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Supporting Information

A Wash-Free SNAP-tag Fluorogenic Probe based on Additive Effects of Quencher Release and Environmental Sensitivity

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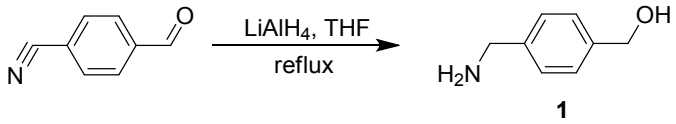
CONTENTS:

1. Materials and instruments

General Chemicals were purchased from Sigma-Aldrich, Aladdin, Adamas and used without further purification. Solvents (DMSO, DMF, tetrahydrofuran, dichloromethane, hexane, ethyl acetate, ethyl alcohol, acetone and methanol) were from Sigma-Aldrich and used without further treatment or distillation. *E. coli* strain BL21 and Trans 5α were purchased from Lucigen. Ampicillin (Amp) and isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Sigma-Aldrich. Ni-NTA agarose and Q Sepharose™ Fast Flow were from GE Healthcare. All water used was from a Millipore water purification system with a minimum resistivity of 18.0 MΩ · cm.

^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker 400 spectrometer with Chemical shifts reported in ppm and coupling constants (*J*) reported in Hz. Mass spectrometry data were performed on a HP1100LC/MSD mass spectrometer and a LC/Q-TOF MS spectrometer. UV-vis absorption spectra were obtained on an Agilent Cary 60 UV-Vis Spectrophotometer. Fluorescence measurements were performed on a VAEIAN CARY Eclipse fluorescence spectrophotometer equipped with a Single Cell Peltier temperature controller. The fluorescence imaging was performed by using ANDOR™ living cell laser scanning confocal microscope (Revolution WD).

2. Synthesis of Compounds



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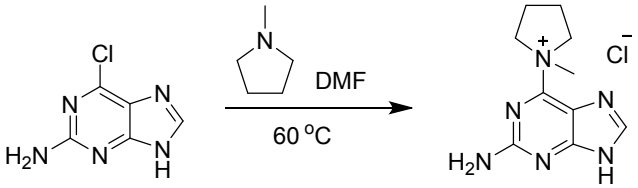
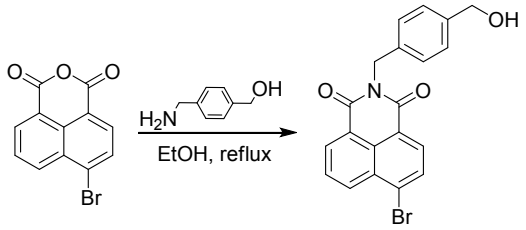
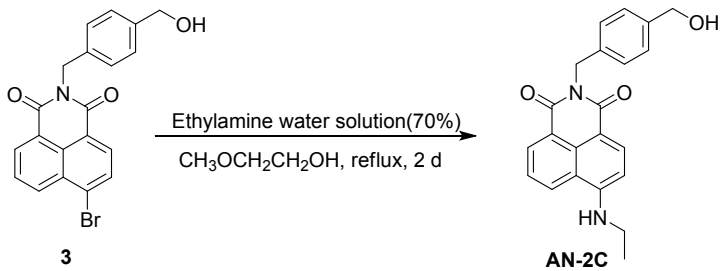
Compound 1. 4-cyanobenzaldehyde (2.85 g, 21.7 mmol) in 20 mL dry THF was added to a solution of LiAlH4 (3.45 g, 87 mmol) in 80 mL THF in ice bath. Then, the solution was slowly heated to reflux. After 10 h, the reaction was quenched with 5 mL water. The mixture was filtered on a pad of celite (washed with EtOAc). Concentration in vacuo gave 4-(aminomethyl) benzyl alcohol as a white (2.94 g, 97%) solid without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 (d, *J* = 8.2 Hz, 2H), 7.30 (d, *J* = 8.2 Hz, 2H), 4.68 (s, 2H), 3.86 (s, 2H), 1.62 (s, 3H).



Compound 2. 2.8 mL 1-methylpyrrole (26.4 mmol) was added to a solution of 6-chloropurine (2.0 g, 11.8 mmol) in 80 mL DMF. Then, the solution was heated to 60 °C overnight. The reaction was cooled to room temperature and filtered on a pad of celite (washed with EtOAc). Concentration in vacuo gave compound **2** as white solid (1.62 g, 52%) solid without further purification. ^{1}H NMR (400 MHz, DMSO) δ 13.41 (s, 1H), 8.35 (s, 1H), 7.12 (s, 2H), 4.67 – 4.48 (m, 2H), 4.03 – 3.89 (m, 2H), 3.65 (s, 3H), 2.30 – 2.19 (m, 2H), 2.06 (dd, *J* = 7.4, 4.7 Hz, 2H).

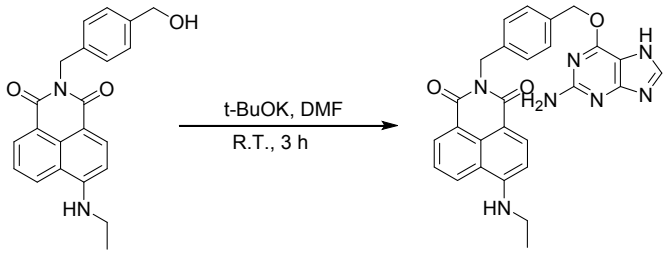


Compound 3. A solution of 4-bromo-1,8-naphthalic anhydride (2.77 g, 10 mmol) in 100 mL EtOH was added 4-(aminomethyl)benzyl alcohol (1.37 g, 10 mmol). The mixture was heated to reflux for 6 h. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography (DCM:MeOH=100:1). The product was obtained as white solid 3.08 g, yield 78%.^{1}H NMR (400 MHz, DMSO) δ 8.57 (d, *J* = 7.2 Hz, 1H), 8.53 (d, *J* = 8.5 Hz, 1H), 8.33 (d, *J* = 7.9 Hz, 1H), 8.20 (d, *J* = 7.9 Hz, 1H), 7.98 (t, *J* = 7.9 Hz, 1H), 7.33 (d, *J* = 7.9 Hz, 2H), 7.25 (d, *J* = 8.0 Hz, 2H), 5.22 (s, 2H), 5.14 (t, *J* = 5.7 Hz, 1H), 4.44 (d, *J* = 5.6 Hz, 2H).



Compound AN-2C. compound **3** (200 mg, 0.51 mmol) in 5 mL 2-methoxyethanol was added to 100 μL ethylamine water solution under N_2. The mixture was slowly heated to 120 °C for 2 d. The solvent was removed under reduced pressure, and the residue was purified by flash column

chromatography (DCM:MeOH=80:1). The product was obtained as yellow solid 150 mg, yield 82%.^{1}H NMR (400 MHz, $CDCl_3$) δ 8.59 (d, *J* = 7.3 Hz, 1H), 8.48 (d, *J* = 8.4 Hz, 1H), 8.09 (d, *J* = 8.3 Hz, 1H), 7.64 – 7.58 (m, 1H), 7.54 (d, *J* = 8.0 Hz, 2H), 7.29 (d, *J* = 8.0 Hz, 2H), 6.73 (d, *J* = 8.4 Hz, 1H), 5.36 (s, 2H), 5.19 (s, 1H), 4.64 (d, *J* = 5.5 Hz, 2H), 3.46 (td, *J* = 12.3, 7.2 Hz, 2H), 1.46 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 164.27, 163.38, 151.19, 141.67, 136.79, 135.03, 131.34, 129.99, 129.30, 127.82, 126.90, 124.73, 122.18, 120.59, 107.77, 104.30, 63.12, 42.73, 38.02, 14.13.



