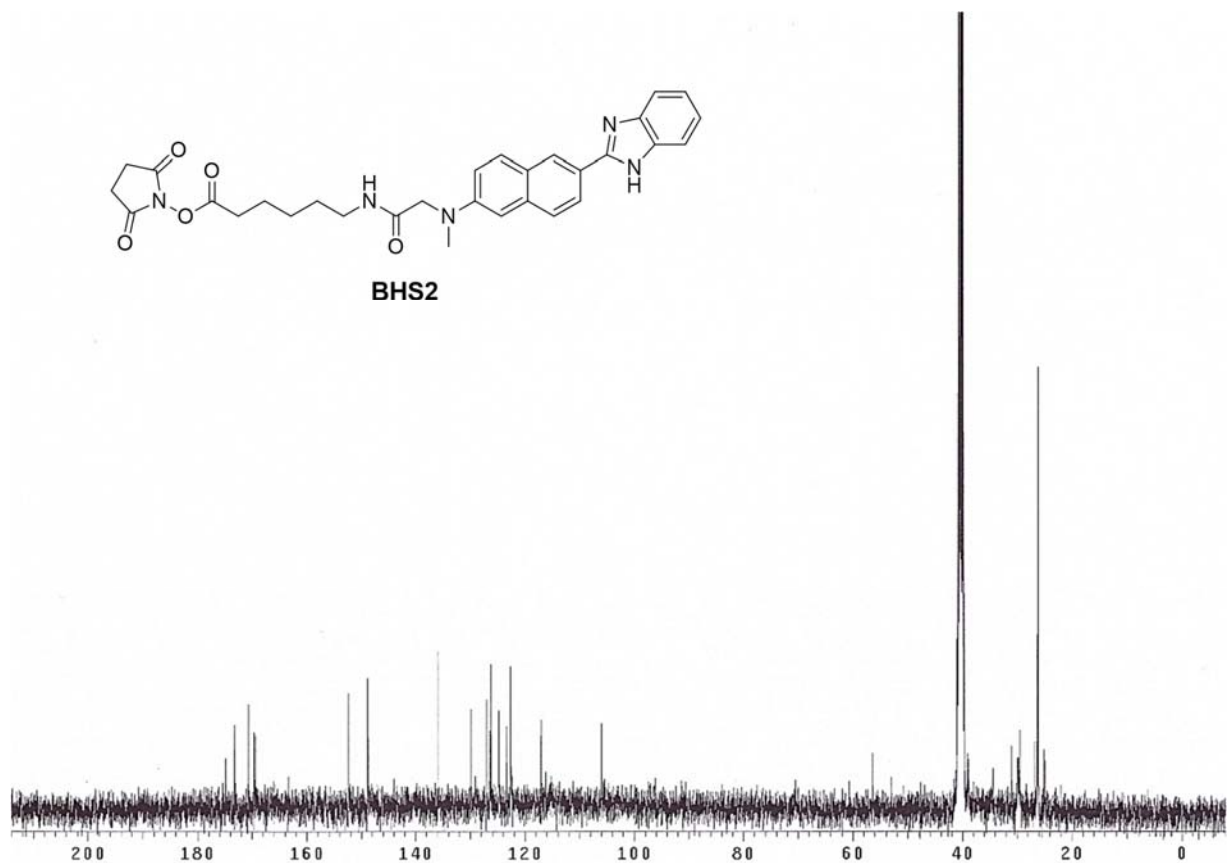
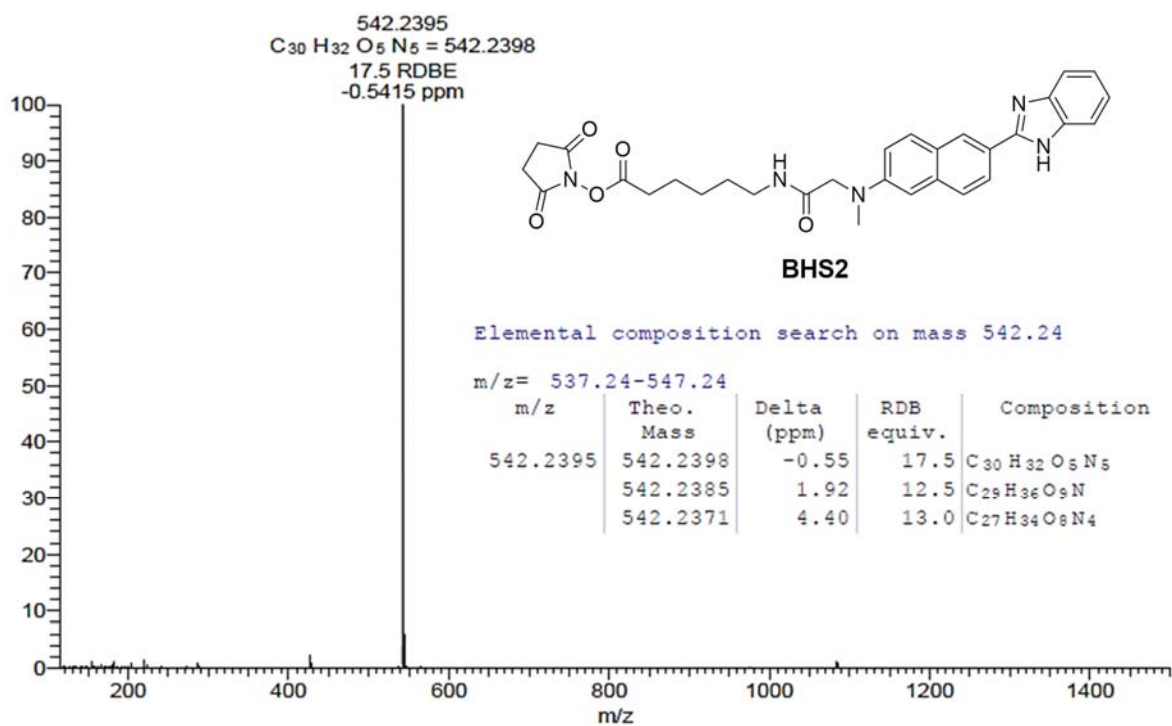


