



Supporting Information

Tetrazole-Based Probes for Integrated Phenotypic Screening, Affinity-Based Proteome Profiling, and Sensitive Detection of a Cancer Biomarker

Ke Cheng, Jun-Seok Lee, Piliang Hao, Shao Q. Yao, Ke Ding, and Zhengqiu Li**

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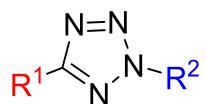
1. General Information

All chemicals were purchased from commercial vendors and used without further purification, unless indicated otherwise. All reactions requiring anhydrous conditions were carried out under argon or nitrogen atmosphere using oven-dried glassware. AR-grade solvents were used for all reactions. Reaction progress was monitored by TLC on pre-coated silica plates (Merck 60 F₂₅₄ nm, 0.25 µm) and spots were visualized by UV, iodine or other suitable stains. Flash column chromatography was carried out using silica gel (Merck 60 F₂₅₄ nm, 0.040-0.063 µm). All NMR spectra (¹H-NMR, ¹³C-NMR) were recorded on Bruker 300 MHz/400 MHz NMR spectrometers. Chemical shifts were reported in parts per million (ppm) referenced with respect to appropriate internal standards or residual solvent peaks (CDCl₃ = 7.26 ppm, DMSO-d₆ = 2.50 ppm). The following abbreviations were used in reporting spectra, br s (broad singlet), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublets). Mass spectra were obtained on Agilent LC-ESI-MS system. All analytical HPLC were carried out on Agilent system. Water with 0.1% TFA and acetonitrile with 0.1% TFA were used as eluents and the flow rate was 0.5 mL/min. Antibodies against NOS2 (ab178945), FLAD1 (ab89968), ANXA2 (ab41803) and the recombinant ANXA2 protein (ab188455) were purchased from Abcam.

2. Cell culture and Western blot

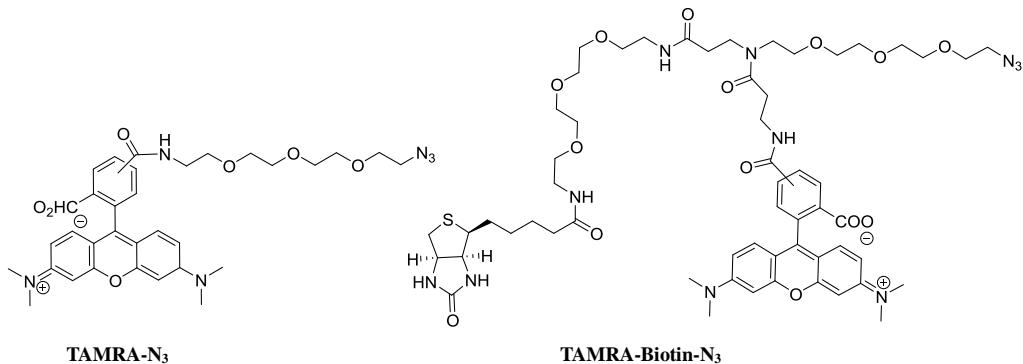
Cell lines were obtained from the National Cancer Institute Developmental Therapeutics Program (NCI-60). Cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) or RPMI 1640 Medium (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), 100 units/mL penicillin, and 100 µg/mL streptomycin (Thermo Scientific) and maintained in a humidified 37 °C incubator with 5% CO₂. To generate protein lysates, cells were washed twice with cold phosphate-buffered saline (PBS), harvested with 1× trypsin or by use of a cell scraper, and collected by centrifugation. Cell pellets were then washed with PBS and lysed with RIPA (Thermo Scientific™, #89900) lysis and extraction buffer (with Pierce™ Protease Inhibitor Tablets, Thermo Scientific™, #A32955). Protein concentration was determined by Pierce™ BCA Protein Assay Kit (Thermo Scientific™, #23252) and Synergy H1 Hybrid Multi-Mode Reader (BioTek). For Western blotting experiments, samples were resolved by SDS-polyacrylamide gels and transferred to poly membranes. Membranes were then blocked with 3% bovine serum albumin (BSA) in TBST (0.1% Tween in Tris-buffered saline) for 1 h at room temperature. After blocking, membranes were incubated with the corresponding primary antibody for another 1 hour. After incubation, membranes were washed with TBST (4×10 min) and then incubated with an appropriate secondary antibody. Finally, blots were washed again with TBST before being developed with SuperSignal West Dura Kit (Thermo Scientific), and finally imaged with Amersham Imager 600(GE Healthcare). Cell Counting Kit-8(CCK-8, DOJINDO, #CK04) was used for cell proliferation assay. Proteome labeling, in-gel fluorescence scanning and cellular imaging experiments were performed as previously reported.^[1,2,3]

3. Structures of the tetrazole-based probes (Tz1-22) used in the current study.

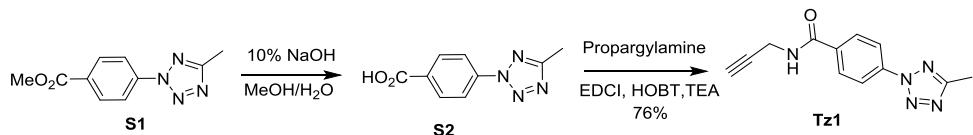


Probe	R ¹	R ²	Probe	R ¹	R ²
Tz1			Tz13		
Tz2			Tz14		
Tz3			Tz15		
Tz4			Tz16		
Tz5			Tz17		
Tz6			Tz18		
Tz7			Tz19		
Tz8			Tz20		
Tz9			Tz21		
Tz10			Tz22		
Tz11					
Tz12					

4. Structures of the reporters



5. Chemical Synthesis

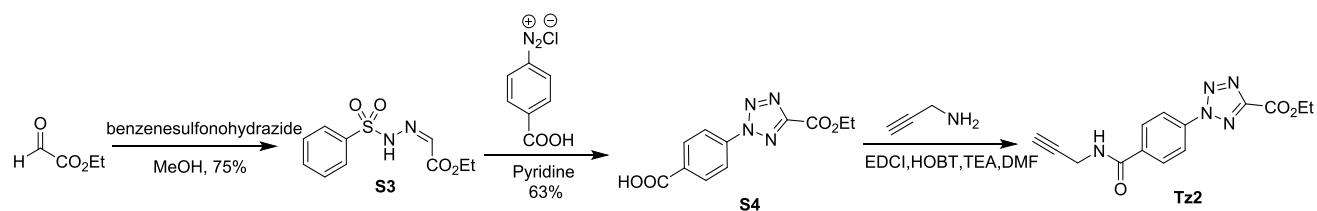


Scheme S1

S1 was synthesized based on previously published procedures.^[4]

(S2). To a solution of **S1** (400 mg, 1.89 mmol) in 10 mL methanol/H₂O (1:1) was added 3 mL 10% NaOH solution. The mixture was heated to reflux for 2 h. After cooled to rt, the mixture was acidified with 1N HCl and subsequently filtered. The residue was dried to yield **S2** (380 mg) which was used in next step directly.

(Tz1). To a stirred solution of **S2** (204 mg, 1 mmol) in 5 mL DMF was added HOBT (270 mg, 2 mmol), EDCI (382 mg, 2 mmol) and TEA (204 mg, 2 mmol). The mixture was stirred for 30 min at r.t followed by addition of propargylamine (55 mg, 1 mmol). The reaction was then stirred at room temperature overnight in the dark. Subsequently, it was quenched by addition of 5 mL water and extracted with 2 × 10 mL of ethyl acetate. The organic layers were combined and washed with 2×10 mL brine and dried over anhydrous Na₂SO₄. Upon solvent evaporation *in vacuo*, the residue was purified by flash column (PE:EA = 20:1, *R_f* = 0.25) to give **Tz1** as a yellow solid (197 mg, 82% yield). ¹H NMR (400 MHz, CDCl₃) δ 8.22 (d, *J* = 8.7 Hz, 2H), 8.00 (d, *J* = 8.7 Hz, 2H), 6.45 (s, 1H), 4.32 (dd, *J* = 5.2, 2.5 Hz, 2H), 2.68 (s, 3H), 2.34 (s, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 165.70, 163.64, 138.92, 134.42, 128.69, 119.68, 79.10, 72.27, 30.01, 11.01. LC-MS (ESI) calcd for [M - 1]⁻ 240.1; Found 240.1.

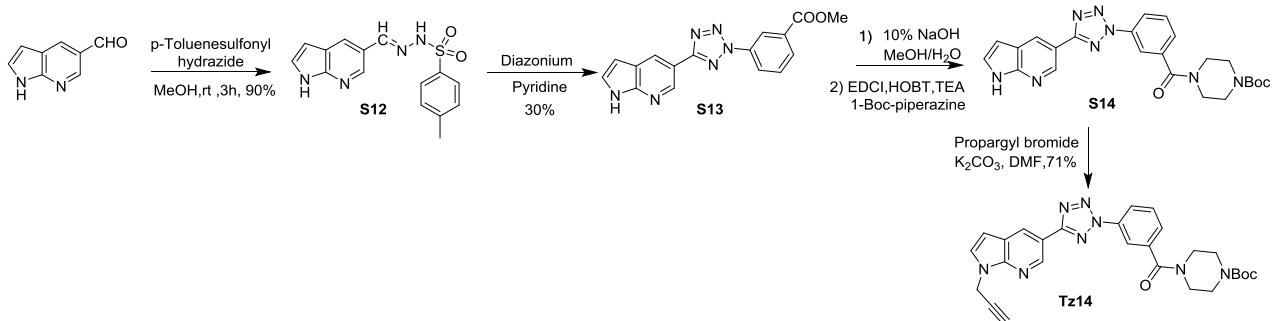


Scheme S2

(S4). Synthesis of the diazonium salt and phenylsulfinylhydrazones (**S3**) was based on previously published procedures^[5]. To a suspension of 4-aminobenzoic acid (121 mg, 1 mmol) in 5 mL H₂O and 5 mL EtOH was added 0.5 mL concentrated HCl, followed by addition of a solution of NaNO₂ (69 mg, 1 mmol) in 1 mL H₂O at 0 °C. After stirring for 10 min, the solution was added dropwise to the solution of **S3** (1 mmol) in 5 mL pyridine over 50 min at -10 °C. The mixture was warmed up to r.t. prior to addition of 30 mL H₂O. The solution was extracted with 2 × 10 mL of ethyl acetate, the combined organic layers were washed successively with 3×100 mL 2 N HCl and 70 mL brine. Upon solvent evaporation *in vacuo*, the residue was purified by flash column (PE : EA = 5:1, *R_f* = 0.3) to give **S4** as a brown solid (165 mg, 63%). ¹H NMR (400 MHz, DMSO) δ 13.39 (s, 1H), 8.28 (d, *J* = 8.8 Hz, 2H), 8.24 – 8.20 (m,

stirred solution of **S10** (5 mmol) in 10 mL pyridine at -5 °C over a period of 30 min. The resulting mixture was stirred at -5 °C for 2 h and then further 2 h at room temperature. The reaction mixture was diluted with 30 mL H₂O and 30 mL EtOAc. The organic phase was separated and washed with 1N HCl (2 × 20 mL), followed by concentration *in vacuo*. The residue was purified by flash chromatography (DCM:MeOH = 100:1) to afford the compound **S11** as a yellow solid (491 mg, 32% yield), *R_f* = 0.5 (PE:EA = 4:1). ¹H NMR (400 MHz, DMSO) δ 8.30 – 8.26 (m, 2H), 8.26 – 8.21 (m, 2H), 7.67 (t, *J* = 1.9 Hz, 1H), 7.08 – 7.02 (m, 1H), 6.68 (dd, *J* = 2.8, 1.7 Hz, 1H), 4.97 (d, *J* = 2.5 Hz, 2H), 3.92 (s, 3H), 3.57 (t, *J* = 2.5 Hz, 1H). ¹³C NMR (101 MHz, DMSO) δ 165.70, 162.75, 139.58, 131.65, 130.71, 123.27, 122.02, 120.09, 110.60, 107.99, 79.48, 76.91, 52.99, 38.69. LC-ESI-MS (ESI) calcd for [M + 1]⁺ 308.1 ; Found 308.2.

(**Tz13**). To a solution of **S11** (307 mg, 1 mmol) in 5 mL MeOH and 5 mL water was added 2 mL 10% NaOH solution. The mixture was heated to reflux for 3 h before cooled to rt and acidified with 1N HCl followed by filtration. The residue was dried to afford a crude product (267 mg, 87%), which was used in next step without further purification. 48 mg (0.163 mmol) of the crude product was then dissolved in 5 mL DMF, followed by addition of EDCI (63 mg, 0.33 mmol), HOBT (44.6 mg, 0.33 mmol) and triethylamine (33.3 mg, 0.33 mmol). The resulting mixture was stirred for 1h at room temperature. Subsequently, 30.3 mg (0.163 mmol) tert-butyl piperazine-1-carboxylate was added, and stirred at room temperature for 24 h prior to addition of 10 mL water. Upon extraction with 2 × 10 mL of ethyl acetate, the organic layers were washed with 2 × 10 mL brine and dried over anhydrous Na₂SO₄. The organic phase was concentrated *in vacuo* and the residue was purified by flash column (DCM:MeOH = 30:1) to afford **Tz13** as a white solid (49 mg, 65% yield), *R_f* = 0.6 (PE:EA = 1:1). ¹H NMR (300 MHz, CDCl₃) δ 8.24 (d, *J* = 8.6 Hz, 1H), 7.60 (d, *J* = 8.7 Hz, 1H), 7.26 (s, 1H), 6.84 (dt, *J* = 4.4, 2.7 Hz, 1H), 4.76 (d, *J* = 2.5 Hz, 1H), 3.61 (d, *J* = 85.4 Hz, 4H), 2.51 (t, *J* = 2.5 Hz, 1H), 1.47 (s, 4H). ¹³C NMR (126 MHz, CDCl₃) δ 169.34, 162.65, 154.60, 137.85, 136.17, 128.70, 122.02, 121.27, 119.86, 111.84, 108.61, 80.57, 77.36, 77.10, 76.85, 74.64, 60.45, 58.47, 53.49, 39.17, 28.43, 21.09, 18.48, 14.25, 1.07. HR-MS (ESI) Calcd for [M+H]⁺ 462.2254; Found: 462.2236.



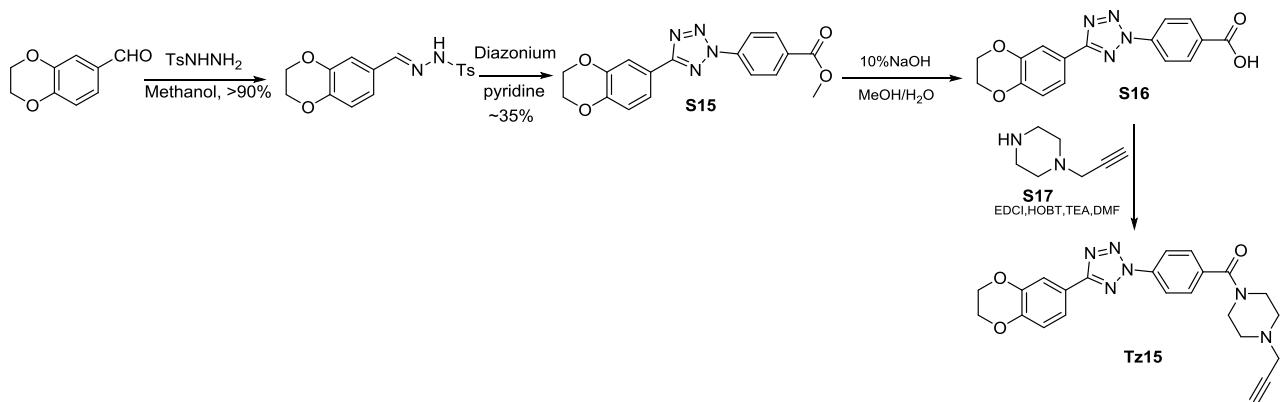
Scheme S5

(**S13**). Synthesis of the diazonium and *p*-toluenesulfonylhydrazones was based on previously published procedures.^[5] The freshly prepared diazonium salt solution (5 mmol) was added dropwise to a stirred solution of **S12** (5 mmol) in 10 mL pyridine at -5 °C over a period of 30 min. The resulting mixture was stirred for 2 h at -5 °C and then further 2 h at room temperature. The reaction mixture was diluted with 30 mL H₂O and 30 mL EtOAc. The organic phase was separated and washed with 1N HCl (2 × 20 mL), followed by concentration *in vacuo*. The residue was purified by flash chromatography (DCM:MeOH = 100:1) to afford the desired tetrazole compound **S13** as a yellow solid (480 mg, 30% yield), *R_f* = 0.5 (PE:EA = 3:1). ¹H NMR (400 MHz, DMSO) δ 12.04 (s, 1H), 9.01 (d, *J* = 1.9 Hz, 1H), 8.71 (d, *J* = 1.7 Hz, 1H), 8.61 (s, 1H), 8.49 – 8.41 (m, 1H), 8.15 (d, *J* = 7.8 Hz, 1H), 7.85 (t, *J* = 8.0 Hz, 1H), 7.68 – 7.60 (m, 1H), 6.64 (dd, *J* = 3.2, 1.5 Hz, 1H), 3.95 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 165.46, 164.82, 149.90, 141.55, 136.78, 131.80, 131.37, 130.78, 128.42, 127.07, 124.58, 120.31, 120.04, 114.92, 101.38, 53.16. LC-MS (ESI) calcd for [M + 1]⁺ 321.1; Found 321.1.

(**S14**). To a solution of **S13** (320 mg, 1 mmol) in 5 mL MeOH and 5 mL water was added 2 mL 10% NaOH solution. The mixture was heated to reflux for 3 h before cooled to rt, and then acidified with 1N HCl followed by filtration and dried to afford a crude product (yellow solid, 271 mg, 90% yield), which was used in next step without further purification. 76 mg (0.25 mmol) of the crude product was then dissolved in 8 mL DMF, followed by addition of EDCI (95.5 mg, 0.5 mmol), HOBT (67.5 mg, 0.5 mmol)

and triethylamine (50.5 mg, 0.5 mmol). The resulting mixture was stirred for 1 h at room temperature. Subsequently, 46.5 mg (0.25 mmol) tert-butyl piperazine-1-carboxylate was added, and stirred at room temperature for 24 h prior to addition of 10 mL water. Upon extraction with 2×10 mL of ethyl acetate, the organic layers were washed with 2×10 mL brine and dried over anhydrous Na_2SO_4 . The organic phase was concentrated *in vacuo* and the residue was purified by flash column (DCM:MeOH = 30:1) to afford **S14** as a yellow solid (70 mg, 59% yield), $R_f = 0.4$ (PE:EA = 1:1). ^1H NMR (400 MHz, CDCl_3) δ 10.45 (s, 1H), 9.25 (s, 1H), 8.89 (d, $J = 1.8$ Hz, 1H), 8.39 – 8.30 (m, 2H), 7.70 (t, $J = 7.8$ Hz, 1H), 7.62 – 7.56 (m, 1H), 7.51 (d, $J = 3.5$ Hz, 1H), 6.71 (d, $J = 3.5$ Hz, 1H), 3.95 – 3.40 (m, 8H), 1.50 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 168.80, 164.49, 154.52, 148.58, 141.08, 137.30, 136.93, 130.29, 128.52, 128.11, 126.88, 120.99, 118.52, 102.16, 80.56, 57.04, 47.62, 42.29, 28.38. LC-MS (ESI) calcd for [M -1]⁻ 473.2; Found 473.2.

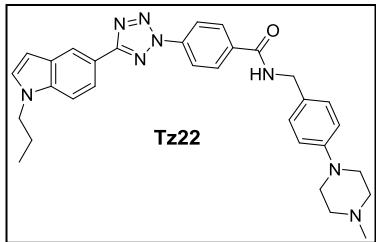
(**Tz14**). To a solution of **S14** (44.6 mg, 0.1 mmol) in 5 mL DMF was added propargyl bromide (12 mg, 0.1 mmol) and potassium carbonate (41.4 mg, 0.3 mmol). The mixture was stirred at rt overnight prior to addition of 20 mL water. Upon extraction with 3×10 mL of ethyl acetate, the organic layers were combined and washed with 2×10 mL brine and concentrated *in vacuo*. The residue was purified by flash column (PE:EA = 5:1) to afford **Tz14** as a white solid (37 mg, 71% yield), $R_f = 0.4$ (PE:EA = 1:1). ^1H NMR (400 MHz, CDCl_3) δ 9.25 (d, $J = 1.7$ Hz, 1H), 8.83 (d, $J = 1.8$ Hz, 1H), 8.42 – 8.29 (m, 2H), 7.69 (t, $J = 7.9$ Hz, 1H), 7.57 (t, $J = 6.0$ Hz, 2H), 6.70 (d, $J = 3.6$ Hz, 1H), 5.23 (d, $J = 2.5$ Hz, 2H), 3.98 – 3.35 (m, 8H), 2.48 (t, $J = 2.5$ Hz, 1H), 1.50 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 168.78, 164.56, 154.50, 147.42, 141.66, 137.29, 136.93, 130.27, 128.81, 128.31, 128.09, 120.99, 120.93, 118.50, 116.04, 101.68, 80.53, 77.57, 73.79, 34.19, 28.38. HR-MS (ESI) Calcd for [M+H]⁺ 513.2363; Found 513.2376.



Scheme S6

(**S15**). Synthesis of **S17** was based on previously published procedures.^[7] The freshly prepared diazonium salt solution (5 mmol) was added dropwise to a stirred solution of corresponding *p*-toluenesulfonohydrazide (1.66 g, 5 mmol) in 10 mL pyridine at -5 °C over a period of 30 min. The resulting mixture was stirred for 2 h at -5 °C and then further 2 h at room temperature. The reaction mixture was diluted with 30 mL H_2O and 30 mL EtOAc. The organic phase was separated and washed with 1N HCl (2×20 mL), followed by concentration *in vacuo*. The residue was purified by flash chromatography (DCM:MeOH = 100:1) to afford the desired tetrazole compound **S15** as a yellow solid (502 mg, 35% yield), $R_f = 0.5$ (PE:EA = 5:1). ^1H NMR (400 MHz, DMSO) δ 8.42 – 8.37 (m, 2H), 8.35 – 8.30 (m, 2H), 7.75 (dd, $J = 8.4, 2.1$ Hz, 1H), 7.70 (d, $J = 2.0$ Hz, 1H), 7.17 (d, $J = 8.4$ Hz, 1H), 4.42 (s, 4H), 4.00 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 165.67, 164.93, 146.43, 144.39, 139.54, 131.67, 131.07, 120.55, 120.40, 119.66, 118.60, 115.78, 64.84, 64.60, 53.03. LC-MS (ESI) calcd for [M -1]⁻ 377.1; Found 377.1.

(**S16**). A solution of **S15** (338 mg, 1 mmol) in 5 mL MeOH and 5 mL water was added 1.5 mL 10% NaOH solution. The mixture was heated to reflux for 3 h and then cooled to rt, and acidified with 1N HCl followed by filtration. The residue was purified by flash chromatography (DCM:MeOH = 20:1) to afford **S16** as a white solid (285 mg, 88% yield), $R_f = 0.4$ (DCM:MeOH = 10:1). ^1H NMR (400 MHz, DMSO) δ 13.32 (s, 1H), 8.24 (dd, $J = 22.7, 8.8$ Hz, 4H), 7.65 (dd, $J = 8.4, 2.0$ Hz, 1H), 7.60 (d, $J = 2.0$ Hz, 1H), 7.08 (d, $J = 8.4$ Hz, 1H), 4.34 (s, 4H). ^{13}C NMR (101 MHz, DMSO) δ 166.72, 164.86, 146.37, 144.35, 139.25, 132.35, 131.72, 120.50, 120.18, 119.69, 118.54, 115.74, 64.82, 64.58. LC-MS (ESI) calcd for [M -1]⁻ 323.1; Found 323.1.

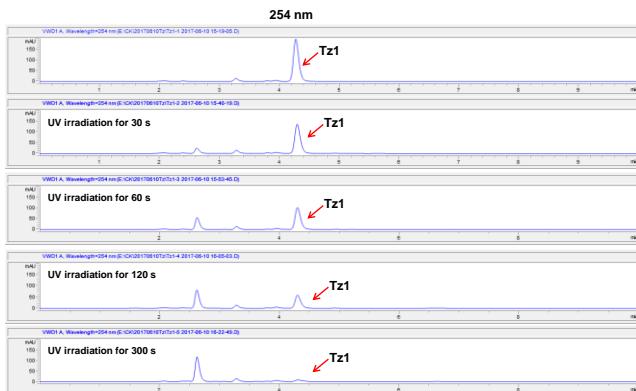


(Tz22). Synthesis of **Tz22** was similar to **Scheme S7**, a yellow solid (51 mg, 64% yield), R_f = 0.4 (DCM:MeOH = 10:1). ^1H NMR (400 MHz, DMSO) δ 9.25 (t, J = 5.8 Hz, 1H), 8.44 (d, J = 1.1 Hz, 1H), 8.28 (d, J = 8.8 Hz, 2H), 8.20 (d, J = 8.8 Hz, 2H), 7.97 (dd, J = 8.6, 1.5 Hz, 1H), 7.71 (d, J = 8.7 Hz, 1H), 7.52 (d, J = 3.1 Hz, 1H), 7.24 (d, J = 8.6 Hz, 2H), 6.94 (d, J = 8.7 Hz, 2H), 6.63 (d, J = 3.0 Hz, 1H), 4.43 (d, J = 5.7 Hz, 2H), 4.21 (t, J = 7.0 Hz, 2H), 3.25 (s, 4H), 2.84 (s, 4H), 2.49 (s, 3H), 1.88 – 1.75 (m, 2H), 0.87 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 165.98, 164.67, 149.27, 137.73, 136.95, 135.26, 130.31, 130.18, 129.10, 128.22, 128.17, 119.52, 119.43, 119.35, 116.94, 115.53, 110.64, 101.46, 53.28, 47.13, 46.97, 43.86, 42.16, 23.14, 11.06. LC- MS (ESI) Calcd for [M+H] $^+$ 535.3; Found 535.3.

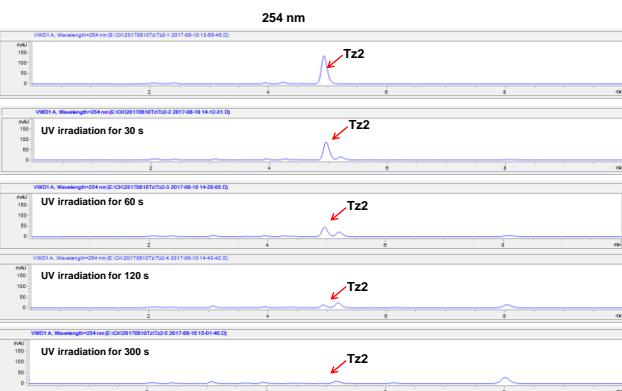
6. HPLC analysis of the photolysis velocities of Tz1-5 in PBS buffer.

Briefly, 1 μ L of **Tz1-5** (10 mM stock concentration in DMSO) was added to 99 μ L PBS buffer, the mixture was subsequently irradiated with UV light (302 nm) for 30 s, 60 s, 120 s, 300 s, respectively. The resulting solution (10 μ L) was then analyzed immediately by HPLC (Agilent Technologies, 1260 Infinity, YMC-Triant C18, 250 \times 4.6 mmL.D. S-5 μ m, 12 nm). Flow phase was 60% ACN and 40% H₂O with flow rate of 0.5 mL/min.