AN-2C **BGAN-2C**

Compound BGAN-2C. A solution of compound **AN-2C** (100 mg, 0.28 mmol), **2** (215 mg, 0.69 mmol) and t-BuOK (189 mg, 1.38 mmol) in 5 mL dry DMF was stirred at room temperature for 3 h under N_2. The solvent was then removed under reduced pressure, and the residue was purified by flash column chromatography (DCM: MeOH=20:1) to give yellow powder 100 mg. Yield 73%.^{1}H NMR (400 MHz, DMSO) δ 12.41 (s, 1H), 8.71 (d, *J* = 8.4 Hz, 1H), 8.44 (d, *J* = 7.2 Hz, 1H), 8.28 (d, *J* = 8.6 Hz, 1H), 7.79 (s, 2H), 7.67 (t, *J* = 7.8 Hz, 1H), 7.43 (d, *J* = 7.7 Hz, 2H), 7.35 (d, *J* = 7.9 Hz, 2H), 6.76 (d, *J* = 8.7 Hz, 1H), 6.28 (s, 1H), 5.43 (s, 2H), 5.23 (s, 2H), 3.42 (dd, *J* = 12.9, 6.6 Hz, 2H), 1.31 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 164.29, 163.38, 160.08, 151.21, 138.27, 135.89, 135.06, 131.38, 130.01, 129.32, 128.99, 128.03, 124.71, 122.15, 120.59, 107.74, 104.30, 66.94, 42.75, 38.02, 14.13.

Compound AN-8C. The synthetic strategy toward **AN-8C** is similar to the synthesis of **AN-2C** (Scheme S1), yield 71%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.31 (d, *J* = 14.7, 7.7 Hz, 1H), 7.84 (d, *J* = 7.9 Hz, 1H), 7.48 (d, *J* = 7.1 Hz, 2H), 7.34 – 7.28 (m, 1H), 7.26 (d, *J* = 6.8 Hz, 2H), 6.53 (d, *J* = 8.2 Hz, 1H), 5.40 (s, 1H), 5.27 (s, 2H), 4.61 (s, 2H), 3.29 (d, *J* = 3.9 Hz, 2H), 2.55 (s, 1H), 1.75 (s, 2H), 1.45 (s, 2H), 1.28 (s, 8H), 0.88 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.58, 164.06, 149.73, 140.17, 137.18, 134.62, 131.13, 129.57, 129.11, 126.99, 126.10, 124.38, 122.55, 119.92, 109.42, 104.14, 65.03, 43.73, 42.98, 31.81, 29.37, 29.25, 28.90, 27.22, 22.66, 14.13.

Compound BGAN-8C. The synthetic strategy toward **BGAN-8C** is similar to the synthesis of **BGAN-2C**, yield 68%. ^{1}H NMR (400 MHz, DMSO) δ 12.42 (s, 1H), 8.70 (d, *J* = 8.3 Hz, 1H), 8.42 (d, *J* = 7.1 Hz, 1H), 8.26 (d, *J* = 8.5 Hz, 1H), 7.79 (s, 2H), 7.66 (t, *J* = 7.7 Hz, 1H), 7.42 (d, *J* = 7.4 Hz, 2H), 7.34 (d, *J* = 7.8 Hz, 2H), 6.75 (d, *J* = 8.6 Hz, 1H), 6.27 (s, 2H), 5.42 (s, 2H), 5.22 (s, 2H), 3.36 (m, 2H), 1.67 (d, *J* = 6.6 Hz, 2H), 1.23-1.38 (10H), 0.82 (T, *J* = 6.5 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 164.28, 163.37, 160.07, 155.65, 151.35, 138.26, 135.89, 135.02, 131.35, 130.04, 129.28, 128.92, 128.02, 124.67, 122.14, 120.60, 107.65, 104.31, 66.95, 43.34, 42.74, 31.70, 29.25, 29.13, 28.28, 27.10, 22.53, 14.38.

Compound AN-12C. The synthetic strategy toward **AN-12C** is similar to the synthesis of **AN-2C**, yield 65%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.47 (d, *J* = 7.3 Hz, 1H), 8.39 (d, *J* = 8.4 Hz, 1H), 7.97 (d, *J* = 8.4 Hz, 1H), 7.49 (dd, *J* = 17.5, 8.1 Hz, 3H), 7.27 (d, *J* = 8.0 Hz, 2H), 6.63 (d, *J* = 8.5 Hz, 1H), 5.32 (s, 3H), 4.62 (s, 2H), 3.35 (d, *J* = 4.5 Hz, 2H), 1.78 (dt, *J* = 14.8, 7.3 Hz, 2H), 1.54 – 1.17 (m, 18H), 0.88 (t, *J* = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 159.89, 159.33, 144.91, 135.25, 132.55, 129.94, 126.48, 125.01, 124.34, 122.27, 121.23, 119.78, 118.12, 115.31, 105.04, 99.53, 60.38, 39.00, 38.28, 27.16, 24.90, 24.88, 24.84, 24.82, 24.64, 24.59, 24.21, 22.44, 17.94, 9.37.

Compound BGAN-12C. The synthetic strategy toward **BGAN-8C** is similar to the synthesis of **BGAN-2C**, yield 66%. ^{1}H NMR (400 MHz, DMSO) δ 12.44 (s, 1H), 8.70 (d, *J* = 8.4 Hz, 1H), 8.42 (d, *J* = 7.2 Hz, 1H), 8.26 (d, *J* = 8.5 Hz, 1H), 7.82 (s, 1H), 7.80 (s, 1H), 7.65 (t, *J* = 7.8 Hz, 1H), 7.43 (d, *J* = 7.9 Hz, 2H), 7.34 (d, *J* = 7.9 Hz, 2H), 6.74 (d, *J* = 8.7 Hz, 1H), 6.25 (s, 2H), 5.42 (s, 2H), 5.22 (s, 2H), 3.35 (d, *J* = 5.9 Hz, 2H), 1.73 – 1.60 (m, 2H), 1.36 (d, *J* = 6.5 Hz, 2H), 1.32 – 1.04 (m, 16H), 0.81 (t, *J* = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 164.28, 163.36, 160.05, 151.34, 138.28, 135.82, 135.02, 131.36, 130.03, 129.28, 128.97, 128.01, 124.67, 122.11, 120.58, 107.60, 104.28, 66.98, 43.30, 42.73, 31.73, 29.49, 29.45, 29.25, 29.15, 28.22, 27.04, 22.54, 14.39.

Compound AN-DM. The synthetic strategy toward **AN-DM** is similar to Ref. S1, yield 78%. 1H-NMR (CDCl3, 400 MHz) δ 3.10 (s, 6H), 3.53 (s, 3H), 7.09 (d, J = 8.4 Hz, 1H), 7.63 (t, J = 8.0 Hz, 1H), 8.42 (d, J = 8.4 Hz, 1H), 8.45 (d, J = 8.0 Hz, 1H), 8.54 (d, J = 7.2 Hz, 1H). 13C-NMR (CDCl3, 100 MHz) δ 26.82, 44.78, 113.27, 114.85, 122.92, 124.84, 125.24, 130.07, 130.94, 131.17, 132.59, 156.94, 164.31, 164.86.

