Supporting Information for

Bioorthogonally Applicable Multicolor Fluorogenic Naphthalimide-Tetrazine Probes with Aggregation-Induced Emission Characters

Yongcheng Wang,‡ Yu Teng,‡ Hong Yang, Xiang Li, Dali Yin, and Yulin Tian*

State Key Laboratory of Bioactive Substances and Function of Natural Medicine, Beijing Key Laboratory of Active Substances Discovery and Drugability Evaluation, Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, 1 Xian Nong Tan Street, Beijing, 100050, China. E-mail: tianyulin@imm.ac.cn

Contents

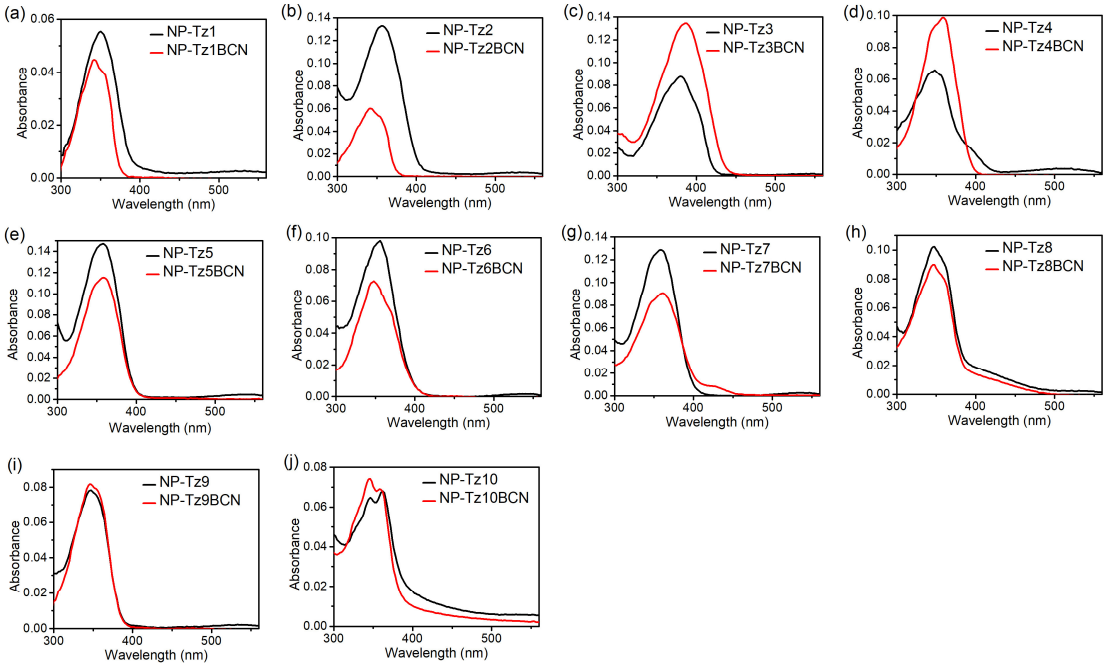


Fig. S1 (a-j) Absorption spectra of 10 μM NP-Tz1～NP-Tz10 and NP-Tz1BCN～NP-Tz10BCN in H_2O.

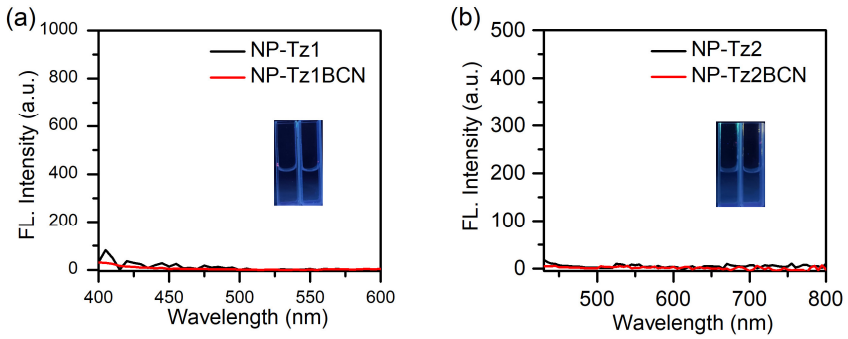


Fig. S2 Fluorescence emission spectra and fluorescent images of NP-Tz1/NP-Tz1BCN (a) and NP-Tz2/NP-Tz2BCN (b) in H_2O (λ_{exc}=350 nm).

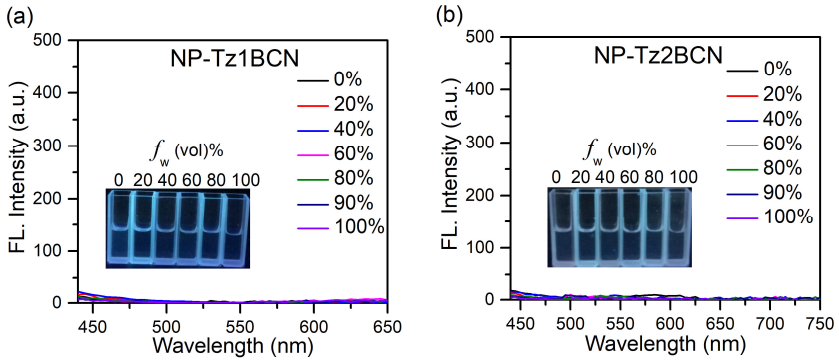


Fig. S3 Fluorescence emission spectra and fluorescent images of NP-Tz1BCN (a) and NP-Tz2BCN (b) in DMSO/H_2O mixed solution (10 μM) with different water fractions (λ_{exc}=350 nm).

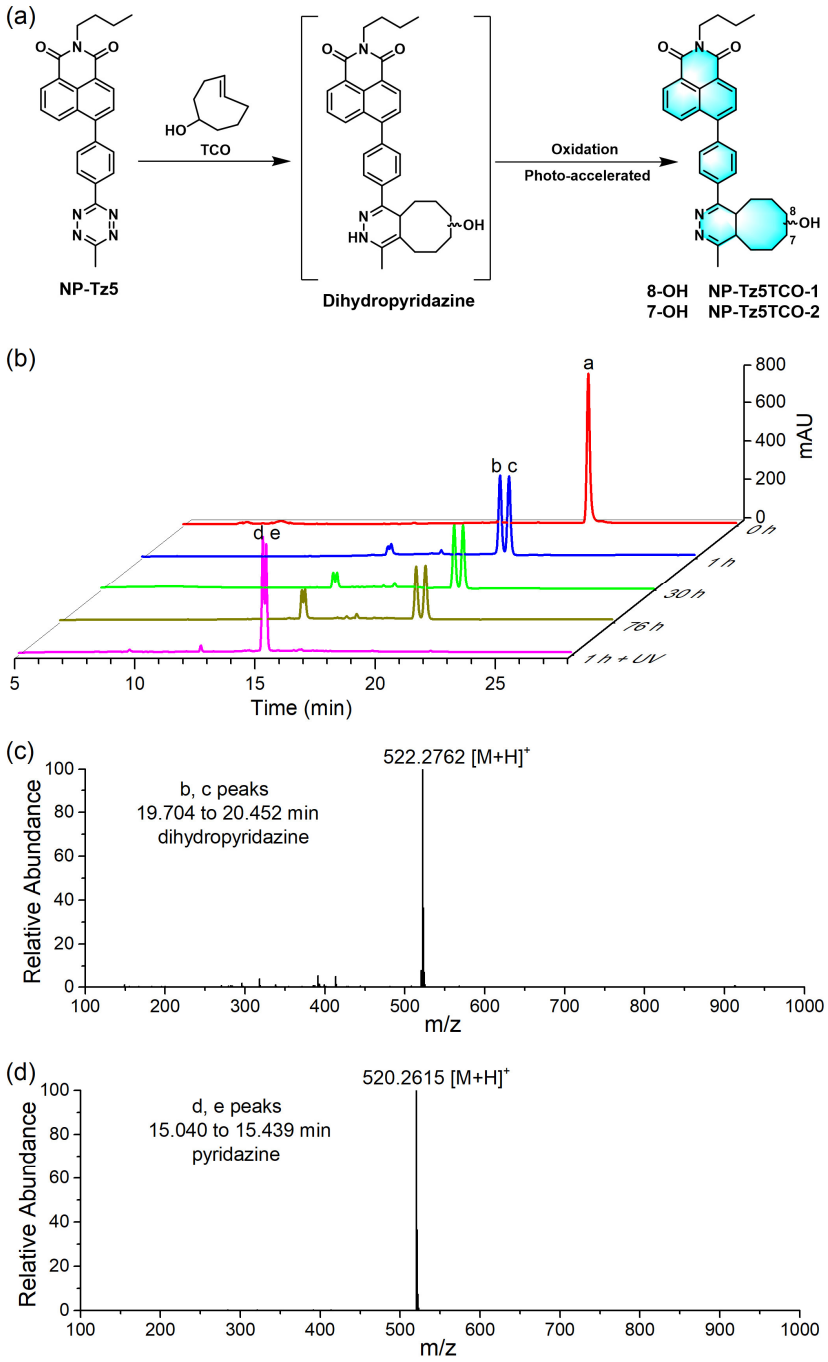


Fig. S4 (a) The iEDDA reaction between NP-Tz5 and TCO. (b) HPLC traces of the iEDDA reaction between NP-Tz5 (10 μM) and TCO (100 μM) in DMSO/H_2O mixed solution (f_w = 90%). Peak a refers to tetrazine (NP-Tz5), peaks b, c refer to dihydropyridazine, peaks d, e refer to pyridazine. After 1 h reaction, the major products are dihydropyridazines. The oxidation of dihydropyridazines to pyridazines in air at r.t. took place rather slowly. After 76 h, there are still quite an amount of dihydropyridazines. When the 1 h reaction mixture was photoirradiated with 365 nm UV light for 10 min, dihydropyridazines were completely converted to pyridazines, revealing a photo-accelerated oxidation procedure. (c) Mass spectrum associated with peaks b, c (dihydropyridazine). (d) Mass spectrum associated with peaks d, e (pyridazine).

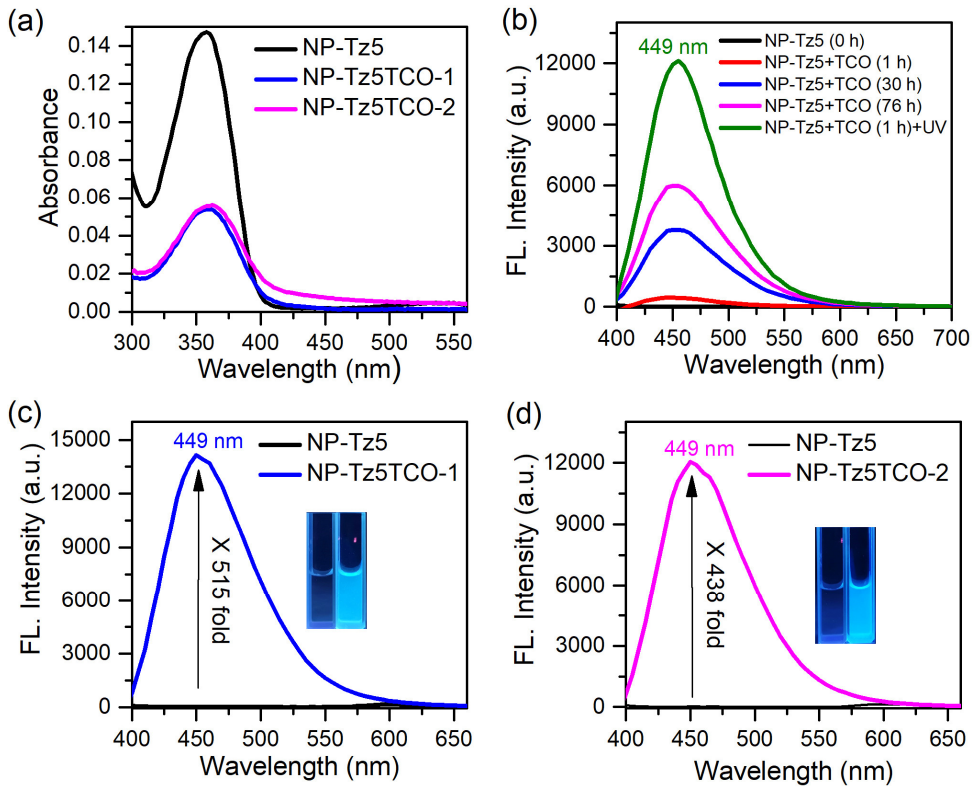


Fig. S5 (a) Absorption spectra of 10 μM NP-Tz5, NP-Tz5TCO-1, and NP-Tz5TCO-2 in H_2O. (b) Fluorescence emission spectra of the reaction mixture of 10 μM NP-Tz5 and 100 μM TCO in DMSO/H_2O mixed solution (f_w = 90%) (λ_{exc}=350 nm). For photo-accelerated oxidation, after 1 h reaction of NP-Tz5 with TCO, the reaction mixture was photoirradiated with 365 nm UV light for 10 min. The fluorescence intensity of dihydropyridazine (after 1 h reaction of TCO with NP-Tz5) is much lower than that of the pyridazine (1 h reaction followed by UV irradiation). The quenching effect of dihydropyridazine may be ascribed to the photoinduced electron transfer (PeT) between the fluorophore and the dihydropyridazine.[1] After oxidation of dihydropyridazine to pyridazine, PeT effect was relieved and the fluorescence emission was fully restored. (c) Fluorescence emission spectra of 10 μM NP-Tz5/NP-Tz5TCO-1 in H_2O (λ_{exc}=350 nm). (d) Fluorescence emission spectra of 10 μM NP-Tz5/NP-Tz5TCO-2 in H_2O (λ_{exc}=350 nm).

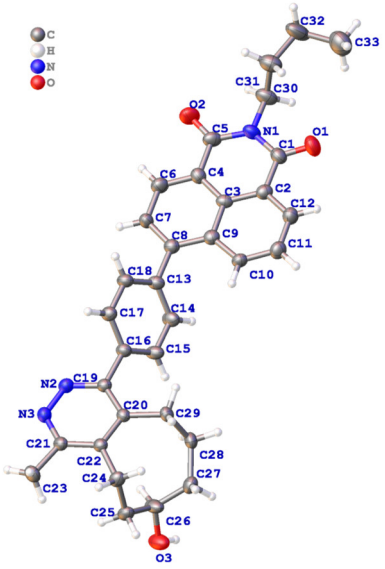


Fig. S6 Thermal ellipsoid plot of the crystal structure of NP-Tz5TCO-2 (50% ellipsoid probability).

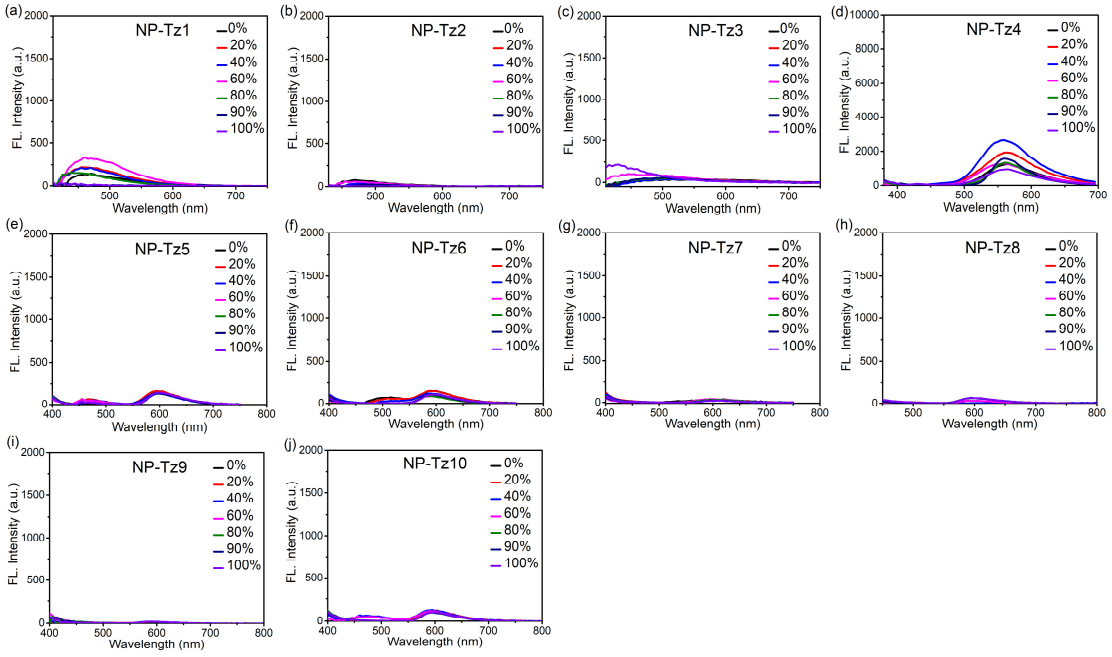


Fig. S7 (a-j) The fluorescence emission spectra of naphthalimide tetrazines, NP-Tz1~NP-Tz10 in DMSO/H_2O mixed solution (10 μM) with different water fractions (λ_{exc}=350 nm).

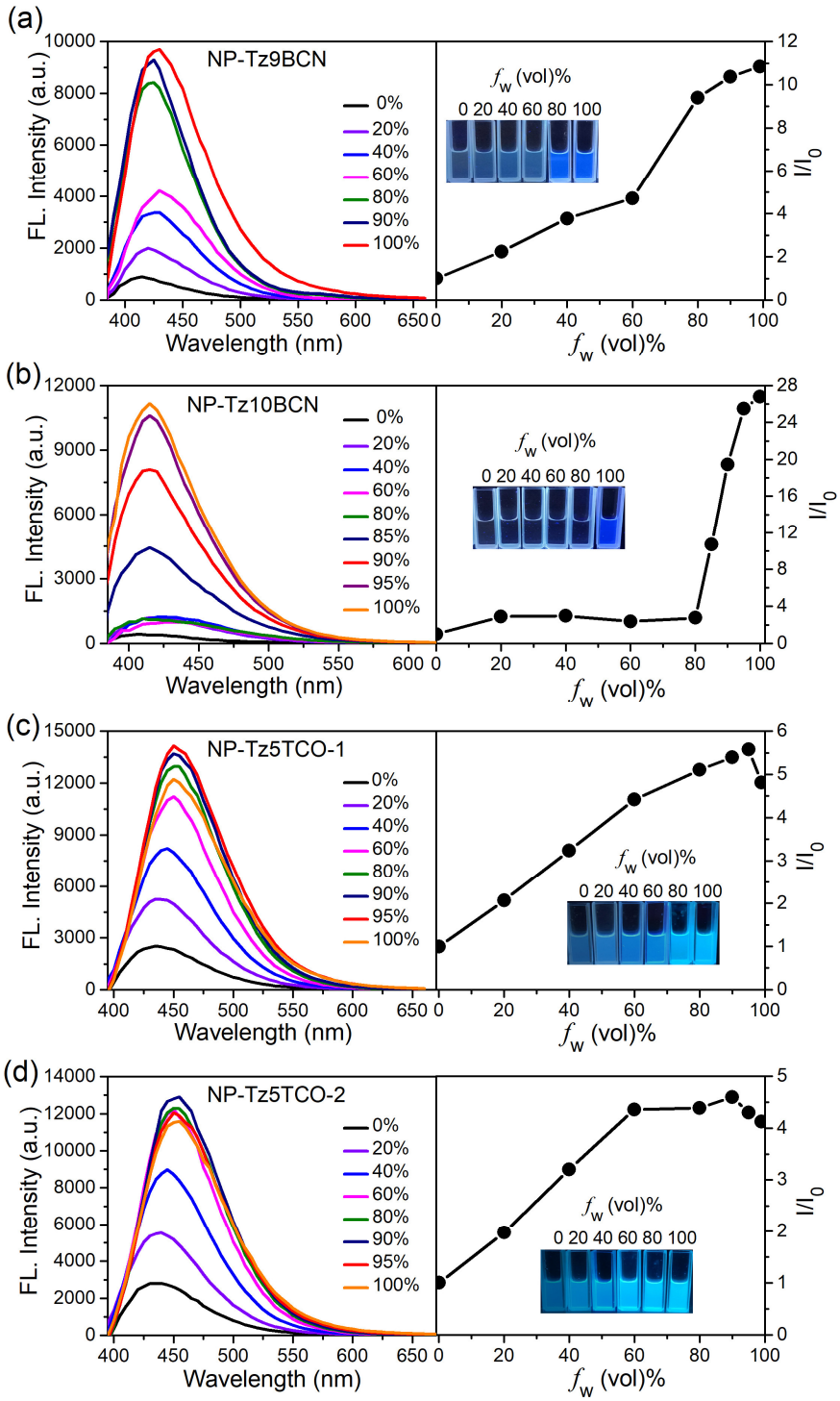


Fig. S8 Left: fluorescence emission spectra of NP-Tz9BCN (a), NP-Tz10BCN (b), NP-Tz5TCO-1 (c), and NP-Tz5TCO-2 (d) in DMSO/H_2O mixed solution (10 μM) with different water fractions (λ_{exc}=350 nm); right: Plot of relative emission intensity (I/I_0) vs water fractions.

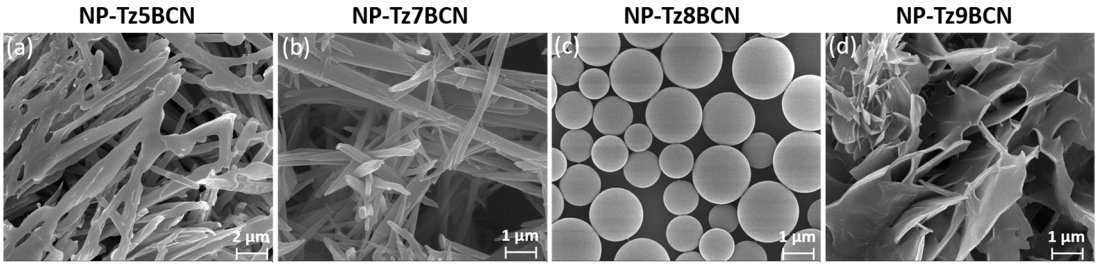


Fig. S9 SEM images for the aggregates of NP-Tz5BCN (a), NP-Tz7BCN (b), NP-Tz8BCN (c), and NP-Tz9BCN (d). Microrods for NP-Tz5BCN and NP-Tz7BCN, smooth spherical shaped particles in diameter of about 0.8–2 μm for NP-Tz8BCN, schistose-like microstructures for NP-Tz9BCN.

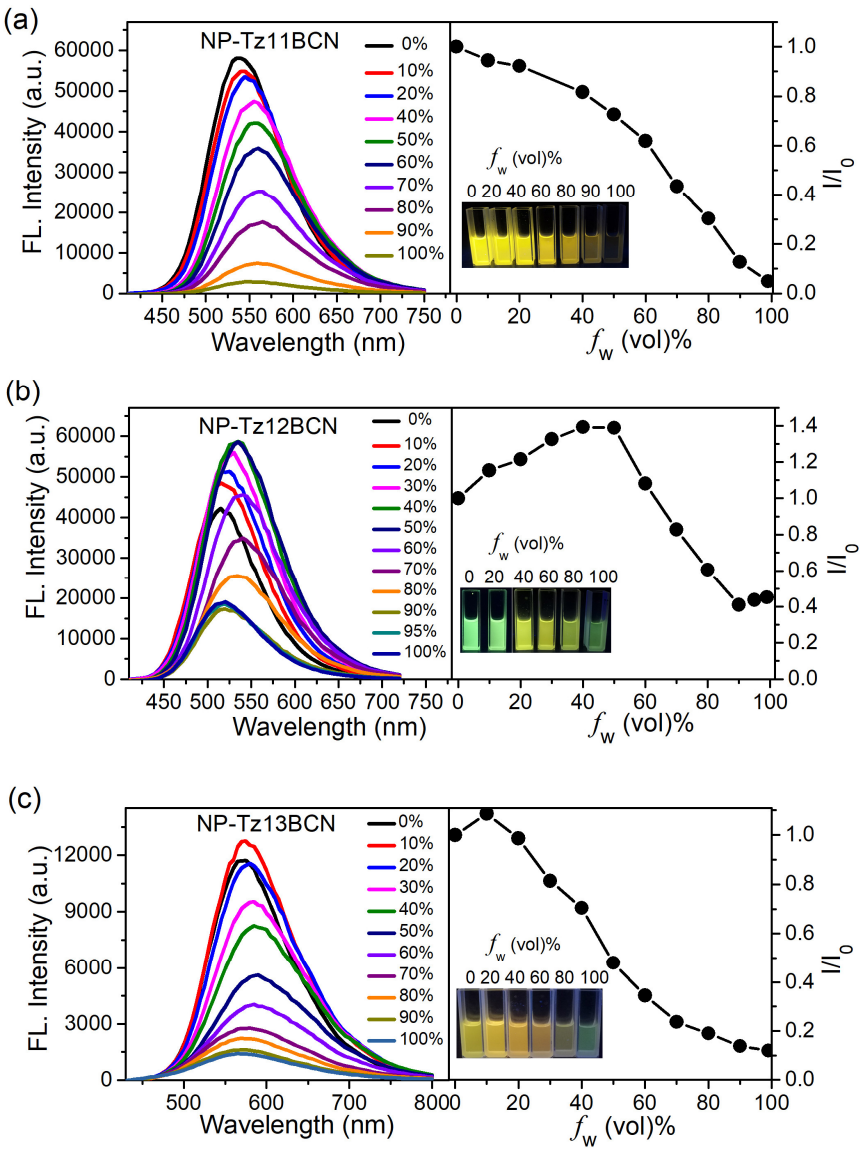


Fig. S10 Left: fluorescence emission spectra of NP-Tz11BCN (a), NP-Tz12BCN (b), and NP-Tz13BCN (c) in DMSO/H_2O mixed solution (10 μM) with different water fractions (λ_{exc}=350 nm); right: Plot of relative emission intensity (I/I_0) vs water fractions.