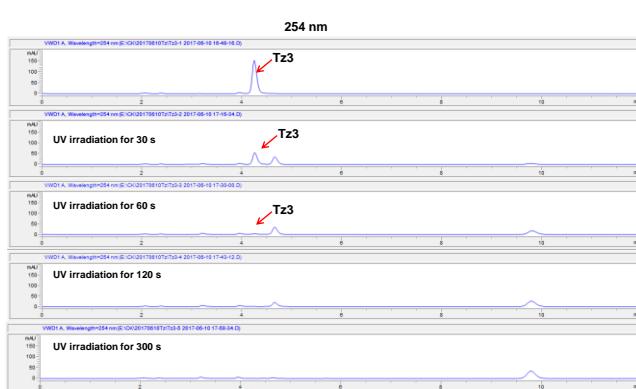
(A)



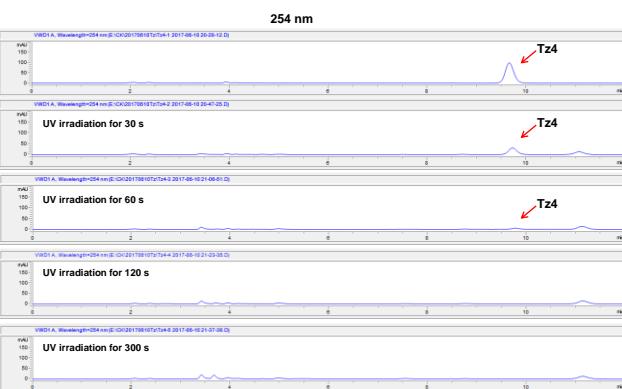
(B)



(C)



(D)



(E)

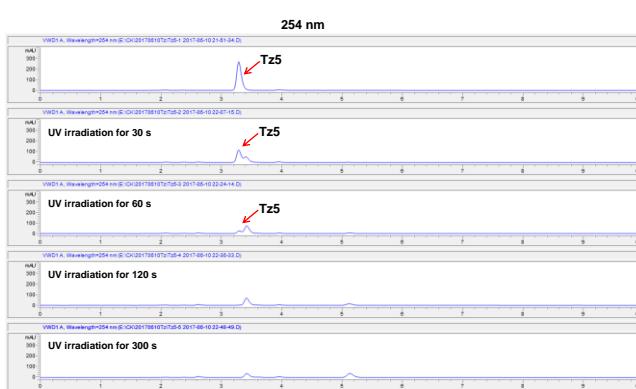
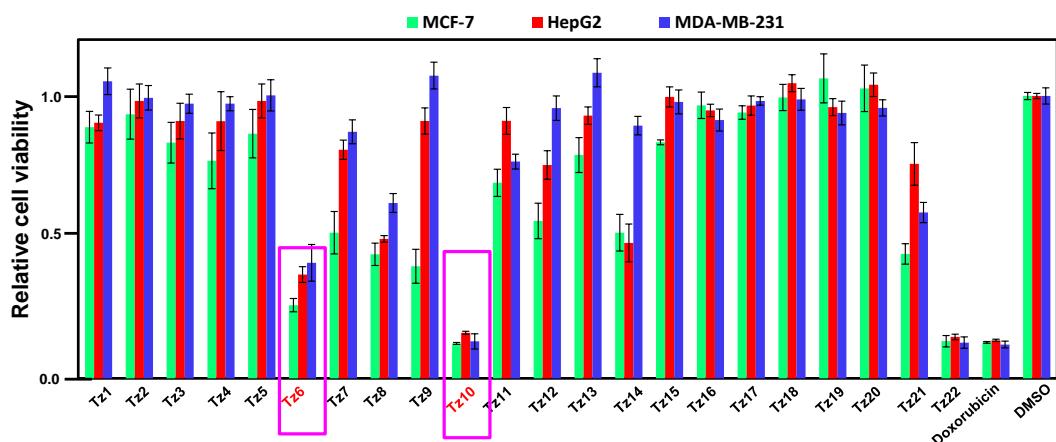


Figure S1. HPLC analysis of **Tz1-5** in PBS after UV irradiation for different periods of time. These results indicated that **Tz1** and **Tz2** can be completely photolysed within 5 min and 2 min, respectively, and **Tz3/4/5** can be photolysed within 1min.

7. Cell Growth Inhibition Assay.

Cell growth inhibition assays were carried out using MCF-7, HepG2, HeLa, H460, A549 and Hs578T cells. Cell viability was determined by CCK8 assay. The procedures were similar to previously published protocols,^[1] 3000 cells per well were seeded in a 96-well plate and incubated for 12 h in a humidified incubator for adherence. **Tz1-22** and Doxorubicin were added to cells at different final concentrations and incubated for 72 h. 10 μ L of CCK-8 reagent was added to each well and incubated for 0.5 h. Following that, the absorbance was measured at 450 nm and 650 nm on a plate reader (Synergy HI, BioTek Instruments, Inc. Vermont, US). Cell viability rate was determined as $VR = (A - A_0)/(As - A_0) \times 100\%$, where A is the absorbance of the experimental group, As is the absorbance of the control group (DMSO was used as the control) and A_0 is the absorbance of the blank group (no cells). IC_{50} values were calculated using GraphPad Prism.

(A)



(B)

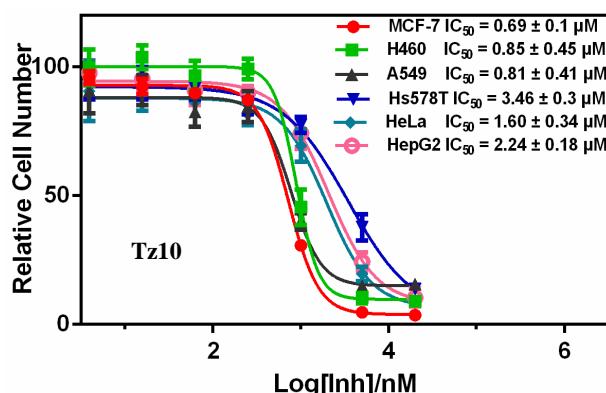


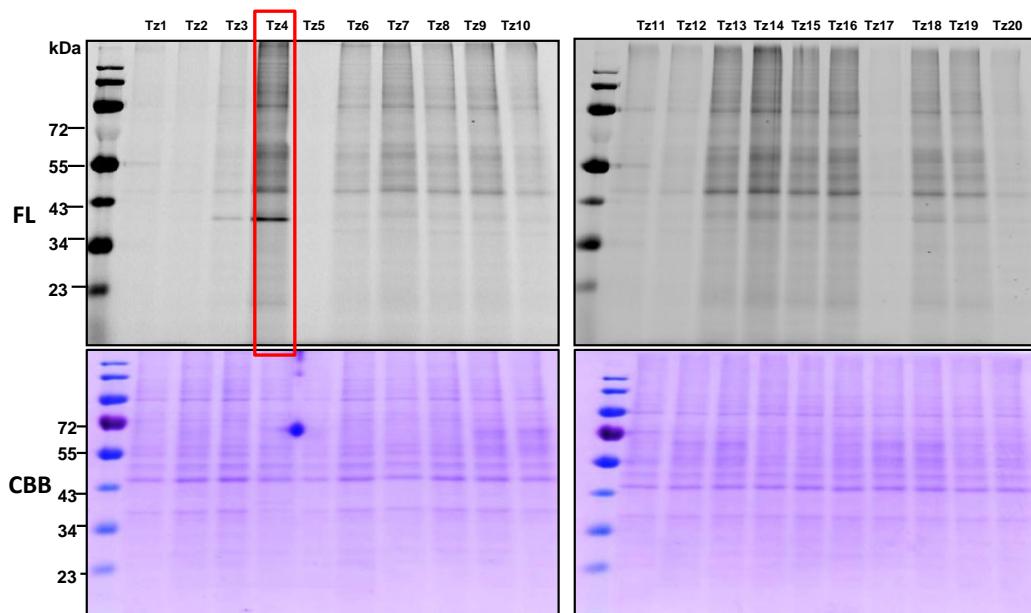
Figure S2. (A) Antiproliferative activities of **Tz1-22** against MCF-7, HepG2 and MDA-MB-231 cancer cell lines at 5 μ M final concentration. (B) IC_{50} values of **Tz10** against MCF-7, H460, A549, Hs578T, HeLa and HepG2 cancer cell lines.

8. In Vitro, In Situ Proteome Labeling.

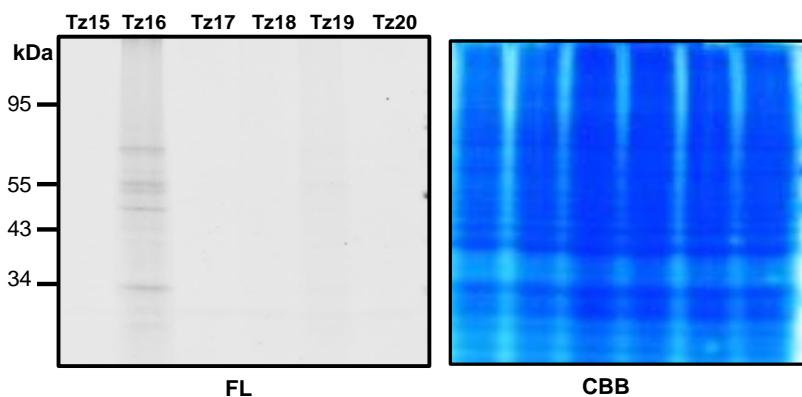
For gel-based protein/cell lysate labeling experiments, procedures were based on previously published protocols with some modifications.^[1-3] For labeling of BSA with **Tz1-5**, to 49.5 μ L of PBS containing 2 μ g BSA protein was added 0.5 μ L probe (stock concentration was 100 μ M in DMSO), the mixture was incubated for 10 min prior to UV irradiation for different periods of time (20 s, 1 min, 5 min), clicked with TAMRA-N₃ under standard click chemistry conditions (30 μ M TAMRA-N₃ from 30 mM stock solution in DMSO, 100 μ M THPTA from 100 mM freshly prepared stock solution in DMSO, 1 mM TCEP from 1 M freshly prepared stock solution in deionized water, and 1 mM CuSO₄ from 1 M freshly prepared stock solution in deionized water). After 2 h of click reaction, 6 \times SDS loading dye was added and the mixture was heated to 90 °C for 2 min. The resulting proteins were resolved by SDS-PAGE. In-gel fluorescence scanning was used to visualize the labeled protein bands. Both in-gel fluorescence scanning (FL) and coomassie staining (CBB) were always carried out on the gels upon SDS-PAGE separation of labeled samples. The procedures of labeling MCF-7 cell lysates with **Tz1-20** were the same as described above (10 min of UV irradiation).

For *in situ* proteome labeling, cells were grown to 80–90% confluence in 6-well plates under conditions as described above. The medium was removed and washed twice with PBS and then treated with 0.5 mL probe-containing medium in the presence or absence of excessive competitors (diluted from DMSO stocks whereby DMSO never exceeded 1% in the final solution). After 1-5 h of incubation, the medium was aspirated and cells were washed twice with PBS to remove excessive probe, followed by UV irradiation (302 nm) for 10 min on ice. The cells were lysed with 200 μ L RIPA lysis buffer (Thermo Scientific™ #89900) containing phosphatase inhibitor (Thermo Scientific™ #88669) on ice for 30 min. A soluble protein solution was obtained by centrifugation for 10 min (14000 rpm, 4 °C). Eventually, the protein concentrations were determined by using the BCA protein assay (Pierce™ BCA protein assay kit) and diluted to 1 mg/mL with PBS. A freshly pre-mixed click chemistry reaction cocktail (50 μ M TAMRA-N₃ from 30 mM stock solution in DMSO, 100 μ M THPTA from 100 mM freshly prepared stock solution in DMSO, 1 mM TCEP from 1 M freshly prepared stock solution in deionized water, and 1 mM CuSO₄ from 1 M freshly prepared stock solution in deionized water). The reaction was further incubated for 2 h prior to addition of pre-chilled acetone (-20 °C). The precipitated proteins were subsequently collected by centrifugation (14000 rpm, 10 min at 4 °C), and washed with 200 μ L of prechilled methanol. The samples were dissolved in 1× SDS loading buffer and heated for 10 min at 95 °C. 20 μ g proteins for each lane were loaded on SDS-PAGE (10% gel) and then visualized by in-gel fluorescence scanning (Typhoon FLA 9500). (see Figures 1E, 2B, 3A and S3 for representative examples).

(A)



(B)



(C)

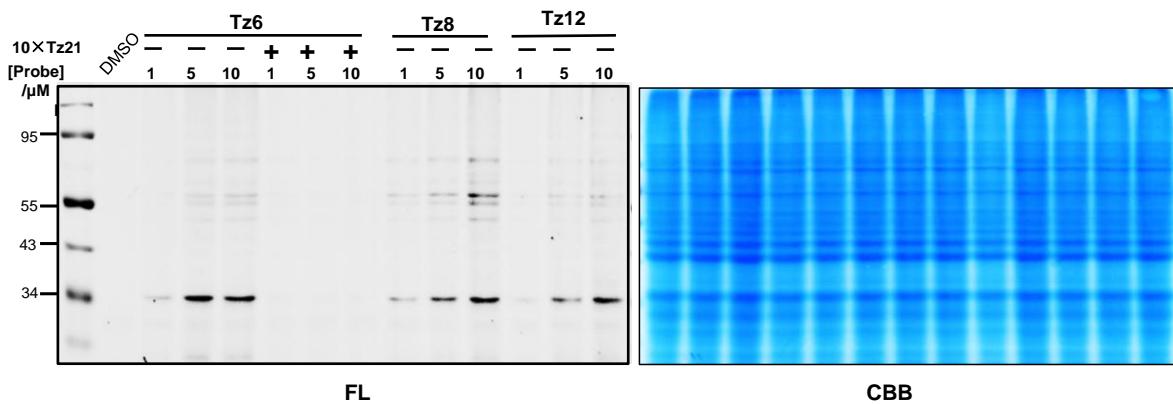


Figure S3. (A) Labeling profiles of MCF-7 cell lysates with **Tz1-20** (2 μ g protein, 1 μ M final concentration of the probes). (B) Labeling profile of MCF-7 cells with **Tz15-20** (5 μ M, the labeling profiles of **Tz1-14** were presented in Figure 1E and 2B of maintext). (C) Concentration-dependent labeling of MCF-7 cells with **Tz6/8/12**.

9. Pull down and targets validation

To identify the interacting cellular targets of **Tz6/10**, pull-down (PD) experiments were carried out, and followed by Western blotting (WB) and LC-MS/MS, where applicable. The general pull-down experiments were based on previously reported procedures,^[1,2,3] with the following optimizations. MCF-7 cells were grown to 80–90% confluence under the conditions described above. The medium was removed and the cells were treated with **Tz6/10**-containing medium in the presence or absence of corresponding competitors (final concentration of the probe was 2 μ M, DMSO never exceeded 1% in the final solution). After 5 h of incubation, the medium was aspirated, and cells were washed twice with PBS to remove excessive probe, followed by UV-irradiation (302 nm) for 10 min on ice. The cells were lysed with RIPA buffer and centrifuged for 10 min (14000 rpm, 4 °C) to get a soluble protein solution. Eventually, the protein concentrations were determined by BCA protein assay and then diluted to 1 mg/mL with PBS. A freshly premixed click chemistry reaction cocktail was added (50 μ M TAMRA-Biotin-N₃ from 30 mM stock solution in DMSO, 100 μ M THPTA from 100 mM freshly prepared stock solution in DMSO, 1 mM TCEP from 1 M freshly prepared stock solution in deionized water, and 1 mM CuSO₄ from 1 M freshly prepared stock solution in deionized water). The reaction was further incubated for 2 h with gentle mixing prior to precipitation by addition of pre-chilled acetone (-20 °C). Precipitated proteins were subsequently collected by centrifugation (13000 rpm \times 10 min at 4 °C) and dissolved in PBS containing 1% SDS. Upon incubation with streptavidin beads for 2 hours at rt, the beads were washed with PBS containing 0.5% SDS (3 \times 1 mL) and PBS (3 \times 1 mL). The enriched proteins were eluted by 1 \times loading buffer at 95 °C for 10 min and separated by SDS-PAGE (10%). Control pull-down experiments using the DMSO and the inactive probe **Tz4** were carried out concurrently with live cells. WB experiments were carried out as previously described using the corresponding antibodies.

For in gel digestion, the enriched proteins were then separated on 10% SDS-PAGE gels, followed by coomassie staining. The whole lane or the specific band was cut to small particles followed by distaining with ammonium bicarbonate buffer (25 mM, ABB) and 50% acetonitrile in ammonium bicarbonate buffer (25 mM) until the blue color disappeared completely. The gel particles were then incubated with DTT solution (10 mM final concentration in 25 mM ABB buffer) for 1h and then incubated with IAA (50 mM final concentration in 25 mM ABB) for 1h. After that the samples were incubated with trypsin (1:30, tripsin/protein in gel) for overnight. Upon extraction from the gel with 50% acetonitrile and 1% formic acid and desalting, the peptides were separated and analyzed on an Easy-nLC 1000 system coupled to a Q Exactive HF (both - Thermo Scientific). About 1 μ g of peptides were separated in an home-made column (75 μ m x 15 cm) packed with C18 AQ (5 μ m, 300 \AA , Michrom BioResources, Auburn, CA, USA) at a flow rate of 300 nL/min. Mobile phase A (0.1% formic acid in 2% ACN) and mobile phase B (0.1% formic acid in 98% ACN) were used to establish a 60 min gradient comprised of 2 min of 5% B, 40 min of 5-26% B, 5 min of 26-30% B, 1 min of 30-35% B, 2 min of 35-90% B and 10 min of 90% B. Peptides were then ionized by electrospray at 1.9 kV. A full MS spectrum (375-1400 m/z range) was acquired at a resolution of 120,000 at m/z 200 and a maximum ion accumulation time of 20 ms. Dynamic exclusion was set to 30 s. Resolution for HCD MS/MS spectra was set to 30,000 at m/z 200. The AGC setting of MS and MS² were set at 3E6 and

1E5, respectively. The 20 most intense ions above a 1.0E3 counts threshold were selected for fragmentation by HCD with a maximum ion accumulation time of 60 ms. Isolation width of 1.6 m/z units was used for MS². Single and unassigned charged ions were excluded from MS/MS. For HCD, normalized collision energy was set to 25%.

The raw data were processed and searched with MaxQuant 1.5.4.1 with MS tolerance of 4.5 ppm, and MS/MS tolerance of 20 ppm. The UniProt human protein database (release 2016_07, 70630 sequences) and database for proteomics contaminants from MaxQuant were used for database searches. Reversed database searches were used to evaluate false discovery rate (FDR) of peptide and protein identifications. Two missed cleavage sites of trypsin were allowed. Carbamidomethylation (C) was set as a fixed modification, and oxidation (M), Acetyl (Protein N-term) and deamidation (NQ) were set as variable modifications. The FDR of both peptide identification and protein identification is set to be 1%.^[8] The options of “Second peptides”, “Match between runs” and “Dependent peptides” were enabled. Label-free quantification was used to quantify the difference of protein abundances between different samples.^[9,10]

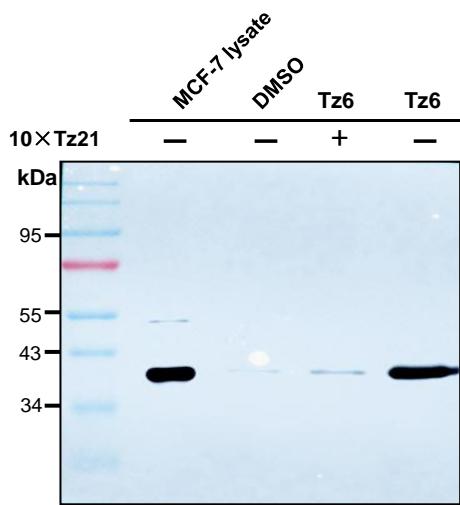


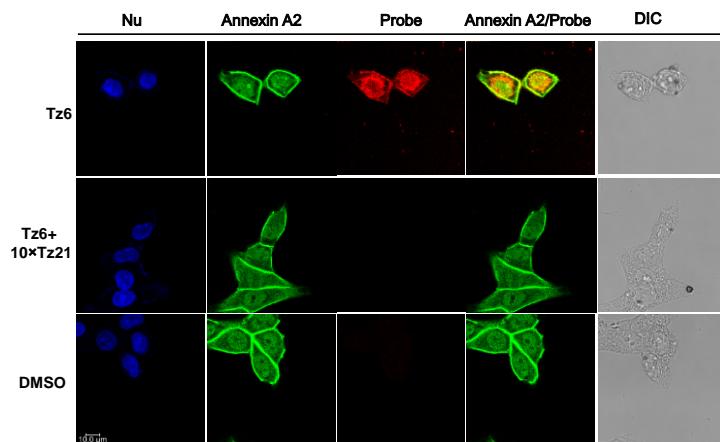
Figure S4. Pull-down/WB analysis with **Tz6** against annexin A2 (2 μ M).

10. Cellular Imaging

To demonstrate the utility of the cell-permeable probes for imaging of potential cellular targets, we performed fluorescence microscopy. The general procedures were similar to what was previously reported.^[1,2,3] MCF-7 cells were seeded in glass bottom dishes (Mattek) and grown until 70–80% confluence. Cells were then treated with 0.5 mL of DMEM with probes (**Tz6/10**) at different final concentrations in the presence or absence of corresponding competitors. After incubation for 2 h, the medium was removed and cells were gently washed twice with PBS, followed by UV irradiation (302 nm UV lamp) for 10 min on ice. The cells were subsequently fixed for 10 min at room temperature with 3.7% formaldehyde in PBS, washed twice with cold PBS again, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were then treated with a freshly premixed click chemistry reaction (50 μ M TAMRA-N₃ from 2.5 mM stock solution in DMSO, 0.1 mM TBTA from 2.5 mM freshly prepared stock solution in deionized water, 1 mM TCEP from 25 mM freshly prepared stock solution in deionized water, and 1 mM CuSO₄ from 25 mM freshly prepared stock solution in deionized water) for 2 h at room temperature with vigorous shaking. Cells were washed with PBS three times and 0.1% Tween 20 in PBS for once. Finally, the cells were stained with Hoechst (1:5000 dilution in PBS) for 10 min at room temperature prior to image. DMSO-treated samples were used as controls concurrently.

For co-localization experiments, cells were further incubated with anti-ANXA2 (1:1000) or anti-NOS2 (1:200) antibody for 1 h at room temperature (or overnight at 4 °C), washed twice with PBS, and then incubated with Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (1:500) for 1 h, following by washing again. Imaging was done with the Leica TCS SP8 confocal microscope system equipped with Leica HCX PL APO 40 \times /1.20 W CORR CS, 405 nm diode laser, white laser (470–670 nm, with 1 nm increments, with eight channels AOTF for simultaneous control of eight laser lines, each excitation wavelength provides 1.5 mV), and a photomultipliertube (PMT) detector ranging from 410 to 700 nm for steady state fluorescence.

(A)



(B)

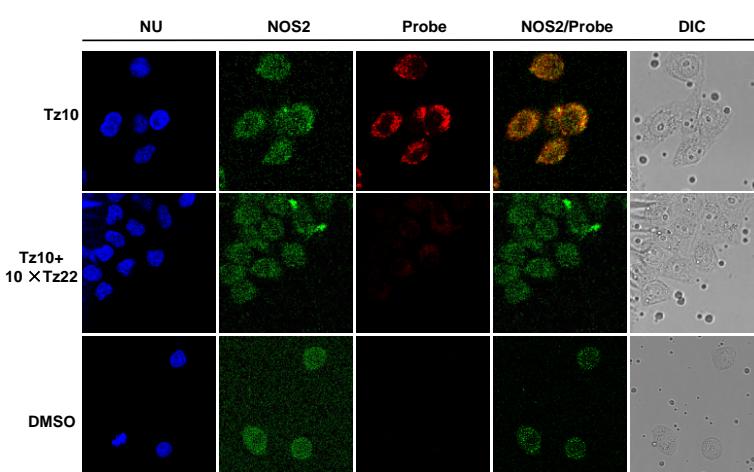


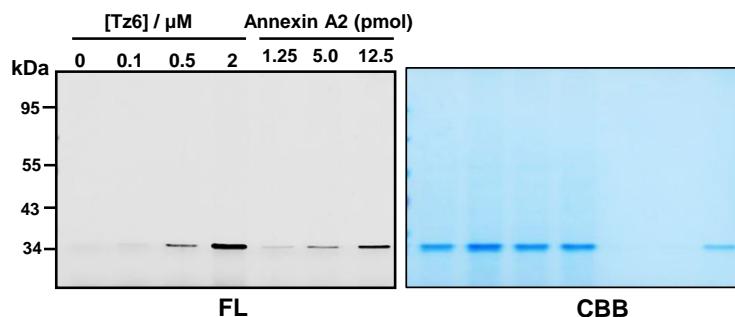
Figure S5. (A) Cellular imaging of probe **Tz6** (2 μM) in MCF-7 cells with or without 10 × **Tz21**, and immunofluorescence (IF) against annexin A2 (ANXA2). Scale bar = 10 μm. (B) Cellular imaging results of MCF-7 cells treated with **Tz10** in the presence or absence of 10 × **Tz22**, and immunofluorescence (IF) against Nitric oxide synthase (NOS2). Parts of the images were reproduced in the main text (Figures 4D).

11. Recombinant protein labeling and LC-MS/MS analysis of the binding sites.

To determine the labeling sites in annexin A2 by **Tz6**, the probe-labeled samples were analyzed by LC-MS/MS to determine the site/residue within the protein that was covalently modified by the probe. Briefly, **Tz6** (0.75 nmol) was incubated with annexin A2 protein (25 μg, 0.63 nmol) in PBS buffer for 30 min at room temperature, then UV-irradiated for 10 min on ice. Negative controls with samples treated with DMSO were concurrently conducted. Upon SDS-PAGE separation of the resulting samples, the gel bands corresponding to the labeled annexin A2 was excised followed by trypsin digestion and peptide extraction, the procedures were same as above-described. The peptides samples were desalted by C₁₈ column and dried. LC-MS/MS analysis was performed on a nanoAcuity (Waters) LC system coupled with Q-Executive Orbitrap mass spectrometer (Thermo Scientific Inc.). Samples were loaded onto a fused silica desalting column packed with 2 cm C₁₈ reverse phase resin (Phenomenex Inc.), and peptides were separated on a reverse phase column (100 μm fused silica packed with 15 cm C₁₈ resin). Peptide separation condition was a gradient 5-50% Buffer B in Buffer A (Buffer A: water, 0.1 % formic acid; Buffer B: acetonitrile, 0.1% formic acid), and the flow rate through the column was set to 250 nL min⁻¹, and spray voltage was set to 1.8 keV. Neither sheath gas nor makeup liquid was used. MS data acquisition began 0.1 min after the sample was injected and continued throughout analysis for data dependent analysis (DDA) mode. DDA parameter was set for each full MS scan (350 – 1800 MW) followed by 10 MS/MS fragmentation scans for most intense ions with dynamic exclusion enabled for 30 sec. The resulting MS/MS data was searched using the in-house Proteome Discoverer server (v1.4.0.288 Thermo Scientific) using IPIHuman proteome sequence database (IPIHuman v3.87), and de novo sequencing was

performed using PEAKS Studio (version 7.0, Bioinformatics Solutions Inc.). A maximum of three missed cleavages were allowed and fully tryptic peptides were considered with dynamic modification of carbamyl at N-terminal. The maximal mass tolerance in MS mode was set to 5 ppm, and fragment MS/MS tolerance was set to 0.36 Da for HCD fragmentation data. Three peptides (“TPAQYDAS”, “YELIDQDAR”, “GSVIDYELIDQDAR”) from annexin A2 were shown to have carried a molecular weight increase which corresponded to the addition of **Tz6**-labeled adducts, and they were further analyzed by MS/MS to map the residue/site of the modification (Figure S6). Results indicated residue ASP¹¹⁰ (D) in annexin A2 was covalently modified by the probe. Docking experiments were performed using AutoDock Vina based on the human annexin A2 crystal structure (PDB ID: 1W7B).¹¹

(A)



(B)

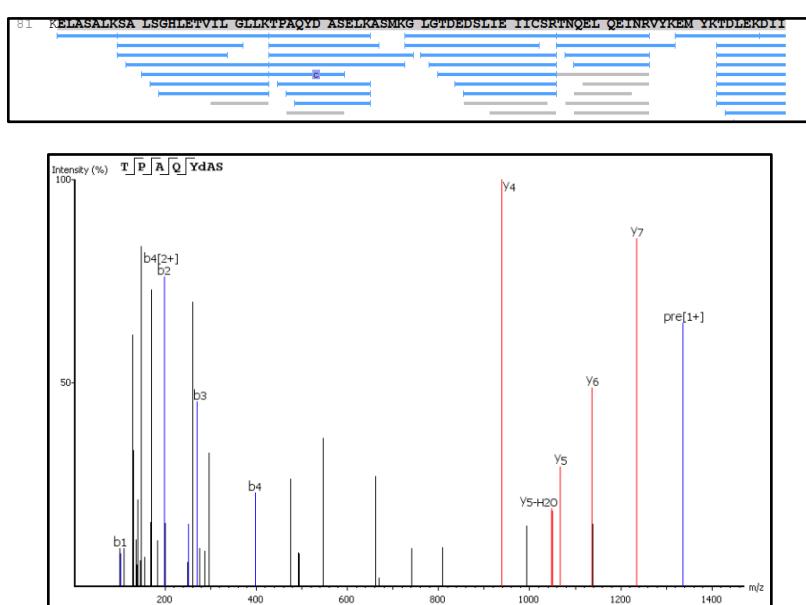


Figure S6. (A) Labeling profiles of recombinant annexin A2 with **Tz6**. (B) LC-MS/MS site-mapping analysis of annexin A2 labeled by **Tz6**. A peptide in annexin was positively identified, indicating labeling site was ASP¹¹⁰.

