Supporting Information

Dual-Locked Cyclopeptide-siRNA Conjugate for Tumor-Specific Gene Silencing

Chen Li,[a,#] Shuaishuai Sun,[b,#] Hao Kong,[a] Xiangqian Xie,[b] Gaolin Liang,[c,*] Yan Zhang,[a,*] Huan Wang,[b,*] and Jinbo Li[a,*]

[a]*State Key Laboratory of Analytical Chemistry for Life Sciences, Jiangsu Key Laboratory of Advanced Organic Materials, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210023, China.*

[b]*State Key Laboratory of Coordination Chemistry, Jiangsu Key Laboratory of Advanced Organic Materials, School of Chemistry and Chemical Engineering, Chemistry and Biomedicine Innovation Center (ChemBIC), Nanjing University, Nanjing 210023, China.*

[c]*State Key Laboratory of Digital Medical Engineering, School of Biological Science and Medical Engineering, Southeast University, Nanjing 211189, China.*

*Emails: gliang@seu.edu.cn; njuzy@nju.edu.cn; wanghuan@nju.edu.cn; jinboli@nju.edu.cn.

Materials and Methods

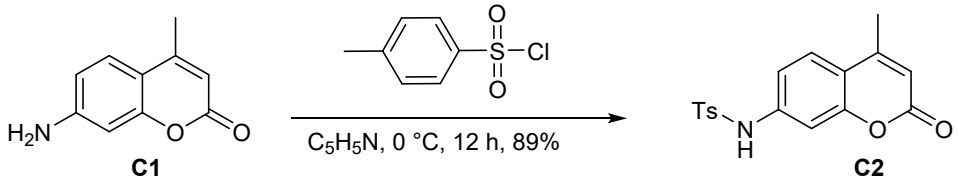
1. General materials and methods

All chemicals and solvents were purchased from J&K chemicals or Sigma-Aldrich. High glucose Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, Lipofectamine 2000 were purchased from Life Technologies. All aqueous solutions were treated by diethylpyrocarbonate (DEPC) before use. The light source was a CEL-PE300-3A lamp purchased from CeauLight (Beijing, China). UV-Vis absorption and fluorescence spectra were recorded at room temperature on Ocean Optics Maya 2000 and Shimadzu RF-5301PC spectrofluorophotometers with a 1 cm standard quartz cell, respectively. Flow cytometry assay was done on FACS Calibur (BD, USA).

^{1}H NMR and ^{13}C NMR spectra were obtained on a 400 MHz Bruker AVANCE III–400 spectrometer. Chemical shifts are reported in δ (ppm) relative to the solvent residual peak. MALDI-TOF-MS was performed on ABSCIEX MALDI-TOF-TOF 4800 PLUS (AB Sciex, USA). The LC-ESI-MS was done on Agilent 6550 iFunnel Q-TOF LC/MS (Agilent, USA). HPLC was carried out on Thermo UltiMate 3000 with CH_3CN/H_2O (0.1 M $CH3COONH^+$) as eluents.

2. Synthesis and characterization of the compounds

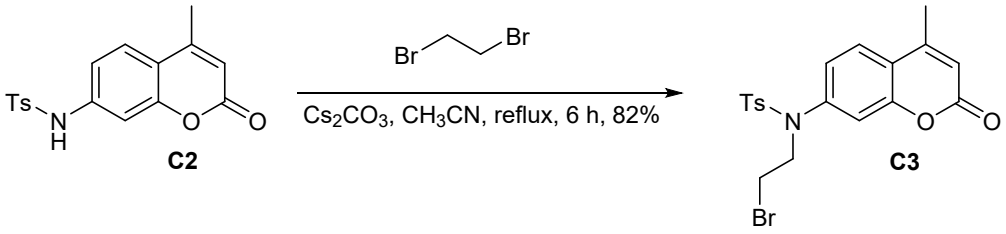
Synthesis of C2



C2 was synthesized according to the method reported by Song et al[1]. 7-Amino-4-methylcoumarin (3.50 g, 20 mmol) was dissolved in CH_2Cl_2 (20 mL), then p-

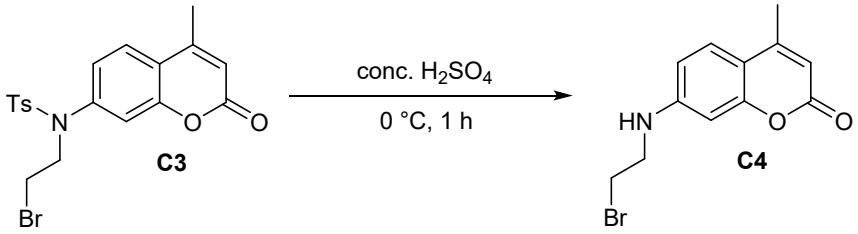
toluenesulfonyl chloride (7.63 g, 40 mmol) in pyridine (20 mL) was slowly added to the solution. The mixture was stirred at room temperature for 12 h. CH_2Cl_2 (150 mL) was then added, and the solution was washed with 10% citrate aq. three times and brine once. The solution was dried with sodium sulfate and evaporated. The crude solid was washed with a small portion of EtOH and dried in vacuo to give a colorless solid **C2** (5.86 g, 89 % yield), ^{1}H NMR (400 MHz, DMSO-*d6*): δ = 10.92 (s, 1 H), 7.74 (d, *J* = 8.3 Hz, 2 H), 7.63 (d, *J* = 8.6 Hz, 1 H), 7.38 (d, *J* = 8.0 Hz, 2 H), 7.09 (dd, *J* = 8.6, 2.1Hz, 1 H), 7.04 (d, *J* = 2.1 Hz, 1 H), 6.23 (s, 1 H), 2.33 (s, 6 H).

Synthesis of C3



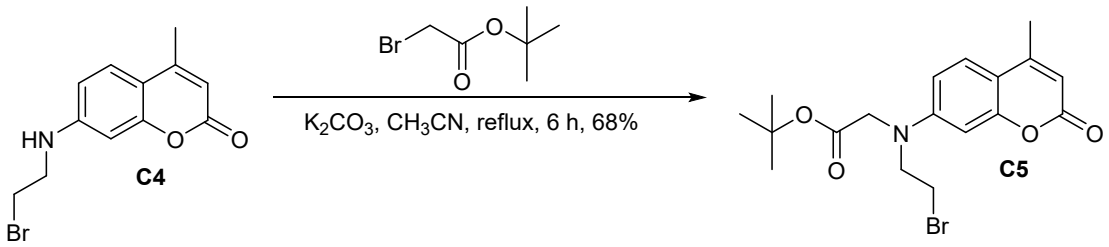
C3 was synthesized as described in the previous work[2]. A mixture of compound **C2** (5.0 g, 15.2 mmol) and 1, 2-dibromoethane (13.2 mL, 152 mmol) was dissolved in 250 mL distilled acetonitrile. Then, cesium carbonate (7.4 g, 22.8 mmol) was added to the solution, and the obtained mixture was refluxed for 6 h. After filtration, the solution was evaporated, and the obtained crude product was recrystallized in C_2H_5OH to give 5.4 g (82 % yield) of **C3** as a gray solid. ^{1}H NMR (400 MHz, DMSO-*d6*): δ = 7.78 (d, *J* = 8.5 Hz, 1 H), 7.50 (d, *J* = 8.3 Hz, 2 H), 7.40 (d, *J* = 8.3 Hz, 2 H), 7.20 (dd, *J* = 8.5, 2.1Hz, 2 H), 7.17 (d, *J* = 2.1Hz, 1 H), 6.42 (s, 1 H), 4.04 (t, *J* = 6.2 Hz, 2 H), 4.47 (t, *J* = 6.2 Hz, 2 H), 2.43 (s, 3 H), 2.40 (s, 3 H).

Synthesis of C4



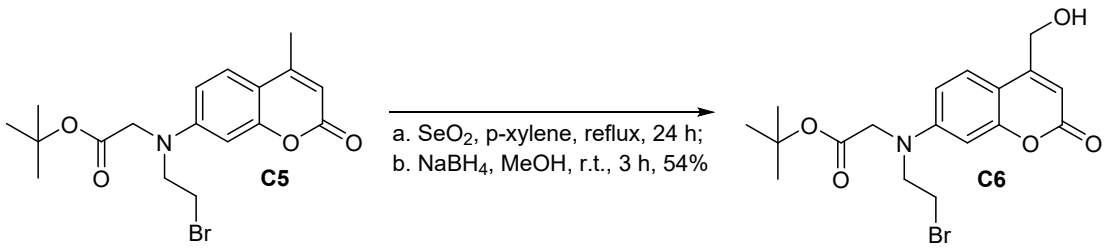
C3 (5 g, 11.5 mmol) was added to 15 mL conc. sulfuric acid and the solution was stirred for 1 h at 0 °C. The reaction mixture was carefully poured into water, neutralized with saturated aqueous sodium bicarbonate and extracted with EtOAc (5 × 50 mL). The organic solution was dried over Na_2SO_4 and evaporated, the solid was used for next step without further purification.

Synthesis of C5



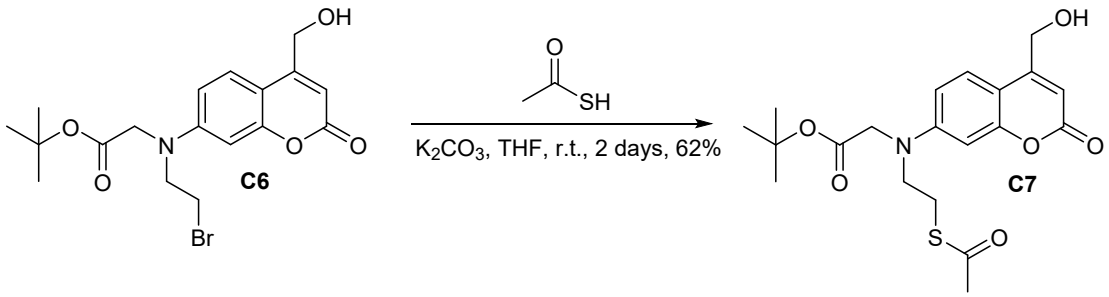
A mixture of **C4** (4.5 g, 16 mmol), bromoethane (29.8 mL, 0.40 mol), K_2CO_3 (3.3 g, 29.7 mmol), NaI (0.2 g, 1.3 mmol), and tetrabutylammonium bromide (0.42 g, 1.3 mmol) in CH_3CN (200 mL) was refluxed for 6 h. The reaction mixture was allowed to cool to room temperature. After filtration, the solvent was removed under vacuum. The residue was then dissolved in CH_2Cl_2 and washed with H_2O (3x100 mL). The organic layer was then dried over Na_2SO_4 and evaporated to remove the solvents. The obtained crude product was purified by column chromatography (EtOAc : Hexane = 1:4) to produce **C5** (4.3 g, 68 % yield) as a yellow solid. ^{1}HNMR ($CDCl_3$, 400 MHz): δ = 7.45 (d, *J* = 9.2 Hz, 1 H), 6.57 (dd, *J* = 9.2, 2.8 Hz, 1 H), 6.48 (d, *J* = 2.8 Hz, 1 H), 6.03 (s, 1 H), 4.06 (s, 2 H), 3.85 (t, *J* = 8.0 Hz, 2 H), 3.55 (t, *J* = 8.0 Hz, 2 H), 2.35 (s, 3 H), 1.47 (s, 9 H); ^{13}C NMR ($CDCl_3$, 100 MHz): 168.85, 161.67, 155.67, 152.58, 149.93, 125.83, 111.13, 110.49, 108.49, 98.73, 82.70, 54.15, 28.07, 27.79, 18.45.

Synthesis of C6



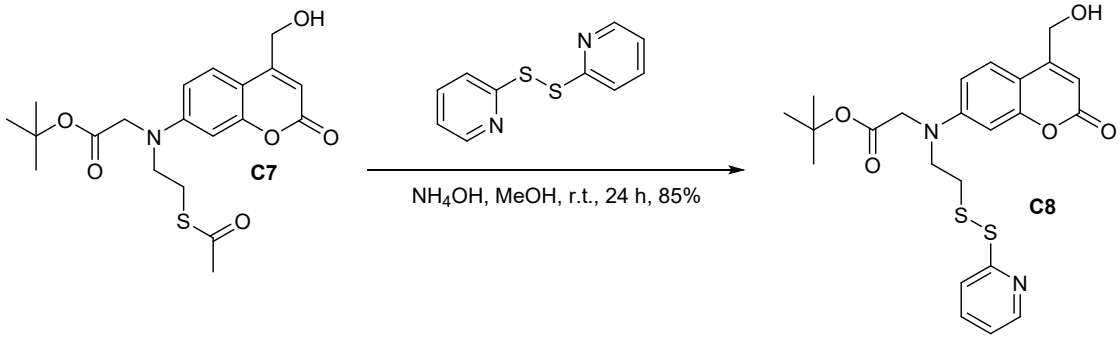
A mixture of SeO_2 (4.6 g, 30.6 mmol) and **C5** (4 g, 10.2 mmol) were suspended in 200 mL p-xylene. The reaction mixture was refluxed under vigorous stirring with the protection under an argon atmosphere. After 24 h, the mixture was filtered and concentrated under reduced pressure. The dark brown residual oil was dissolved in methanol (200 mL), then sodium borohydride (0.78 g, 20.4 mmol) was added. The solution was stirred for 3 h at room temperature. Thereafter, the suspension was carefully neutralized with 1 M HCl, diluted with H_2O, and partially concentrated under reduced pressure to remove methanol. The mixture was extracted with CH_2Cl_2 and the obtained organic phase was washed with H_2O and brine, dried over Na_2SO_4, and concentrated in vacuum. The obtained oil was purified by column chromatography (n-Hexane/EtOAc = 2/1) to yield 2.3 g (54 %) of C6 as a brown solid. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.36 (d, *J* = 9.2 Hz, 1 H), 6.73 (dd, *J* = 9.2, 2.8 Hz, 1 H), 6.54 (d, *J* = 2.8 Hz, 1 H), 6.36 (s, 1 H), 4.80 (s, 2 H), 4.25 (br, 1 H), 4.04 (s, *J* = 7.2 Hz, 2 H), 3.57 (t, *J* = 7.6 Hz, 2 H), 3.10 (*J* = 7.6 Hz, 2 H), 1.47 (s, 9 H); ^{13}C NMR ($CDCl_3$, 100 MHz): 196.10, 169.15, 163.20, 155.56, 155.22, 150.52, 124.58, 109.27, 108.00, 106.41, 98.71, 82.86, 60.72, 53.79, 52.04, 28.05, 26.18.

