Electronic Supplementary Material (ESI) for Analyst.

Supporting Information to Accompany **"Design, synthesis and biological evaluation of small molecular fluorescent probes targeting EGFR for tumor detection and treatment"**

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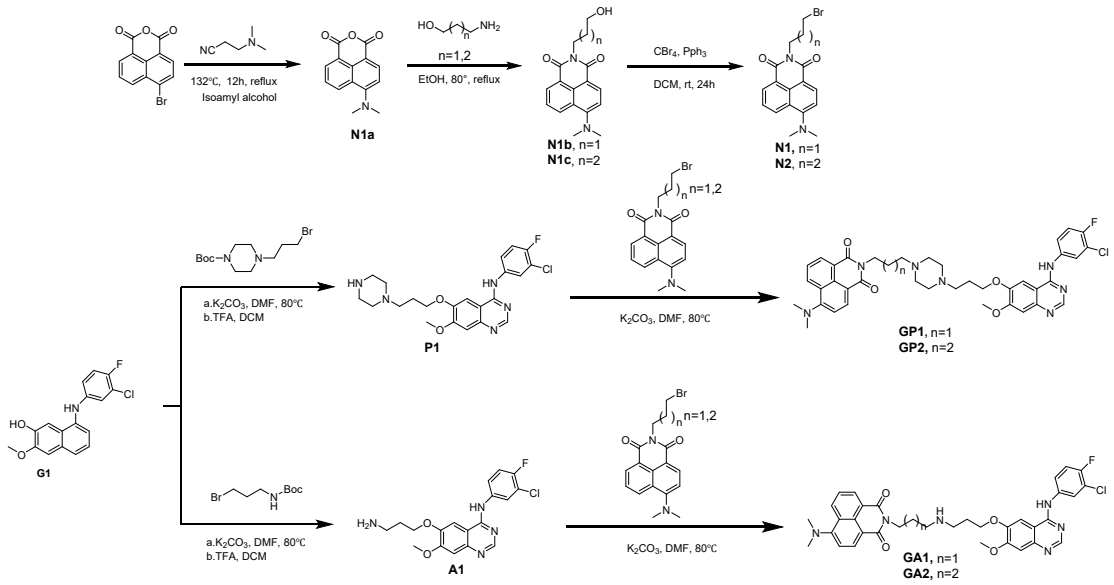
Synthesis of GP and GA Probes.



Scheme S1. Synthetic route of **GP** and **GA** series probes

Synthesis of **N1-N2.**

Compound 4-Bromo-1,8-naphthalic anhydride (1200 mg, 4.44 mmol, 1.0 eq) and 3-(dimethylamino) propanenitrile (1700 mg, 17.32 mmol, 4.0 eq) were dissolved in isoamyl alcohol (30 mL), heated to 132 ℃ and stirred for 12 h. Then the reaction solution was cooled to room temperature, filtered, washed in cold water and dried to obtain yellow solid **N1a** (950 mg, 91% yield). Intermedia **N1a** (950 mg, 3.94 mmol, 1.0 eq) and 3-aminopropan-1-ol (5024 mg, 66.99 mmol,17.0 eq) were dissolved in ethanol (30 mL), heated to 80 ℃ under nitrogen protection and stirred for 12 h. After the reaction solution was cooled to room temperature, water (50 mL) was added and extracted with ethyl acetate (20 mL × 3), washed with brine (50 mL), dried with anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by silica gel (petroleum ether: ethyl acetate = 3:1) to obtain yellow solid **N1b** (1000 mg, 81%) yield. Intermedia **N1b** (1000 mg, 3.20 mmol, 1.0 eq) was dissolved in dichloromethane (35 mL), carbon tetrabromide (2122 mg, 6.4 mmol, 2.0 eq) and triphenylphosphorus (1678 mg, 6.4 mmol, 2.0 eq) were added, and stirred at room temperature for 24 h under nitrogen protection. After the reaction was completed, water (50mL) was added and extracted with ethyl acetate (20 mL × 3), washed in brine (30 mL), dried with anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by silica gel (petroleum ether: ethyl acetate = 2.5:1) to obtain yellow solid **N1** (900 mg, 78%). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.60-8.63 (m, 1H, Ar-H), 8.55-8.42 (m, 2H, Ar-H), 7.73-7.68 (m, 1H, Ar-H), 7.55-7.48 (m, 1H, Ar-H), 7.16 (d, J = 8.2 Hz, 1H, Ar-H), 4.35 (t, J = 7.0 Hz, 2H, CH_2), 3.58-3.48 (m, 2H, CH_2), 3.15 (d, J = 1.0 Hz, 6H, -N(CH_3)$_2$), 2.47-2.29 (m, 2H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 164.61, 164.10, 157.04, 154.95, 148.67, 131.37, 130.99, 124.79, 124.11, 113.14, 52.84, 52.27, 44.71, 29.66.

For the synthesis of compound **N2**, refer to the same method above.

N2: yellow solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.59 (dd, J = 7.3, 1.2 Hz, 1H, Ar-H), 8.53-8.42 (m, 2H, Ar-H), 7.68-7.71 (m, 1H, Ar-H), 7.14 (d, J = 8.2 Hz, 1H, Ar-H), 4.24 (t, J = 6.9 Hz, 2H, CH_2), 3.51 (t, J = 6.5 Hz, 2H, CH_2), 3.14 (s, 6H, -N(CH_3)$_2$), 2.06-1.88 (m, 4H, CH_2). ^{13}C

NMR (75 MHz, $CDCl_3$) δ ppm 163.62, 163.08, 156.06, 153.92, 131.70, 130.31, 130.04, 123.85, 123.17, 112.26, 43.74, 38.58, 30.57, 28.68, 21.63.

Synthesis of **GP1-GP2**

Compound **P1** was synthesized according to reports in the published literature[1].

The compound **P1** (900 mg, 2.0 mmol, 1.0 eq) and **N1** (728 mg, 2.0 mmol, 1.0 eq) were dissolved in DMF (30 mL) and stirred at 80 °C for 12 h after adding potassium carbonate (1382 mg, 10.0 mmol, 5.0 eq). The reaction solution was cooled to room temperature, vacuum concentrated and added with water (50 mL), extracted with ethyl acetate (20 mL × 3), washed with brine (50 mL), dried with anhydrous sodium sulfate and vacuum concentrated. The residue was purified by silica gel (dichloromethane: methanol = 10:1) to obtain yellow solid **GP1** (200 mg, 14%). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.68 (s, 1H, Ar-H), 8.61-8.56 (m, 1H, Ar-H), 8.51 -8.44 (m, 2H, Ar-H), 8.01-8.11 (m, 1H, Quinoline-H), 7.73-7.63 (m, 2H, Ar-H), 7.57 (s, 1H, Ar-H), 7.23 (s, 1H, Ar-H), 7.11-7.16 (m, 2H, Ar-H), 4.27-4.19 (m, 2H, CH_2), 4.12 (t, J = 6.7 Hz, 2H, CH_2), 3.97 (s, 3H, -OCH_3), 3.14 (s, 6H, -$N(CH_3)_2$), 2.51-2.56 (m, 12H, CH_2), 2.10-2.04 (m, 2H, CH_2), 1.90-1.93 (m, 2H, CH_2). ^{13}C NMR (75 MHz, DMSO) δ ppm 164.19, 163.55, 156.93, 156.43, 154.89, 153.57 (d, $J_{C\text{-}F}$ = 241.5 Hz),153.02, 148.71, 147.34, 137.24 (d, $J_{C\text{-}F}$ = 3.0 Hz), 132.63, 131.84, 130.91, 130.06, 125.39, 124.64, 123.89, 122.74 (d, $J_{C\text{-}F}$ = 6.75 Hz), 119.19 (d, $J_{C\text{-}F}$ = 18.75 Hz), 116.91 (d, $J_{C\text{-}F}$ = 21.75 Hz), 113.92, 113.38, 109.21, 107.64, 102.94, 67.53, 56.30, 55.78, 54.79, 52.71, 44.81, 29.47, 26.31, 24.67. HRMS (ESI) calcd. for $C_{39}H_{42}ClFN_7O_4$ $[M+H]^+$ 726.2965, found 726.2960.