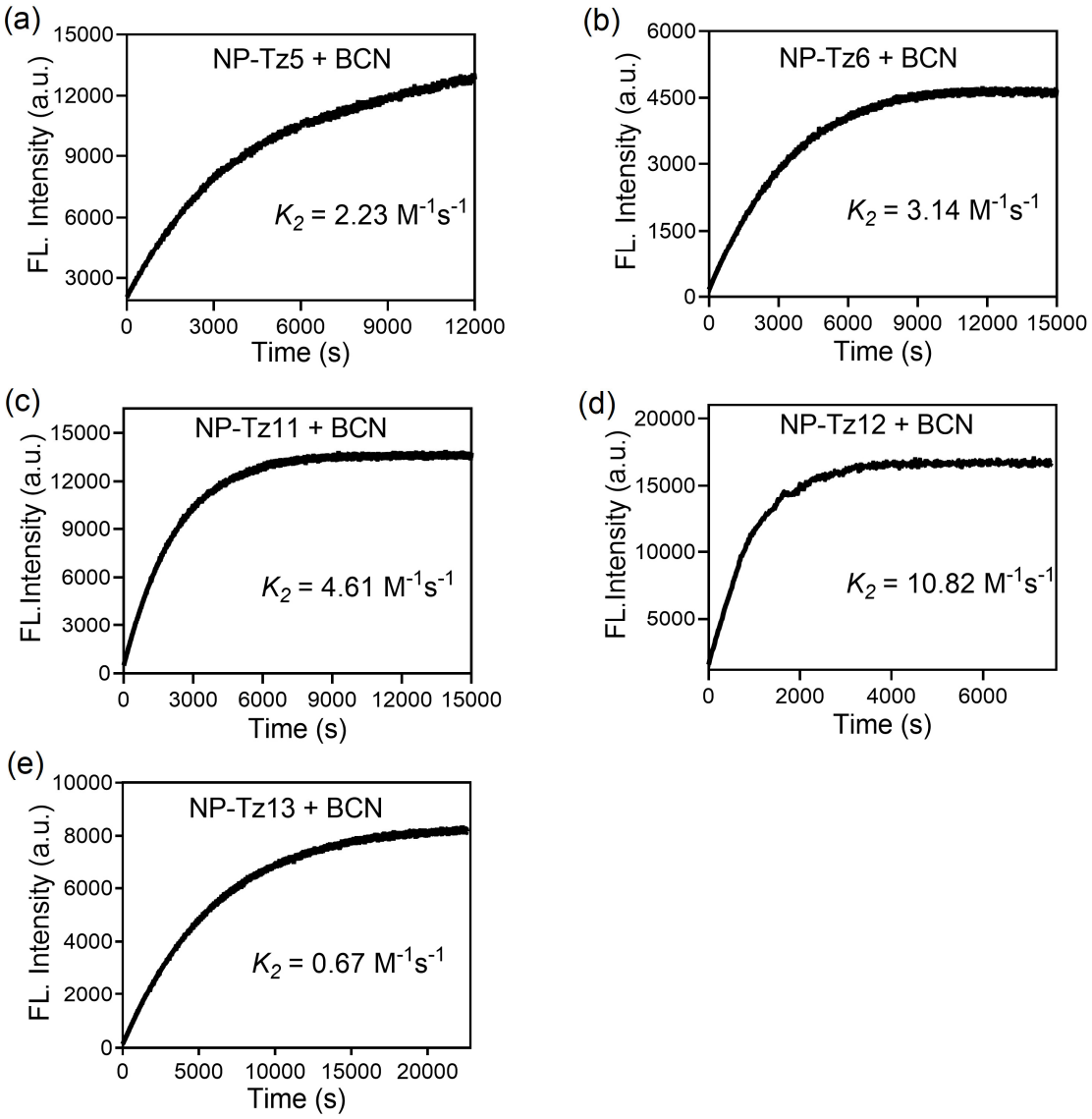


Fig. S11 To confirm that NP-Tz series are able to undergo rapid bioorthogonal reactions needed for biomedical applications, we evaluated the reaction kinetics of NP-Tz5 (a), NP-Tz6 (b), NP-Tz11 (c), NP-Tz12 (d), and NP-Tz13 (e) in DMSO by tracking the change in fluorescence intensity over time, after treatment with BCN. All of these NP-Tzs showed fast reaction kinetics, with the second-order rate constant (k_2) ranging from 0.67 to 10.82 M^{-1} s^{-1}.

Table S1. Photophysical properties of iEDDA cycloadducts[a]

Probes	λ_{abs} (nm)	λ_{em}[d] (nm)	ε[e] [$M^{-1}cm^{-1}$]	Φ_F[f]	Turn-on[g]
NP-Tz1BCN	342	---	9780	0.004	---
NP-Tz2BCN	342	---	14500	0.013	---
NP-Tz3BCN	387	463	13643	0.014	---
NP-Tz4BCN	358	396	15963	0.59	174
NP-Tz5BCN	359	450	19230	0.49	927
NP-Tz6BCN	347	466	15337	0.14	1779
NP-Tz7BCN	362	457	17360	0.27	1336
NP-Tz8BCN	345	601	15283	0.065	44
NP-Tz9BCN	346	433	13507	0.22	570
NP-Tz10BCN	344	415	18850	0.14	224
NP-Tz11BCN[b]	406	542	9967	0.59	234
NP-Tz12BCN[c]	387	537	15227	0.69	237
NP-Tz13BCN[b]	416	571	14480	0.17	106
NP-Tz5TCO-1	359	449	16117	0.41	515
NP-Tz5TCO-2	361	449	14723	0.41	438

[a] Unless noted otherwise the samples were prepared at the concentration of 10 μM in H_2O. [b] The samples were prepared at the concentration of 10 μM in DMSO. [c] The samples were prepared at the concentration of 10 μM in DMSO/H_2O (1:1, v/v). [d] Determined at λ_{exc} = 350 nm. [e] Extinction coefficient. [f] Fluorescence quantum yield, quinine sulfate in aqueous 0.1 N H_2SO_4 as standard. [g] Fluorescence turn-on ratios of iEDDA cycloadducts towards corresponding tetrazines.

Table S2. Crystal data and structure refinement for NP-Tz5TCO-2.

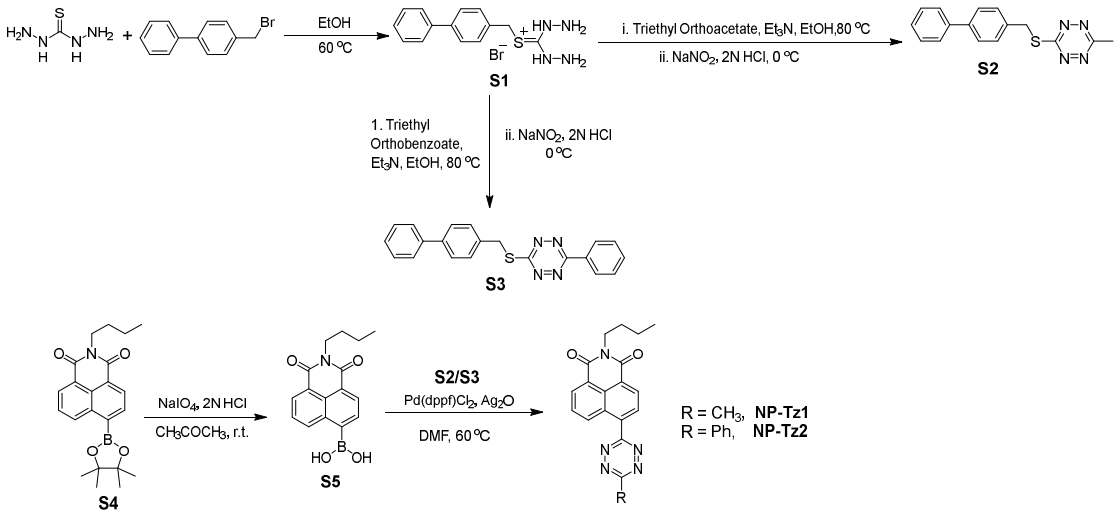
	Single crystal of NP-Tz5TCO-2
Empirical formula	$C_{33}H_{33}N_3O_3$
Formula weight	519.62
Temperature/K	292.98(10)
Crystal system	monoclinic
Space group	C2/c
a/Å	32.443(6)
b/Å	9.3730(5)
c/Å	23.143(4)
α/°	90
β/°	130.84(3)
γ/°	90
Volume/Å^3	5324(2)
Z	8
ρ_{calc}g/cm^3	1.297
μ/mm^{-1}	0.664
F(000)	2208.0
Crystal size/mm^3	0.14 × 0.12 × 0.11
Radiation	Cu Kα (λ = 1.54184)
2Θ range for data collection/°	7.204 to 133.202
Index ranges	-29 ≤ h ≤ 38, -7 ≤ k ≤ 11, -27 ≤ l ≤ 21
Reflections collected	9064
Independent reflections	4687 [R_{int} = 0.0423, R_{sigma} = 0.0523]
Data/restraints/parameters	4687/0/365
Goodness-of-fit on F^2	1.043
Final R indexes [I>=2σ (I)]	R_1 = 0.0760, wR_2 = 0.2175
Final R indexes [all data]	R_1 = 0.1132, wR_2 = 0.2520
Largest diff. peak/hole / e Å^{-3}	0.35/-0.31

General Information

Solvents and chemicals were purchased from commercial sources and used directly without further purification.[1] H NMR and ^{13}C NMR spectra were recorded on a Varian 400 MHz, 500 MHz, or 600 MHz spectrometer. Chemical shifts are referenced to the residual solvent peak and reported as δ units in ppm (in NMR description, s = singlet, d = doublet, t = triplet, q = quartet and m = multiple), and all coupling constant (J) values are given in hertz. ESI-HRMS data were measured on Thermo LCQ Deca XP Max mass spectrometer equipped with an ion trap mass analyzer. Silica gel flash column chromatography was performed on Biotage Isolera one. Fluorescence emission spectra and full wavelength absorption spectra were recorded on Tecan Spark™ 10M Multimode Microplate Reader. SEM images were obtained on a Zeiss Sigma 300 scanning electron microscope. Confocal laser scanning microscope imaging was conducted using a Leica TCS SP8 X Confocal Microscope.

Experimental Procedures and Characterization Data

Synthetic Route to NP-Tz1 and NP-Tz2



Synthesis of ([1,1'-biphenyl]-4-ylmethyl)thiocarbohydrazide bromide (S1)[2]

To a solution of thiocarbohydrazide (2120 mg, 20 mmol) in ethanol (50 mL) was added 4-bromomethyl biphenyl (4940 mg, 20 mmol). The mixture was stirred at 60 °C using an oil bath for 16 h under argon protection. A thick white slurry forms during the reaction. The reaction was brought to room temperature and the slurry was broken up with 50 mL diethyl ether. The white solids were isolated by filtration, washed with diethyl ether, and then dried under rotary evaporation to give the titled compound as white solid (4750 mg, 67%). ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.67 – 7.60 (m, 4H), 7.52 – 7.48 (m, 2H), 7.47 – 7.41 (m, 2H), 7.38 – 7.32 (m, 1H), 4.29 (s, 2H).

Synthesis of 3-(([1,1'-biphenyl]-4-ylmethyl)thio)-6-methyl-1,2,4,5-tetrazine (S2)[2]

To a solution of **S1** (1414 mg, 4.00 mmol) in EtOH (20 mL) was added

triethylorthoacetate (972 mg, 6.00 mmol). After stirred at room temperature for 10 min, Et_3N (404 mg, 4.00 mmol) was added dropwise, and the reaction mixture was stirred at 80 °C using an oil bath for 2 h, then cooled to 0 °C. Sodium nitrite (552 mg, 8.00 mmol) was added to the solution followed by 2 N HCl until the gas evolution ceased and the pH value was 3–4. The reaction mixture was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash column chromatography (PE/EtOAc = 4:1) to afford the titled compound as bright red solid (880 mg, 75% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 – 7.57 (m, 1H), 7.57 – 7.54 (m, 5H), 7.46 – 7.41 (m, 2H), 7.37 – 7.32 (m, 1H), 4.59 (s, 2H), 2.98 (s, 3H); HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{16}H_{15}N_4S$ 295.1012; Found 295.1008.

Synthesis of 3-(([1,1'-biphenyl]-4-ylmethyl)thio)-6-phenyl-1,2,4,5-tetrazine (S3)[2]

To a solution of **S1** (1414 mg, 4.00 mmol) in EtOH (20 mL) was added triethyl orthobenzoate (1346 mg, 6.00 mmol). After stirring at room temperature for 10 min, Et_3N (404 mg, 4.00 mmol) was added dropwise, and the mixture was stirred at 80 °C using an oil bath for 2 h, then cooled to 0 °C. Sodium nitrite (552 mg, 8.00 mmol) was added to the solution followed by 2 N HCl until the gas evolution ceased and the pH value was 3–4. The reaction mixture was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash column chromatography (PE/EtOAc = 4:1) to afford the titled compound as bright red solid (725 mg, 51% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.44 – 8.40 (m, 2H), 7.69 – 7.60 (m, 9H), 7.46 (t, J = 7.6 Hz, 2H), 7.39 – 7.33 (m, 1H), 4.73 (s, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.8, 162.7, 141.0, 140.7, 134.8, 132.5, 131.7, 129.8, 129.4, 128.9, 127.7, 127.6, 127.6, 127.2, 34.7; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{21}H_{17}N_4S$ 357.1168; Found 357.1171.

Synthesis of (2-butyl-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-6-yl) boronic acid (S5)[3]

To a solution of **S4**[4] (600 mg, 1.58 mmol) and sodium periodate (1016 mg, 4.75 mmol) in acetone (10 mL) was added 2 N HCl (2 mL, 4.00 mmol) dropwise. After stirred at room temperature for 1 h, the mixture was filtrated to give the titled compound as white solid (390 mg, 83% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.80 (d, J = 8.4 Hz, 1H), 8.51 – 8.42 (m, 2H), 8.05 (d, J = 7.2 Hz, 1H), 7.90 – 7.83 (m, 1H), 4.12 – 3.97 (m, 2H), 1.74 – 1.56 (m, 2H), 1.40 – 1.31 (m, 2H), 0.93 (t, J = 7.6 Hz, 3H); HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{16}H_{17}O_4NB$ 298.1245; Found 298.1231.

Synthesis of 2-butyl-6-(6-methyl-1,2,4,5-tetrazin-3-yl)-1*H*-benzo[*de*]isoquinoline -1,3(2*H*)-dione (NP-Tz1)[2]

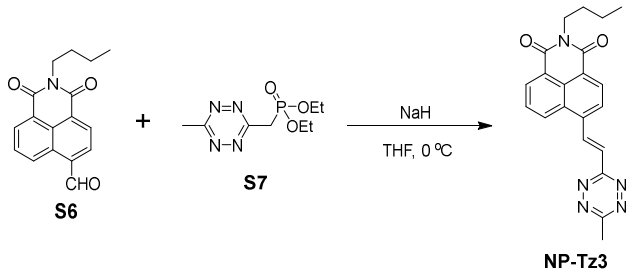
To a solution of **S2** (35 mg, 0.12 mmol) in DMF (5 mL) was added **S5** (71 mg, 0.24 mmol), $Pd(dppf)_2Cl_2$ (7 mg, 0.01 mmol) and silver oxide (68 mg, 0.30 mmol). The reaction mixture was stirred at 60 °C using an oil bath for 3 h under argon, then concentrated. The residue was purified by silica gel flash column chromatography

(PE/EtOAc = 3:1) to give the titled compound as pink solid (21 mg, 50% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.04 (dd, J = 8.8, 0.8 Hz, 1H), 8.79 (d, J = 7.6 Hz, 1H), 8.71 (dd, J = 7.2, 0.8 Hz, 1H), 8.59 (d, J = 7.6 Hz, 1H), 7.87 (dd, J = 8.8, 7.2 Hz, 1H), 4.22 (t, J = 7.6 Hz, 2H), 3.23 (s, 3H), 1.82 – 1.70 (m, 2H), 1.51 – 1.44 (m, 2H), 1.00 (t, J = 7.6 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.4, 166.3, 164.0, 163.7, 134.8, 131.9, 131.9, 130.8, 130.4, 129.4, 129.0, 128.6, 125.7, 123.3, 40.7, 30.3, 21.5, 20.5, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{19}H_{18}O_2N_5$ 348.1455; Found 348.1444.

Synthesis of 2-butyl-6-(6-phenyl-1,2,4,5-tetrazin-3-yl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz2)[2]

To a solution of **S3** (71 mg, 0.20 mmol) in DMF (5 mL) was added **S5** (119 mg, 0.40 mmol), $Pd(dppf)_2Cl_2$ (15 mg, 0.02 mmol) and silver oxide (116 mg, 0.50 mmol). The reaction mixture was stirred at 60 °C using an oil bath for 3 h under argon, then concentrated. The residue was purified by silica gel flash column chromatography (PE/EtOAc = 2:1) to give the titled compound as pink solid (36 mg, 44% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.14 (d, J = 8.6 Hz, 1H), 8.77 (d, J = 7.7 Hz, 1H), 8.74 – 8.60 (m, 4H), 7.87 (t, J = 7.9 Hz, 1H), 7.75 – 7.57 (m, 3H), 4.21 (t, J = 7.2 Hz, 2H), 1.81 – 1.69 (m, 2H), 1.55 – 1.38 (m, 2H), 1.00 (t, J = 7.2 Hz, 3H), ^{13}C NMR (151 MHz, $CDCl_3$) δ 166.1, 164.0, 163.6, 163.4, 134.7, 133.5, 132.0, 131.8, 131.3, 130.8, 130.4, 129.6, 129.4, 129.0, 128.6, 128.6, 125.7, 123.3, 40.6, 30.3, 20.5, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{24}H_{20}O_2N_5$ 410.1612; Found 410.1615.

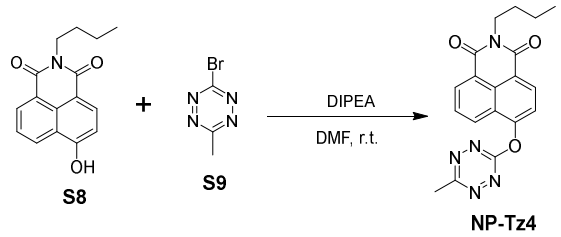
Synthesis of (*E*)-2-butyl-6-(2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)-1*H*-benzo[*de*] isoquinoline-1,3(2*H*)-dione (NP-Tz3)[5]



To a solution of **S6**[6] (84 mg, 0.30 mmol) and **S7**[5] (88 mg, 0.36 mmol) in anhydrous THF (5 mL) was added sodium hydride (24 mg, 0.60 mmol, 60% dispersed in mineral oil) at 0 °C. The reaction mixture was stirred at 0 °C using an oil bath for 2 h, then quenched with 10 ml of saturated NH_4Cl solution. The reaction mixture was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash column chromatography (PE/EtOAc = 4:1) to afford the titled compound as a pink solid (39 mg, 35% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.11 (d, J = 16.0 Hz, 1H), 8.65 (t, J = 8.4 Hz, 3H), 8.17 (d, J = 7.6 Hz, 1H), 7.85 (t, J = 7.6 Hz, 1H), 7.69 (d, J = 16.0 Hz, 1H), 4.19 (t, J = 7.2 Hz, 2H), 3.12 (s, 3H), 1.83 – 1.64 (m, 2H), 1.56 – 1.33 (m, 2H), 0.98 (t, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.1, 164.4, 164.1, 163.8, 138.5, 135.6, 131.6, 130.8, 129.7, 128.7, 127.7, 126.7, 125.4, 123.8, 123.4, 110.1, 40.5, 30.3, 21.5, 20.5, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for

$C_{21}H_{20}O_2N_5$ 374.1612; Found 374.1597.

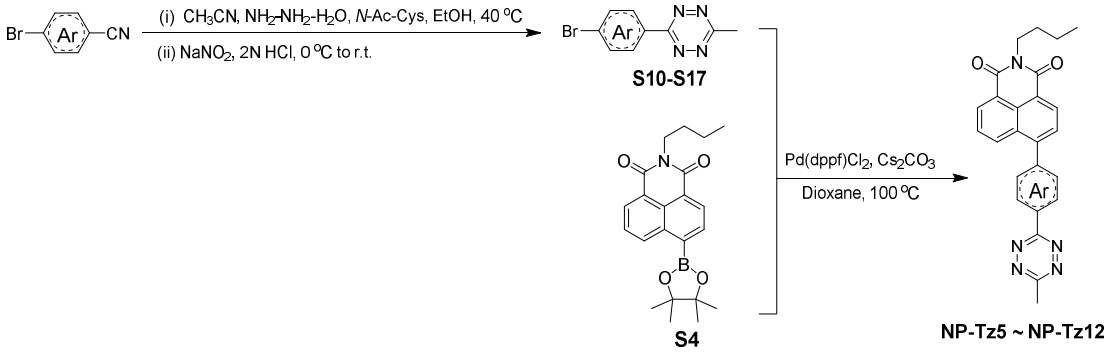
Synthesis of 2-butyl-6-((6-methyl-1,2,4,5-tetrazin-3-yl)oxy)-1*H*-benzo[*de*] isoquinoline-1,3(2*H*)-dione (NP-Tz4)



S8 S9 NP-Tz4

To a solution of **S8**[7] (27 mg, 0.10 mmol) and **S9**[8] (18 mg, 0.10 mmol) in DMF (3 mL) was added DIPEA (26 mg, 0.20 mmol). After stirred at room temperature for 2 h, the reaction mixture was concentrated. The residue was purified by silica gel flash column chromatography (PE/EtOAc = 4:1) to afford the titled compound as red solid (20 mg, 55% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.65 (d, J = 8.0 Hz, 2H), 8.35 (dd, J = 8.4, 0.8 Hz, 1H), 7.83 – 7.73 (m, 1H), 7.64 (d, J = 8.0 Hz, 1H), 4.19 (t, J = 7.6 Hz, 2H), 3.07 (s, 3H), 1.77 – 1.65 (m, 2H), 1.53 – 1.37 (m, 2H), 0.98 (t, J = 7.6 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.3, 167.4, 164.0, 163.4, 152.8, 132.1, 131.9, 129.9, 127.9, 127.5, 125.0, 123.4, 121.3, 118.3, 40.5, 30.3, 20.5, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{19}H_{18}O_3N_5$ 364.1404; Found 364.1412..

Synthetic Route to NP-Tz5 ~ NP-Tz12

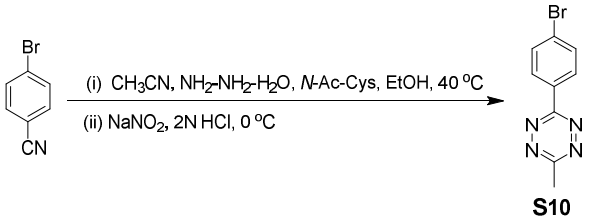


S10-S17 S4 NP-Tz5 ~ NP-Tz12

General procedure A for the synthesis of intermediates S10 ~ S17[5]

To a 25 mL flask equipped with a stir bar, nitrile substrate (1 eq), acetonitrile (10 eq), *N*-acetyl-cysteine (1 eq), and hydrazine hydrate (16 eq) were added. The reaction mixture was stirred at 40 °C using an oil bath under argon. Upon completion, the reaction solution was cooled to 0 °C, then an ice water solution of sodium nitrite (15 eq) was slowly added, followed by slow addition of 2 N HCl during which the solution was stirred intensely and turned bright red, and gas evolved. Addition of 2 N HCl continued until gas evolution ceased and the pH value was 3–4. Then the reaction mixture was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash column chromatography (PE/EtOAc = 10:1) to afford the desired product.

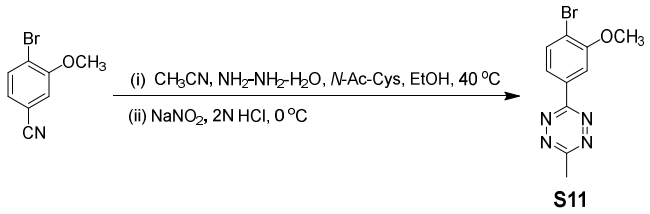
Synthesis of 3-(4-bromophenyl)-6-methyl-1,2,4,5-tetrazine (S10)[5]



S10

The title compound was synthesized according to General Method A. Pink solid; 32% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.47 (d, J = 8.8 Hz, 2H), 7.73 (d, J = 8.8 Hz, 2H), 3.10 (s, 3H); HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_9H_8N_4Br$ 250.9927; Found 250.9931.

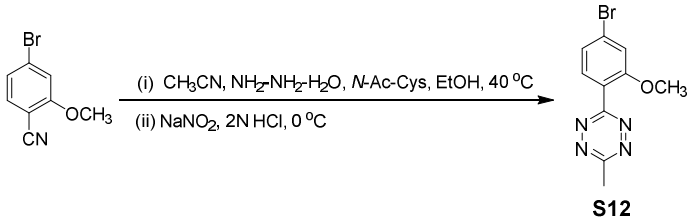
Synthesis of 3-(4-bromo-3-methoxyphenyl)-6-methyl-1,2,4,5-tetrazine (S11)



S11

The title compound was synthesized according to General Method A. Pink solid; 55% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (d, J = 2.0 Hz, 1H), 8.09 (dd, J = 8.4, 2.0Hz, 1H), 7.75 (d, J = 8.4 Hz, 1H), 4.04 (s, 3H), 3.10 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.6, 163.7, 156.8, 134.4, 132.3, 121.4, 117.3, 110.6, 56.7, 21.4; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{10}H_{10}ON_4Br$ 281.0033; Found 281.0042.

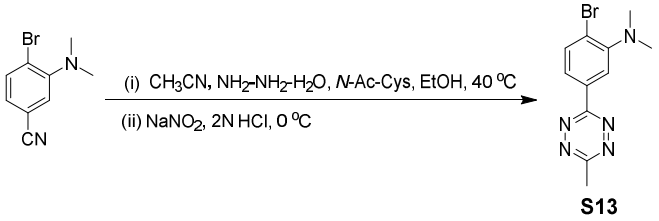
Synthesis of 3-(4-bromo-2-methoxyphenyl)-6-methyl-1,2,4,5-tetrazine (S12)



S12

The title compound was synthesized according to General Method A. Pink solid; 47% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (d, J = 8.4 Hz, 1H), 7.32 (dd, J = 8.4, 2.0 Hz, 1H), 7.26 (d, J = 2.0 Hz, 1H), 3.90 (s, 3H), 3.10 (s, 3H); HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{10}H_{10}ON_4Br$ 281.0033; Found 281.0045.

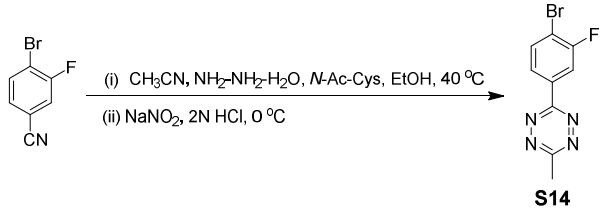
Synthesis of 2-bromo-*N*, *N*-dimethyl-5-(6-methyl-1,2,4,5-tetrazin-3-yl)aniline (S13)



S13

The title compound was synthesized according to General Method A. Pink solid; 61% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.29 (d, J = 2.0 Hz, 1H), 8.09 (dd, J = 8.0, 2.0 Hz, 1H), 7.76 (d, J = 8.0 Hz, 1H), 3.09 (s, 3H), 2.91 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.5, 163.8, 153.0, 135.1, 131.8, 124.3, 123.1, 119.7, 44.3, 21.3; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{26}H_{24}O_3N_5$ 454.1874; Found 454.1880.