Compound BGAN-DM. The synthetic strategy toward **BGAN-DM** is similar to the synthesis of **BGAN-2C**, yield 80%. ^{1}H NMR (400 MHz, DMSO) δ 12.41 (s, 1H), 8.54 – 8.50 (m, 1H), 8.54 – 8.49 (m, 1H), 8.49 – 8.44 (m, 1H), 8.35 (d, *J* = 8.3 Hz, 1H), 7.79 (s, 1H), 7.78 – 7.71 (m, 1H), 7.44 (d, *J* = 8.0 Hz, 2H), 7.36 (d, *J* = 8.1 Hz, 2H), 7.20 (d, *J* = 8.4 Hz, 1H), 6.27 (s, 2H), 5.43 (s, 2H), 5.24 (s, 2H), 3.10 (s, 6H). ^{13}C NMR (101 MHz, DMSO) δ 164.17, 163.48, 160.09, 157.23, 155.65, 138.24, 135.98, 133.05, 132.29, 131.31, 130.22, 129.01, 127.98, 125.47, 124.64, 122.63, 113.43, 66.91, 56.50, 44.84.

3. Preparation of the SNAP-tag

pSNAP-tag(T7)-2 vector (NEB) plasmid was digested by restriction enzymes Nde I and Xho I. And then the SNAP-tag gene was cloned into pET-22b (pET-22b-SNAP) with C-terminal His-tag. The plasmid was transformed into *E. coli* strain BL21 and cultured at 37 °C in 1 L LB medium containing 50 mg/ml ampicillin. The protein expression was induced by the addition of 1 mM IPTG when OD_{600} reached 0.8. After an additional growing at 28 °C for 4 hours, The cells were harvested by centrifugation. The protein was extracted by sonicating in 20 mM PBS buffer, pH 7.4, including 1 mM PMSF and 50 mM NaCl. The supernatant liquid was loaded into Q Sepharose™ Fast Flow running in 20 mM PBS buffer, pH 7.0, and eluted with 200 mM NaCl. The protein was further purified by Ni-NTA column in 20 mM PBS buffer pH=7.4. Total protein was measured by the Bradford protein assay using BSA as a standard. Electrophoresis (SDS-PAGE) was carried out to check the purification of the protein according to the protocol of the manufacturer.

4. Experimental Procedures

Computational design of the interaction between BGAN-R and SNAP-tag

The substrate BGAN-R was built using GaussView 5.0 and optimized at the B3LYP/6-31G level by the Gaussian 03 program.[2] Automated docking simulations were conducted with Auto-Dock 4.2 in the presence of the target protein SNAP-tag (PDB ID: 3KZY). The best conformation of the docking complex was generated based on the scoring functions and visualized via PyMOL.

Fluorometric analysis *in vitro*

All fluorescent probes were dissolved in DMSO to obtain 10 mM stock solution. The probes were diluted to the desired concentrations with an appropriate aqueous buffer solution for measurement. The

general procedure for SNAP-tag protein labeling was to incubate 5 μM fluorescence probe and 10 μM SNAP-tag protein in PBS buffer containing 1% (v/v) DMSO at 37 °C for 30 minutes. The samples were excited at 460 nm. Relative fluorescence quantum yields of the compounds were obtained via the relative determination method, with Coumarin 153 as a reference compound,[3] which compared the area under the emission spectrum of the sample with that of Coumarin 153 in ethyl alcohol (λ = 0.53 when excited at 440 nm) for derivatives.

SDS-PAGE analysis of the SNAP-tag labeled by BGAN-R

Purified SNAP-tag (10 μM) was incubated with fluorescent probes (20 μM) at 37 °C for 30 min. The reaction mixtures were heated at 95 °C for 10 min and analyzed by SDS-PAGE. The gels were then stained with Coomassie Brilliant Blue and photographed.

Kinetic study of the SNAP-tag labeled by BGAN-R

The time required for labeling half of proteins, $t_{1/2}$ was estimated by monitoring the increase in the fluorescence intensity of the probes. It was determined under the condition of 5 μM **BGAN-R** and 5 μM SNAP-tag protein in PBS buffer containing 1 % DMSO at room temperature. The excitation wavelengths were 460 nm, 460 nm, and 450 nm for **BGAN-2C**, **BGAN-8C**, and **BGAN-12C**. The emission wavelengths were 538 nm, 530 nm, and 520 nm, respectively. The fluorescence data were converted to labeled fractions by using the following equation:

$$[\text{Labeled fraction}] = (F_t - F_0) / (F_{max} - F_0)$$

where F_t, F_{max}, and F_0 represent the observed, maximum and initial fluorescence intensities.

The pseudo-first-order rate constant, k_{obs}, was obtained by fitting the fluorescence data with the following equation:

$$[\text{Labeled fraction}] = 1 - \exp(-k_{obs}\, t)$$

Then, the pseudo-first-order rate constant was plotted against the protein concentration. By fitting the plot with the equation: $k_{obs} = k_2[\text{probe}]$, the second-order-rate constant, k_2 was acquired.

Cell Toxicity Assay

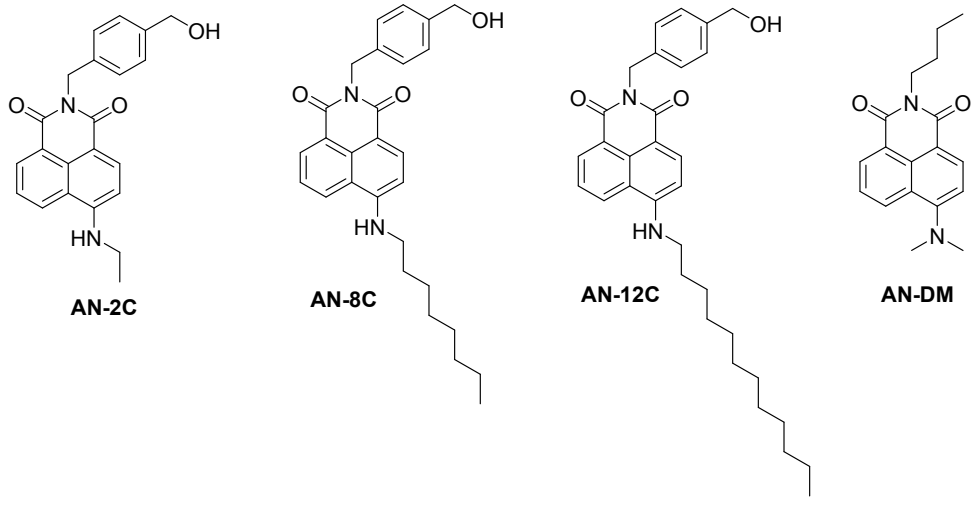
The cytotoxicity of the fluorescence probe was determined using the MTT cell proliferation and Cytotoxicity Detection Kit (KeyGEN BioYECH,) The HEK 293 cells were seeded into 96-well plate at

a density of 1×10^4 cells/well in 200 μL DMEM (10 % FBS) and then treated with fluorescence probe **BGAN-2C** (at a final concentration of 0, 1, 2, 5, 10 and 15 μM) 12 hours under 5 % CO_2, 37 °C. After incubation, the cell supernatant was removed and cells were supplemented with fresh 150 μL medium and 50 μL 1x MTT per well and incubated for another 4 h (5 % CO_2, 37 °C). Then the cell supernatant was removed and the cells were resuspended with 150 μL DMSO .The absorption was recorded at 570 and 620 nm using a UV-Vis microplate reader. The cell viability was determined by comparing the probe treated cells and the untreated control.