Synthesis of C7



A mixture of **C6** (2.0 g, 4.9 mmol), thioacetic acid (1.1 g, 14.7 mmol) and K_2CO_3 (1.4 g, 9.8 mmol) in CH_3CN (100 mL) was stirred at room temperature for 2 days. The reaction mixture was filtered and the solvent was removed under vacuum. The residual oil was purified with column chromatography (EtOAc/CH_2Cl_2 = 1/3~1/1) to give 1.2 g (62 % yield) of **C7** as a yellow solid. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.36 (d, J = 9.0 Hz, 1 H), 6.74 (dd, J = 9.0, 2.6 Hz, 1 H), 6.55 (d, J = 2.6 Hz, 1 H), 6.21 (s, 1 H), 5.22 (s, 2 H), 4.04 (s, 2 H), 3.58 (t, J = 7.6 Hz, 2 H), 3.11 (t, J = 7.8 Hz, 2 H), 2.39 (s, 3 H), 2.19 (s, 3 H), 1.47 (s, 9 H); ^{13}C NMR ($CDCl_3$, 100 MHz): 195.72, 170.20, 168.84, 161.56, 155.88, 150.59, 149.28, 124.64, 109.09, 107.99, 98.85, 82.64, 61.27, 53.83, 52. 08, 30.72, 28.07, 26.21, 20.74.

Synthesis of C8



A solution of the **C7** (100 mg, 0.25 mmol) and 2, 2'-dithiodipyridine (83 mg, 0.38 mmol) in MeOH (50 mL) was degassed by repeatedly subjecting it to vacuum and argon purge following by addition of 2 mL NH_4OH aqueous. The reaction was left stirring at room temperature for 24 h. Then, MeOH was removed by rotary evaporation, and the residue was extracted with CH_2Cl_2, washed with saturated NaCl solution (50 mL × 3), and dried over Na_2SO_4. After evaporation of the solvent, the residue was purified with column chromatography eluting with (silica gel, EtOAc/Hexane = 1/1) to give 97 mg of **C8** (0.21 mmol, 85% yield) as a yellow oil. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.52 (s, 1 H), 7.72 (m, 2 H), 7.27 (m, 1 H), 7.16 (m, 1 H), 6.54 (dd, J = 8.8, 2.1 Hz, 1 H), 6.45 (d, J = 2.1 Hz, 1 H), 6.32 (s, 1 H), 4.79 (s, 2 H), 4.04 (s, 2 H), 3.81 (t, J = 7.2 Hz,

2 H), 3.06 (t, *J* = 7.2 Hz, 2 H), 1.44 (s, 9 H); ^{13}C NMR ($CDCl_3$, 100 MHz): 169.01, 162.01, 159.15, 155.71, 154.38, 150.14, 149.44, 137.73, 124.56, 121.30, 120.70, 108.71, 108.05, 106.96, 98.69, 82.63, 60.80, 54.09, 51.72, 35.10, 28.06.

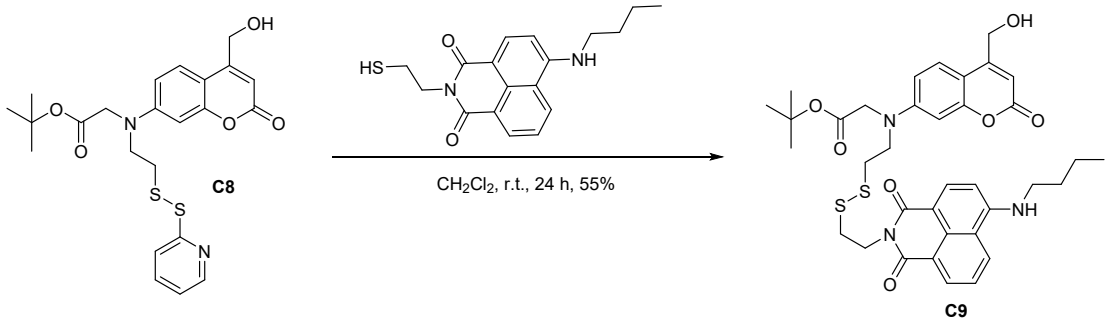
Synthesis of 4-amino-N-n-butyl-1,8-naphthalenedicarboxylic acid anhydride

4-bromo-1,8-naphthalene anhydride (2.77 g, 10 mmol) and N-butylamine were mixed in 2-methoxy-ethanol, and the mixture was refluxed for about 12 h. The reaction solution was poured into water (200 mL). Ethyl acetate was used for extraction. The product was purified by flash column chromatography on silica gel with PE/EA (10 /1) to afford a yellow powder (2.52 g, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.62 (dd, *J* = 7.82, 0.8 Hz, 1 H), 8.52 (dd, *J* = 8.4, 0.8 Hz, 1 H), 8.38 (d, *J* = 7.9, 1 H), 8.00 (d, *J* = 7.9 Hz, 1 H), 7.82 (t, *J* = 8.4, 1 H), 4.17 (t, *J* = 7.5 Hz, 2 H), 1.71 (m, 2 H), 1.44 (m, 2 H), 0.98 (t, *J* = 7.3 Hz, 3 H).

Synthesis of 6-(butylamino)-2-(2-mercaptoethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione

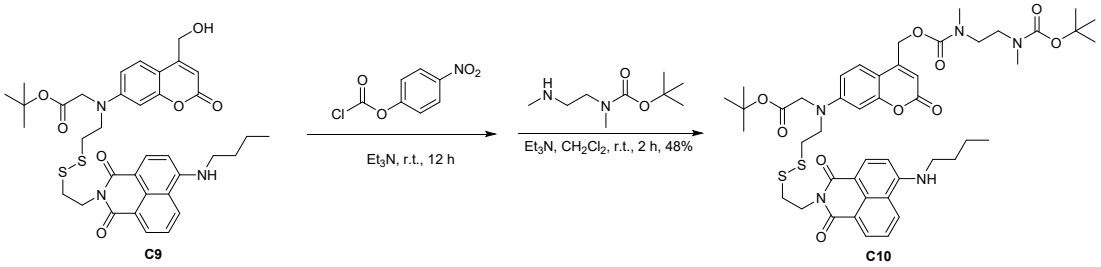
Under a nitrogen atmosphere, 20 mL of pyridine was added to a three-necked flask containing cysteine hydrochloride (5.86 g, 37.2 mmol), and triethylamine (4.83 ml, 37.2 mmol) was added to the mixture, stirred at room temperature for 10 min. Then, 4-amino-N-n-butyl-1,8-naphthalenedicarboxylic acid anhydride (2.5 g, 9.30 mmol) was added, and the mixture was heated to 125℃ for 20 h. The unreacted cysteine hydrochloride was removed by cooling and filtration. The target compound was purified by flash column chromatography on silica gel with DCM to afford a yellow powder (2.8 g, 92%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.52 (dd, *J* = 7.4, 0.8 Hz, 1 H), 8.39 (d, *J* = 8.4 Hz, 1 H), 8.07 (dd, *J* = 8.4, 0.8 Hz,1 H), 7.58 (d, *J* = 7.4 Hz, 1 H), 6.67 (d, *J* = 8.4, 1 H), 4.09 (t, *J* = 7.5 Hz, 2 H), 3.57 (t, *J* = 6.3 Hz, 2 H), 3.21 (m, 2 H), 1.63 (m, 2 H), 1.37 (m, 2 H), 0.90 (t, *J* = 7.3 Hz, 3 H).

Synthesis of C9



C8 (95 mg, 0.2 mmol) and 6-(butylamino)-2-(2-mercaptoethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (79 mg, 0.24 mmol) were mixed in DCM (15 mL) and stirred at room temperature 12 h. After completion of the reaction monitored by TLC, the mixture was concentrated under reduced pressure to afford the crude product, which was purified by silica gel chromatography to obtain the desired product **C9** (76 mg, 55% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.54 (d, *J* = 7.3 Hz, 1 H), 8.36 (d, *J* = 8.4 Hz, 1 H), 8.13 (d, *J* = 8.3 Hz, 1 H), 7.60 (t, *J* = 8.3 Hz, 2 H), 6.70 (d, *J* = 2.5 Hz, 1 H), 6.57 (t, *J* = 8.0 Hz, 1 H), 6.46 (dd, *J* = 8.9, 2.5 Hz, 1 H), 6.32 (s, 1 H), 4.76 (s, 2 H), 4.15 (t, *J* = 7.5 Hz, 2 H), 4.00 (s, 2 H), 3.85-3.70 (m, 4 H), 3.17 (t, *J* = 6.4 Hz, 2 H), 2.97 (t, *J* = 7.5 Hz, 2 H), 1.75 – 1.65 (m, 2 H), 1.45 (s, 9 H), 1.26 (s, 2 H), 0.97 (t, *J* = 7.3 Hz, 3 H).

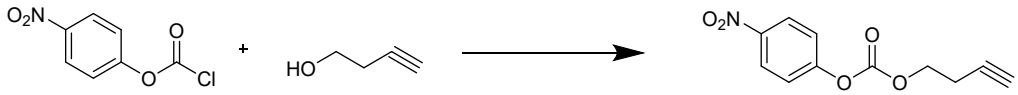
Synthesis of C10



To a solution of **C9** (75 mg, 0.11 mmol) in 10 mL DCM, 4-nitrophenyl carbonochloridate (53 mg, 0.16 mmol) and Et_3N were added. The mixture was stirred at room temperature for 12 h. After completion of the reaction monitored by TLC, the mixture was concentrated under reduced pressure, then tert-butyl methyl(2-(methylamino) ethyl) carbamate (81 mg, 0.43 mmol) and Et_3N were added. The mixture

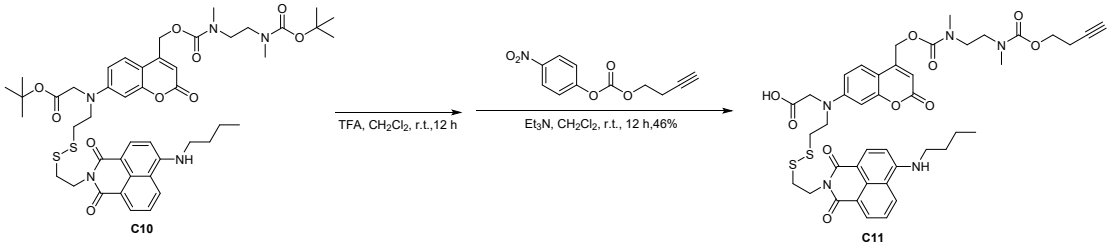
was purified by HPLC to obtain the desired product **C10** (47 mg, 48% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.49 (d, *J* = 7.28 Hz, 1 H), 8.38 (dd, *J* = 8.4 Hz, 1 H), 8.16 (d, *J* = 8.4 Hz, 1 H), 7.54 (t, *J* = 7.8, 2 H), 7.26 (m, 1 H), 6.70 (d, *J* = 8.4 Hz, 1 H), 6.55 (s, 1 H), 6.48 (d, *J* = 8.92 Hz, 1 H), 6.11 (s, 1 H), 5.17 (d, *J* = 6.4 Hz, 2 H), 4.08 (t, *J* = 7.56, 2 H), 3.96 (s, 2 H), 3.77-3.71 (m, 4 H), 3.39-3.31 (m, 4 H), 3.08 (t, *J* = 6.4, 2 H), 2.96-2.79 (m, 8 H), 1.38 (m, 22 H), 0.9 (t, *J* = 7.3 Hz, 3 H). Calc'd 928.3707 $[M + Na]^+$; found 928.3579.

Synthesis of the derivative of 3-butyn-1-ol



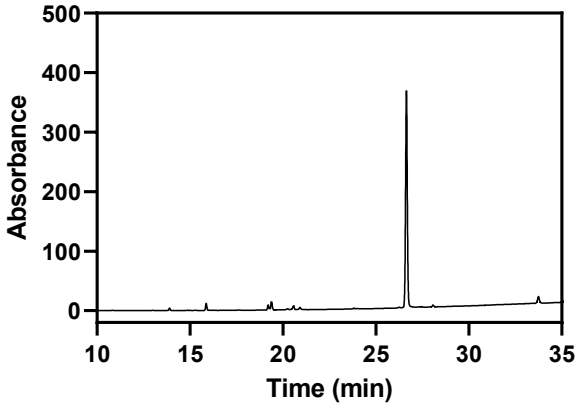
3-Butyn-1-ol was dissolved in a pyridine solution. Pyridine was used both as the solvent and as a base catalyst, p-Nitrophenyl chloroformate was then added dropwise to the solution. Then, the mixture was stirred at room temperature for 12 h. After completion of the reaction monitored by TLC, the mixture was concentrated under reduced pressure to afford the crude product, which was purified by silica gel chromatography to obtain the desired product (189 mg, 45% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.29 (dd, *J* = 7.2, 2.4 Hz, 2 H), 7.40 (dd, *J* = 7.2, 2.4 Hz, 2 H) 4.40 (t, *J* = 6.4 Hz, 2 H), 2.68 (dt, *J* = 6.4, 2.8 Hz, 2 H), 2.08 (t, *J* = 2.8 Hz, 1 H); ^{13}C NMR ($CDCl_3$, 100 MHz): 155.41, 152.25, 145.48, 125.34, 121.76, 78.93, 70.67, 66.67, 18.98.

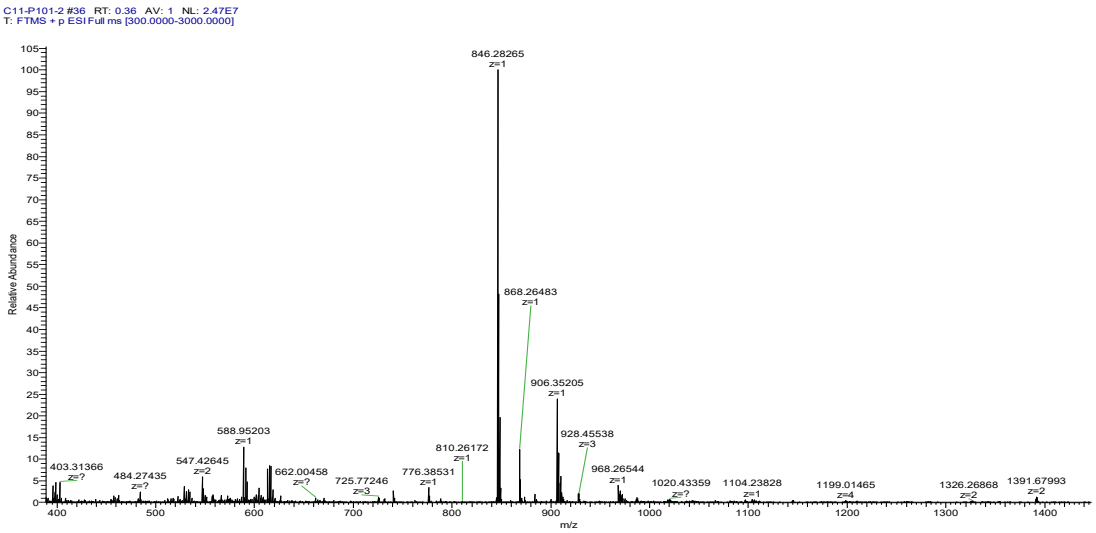
Synthesis of C11



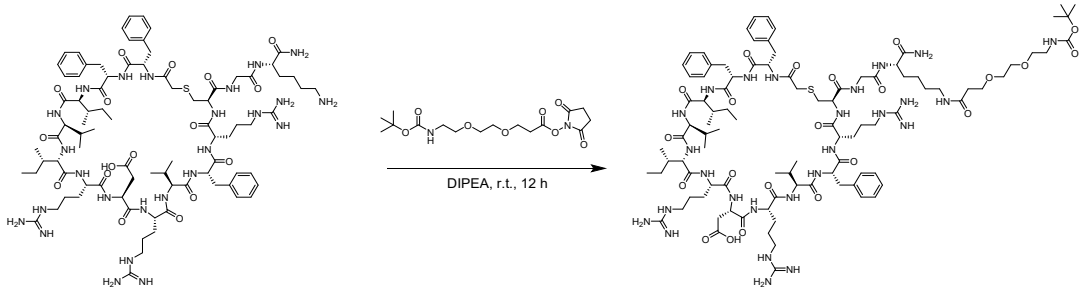
To a solution of **C10** (40 mg, 0.04 mmol) in DCM (6 mL), TFA (3 mL) was added. The mixture was stirred 12 h at room temperature. The solution was concentrated under

reduced pressure and vacuum pump for 4 h. The middle product was dissolved in DCM, and but-3-yn-1-yl (4-nitrophenyl) carbonate (18.82 mg, 0.08 mmol) was added. The mixture was stirred at room temperature for 12 h. The mixture was purified by HPLC to obtain the desired product **C11** (26 mg, 78% yield). Calc'd 846.2844 $[M + H]^+$; found 846.2827.