For the synthesis of compound **GP2**, refer to the same method above.

GP2: yellow solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.67 (d, J = 2.2 Hz, 1H, Ar-H), 8.56-8.58 (m, 1H, Ar-H), 8.49-8.43 (m, 2H, Ar-H), 7.92-7.94 (m, 1H, Quinoline-H), 7.70-7.56 (m, 2H, Ar-H), 7.51 (s, 1H, Ar-H), 7.22 (s, 1H, Ar-H), 7.15-7.07 (m, 2H, Ar-H), 4.18 (t, J = 7.4 Hz, 2H, CH_2), 4.04 (t, J = 6.7 Hz, 2H, CH_2), 3.95 (s, 3H, -OCH_3), 3.14 (s, 6H, -$N(CH_3)_2$), 2.54-2.27 (m, 12H, CH_2), 2.01-2.03 (m, 2H,CH_2), 1.74 (t, J = 7.5 Hz, 2H, CH_2), 1.62-1.52 (m, 2H, CH_2). ^{13}C NMR (75 MHz, DMSO) δ ppm 164.05, 163.41, 156.93, 156.43, 154.89, 153.53 (d, $J_{C\text{-}F}$ = 240.75 Hz)152.97, 148.89, 147.35, 137.35 (d, $J_{C\text{-}F}$ = 3.0 Hz), 132.66, 131.88, 130.93, 130.01, 125.36, 124.63, 123.84, 122.68 (d, $J_{C\text{-}F}$ = 7.5 Hz), 119.18(d, $J_{C\text{-}F}$ = 18 Hz), 116.88 (d, $J_{C\text{-}F}$ = 21 Hz), 113.77, 113.36, 109.27, 107.65, 102.84, 66.43, 57.75, 56.27, 55.37, 44.80, 32.51, 22.91. HRMS (ESI) calcd. for $C_{40}H_{44}ClFN_7O_4$ $[M+H]^+$ 740.3122, found 740.3120.

Synthesis of **GA1-GA2**

Compound **A1** was synthesized according to reports in the published literature[2].

The compounds **A1** (800 mg, 2.12 mmol, 1.0 eq) and **N1** (763 mg, 2.12 mmol, 1.0 eq) were dissolved in DMF (25 mL) and stirred at 80 °C for 12 h after adding potassium carbonate (1465 mg, 10.6 mmol, 5.0 eq). The reaction solution was cooled to room temperature, vacuum concentrated and added with water (45 mL), extracted with ethyl acetate (15 mL × 3), washed with brine (30 mL), dried with anhydrous sodium sulfate and vacuum concentrated. The residue was purified by silica gel (dichloromethane: methanol = 10:1) to obtain yellow solid **GA1** (230 mg, 17%). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.53 (s, 1H, Ar-H), 8.29 (t, J = 7.2 Hz, 2H, Ar-H), 8.15 (d, J = 8.3 Hz, 1H, Ar-H), 7.80 (s, 1H, Ar-H), 7.70-7.73 (m, 1H, Ar-H), 7.59-7.62 (m, 1H,

Quinoline-H), 7.47-7.39 (m, 1H, Ar-H), 7.10 (s, 1H,Ar-H), 6.83-6.73 (m, 2H, Ar-H), 4.51 (t, J = 6.5 Hz, 2H, CH_2), 4.30 (t, J = 6.1 Hz, 2H, CH_2), 3.96 (s, 3H, -OCH_3), 3.27 (t, J = 5.9 Hz, 2H, CH_3), 3.11-3.13 (m, 9H, -$N(CH_3)_2$, NH, CH_2), 2.41-2.43(m, 4H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 1164.65, 164.15, 157.05, 156.41, 154.40, 154.10 (d, $J_{C\text{-}F}$ = 243.75 Hz), 153.20, 147.88, 146.99, 135.90(d, $J_{C\text{-}F}$ = 3 Hz), 132.68, 131.50, 130.96, 129.99, 124.68, 124.54, 123.93, 122.26, 121.66(d, $J_{C\text{-}F}$ = 6.75 Hz), 120.25 (d, $J_{C\text{-}F}$ = 18.75 Hz), 115.88 (d, $J_{C\text{-}F}$ =21.75 Hz), 113.65, 112.85, 109.16, 107.24, 102.89, 67.11, 56.02, 48.31, 44.66, 39.20, 29.72, 25.21, 24.62. HRMS (ESI) calcd. for $C_{35}H_{35}ClFN_6O_4$ $[M+H]^+$ 657.2387, found 657.2390.

For the synthesis of compound **GA2**, refer to the same method above.

GA2: yellow solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.42 (s, 1H, Ar-H), 8.23 (t, J = 8.5 Hz, 2H, Ar-H), 8.11 (d, J = 8.3 Hz, 1H, Ar-H), 7.90-7.92(m, 1H, Ar-H), 7.76 (s, 1H, Ar-H), 7.60-7.62 (m, 1H, Quinoline-H), 7.43-7.35 (m, 1H, Ar-H), 6.95 (s, 1H, Ar-H), 6.86-6.78 (m, 2H, Ar-H), 4.39 (t, J = 6.7 Hz, 2H, CH_2), 4.07 (t, J = 6.8 Hz, 2H, CH_2), 3.91 (s, 3H, -OCH_3), 3.29 (t, J = 6.2 Hz, 2H), 3.17 (q, J = 6.4 Hz, 2H), 3.08 (s, 6H), 2.43 (t, J = 6.6 Hz, 2H), 2.08 (s, 2H). 1.94-1.97 (m, 3H, NH, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 163.94, 163.50, 156.25, 155.51, 153.75, 153.14 (d, $J_{C\text{-}F}$ = 243.75 Hz) 152.29, 147.39, 146.14, 134.86(d, $J_{C\text{-}F}$ = 3.75 Hz), 131.98, 130.69, 130.23, 129.17, 123.75, 123.62, 122.94, 121.30, 120.62(d, $J_{C\text{-}F}$ = 6.75 Hz), 119.25 (d, $J_{C\text{-}F}$ = 18.75 Hz), 114.94 (d, $J_{C\text{-}F}$ = 21.75 Hz), 112.61, 111.90, 108.28, 106.38, 101.52, 66.32, 55.05, 45.49, 43.63, 30.91, 28.68, 21.67, 13.10. HRMS (ESI) calcd. for $C_{36}H_{37}ClFN_6O_4$ $[M+H]^+$ 671.2543, found 671.2540.