Fluorescence Imaging of HEK 293 Cells

We transfected HEK 293 Cells (1,000 cells/ well) seeded in plates with plasmids $pSNAP_f$ vector (NEB), $pSNAP_f$-Cox8A (NEB) and SNAP-H2B (a gift from Pro. Qingkai Yang, Dalian Medical University) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After the incubation of the cells at 5 % CO_2 37 °C for 24 h, the cells were washed once with Hank's balanced salt solution (HBSS) and incubated with 5 μM fluorescence probe in HBSS at 37 °C for 30 min. The cell nuclei were co-stained with 100 ng mL^{-1} Hoechst 33342. Microscopic images of the cells were recorded using a confocal laser scanning microscope. In addition, both SNAP and $SNAP_f$ variant based on hAGT mutant.[4] And SNAP has been used for qualitative analysis of compounds in vitro.

5. Supplementary Schemes and Figures



Scheme S1 Structure of control compounds.

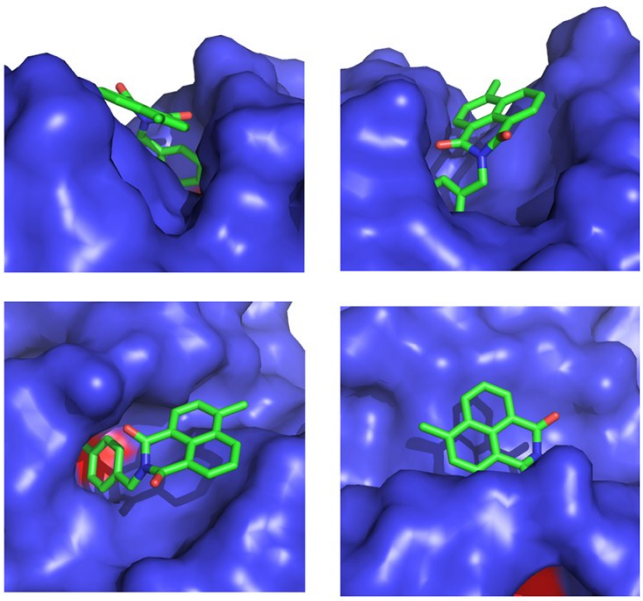


Fig. S1 Computer simulations image of SNAP-tag binding with **BGAP-R.** The probe attached in the hydrophobic pocket of the protein with 4-substituted group of 1, 8-naphthalimide exposed at the outside of the cavity.

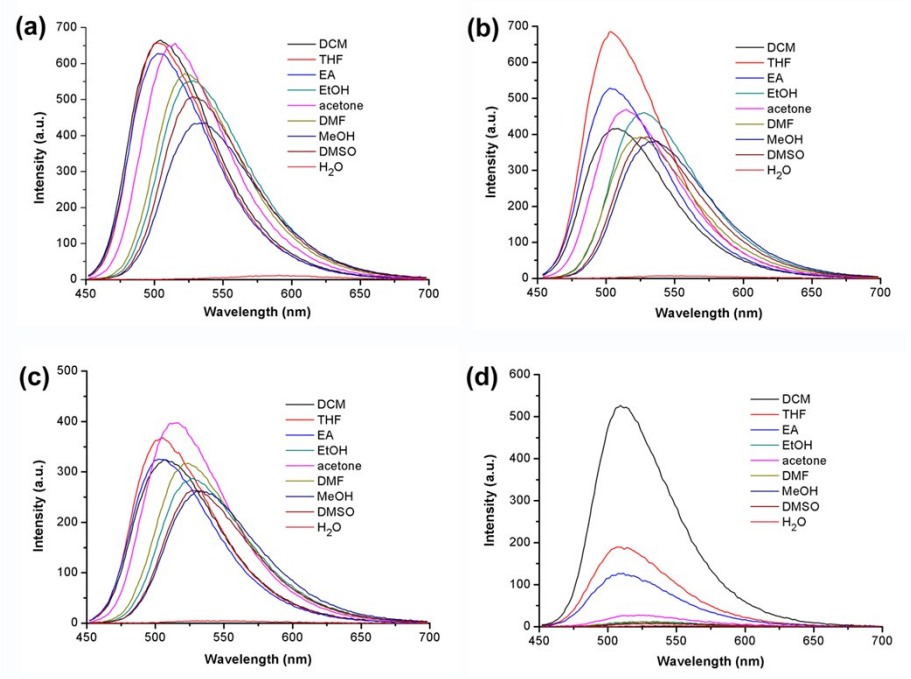


Fig. S2 Comparison of fluorescence response of probes in various organic solvents. (a) **BGAN-2C**, (b) **BGAN-8C**, (c) **BGAN-12C**, and (d) **BGAN-DM**.

Table S1 The ratio of fluorescent intensity between polar solvent DCM and nonpolar solvent H_2O

	BGAN-2C	**BGAN-8C**	**BGAN-12C**	**BGAN-DM**

The ratio of fluorescence intensity between DCM and H_2O	66	64	81	210

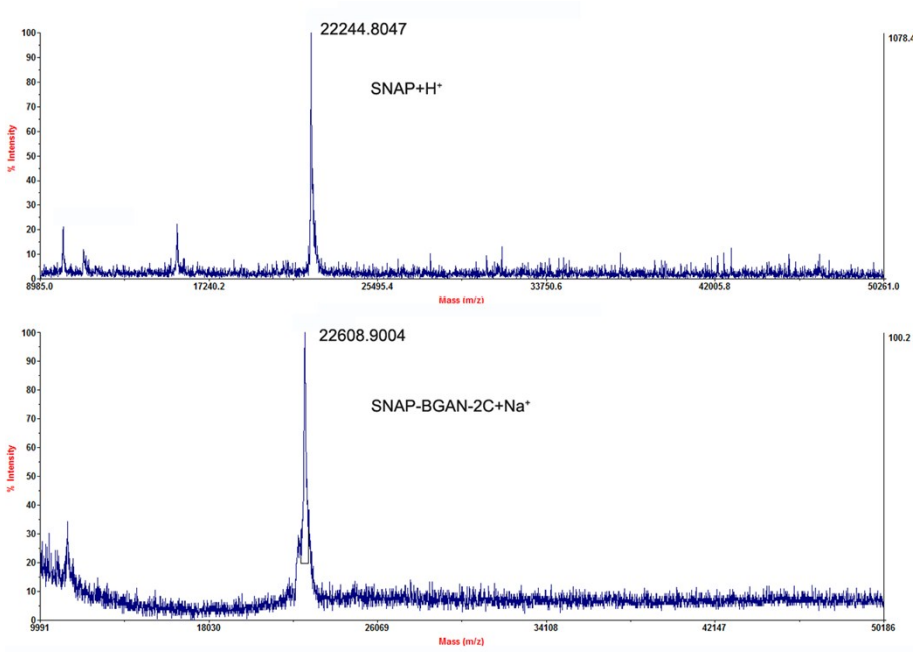


Fig. S3 MALDI-TOF mass spectrometry analysis of SNAP-tag with and without binding with **BGAN-2C**.