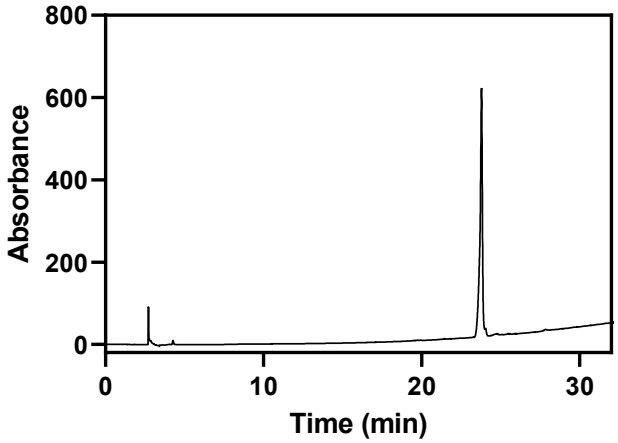


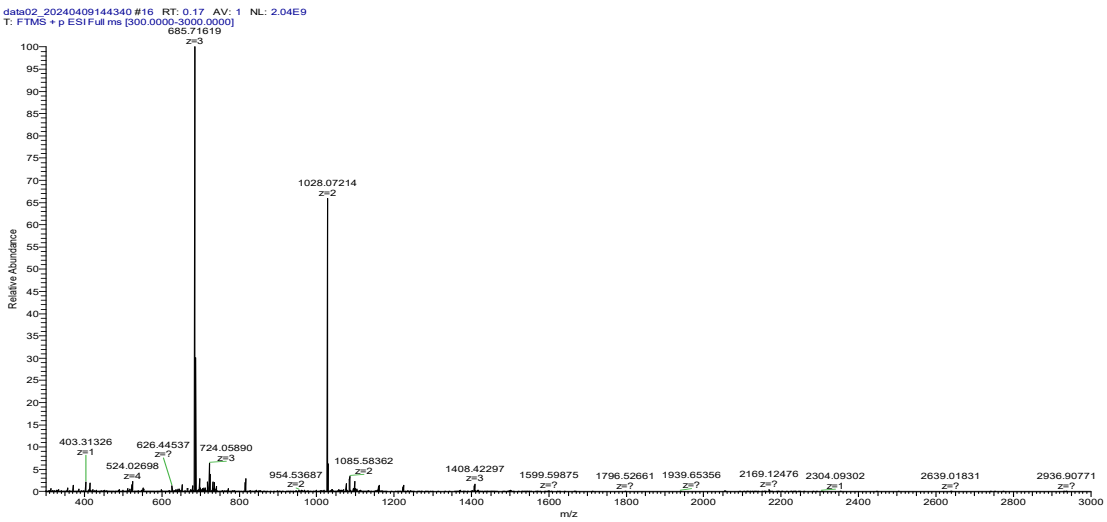


Synthesis of Kp101 peptide-PEG_2-Boc

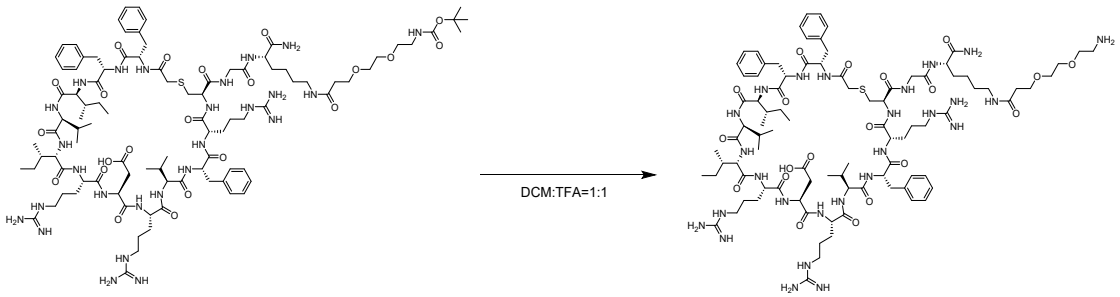


To a solution of **Kp101 cyclopeptide** (20 mg, 0.011 mmol) in DMSO (2 ml), Boc-NH-PEG_2-C2-NHS ester and DIPEA was added. The mixture was stirred for 12 h at room temperature. The mixture was purified by HPLC to obtain the desired product **Kp101 peptide-PEG_2-Boc** (18 mg, 80 % yield). ESI-HRMS m/z Calcd. 685.7190 $[M + 3H]^{3+}$, Found 685.7162; Calcd. 1028. 0745 $[M + 2H]^{2+}$, Found 1028.0721.

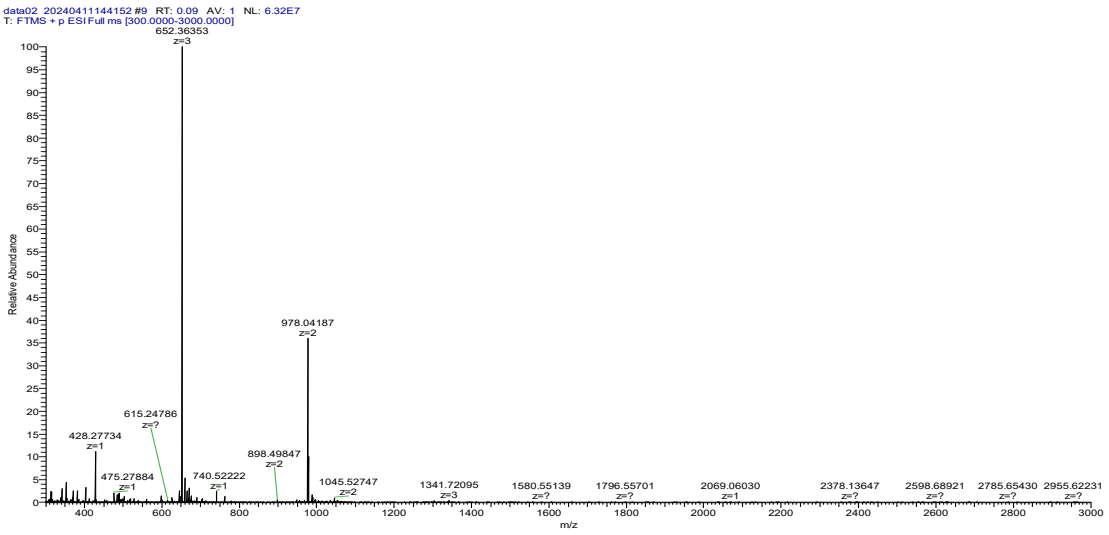




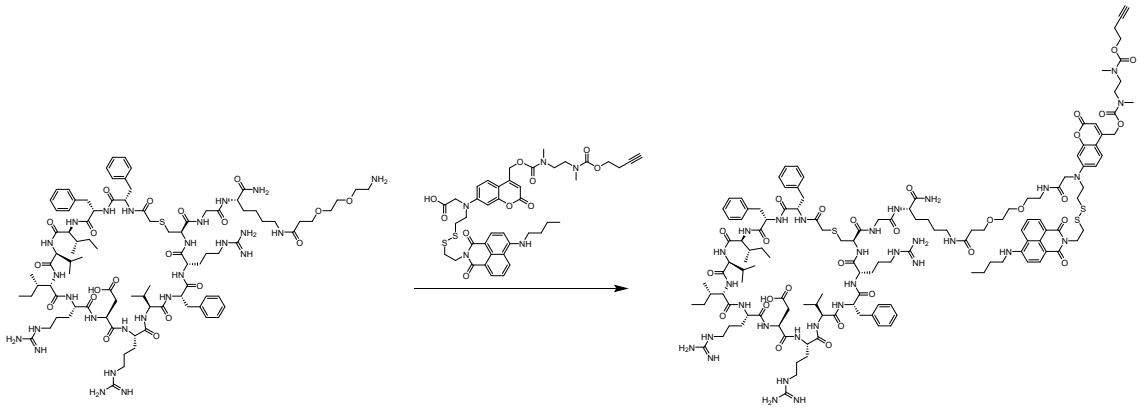
Synthesis of Kp101 peptide-PEG_2-NH_2



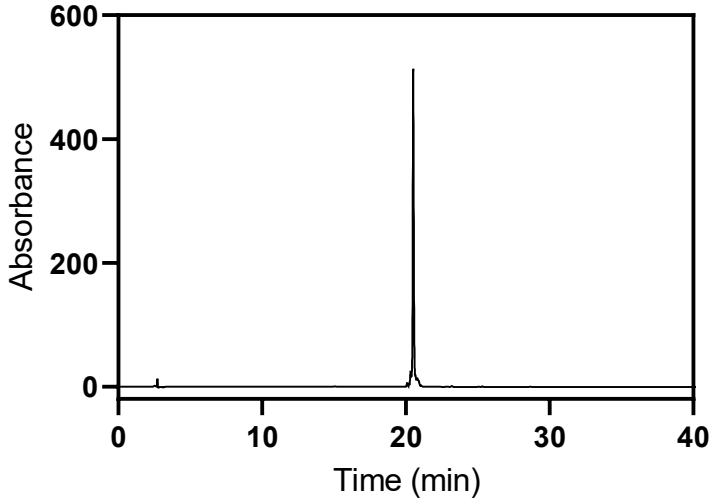
To a solution of **Kp101 peptide-PEG$_2$-Boc** (18 mg, 0.009 mmol) in DMSO (2 mL), TFA (1 mL) was added. The mixture was stirred for 12 h at room temperature. The solution was concentrated under reduced pressure and vacuum pump for 4 h. **Kp101 peptide-PEG$_2$-NH$_2$** was obtained as a white solid (16 mg, 91 % yield). ESI-HRMS m/z Calcd. 652.3601 $[M + 3H]^{3+}$, Found 652.3635; Calcd. 978.0402 $[M + 2H]^{2+}$, Found 978.0419.

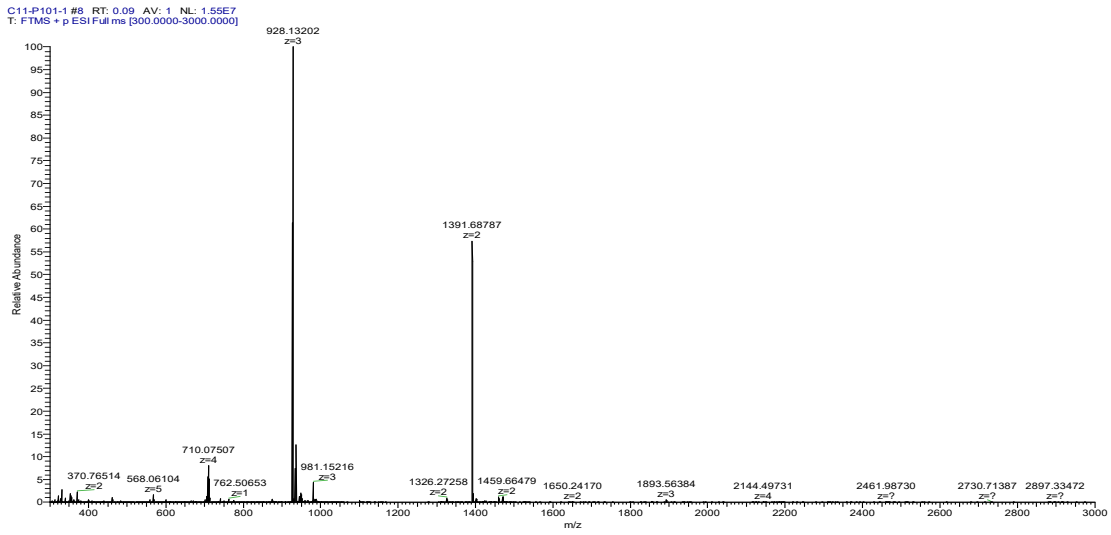


Synthesis of Kp101 peptide-c11



A mixture of **Kp101 peptide-PEG2-NH_2** (16 mg, 0.0082 mmol), **C11** (9 mg, 0.01 mmol), HBTU (9.33 mg, 0.0246 mmol) and DIPEA (38 μL, 0.0246 mmol) in 3 mL anhydrous DMF was stirred at 25 °C for 12 h. The resulting residue was purified by semipreparative HPLC to give desired product as a light yellow solid (12 mg, 53%). ESI-HRMS m/z Calcd. 928.1234 $[M + 3H]^{3+}$, Found 928.1320; 1391.6812 $[M + 2H]^{2+}$, Found 1391.6879.





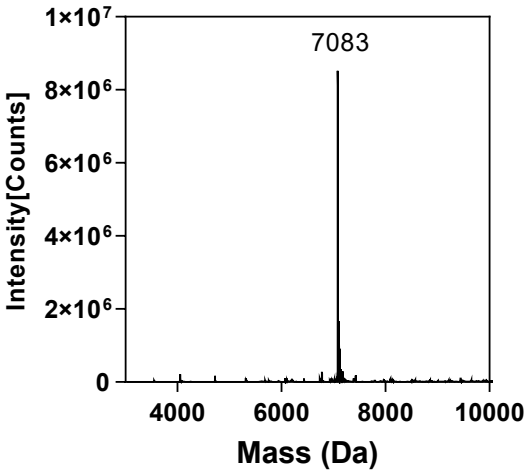
Oligonucleotide synthesis and sequences.