Spectroscopic properties of the GP and GA probes.

The UV-vis absorption and fluorescence emission spectrum of **GP** and **GA** probes (10 μM) in different solvents (dichloromethane, methanol, ethyl acetate, toluene, tetrahydrofuran, acetonitrile, 1,4-dioxane, PBS, DMF, DMSO, containing 1% DMSO) were measured. The fluorescence emission spectrum and ultraviolet absorption spectrum of the solution were detected by Agilent CARY ECLIPSE fluorescence spectrophotometer and Shimazu UV-2600 ultraviolet spectrophotometer, respectively.

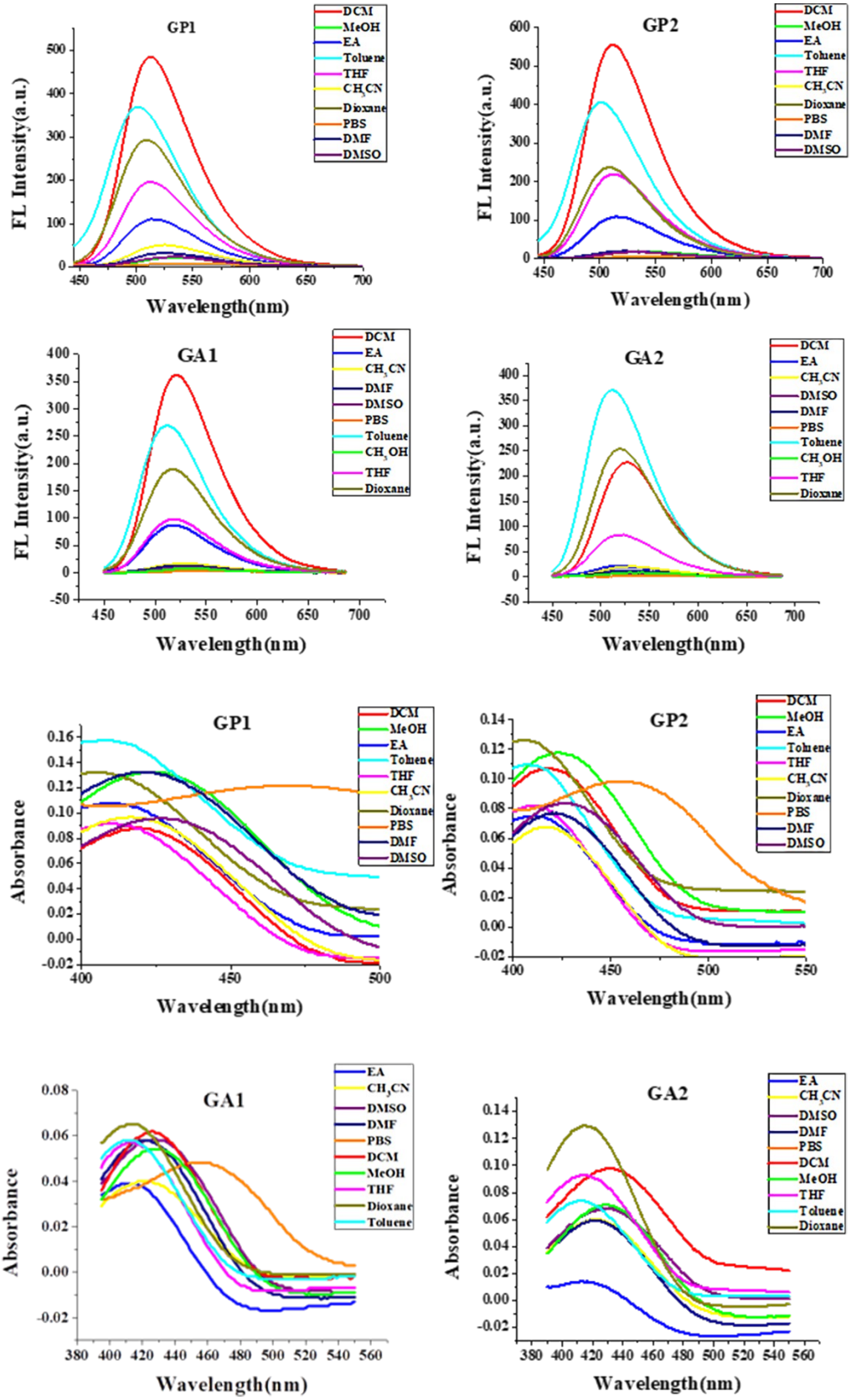


Fig S1. UV absorption and fluorescence emission of probes **GP** and **GA** in different solvents.

Western blotting

MDA-MB-231, MCF-7, HCT-116 and HT-29 cell lines were incubated with probe **GP1** for 24h, and the total protein was determined with BCA detection kit (Beyotime, China) after sample collection. The total lysate was denatured in SDS-loading buffer solution at 100 ℃ for 5 min, and the total protein was separated by 8% SDS-PAGE for 2 h and transferred to PVDF membrane. Use 5% skimmed milk to seal PVDF membrane in TBST at room temperature for 1 h, and then incubate it with corresponding antibody and enzyme labeled secondary antibody. Finally, ECL reagent (Beyotime, Jiangsu, China) was used to detect protein content. To further verify the mechanism of **GP1** probe on EGFR protein, select a cell line with stable and high expression, and use different concentrations of **GP1** (2.5, 5, 15, 20 μM). Incubate cells for 24 h and repeat the above operations required for protein content detection.

Cytotoxicity assay

The cytotoxicity of gefitinib, **GP** and **GA** probes to different tumor cell lines (MCF-7, MDA-MB-231, HT-29, HCT-116, CT-26, HepG-2) was detected by MTT assay. The above tumor cells were inoculated into 96 well plates, each containing 5000 cells and 10% fetal bovine serum medium, and then the well plates were placed in the incubator for 12 h. After incubating in the incubator for 72 h, 100 μL of the tested substance was added to each well to obtain MTT solution (1 mg/mL). The solution was incubated in the incubator for 4 h, incubated in the shaker at room temperature for 10 min, and DMSO solution was obtained by adding 100 μL to each well, incubated in the oven at 37 ℃ for 15 min. Use the microplate reader to detect the absorbance at 490 nm wavelength and calculate the IC_{50} value. Each group of experiments should be conducted three times in parallel to ensure the accuracy of the experimental results.

Selectivity and sensitivity

In order to evaluate the selectivity and sensitivity of probes in high and low EGFR expressing cell lines, the fluorescence response values of **GP** and **GA** probes in MDA-MB-231, MCF-7, HCT-116 and HT-29 cell lines were measured. All probes were configured into solutions of different concentrations (2.5, 5, 10, 20 μM). After the cells were fully adherent to the wall, culture medium and probes of different concentrations were added and incubated for 1 h. 50 μL pancreatin was added into each well of the 96-well plate. After incubation for a period of time, 100 microliter DMEM complete medium containing 10% FBS and 1% double antibody was added into each well. Suck the liquid from each hole and add it to each group of EP tubes for centrifugation for 5 min (1500 r/min). PBS was added for re-suspension (1 mL per group), fluorescence intensity (λex = 469 nm and λem = 521 nm) was measured by microplate reader.