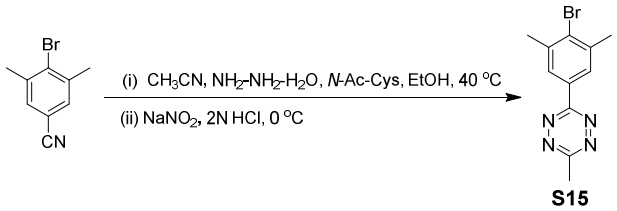
Synthesis of 3-(4-bromo-3-fluorophenyl)-6-methyl-1,2,4,5-tetrazine (S14)



S14

The title compound was synthesized according to General Method A. Pink solid; 44% yield; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.31 (d, J = 9.6 Hz, 1H), 8.22 (d, J = 8.0 Hz, 1H), 8.01 (t, J = 7.6 Hz, 1H), 3.01 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.5, 162.1, 162.1, 160.0, 157.6, 134.8, 133.7, 133.6, 124.8, 124.8, 115.1, 114.9, 113.0, 112.8, 20.9; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_9H_7N_4BrF$ 268.9833; Found 268.9830.

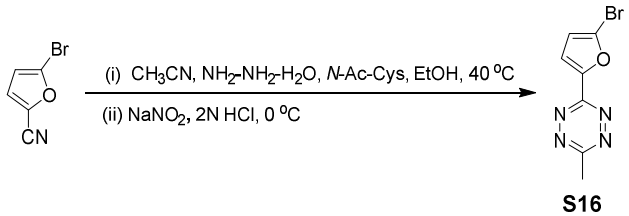
Synthesis of 3-(4-bromo-3,5-dimethylphenyl)-6-methyl-1,2,4,5-tetrazine (S15)



S15

The title compound was synthesized according to General Method A. Pink solid; 58% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.28 (s, 2H), 3.09 (s, 3H), 2.54 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.4, 163.9, 139.8, 133.0, 130.1, 127.3, 24.2, 21.3; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{11}H_{12}N_4Br$ 279.0240; Found 279.0252.

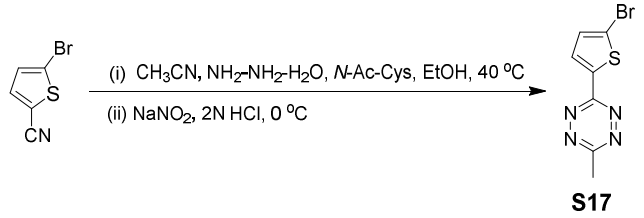
Synthesis of 3-(5-bromofuran-2-yl)-6-methyl-1,2,4,5-tetrazine (S16)



S16

The title compound was synthesized according to General Method A. Pink solid; 44% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 (d, J = 3.6 Hz, 1H), 6.60 (d, J = 3.6 Hz, 1H), 3.03 (s, 3H); HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_7H_6ON_4Br$ 240.9719; Found 240.9723.

Synthesis of 3-(5-bromothiophen-2-yl)-6-methyl-1,2,4,5-tetrazine (S17)



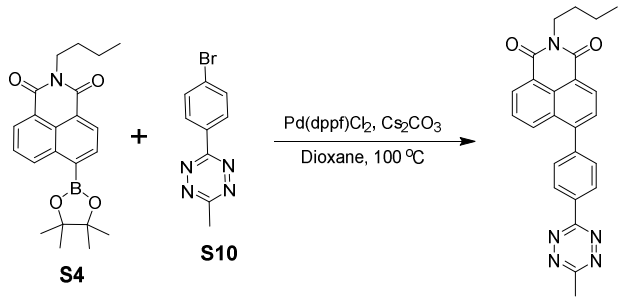
S17

The title compound was synthesized according to General Method A. Red solid; 37% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, J = 4.0 Hz, 1H), 7.21 (d, J = 4.0 Hz, 1H), 3.04 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.1, 161.5, 137.1, 132.1, 131.4, 120.7, 21.4; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_7H_6N_4BrS$ 256.9491; Found 256.9494.

General procedure B for the synthesis of NP-Tz5 ~ NP-Tz12[9]

To a solution of **S10-S17** (1 eq) in 1,4-dioxane (10 mL) was added **S4**[3] (1 eq), $Pd(dppf)Cl_2$ (0.1eq) and Cs_2CO_3 (2 eq). The reaction mixture was stirred at 100 °C using an oil bath for 3 h under argon, then concentrated. The residue was purified by silica gel flash column chromatography (PE/EtOAc = 5:1) to afford the titled compound.

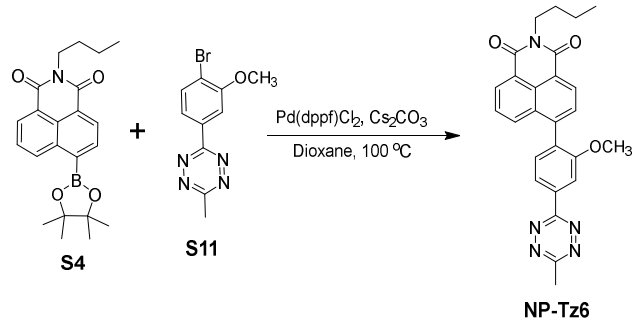
Synthesis of 2-butyl-6-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-1*H*-benzo[*de*] isoquinoline-1,3(2*H*)-dione (NP-Tz5)



S4 **S10** **NP-Tz5**

The title compound was synthesized according to General Method B. Magenta solid; 31% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.78 (d, J = 8.4 Hz, 2H), 8.69 (d, J = 7.6 Hz, 1H), 8.66 (dd, J = 7.2, 0.8 Hz, 1H), 8.28 (dd, J = 8.8, 0.8 Hz, 1H), 7.80 – 7.72 (m, 4H), 4.22 (t, J = 7.6 Hz, 2H), 3.15 (s, 3H), 1.82 – 1.69 (m, 2H), 1.54 – 1.41 (m, 2H), 1.00 (t, J = 7.6 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.7, 164.4, 164.1, 164.0, 145.6, 143.2, 132.3, 132.1, 131.5, 131.0, 130.9, 130.0, 128.9, 128.3, 128.0, 127.4, 123.3, 122.6, 40.5, 30.4, 21.4, 20.6, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{25}H_{22}O_2N_5$ 424.1768; Found 424.1766.

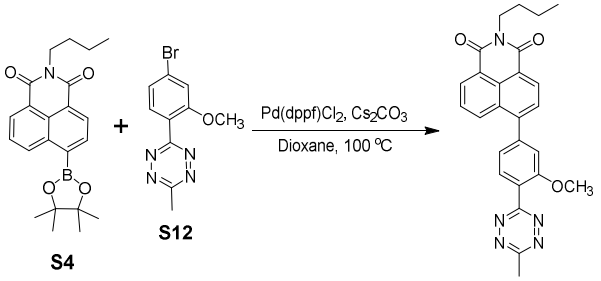
Synthesis of 2-butyl-6-(2-methoxy-4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz6)



S4 S11 NP-Tz6

The title compound was synthesized according to General Method B. Red solid; 28% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.68 (d, J = 7.6 Hz, 1H), 8.63 (d, J = 7.2 Hz, 1H), 8.39 (d, J = 8.0 Hz, 1H), 8.32 (s, 1H), 7.98 (d, J = 8.4 Hz, 1H), 7.73 (d, J = 7.6 Hz, 1H), 7.68 (t, J = 8.0 Hz, 1H), 7.52 (d, J = 7.6 Hz, 1H), 4.22 (t, J = 7.6 Hz, 2H), 3.85 (s, 3H), 3.15 (s, 3H), 1.81 – 1.68 (m, 2H), 1.54 – 1.40 (m, 2H), 1.00 (t, J = 7.6 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.7, 164.4, 164.3, 163.9, 157.7, 142.8, 133.8, 132.7, 132.5, 132.2, 131.3, 130.8, 130.5, 128.5, 126.9, 123.1, 122.6, 120.7, 110.1, 56.0, 40.4, 30.4, 21.4, 20.6, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{26}H_{24}O_3N_5$ 454.1874; Found 454.1881.

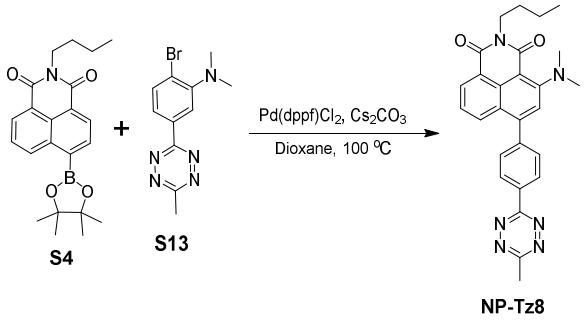
Synthesis of 2-butyl-6-(3-methoxy-4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz7)



S4 S12 NP-Tz7

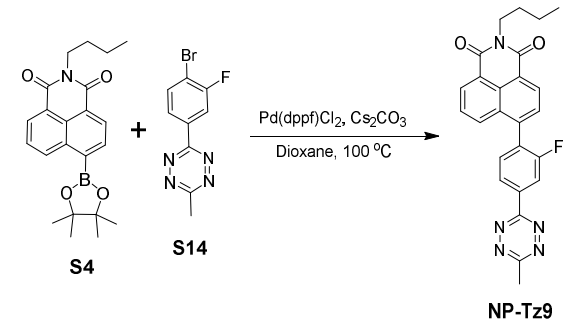
The title compound was synthesized according to General Method B. Red solid; 34% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.71 – 8.62 (m, 2H), 8.31 (dd, J = 8.5, 1.0 Hz, 1H), 8.09 (d, J = 7.8 Hz, 1H), 7.81 – 7.71 (m, 2H), 7.30 (dd, J = 7.8, 1.4 Hz, 1H), 7.22 (d, J = 1.2 Hz, 1H), 4.22 (t, J = 7.6 Hz, 2H), 3.94 (s, 3H), 3.15 (s, 3H), 1.80 – 1.69 (m, 2H), 1.53 – 1.40 (m, 2H), 1.00 (t, J = 7.6 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.6, 165.8, 164.3, 164.1, 158.5, 145.7, 143.9, 132.3, 132.1, 131.5, 130.8, 130.1, 128.8, 127.8, 127.4, 123.2, 122.8, 122.6, 122.4, 113.9, 56.5, 40.5, 30.4, 21.4, 20.5, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{26}H_{24}O_3N_5$ 454.1874; Found 454.1880.

Synthesis of 2 – buty l- 6 - (2-(dimethylamino) - 4 - (6-methyl-1,2,4,5-tetrazin-3-yl) phenyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz8)



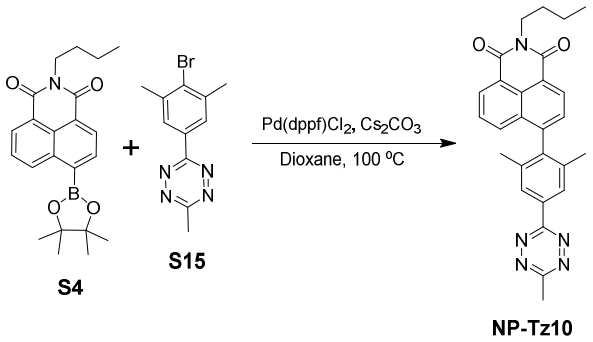
The title compound was synthesized according to General Method B. Red solid; 32% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.67 (d, J = 7.6 Hz, 1H), 8.62 (dd, J = 7.2, 1.2 Hz, 1H), 8.35 (d, J = 1.6 Hz, 1H), 8.28 (dd, J = 8.0, 1.6 Hz, 1H), 8.08 (dd, J = 8.4, 1.2 Hz, 1H), 7.83 (d, J = 7.6 Hz, 1H), 7.67 (dd, J = 8.4, 7.2 Hz, 1H), 7.43 (d, J = 8.0 Hz, 1H), 4.21 (t, J = 7.6 Hz, 2H), 3.13 (s, 3H), 2.53 (s, 6H), 1.81 – 1.70 (m, 2H), 1.53 – 1.41 (m, 2H), 0.99 (t, J = 7.6 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.5, 164.4, 164.2, 164.1, 152.8, 146.1, 134.5, 133.6, 133.0, 131.5, 131.1, 129.5, 128.8, 128.1, 126.8, 123.1, 122.2, 120.6, 117.2, 110.1, 43.2, 40.4, 30.4, 21.4, 20.6, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{27}H_{27}O_2N_6$ 467.2190; Found 467.2192.

Synthesis of 2 – buty l- 6 - (2-(dimethylamino) - 4 - (6-methyl-1,2,4,5-tetrazin-3-yl) phenyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz9)



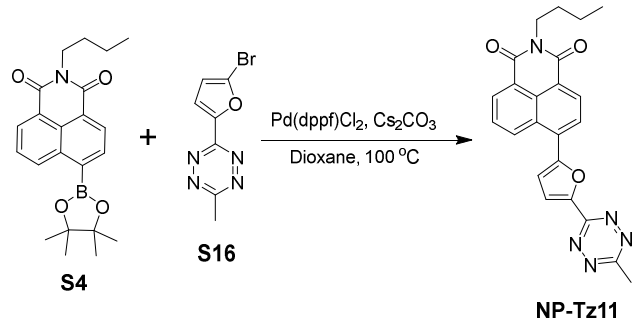
The title compound was synthesized according to General Method B. Red solid; 22% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.70 (d, J = 7.6 Hz, 1H), 8.67 (dd, J = 7.2, 1.0 Hz, 1H), 8.60 (dd, J = 8.0, 1.6 Hz, 1H), 8.52 (dd, J = 10.4, 1.6 Hz, 1H), 8.10 – 8.04 (m, 1H), 7.80 (d, J = 7.6 Hz, 1H), 7.75 (dd, J = 8.4, 7.2 Hz, 1H), 7.67 (t, J = 7.6 Hz, 1H), 4.23 (d, J = 7.2 Hz, 2H), 3.17 (s, 3H), 1.81 – 1.70 (m, 2H), 1.53 – 1.41 (m, 2H), 1.00 (t, J = 7.2 Hz, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 168.1, 164.3, 164.1, 163.2, 163.2, 161.2, 159.5, 139.4, 134.7, 134.7, 133.1, 133.1, 132.0, 131.6, 130.7, 130.6, 130.1, 128.8, 128.6, 127.5, 124.0, 124.0, 123.3, 123.3, 115.7, 115.5, 40.5, 30.4, 21.5, 20.6, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{25}H_{21}O_2N_5F$ 442.1674; Found 442.1666.

Synthesis of 2-butyl-6-(2,6-dimethyl-4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-1*H* -benzo[*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz10)



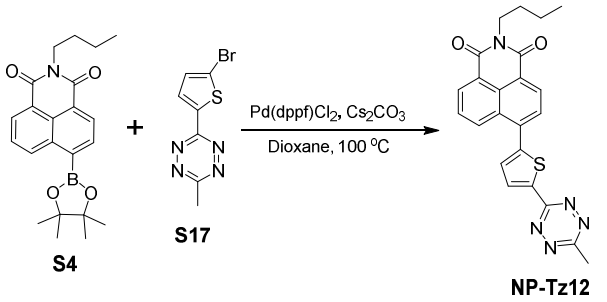
The title compound was synthesized according to General Method B. Red solid; 27% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.70 (d, J = 7.2 Hz, 1H), 8.63 (dd, J = 7.2, 1.2 Hz, 1H), 8.43 (s, 2H), 7.73 (dd, J = 8.4, 1.2 Hz, 1H), 7.66 (dd, J = 8.4, 7.2 Hz, 1H), 7.60 (d, J = 7.6 Hz, 1H), 4.22 (t, J = 7.6 Hz, 2H), 3.13 (s, 3H), 2.02 (s, 6H), 1.80 – 1.70 (m, 2H), 1.53 – 1.41 (m, 2H), 0.99 (t, J = 7.6 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.5, 164.3, 164.2, 164.1, 145.0, 142.4, 138.0, 131.8, 131.5, 131.4, 131.2, 129.9, 128.7, 127.5, 127.5, 127.2, 123.5, 122.5, 40.4, 30.4, 21.4, 20.7, 20.5, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{27}H_{26}O_2N_5$ 452.2081; Found 452.2093.

Synthesis of 2-butyl-6-(5-(6-methyl-1,2,4,5-tetrazin-3-yl)furan-2-yl)-1*H*-benzo[*de*] isoquinoline-1,3(2*H*)-dione (NP-Tz11)



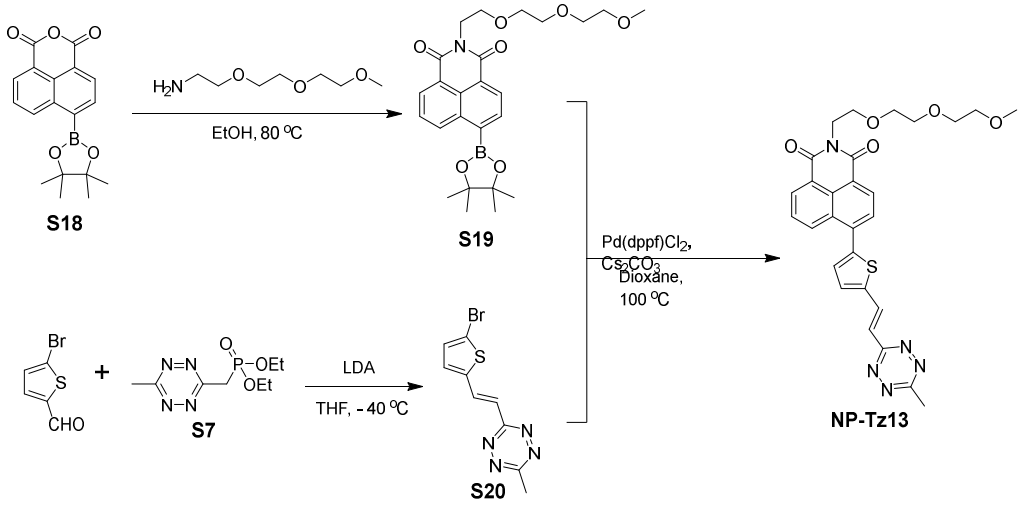
The title compound was synthesized according to General Method B. Red solid; 28% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.01 (d, J = 8.4 Hz, 1H), 8.67 (t, J = 8.4 Hz, 2H), 8.19 (d, J = 8.0 Hz, 1H), 7.92 – 7.82 (m, 2H), 7.22 (d, J = 3.6 Hz, 1H), 4.20 (t, J = 7.6 Hz, 2H), 3.12 (s, 3H), 1.81 – 1.68 (m, 2H), 1.54 – 1.38 (m, 2H), 0.99 (t, J = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.9, 164.2, 163.8, 158.8, 156.5, 148.3, 132.5, 131.9, 131.7, 130.7, 129.1, 128.4, 128.1, 127.1, 123.3, 123.2, 118.3, 114.5, 40.5, 30.4, 21.5, 20.6, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{23}H_{20}O_3N_5$ 414.1561; Found 414.1569.

Synthesis of 2-butyl-6-(5-(6-methyl-1,2,4,5-tetrazin-3-yl)thiophen-2-yl)-1*H*-benzo [*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz12)



The title compound was synthesized according to General Method B. Red solid; 24% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.71 – 8.59 (m, 3H), 8.38 (d, J = 3.9 Hz, 1H), 7.91 (d, J = 7.6 Hz, 1H), 7.81 (dd, J = 8.4, 7.6 Hz, 1H), 7.48 (d, J = 3.9 Hz, 1H), 4.21 (t, J = 7.6 Hz, 2H), 3.09 (s, 3H), 1.81 – 1.68 (m, 2H), 1.54 – 1.39 (m, 2H), 0.99 (t, J = 7.6 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.2, 164.2, 163.9, 162.1, 146.7, 137.8, 137.5, 131.8, 131.7, 131.6, 130.7, 130.5, 129.9, 128.9, 128.9, 127.8, 123.3, 123.1, 40.5, 30.3, 21.4, 20.5, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{23}H_{20}O_2N_5S$ 430.1332; Found 430.1335.

Synthetic Route to NP-Tz13



Synthesis of 2-(2-(2-(2-methoxyethoxy)ethoxy)ethyl)-6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (S19)

To a solution of **S18**[10] (180 mg, 0.56 mmol) in EtOH (10 mL) was added 2-(2-(2-methoxyethoxy)ethoxy)ethan-1-amine (100 mg, 0.61 mmol). The reaction mixture was stirred at 80 °C using an oil bath for 3 h, then concentrated. The residue was purified by silica gel flash column chromatography (PE/EtOAc = 1:1) to afford the titled compound as white solid (150 mg, 57% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.11 (dd, J = 8.5, 1.2 Hz, 1H), 8.58 (dd, J = 7.3, 1.2 Hz, 1H), 8.55 (d, J = 7.3 Hz, 1H), 8.29 (d, J = 7.3 Hz, 1H), 7.77 (dd, J = 8.5, 7.3 Hz, 1H), 4.43 (t, J = 6.2 Hz, 2H), 3.82 (t, J = 6.2 Hz, 2H), 3.72 – 3.69 (m, 2H), 3.63 – 3.60 (m, 2H), 3.59 – 3.56 (m, 2H), 3.45 – 3.41 (m, 2H), 3.30 (s, 3H), 1.45 (s, 12H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.5, 135.9, 135.4, 135.1, 131.0, 129.9, 128.1, 127.2, 124.8, 122.7, 84.7, 72.0, 70.7, 70.6,

70.3, 68.1, 59.1, 39.3, 25.1; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{25}H_{33}O_7NB$ 470.2345; Found 470.2348.

Synthesis of (*E*)-3-(2-(5-bromothiophen-2-yl)vinyl)-6-methyl-1,2,4,5-tetrazine (S20)[5]

To a solution of 5-bromothiophene-2-carbaldehyde (191 mg, 1.00 mmol) and **S7**[5] (270 mg, 1.10 mmol) in anhydrous THF (10 mL) was added LDA solution (0.80 mL, 1.60 mmol, 2 M in THF) dropwise at -40 °C under argon. The reaction mixture was stirred at -40 °C for 3 h, then quenched with 20 ml of saturated NH_4Cl solution. The mixture was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel flash column chromatography (PE/EtOAc = 4:1) to afford the titled compound as a pink solid (97 mg, 35% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.27 (d, J = 16.0 Hz, 1H), 7.13 (d, J = 16.0 Hz, 1H), 7.09 (d, J = 4.0 Hz, 1H), 7.06 (d, J = 4.0 Hz, 1H), 3.04 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.3, 164.6, 142.3, 132.6, 131.3, 131.1, 119.9, 116.2, 21.3; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_9H_8N_4SBr$ 282.9648; Found 282.9645.

Synthesis of (*E*)-2-(2-(2-(2-methoxyethoxy)ethoxy)ethyl)-6-(5-(2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)thiophen-2-yl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz13)[9]

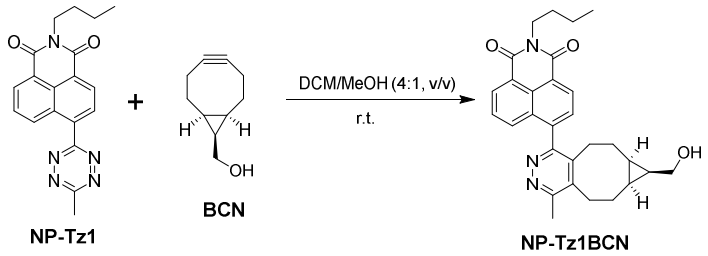
To a solution of **S19** (94 mg, 0.20 mmol) in 1,4-dioxane (10 mL) was added **S20** (57 mg, 0.20 mmol), $Pd(dppf)Cl_2$ (15 mg, 0.02 mmol) and Cs_2CO_3 (195 mg, 0.60 mmol). The reaction mixture was stirred at 100 °C using an oil bath for 3 h under argon, then concentrated. The residue was purified by silica gel flash column chromatography (PE/EtOAc = 1:1) to afford the titled compound as pink solid (36 mg, 33% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.68 – 8.63 (m, 2H), 8.62 (d, J = 7.6 Hz, 1H), 8.44 (d, J = 16.0 Hz, 1H), 7.87 (d, J = 7.6 Hz, 1H), 7.80 (dd, J = 8.4, 7.6 Hz, 1H), 7.48 (d, J = 3.8 Hz, 1H), 7.35 (d, J = 3.8 Hz, 1H), 7.32 (d, J = 16.0 Hz, 1H), 4.46 (t, J = 6.1 Hz, 2H), 3.84 (t, J = 6.1 Hz, 2H), 3.75 – 3.68 (m, 2H), 3.66 – 3.61 (m, 2H), 3.61 – 3.56 (m, 2H), 3.49 – 3.41 (m, 2H), 3.32 (s, 3H), 3.06 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.4, 164.7, 164.2, 163.9, 142.7, 142.7, 138.3, 132.9, 132.1, 131.7, 131.5, 130.8, 130.1, 129.9, 129.1, 128.8, 127.6, 123.2, 122.6, 120.7, 72.0, 70.8, 70.7, 70.4, 68.1, 59.1, 39.4, 21.4; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{28}H_{28}O_5N_5S$ 546.1806; Found 546.1807.

General procedure C for the synthesis of Tz1BCN ~ Tz13BCN

To a solution of **NP-Tz1** ~ **NP-Tz13** (1 eq) in DCM/MeOH (5 mL, 4:1) was added bicyclononyne (BCN, 2 eq). The reaction mixture was stirred at room temperature for 10 min, then concentrated. The residue was purified by silica gel flash column chromatography (DCM/MeOH = 20:1) to afford the titled compound.

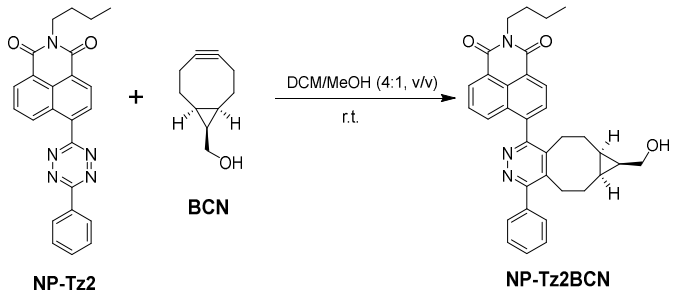
Synthesis of 2-butyl-6-(7-(hydroxymethyl)-4-methyl-6,6a,7,7a,8,9-hexahydro-5*H*-cyclopropa[5,6]cycloocta[1,2-*d*]pyridazin-1-yl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)

-dione (NP-Tz1BCN)



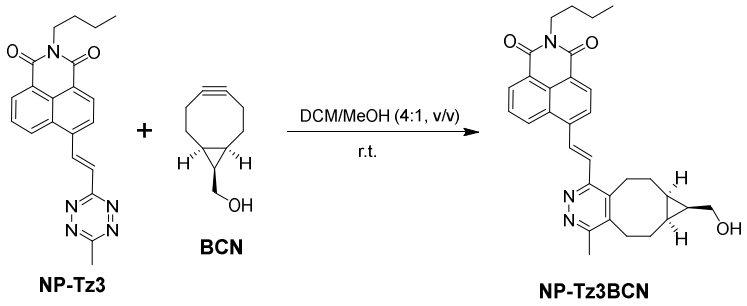
The title compound was synthesized according to General Method C. Yellow solid; 64% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.69 (t, J = 7.6 Hz, 1H), 8.62 (d, J = 7.2 Hz, 1H), 7.80 – 7.61 (m, 3H), 4.26 – 4.17 (m, 2H), 3.70 (dd, J = 10.0, 8.4 Hz, 2H), 3.16 – 2.88 (m, 2H), 2.86 (d, J = 4.2 Hz, 3H), 2.80 – 2.63 (m, 1H), 2.62 – 2.36 (m, 2H), 1.95 – 1.84 (m, 1H), 1.83 – 1.66 (m, 3H), 1.61 – 1.38 (m, 4H), 1.16 – 1.09 (m, 1H), 0.99 (t, J = 7.4 Hz, 3H), 0.92 – 0.70 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.2, 164.1, 159.2, 158.9, 142.2, 141.9, 140.9, 140.3, 131.9, 131.6, 130.7, 130.5, 128.3, 128.1, 127.5, 123.2, 59.3, 50.9, 40.5, 30.4, 28.6, 27.9, 27.5, 27.0, 21.7, 20.8, 20.8, 20.5, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{29}H_{32}O_3N_3$ 470.2438; Found 470.2436.