12. Photocrosslinking efficiency evaluation of **Tz6** with annexin A2

To 30 μL PBS buffer containing 0.5 nmol of recombinant annexin A2 was added 0.5 nmol of **Tz6**. The mixture was incubated for 30 min at room temperature with gentle shaking. Subsequently, the reactions were UV-irradiated (302 nm UV lamp) for 10 min on ice. The labeled protein was then subjected to click reaction with biotin azide, acetone precipitation, and resolubilized in 1% SDS-containing PBS. The resuspended samples were then incubated with avidin-agarose beads (10 μL) for 4 h at room temperature. After centrifugation, supernatant was transferred to another tube, the beads were washed with 0.1% SDS in PBS for 3 times and PBS for 3 times. The washing solution was combined with supernatant fraction. After washing, the beads were boiled in 1× SDS loading buffer (200 mM Tris pH 6.8, 400 mM DTT, 8% SDS) for 15 min to get the Pull-down (PD) samples. Same amount of supernatant fraction and PD samples were loaded on same gel to evaluate the labeled and unlabeled protein by western blot (WB) and coomassie staining (CBB). As shown in **Figure S7**, under such conditions, very little unlabeled ANXA2 was detected in the supernatants (except in DMSO control reactions) from reactions labeled by **Tz6**, indicating highly efficient protein cross-linking.

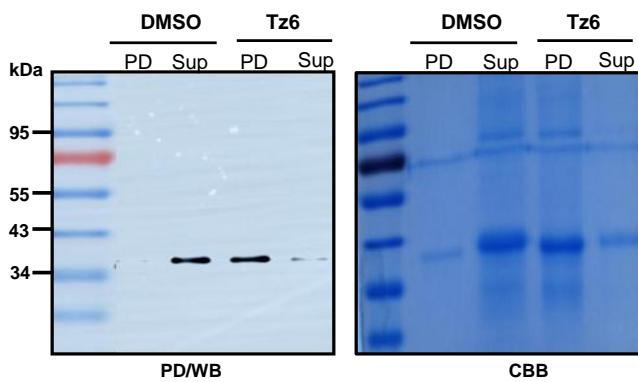
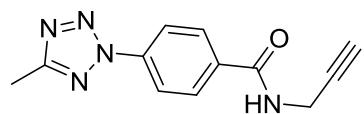
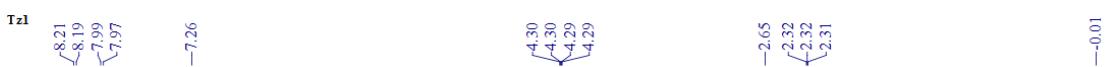
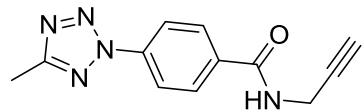
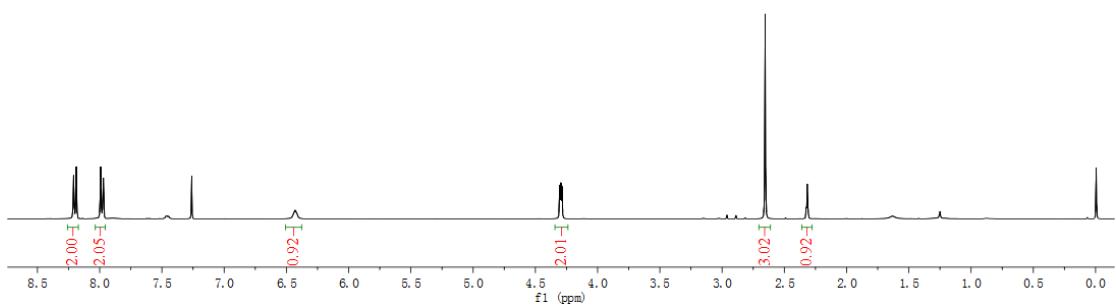


Figure S7. Evaluation of the labeled recombinant annexin A2 and unlabeled protein by **Tz6** through PD/WB and PD/CBB; “Sup” means supernatant fraction, “PD” means pull-down sample.

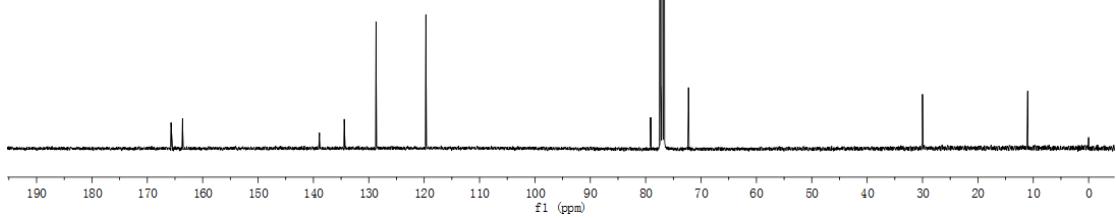
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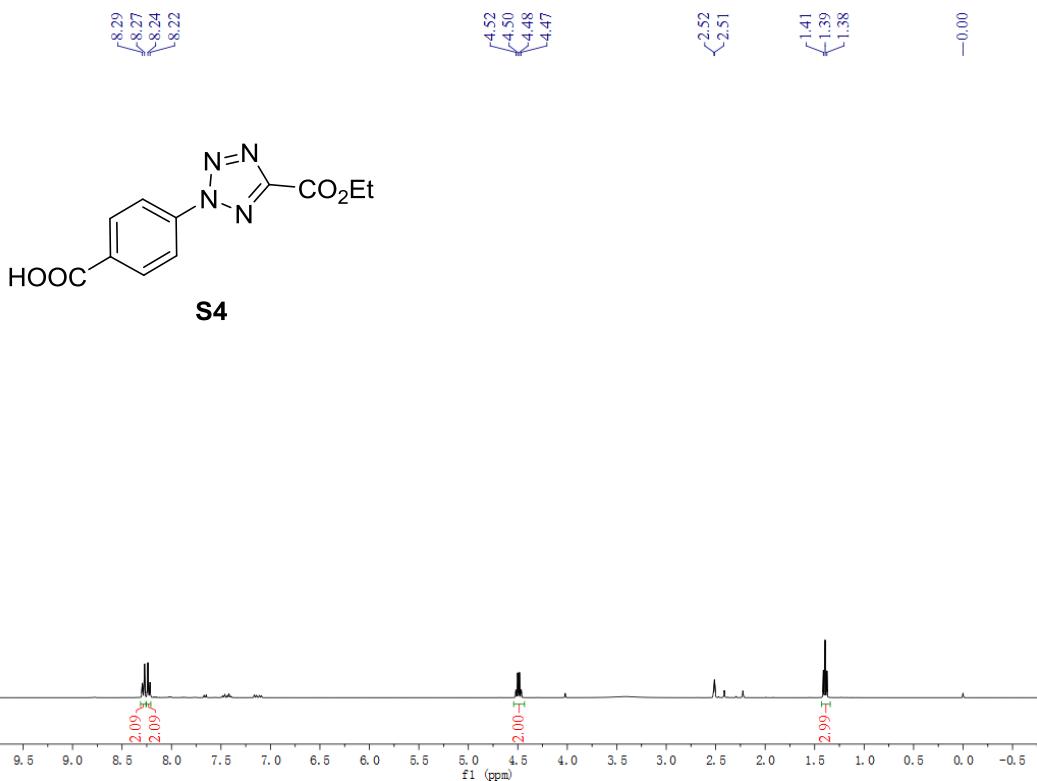
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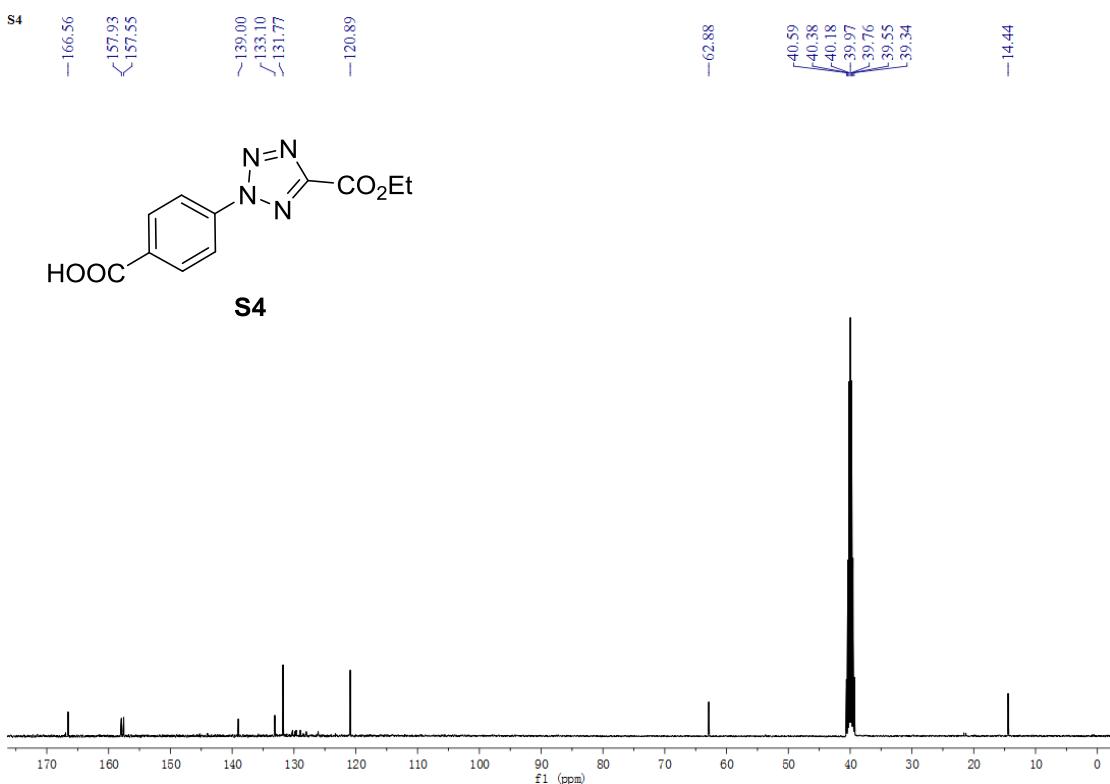
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S4

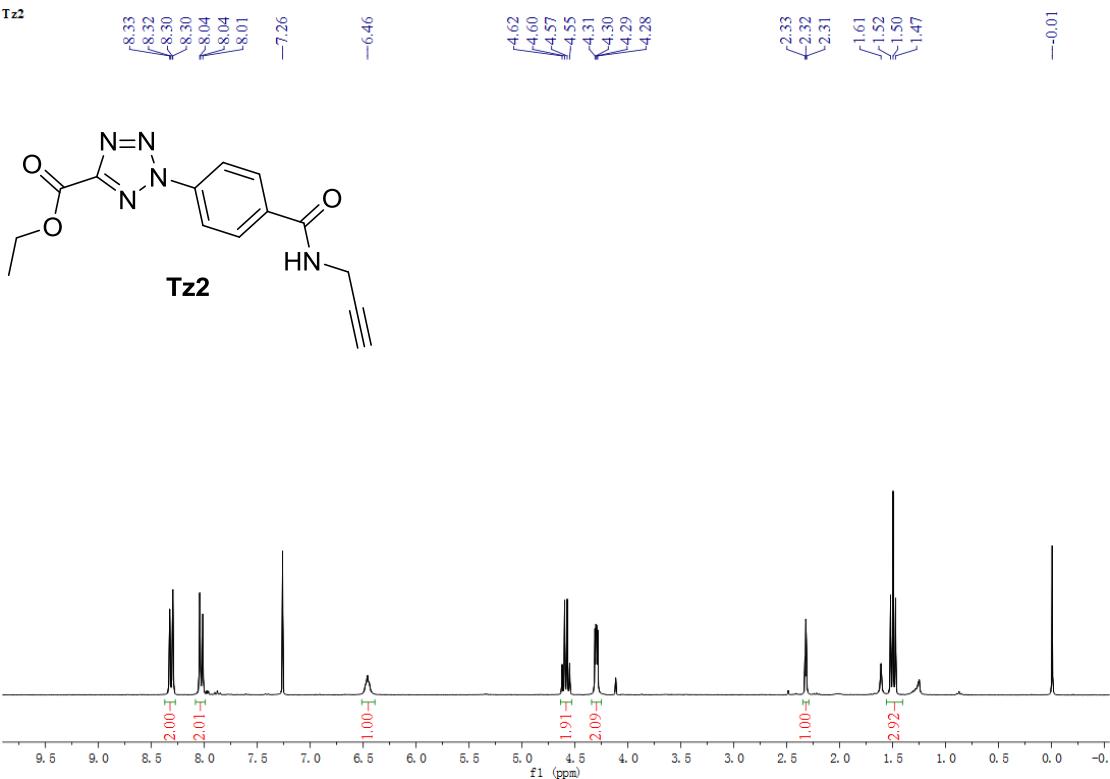


S4

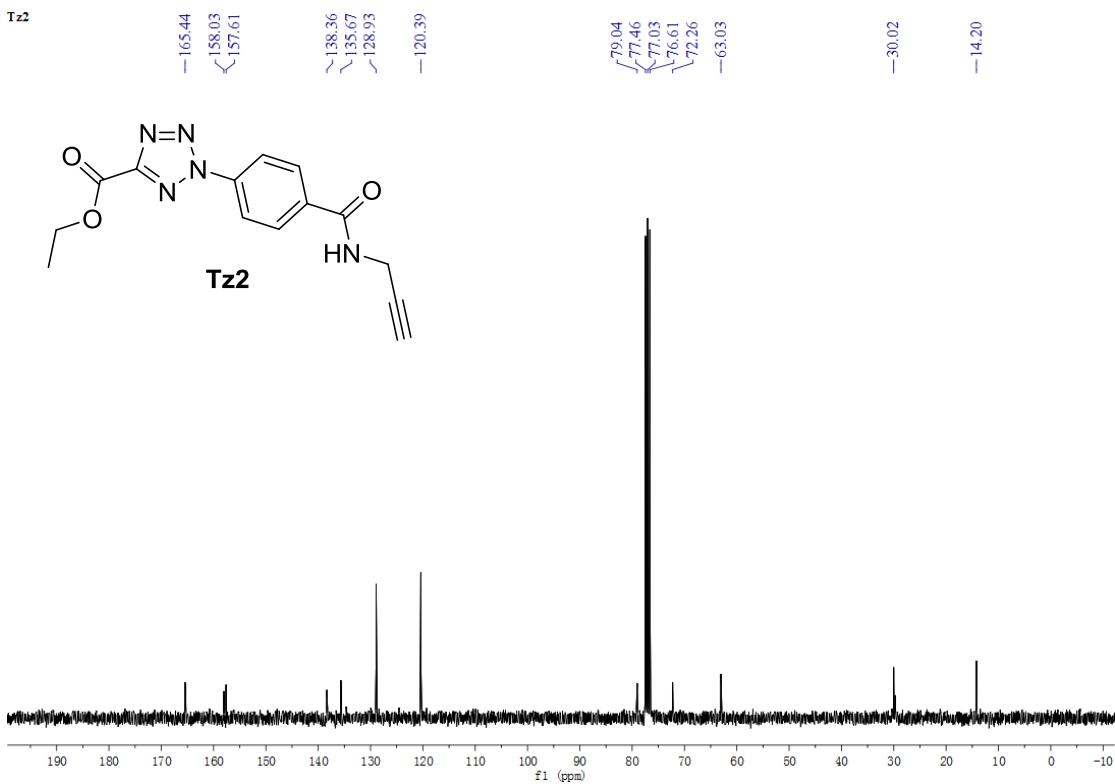


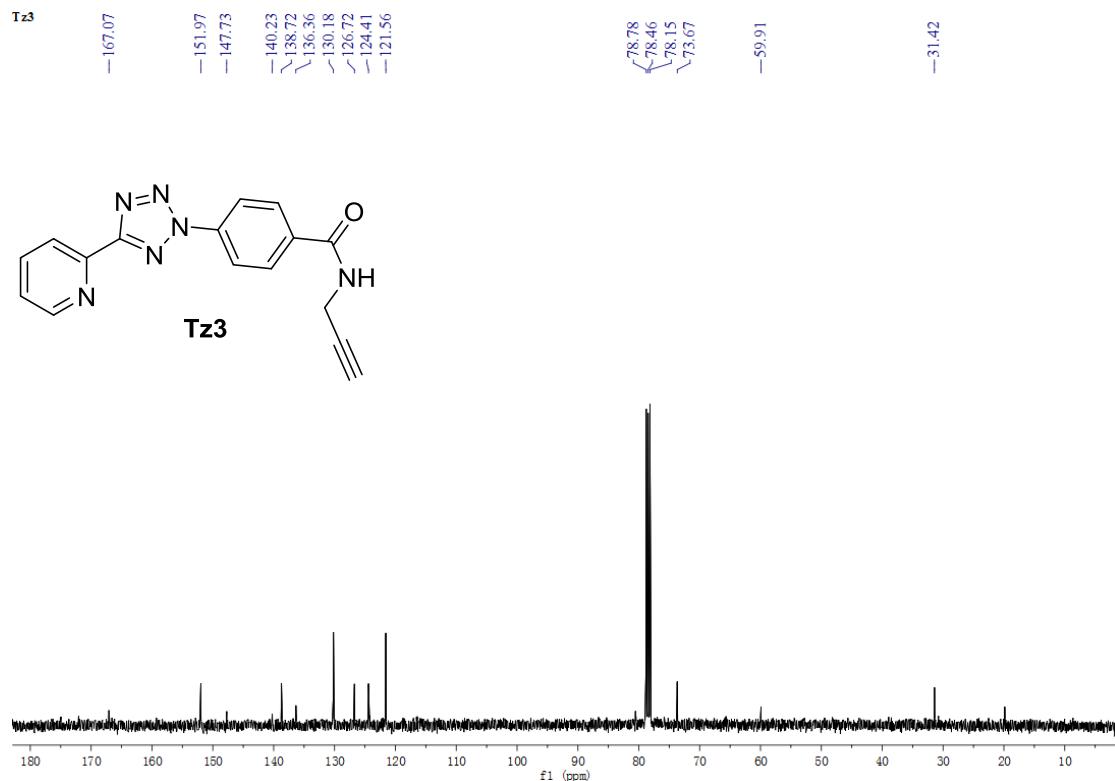
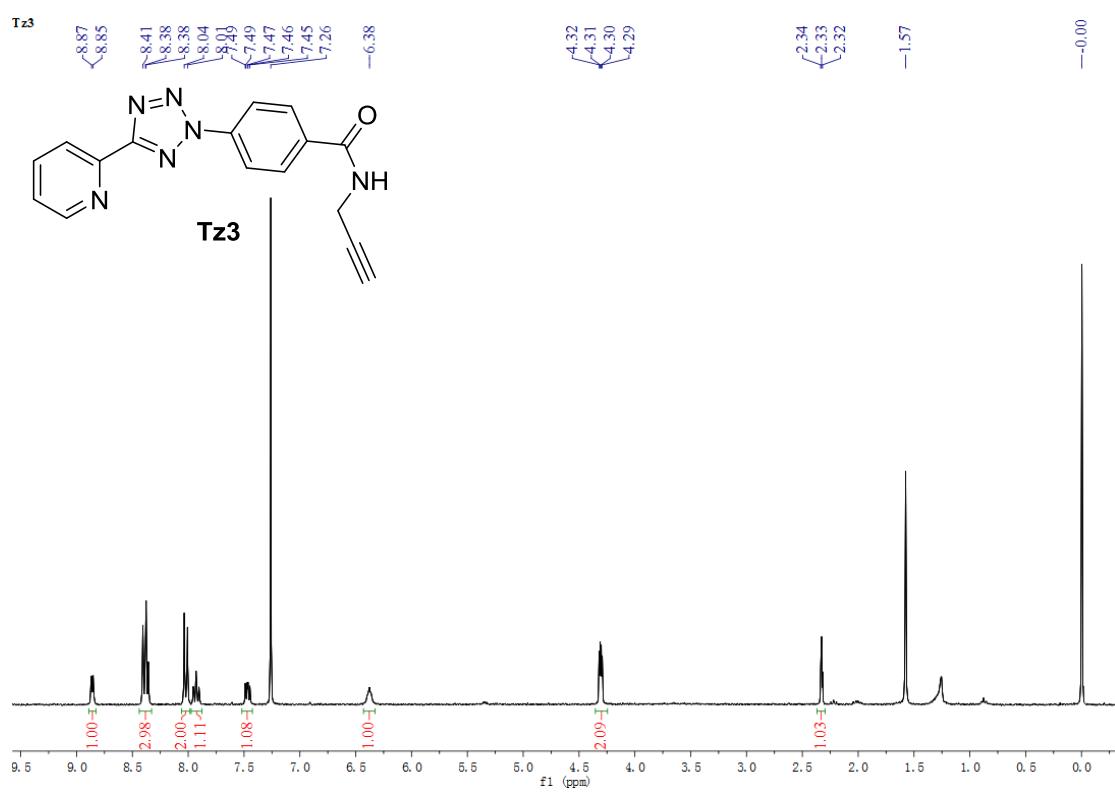
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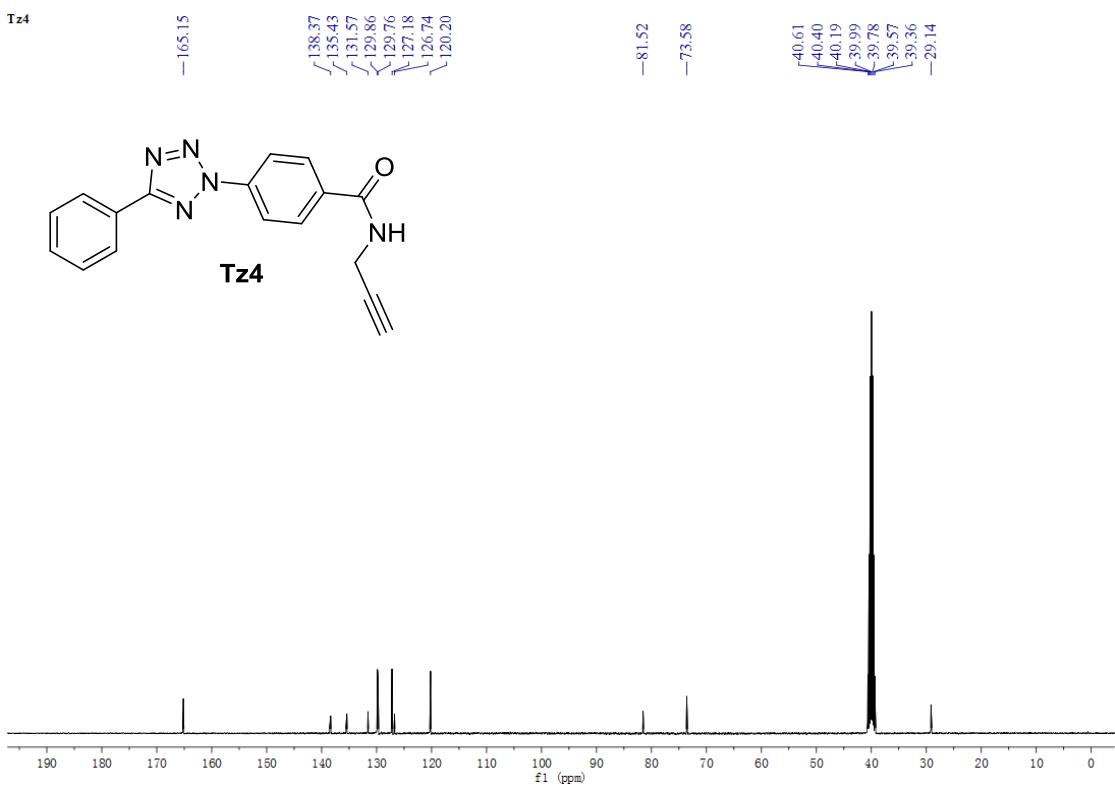
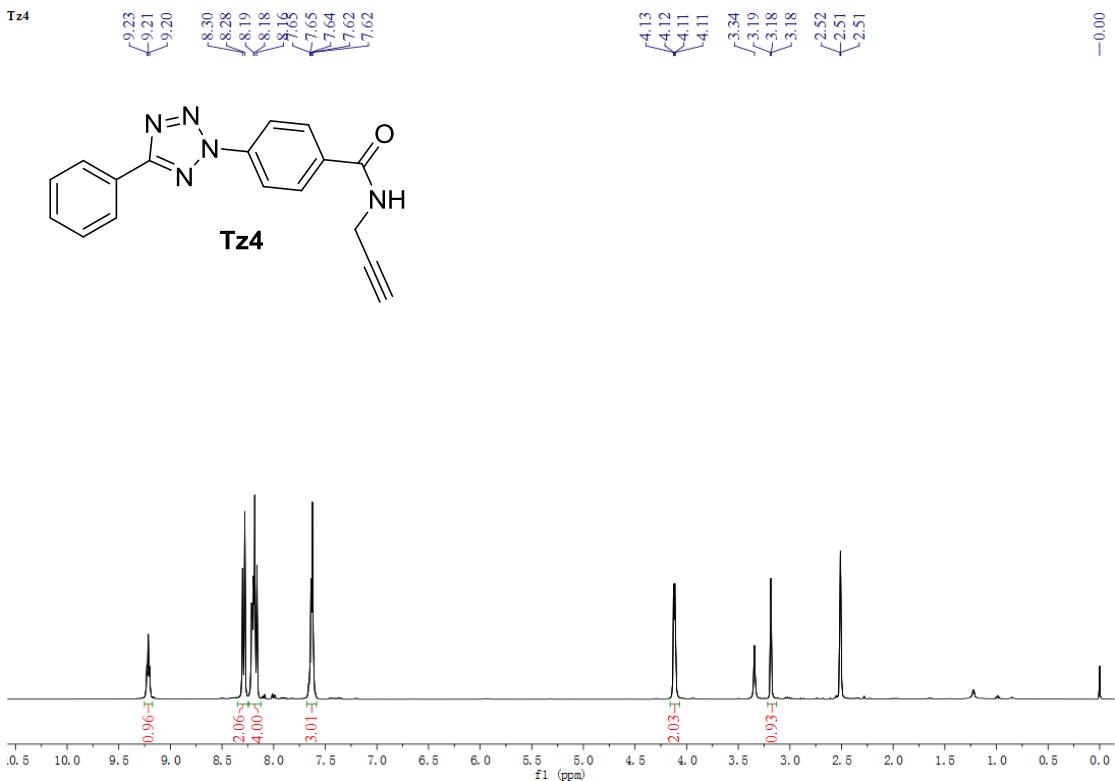
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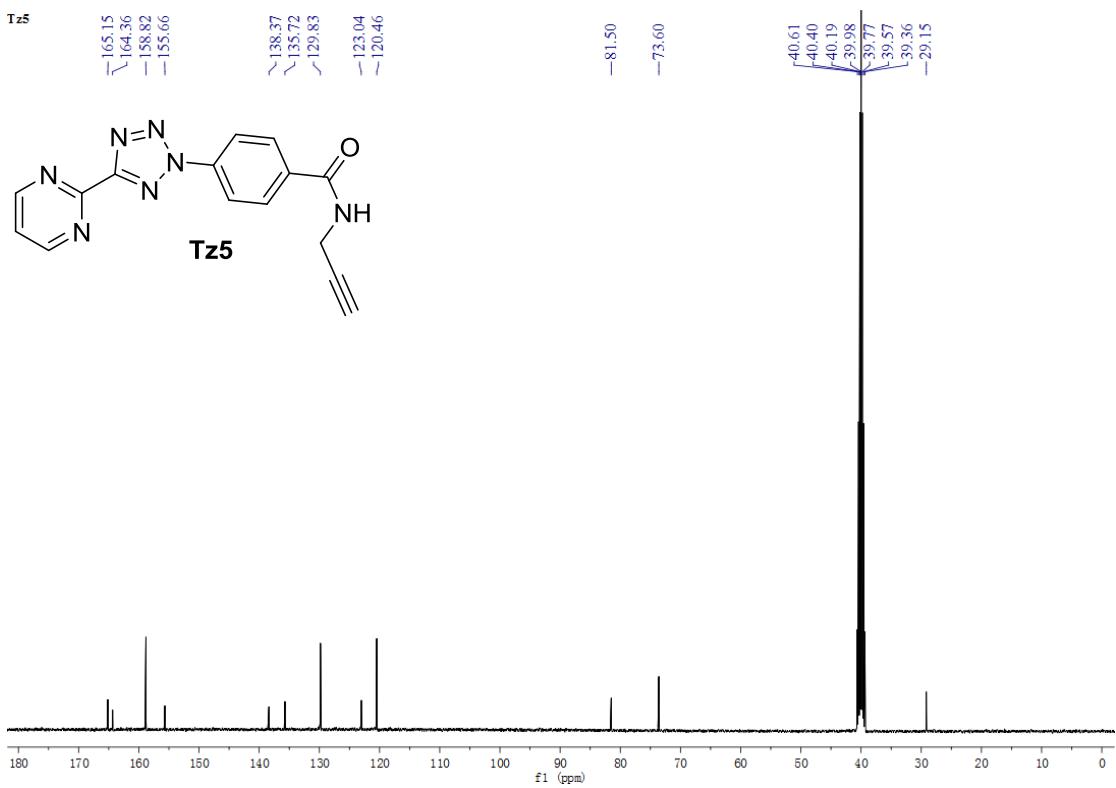
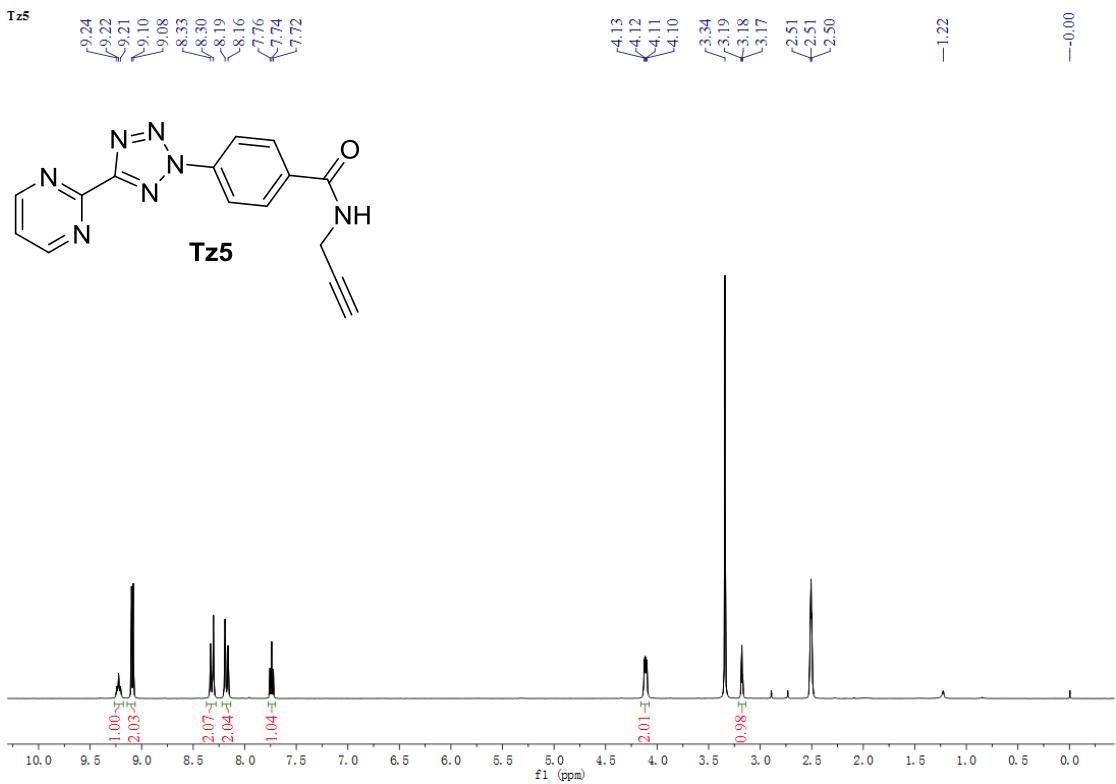