Competitive binding experiment

In order to detect the binding specificity of probe **GP1**, two tumor cell lines with high EGFR expression (MDA-MB-231, HCT-116) were used for competitive binding research, and the experiment was divided into two groups. In the first group of experiments, **GP1** solution with a concentration of 10 μM and gefitinib solution with different concentrations (0, 2, 4, 6 and 8 μM). After MDA-MB-231 cells were fully adherent, medium containing **GP1** and different concentrations of gefitinib were added and incubated for 60 min. 50 μM pancreatin was added into each well of the 96-well plate. After incubation for a period of time, 100 microliter DMEM complete medium containing 10% FBS and 1% double antibody was added into each well. The liquid obtained from each well was absorbed and added to the EP tube of each group for centrifugation for 5 min (1500 r/min). PBS was added for re-suspension (1 mL per group), fluorescence intensity (λex = 469 nm and λem = 521 nm) was measured by microplate reader. In the second group of experiments, except that the concentration of probe **GP1** was 0.5 μM and the concentration of gefitinib was 0, 0.5, 1, 1.25, 1.5, 1.75, 2, 2.25 and 2.5 μM, the other experimental operations were performed according to the first group.

In vivo fluorescence imaging

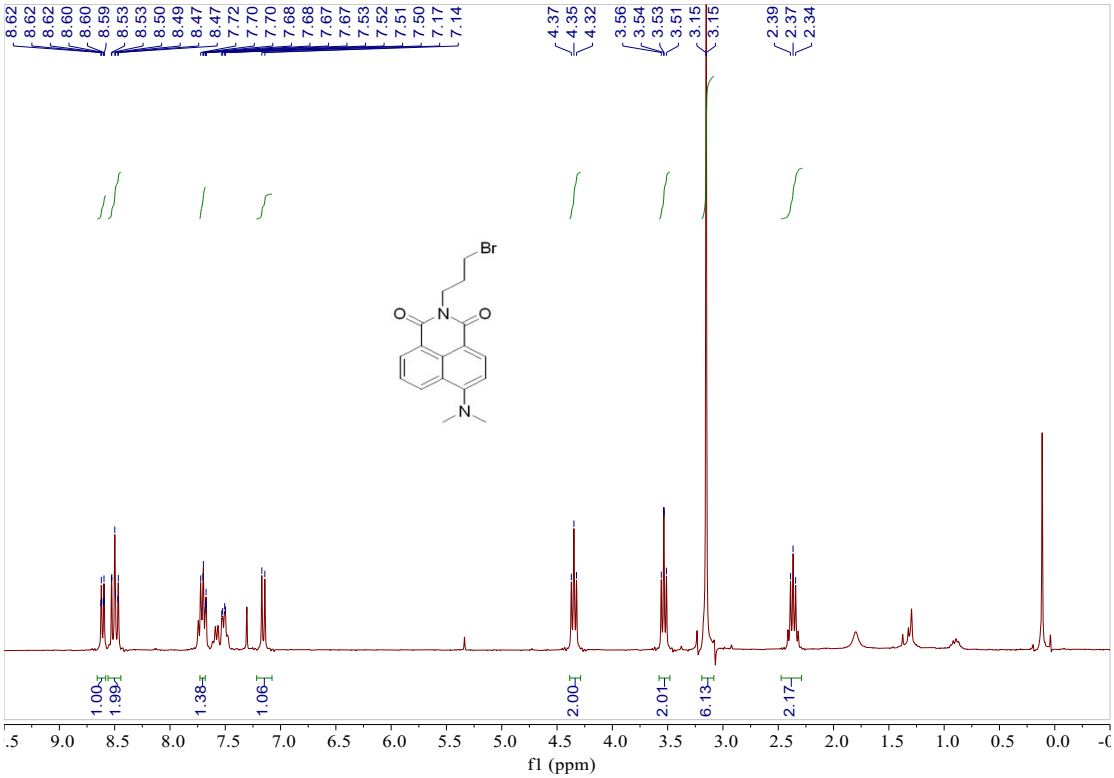
The male balb/c mice at 4-5 weeks of age were purchased from Vitong Lihua. CT-26 tumor cells were inoculated subcutaneously into the armpits of experimental mice, and tumor size was observed 2-3 days later. When the tumor diameter reached 2-3 cm, **GP1** (15 mg/kg) and PBS (negative control) were injected intraperitoneally, and fluorescence imaging was performed at λex 488 and λem 520 nm with an imaging system at different time points (0, 2, 12, 24 h) after injection. Image analysis was performed using Live-Image software. After the *in vivo* imaging capture was completed, the mice were killed, and the tumors and major organs (heart, liver, spleen, lung and kidney) were photographed separately according to the above methods.

Anti-tumor activity

To further determine the anti-tumor activity of **GP1** *in vivo*, mice were divided into three groups (8 mice in each group) and inoculated subcutaneously with CT-26 tumor cells in axilla. According to the activity of the mice, **GP1** (15 mg/kg), gefitinib (100 mg/kg) and PBS were administrated into the three groups on the 7th, 9th, 11th and 13th days after CT-26 cells were inoculated. On the 20th day, the mice were killed, and the tumor tissue was weighed and the tumor layout map was taken.

NMR and ESI-MS Spectra

^{1}H NMR of **N1**.