Synthesis of 2-butyl-6-(7-(hydroxymethyl)-4-phenyl-6,6a,7,7a,8,9-hexahydro-5*H*-cyclopropa[5,6]cycloocta[1,2-*d*]pyridazin-1-yl)-1H-benzo[*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz2BCN)



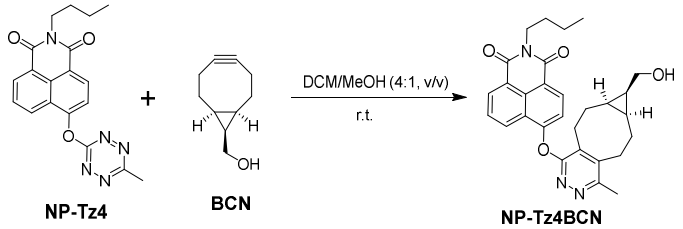
The title compound was synthesized according to General Method C. Yellow solid; 70% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.74 – 8.57 (m, 2H), 7.91 – 7.80 (m, 1H), 7.80 – 7.64 (m, 2H), 7.60 (brs, 2H), 7.58 – 7.47 (m, 3H), 4.30 – 4.18 (m, 2H), 3.72 (d, J = 7.6 Hz, 2H), 3.09 – 2.61 (m, 4H), 2.51 – 2.15 (m, 2H), 1.98 – 1.67 (m, 5H), 1.51 – 1.45 (m, 2H), 1.22 – 1.10 (m, 2H), 1.01 (t, J = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.2, 164.0, 161.2, 158.8, 141.6, 135.7, 132.1, 131.7, 131.6, 130.8, 130.6, 130.4, 130.4, 129.5, 129.2, 128.6, 128.4, 128.2, 127.6, 123.5, 123.4, 123.1, 59.4, 45.3, 40.5, 30.4, 29.8, 28.2, 25.1, 22.8, 22.3, 20.6, 18.7, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{34}H_{34}O_3N_3$ 532.2595; Found 532.2605.

Synthesis of (*E*)-2-butyl-6-(2-(7-(hydroxymethyl)-4-methyl-6,6a,7,7a,8,9-hexahydro-5*H*-cyclopropa[5,6]cycloocta[1,2-*d*]pyridazin-1-yl)vinyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz3BCN)



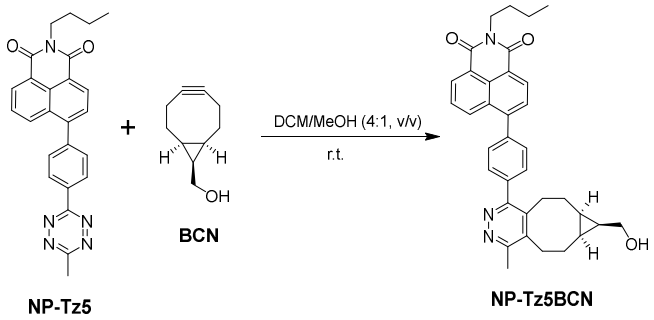
The title compound was synthesized according to General Method C. Yellow solid; 55% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.80 (d, J = 15.6 Hz, 1H), 8.68 (d, J = 8.4 Hz, 1H), 8.63 (dd, J = 7.2, 0.8 Hz, 1H), 8.60 (d, J = 7.6 Hz, 1H), 8.04 (d, J = 7.6 Hz, 1H), 7.79 (dd, J = 8.4, 7.6 Hz, 1H), 7.59 (d, J = 15.6 Hz, 1H), 4.23 – 4.14 (m, 2H), 3.74 (d, J = 7.6 Hz, 2H), 3.16 – 2.87 (m, 4H), 2.77 (s, 3H), 2.50 – 2.33 (m, 2H), 1.80 – 1.55 (m, 5H), 1.52 – 1.37 (m, 3H), 1.19 – 1.09 (m, 1H), 0.98 (t, J = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.3, 164.2, 158.0, 154.4, 141.0, 140.3, 139.1, 131.5, 131.0, 130.7, 130.5, 130.1, 128.7, 127.9, 127.2, 124.5, 123.2, 122.4, 59.5, 40.4, 30.4, 29.8, 27.3, 26.3, 23.4, 23.0, 22.2, 21.2, 20.5, 19.0, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{31}H_{34}O_3N_3$ 496.2595; Found 496.2629.

Synthesis of 2-butyl-6-((7-(hydroxymethyl)-4-methyl-6,6a,7,7a,8,9-hexahydro-5*H*-cyclopropa[5,6]cycloocta[1,2-*d*]pyridazin-1-yl)oxy)-1*H*-benzo[*de*]isoquinoline-1,3(2H)-dione (NP-Tz4BCN)



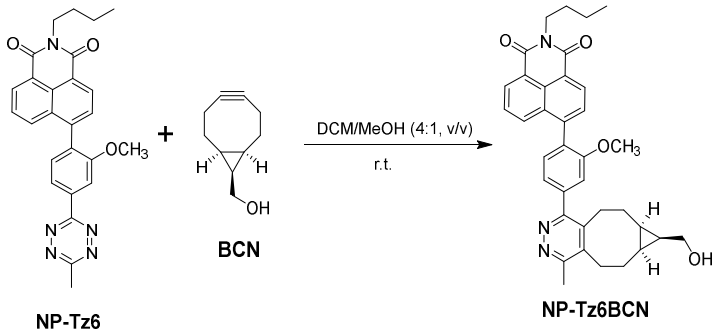
The title compound was synthesized according to General Method C. Yellow solid; 62% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.62 (d, J = 8.0 Hz, 1H), 8.54 (d, J = 8.4 Hz, 1H), 8.41 (d, J = 8.4 Hz, 1H), 7.77 – 7.68 (m, 1H), 7.33 (d, J = 8.2 Hz, 1H), 4.21 – 4.13 (m, 2H), 3.76 (d, J = 7.8 Hz, 2H), 3.25 – 3.11 (m, 1H), 3.11 – 2.98 (m, 2H), 2.97 – 2.83 (m, 1H), 2.67 (s, 3H), 2.47 – 2.33 (m, 2H), 1.78 – 1.54 (m, 5H), 1.51 – 1.38 (m, 2H), 1.22 – 1.12 (m, 1H), 1.01 – 0.88 (m, 5H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.4, 163.8, 163.6, 157.7, 156.4, 144.3, 132.4, 132.1, 131.8, 129.8, 128.3, 126.9, 125.1, 123.1, 118.8, 116.0, 59.5, 40.4, 30.4, 27.7, 24.3, 23.2, 22.6, 22.0, 20.6, 20.5, 18.8, 18.5, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{29}H_{32}O_4N_3$ 486.2387; Found 486.2382.

Synthesis of 2-butyl-6-(4-(7-(hydroxymethyl)-4-methyl-6,6a,7,7a,8,9-hexahydro-5*H*-cyclopropa[5,6]cycloocta[1,2-*d*]pyridazin-1-yl)phenyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz5BCN)



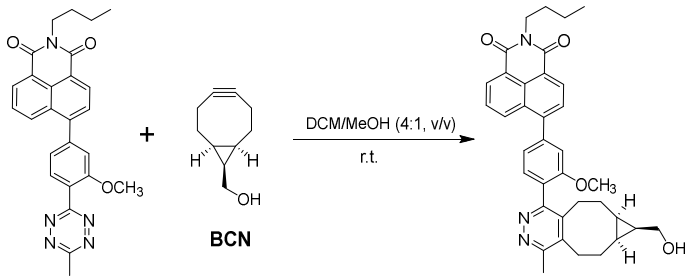
The title compound was synthesized according to General Method C. White solid; 59% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.65 (t, J = 7.6 Hz, 2H), 8.34 (d, J = 9.2 Hz, 1H), 7.77 – 7.69 (m, 2H), 7.68 – 7.59 (m, 4H), 4.27 – 4.16 (m, 2H), 3.76 (d, J = 7.8 Hz, 2H), 3.14 – 2.84 (m, 4H), 2.80 (s, 3H), 2.52 – 2.37 (m, 1H), 2.37 – 2.21 (m, 1H), 1.85 – 1.67 (m, 3H), 1.52 – 1.39 (m, 3H), 1.21 – 1.13 (m, 1H), 1.07 – 0.91 (m, 5H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.4, 164.3, 160.5, 158.1, 146.3, 140.9, 139.3, 139.1, 138.7, 132.7, 131.4, 130.9, 130.2, 130.0, 129.8, 128.8, 128.0, 127.1, 123.1, 122.2, 59.5, 40.4, 30.4, 29.8, 28.3, 27.4, 27.3, 27.3, 22.6, 22.5, 20.9, 20.5, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{35}H_{36}O_3N_3$ 546.2751; Found 546.2757.

Synthesis of 2-butyl-6-(4-(7-(hydroxymethyl)-4-methyl-6,6a,7,7a,8,9-hexahydro-5*H*-cyclopropa[5,6]cycloocta[1,2-*d*]pyridazin-1-yl)-2-methoxyphenyl)-1*H*-benzo[de]isoquinoline-1,3(2*H*)-dione (NP-Tz6BCN)



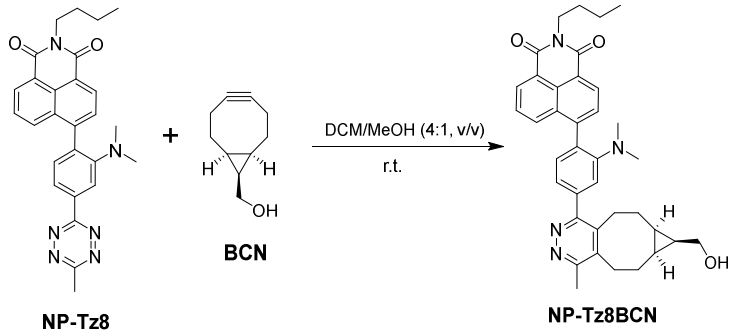
The title compound was synthesized according to General Method C. White solid; 52% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.70 – 8.56 (m, 2H), 8.03 (d, J = 8.4 Hz, 1H), 7.75 – 7.63 (m, 2H), 7.40 – 7.31 (m, 1H), 7.26 – 7.20 (m, 1H), 7.15 (t, J = 7.2 Hz, 1H), 4.27 – 4.12 (m, 2H), 3.79 – 3.68 (m, 5H), 3.14 – 2.83 (m, 4H), 2.79 (s, 3H), 2.51 – 2.36 (m, 1H), 2.36 – 2.18 (m, 1H), 1.77 – 1.69 (m, 2H), 1.61 (s, 2H), 1.51 – 1.41 (m, 2H), 1.21 – 1.15 (m, 1H), 1.08 – 0.91 (m, 5H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.5, 164.4, 160.6, 158.1, 156.9, 143.6, 141.0, 140.4, 139.4, 133.2, 131.2, 130.9, 130.7, 128.6, 128.5, 127.8, 126.7, 123.0, 122.2, 121.8, 121.8, 112.6, 59.5, 55.8, 40.4, 32.1, 30.4, 29.8, 28.3, 28.2, 27.3, 22.8, 22.6, 20.9, 20.6, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{36}H_{38}O_4N_3$ 576.2857; Found 576.2861.

Synthesis of 2-butyl-6-(4-(7-(hydroxymethyl)-4-methyl-6,6a,7,7a,8,9-hexahydro-5*H*-cyclopropa[5,6]cycloocta[1,2-*d*]pyridazin-1-yl)-3-methoxyphenyl)-1H-benzo[*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz7BCN)



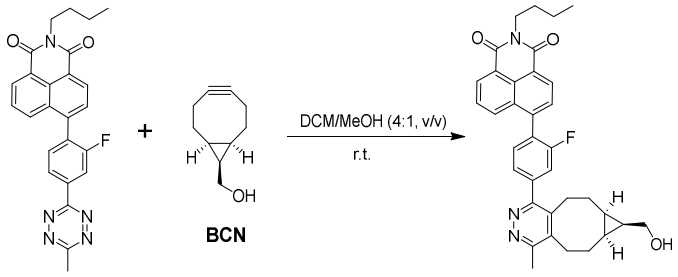
The title compound was synthesized according to General Method C. White solid; 60% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.70 – 8.62 (m, 2H), 8.38 (d, J = 8.4 Hz, 1H), 7.80 – 7.70 (m, 2H), 7.51 – 7.38 (m, 1H), 7.23 – 7.17 (m, 1H), 7.09 (dd, J = 9.6, 1.2 Hz, 1H), 4.27 – 4.18 (m, 2H), 3.83 – 3.70 (m, 5H), 3.14 – 2.98 (m, 1H), 2.98 – 2.83 (m, 2H), 2.83 – 2.67 (m, 4H), 2.52 – 2.36 (m, 1H), 2.23 – 2.05 (m, 1H), 1.81 – 1.71 (m, 3H), 1.71 – 1.56 (m, 2H), 1.54 – 1.40 (m, 3H), 1.19 – 1.09 (m, 1H), 0.99 (t, J = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.5, 164.3, 158.8, 158.3, 157.3, 157.0, 146.6, 141.0, 132.8, 131.4, 131.2, 130.9, 130.2, 128.8, 127.9, 127.9, 127.1, 123.1, 122.7, 122.2, 112.6, 112.2, 59.6, 55.7, 40.5, 30.4, 29.8, 28.4, 28.3, 27.3, 27.1, 23.9, 22.1, 20.8, 20.6, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{36}H_{38}O_4N_3$ 576.2857; Found 576.2866.

Synthesis of 2-butyl-6-(2-(dimethylamino)-4-(7-(hydroxymethyl)-4-methyl-6,6a,7,7a,8,9-hexahydro-5H-cyclopropa[5,6]cycloocta[1,2-d]pyridazin-1-yl)phenyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (NP-Tz8BCN)



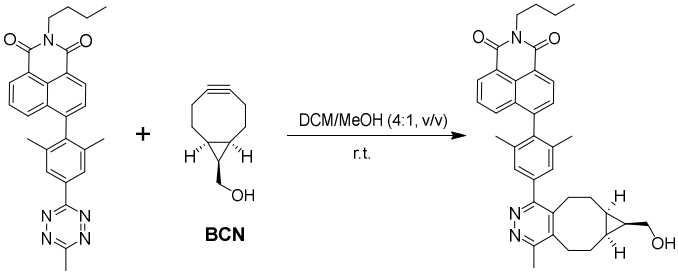
The title compound was synthesized according to General Method C. Yellow solid; 63% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.66 (d, J = 7.6 Hz, 1H), 8.62 (d, J = 6.4 Hz, 1H), 8.18 – 8.12 (m, 1H), 7.82 (d, J = 7.6 Hz, 1H), 7.69 – 7.63 (m, 1H), 7.28 – 7.23 (m, 2H), 7.10 (td, J = 7.6, 7.2, 1.6 Hz, 1H), 4.27 – 4.15 (m, 2H), 3.76 (d, J = 7.6 Hz, 2H), 3.14 – 2.84 (m, 4H), 2.79 (s, 3H), 2.53 – 2.39 (m, 7H), 2.37 – 2.23 (m, 1H), 1.83 – 1.70 (m, 3H), 1.53 – 1.37 (m, 3H), 1.20 – 1.12 (m, 1H), 1.08 – 0.92 (m, 5H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.5, 164.3, 161.0, 157.9, 151.8, 146.8, 140.9, 139.6, 139.4, 133.4, 132.4, 131.4, 131.2, 130.4, 129.7, 128.8, 128.2, 126.6, 122.9, 121.9, 121.7, 119.2, 59.4, 43.2, 40.4, 30.4, 29.8, 29.4, 28.3, 27.3, 24.7, 22.8, 22.6, 20.8, 20.5, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{37}H_{41}O_3N_4$ 589.3173; Found 589.3159.

Synthesis of 2-butyl-6-(2-fluoro-4-(7-(hydroxymethyl)-4-methyl-6,6a,7,7a,8,9-hexahydro-5*H*-cyclopropa[5,6]cycloocta[1,2-*d*]pyridazin-1-yl)phenyl)-1*H*-benzo[de]isoquinoline-1,3(2*H*)-dione (NP-Tz9BCN)



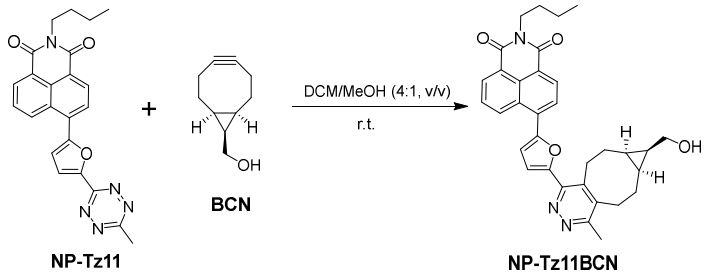
The title compound was synthesized according to General Method C. White solid; 49% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.68 (d, J = 7.2 Hz, 1H), 8.65 (d, J = 7.2 Hz, 1H), 8.15 – 8.09 (m, 1H), 7.80 – 7.70 (m, 2H), 7.53 (t, J = 7.6 Hz, 1H), 7.48 – 7.37 (m, 2H), 4.26 – 4.17 (m, 2H), 3.76 (d, J = 8.0 Hz, 2H), 3.14 – 2.84 (m, 4H), 2.81 (s, 3H), 2.55 – 2.38 (m, 1H), 2.38 – 2.23 (m, 1H), 1.82 – 1.67 (m, 3H), 1.53 – 1.34 (m, 3H), 1.22 – 1.14 (m, 1H), 1.08 – 0.90 (m, 5H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.3, 164.2, 160.3, 159.5, 158.7, 158.4, 141.3, 141.3, 141.1, 140.1, 139.2, 132.4, 132.0, 131.9, 131.5, 130.7, 130.4, 128.9, 128.6, 127.3, 126.5, 126.4, 125.7, 123.2, 122.9, 117.5, 117.3, 59.5, 40.5, 30.4, 29.8, 28.2, 27.4, 24.5, 22.6, 20.9, 20.5, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{35}H_{35}O_3N_3F$ 564.2657; Found 564.2662.

Synthesis of 2-butyl-6-(4-(7-(hydroxymethyl)-4-methyl-6,6a,7,7a,8,9-hexahydro-5*H*-cyclopropa[5,6]cycloocta[1,2-*d*]pyridazin-1-yl)-2,6-dimethylphenyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz10BCN)



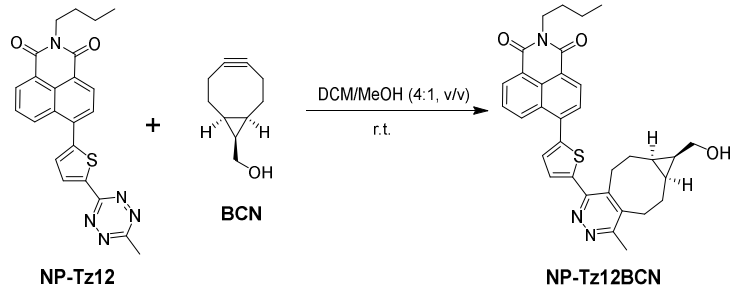
The title compound was synthesized according to General Method C. White solid; 56% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.69 (d, J = 7.2 Hz, 1H), 8.63 (dd, J = 7.2, 1.2 Hz, 1H), 7.80 (dd, J = 8.4, 1.2 Hz, 1H), 7.68 – 7.62 (m, 1H), 7.60 (d, J = 7.6 Hz, 1H), 7.29 (d, J = 7.6 Hz, 2H), 4.27 – 4.16 (m, 2H), 3.76 (d, J = 7.6 Hz, 2H), 3.14 – 2.82 (m, 4H), 2.78 (s, 3H), 2.50 – 2.35 (m, 1H), 2.35 – 2.17 (m, 1H), 1.94 (s, 6H), 1.78 – 1.71 (m, 2H), 1.61 (s, 2H), 1.52 – 1.42 (m, 2H), 1.20 – 1.12 (m, 1H), 1.07 – 0.90 (m, 5H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.4, 164.3, 160.9, 157.9, 145.7, 140.8, 139.3, 138.4, 138.0, 136.6, 136.5, 131.9, 131.5, 131.2, 130.3, 128.6, 127.8, 127.3, 123.3, 122.2, 59.5, 58.5, 53.6, 40.4, 30.4, 28.3, 27.3, 24.5, 22.4, 20.8, 20.7, 20.5, 18.6, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{37}H_{40}O_3N_3$ 574.3064; Found 574.3097.

Synthesis of 2-butyl-6-(5-(7-(hydroxymethyl)-4-methyl-6,6a,7,7a,8,9-hexahydro-5*H*-cyclopropa[5,6]cycloocta[1,2-*d*]pyridazin-1-yl)furan-2-yl)-1H-benzo[*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz11BCN)



The title compound was synthesized according to General Method C. Yellow solid; 51% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.87 (dd, J = 8.8, 1.2 Hz, 1H), 8.63 (dd, J = 7.2, 1.2 Hz, 1H), 8.61 (d, J = 8.0 Hz, 1H), 8.01 (d, J = 8.0 Hz, 1H), 7.79 (dd, J = 8.8, 7.2 Hz, 1H), 7.31 (d, J = 3.6 Hz, 1H), 7.11 (d, J = 3.6 Hz, 1H), 4.23 – 4.12 (m, 2H), 3.73 (d, J = 8.0 Hz, 2H), 3.20 (s, 2H), 3.10 – 2.99 (m, 1H), 2.98 – 2.85 (m, 1H), 2.77 (s, 3H), 2.51 – 2.35 (m, 2H), 1.85 – 1.66 (m, 4H), 1.52 – 1.36 (m, 3H), 1.20 – 1.10 (m, 1H), 1.10 – 0.89 (m, 5H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.2, 163.9, 157.9, 153.9, 152.8, 150.7, 141.5, 138.9, 133.7, 132.0, 131.5, 130.8, 129.1, 128.2, 127.5, 126.0, 123.2, 122.1, 114.3, 59.4, 40.4, 30.3, 29.8, 27.7, 27.3, 24.2, 22.8, 21.1, 20.5, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{33}H_{34}O_4N_3$ 536.2544; Found 536.2533.

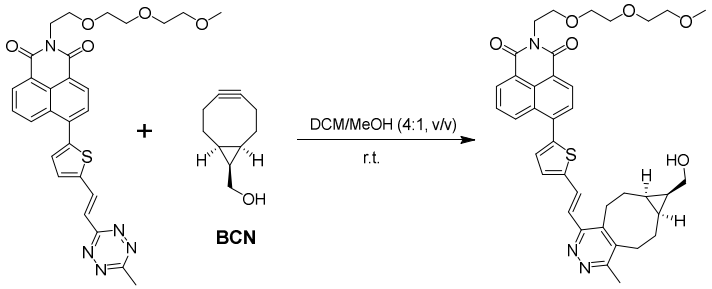
Synthesis of 2-butyl-6-(5-(7-(hydroxymethyl)-4-methyl-6,6a,7,7a,8,9-hexahydro-5*H*-cyclopropa[5,6]cycloocta[1,2-*d*]pyridazin-1-yl)thiophen-2-yl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz12BCN)



The title compound was synthesized according to General Method C. Yellow solid; 54% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.73 (dd, J = 8.4, 0.8 Hz, 1H), 8.65 (dd, J = 7.2, 0.8 Hz, 1H), 8.62 (d, J = 7.6 Hz, 1H), 7.89 (d, J = 7.6 Hz, 1H), 7.78 (dd, J = 8.4, 7.2 Hz, 1H), 7.44 (d, J = 3.6 Hz, 1H), 7.37 (d, J = 3.6 Hz, 1H), 4.26 – 4.14 (m, 2H), 3.76 (d, J = 7.7 Hz, 2H), 3.20 (brs, 2H), 3.08 (dd, J = 14.5, 8.7 Hz, 1H), 2.95 – 2.83 (m, 1H), 2.77 (s, 3H), 2.52 – 2.37 (m, 2H), 1.78 – 1.70 (m, 2H), 1.68 – 1.52 (m, 2H), 1.52 – 1.39 (m, 3H), 1.23 – 1.17 (m, 1H), 1.08 – 0.95 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.3, 164.0, 157.6, 154.2, 142.9, 142.2, 141.4, 139.0, 138.7, 132.4, 131.6, 130.8, 130.0, 129.1, 129.0, 128.7, 128.7, 127.4, 123.1, 122.3, 59.5, 40.5, 32.1, 31.6, 30.4, 29.8, 28.1, 27.4, 25.0, 22.8, 20.9, 20.5, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{33}H_{34}O_3N_3S$ 552.2315; Found 552.2316.

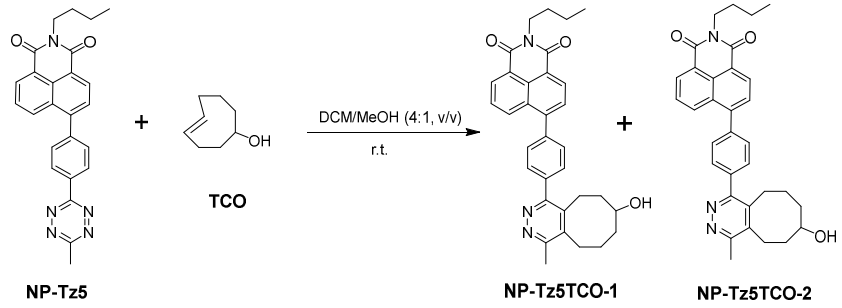
Synthesis of (*E*)-6-(5-(2-(7-(hydroxymethyl)-4-methyl-6,6a,7,7a,8,9-hexahydro-

5*H*-cyclopropa[5,6]cycloocta[1,2-*d*]pyridazin-1-yl)vinyl)thiophen-2-yl)-2-(2-(2-(2-methoxyethoxy)ethoxy)ethyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz13BCN)



The title compound was synthesized according to General Method C. Yellow solid; 61% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.70 (dd, J = 8.4, 1.2 Hz, 1H), 8.65 (dd, J = 7.6, 1.2 Hz, 1H), 8.60 (d, J = 7.6 Hz, 1H), 8.16 (d, J = 15.2 Hz, 1H), 7.85 (d, J = 7.6 Hz, 1H), 7.79 (dd, J = 8.4, 7.6 Hz, 1H), 7.35 – 7.29 (m, 2H), 7.25 (d, J = 15.2 Hz, 1H), 4.46 (t, J = 6.0 Hz, 2H), 3.84 (t, J = 6.0 Hz, 2H), 3.77 – 3.69 (m, 4H), 3.65 – 3.61 (m, 2H), 3.61 – 3.56 (m, 2H), 3.47 – 3.43 (m, 2H), 3.31 (s, 3H), 3.10 – 2.86 (m, 4H), 2.75 (s, 3H), 2.47 – 2.33 (m, 2H), 1.36 – 1.25 (m, 1H), 1.17 – 1.11 (m, 1H), 0.94 – 0.76 (m, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.3, 164.0, 149.8, 144.7, 139.9, 139.1, 138.9, 132.4, 132.4, 131.7, 130.8, 130.0, 129.9, 129.5, 129.1, 128.6, 128.3, 127.5, 123.1, 122.2, 121.9, 121.8, 72.0, 70.8, 70.7, 70.4, 68.1, 59.5, 59.1, 39.4, 29.8, 27.3, 27.2, 26.3, 21.0, 18.9, 18.4, 17.8; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{38}H_{42}O_3N_6S$ 668.2789; Found 668.2777.