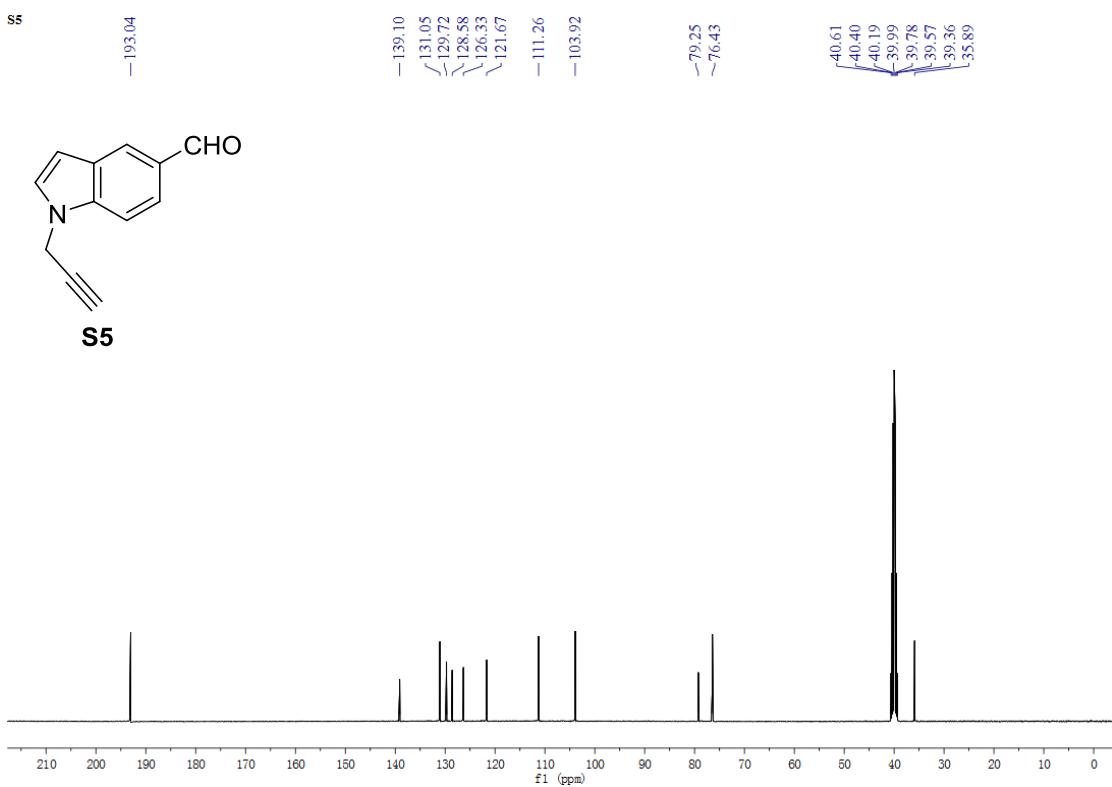
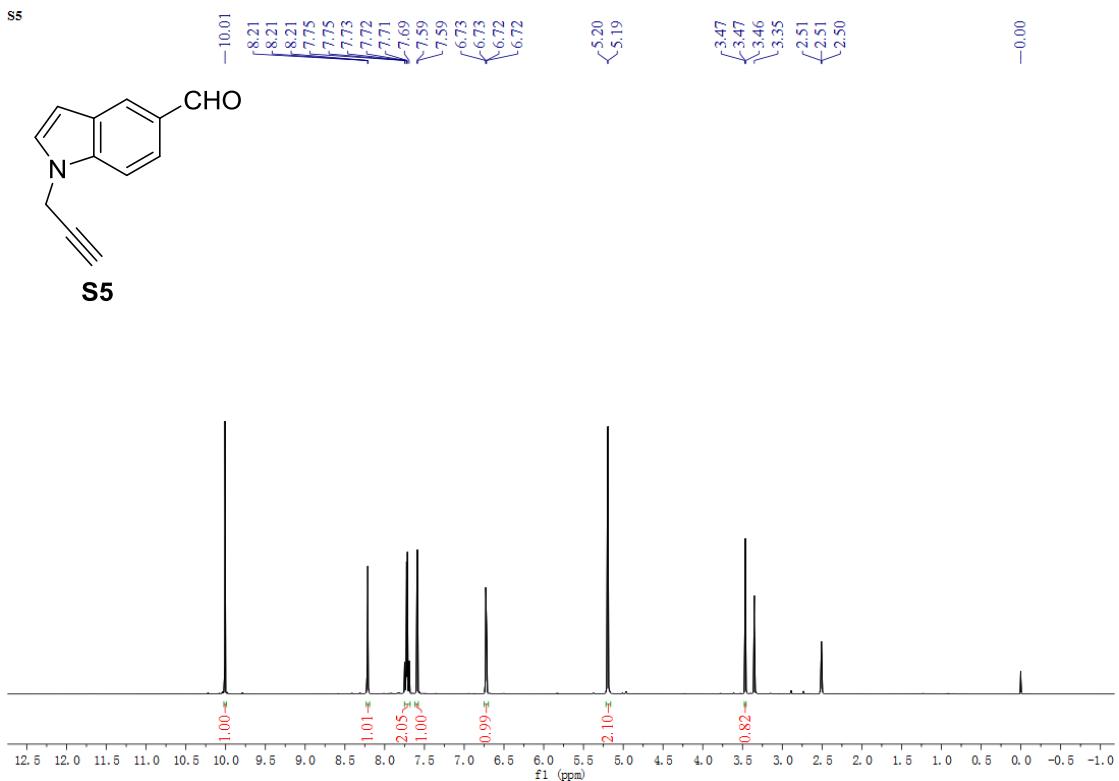
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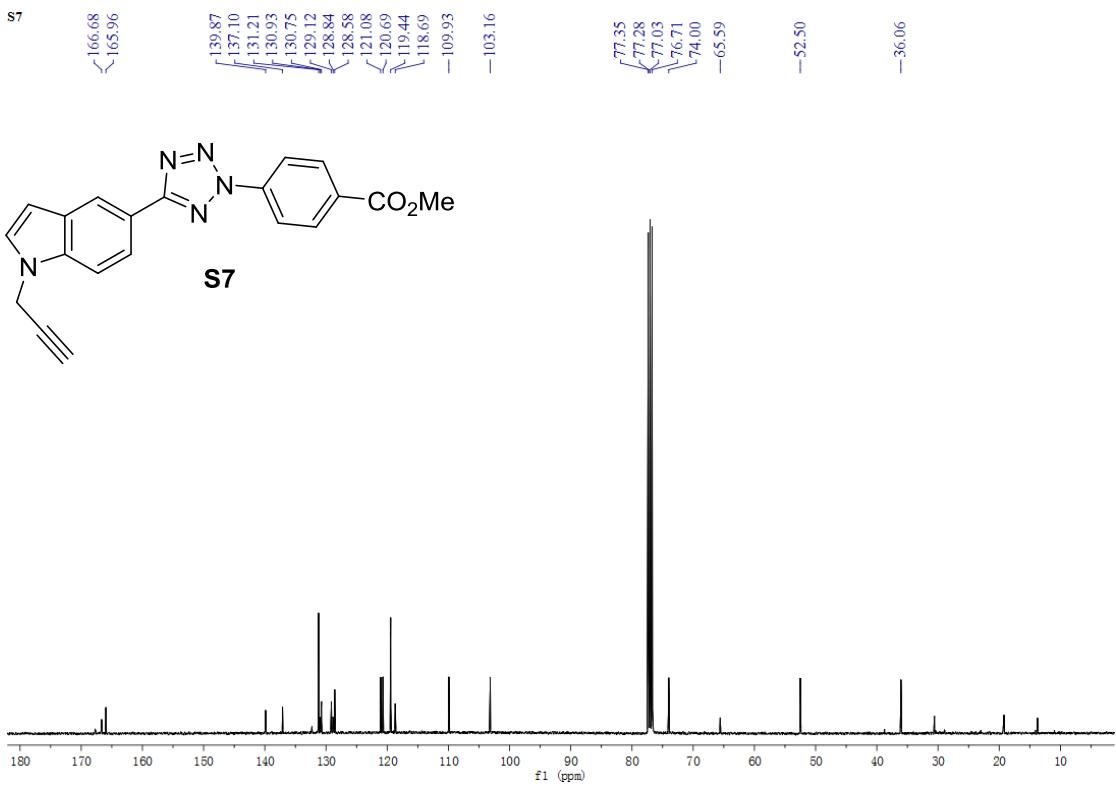
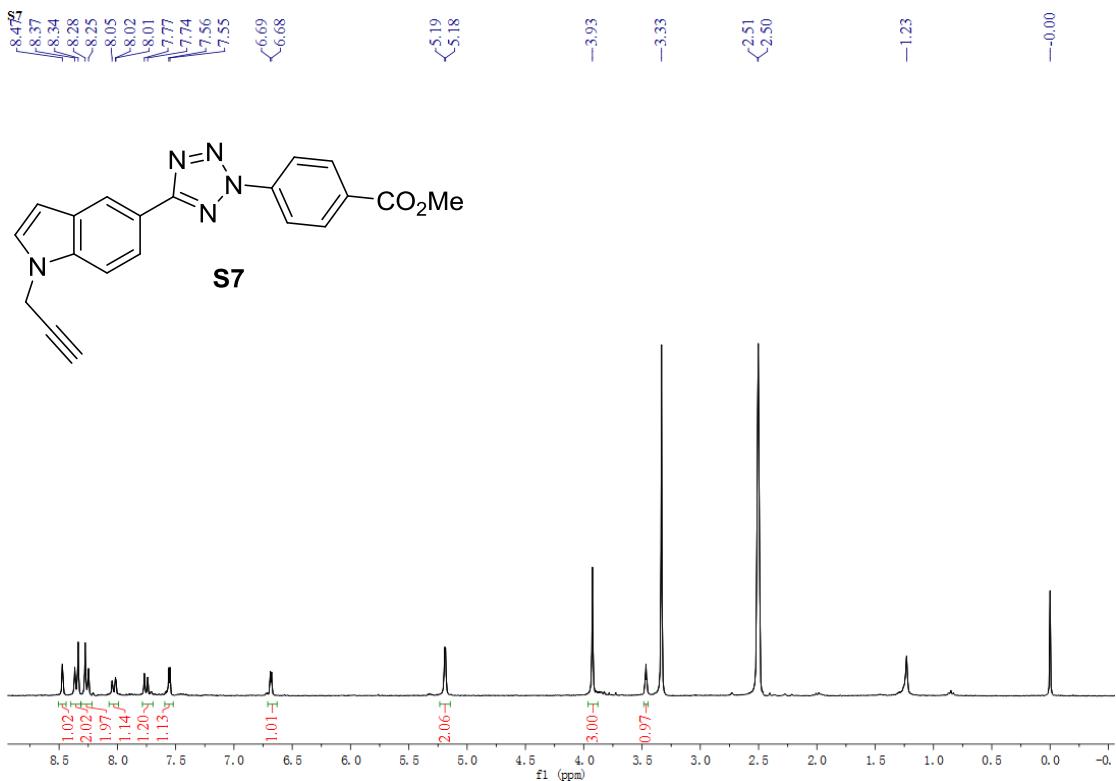


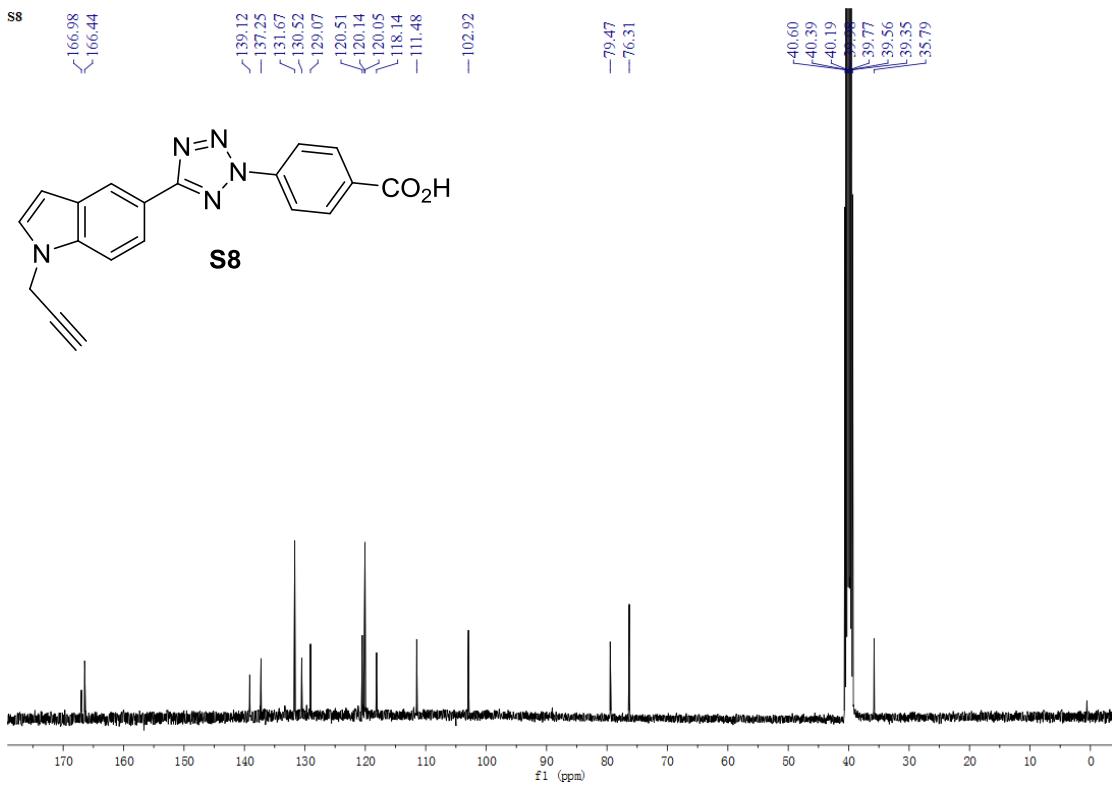
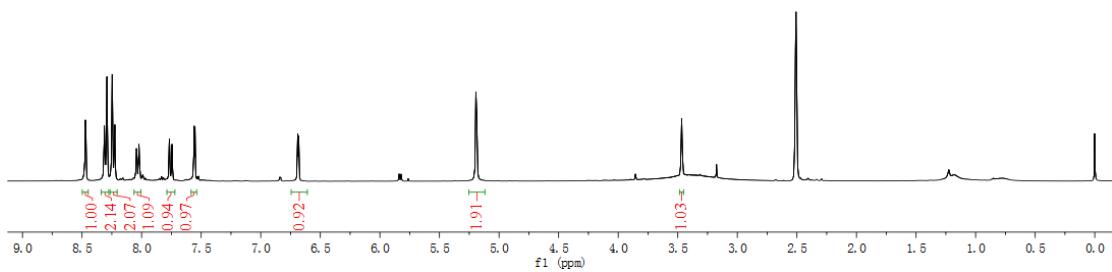
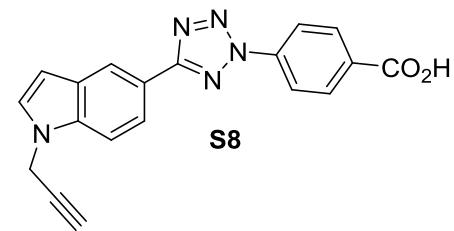
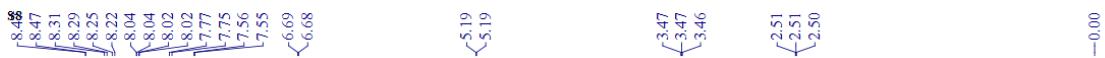


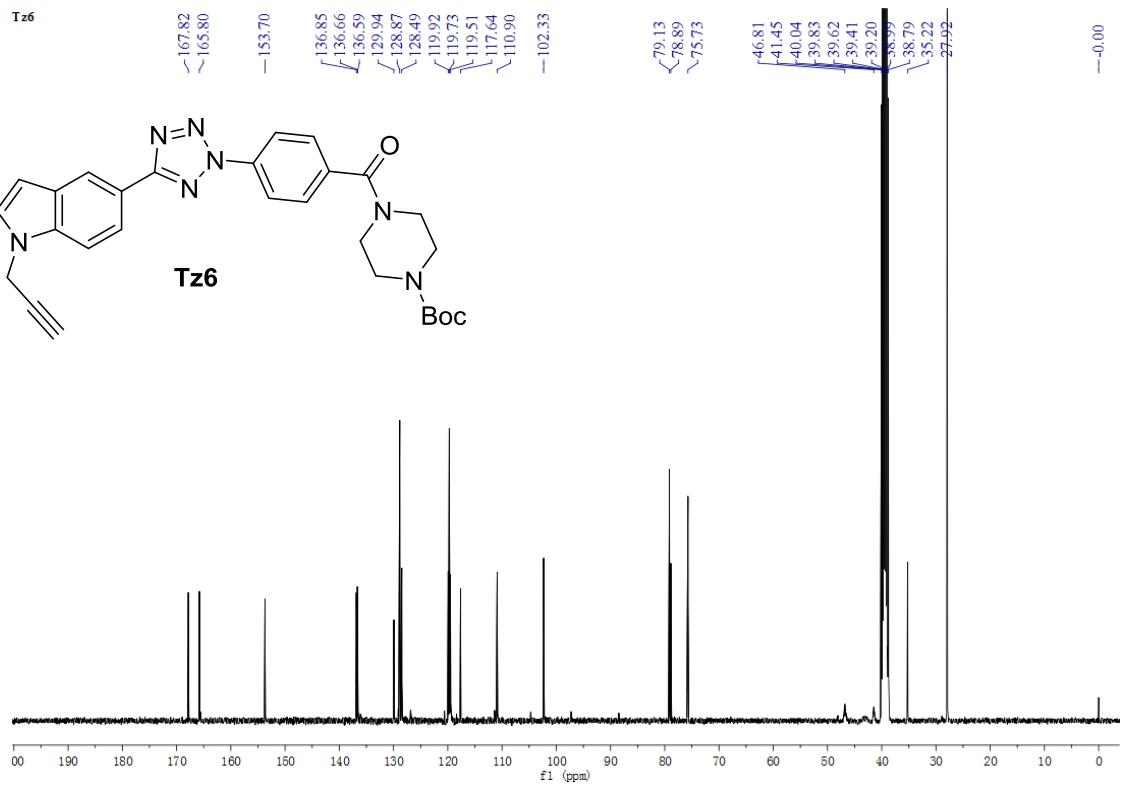
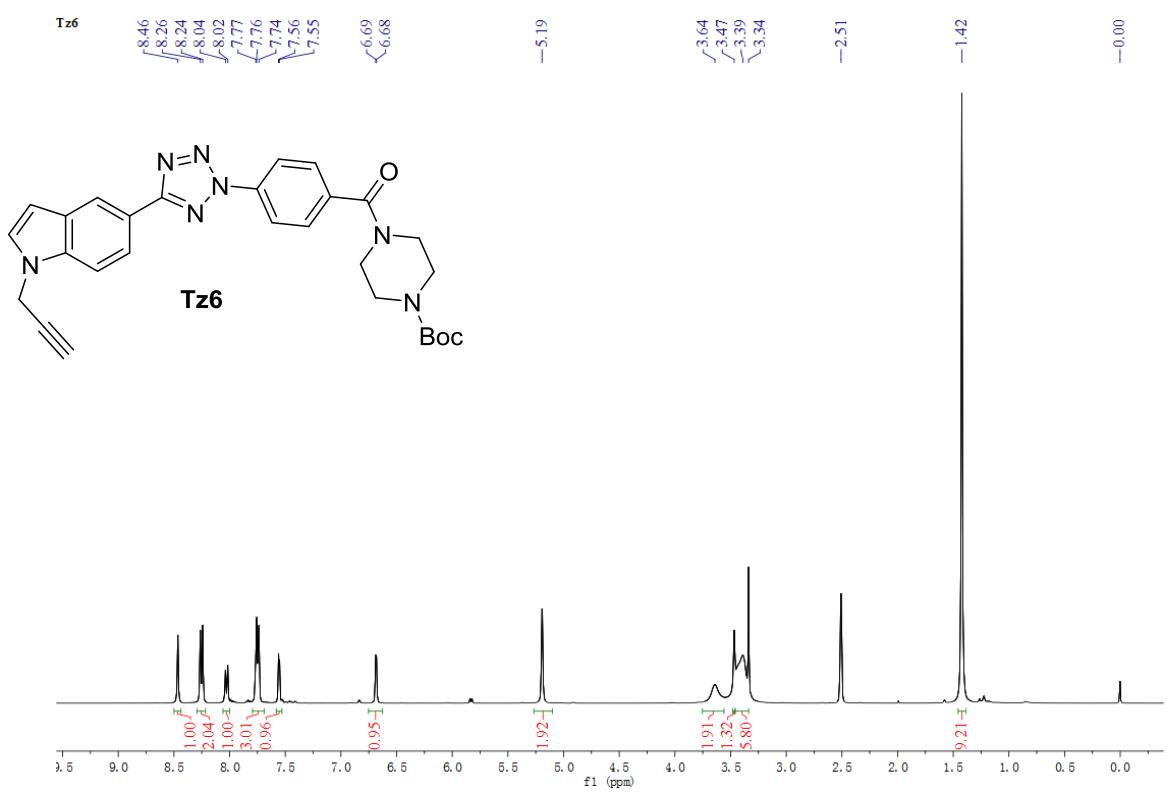