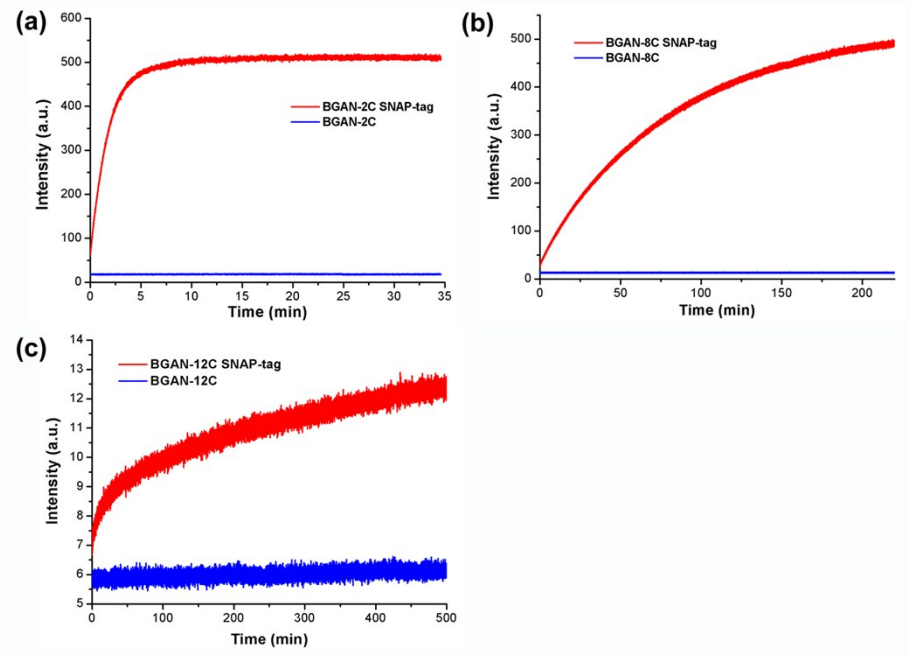


Fig. S4 Time course of fluorescence intensity of BGAN-2C (a), BGAN-8C (b), and BGAN-12C (c) in the presence or absence of SNAP-tag. The reaction was carried out in the PBS buffer at 37 °C. The

protein and probe concentrations were 5 μM, respectively. The excitation wavelengths were 460 nm, 460 nm, and 450 nm for BGAN-2C, BGAN-8C, and BGAN-12C. The emission wavelengths were 538 nm, 530 nm, and 520 nm, respectively.

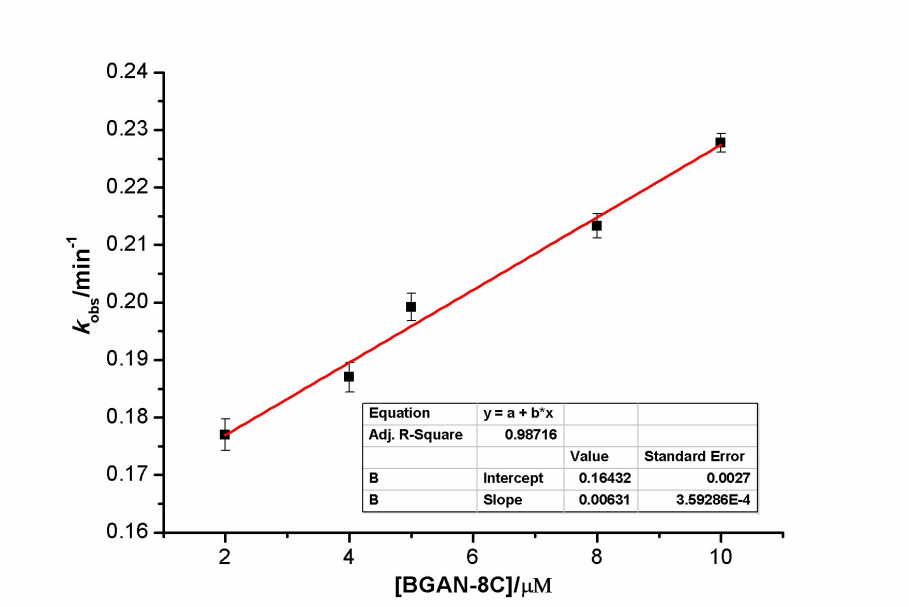


Fig. S5 Plots of BGAN-8C concentration versus the pseudo-first-order rate constant. [BGAN-8C]= 2 μM, 4 μM, 5 μM, 8 μM, and 10 μM. [SNAP-tag]= 500 nM.

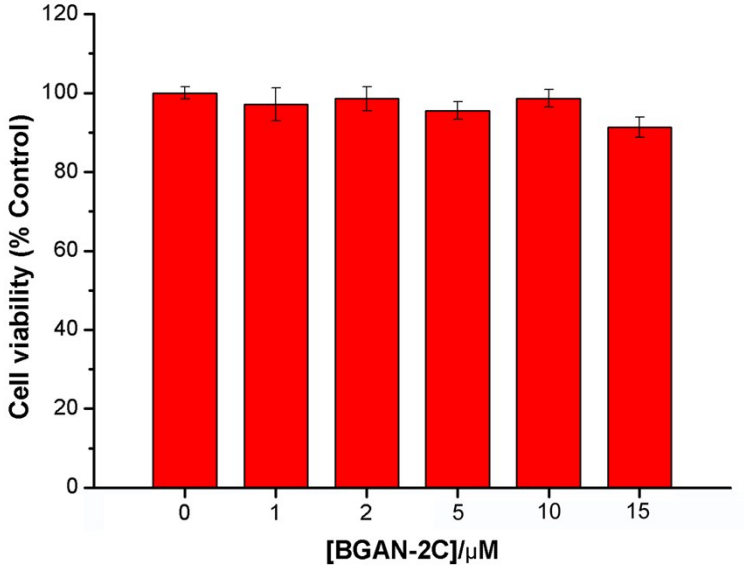


Fig. S6 Toxicity test of BGAN-2C probe with HEK 293 cells.

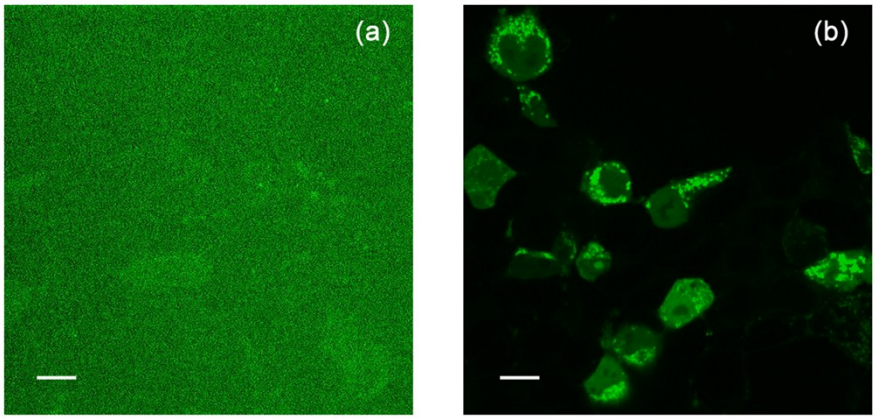


Fig. S7 The live-cell imaging of conventional probe SNAP-Cell® 505-Star for labeling $pSNAP_f$-Cox8A before (a) and after (b) washing with HBSS. Live HEK 293 cells transiently expressing $pSNAP_f$-Cox8A were incubated for 30 min at 37ºC with SNAP-Cell® 505-Star. Then cells were washed with HBSS three times before fluorescence image (b) was taken. Scale bars: 20 μm.