The PD-L1 and Bcl-2 targeted siRNA sequences were purchased from Genepharma (Suzhou, China) and Sangon Biotech (Shanghai, China), respectively, whose sequences were obtained from a previous report[1]. Cy3 siBcl-2 passenger strand and 3'-aminated siBcl-2 guide strand were synthesized on a K&A H-8 DNA/RNA synthesizer. 3'-Amino-modifier CPG was used for the synthesis of 3'- aminated Bcl-2. All phosphonamidites and oligonucleotide synthesis reagents were purchased from Genepharma (Suzhou, China) and used as per manufacturer's instructions. We directly used Cy3 CE phosphonamidite as a monomer to synthesize 5′-Cy3-labeled siBcl-2 passenger strand. After the standard solid-phase synthesis, cleavage from CPG, and deprotection, oligonucleotides were purified by HPLC with CH_3CN/H_2O (0.1 M CH_3COONH^+) as eluents (CH_3CN from 2% to 40% in 30 min). The collected products were desalted and lyophilized. Then, oligonucleotides were dissolved in ddH_2O, and their concentrations were determined using NanoDrop 2000 (Thermo Scientific). (Cy3) siBcl-2: 5'-GUGAAGUCAACAUGCCUGCdTdT-3' (sense); (3'-aminated) siBcl-2: 5'-GCAGGCAUGUUGACUUCACdTdT-3' (antisense); sictrl: 5'-UUCUCCGAACGUGUCACGUUUdTdT-3' (sense); sictrl: 5'-AAACGUGACACGUUCGGAGAAdTdT-3' (antisense); siPD-L1: 5'-

GUGGCAUCCAAGAUACAAAdTdT-3' (sense); siPD-L1: 5'-UUUGUAUCUUGGAUGCCACdTdT-3' (antisense); The oligonucleotides were then desalted and lyophilized. After dispersion in DEPC-treated H_2O, the concentrations of synthesized oligonucleotides were determined by NanoDrop 2000 (Thermo Scientific). The oligonucleotides were further characterized by LC-ESI-MS. Passenger strand of siBcl-2: calculated 6670; found 6669. Guide strand of siBcl-2: calculated 6646; found 6645. Guide strand of 3'-aminated siBcl-2: calculated 6856; found 6853. Passenger strand of siPD-L1: calculated 6700; found 6700. Guide strand of siPD-L1: calculated 6585; found 6585. Passenger strand of siNC: calculated 7391; found 7391. Guide strand of siNC: calculated 7195; found 7196. 5′-Cy3-labeled passenger strand of siBcl-2: calculated 7273; found 7275.

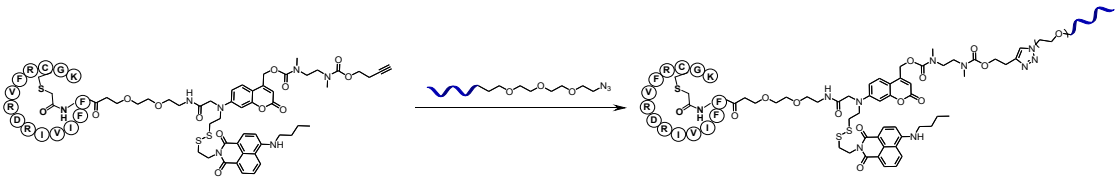
siBcl-2 labeling with NHS-PEG_3-N_3

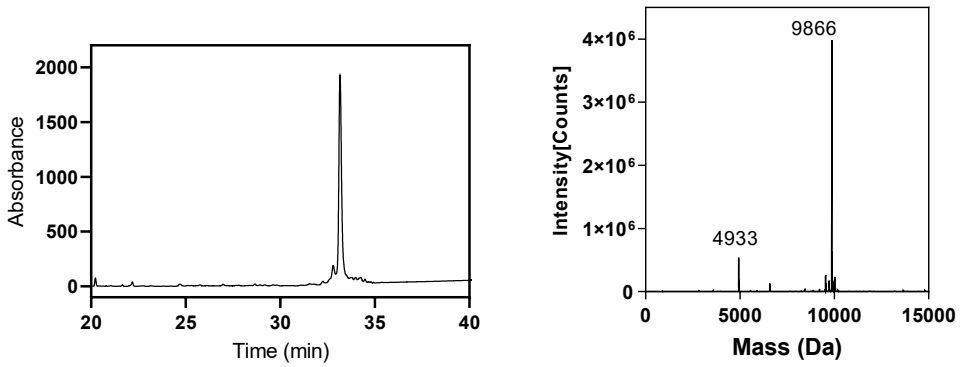
1 mL of a 0.1 mM solution of 3'-aminated siBcl-2 (in DMSO) was added 50 equiv. NHS-PEG_3-N_3 (172 μL of a 10 mg/mL stock in DMSO). The reaction was allowed to incubate for 48 h at room temperature, then purified by HPLC with CH_3CN/H_2O (0.1 M CH_3COONH^+) as eluents (CH_3CN from 2% to 50% in 30 min). The collected products were desalted and lyophilized. Then, **Bcl-2- PEG_3-N_3** was dissolved in ddH_2O, and its concentration was determined using NanoDrop 2000 (Thermo Scientific). The guide strand of siBcl-2-PEG_3-N_3 was characterized by LC-ESI-MS. calculated 7085; found 7083.



Synthesis of Kp101 peptide-C11- siBcl-2

Kp101 peptide-C11 (0.017 mmol) and azide-labeled siBcl-2 (0.0374 mmol) were dissolved in DMSO (1 mL). Then, a solution of $CuSO_4$ (0.0085 mmol), sodium ascorbate (0.0085 mmol), THPTA (0.0085 mmol) dissolved in water (200 μL) was added. The reaction solution was kept stirring at room temperature for 12 h. The mixture was purified by HPLC with CH_3CN/H_2O (0.1 M CH_3COONH^+) as eluents (CH_3CN from 2% to 50% in 60 min). **Kp101 peptide-C11-siBcl-2** was dissolved in ddH_2O, and its concentration was determined using NanoDrop 2000 (Thermo Scientific). The Kp101 peptide-C11 was characterized by LC-ESI-MS. Calculated 9865; found 9866.





3. Cell Lines and Cell Culture

MDA-MB-231 and LO2 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in DMEM supplemented with 10% FBS in a humidified environment at 37 ℃ which contains 5% CO_2 and 95% air.

4. MTT assays

The MTT assay was conducted to evaluate the cell cytotoxicity. The cells were cultivated in a 96-well plate for 12 h for adherence, and then incubated with different concentration of DPRC (0-1 μM) for 48 h. Then 20 μL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL) was added. After incubation for 4 h and removing the remaining MTT, 150 μL DMSO was added to dissolve the formazan. The absorbance of each well at 570 nm was measured by a microplate reader, the cell viability was calculated using the follow formula: (a-b)/(c-b) × 100%, where a, b and c are absorbance of treated wells, control wells and untreated wells, respectively.

5. Confocal fluorescence imaging

To evaluate the cellular uptake and lysosomal escape of DPRC, MDA-MB-231 cells were incubated with DPRC, after each incubation procedure, the cells were washed with PBS buffer three times. To visualize the intracellular disassembly, cells

were exposure to visible photoirradiation (420 nm, 100 mW/cm^2) for 15 min at 4 h. At indicated incubation time, these cells were washed using PBS for 3 times, incubated with Hoechst and LysoTracker Deep Red (Invitrogen, L12492) following the manufacturer's instructions, and fixed using 4 % paraformaldehyde. Images were captured using Leica SP8 STED 3X confocal super-resolution microscope. ImageJ was used to quantify the fluorescence intensities.

6. RT-qPCR

After reaching ~80% confluency, MDA-MB-231 cells were transfected with siPD-L1 (200 nM) or si-ctrl (200 nM) using Lipofectamine 2000 according to the manufacturer's protocol. Or, MDA-MB-231 were treated with DPRC (0-500 nM). For light (+) group, cells were exposure to 420 nm light irradiation (100 mW/cm2) for 15 min at 4 h post incubation. After incubation for 48 h, total RNA of the samples was separated by Trizol reagent (Invitrogen, Shanghai, China) following the manufacturer's protocols. Reverse transcriptase reactions were performed using HiScript® III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). RT-qPCR was done using Quantitative PCR with ChamQTM Universal SYBR QPCR Master Mix (Vazyme, Nanjing, China). Eventually, the product was analyzed on the Applied Biosystem StepOnePlusTM Real-Time PCR system. The quantitative measures were obtained using the ΔΔCT method and was normalized to GAPDH or U6 mRNA levels. Primers for GAPDH (F): 5'-CGAGCCACATCGCTCAGACA; GAPDH (R): 5'-GTGGTGAAGACGCCAGTGGA. Primers for Bcl-2 (F): 5'-CCCTGTGGATGACTGAGTACCTG; Bcl-2 (R): 5'-GTGATGCAAGCTCCCACCAG. Primers for PD-L1 (F): 5'-TTTGCTGAACGCCCCATA; PD-L1 (R): 5'- TGCTTGTCCAGATGACTTCG.

7. Flow cytometric analysis

To evaluate cellular uptake, MDA-MB-231 cells were plated 1 d before the experiment. After reaching ~70% confluency, MDA-MB-231 cells were incubated with 500 nM Cy3-DPRC for 0-6 h, then washed 3 times with PBS, followed by analysis on CytoFLEX (Beckman Coulter Life Sciences). FlowJo software (V 10.0.8r1) was used for data analysis.

To evaluate cell apoptosis, MDA-MB-231 cells were plated 1 day before the experiment. After reaching ~70% confluency, MDA-MB-231 cells were incubated with 500 nM DPRC. For + Light groups, cells were subjected to 420 nm light irradiation (100 mW/cm^2) for 15 min at the 4 h time point post incubation. After a total culture time of 48 h, supernatants and cells were collected according to the instruction of Annexin V-FITC Apoptosis detection Kit (Beyotime, Shanghai), and then were gently resuspended in 195 μL binding solution. Then, 5 μL Annexin V-FITC and 10 μL propidium iodide staining solution were added and mixed for 10 min, followed by analysis on CytoFLEX (Beckman Coulter Life Sciences).

To evaluate the binding affinity potential of DPRC to cell membrane PD-L1, MDA-MB-231 cells were plated 1 d before the experiment. For the PD-L1 (negative) group, MDA-MB-231 cells were transfected with siPD-L1 (200 nM) using Lipofectamine 2000 according to the manufacturer's protocol. For saturation binding experiments, after reaching ~70% confluency, MDA-MB-231 cells were incubated with DPRC in a range of 0 - 2000 nM in 50 μL of 0.1% bovine serum albumin (BSA) in PBS. After 1 h of incubation at 4 °C, the cells were washed two times with 300 μL of 0.1% BSA in PBS and re-suspended in 300 μL of 0.1% BSA. Cells were gated on Forward Scatter and Side Scatter, and a minimum of 10,000 viable cells were analyzed per sample. All experiments were performed in triplicate. The mean fluorescence intensity of the cell−DPRC complexes was used to evaluate the binding affinity. The dissociation constants (K_d) were obtained by fitting the dependence of fluorescence

intensity (Y) and the concentrations of DPRC (X) into the one site saturation equation Y = Bmax X/(Kd + X), using GraphPad Prism 8.0.

8. Microscale thermophoresis (MST) assay

MST experiments were carried out on a Monolith NT.115 (NanoTemper, Germany) instrument. All experiments were carried out in MST buffer (20 mM HEPES buffer, pH 7.5 and 0.05% Tween-20). The recombinant human PD-L1-His was purchased from MedChemExpress (MCE). The concentration of the labeled protein was quantified spectrophotometrically. The final concentration of the protein was 50 nM, and the experiments consisted of 15-point titration series in which Kp101 or DRPC concentrations ranged from 0.6 to 10000 nM. All samples were incubated in the dark for 20 min prior to being loaded into premium-coated capillary tubes (NanoTemper). A typical MST experiment featured LED illumination at 50% power and IR illumination at 80% power. The pre-IR phase was 5 s, followed by an IR-on phase of 10 s and an IR-off phase of 5 s. All data were analyzed using MO. Affinity Analysis.

9. Western blot analysis

MDA-MB-231 cells were seeded on 35 mm dishes. After reaching ~70% confluency, cells were treated with DPRC at different concentrations. After 4 h, cells were irradiated with 15 min of 420 nm light irradiation (100 mW/cm^2). Cells were kept at 37°C for 48 h. Then, cells were washed with PBS for three times, and cellular lysates were extracted using RIPA lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.5, 1% NP40, 1% deoxycholate, 0.1% SDS, 1 × protease inhibitor cocktail). Protein concentration was determined using the BCA protein assay kit. A total of 20 μg of protein was then denatured in 1 × SDS loading buffer and resolved by 10% SDS-PAGE, followed by transferring to a PVDF membrane and blocking in 5 % nonfat milk in TBST. The PVDF membrane was then stained with primary antibody for Bcl-2. After wash, the membrane was further incubated with the appropriate secondary antibody and finally visualized

using ECL reagents. To visualize vinculin that served as the internal control, the membrane was further washed with TBST for 12 h, blocked, incubated with vinculin antibody. After wash, the membrane was then incubated with the secondary antibody and was finally visualized using ECL reagents. The band intensities were analyzed using Image J.

10. Tumor spheroid preparation and treatment

To prepare the cellular tumor spheroids made of MDA-MB-231 cells, agarose-coated 96-well plates were prepared in advance, 0.09 g agarose was added into a glass bottle without lid containing 6 mL DMEM medium. Then the solution was heated to 115 °C and maintained for 30 min, before cooling and solidification, 60 μL of sterile 1.5 % agarose (w/v) solution was dispensed into 96 well plates. Next, 0.3 mL Matrigel was added to 14 mL DMEM medium containing MDA-MB-231 cells, then 200 μL cell suspension was injected to each well of agarose-coated plates. After centrifugation at 1200 rpm for 10 min, the plate was placed in atmosphere with 5% CO_2 and at 37 °C for several days until the tumor sphere formed. For penetration study, the medium was replaced by a fresh medium supplemented with 500 nM Cy3-DPRC. At the 4 h time point, the spheroids were exposure to 420 nm light irradiation (100 mW/cm^2) for 15 min, then the spheroids were transferred to a glass-bottom tissue culture dish, and immediately imaged by laser confocal microscopy.

For the apoptosis assay, the medium of tumor spheroids was replaced by the fresh medium supplemented with 500 nM DPRC. At 4 h time point, MDA-MB-231 cells were exposure to 420 nm light irradiation (100 mW/cm^2) for 5 min. After a total culture time of 48 h, tumor spheroids were washed with PBS for three times. The apoptotic cells in tumor spheroids were stained using an Annexin V-FITC/PI staining kit according to manufacturer's protocol, followed by flow cytometry detection.

11. Statistical analysis

Statistical comparisons between groups were evaluated by Student's *t*-test. Data are shown as mean ± SEM. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ were considered to be statistically significant.