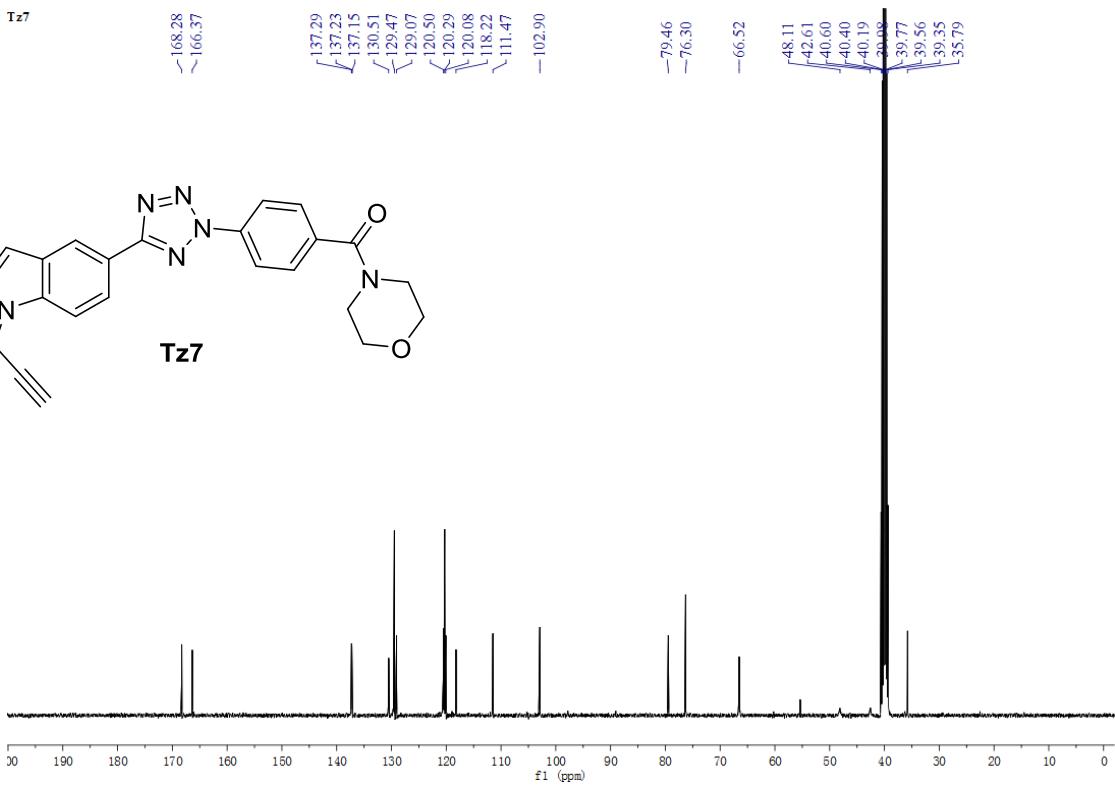
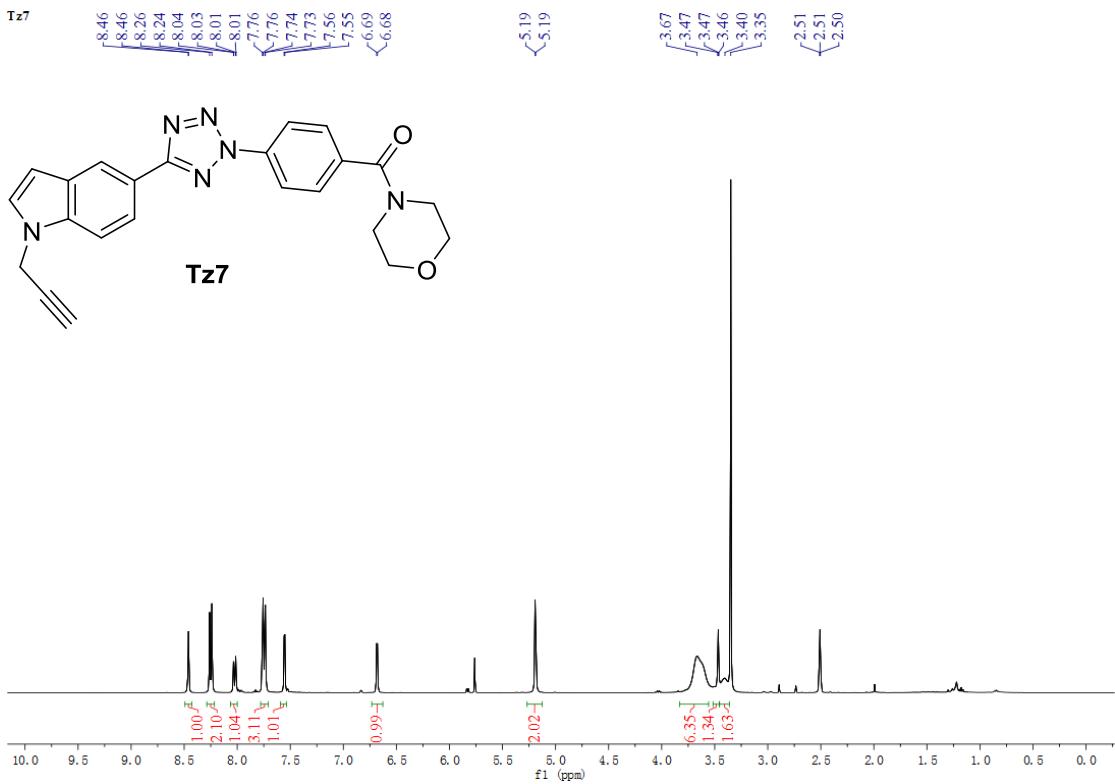












Tz8

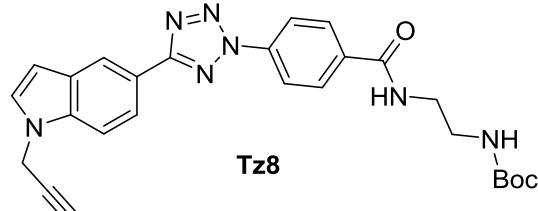
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-5.19

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-2.50

-1.38



Tz8

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165.58

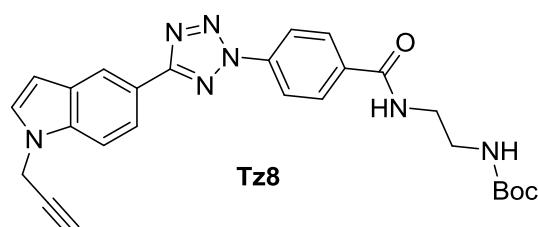
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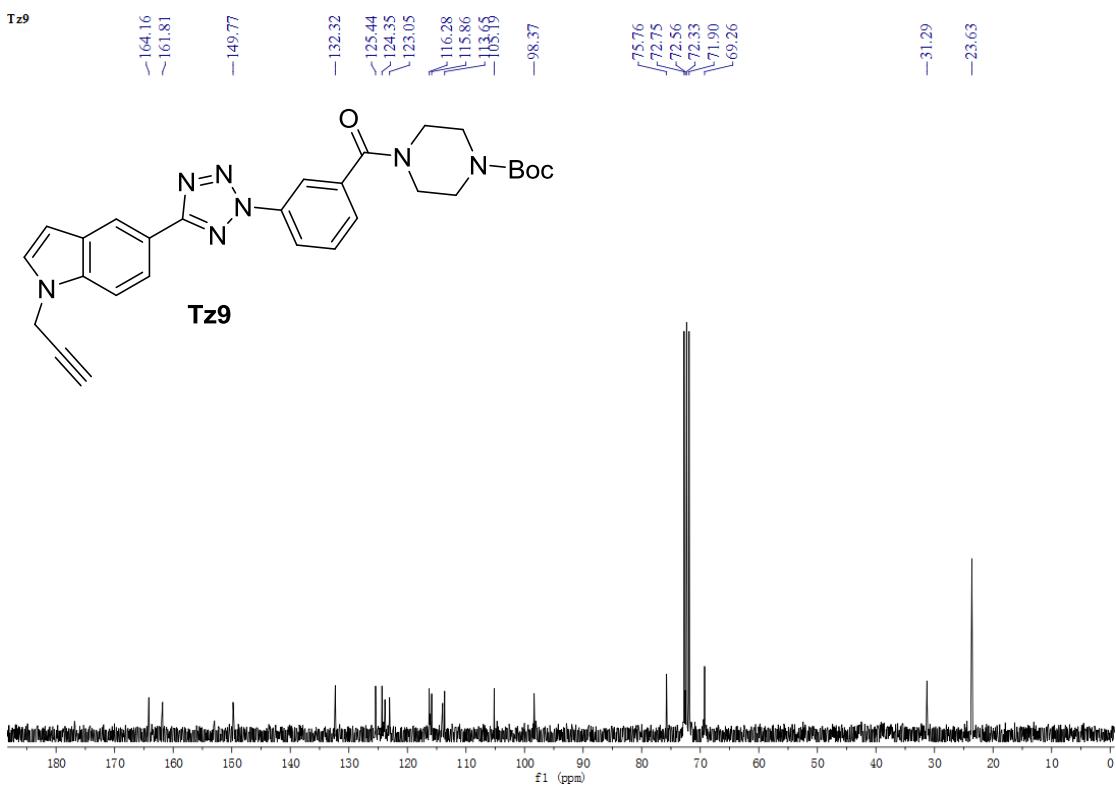
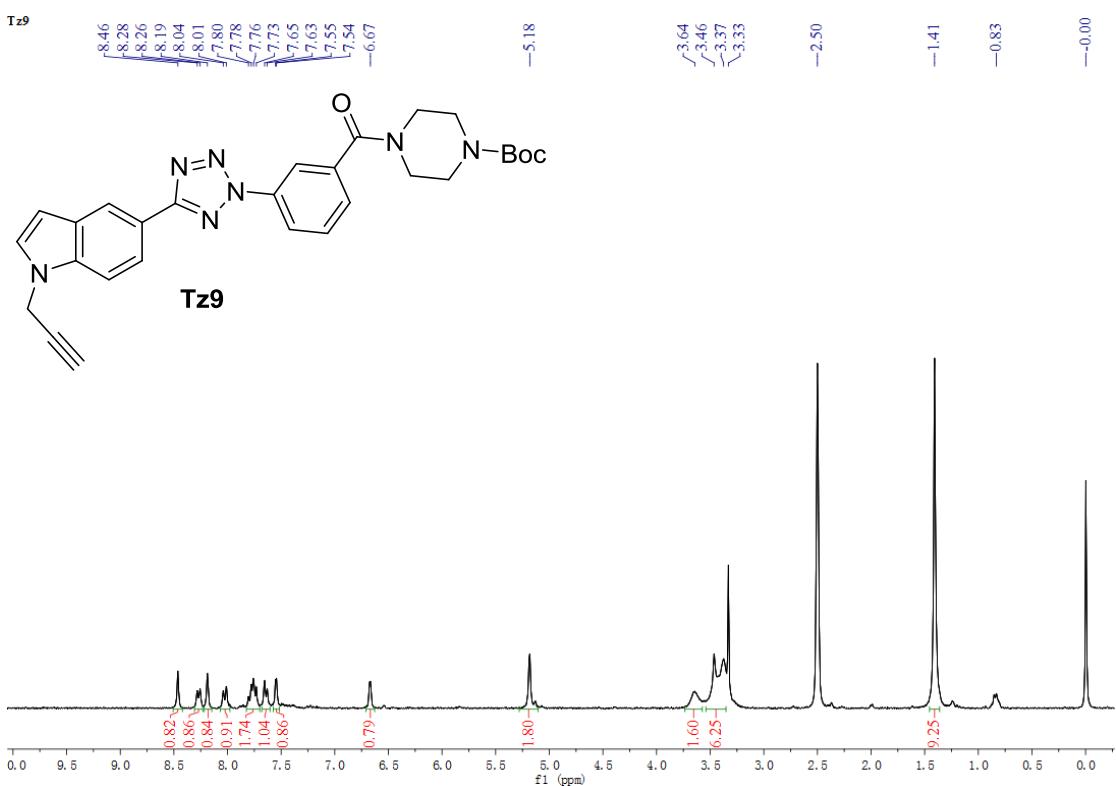
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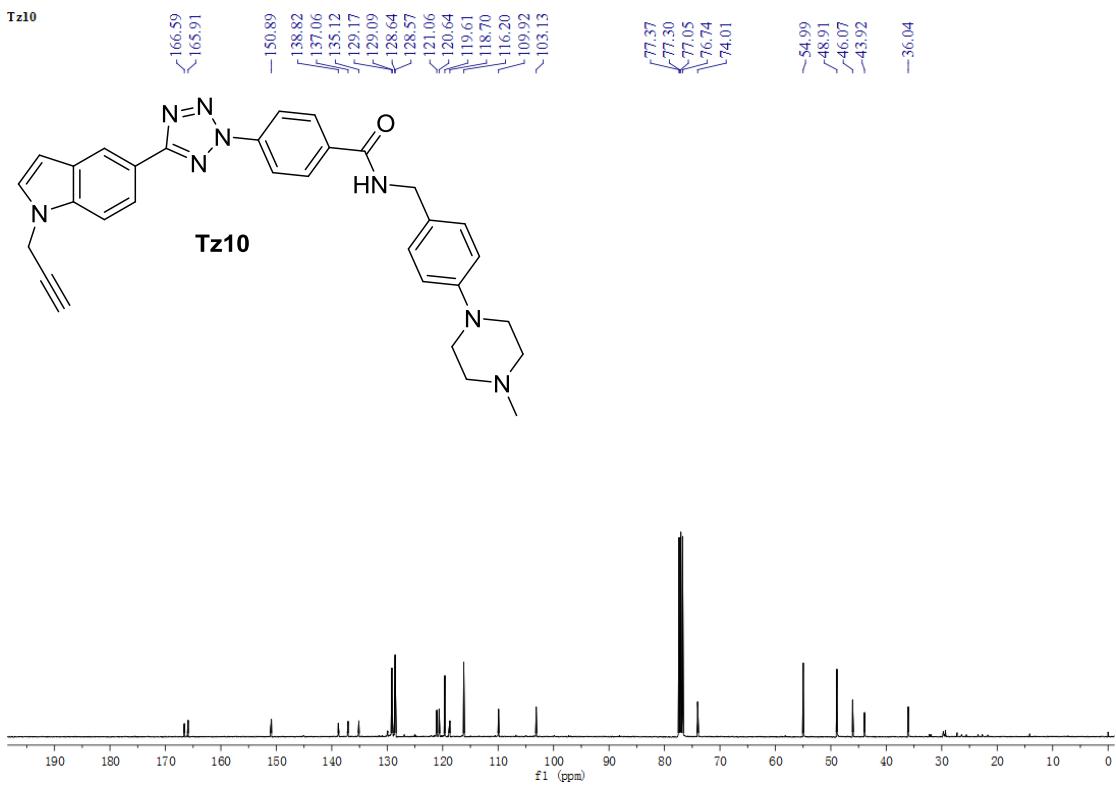
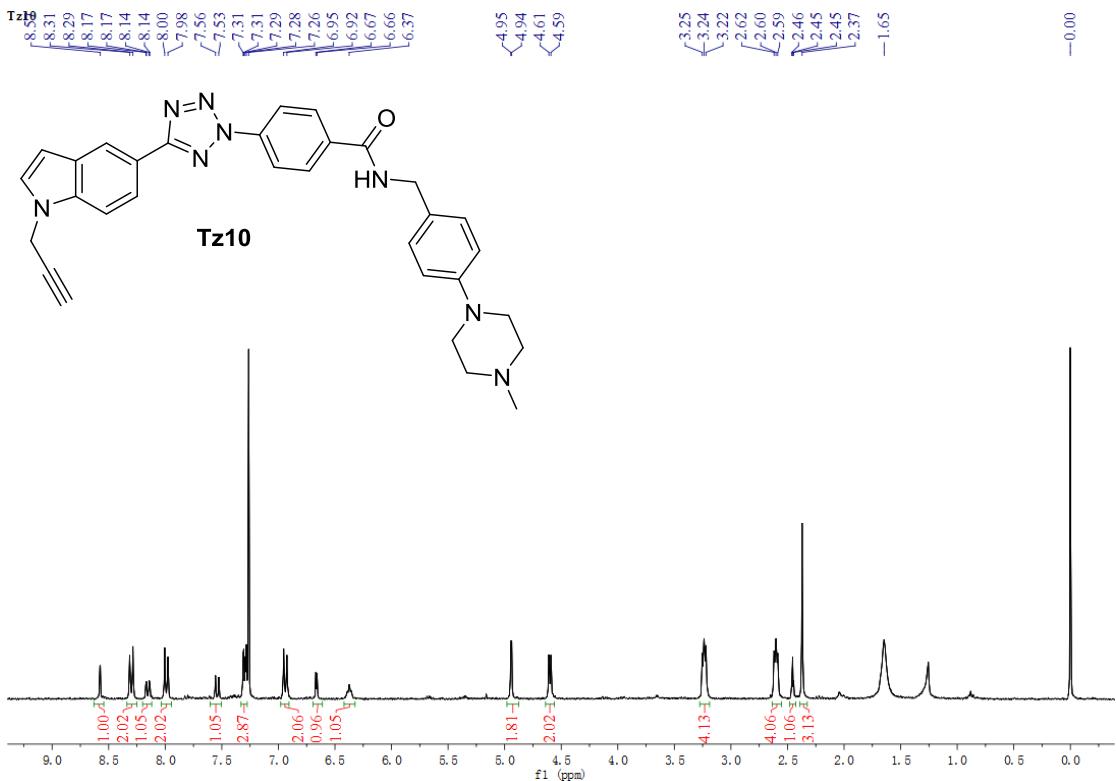
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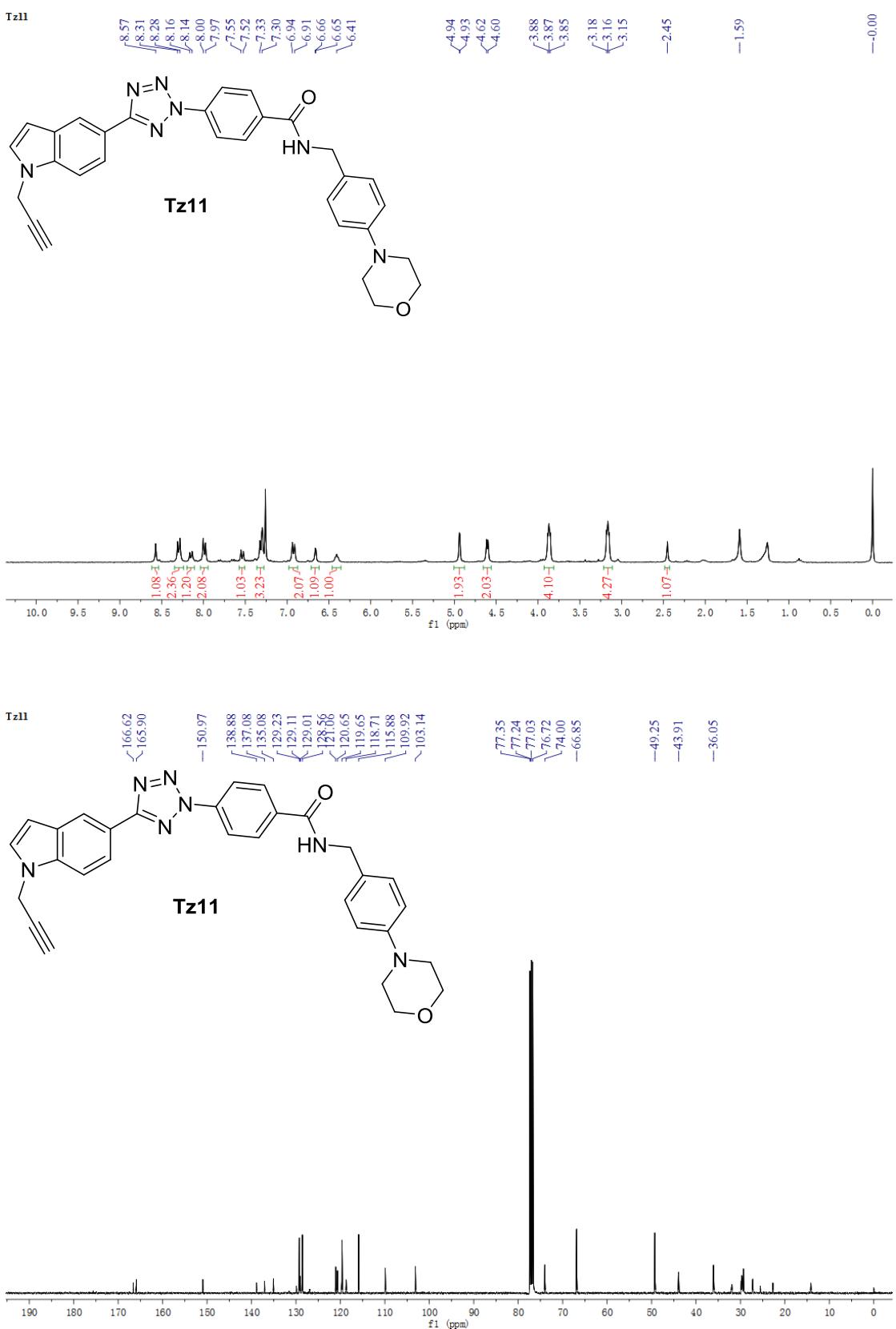
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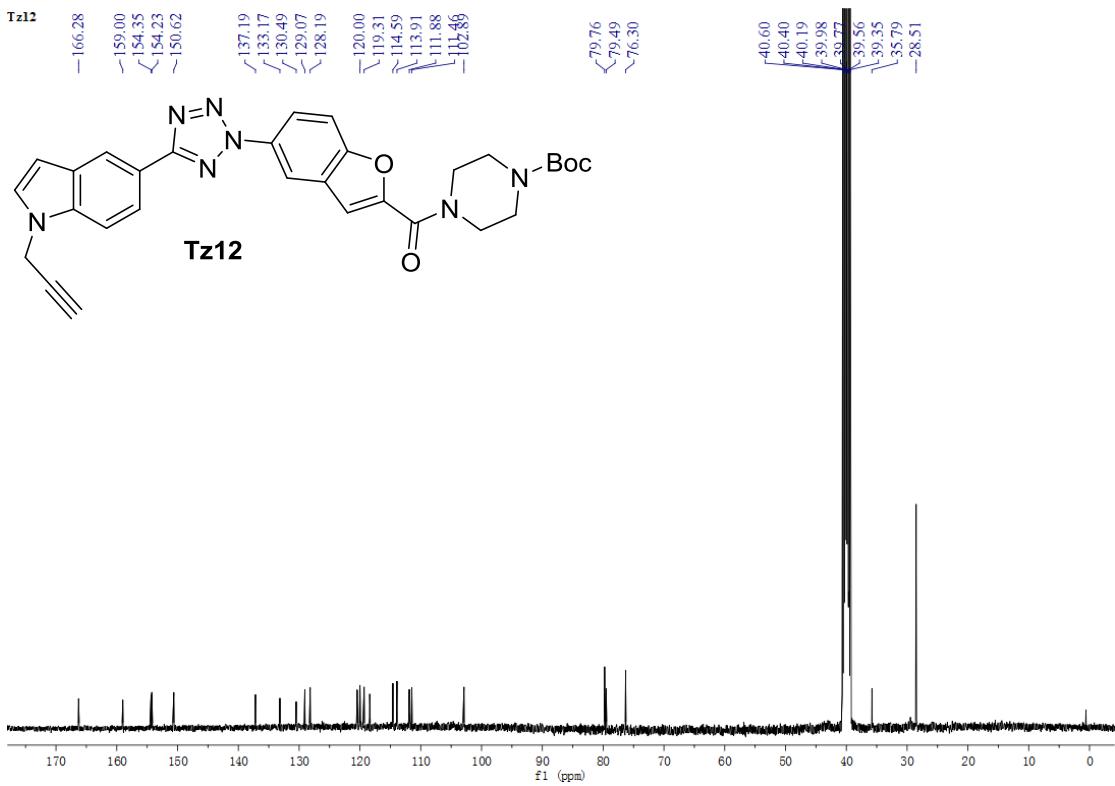
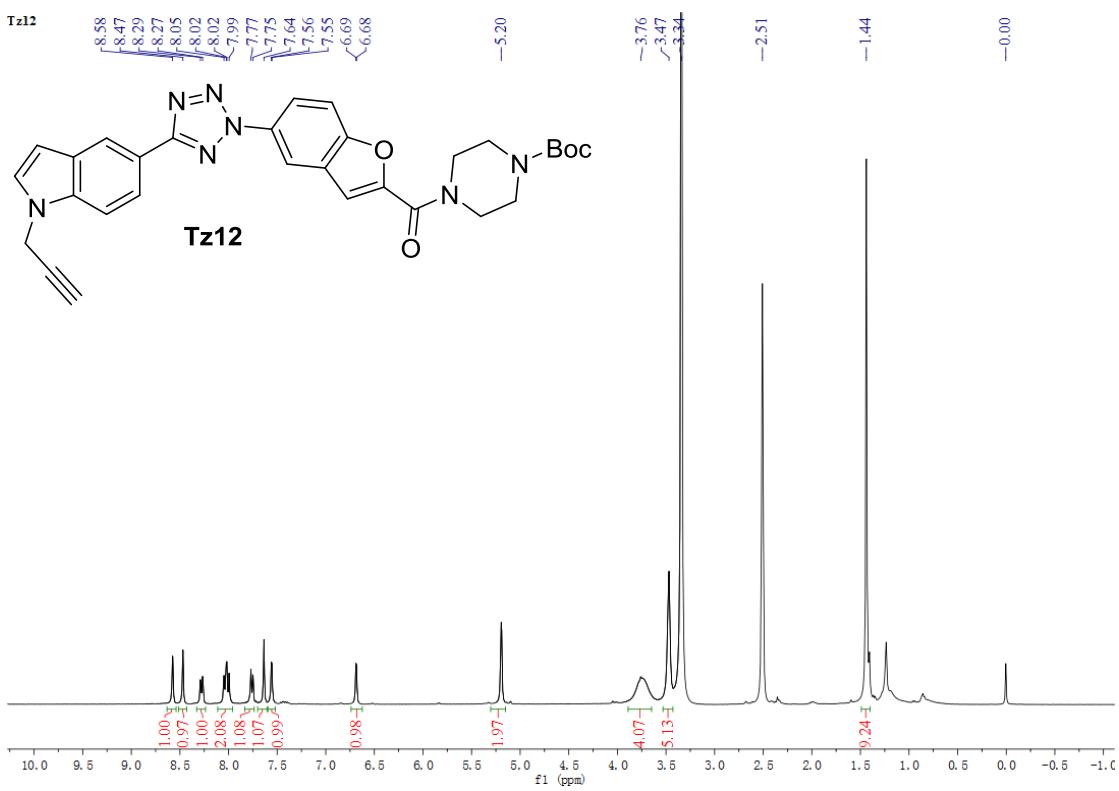
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40.52
40.25
39.97
39.69
39.41
39.13
33.79
-28.71

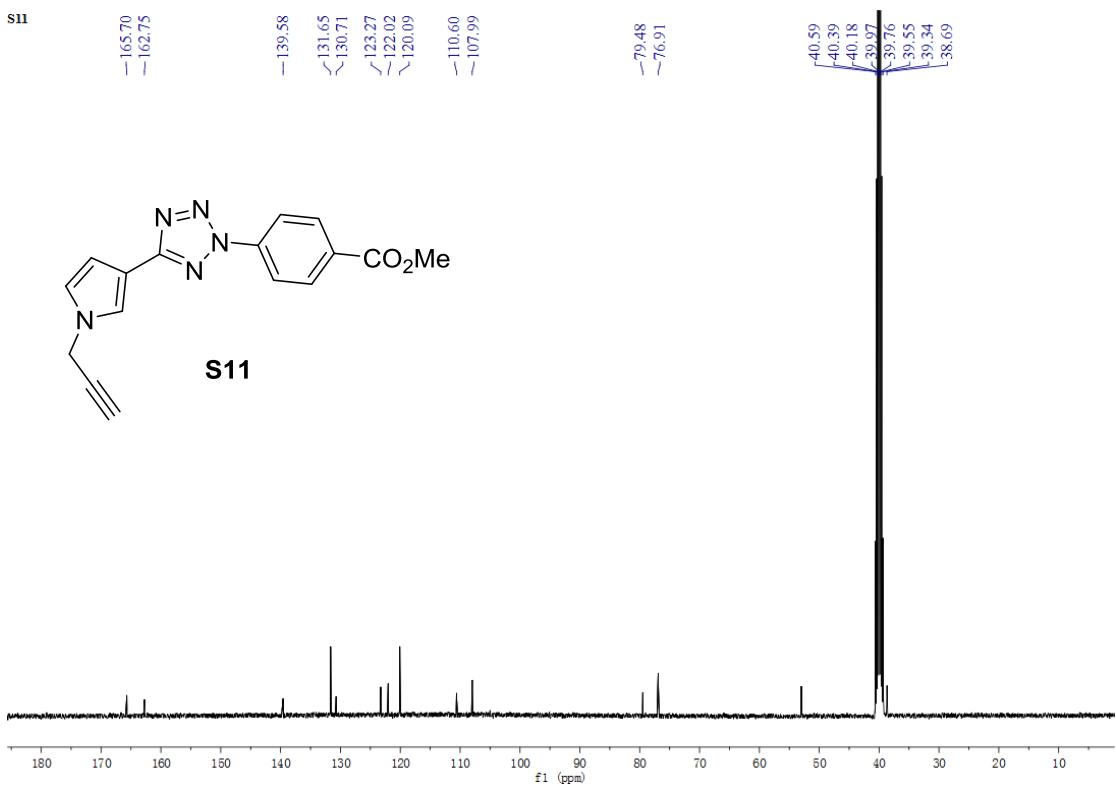
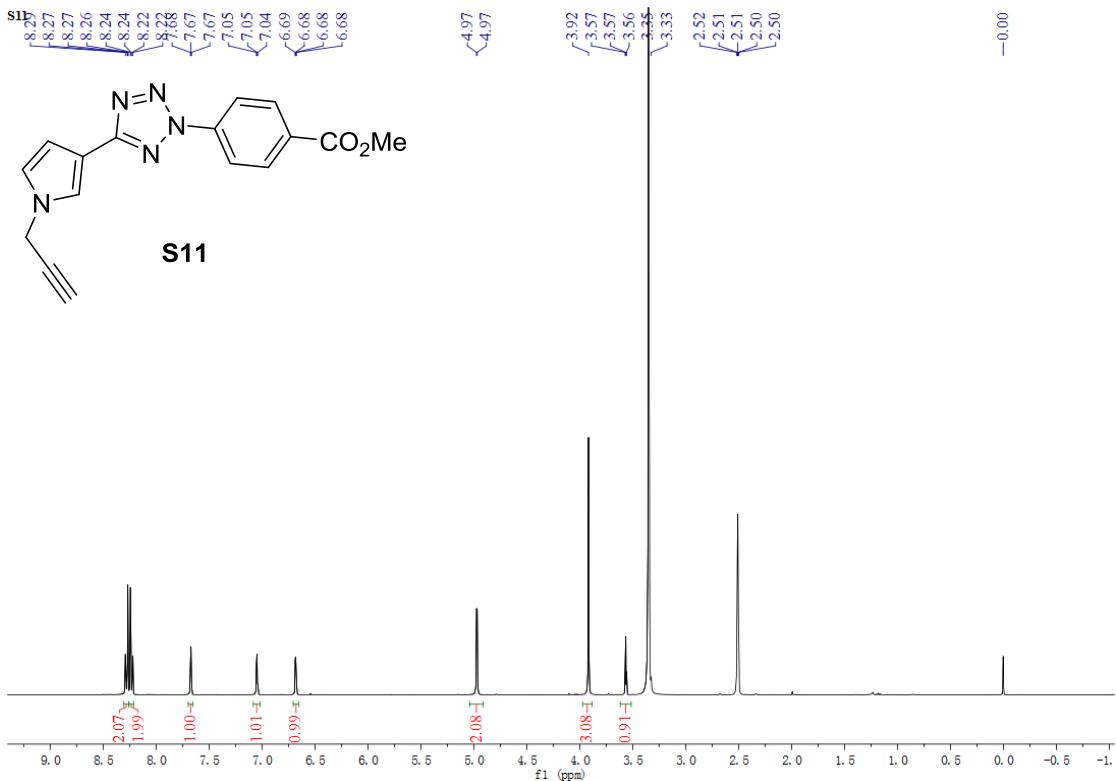