Synthesis of 2-butyl-6-(4-(7/8-hydroxy-4-methyl-5,6,7,8,9,10-hexahydrocycloocta[*d*]pyridazin-1-yl)phenyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (NP-Tz5TCO-1/2)



To a solution of **NP-Tz5** (17 mg, 0.04 mmol) in DCM/MeOH (5 mL, 4:1) was added trans-cyclooctene (TCO, 10 mg, 0.08 mmol). The reaction mixture was stirred at room temperature for 10 min, then concentrated. The residue was loaded on the preparative thin layer chromatography (Pre-TLC) plate, photoirradiated with 365 nm UV light for 10 min, then eluted (DCM/MeOH = 30:1) to afford the titled compounds.

NP-Tz5TCO-1: White solid; R_f = 0.5; 28% yield (6 mg); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.66 (t, J = 7.2 Hz, 2H), 8.33 (d, J = 8.4 Hz, 1H), 7.78 – 7.70 (m, 2H), 7.68 – 7.59 (m, 4H), 4.27 – 4.17 (m, 2H), 3.77 – 3.66 (m, 1H), 3.03 – 2.94 (m, 1H), 2.91 (t,

J = 6.4 Hz, 2H), 2.87 – 2.80 (m, 1H), 2.79 (s, 3H), 2.07 – 1.95 (m, 2H), 1.89 – 1.81 (m, 1H), 1.80 – 1.70 (m, 5H), 1.68 – 1.63 (m, 1H), 1.52 – 1.43 (m, 2H), 1.00 (t, J = 7.6 Hz, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.4, 164.3, 160.3, 158.0, 146.3, 139.2, 138.7, 138.4, 138.0, 132.6, 131.4, 130.9, 130.1, 130.1, 129.6, 128.8, 128.0, 127.2, 123.1, 122.2, 71.6, 40.5, 39.4, 35.9, 30.4, 29.8, 27.2, 24.4, 24.2, 20.6, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{33}H_{34}O_3N_3$ 520.2595; Found 520.2593.

NP-Tz5TCO-2: White solid; R_f = 0.45; 33% yield (7 mg); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.66 (t, J = 7.6 Hz, 2H), 8.34 (dd, J = 8.4, 1.2 Hz, 1H), 7.78 – 7.70 (m, 2H), 7.67 – 7.59 (m, 4H), 4.27 – 4.18 (m, 2H), 3.75 – 3.64 (m, 1H), 3.11 – 3.00 (m, 1H), 2.91 – 2.85 (m, 2H), 2.83 (s, 3H), 2.26 – 2.14 (m, 1H), 1.89 – 1.81 (m, 2H), 1.78 – 1.63 (m, 7H), 1.51 – 1.45 (m, 2H), 1.00 (t, J = 7.6 Hz, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.5, 164.3, 160.4, 157.8, 146.3, 139.2, 138.3, 137.8, 132.7, 131.4, 130.9, 130.1, 130.0, 129.6, 128.9, 128.0, 127.2, 123.1, 122.2, 71.9, 40.5, 37.2, 35.8, 30.4, 29.9, 27.4, 26.5, 24.1, 20.6, 14.0; HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{33}H_{34}O_3N_3$ 520.2595; Found 520.2589.

Absorption and fluorescence spectra of probes

The stock solution of each 1,8-naphthalimide probe was prepared at 10 mM in DMSO. A fresh work solution of corresponding probe was prepared by diluting the stock solution to DMSO/H_2O mixture solution (f_w = 0 to 100%) to make a final concentration of 10 μM. The absorption and fluorescence spectra were recorded using a Tecan Spark™ 10M Multimode Microplate Reader. The data were subtracted from the background absorbance or fluorescence signal of solvent for the same time period.

X-ray single crystal diffraction

Suitable crystals of NP-Tz5TCO-2 were obtained by slowly evaporating a mixture of chloroform and methanol solution (1:1, v/v) at ambient temperature. A colorless crystal was mounted on a glass fiber at a random orientation. The data were collected at 100 K by a diffractometer Rigaku Oxford Diffraction Supernova Dual Source, Cu at Zero equipped with an AtlasS2 CCD using Cu Kα radiation (1.54178 Å), and processed using CrysAlisPro. The structures were solved by direct methods using Olex2 software, and the nonhydrogen atoms were located from the trial structure and then refined anisotropically with SHELXL-2018 using a full-matrix least squares procedure based on F^2. The weighted R factor, wR and goodness-of-fit S values were obtained based on F^2. The hydrogen atom positions were fixed geometrically at the calculated distances and allowed to ride on the parent atoms. Crystallographic data for the structure reported in this paper have been deposited at the Cambridge Crystallographic Data Center and allocated with the deposition numbers: CCDC 2121898.

SEM images

The samples of NP-Tz5BCN, NP-Tz7BCN, NP-Tz8BCN, and NP-Tz9BCN were obtained by preparing DMSO/H_2O mixed solution (f_w = 99%) and still standing for 1 h, then casting one drop of the solution onto the surface of cleaned glass. The samples

were air-dried at room temperature, then submitted for SEM imaging.

Reaction kinetics measurement

The iEDDA reaction kinetics measurements were performed at room temperature in TECAN fluorescence plate reader equipped with automatic injection unit. To a solution of 10 μM NP-Tz5/6/11/12/13 in DMSO in 96-well plate was added 10-fold excess of BCN. The well plate was inserted into plate reader and the measurement was immediately started. The solution was excited at the 350 nm, and the fluorescence intensities were recorded over every 15 seconds at λ_{em} of each probe. The reaction kinetics was hypothesized as pseudo-first order reaction, and the observed rate constants (k_{obs}) were calculated using the one phase exponential association equation. The second order rate constants (k_2) were calculated using the equation: $k_2 = k_{obs}/[BCN]$.

BSA protein labeling

BSA-BCN conjugate (BCN-modified BSA protein) was prepared according to the literature procedure.[11] For fluorogenic BSA labeling, 1 μL of 10 mM NP-Tz5/6/7/8/11/12/13 in DMSO was mixed with 10 μL of unmodified BSA or BSA-BCN conjugate (2 mg/mL, 30 μM) in PBS (pH 7.4), then incubated at r.t. for 1 h. The mixture was analysed by SDS-PAGE and the fluorescence images were recorded. Finally, the gel was subjected to coomassie blue staining.

Confocal fluorescence imaging experiments

HeLa cells were seeded into 35-mm glass-bottom dishes and cultured for 24 h, followed by incubation with MitoTracker red (200 nM) and BCN-TPP (1 μM) in DMEM at 37 °C and 5% CO_2 for 1 h. Then the medium was removed and the cells were washed with PBS (pH 7.4) three times. After incubation with NP-Tz11 or NP-Tz13 (1 μM) in DMEM for another 1 h, the cells were directly submitted for laser scanning confocal microscopy without washing steps. The confocal fluorescence images were acquired using Leica TCS SP8 X equipped with 63×objective and appropriate filter (NP-Tz11: excitation = 405 nm, emission = 488~551 nm; NP-Tz13: excitation = 405 nm, emission = 515~600 nm; MitoTracker red: excitation = 579 nm, emission = 585~670 nm). The control cells were treated with MitoTracker red (200 nM) and NP-Tz11 or NP-Tz13 (1 μM) in DMEM in the absence of BCN-TPP.

Reference

[1] B. Pinto-Pacheco, W. P. Carbery, S. Khan, D. B. Turner and D. Buccella, *Angew. Chem. Int. Ed.*, 2020, 59, 22140-22149.

[2] W. D. Lambert, Y. Fang, S. Mahapatra, Z. Huang, C. W. Am Ende and J. M. Fox, *J. Am. Chem. Soc.*, 2019, **141**, 17068-17074.

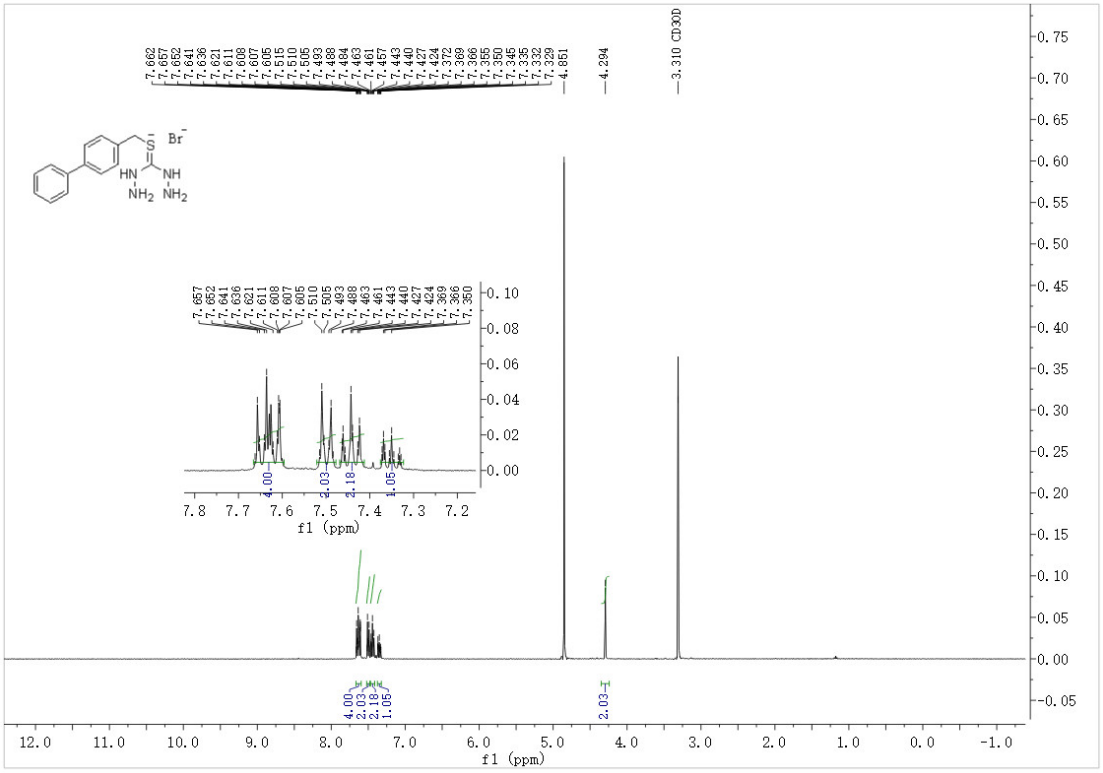
[3] W. Sun, Z. Ma, J. Li, W. Li, L. Du and M. Li, *Sci. Chin. Chem.*, 2013, **56**, 1440-1445.

[4] Y. Cai, Z. Guo, J. Chen, W. Li, L. Zhong, Y. Gao, L. Jiang, L. Chi, H. Tian and W. H. Zhu, *J. Am. Chem. Soc.*, 2016, **138**, 2219-2224.

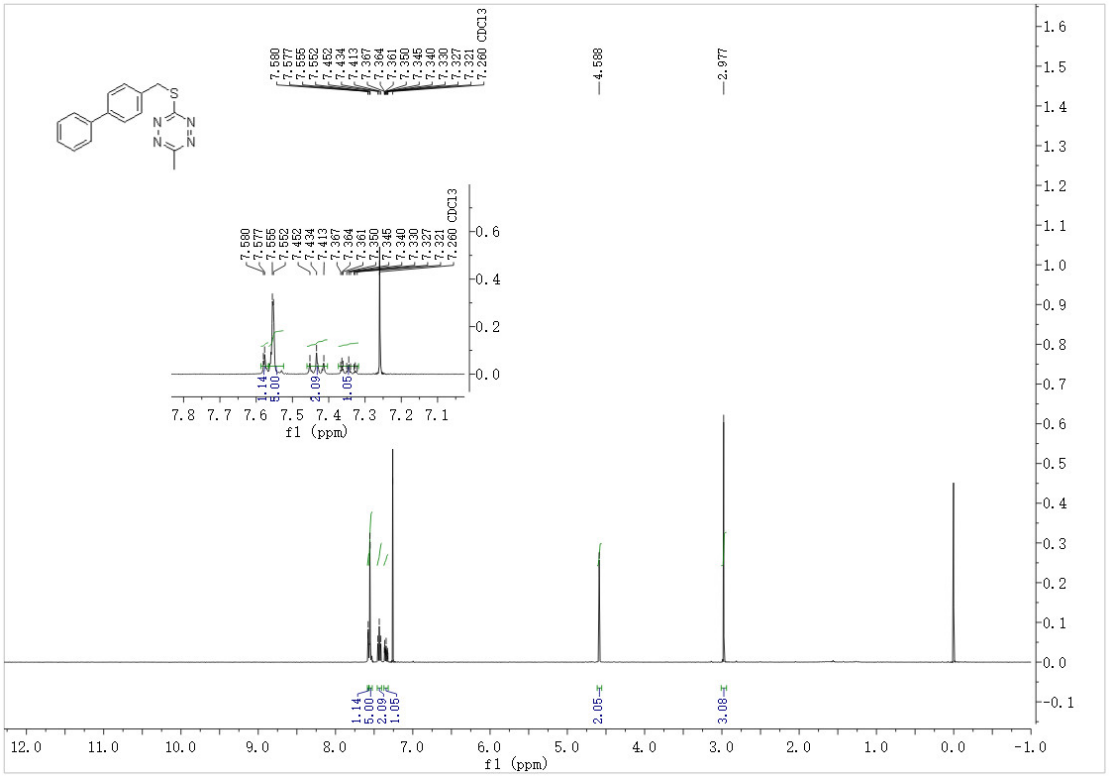
[5] W. Mao, W. Shi, J. Li, D. Su, X. Wang, L. Zhang, L. Pan, X. Wu and H. Wu, *Angew. Chem.*

Int. Ed., 2019, **58**, 1106-1109.
[6] H. Lan, B. Liu, G. Lv, Z. Li, X. Yu, K. Liu, X. Cao, H. Yang, S. Yang and T. Yi, *Sensor. Actuat. B-Chem.*, 2012, **173**, 811-816.
[7] W. Shu, L. Yan, J. Liu, Z. Wang, S. Zhang, C. Tang, C. Liu, B. Zhu and B. Du, *Ind. Eng. Chem. Res.*, 2015, **54**, 8056-8062.
[8] A. Wieczorek, P. Werther, J. Euchner and R. Wombacher, *Chem. Sci.*, 2017, **8**, 1506-1510.
[9] E. Kozma, G. Estrada Girona, G. Paci, E. A. Lemke and P. Kele, *Chem. Commun.*, 2017, **53**, 6696-6699.
[10] X. Liang, X. Xu, D. Qiao, Z. Yin and L. Shang, *Chem. Asian. J.*, 2017, **12**, 3187-3194.
[11] C. Favre and F. Friscourt, *Org. Lett.*, 2018, **20**, 4213-4217.

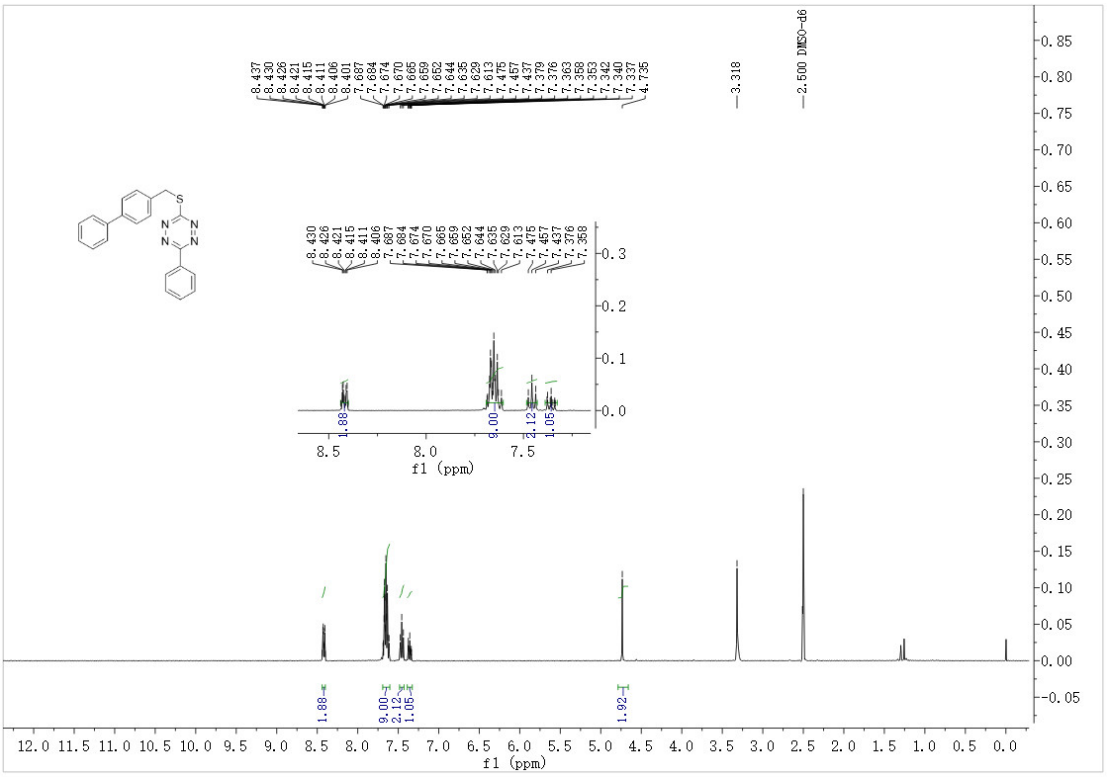
^{1}H NMR of compound **S1** (400 MHz in Methanol-d_4)



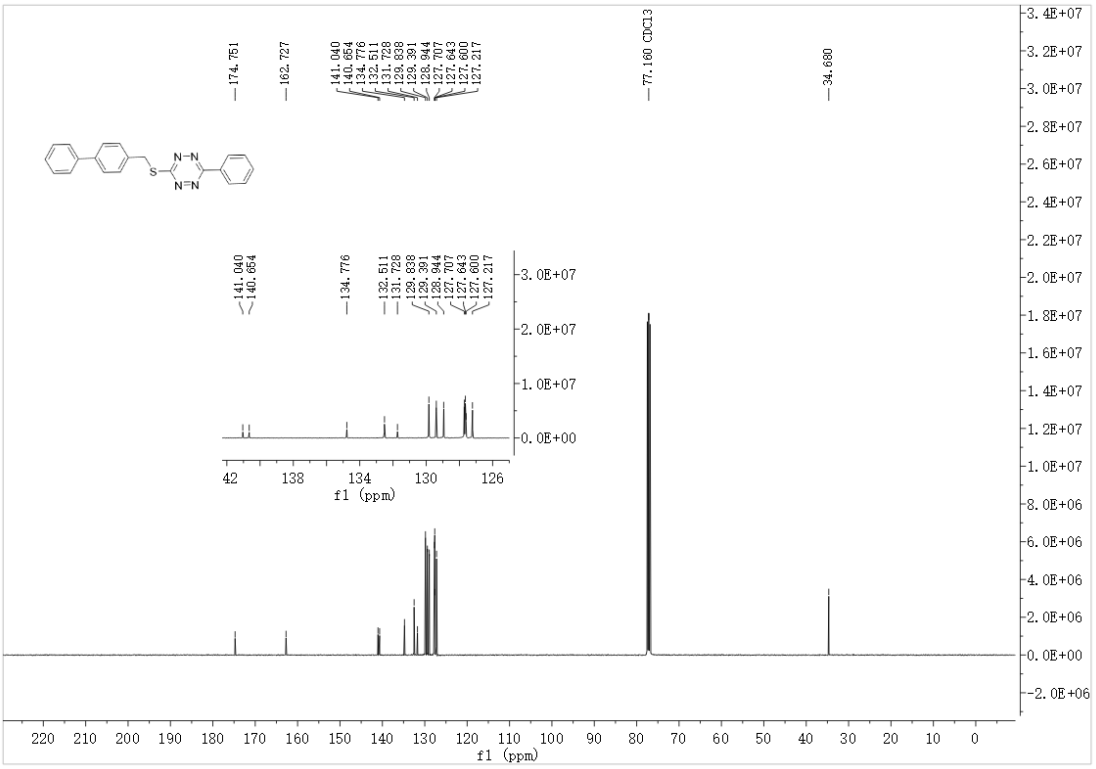
^{1}H NMR of compound **S2** (400 MHz in $CDCl_3$)



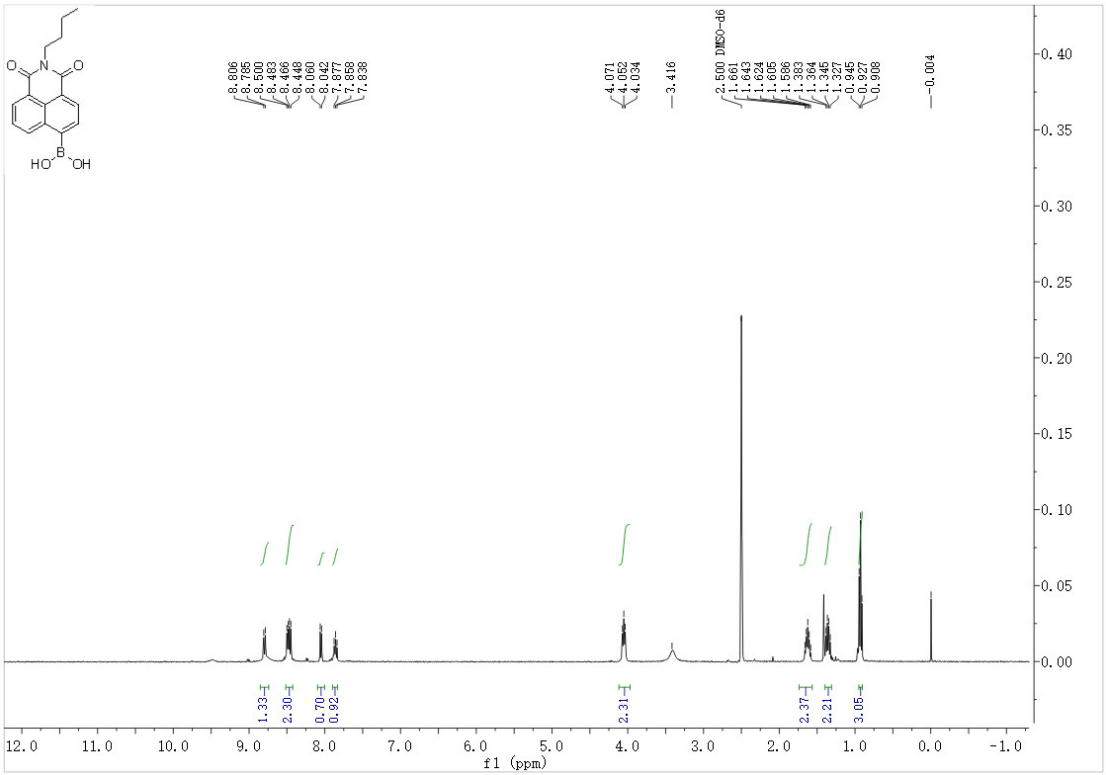
^{1}H NMR of compound **S3** (400 MHz in DMSO-d_6)



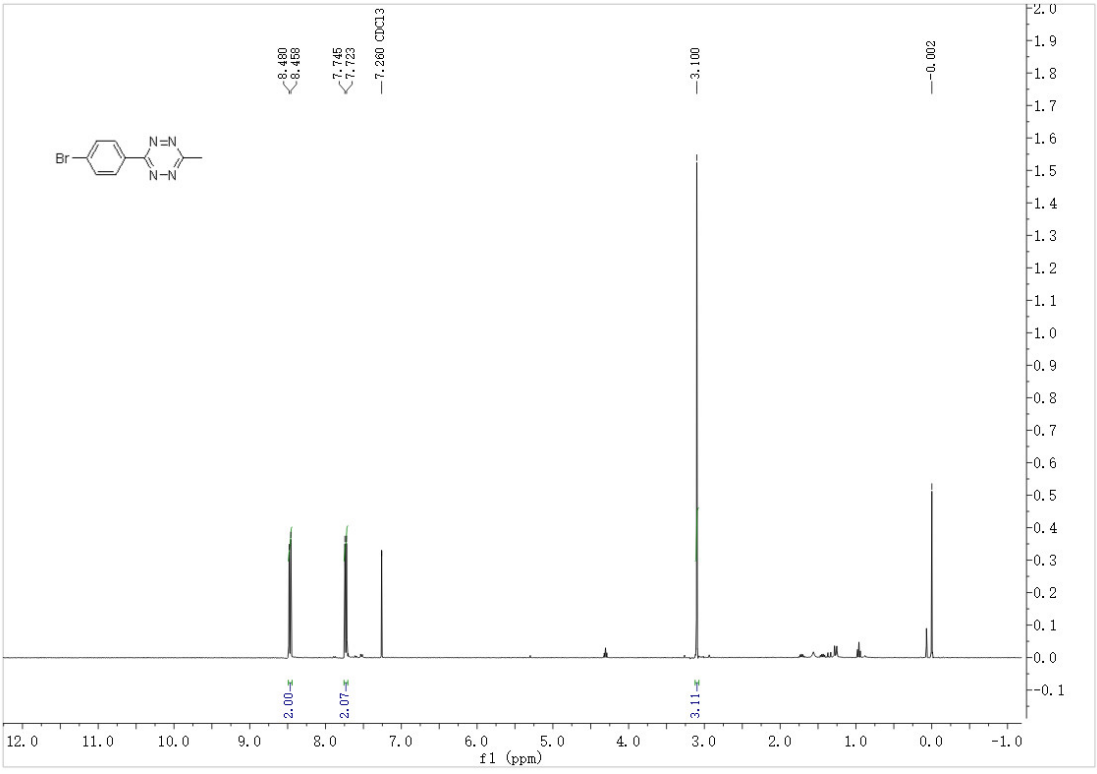
^{13}C NMR of compound **S3** (101 MHz in $CDCl_3$)