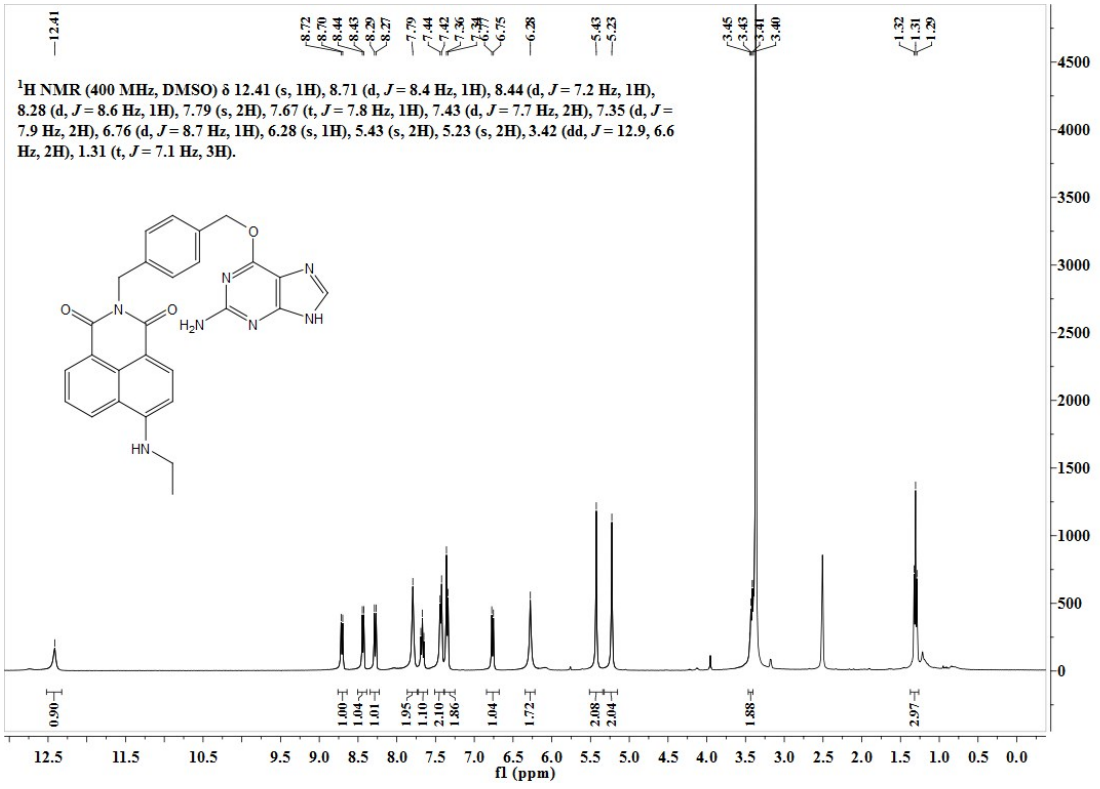


Fig. S8 ^{1}H-NMR of **BGAN-2C** in $DMSO_{d6}$.

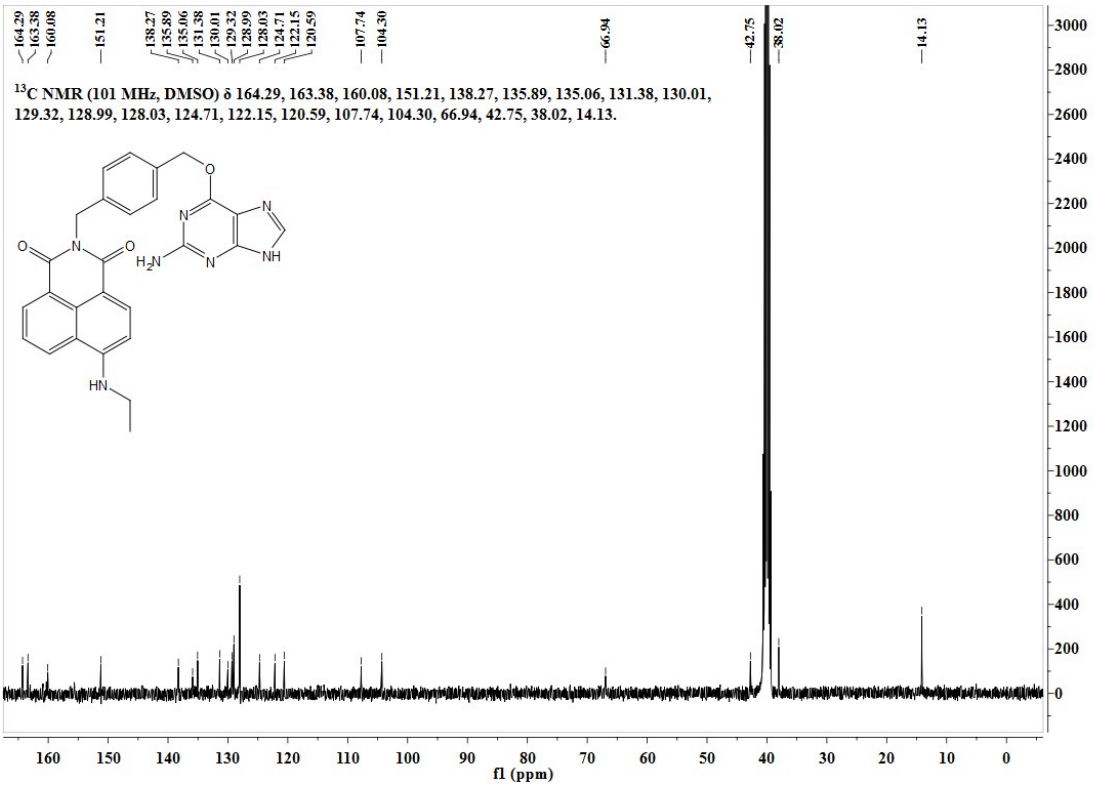


Fig. S9 ^{13}C-NMR of **BGAN-2C** in DMSO$_{d6}$.