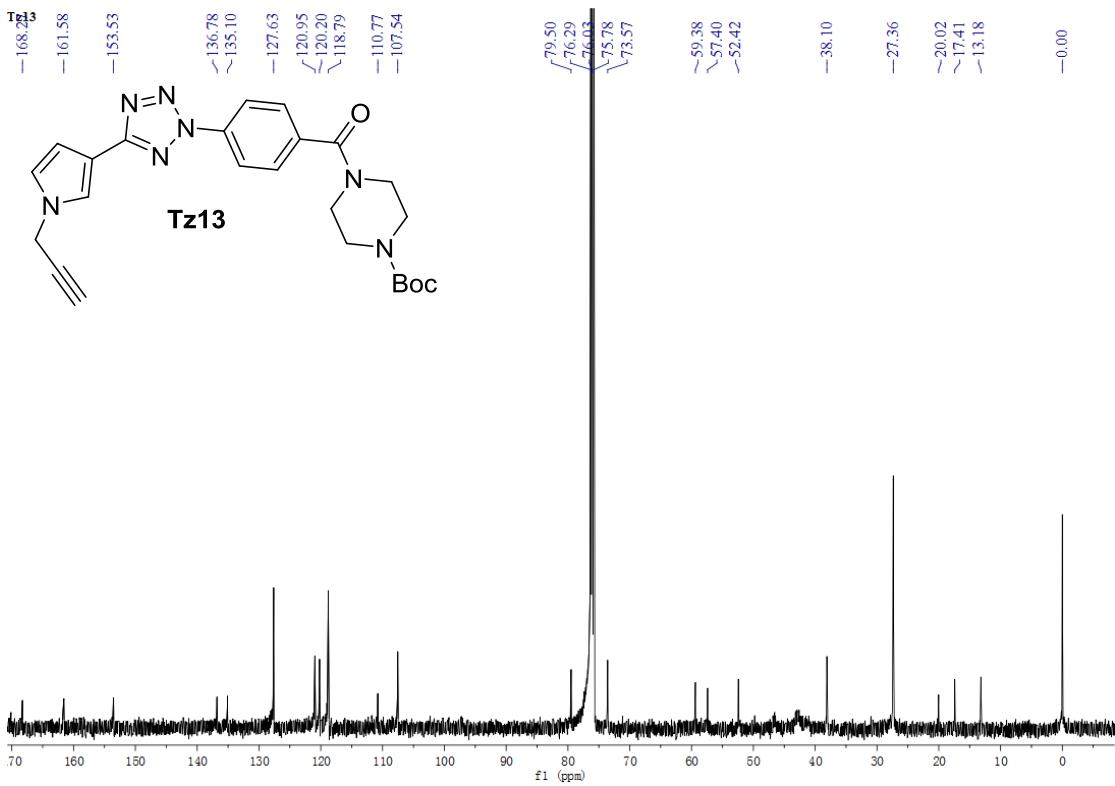
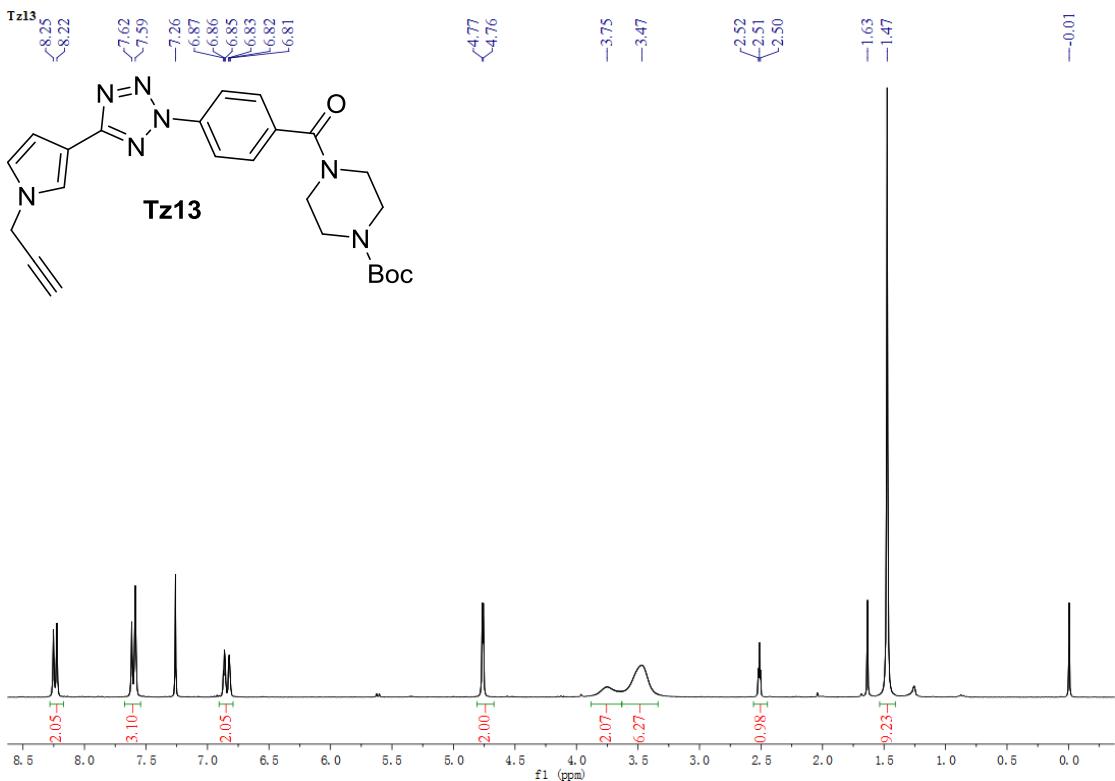




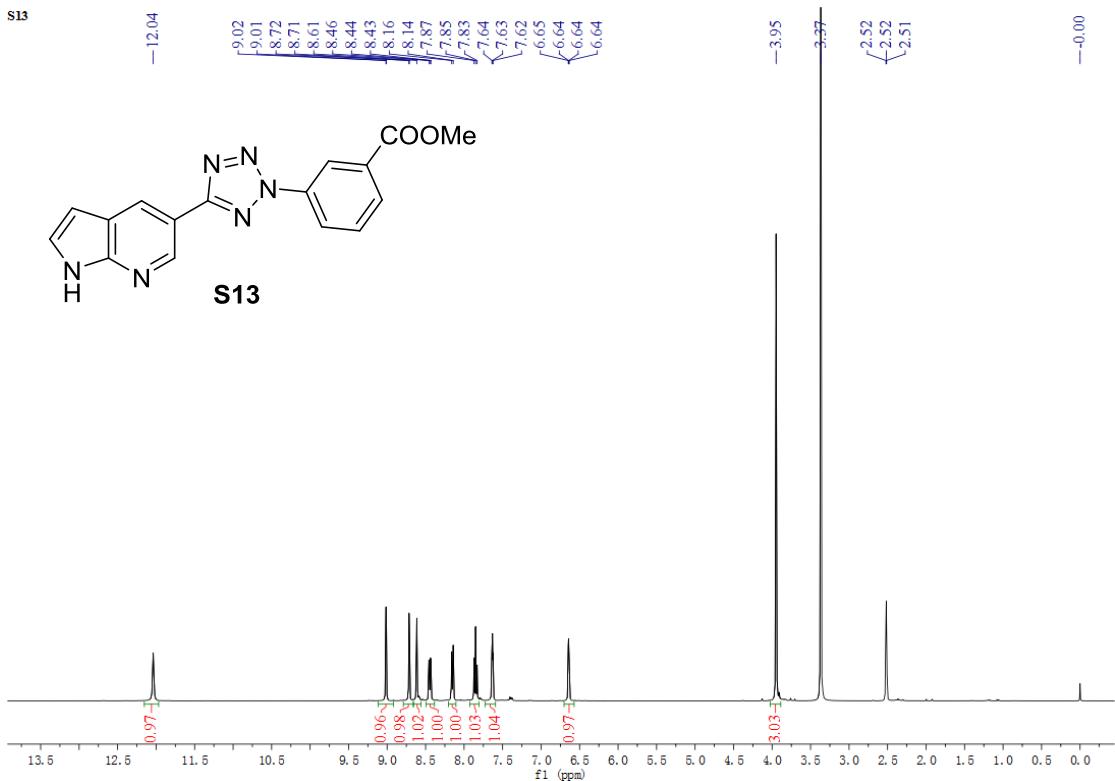




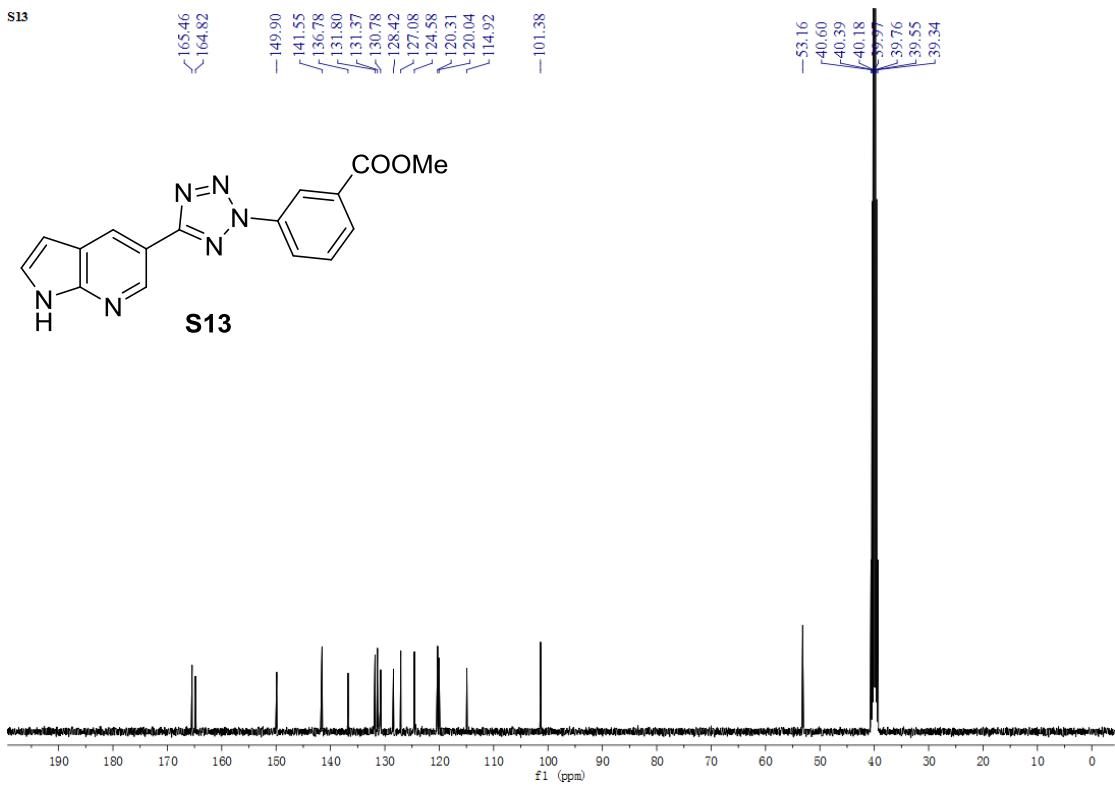
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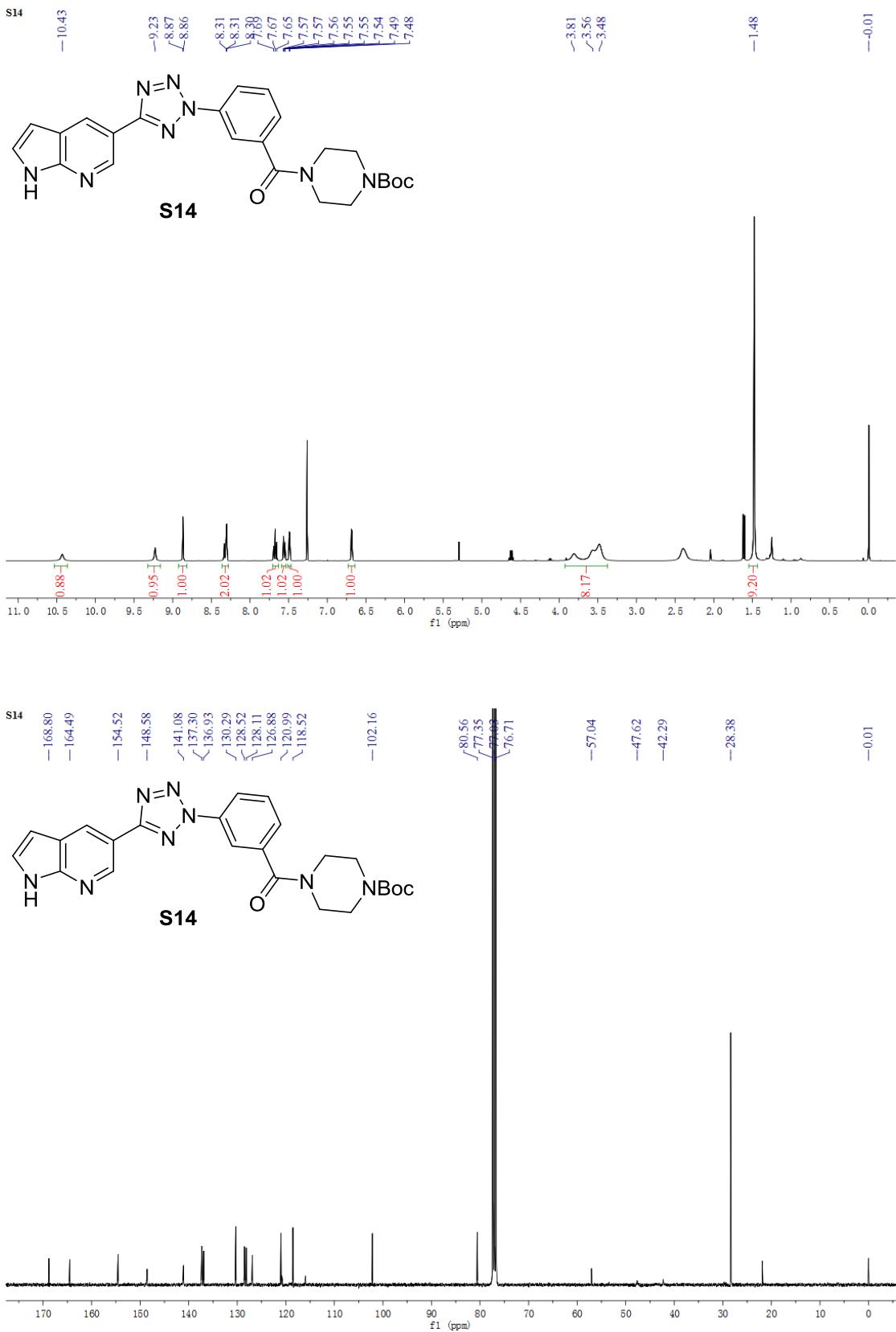


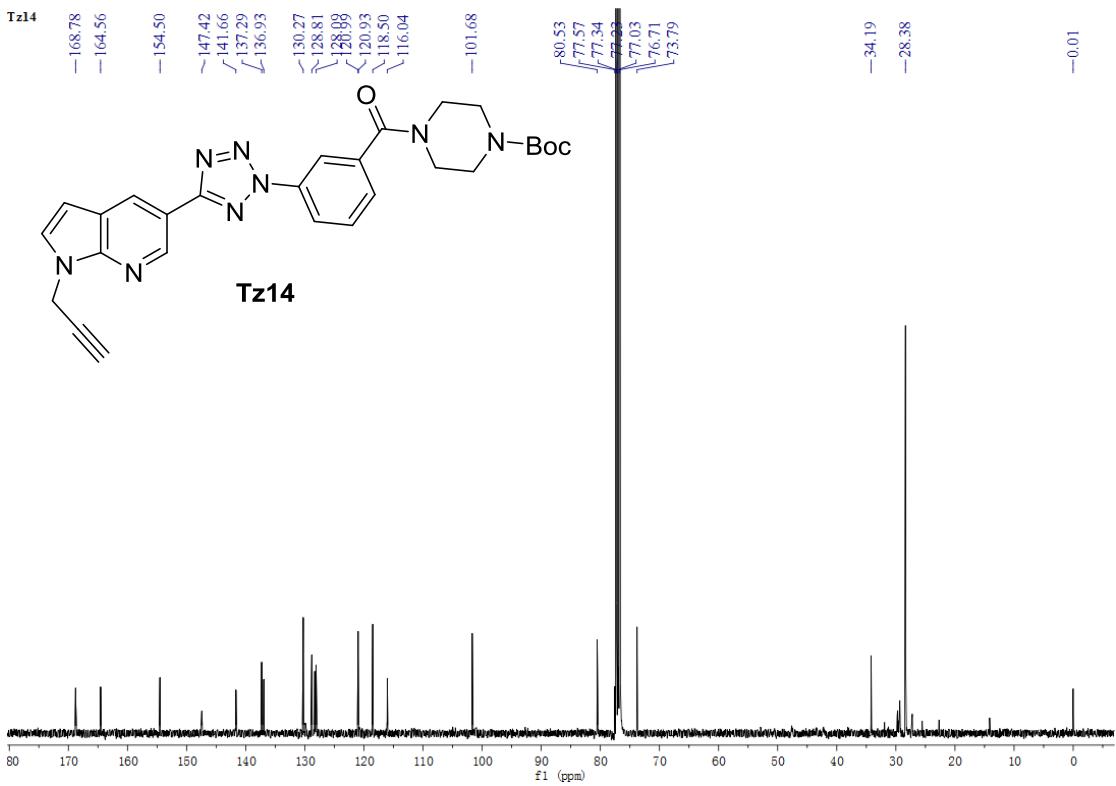
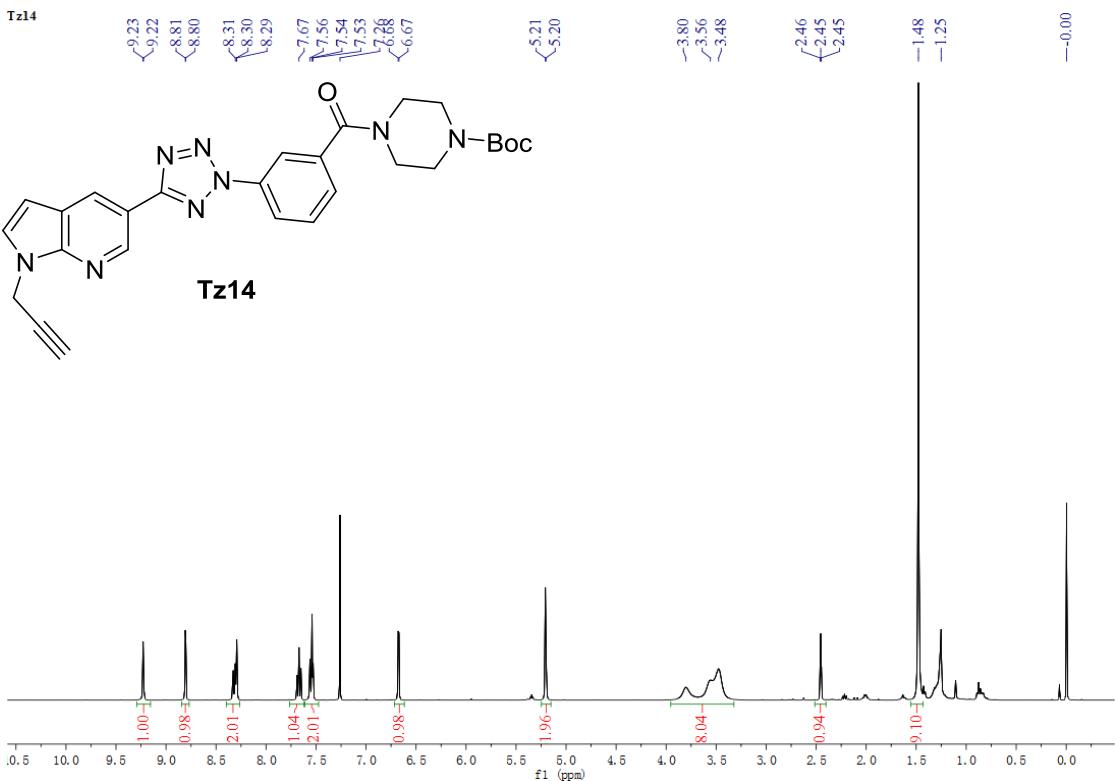
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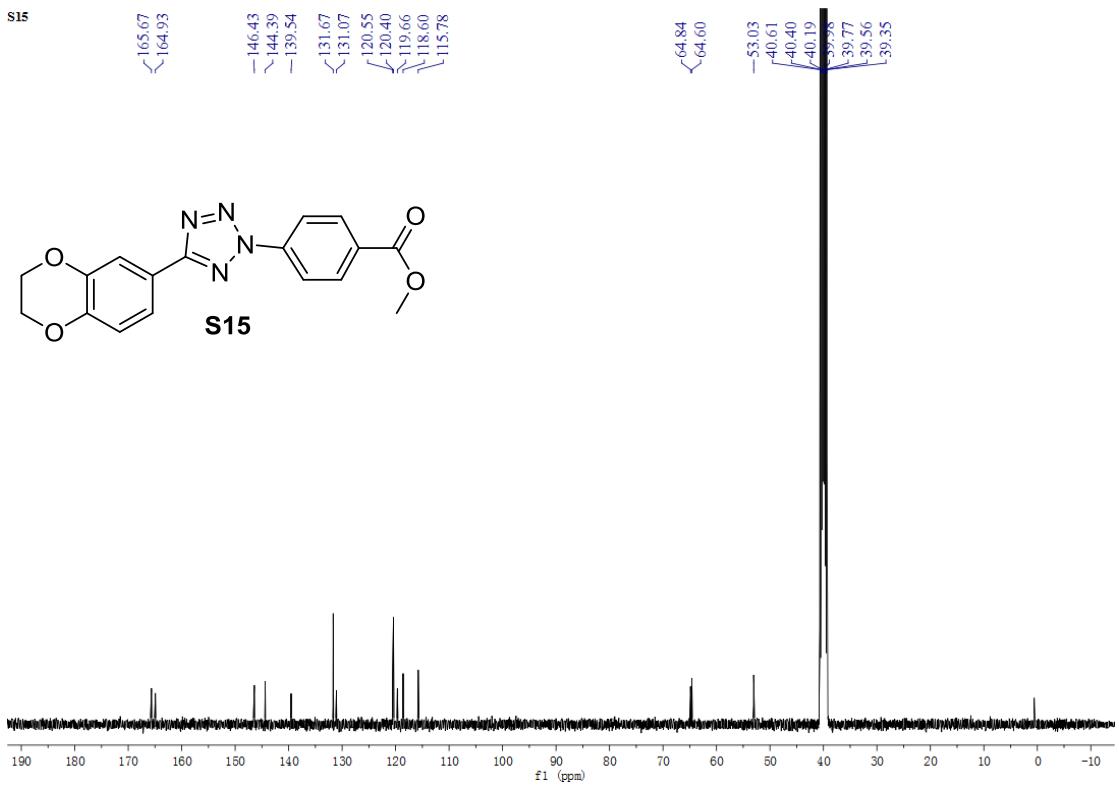
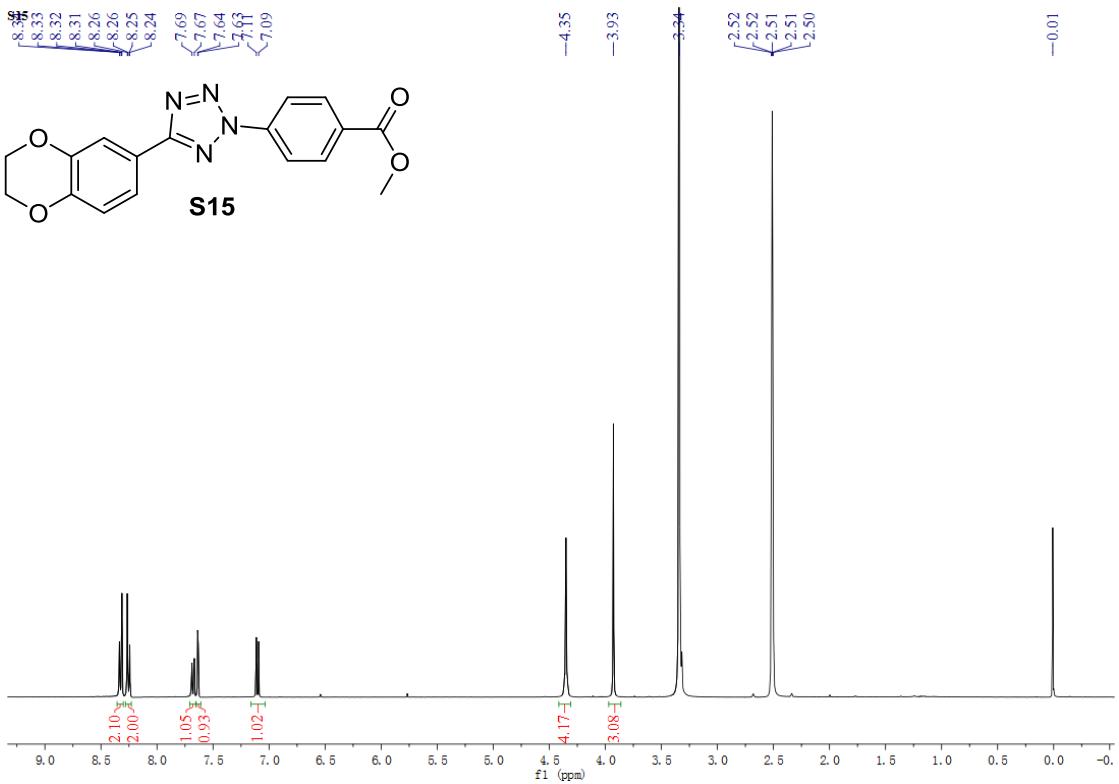