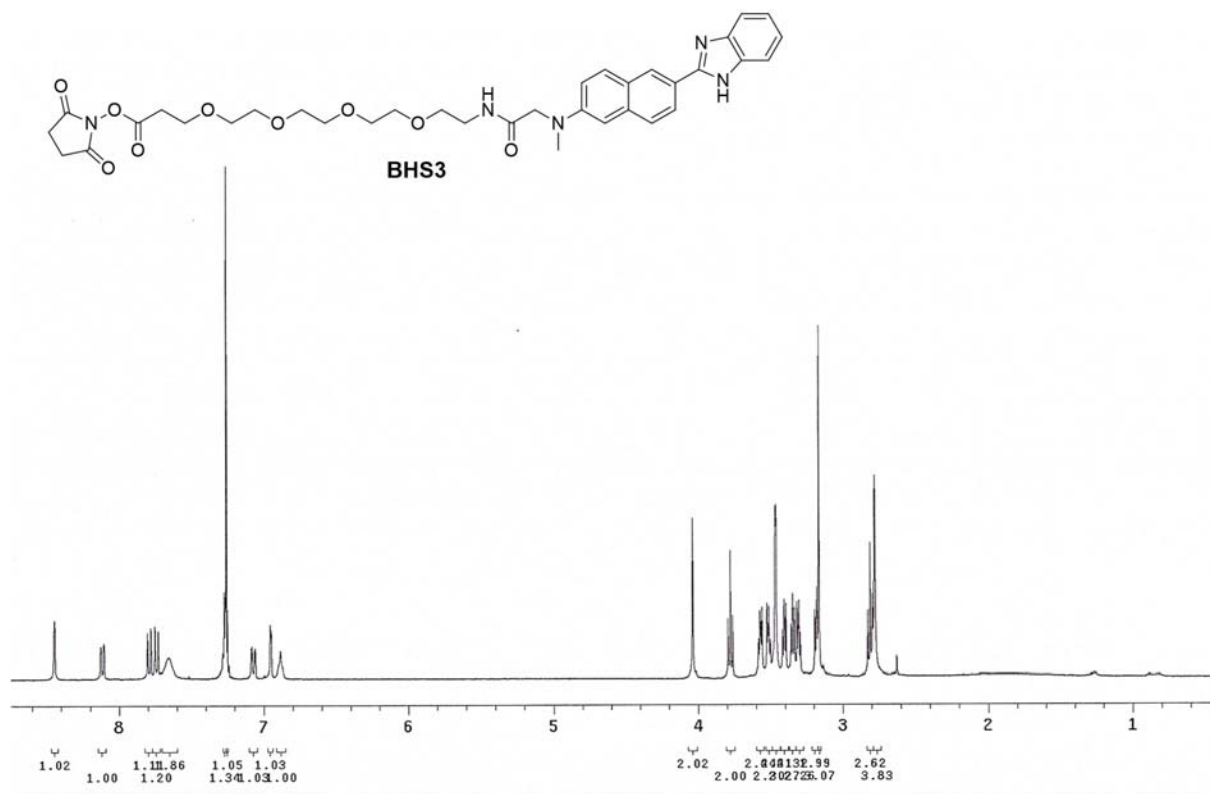
Figure S8.  $^1H$ -NMR spectrum (400 MHz) of **BHS2** in  $d_6$ -DMSO.