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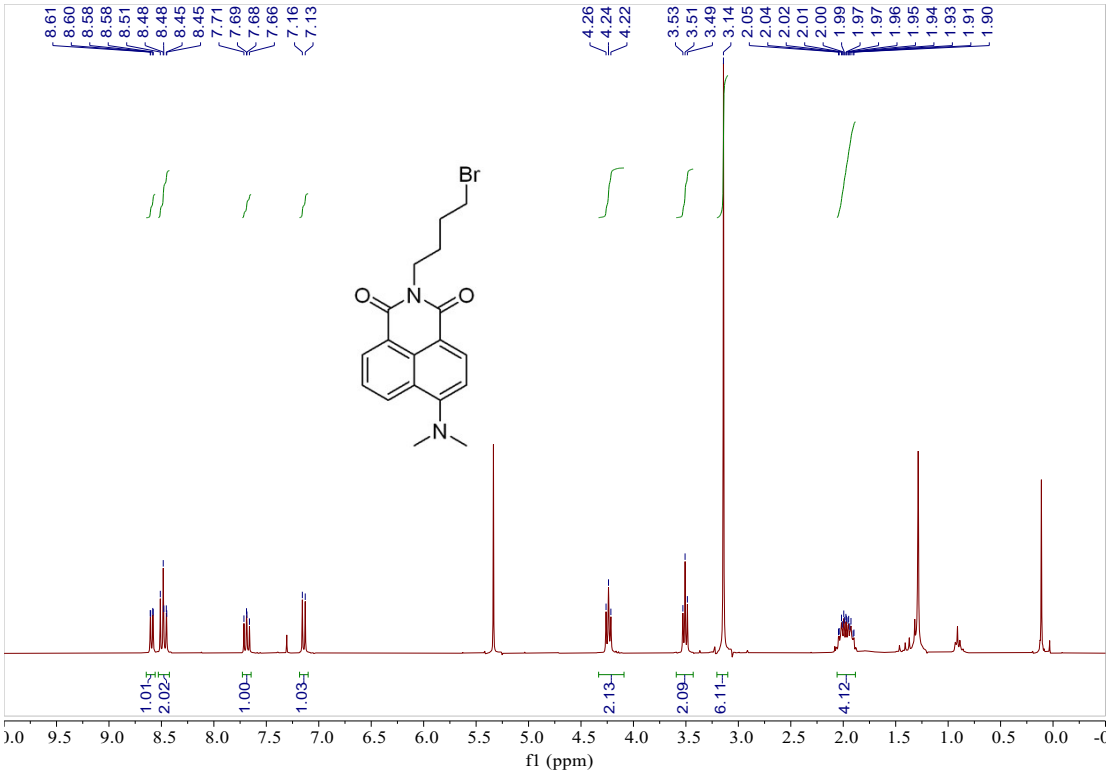
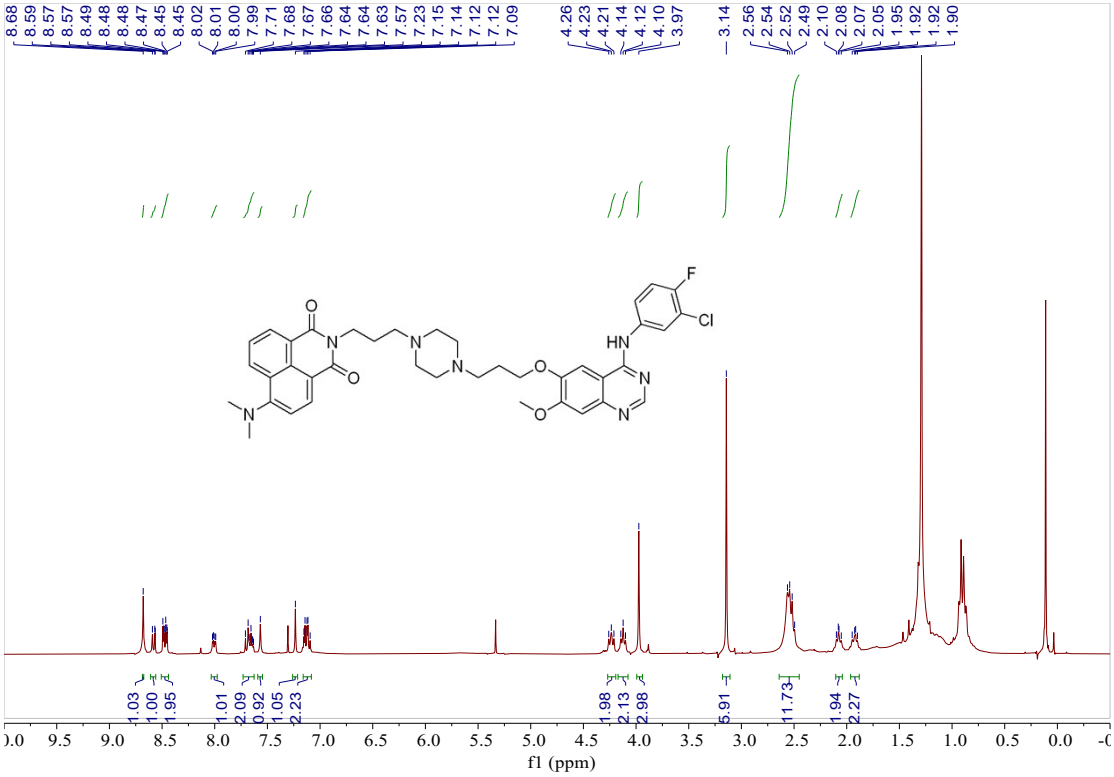
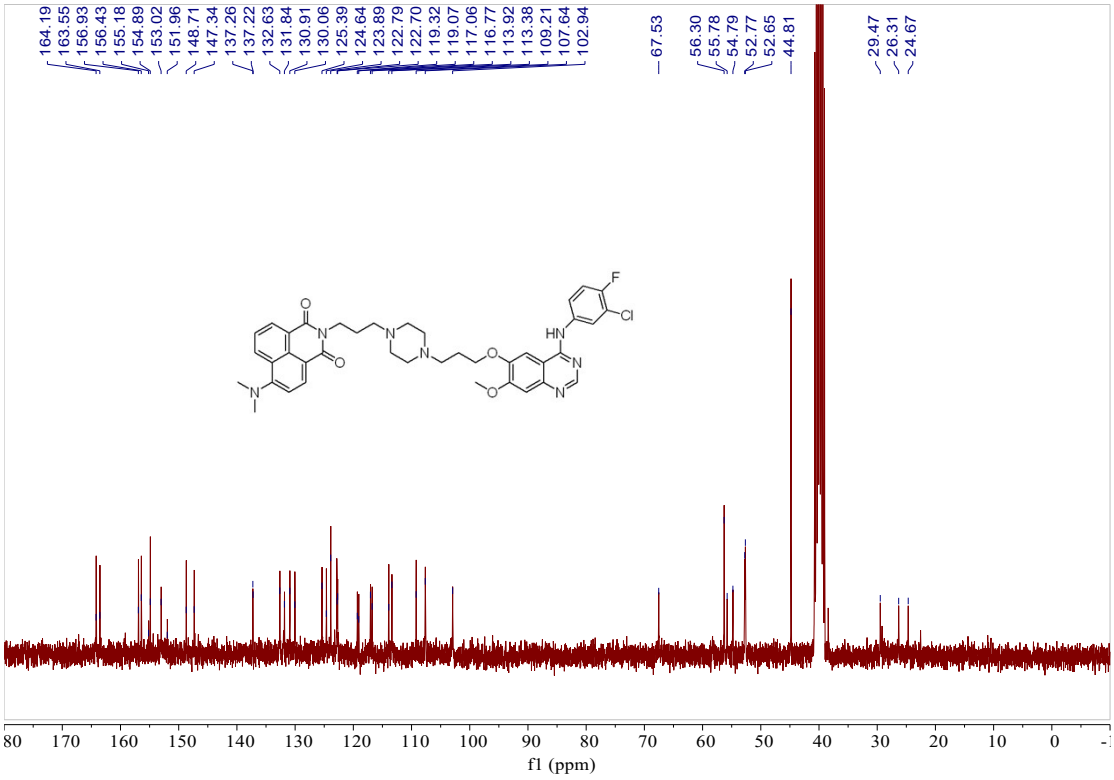