S13



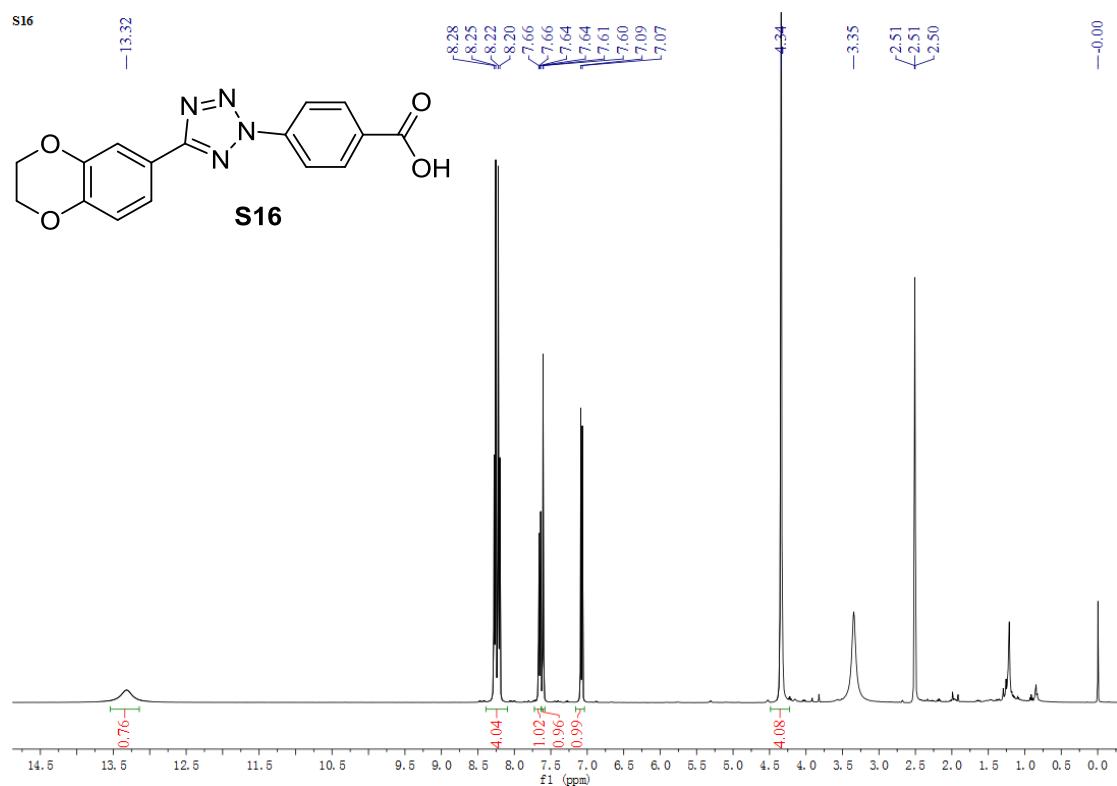




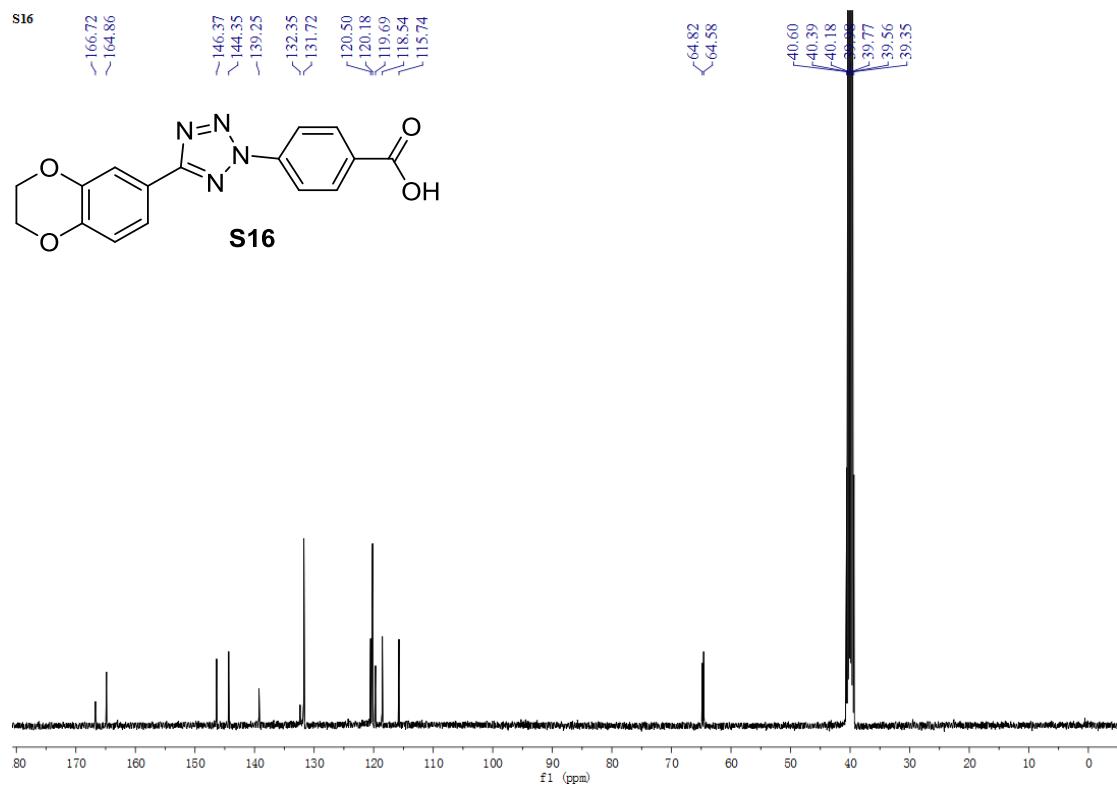


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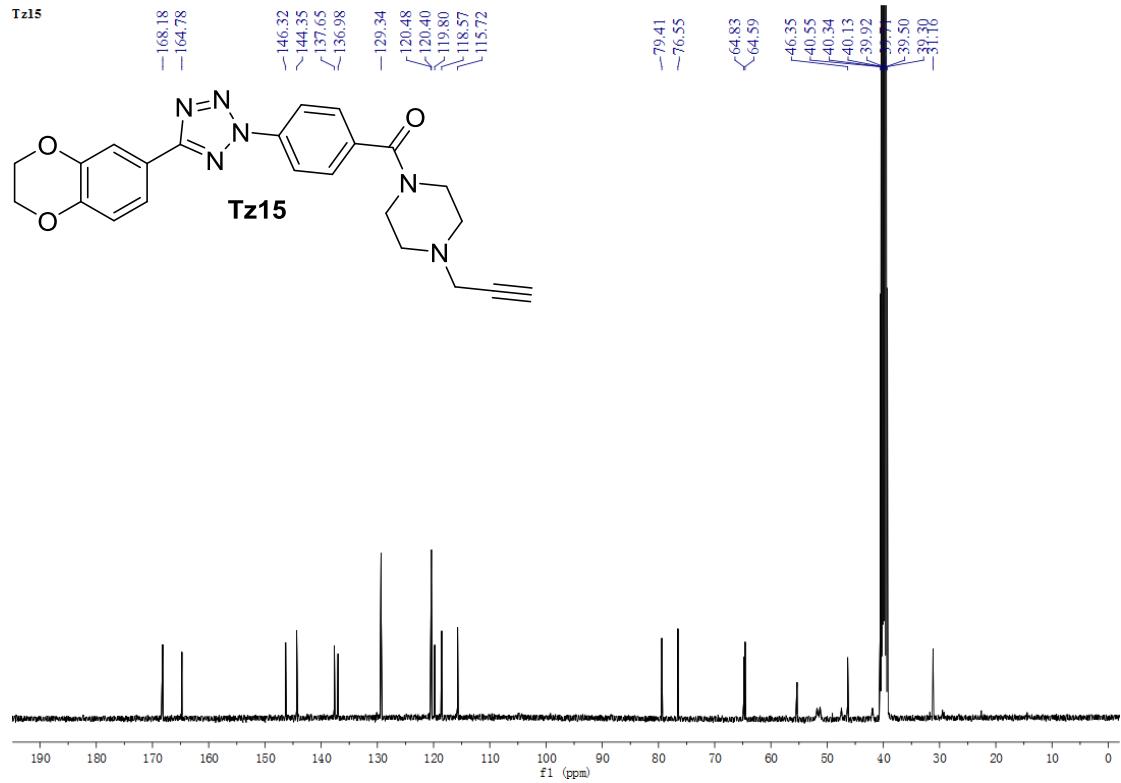
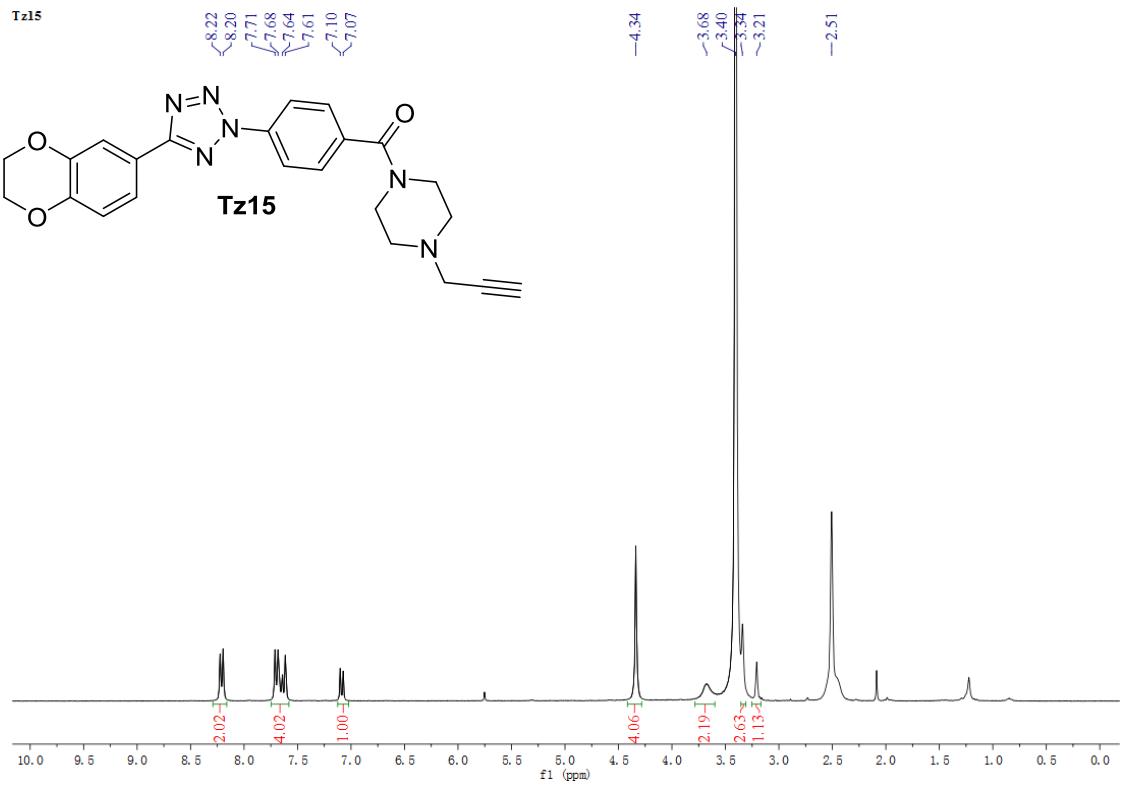
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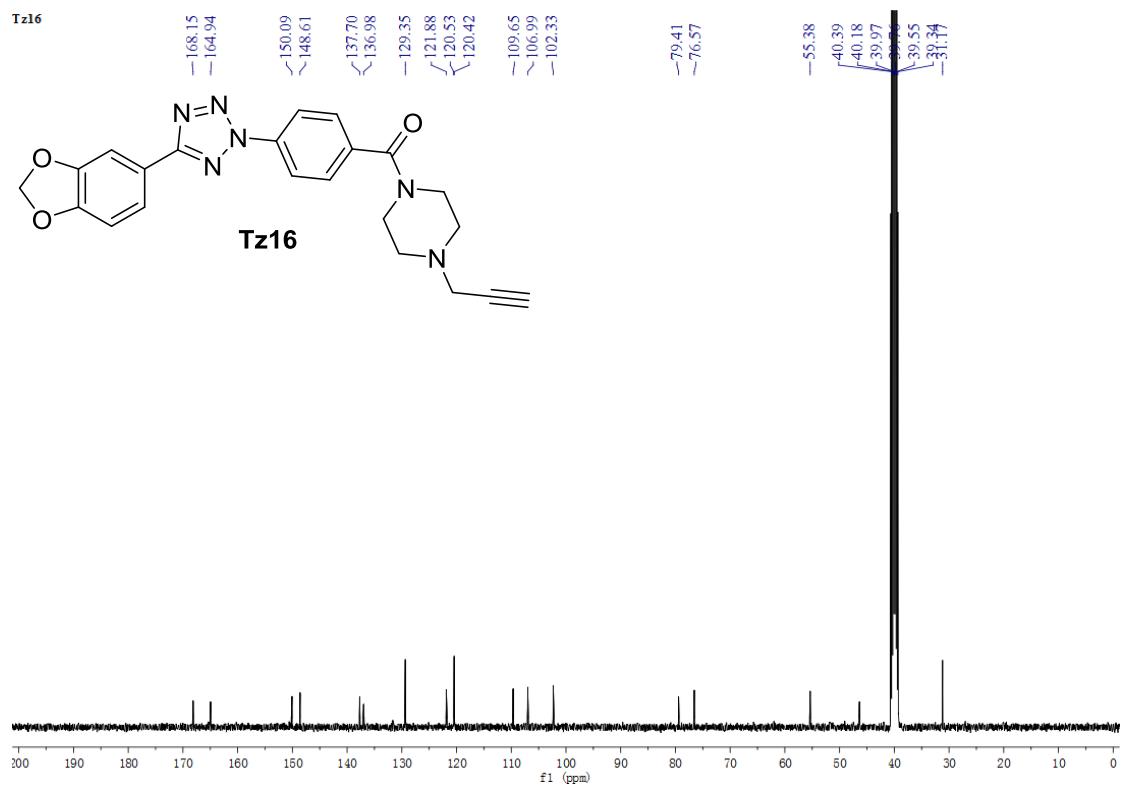
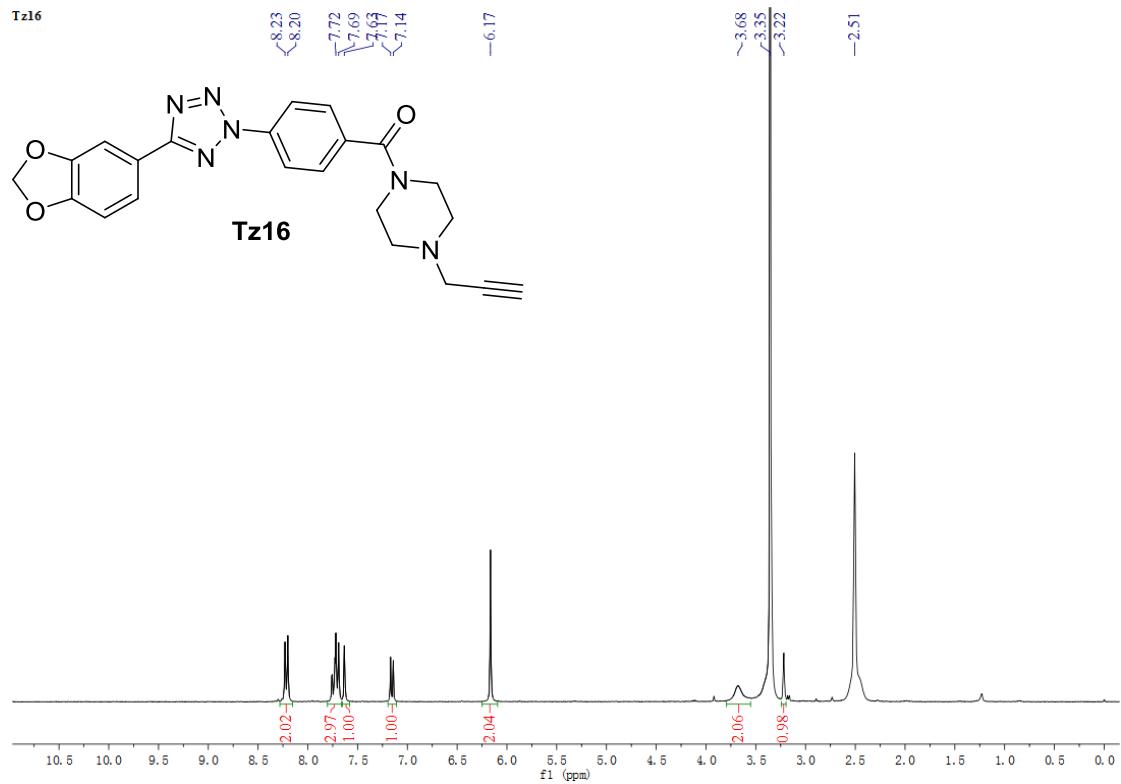


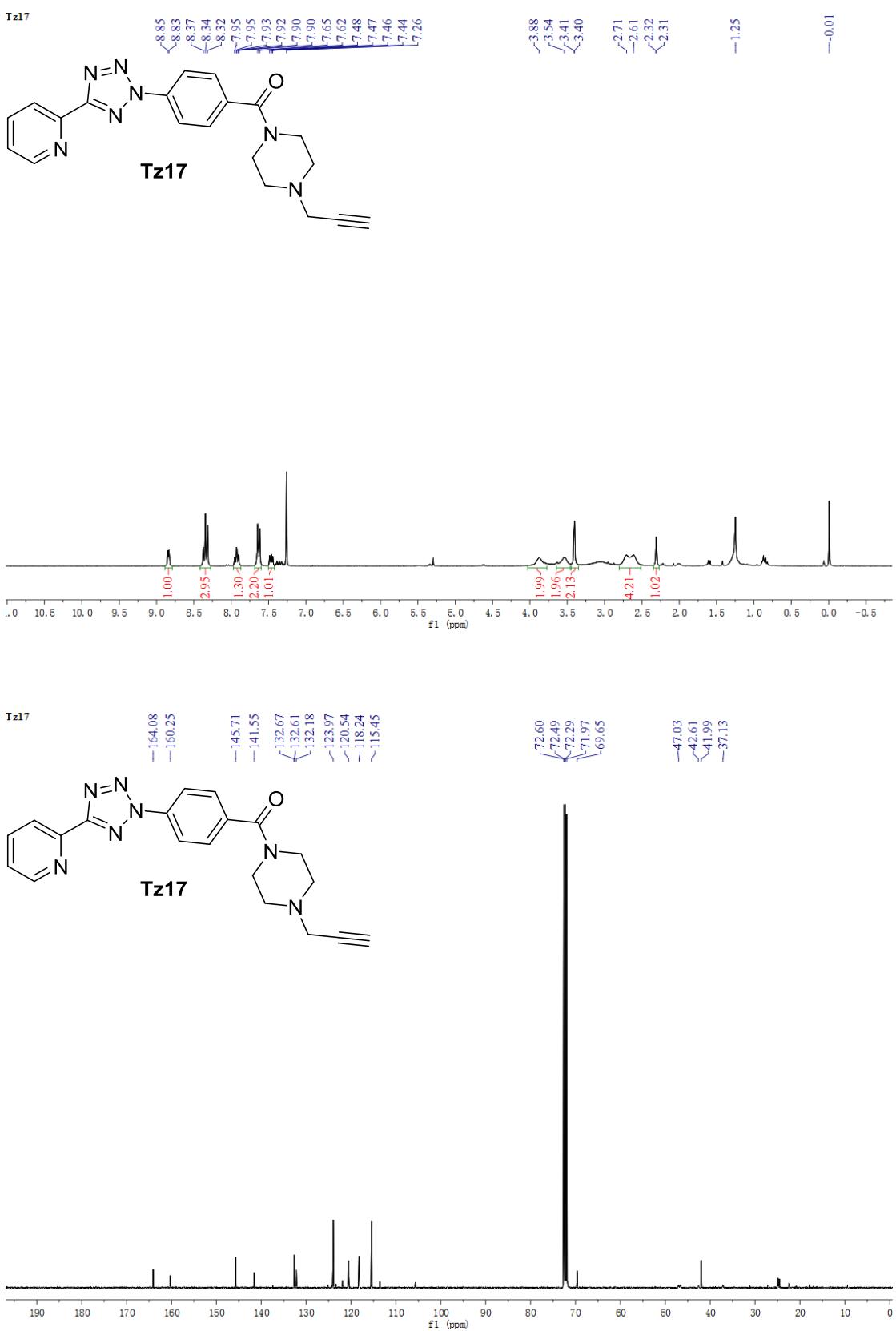
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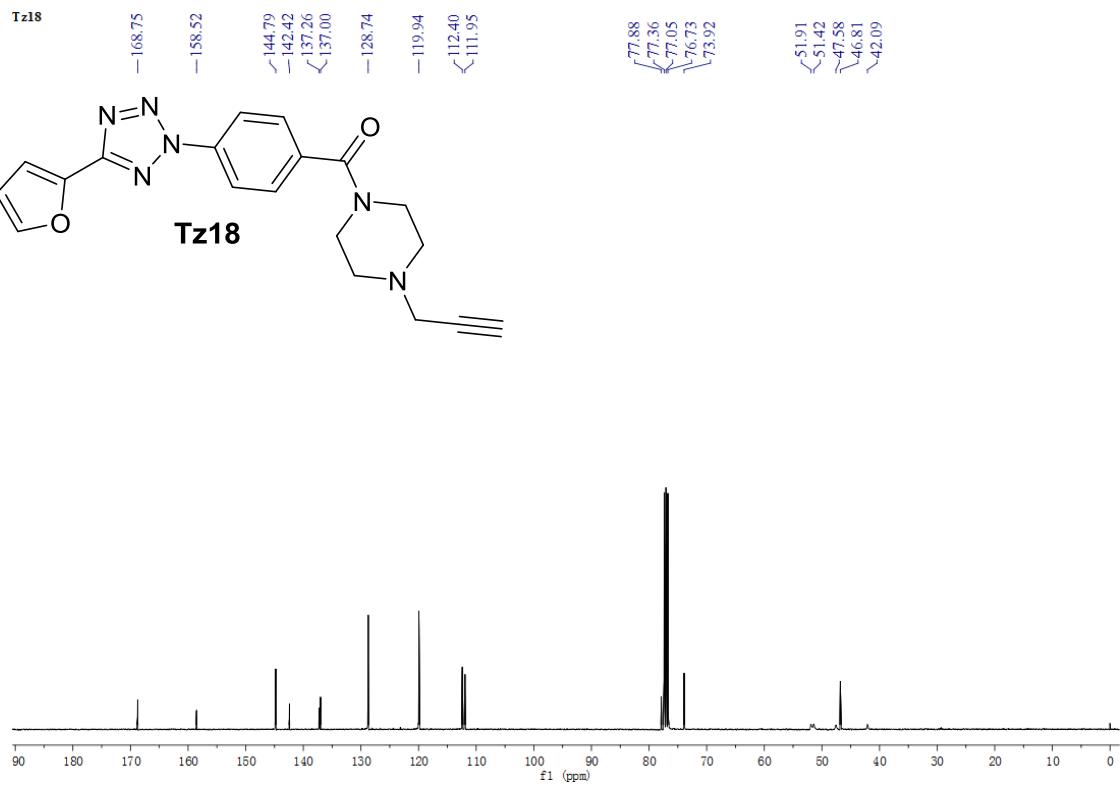
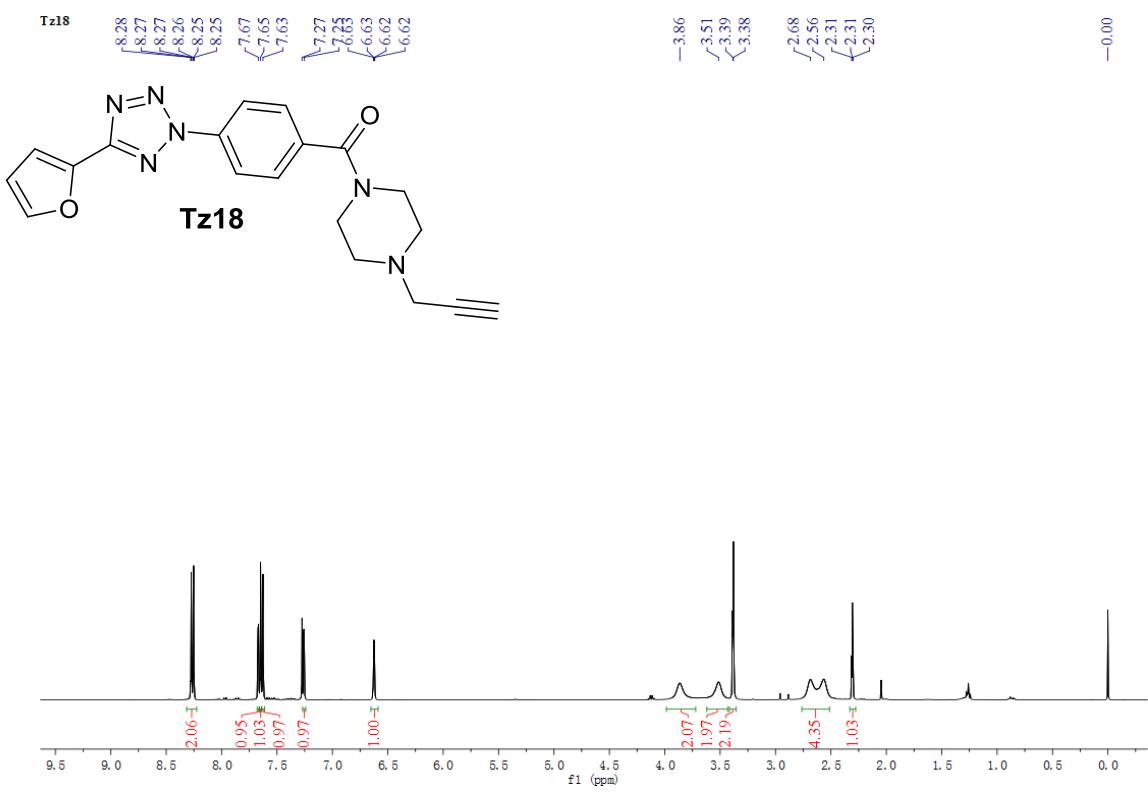