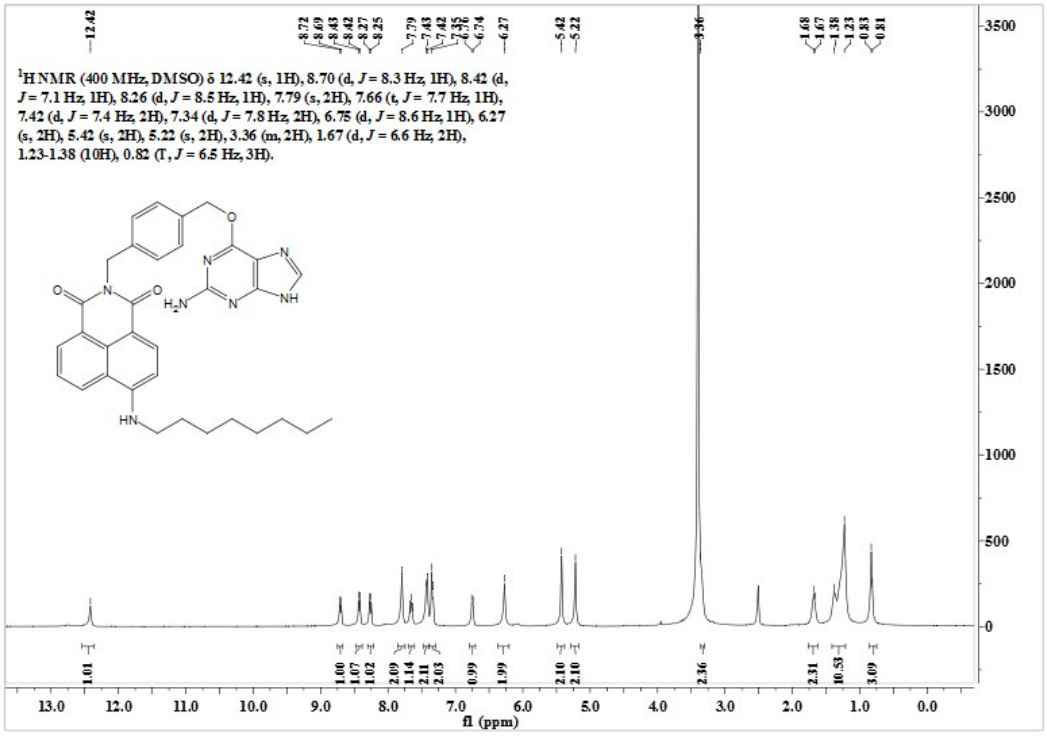


Fig. S10 ^{1}H-NMR of **BGAN-8C** in DMSO$_{d6}$.

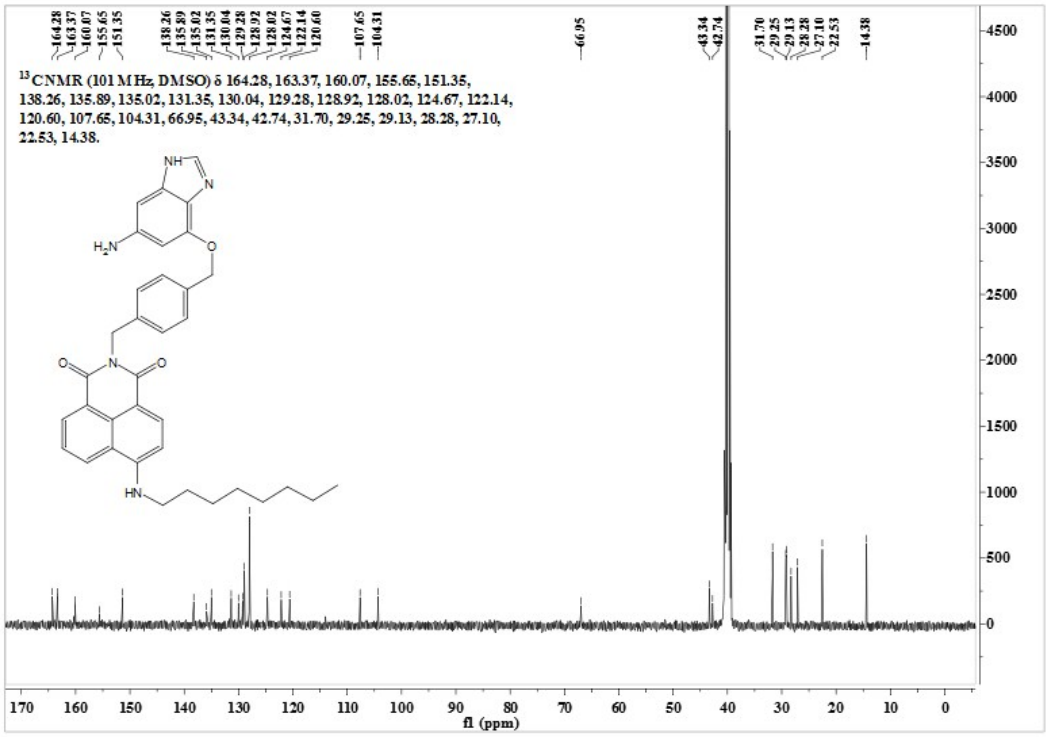


Fig. S11 ^{13}C-NMR of **BGAN-8C** in DMSO$_{d6}$.

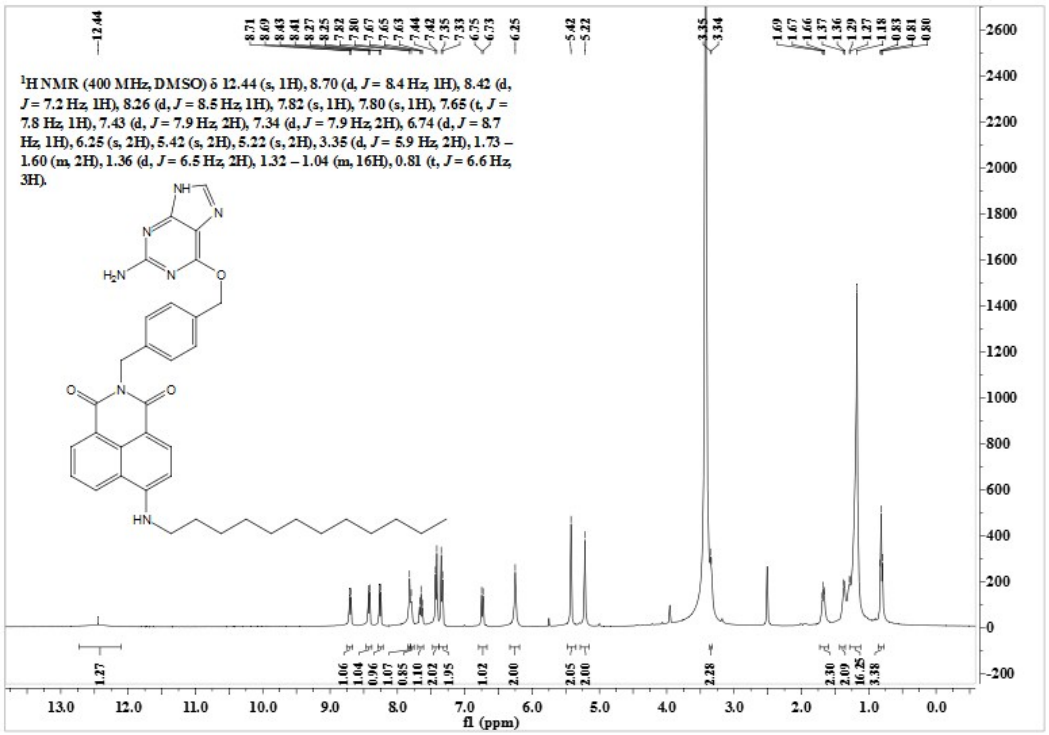


Fig. S12 ^{1}H-NMR of **BGAN-12C** in DMSO$_{d6}$.