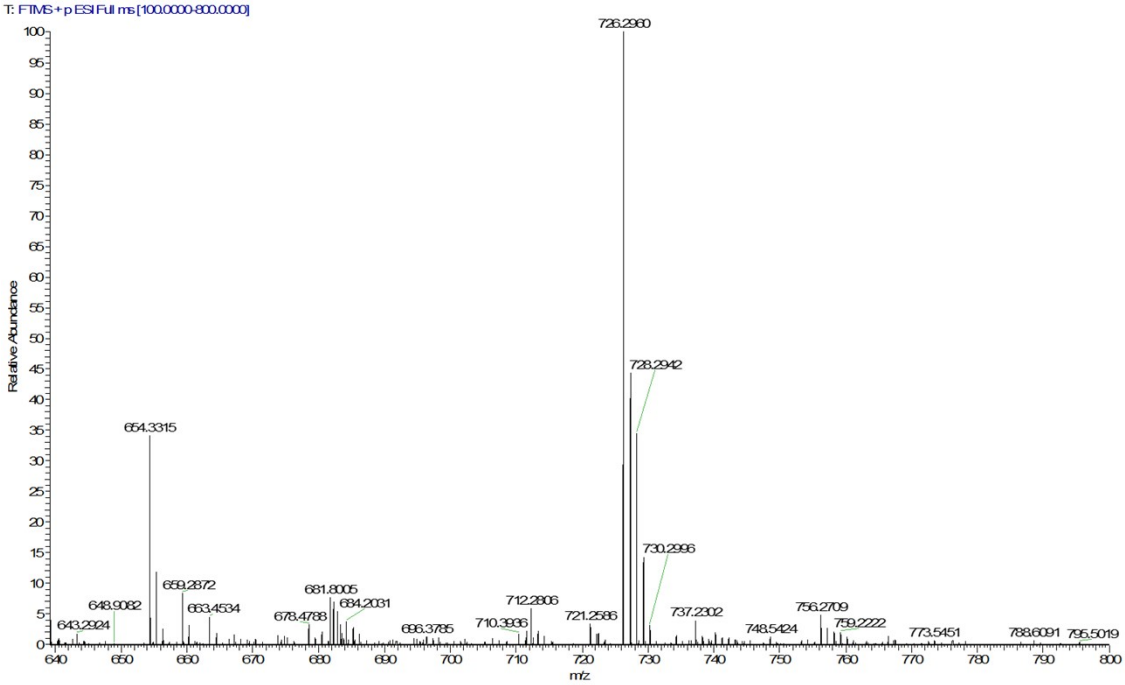
^{1}H NMR of **GP1**.



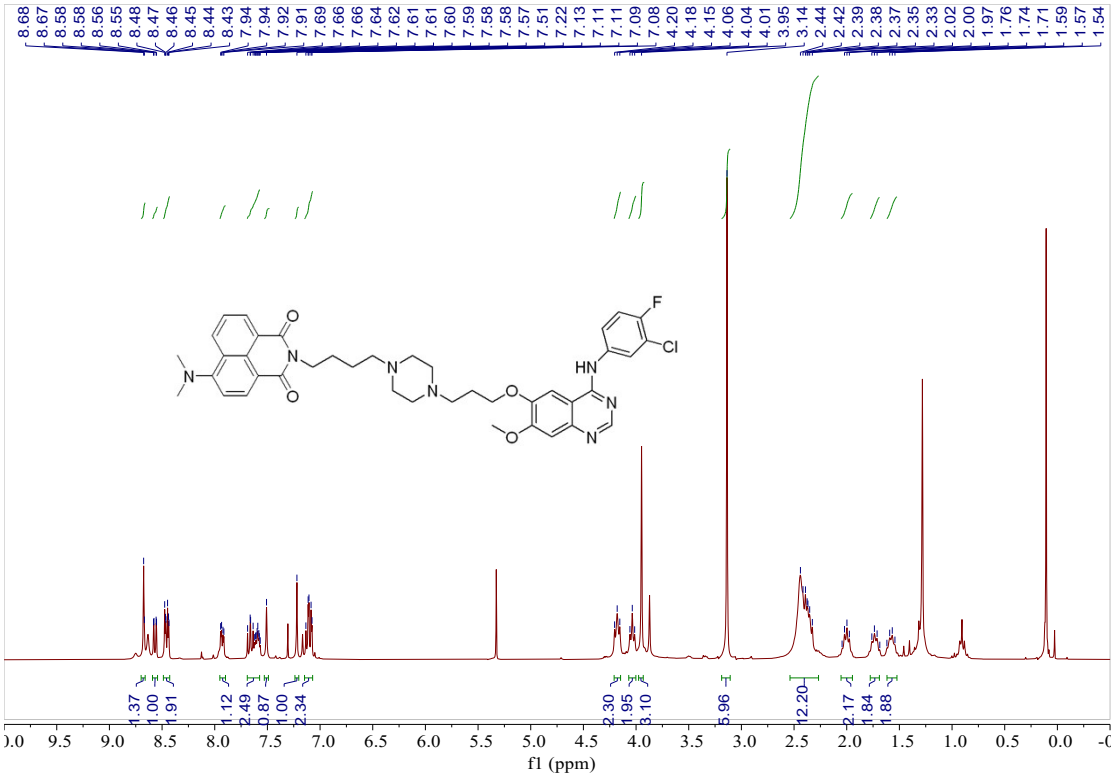
^{13}C NMR of **GP1**.



ESI-MS spectrum of **GP1.**



^{1}H NMR of **GP2**.