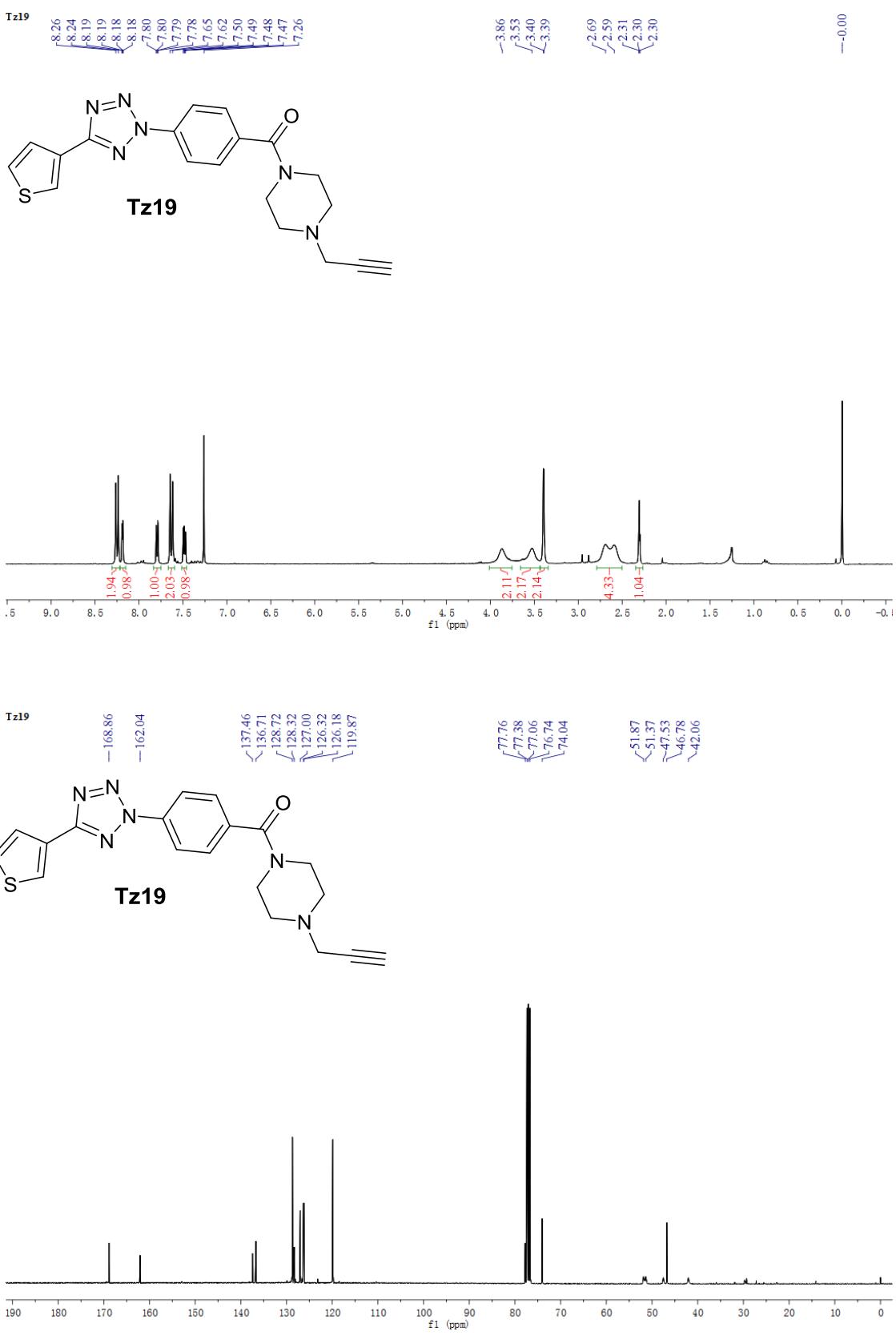
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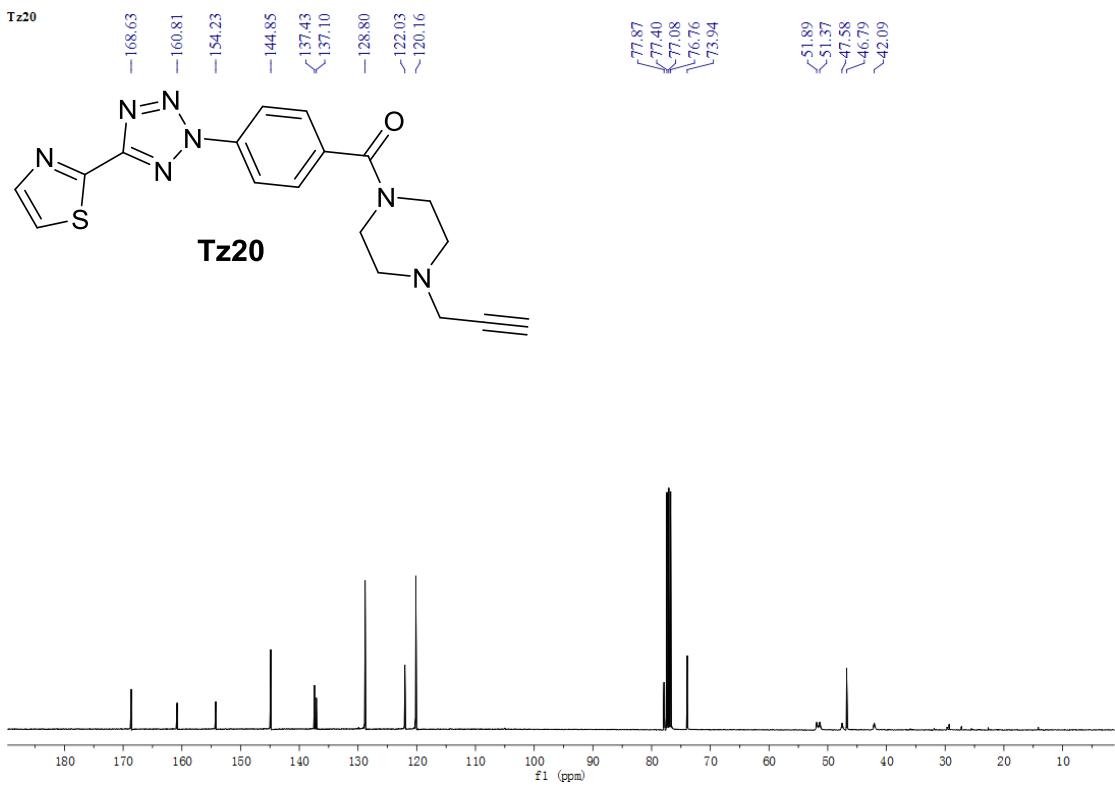
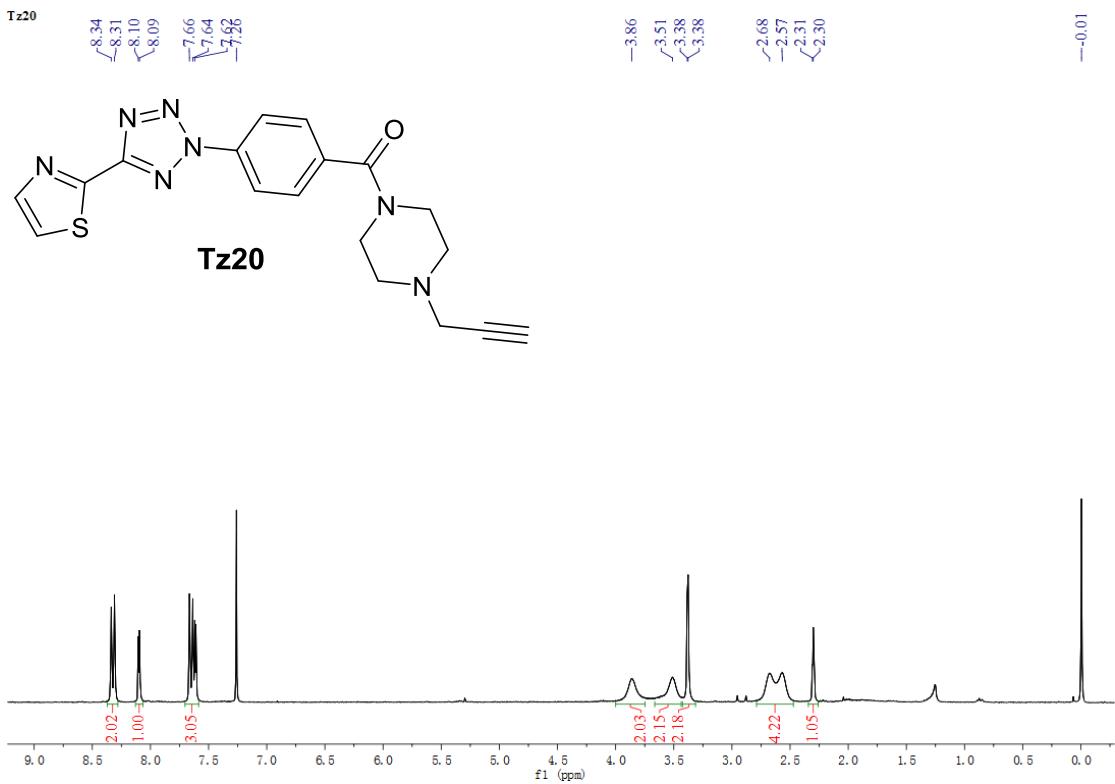




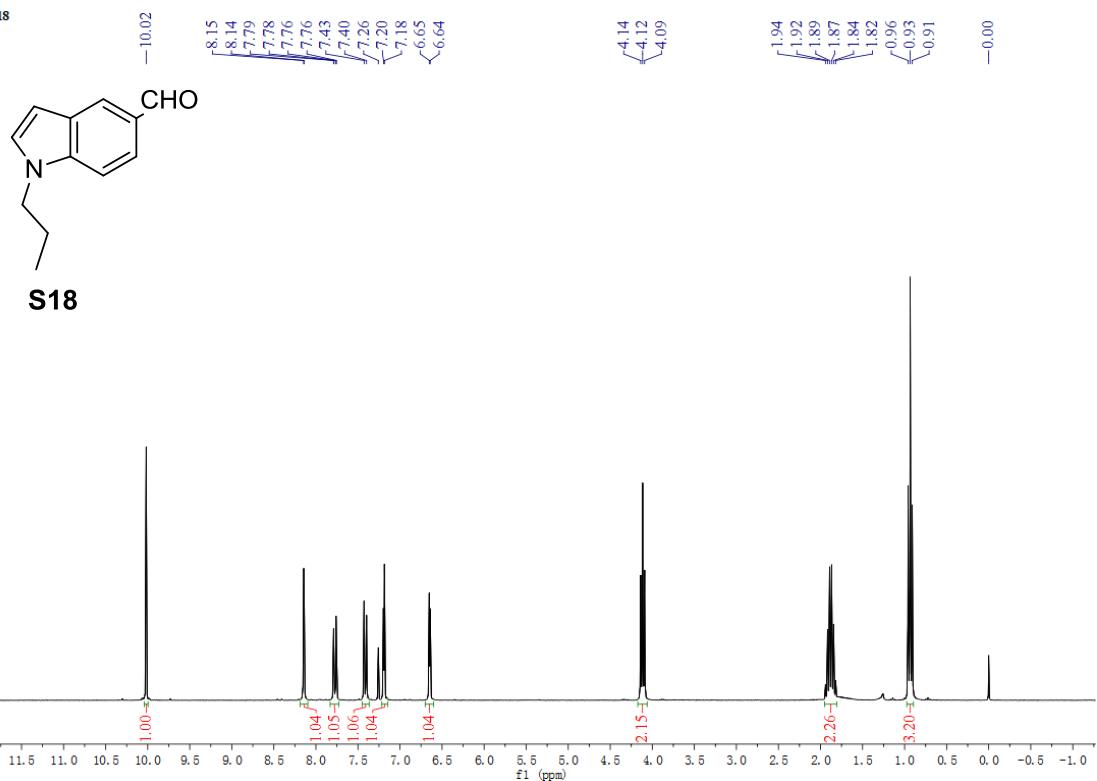


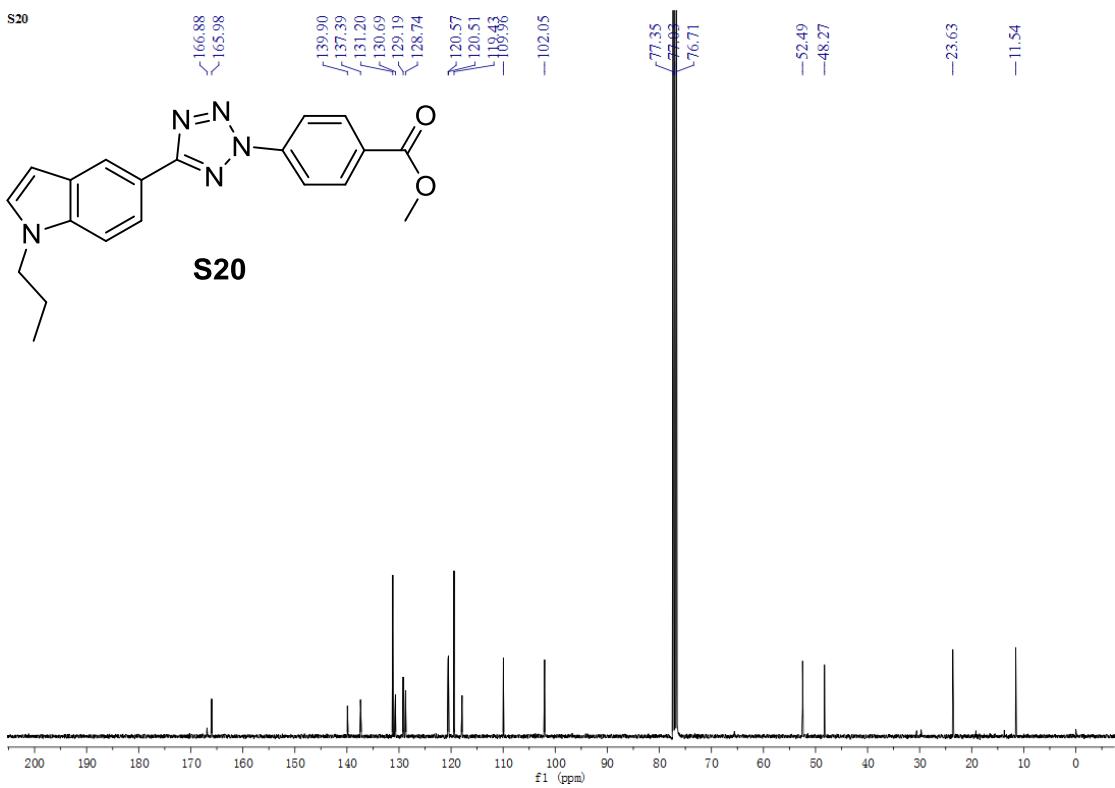
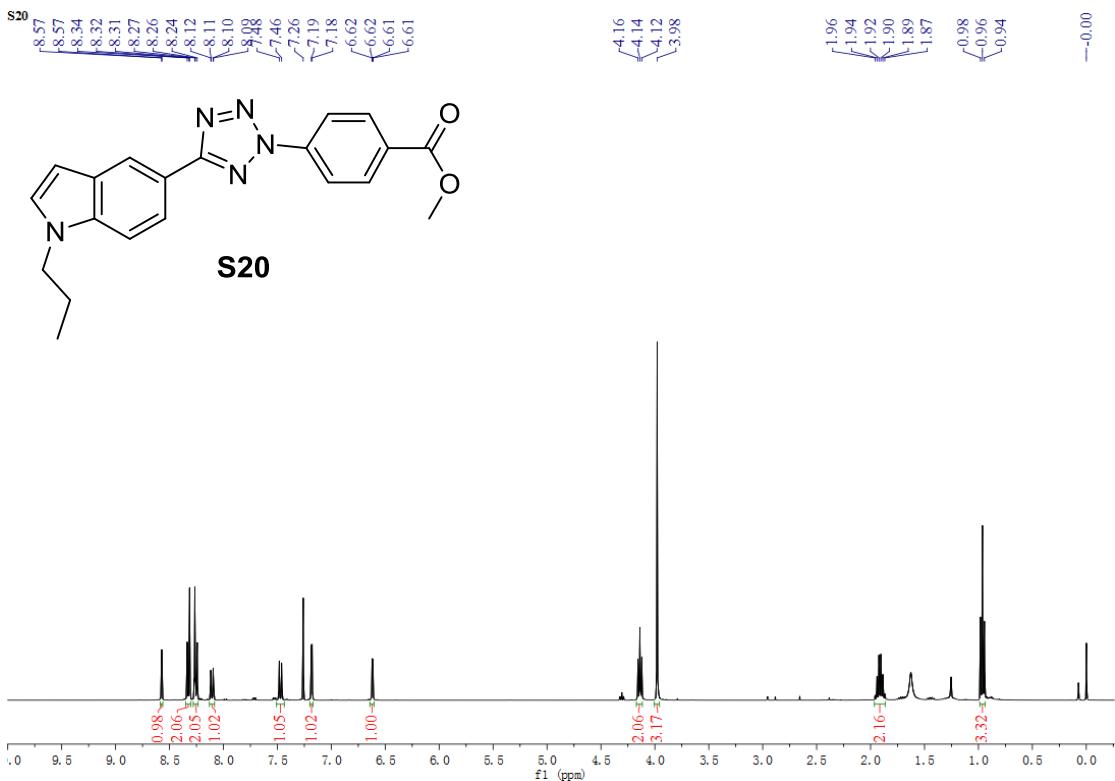




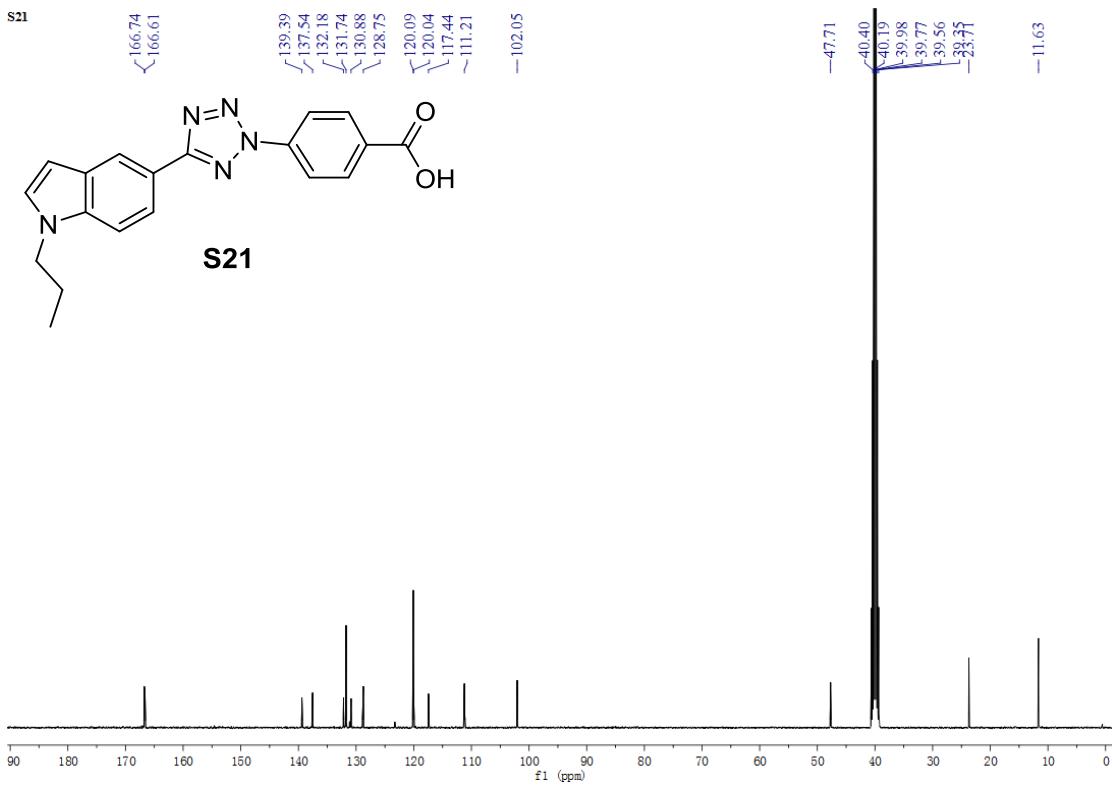
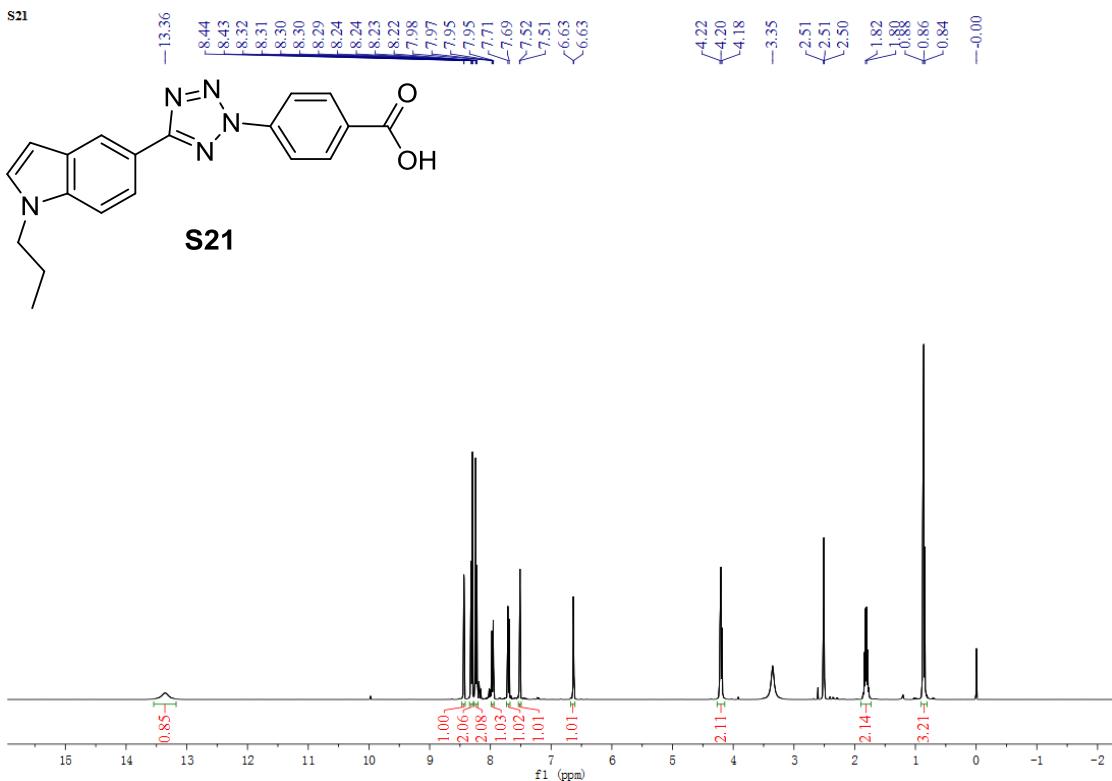


S18



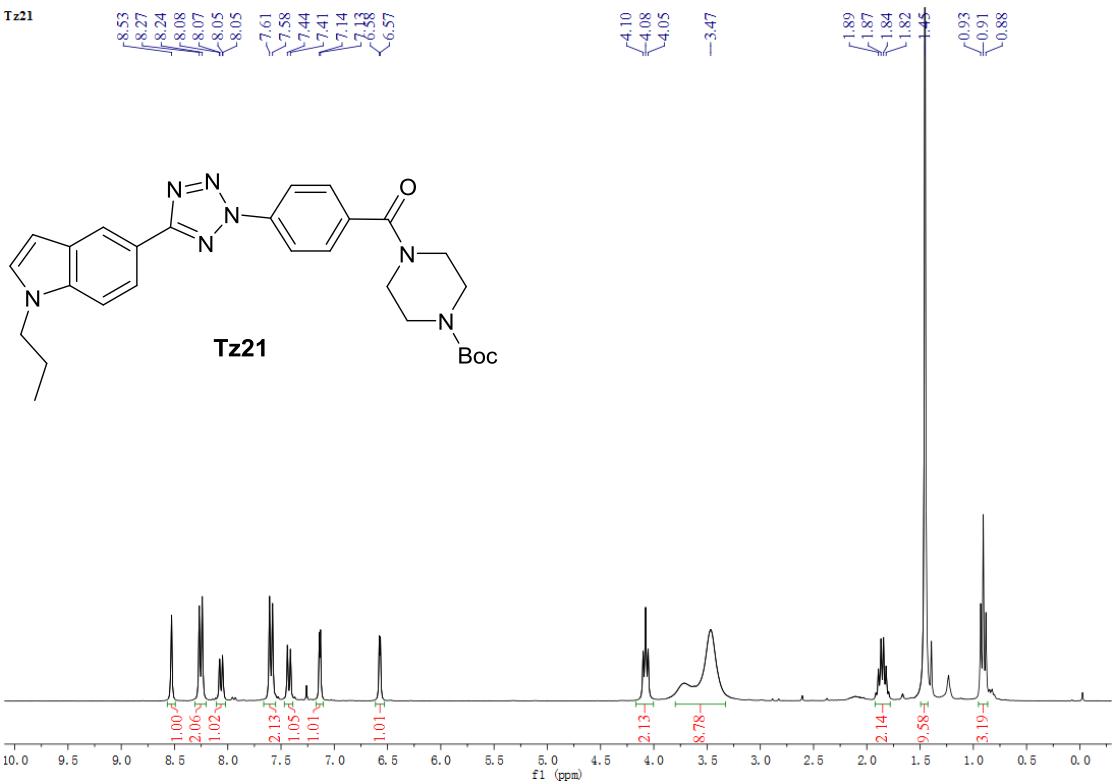


S21



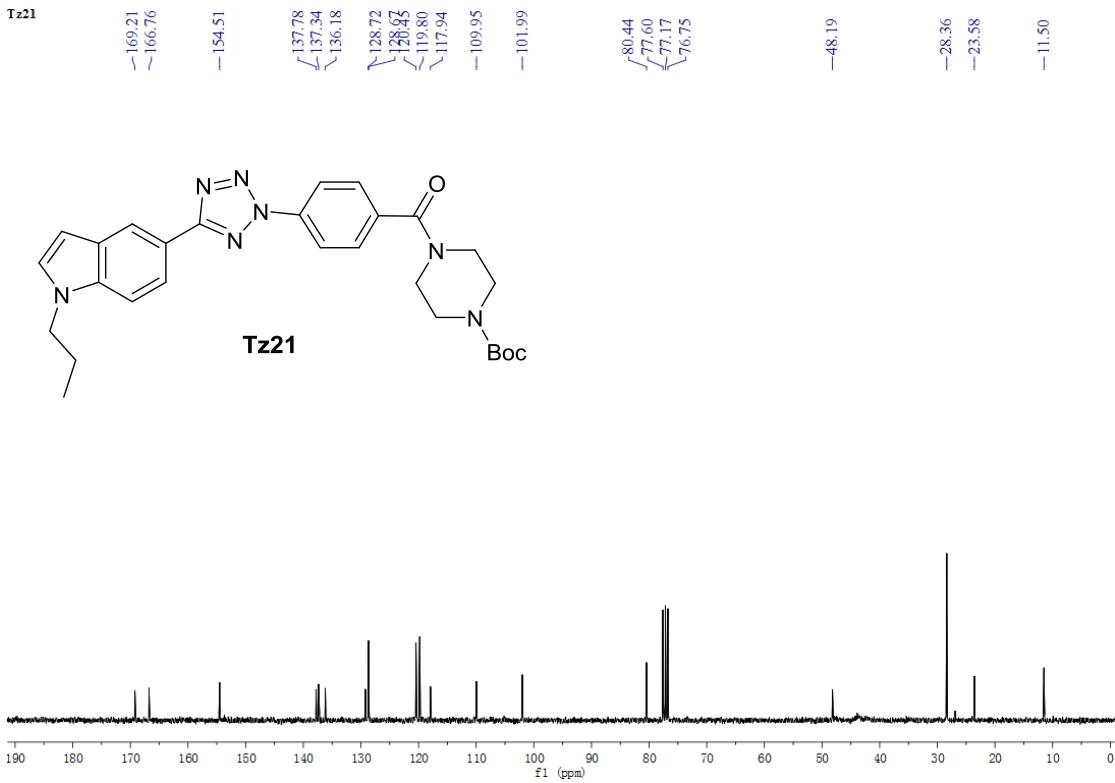
S1_61

Tz21



Tz21

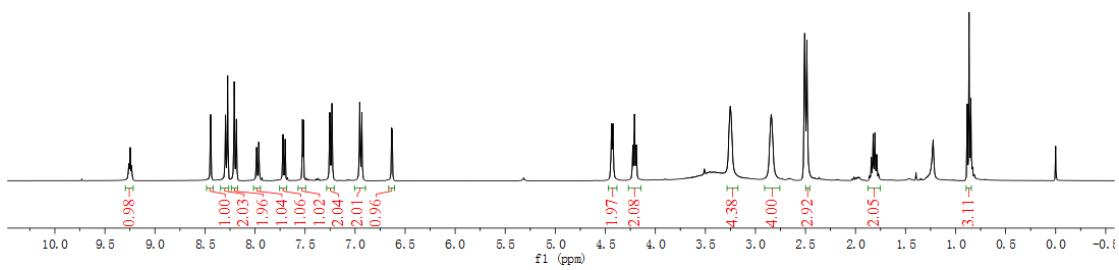
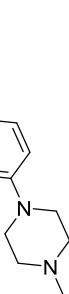
Tz21



Tz22



Tz22



Tz22



Tz22