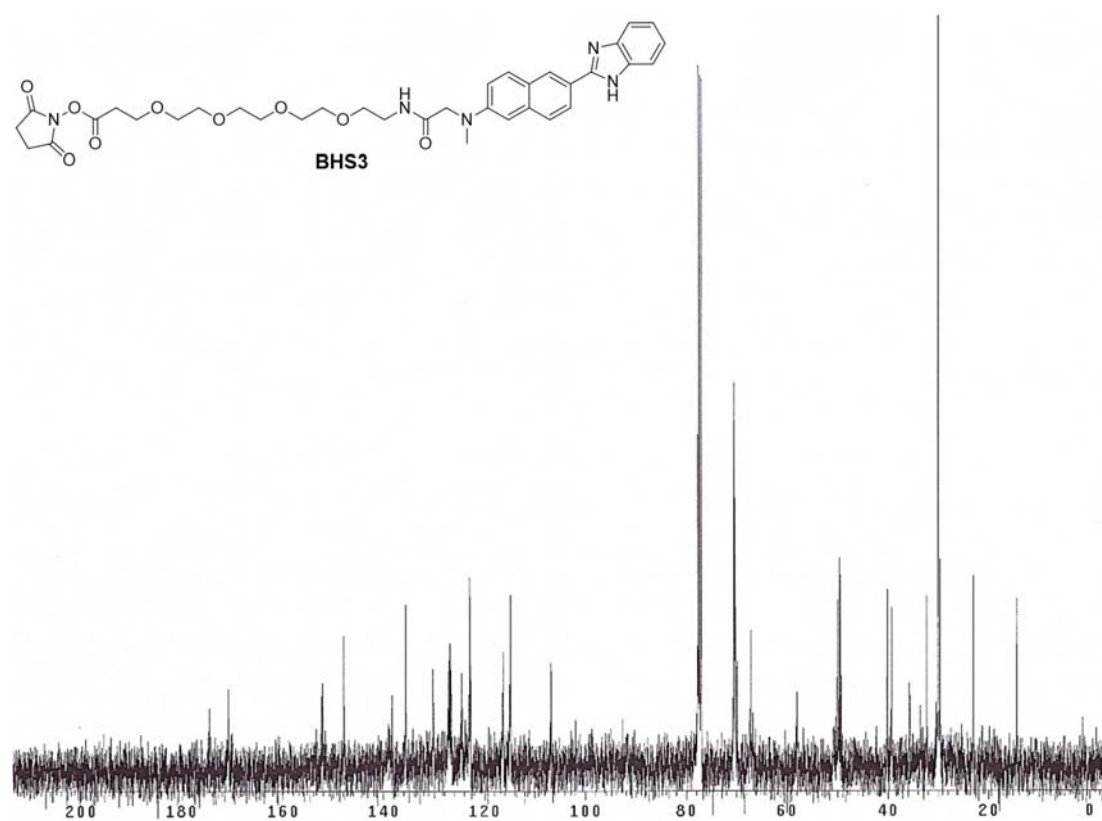
**Figure S9.**  $^{13}\text{C}$ -NMR spectrum (100 MHz) of **BHS2** in  $d_6$ -DMSO.