Supporting Figures

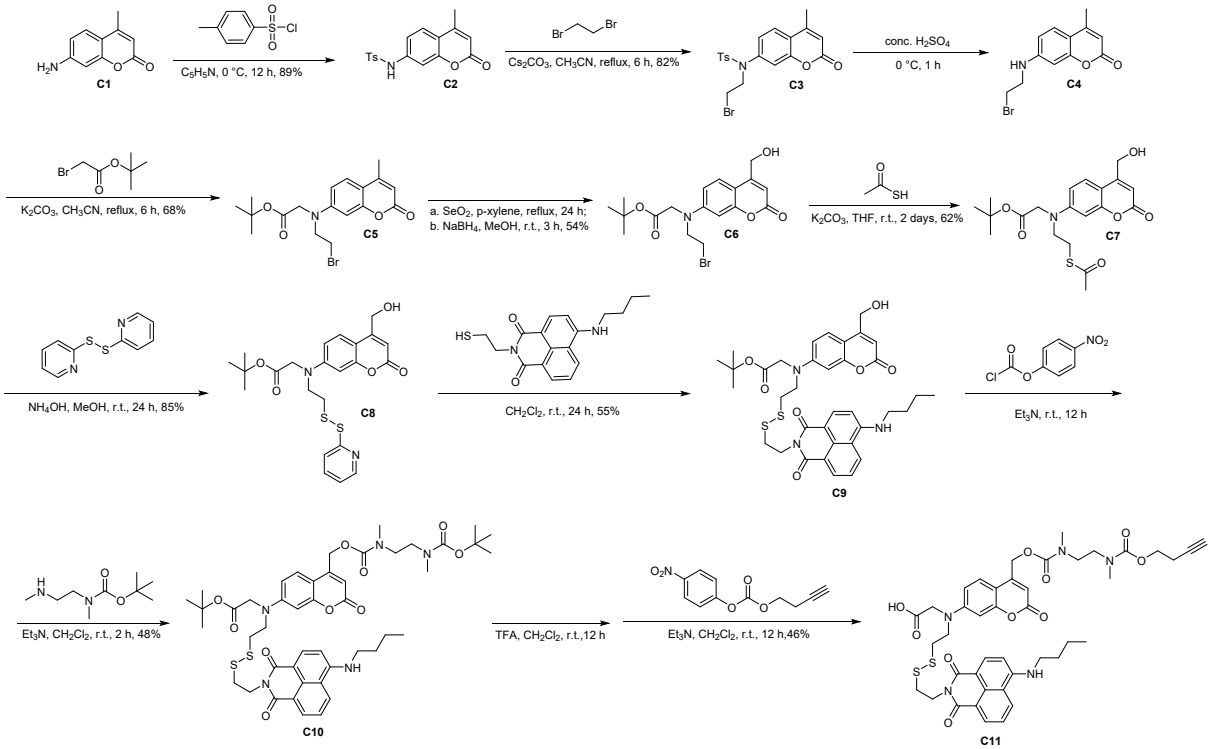


Figure S1. Synthetic routes for C11.

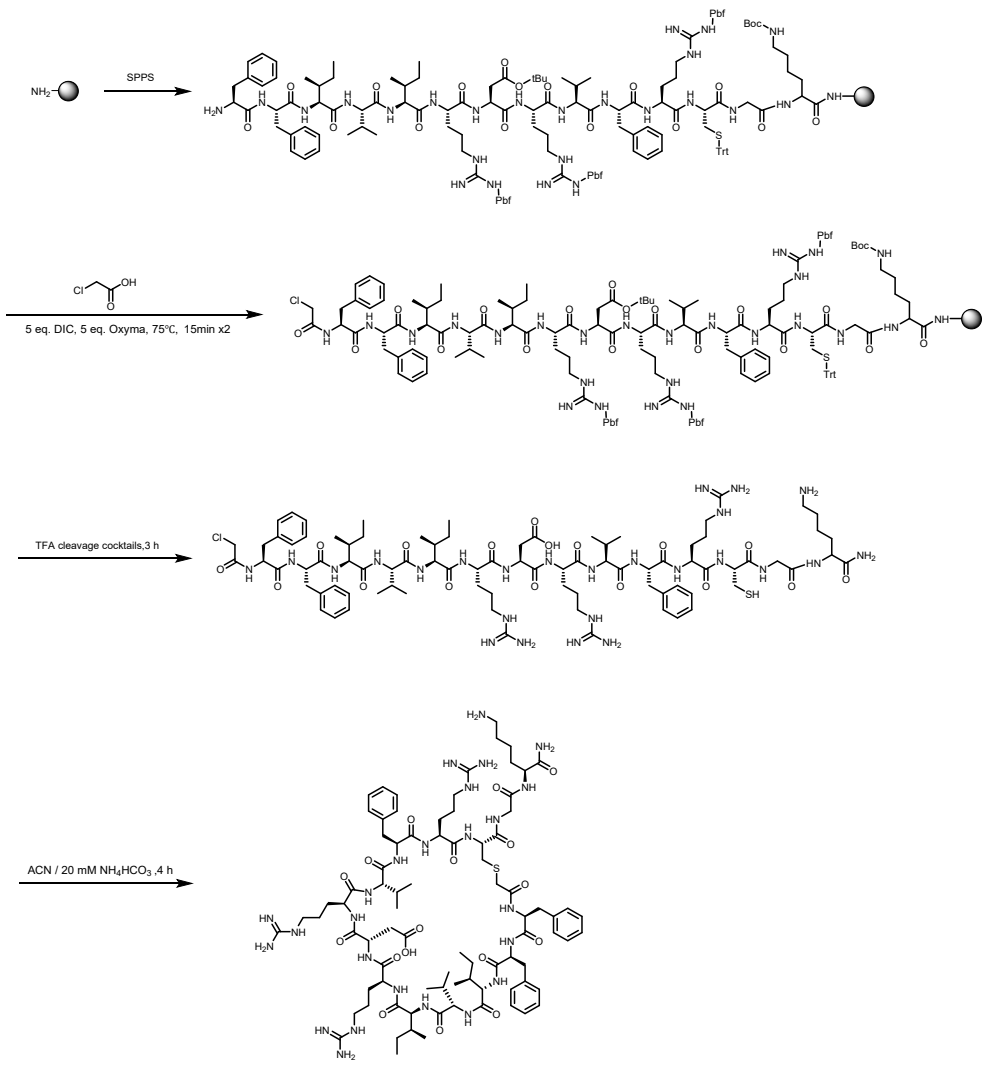


Figure S2. Synthetic routes for Kp101 cyclopeptide

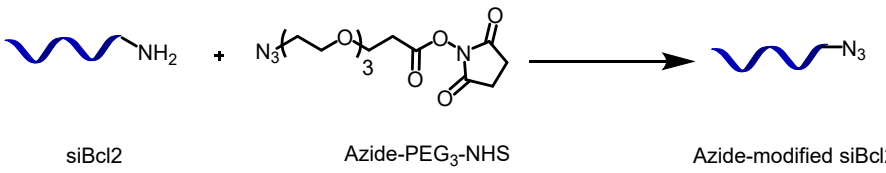


Figure S3. Synthetic route for siBcl-2 guide strand with 3'-azide modification.

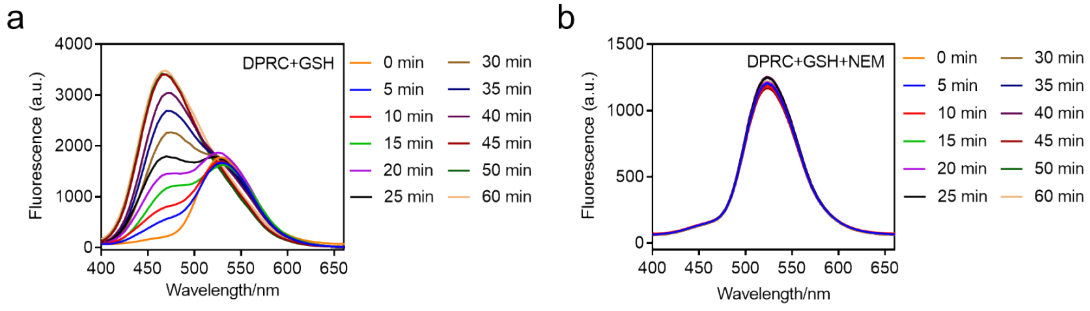


Figure S4. Fluorescence spectra of DPRC after treatment with (**a**) GSH (1 mM) or (**b**) GSH (1 mM) plus NEM (1 mM) for 0-60 min. Ex = 390 nm.

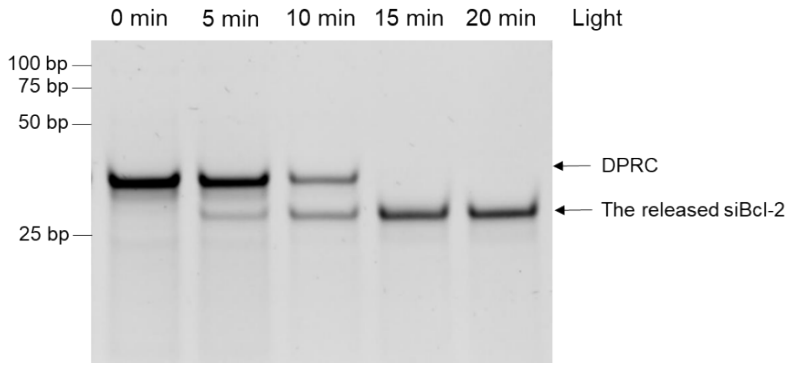


Figure S5. Native PAGE analysis of DPRC pre-treated with 1 mM GSH for 1 h followed by photoirradiation (420 nm, 100 mW/cm^2) for indicated time points.

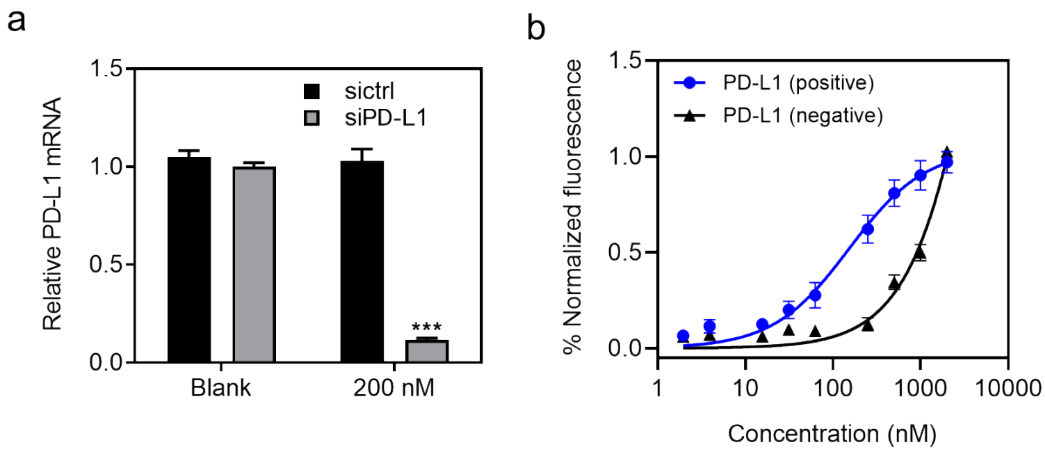


Figure S6. (**a**) RT-qPCR analysis of PD-L1 mRNA levels in MDA-MB-231 cells transfected with 200 nM sictrl or siPD-L1 for 48 h. (b) Binding of DPRC towards native (PD-L1-positive) MDA-MB-231 cells or PD-L1-negative MDA-MB-231 cells. PD-L1-negative MDA-MB-231 cells were prepared by siPD-L1 transfection. Fluorescence intensity was normalized to Maximum. Data are shown as mean ± SEM (n=3). ***$P <$ 0.001.

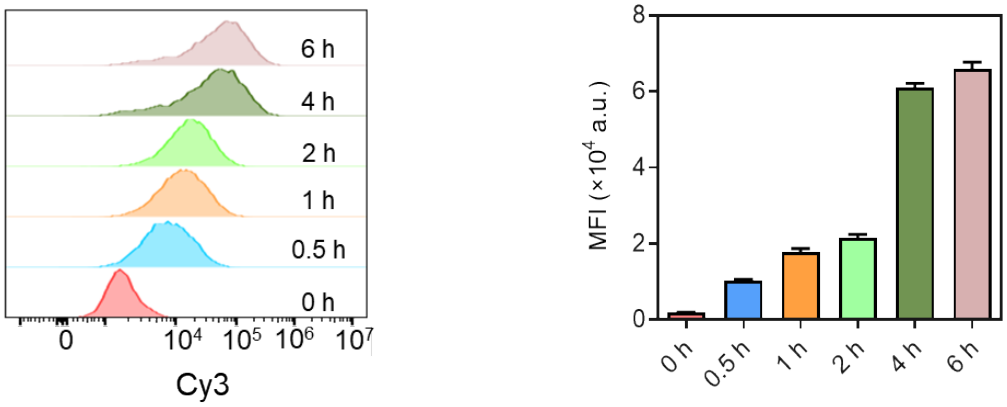


Figure S7. Representative flow cytometry analysis and quantification data of MDA-MB-231 cells treated with Cy3-DPRC (500 nM) for 0, 0.5, 1, 2, 4, or 6 h. Data are shown as mean ± SEM (n=3).

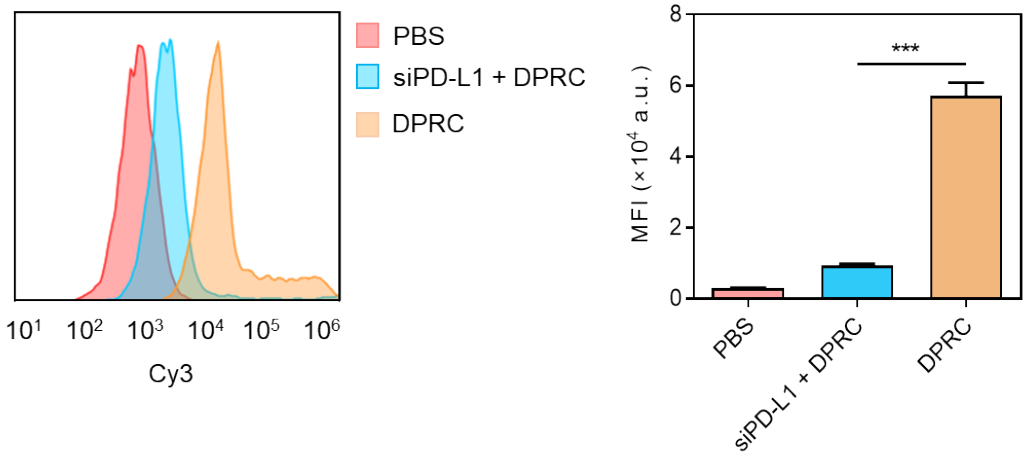


Figure S8. Representative flow cytometry analysis and quantification data of MDA-MB-231 cells or cells pre-treated with 200 nM siPD-L1 for 48 h, followed by treatment with Cy3-DPRC (500 nM) for 4 h. Data are shown as mean ± SEM (n=3). ***P < 0.001.