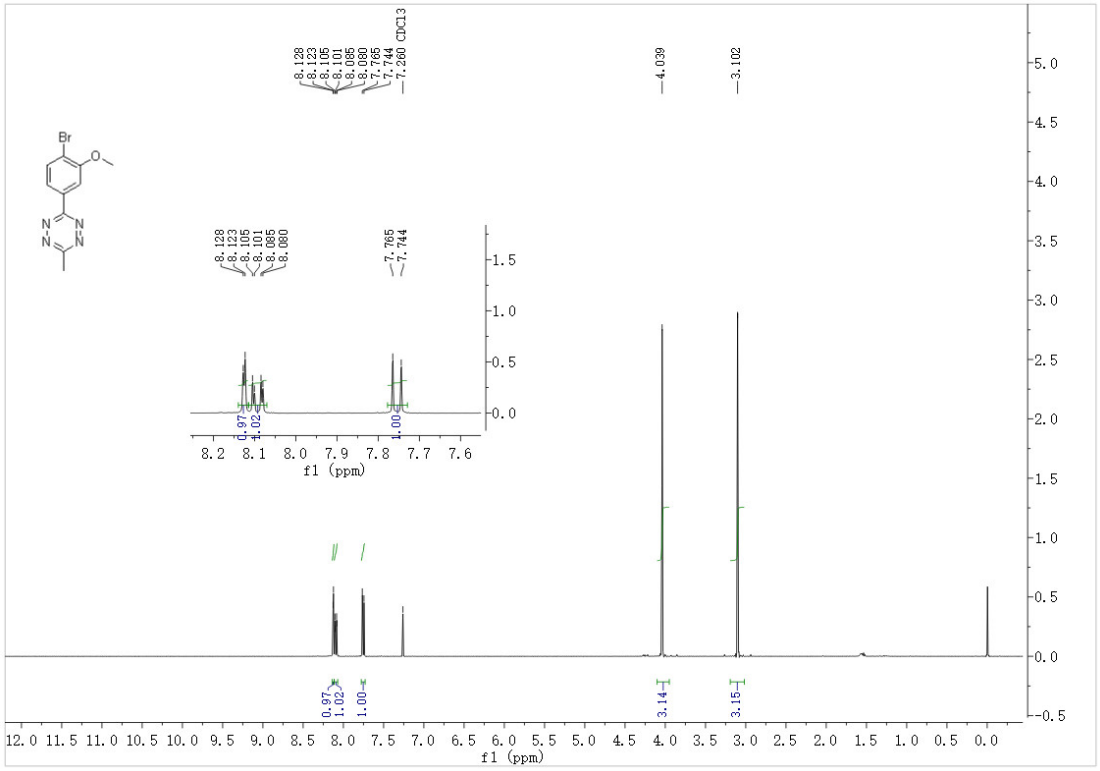
^{1}H NMR of compound **S5** (400 MHz in DMSO-d_6)



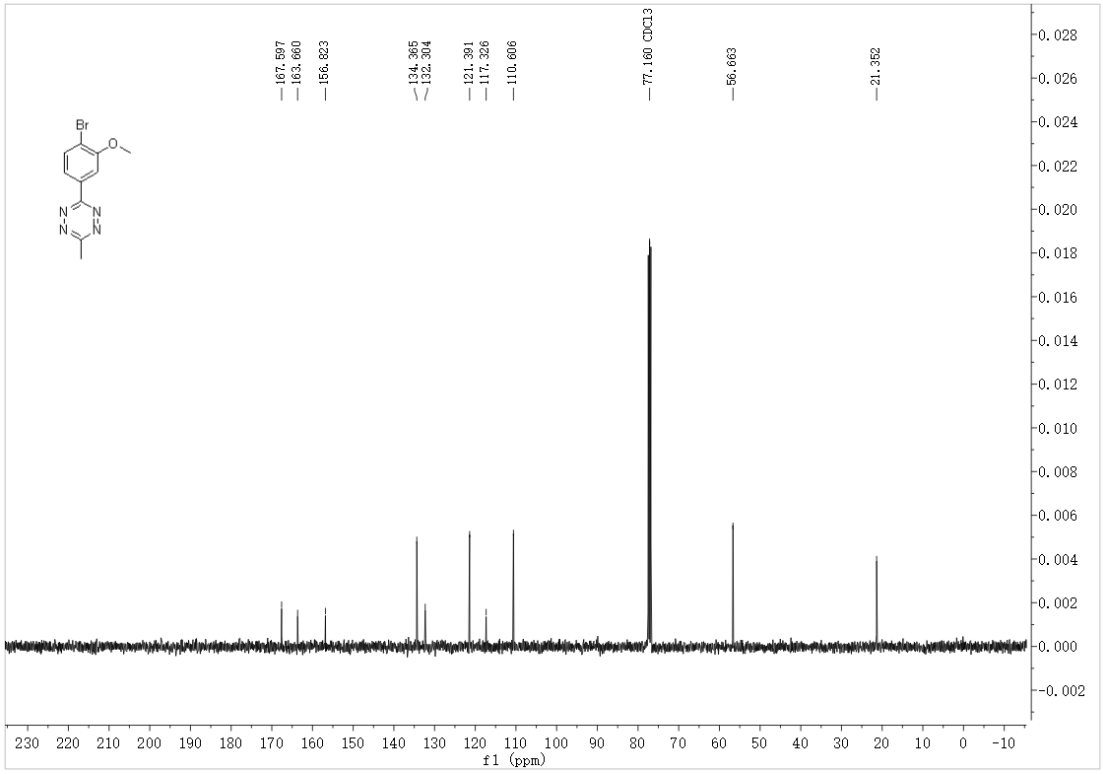
^{1}H NMR of compound **S10** (400 MHz in $CDCl_3$)



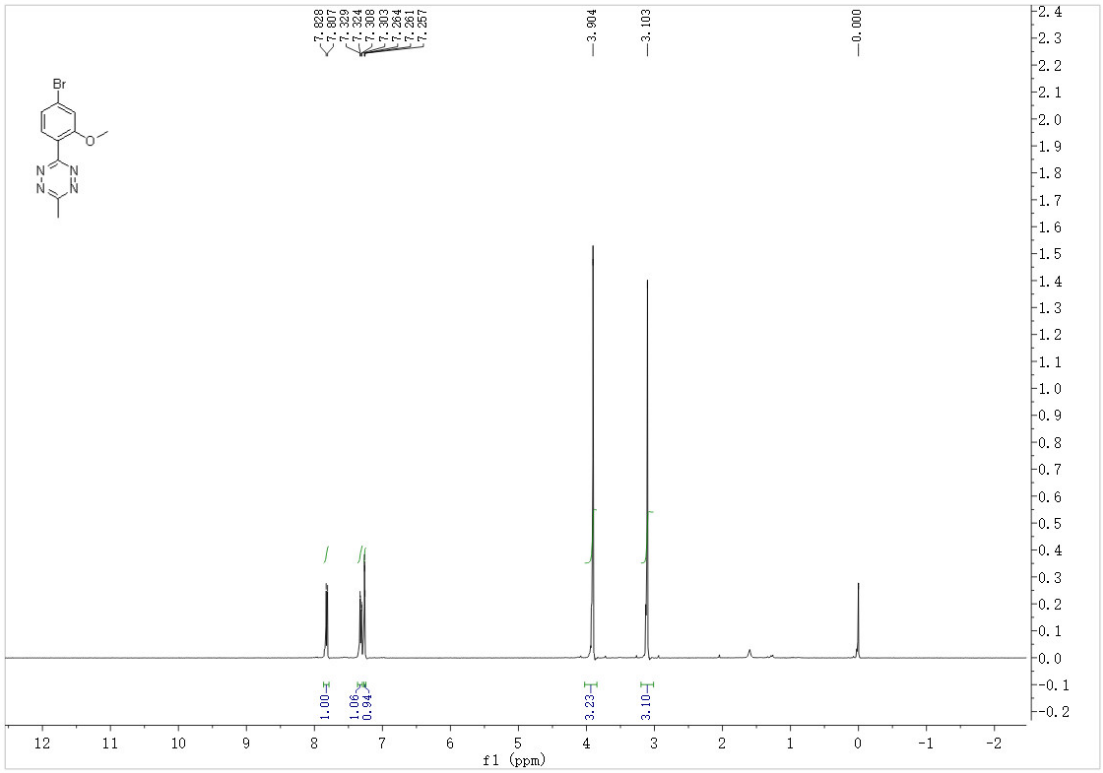
^{1}H NMR of compound **S11** (400 MHz in $CDCl_3$)



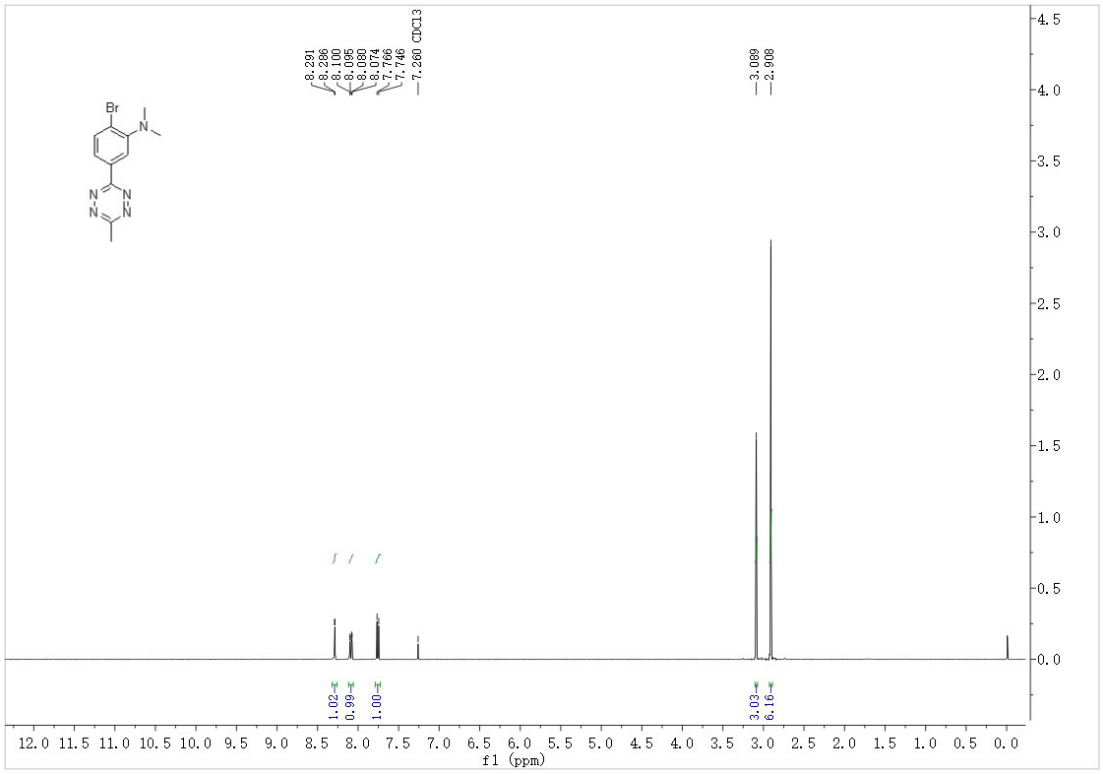
^{13}C NMR of compound **S11** (101 MHz in $CDCl_3$)



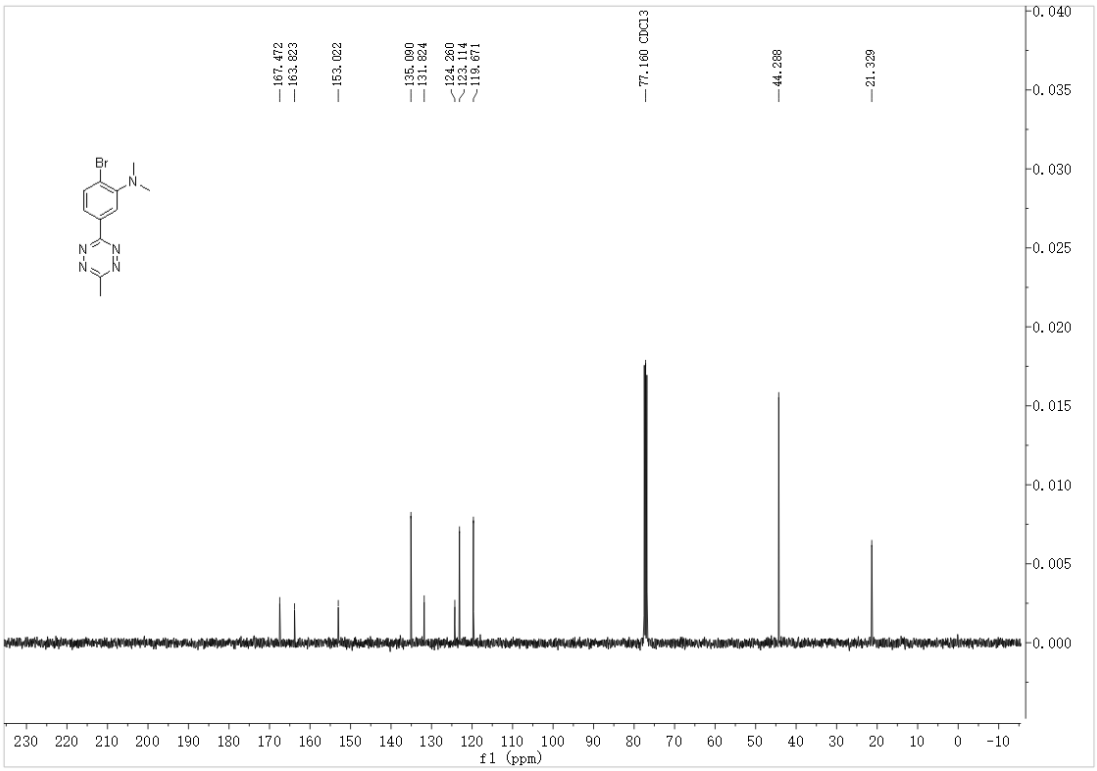
^{1}H NMR of compound **S12** (400 MHz in $CDCl_3$)



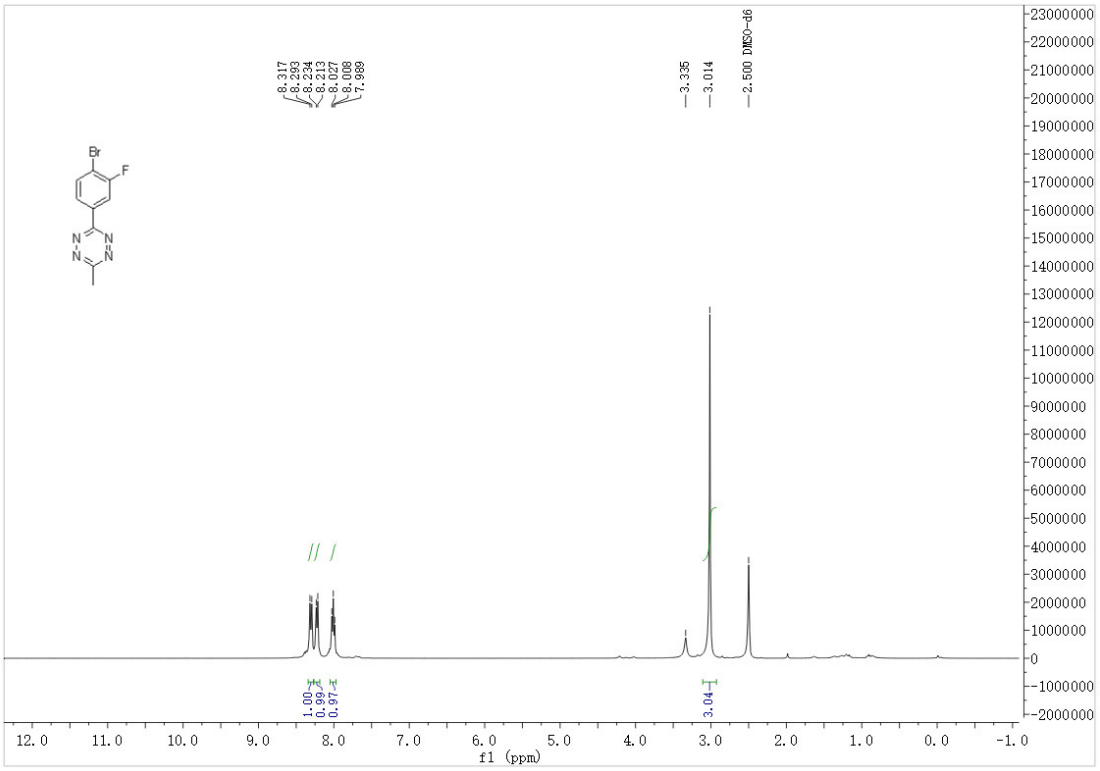
^{1}H NMR of compound **S13** (400 MHz in $CDCl_3$)



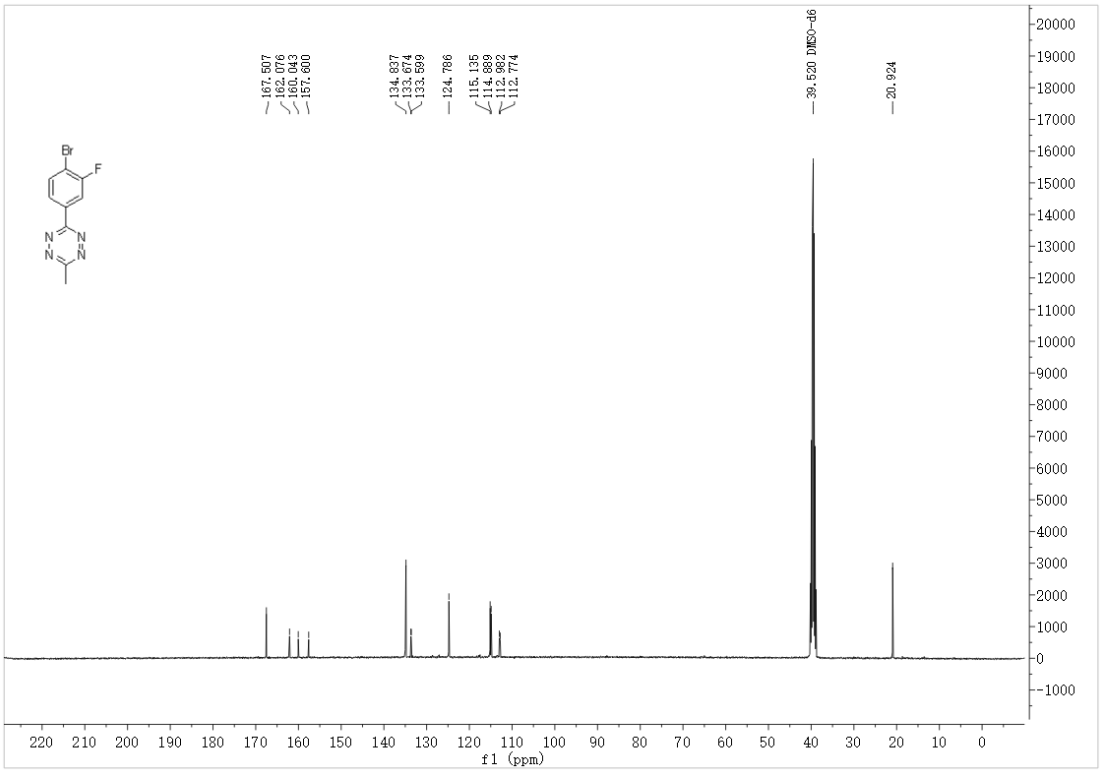
^{13}C NMR of compound **S13** (101 MHz in $CDCl_3$)



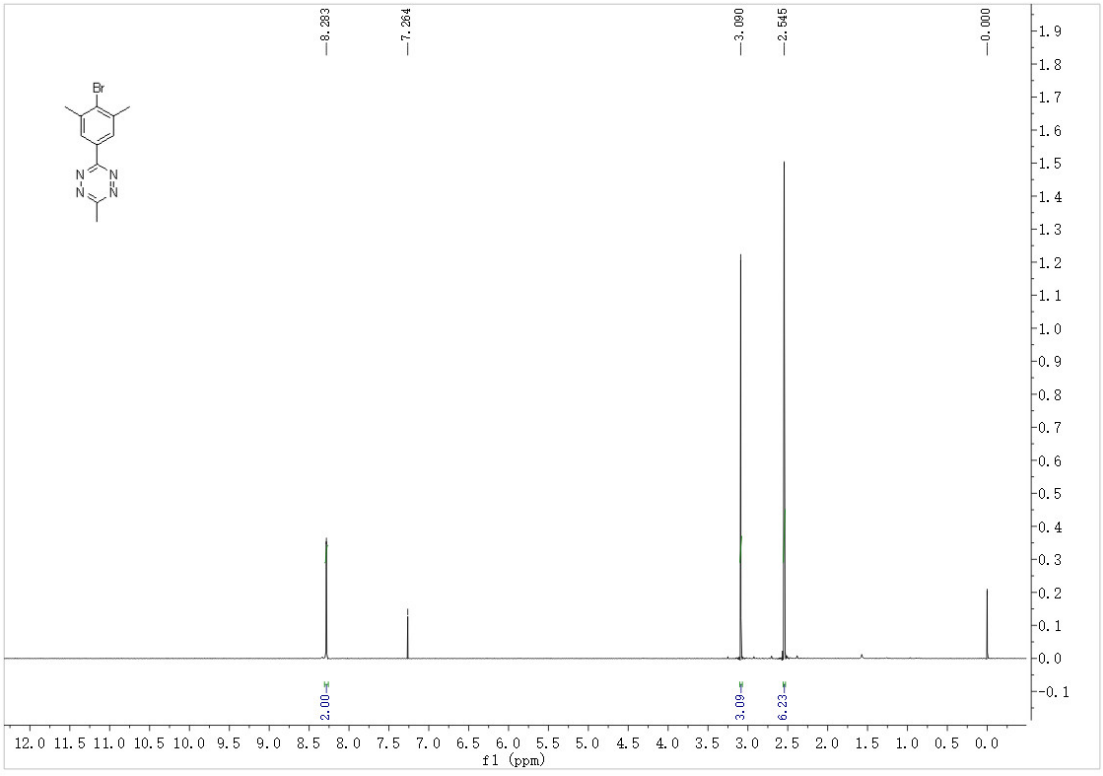
^{1}H NMR of compound **S14** (400 MHz in DMSO-d_6)



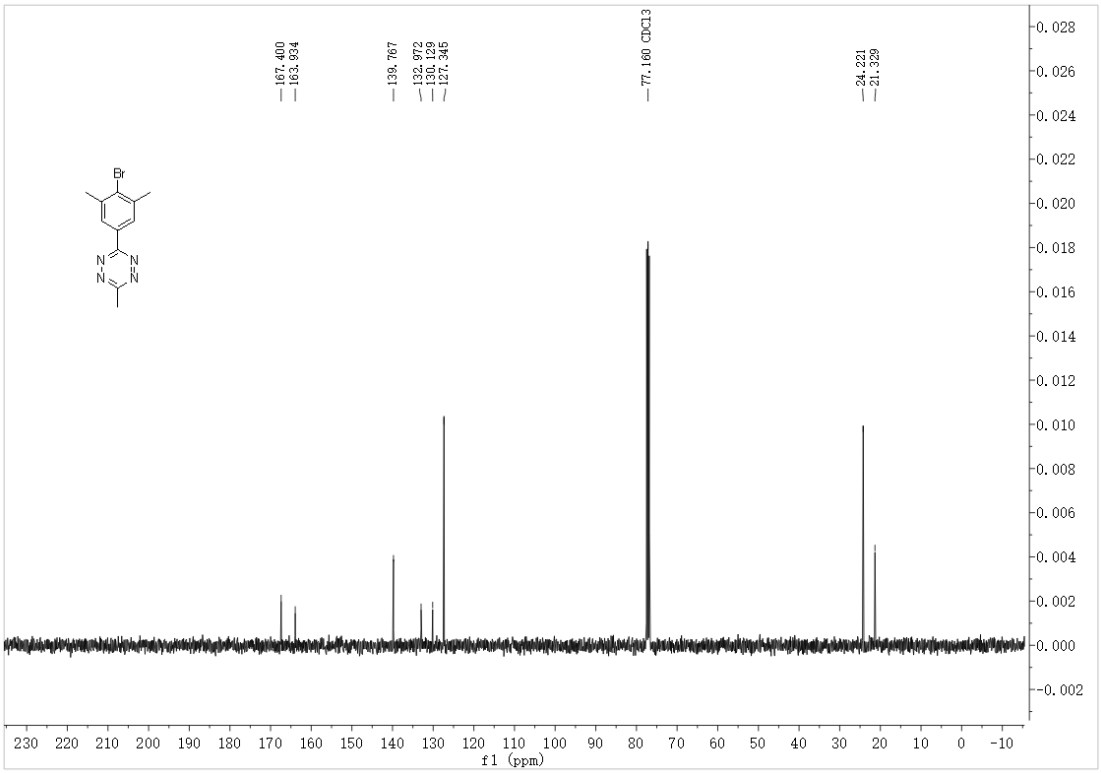
^{13}C NMR of compound **S14** (101 MHz in DMSO-d_6)



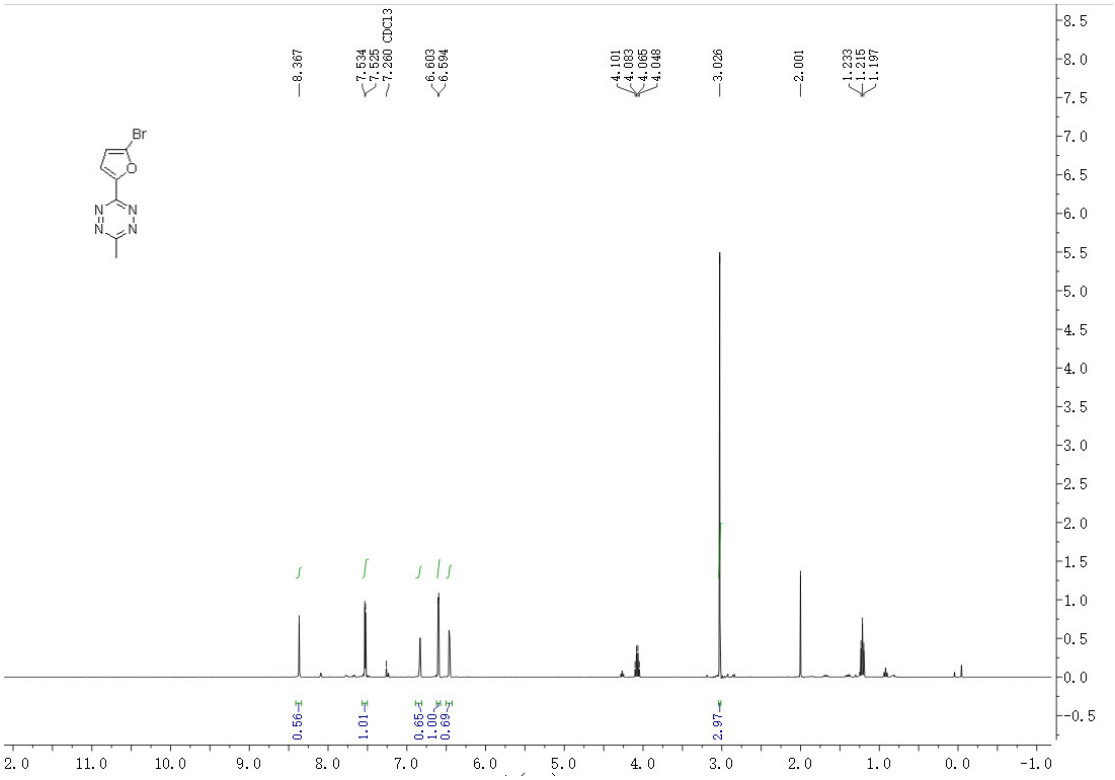
^{1}H NMR of compound **S15** (400 MHz in $CDCl_3$)