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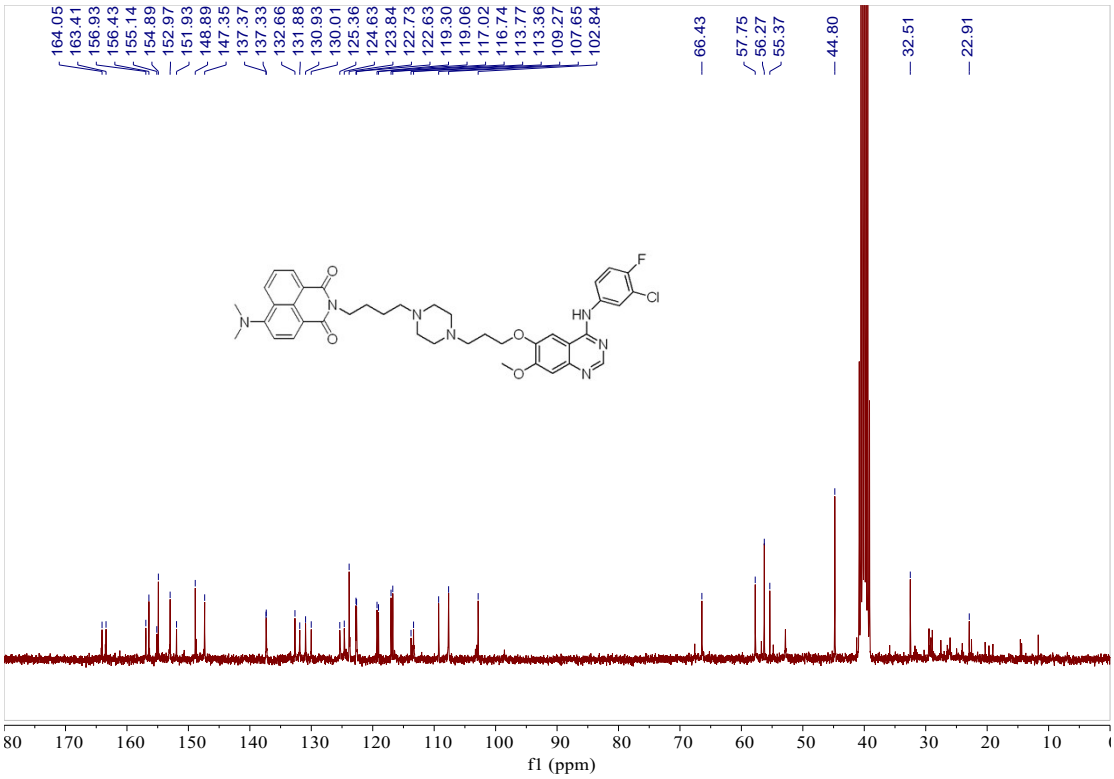
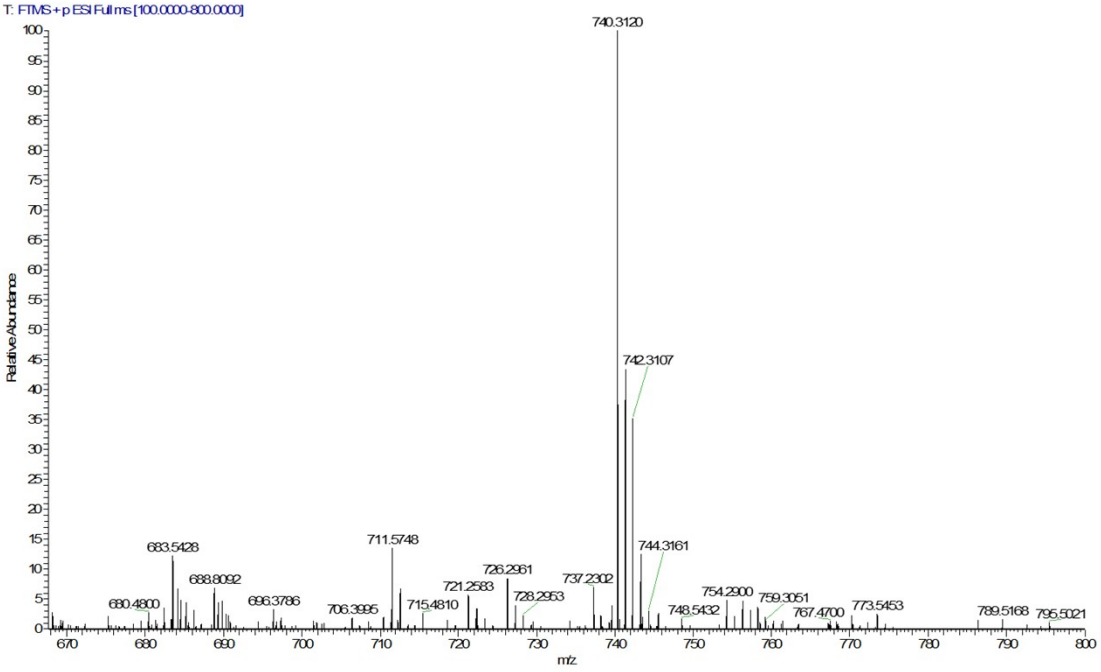
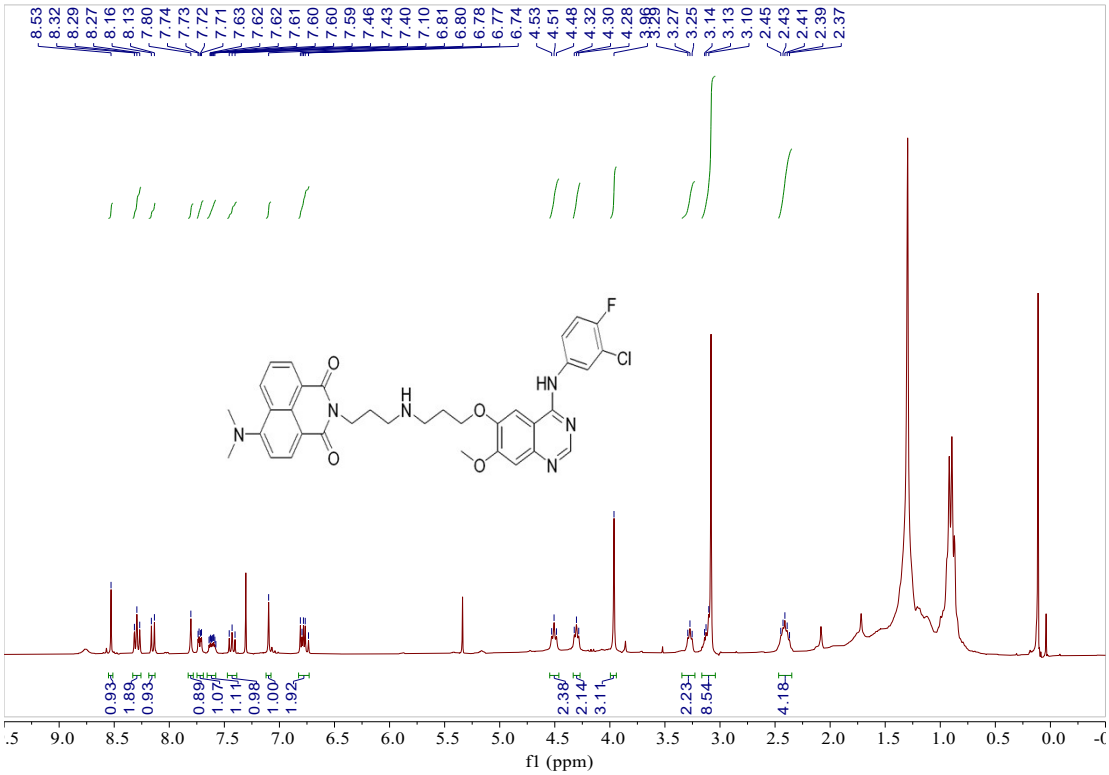


ESI-MS spectrum of **GP2.**



^{1}H NMR of **GA1**.



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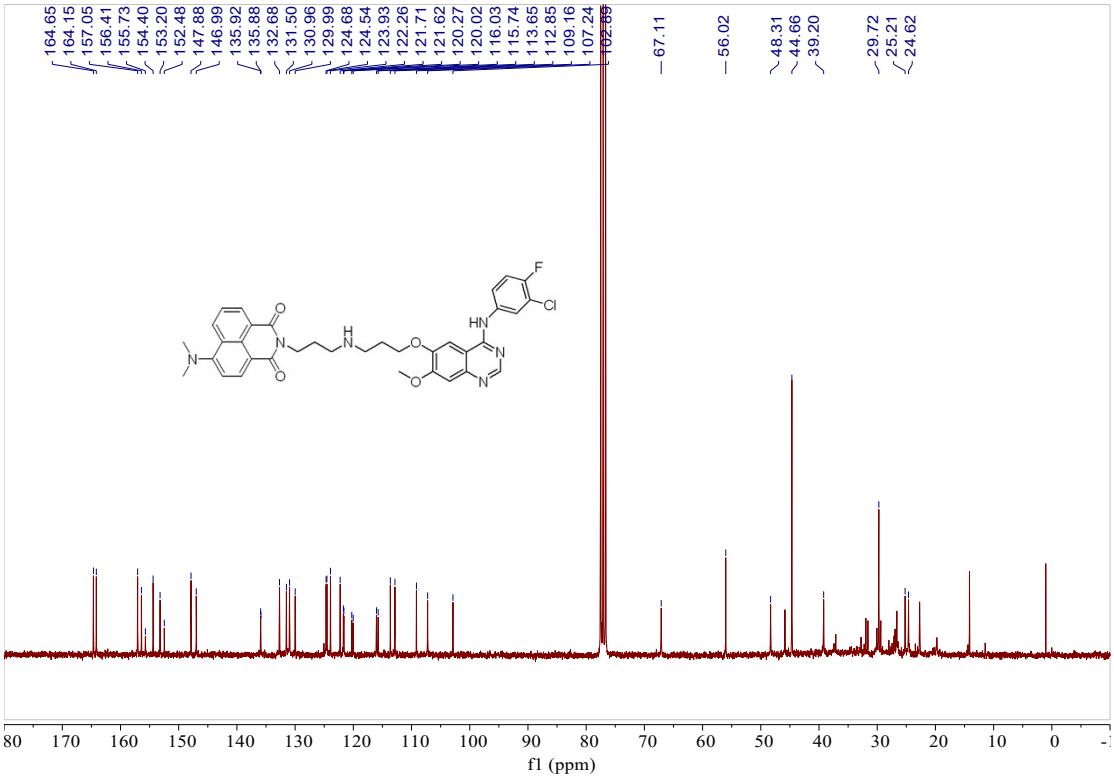