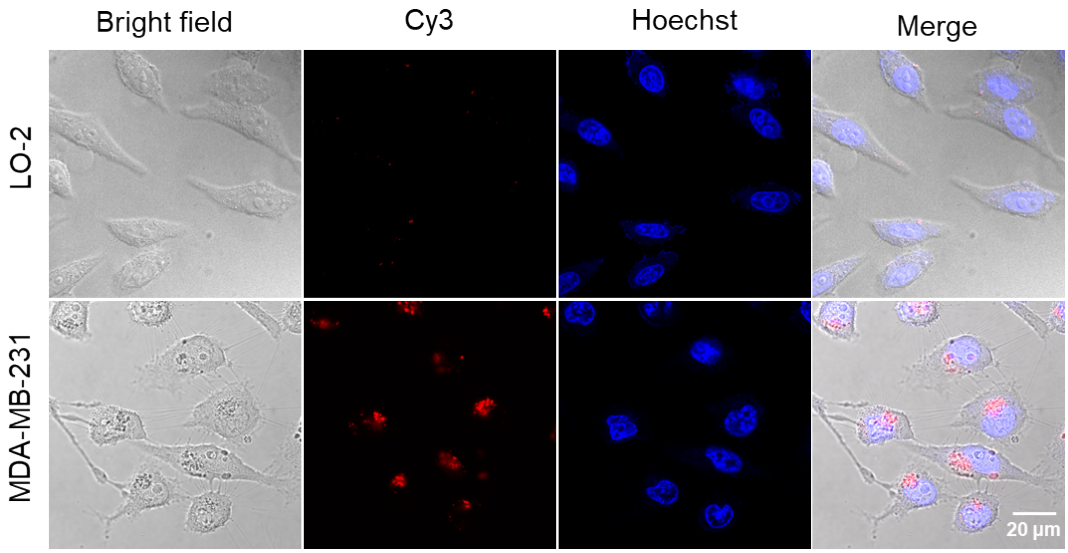


Figure S9. Confocal fluorescence images of LO-2 or MDA-MB-231 cells treated with Cy3-DPRC (500 nM) for 4 h. Hoechst was used to stain the nucleus.

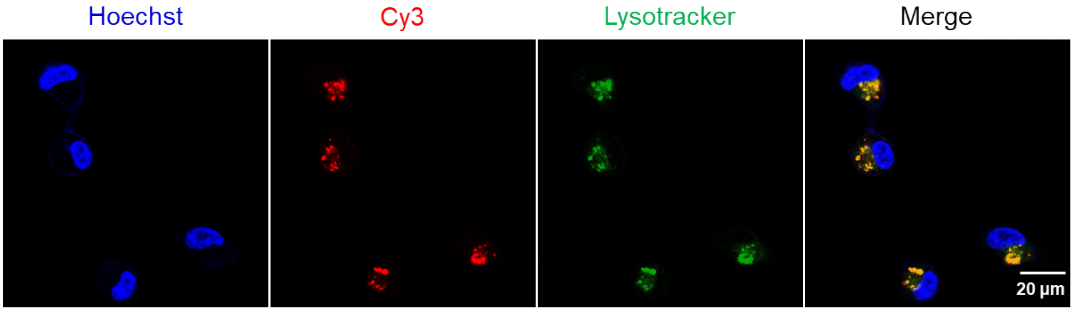


Figure S10. Confocal fluorescence images of MDA-MB-231 cells pre-treated with NEM (50 μM) for 1 h followed by incubation with Cy3-DPRC (500 nM) for 4 h and exposure to 420 nm light irradiation (100 mW/cm^2) for 15 min. The nucleus and lysosome were stained with Hoechst and Lysotracker, respectively.

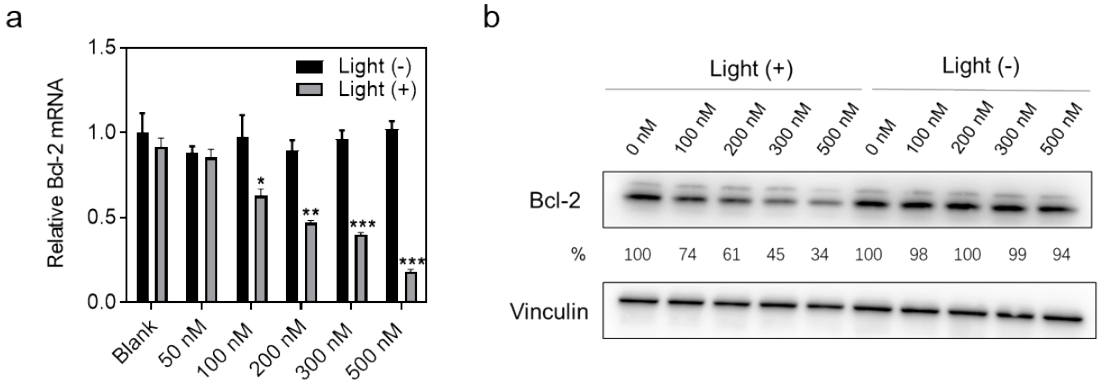


Figure S11. (**a**) RT-qPCR analysis of Bcl-2 mRNA levels in MDA-MB-231 cells treated with DPRC (0, 50, 100, 200, 300, 500 nM) for 48 h. (**b**) Western blot analysis of Bcl-2 protein levels in MDA-MB-231 cells treated with DPRC (0, 50, 100, 200, 300, 500 nM) for 48 h. For + Light group, cells were exposure to 420 nm photoirradiation

(100 mW/cm^2, 15 min) at 4 h post incubation. Data are shown as mean ± SEM (n=3). *P < 0.05, **P < 0.01, and ***P < 0.001, relative to Light (-) groups.

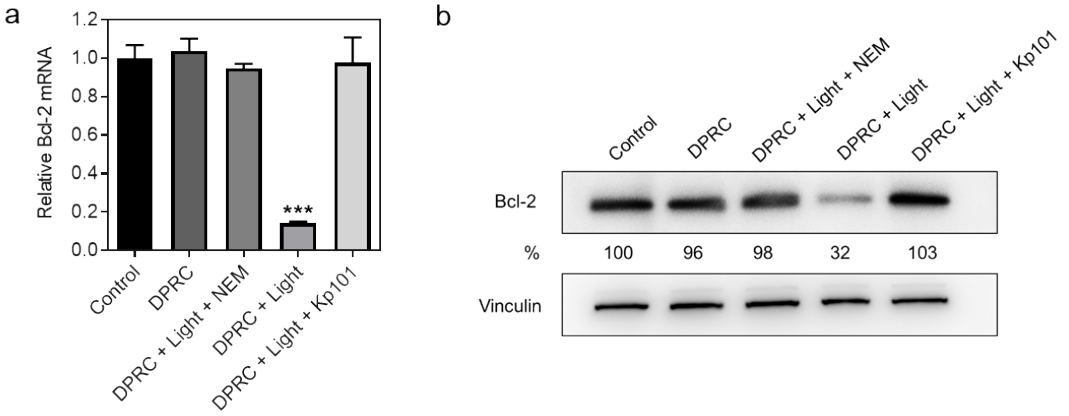


Figure S12. (**a**) RT-qPCR analysis of Bcl-2 mRNA levels and (**b**) western blot analysis of Bcl-2 protein levels in MDA-MB-231 cells treated with DPRC (500 nM), DPRC (500 nM) + NEM (50 μM), or DPRC (500 nM) + Kp101 (50 μM). For + Light group, cells were exposure to 420 nm photoirradiation (100 mW/cm^2, 15 min) at 4 h post incubation. Data are shown as mean ± SEM (n = 3). ***P < 0.001, relative to other groups.

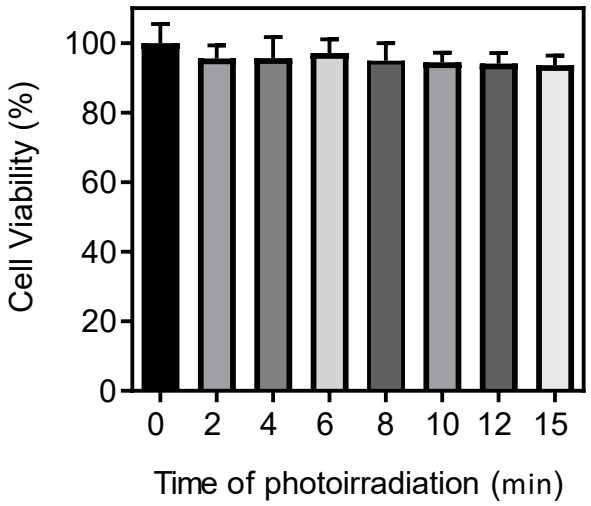


Figure S13. MTT analysis of MDA-MB-231 cell viability following 0-15 minutes of photoirradiation.

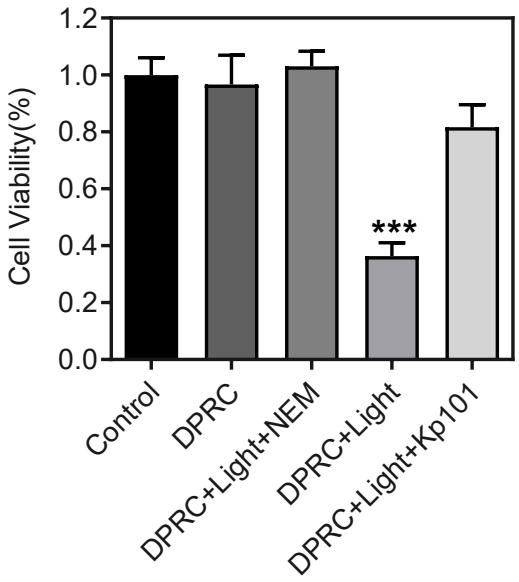


Figure S14. MTT analysis of the viability of MDA-MB-231 cells treated with DPRC (500 nM), DPRC (500 nM) + NEM (50 μM), or DPRC (500 nM) + Kp101 (50 μM). For + Light group, cells were exposure to 420 nm photoirradiation (100 mW/cm^2, 15 min) at 4 h post incubation. Data are shown as mean ± SEM (n = 3). ***$P < 0.001$, relative to other groups.

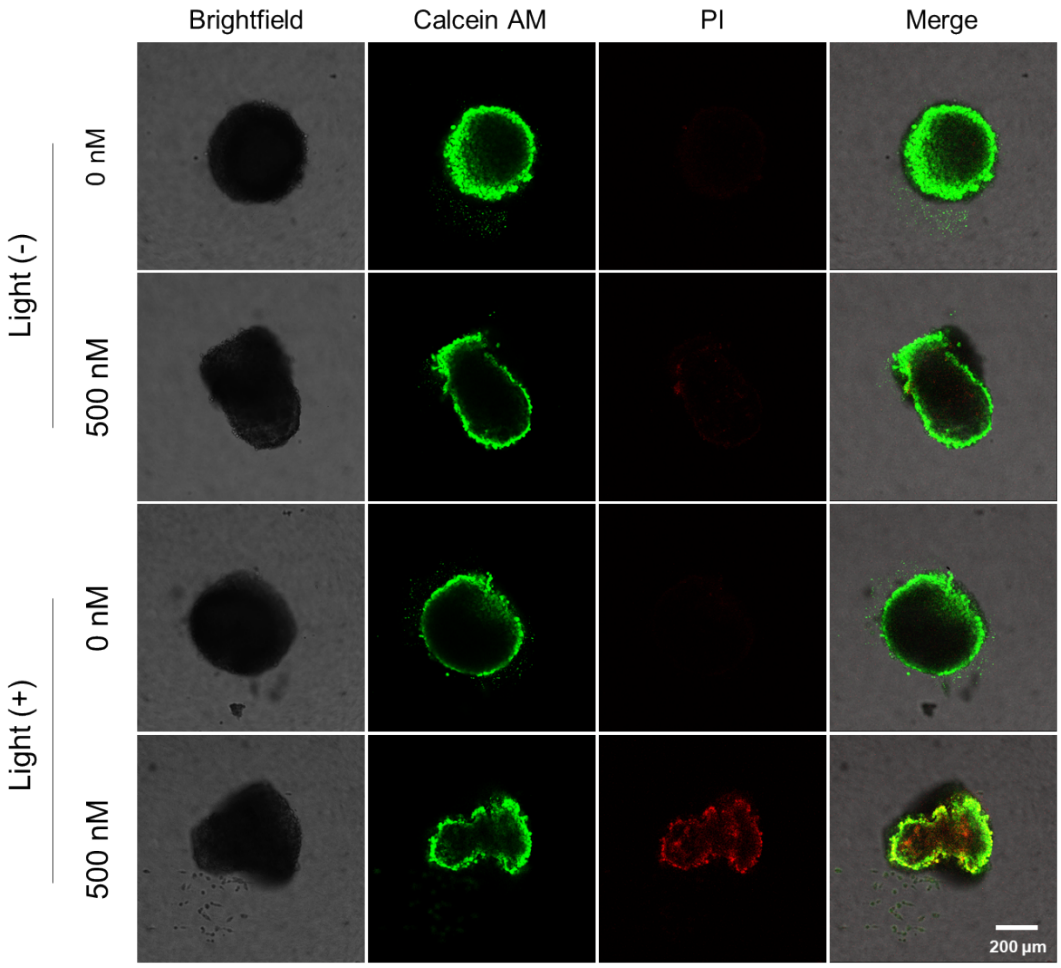


Figure S15. Confocal fluorescence images of MDA-MB-231 3DTSs treated with PBS or DPRC (500 nM) for 48 h, followed by staining with calcein-AM/PI. For + Light group, cells were exposure to 420 nm photoirradiation (100 mW/cm^2, 15 min) at 4 h post incubation.

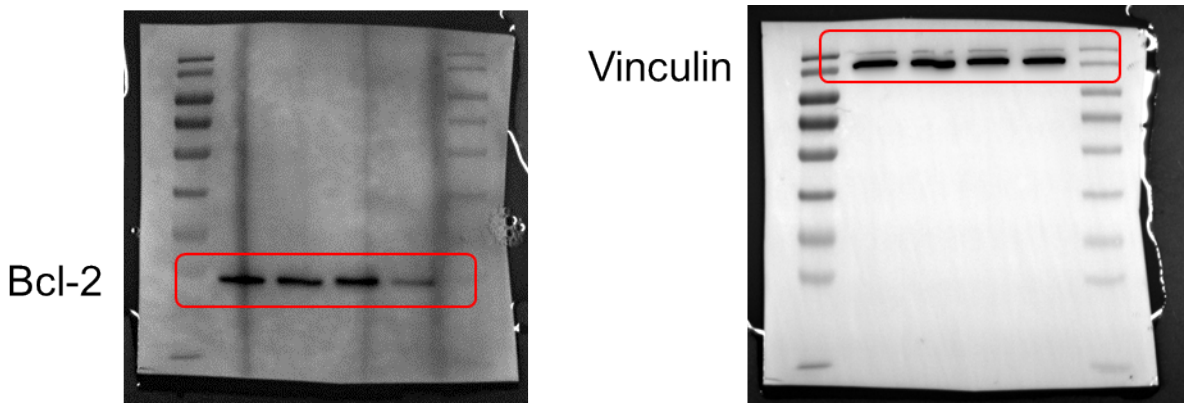


Figure S16. Source western blotting images of Figure 3b.