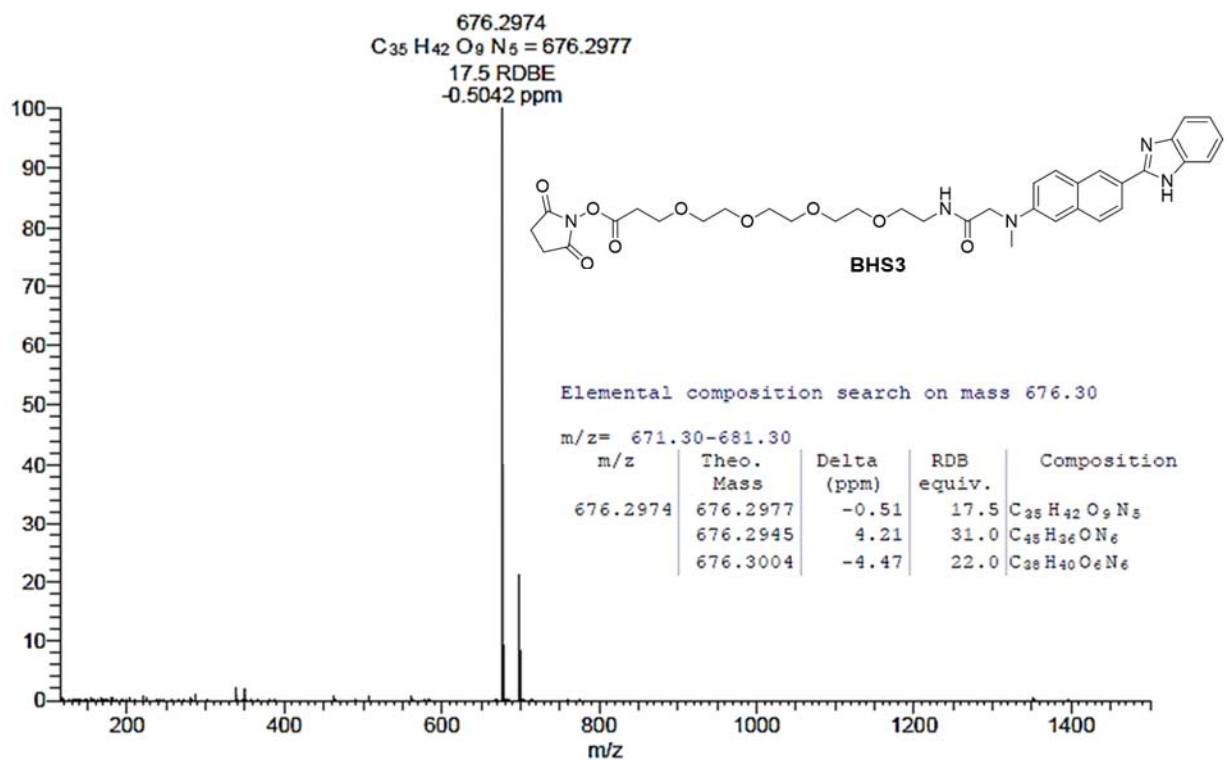
**Figure S10.** HRMS spectrum of **BHS2**.



**Figure S11.**  $^1\text{H-NMR}$  spectrum (400 MHz) of **BHS3** in  $\text{CDCl}_3$ .



**Figure S12.**  $^{13}\text{C-NMR}$  spectrum (100 MHz) of **BHS3** in  $\text{CDCl}_3$  contained 2-drops of  $\text{CD}_3\text{OD}$ .



**Figure S13.** HRMS spectrum of **BHS3**.



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