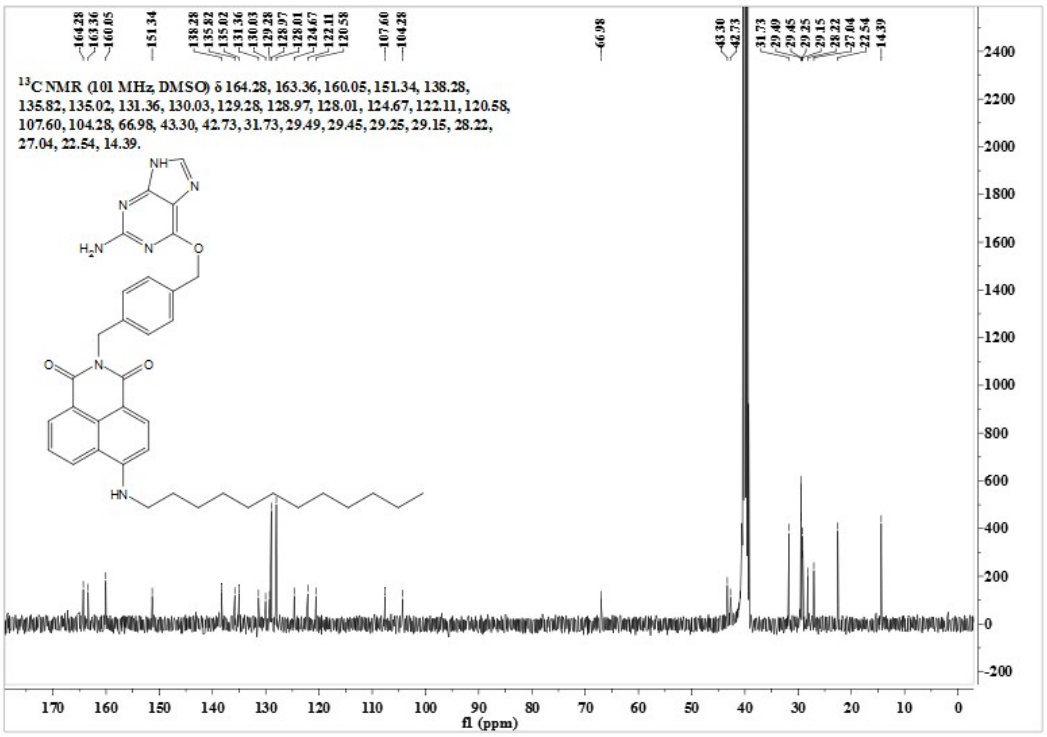


Fig. S13 ^{13}C-NMR of **BGAN-12C** in DMSO$_{d6}$.

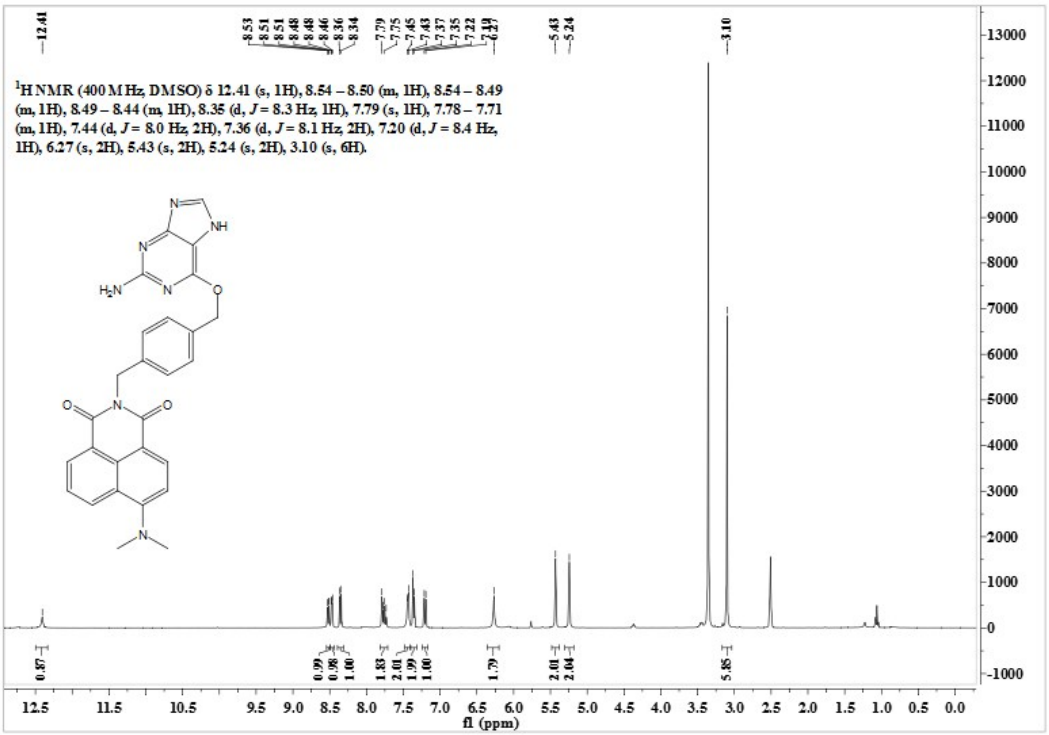


Fig. S14 ^{1}H-NMR of **BGAN-DM** in DMSO$_{d6}$.

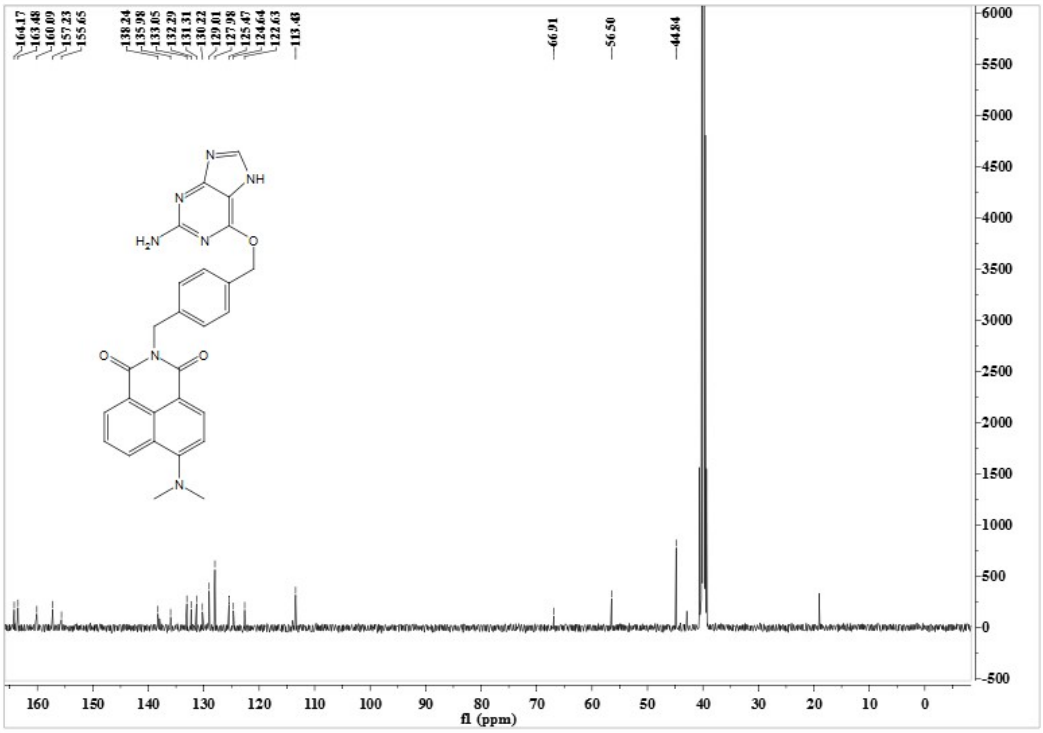


Fig. S15 ^{13}C-NMR of **BGAN-DM** in $DMSO_{d6}$.

6. Reference

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