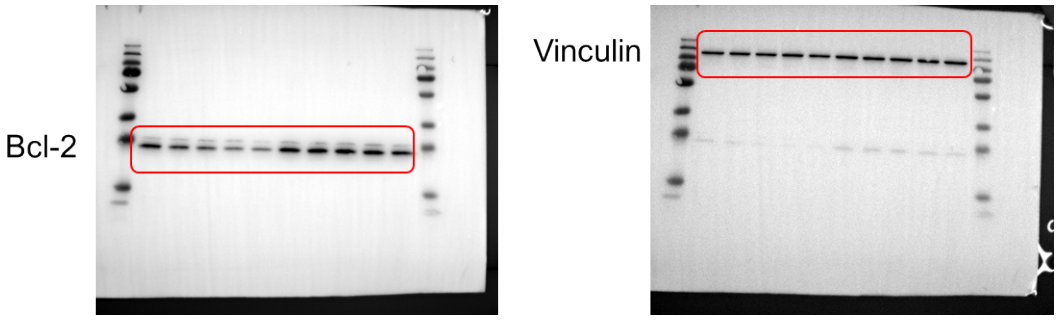


Figure S17. Source western blotting images of Figure S11b.

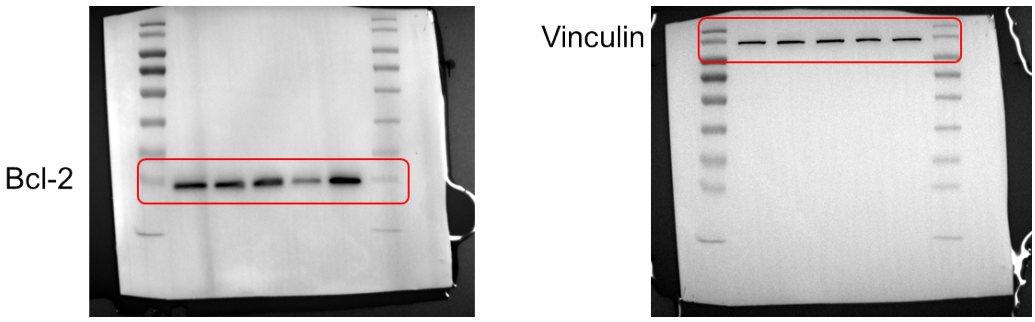


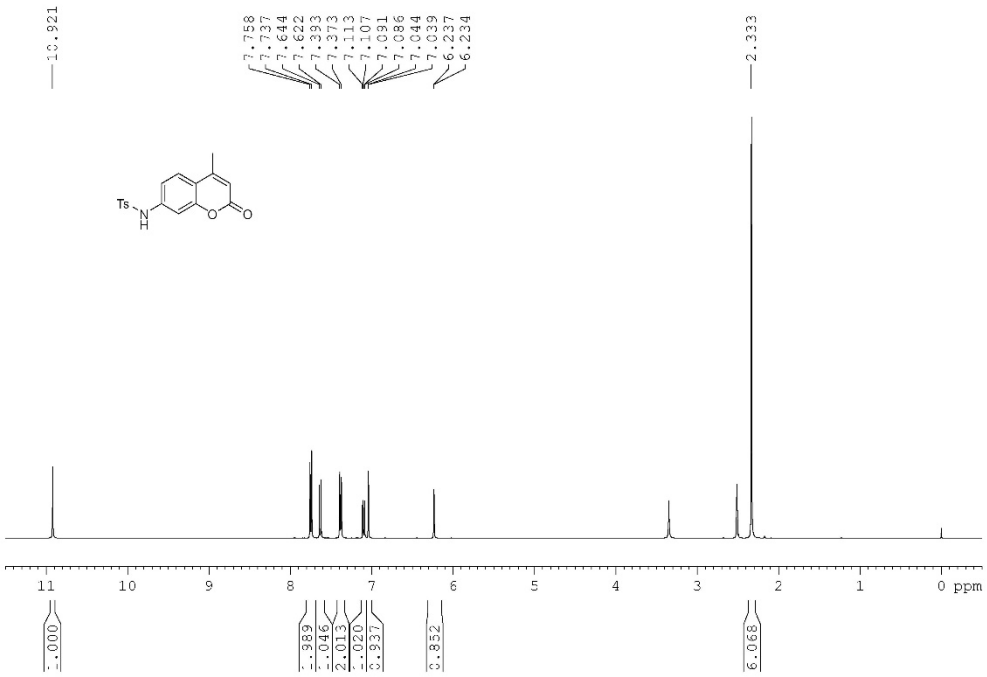
Figure S18. Source western blotting images of Figure S12b.

Reference

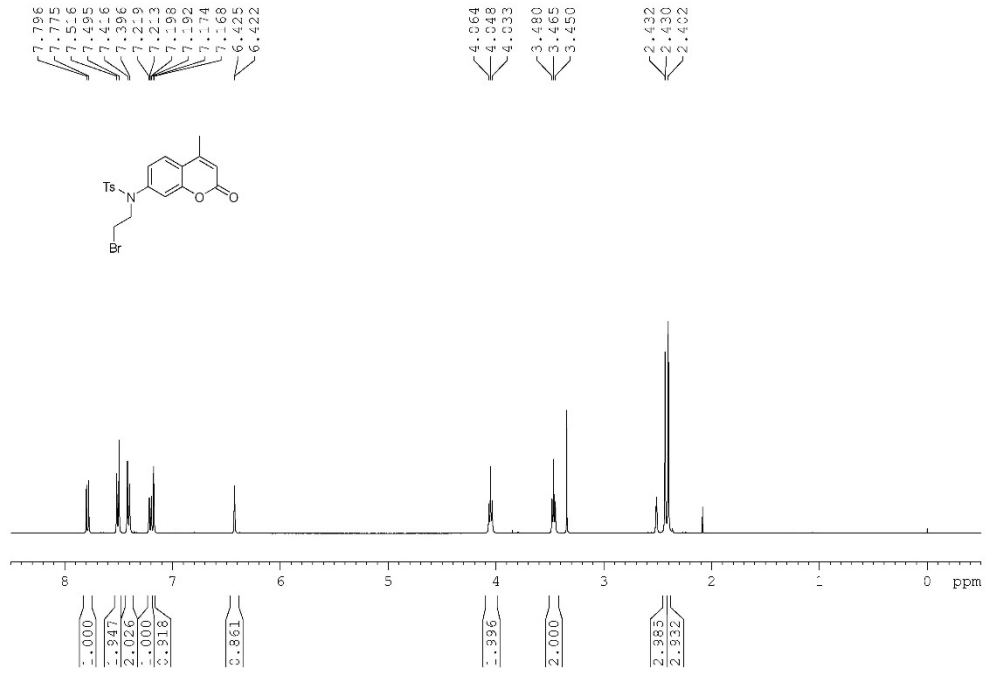
1. M. Mao and Q. H. Song, *Dyes Pigments*, 2012, **92**, 975-981.
2. Q. Lin, C. Bao, S. Cheng, Y. Yang, W. Ji and L. Zhu, *J Am Chem Soc*, 2012, **134**, 5052-5055.