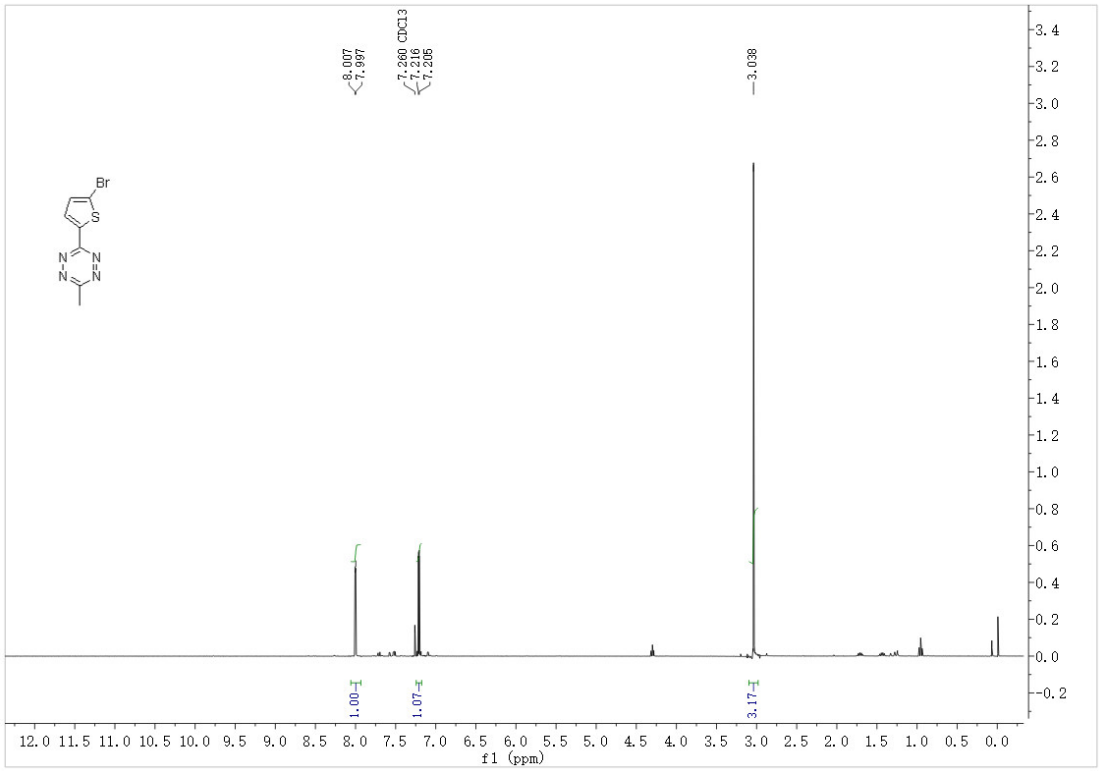
^{13}C NMR of compound **S15** (101 MHz in $CDCl_3$)



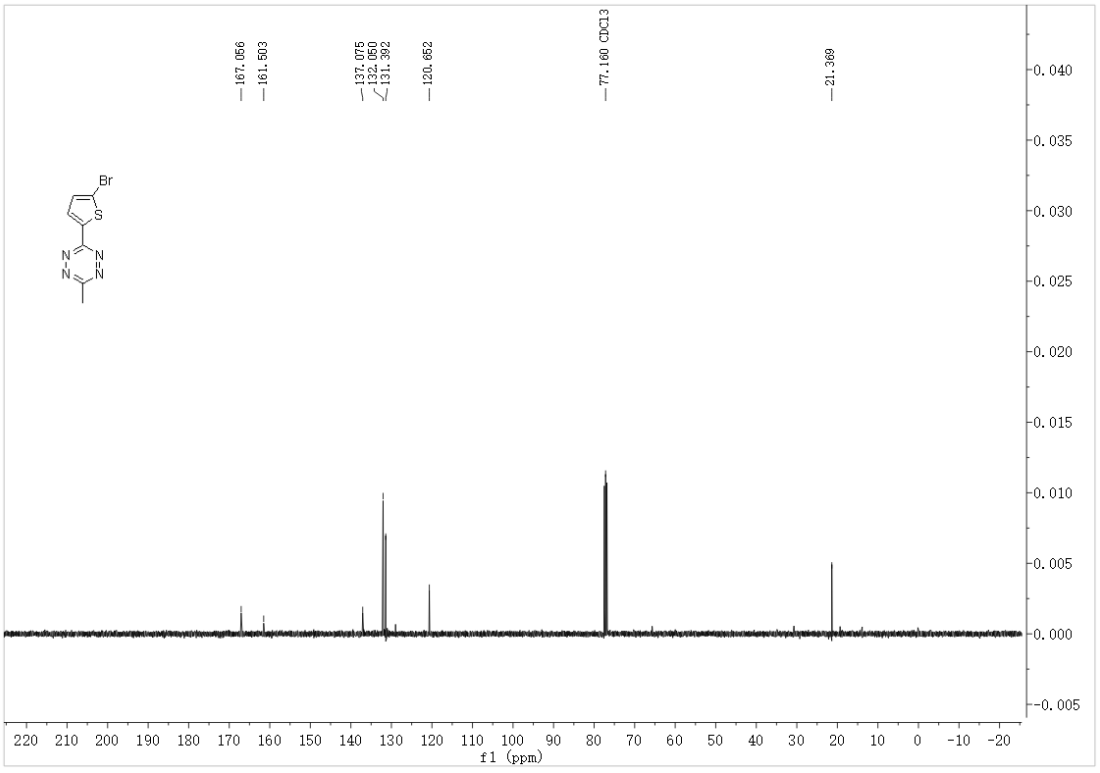
^{1}H NMR of compound **S16** (400 MHz in $CDCl_3$)



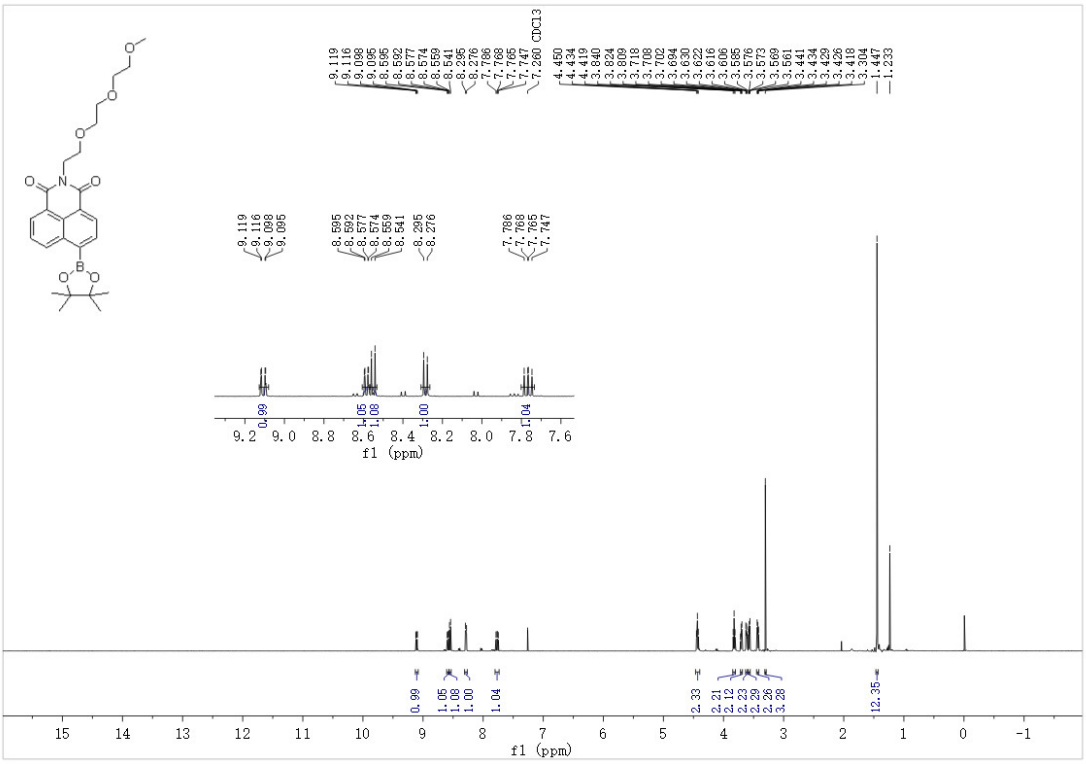
^{1}H NMR of compound **S17** (400 MHz in $CDCl_3$)



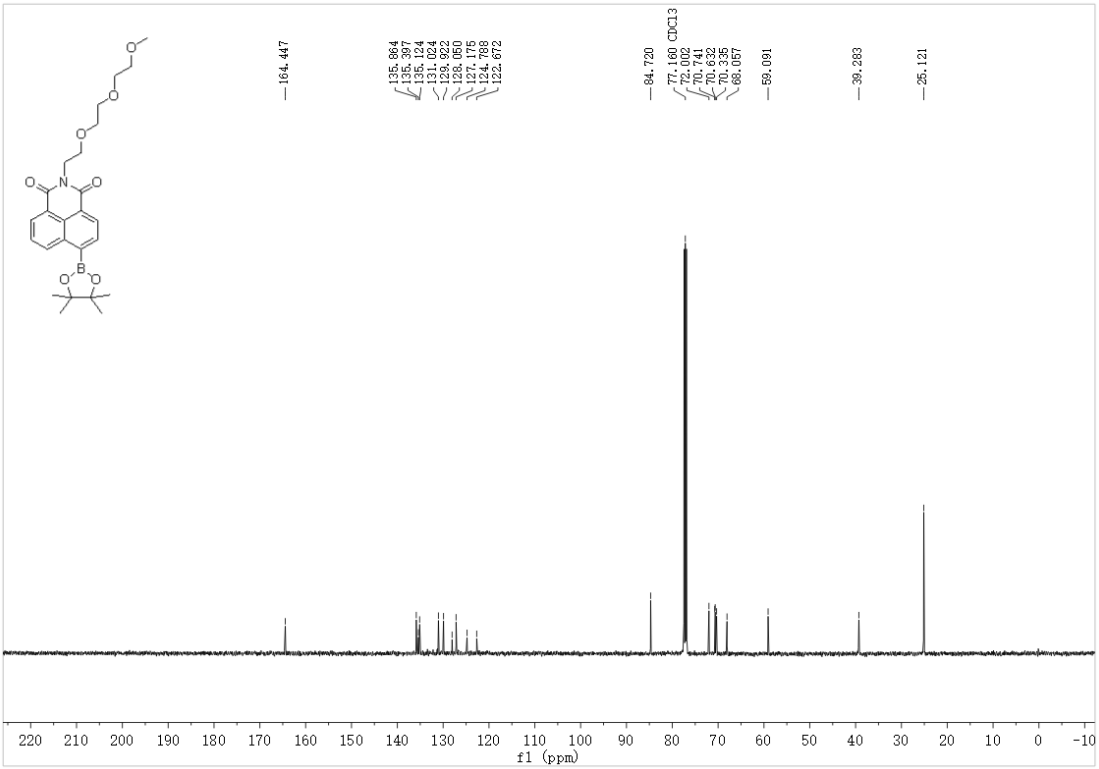
^{13}C NMR of compound **S17** (101 MHz in $CDCl_3$)



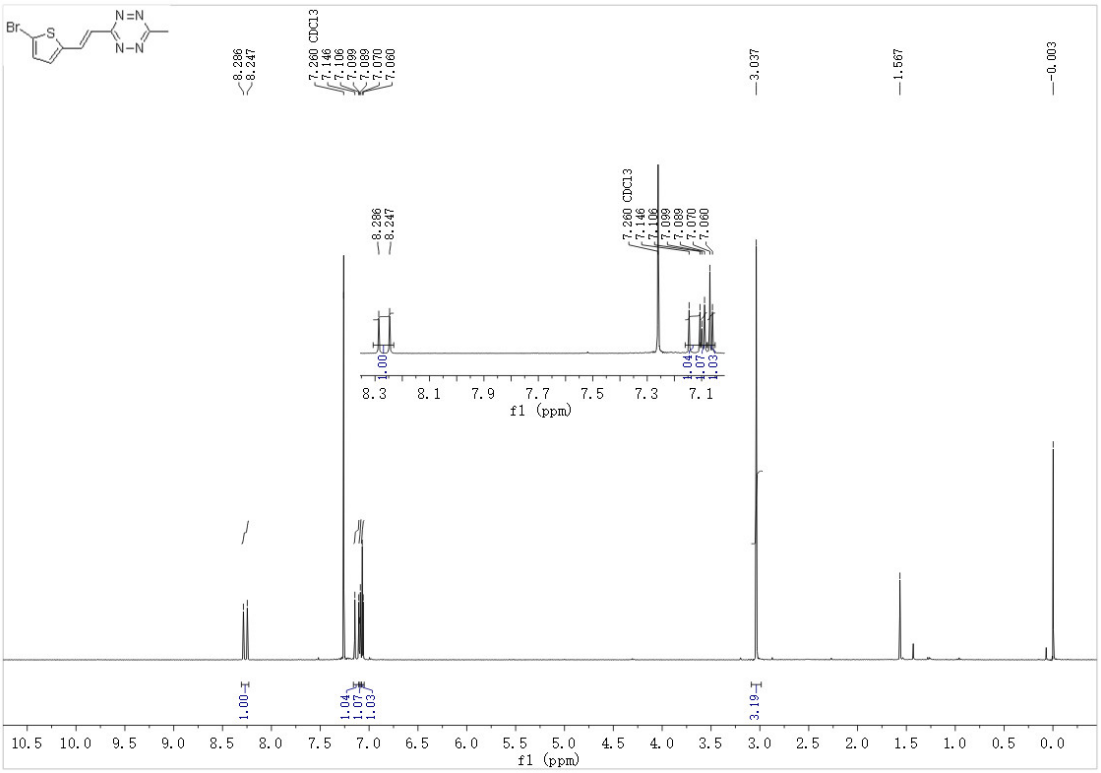
^{1}H NMR of compound S**19** (400 MHz in $CDCl_3$)



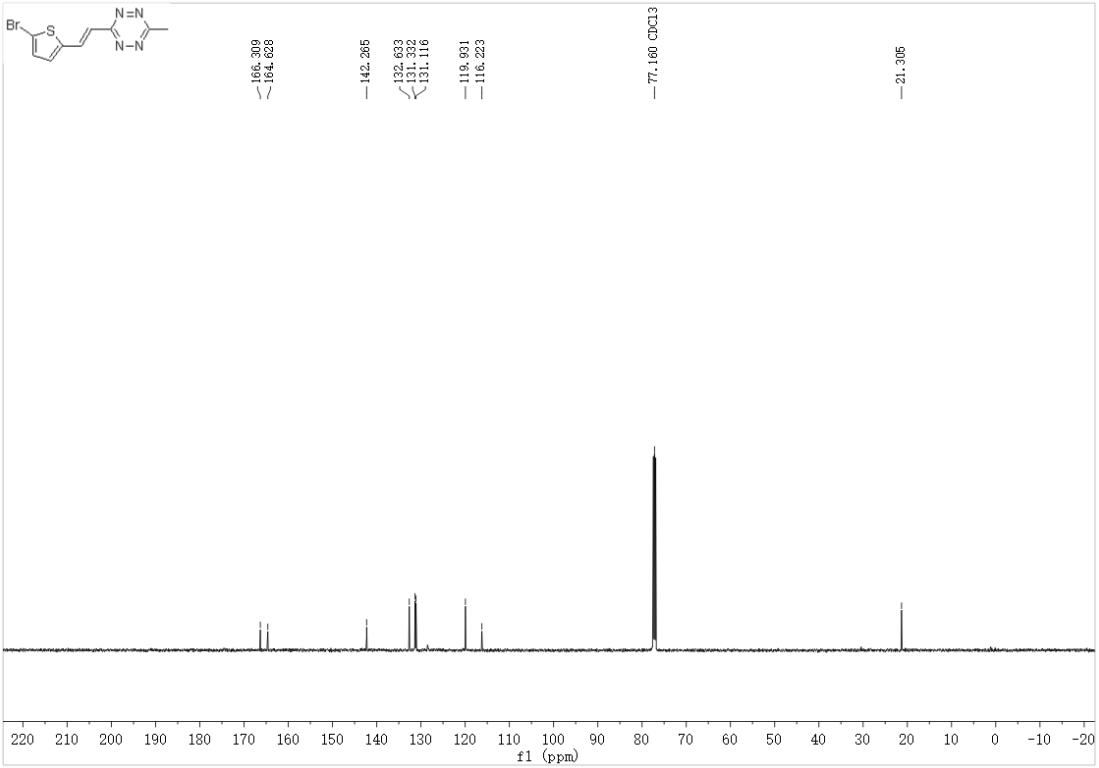
^{13}C NMR of compound **S19** (126 MHz in $CDCl_3$)



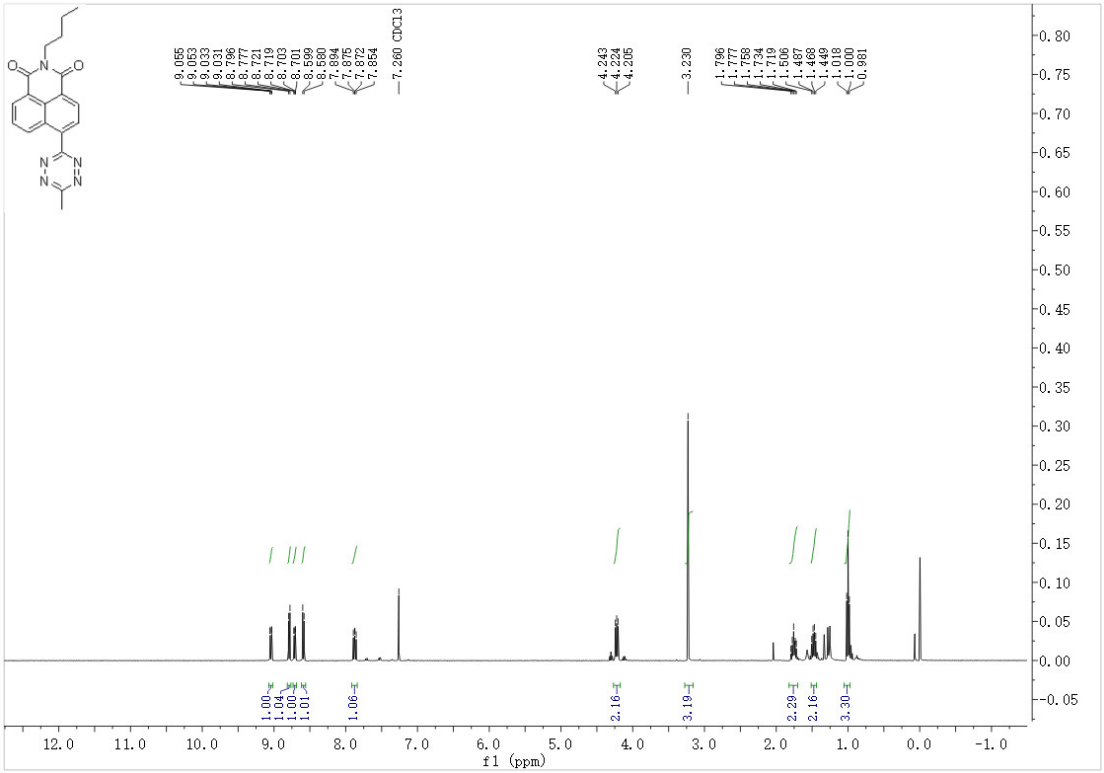
^{1}H NMR of compound **S20** (400 MHz in $CDCl_3$)



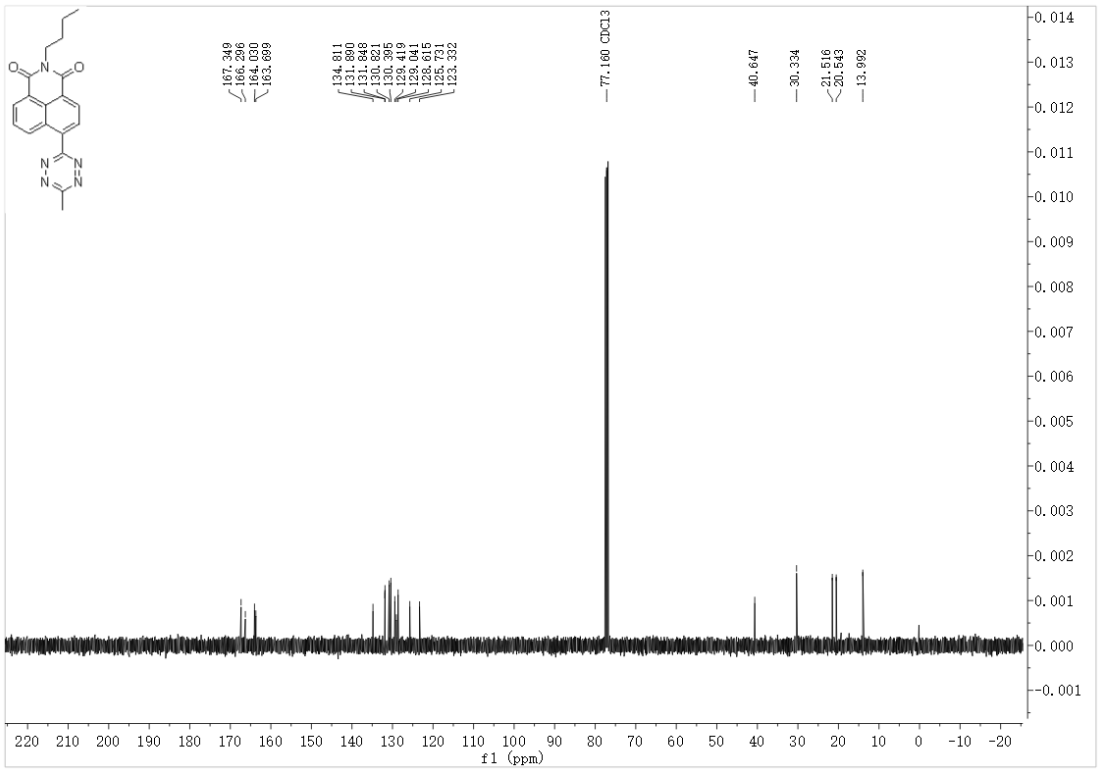
^{13}C NMR of compound **S20** (126 MHz in $CDCl_3$)



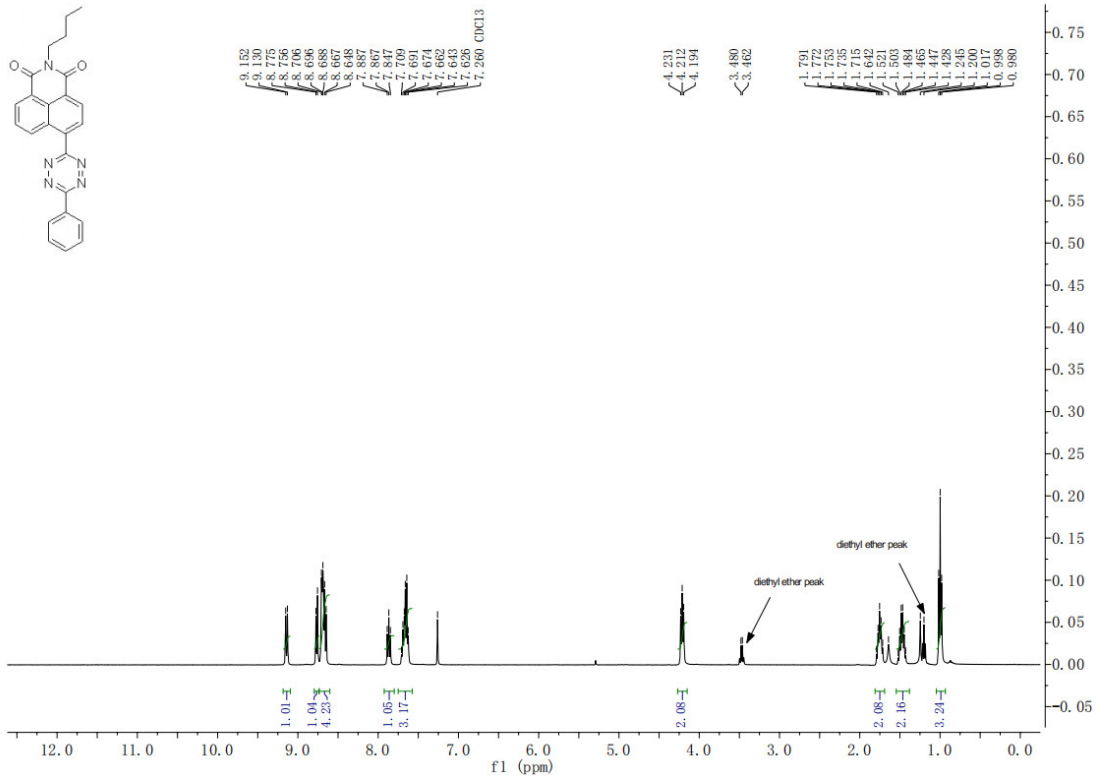
^{1}H NMR of compound **NP-Tz1** (400 MHz in $CDCl_3$)