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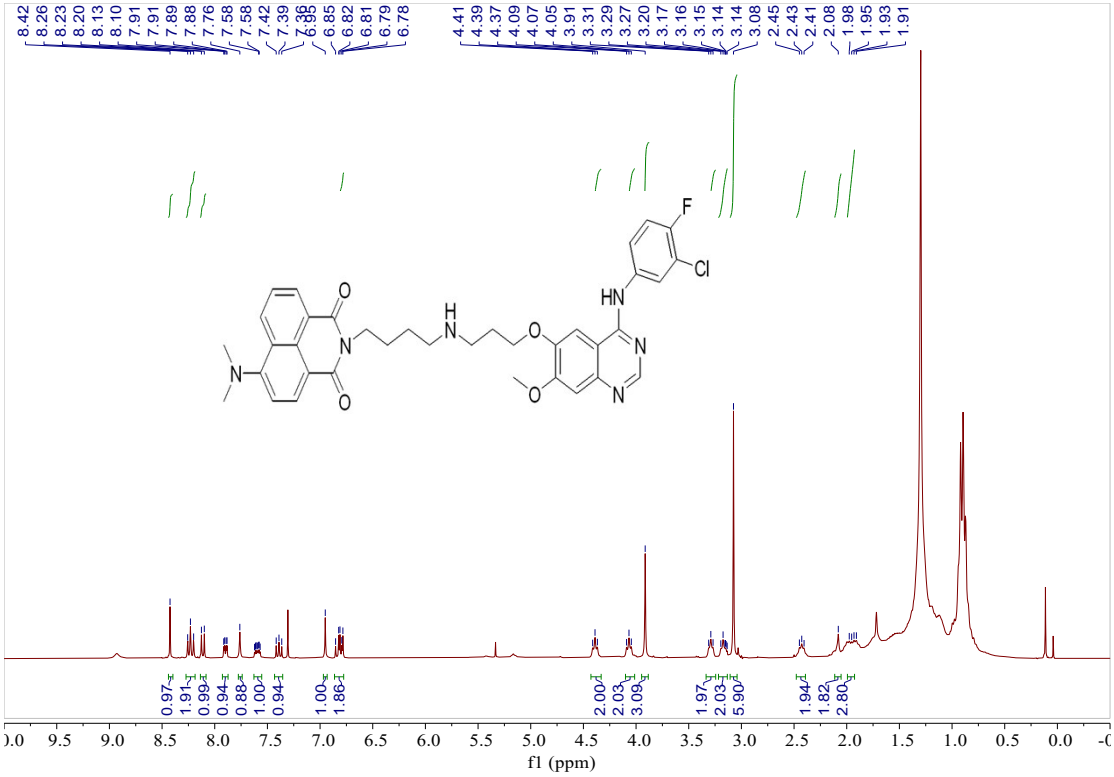

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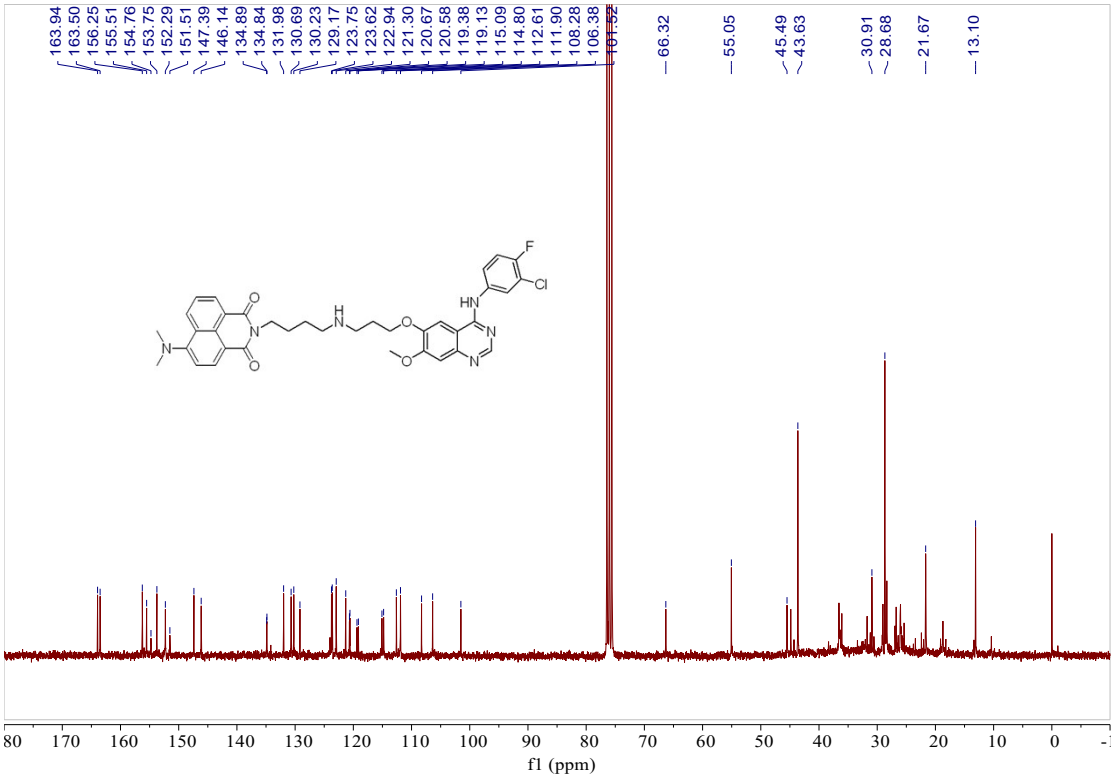

Reference

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2. Yoo B, Ma K, Zhang L, Burns A, Sequeira S, Mellinghoff I, Brennan C, Wiesner U, Bradbury MS: **Ultrasmall dual-modality silica nanoparticle drug conjugates: Design, synthesis, and characterization**. *Bioorg Med Chem* 2015, **23**(22):7119-7130.