NMR Spectra

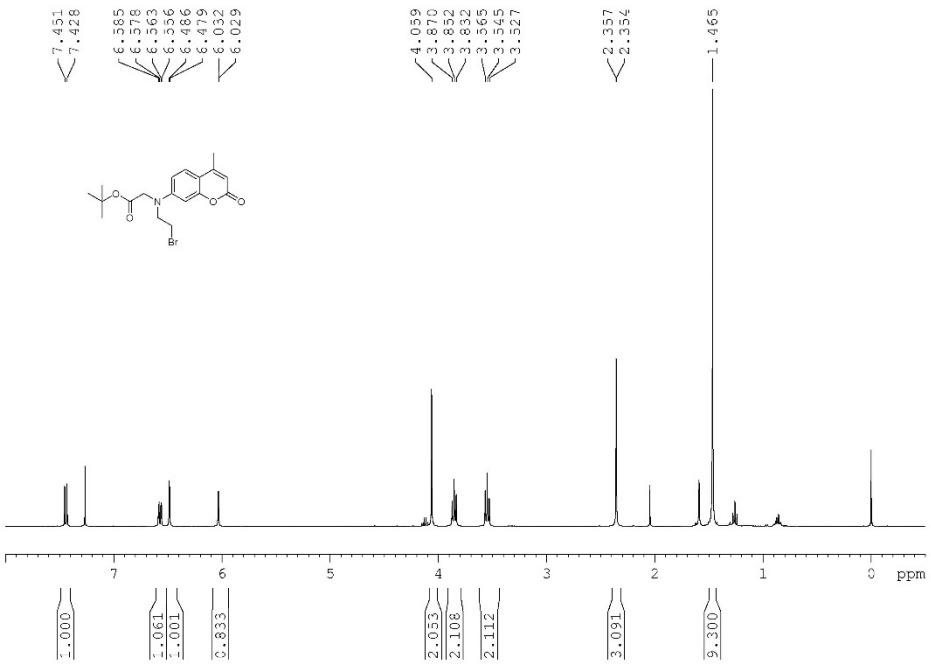
^{1}H NMR of C2



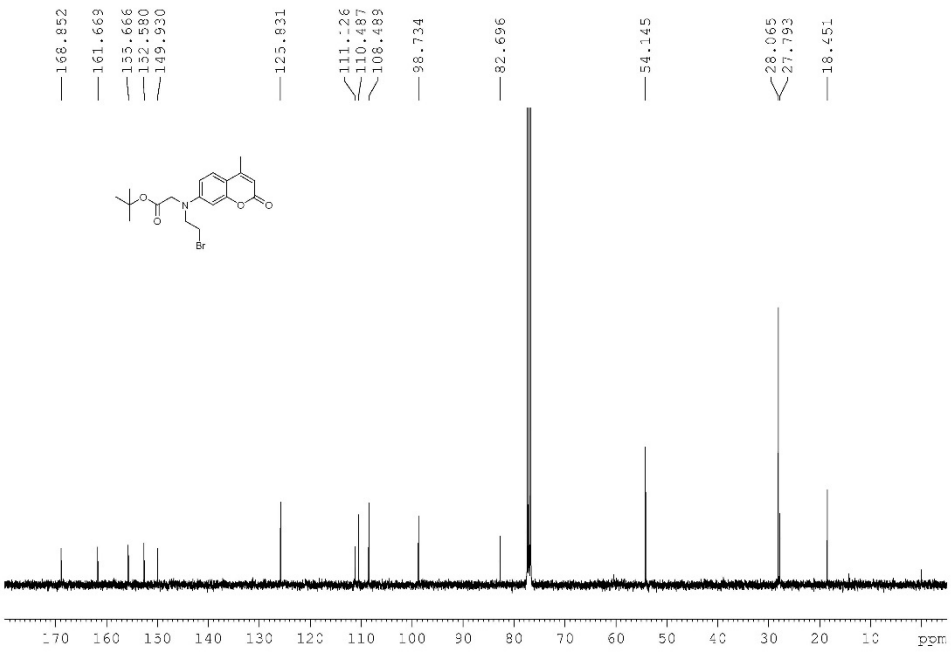
^{1}H NMR of C3



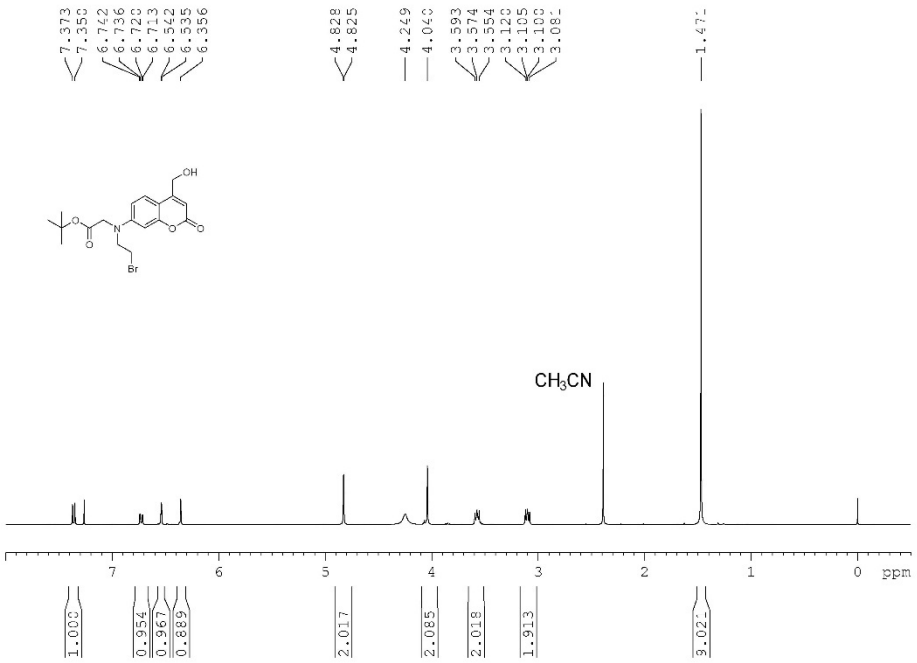
^{1}H NMR of C5



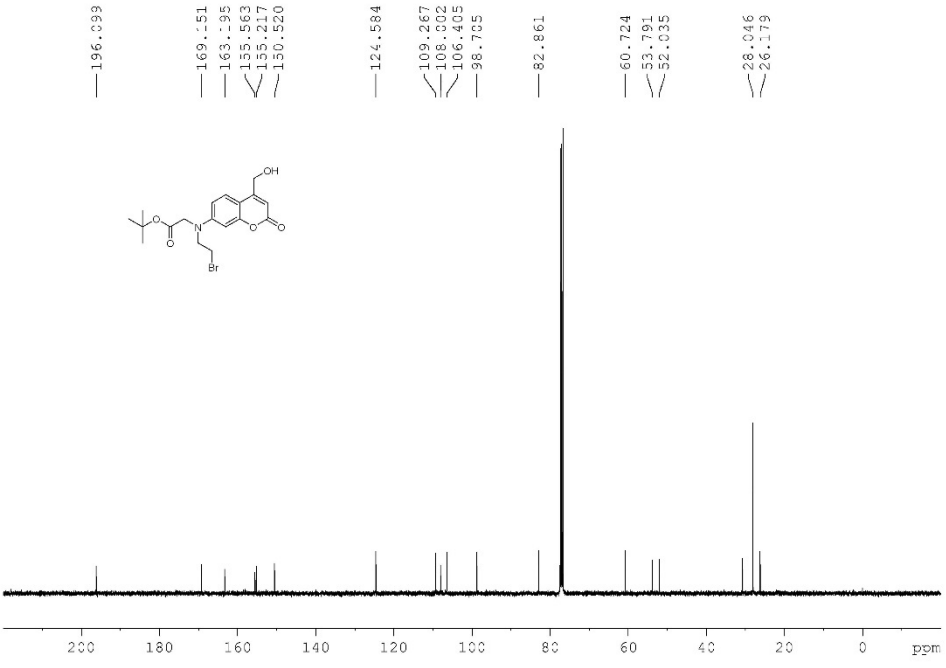
^{13}C NMR of C5



^{1}H NMR of C6

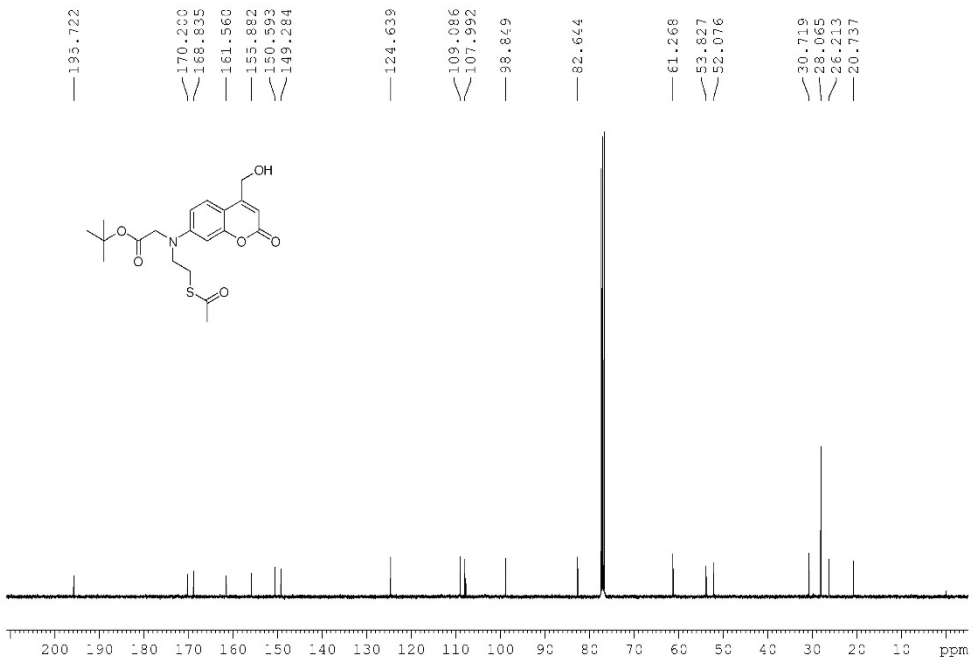


^{13}C NMR of C6



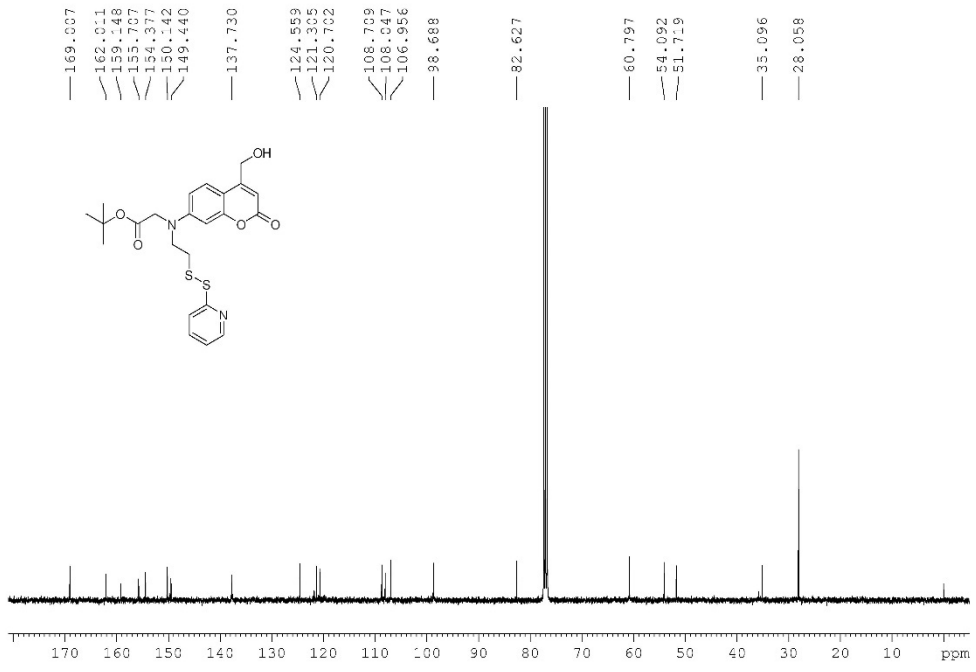
^{1}H NMR of C7

^{13}C NMR of C7

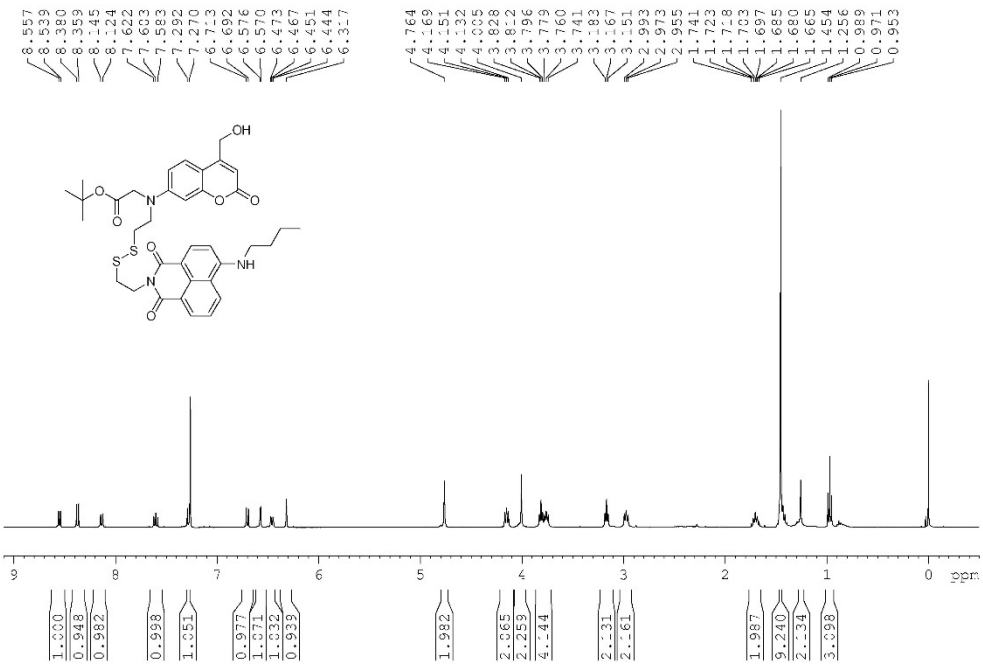


^{1}H NMR of C8

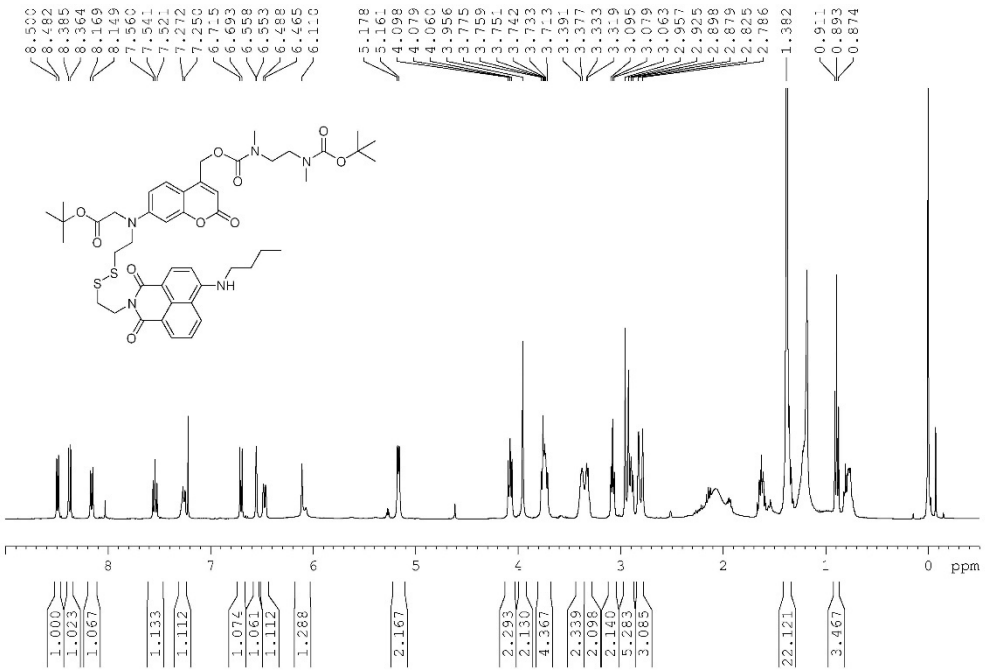
^{13}C NMR of C8



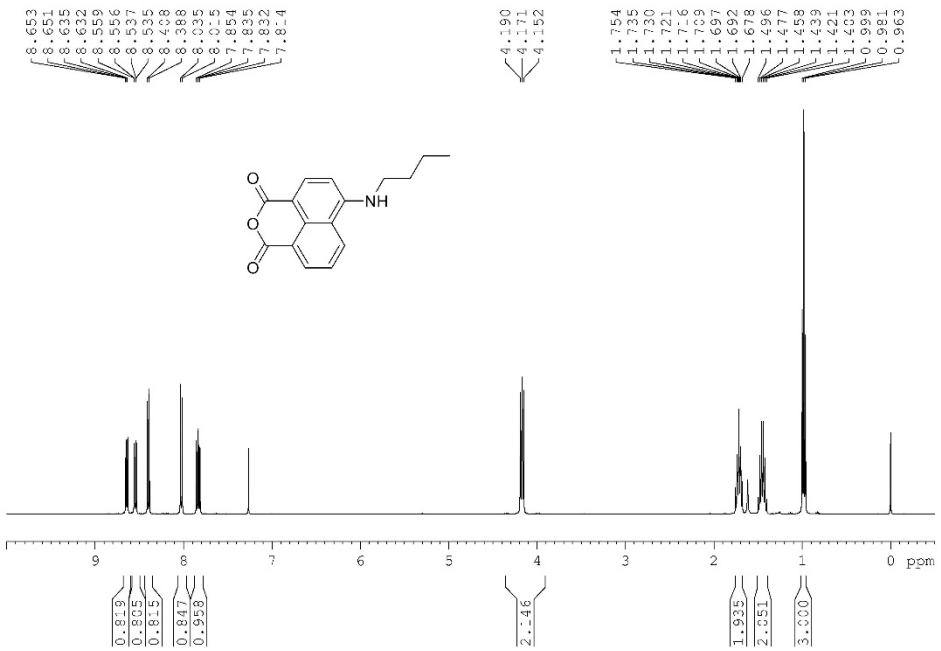
^{1}H NMR of C9



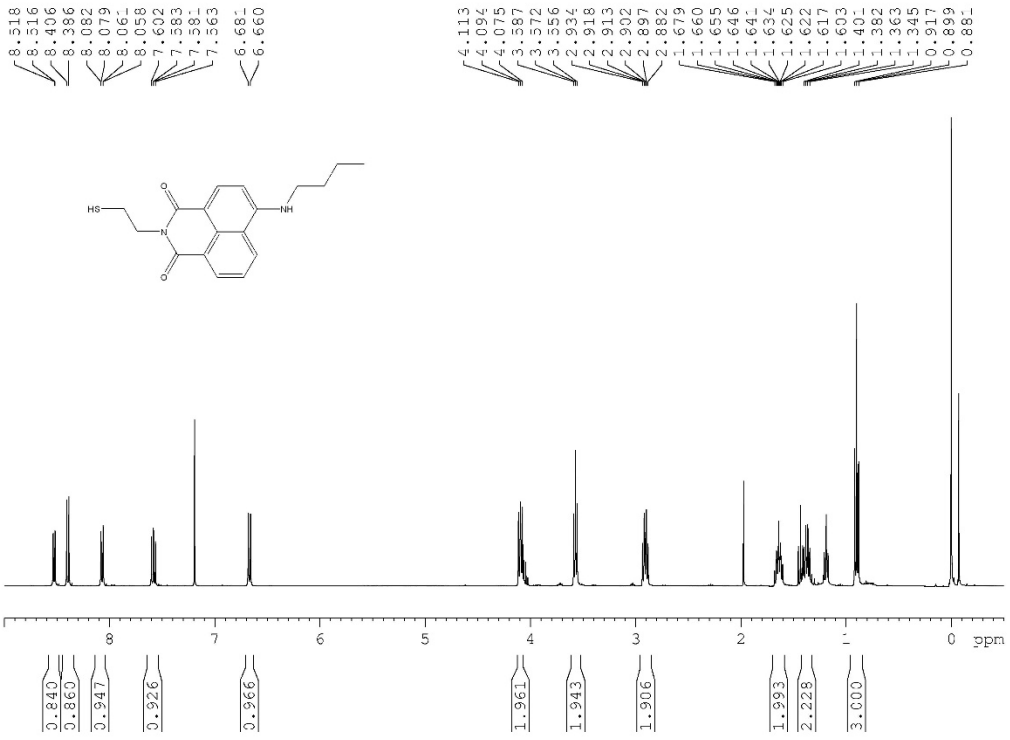
^{1}H NMR of C10



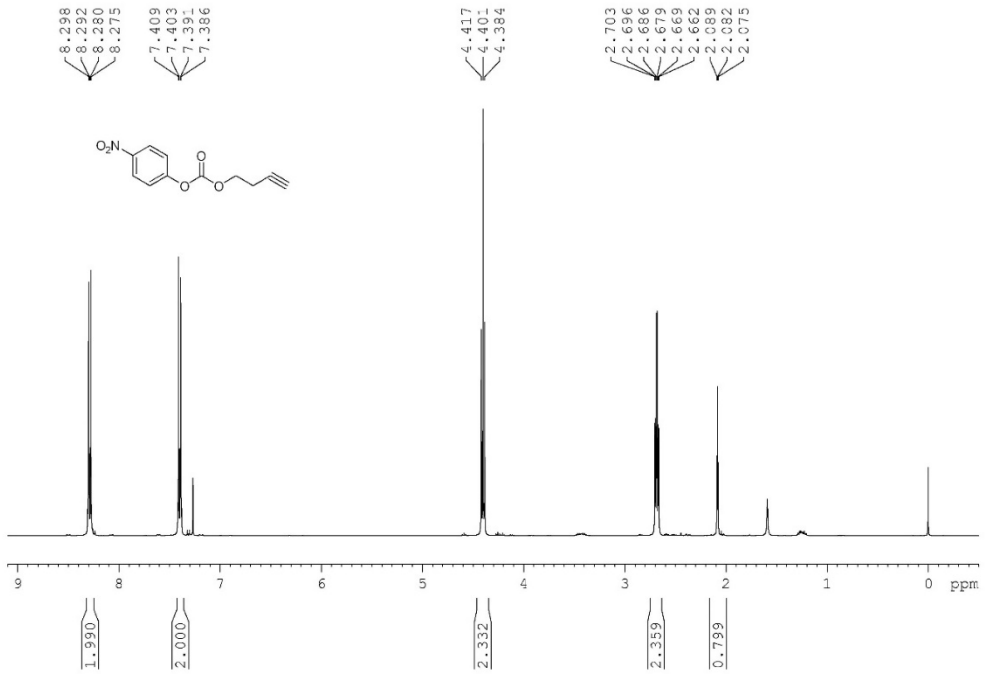
^{1}H NMR of 4-amino-N-n-butyl-1,8-naphthalenedicarboxylic acid anhydride



^{1}H NMR of 6-(butylamino)-2-(2-mercaptoethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione



^{1}H NMR of 3-butyn-1-ol



^{13}C NMR of 3-butyn-1-ol