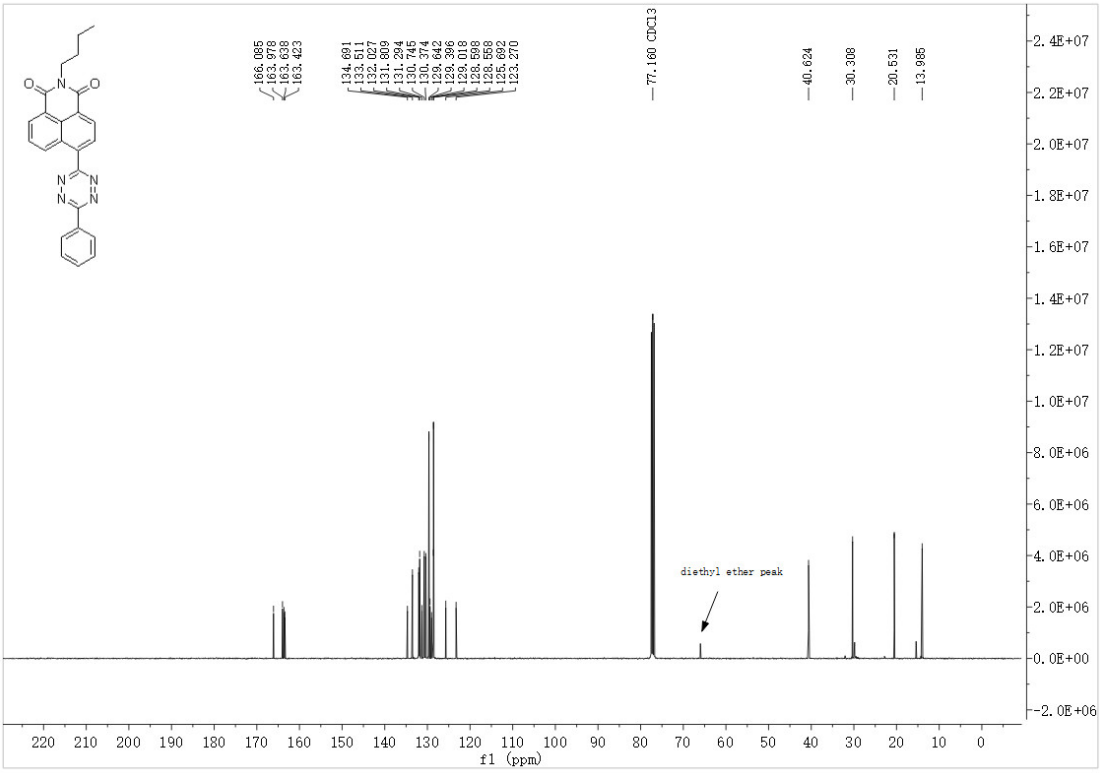
^{13}C NMR of compound **NP-Tz1** (101 MHz in $CDCl_3$)



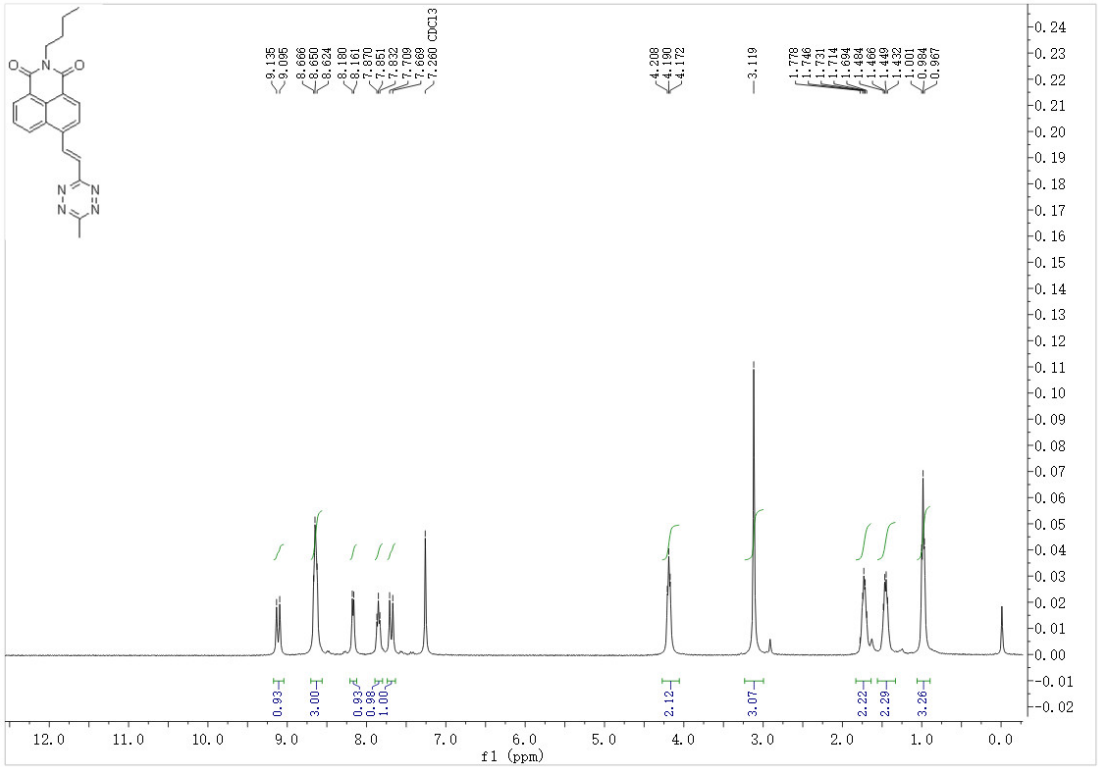
^{1}H NMR of compound **NP-Tz2** (400 MHz in $CDCl_3$)



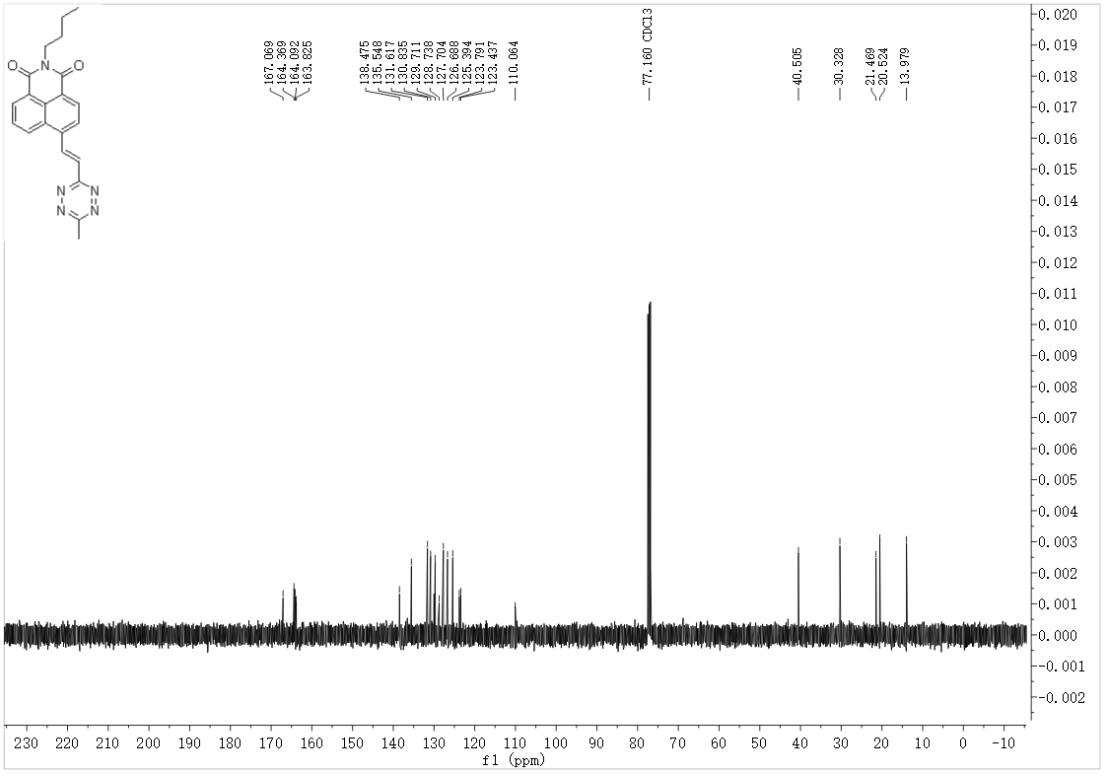
^{13}C NMR of compound **NP-Tz2** (151 MHz in $CDCl_3$)



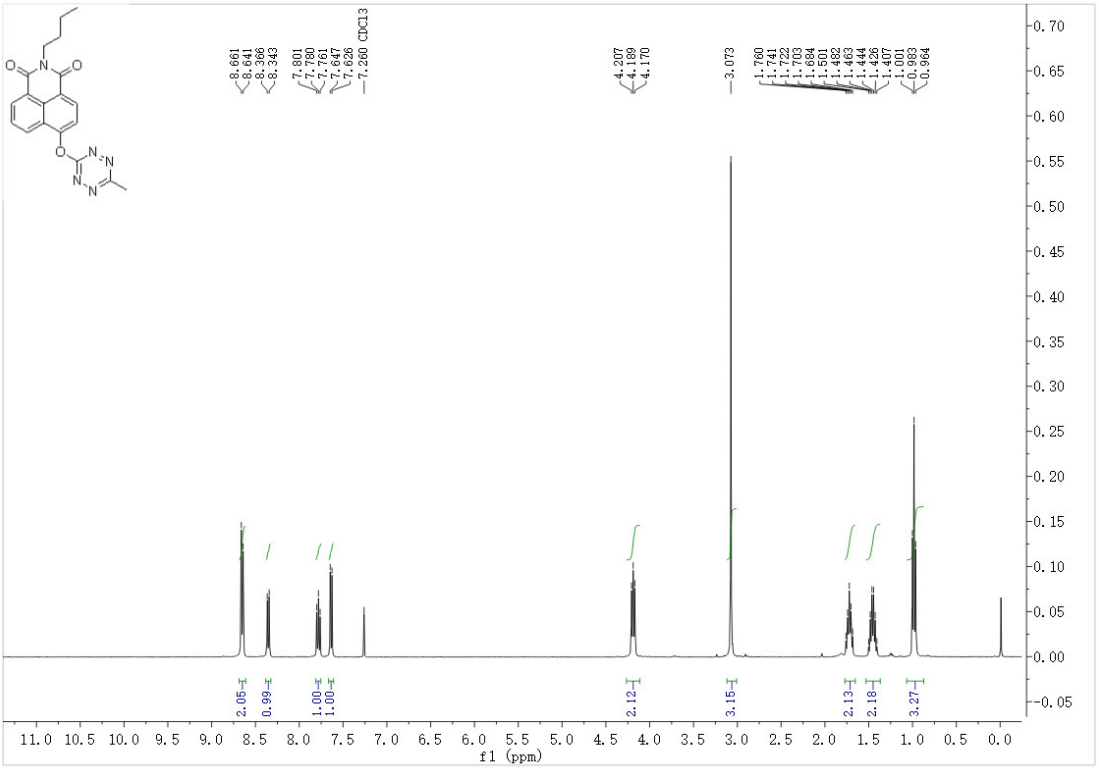
^{1}H NMR of compound **NP-Tz3** (400 MHz in $CDCl_3$)



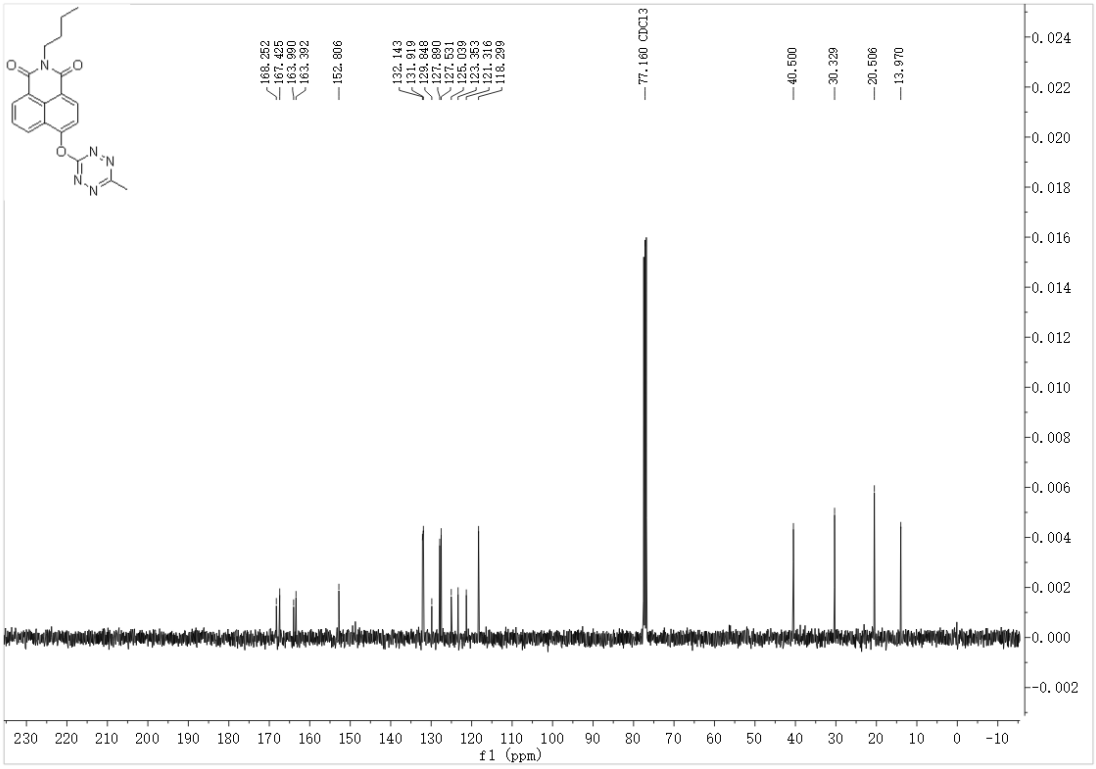
^{13}C NMR of compound **NP-Tz3** (101 MHz in $CDCl_3$)



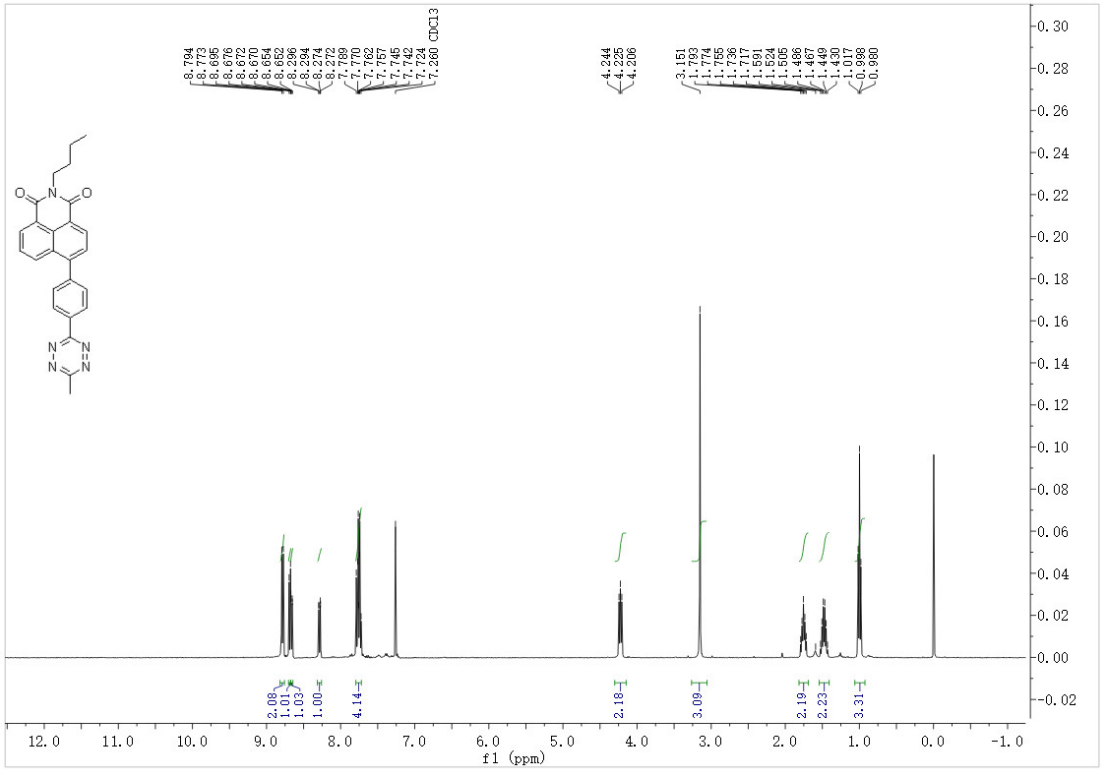
^{1}H NMR of compound **NP-Tz4** (400 MHz in $CDCl_3$)



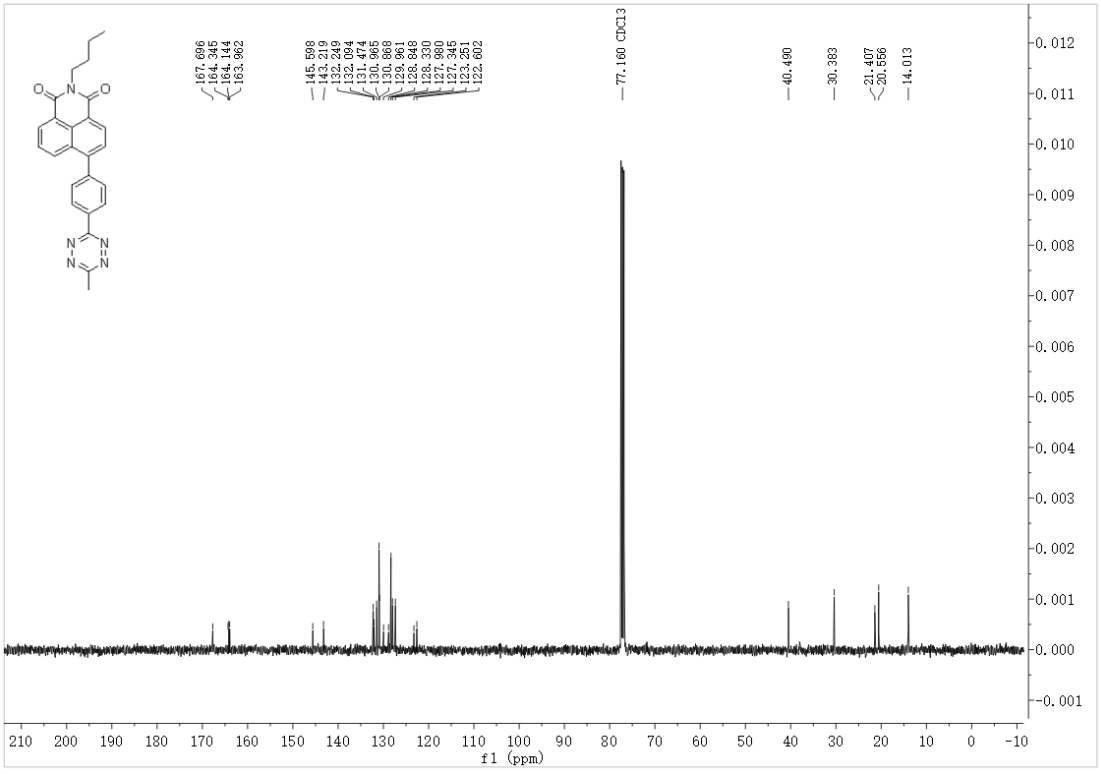
^{13}C NMR of compound **NP-Tz4** (101 MHz in $CDCl_3$)



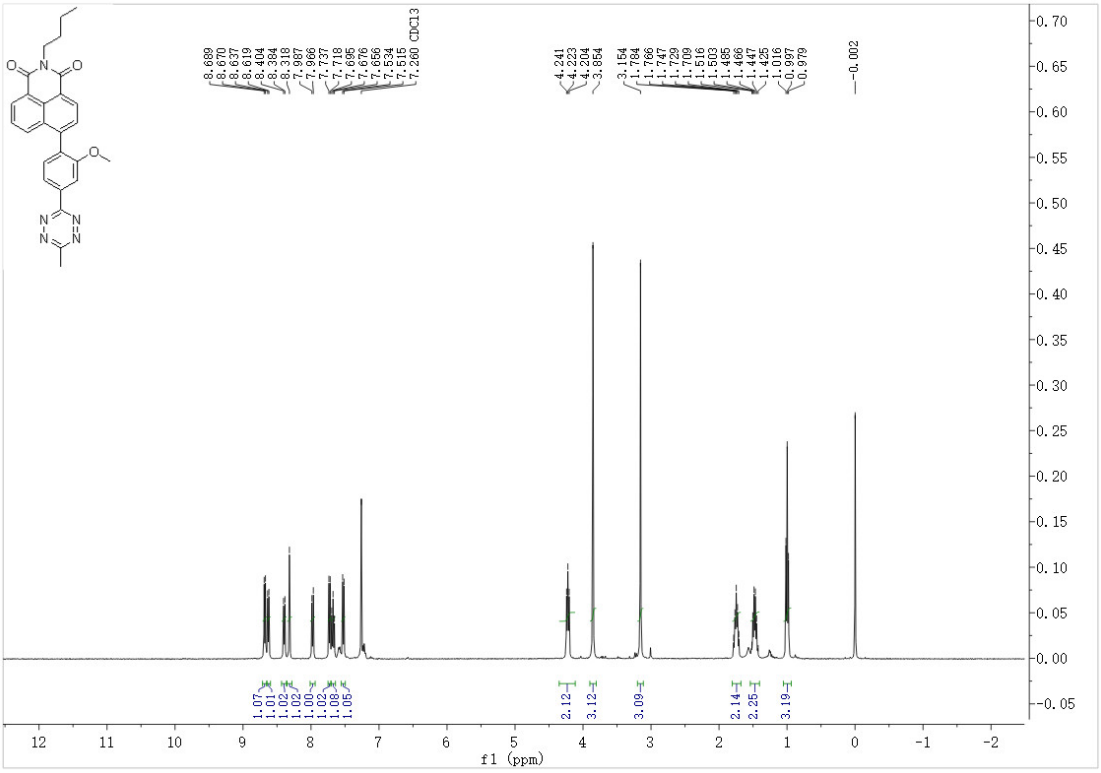
^{1}H NMR of compound **NP-Tz5** (400 MHz in $CDCl_3$)



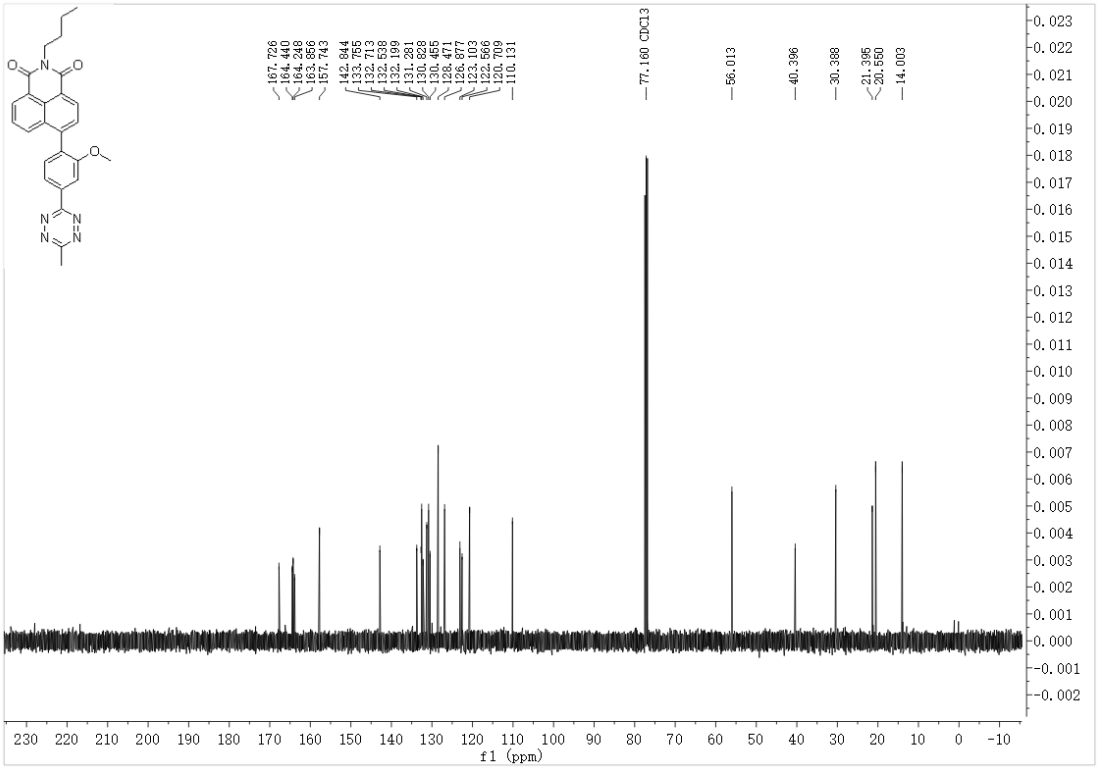
^{13}C NMR of compound **NP-Tz5** (101 MHz in $CDCl_3$)



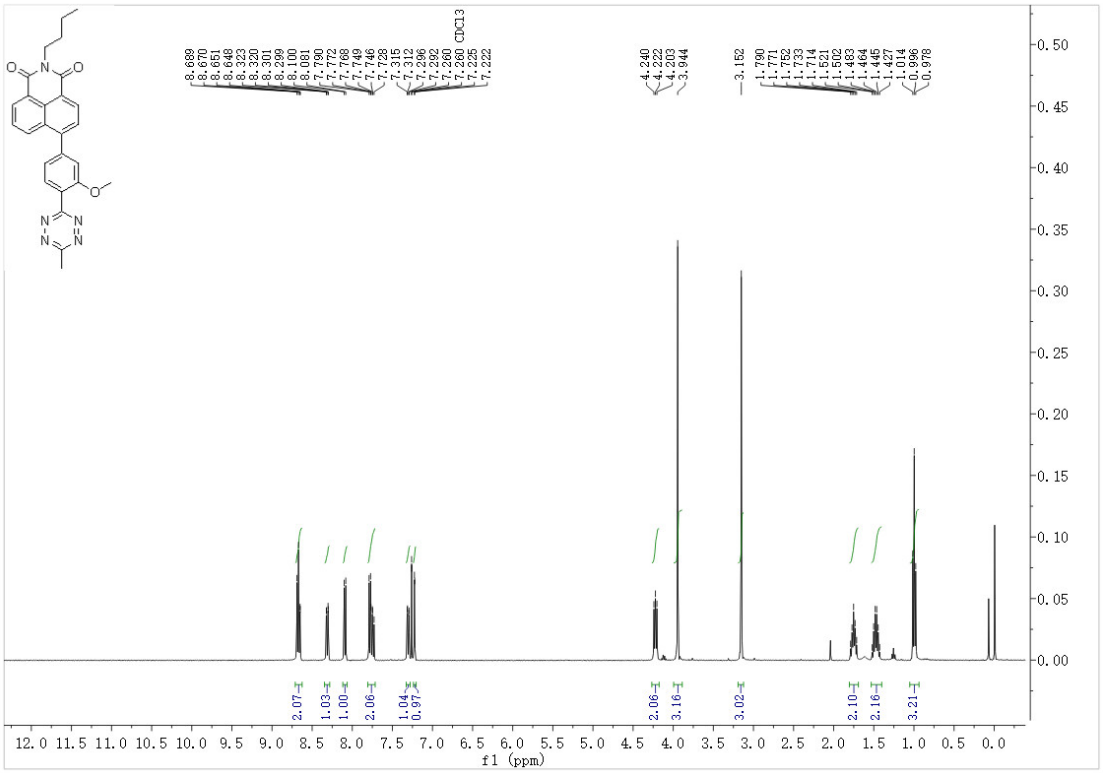
^{1}H NMR of compound **NP-Tz6** (400 MHz in $CDCl_3$)



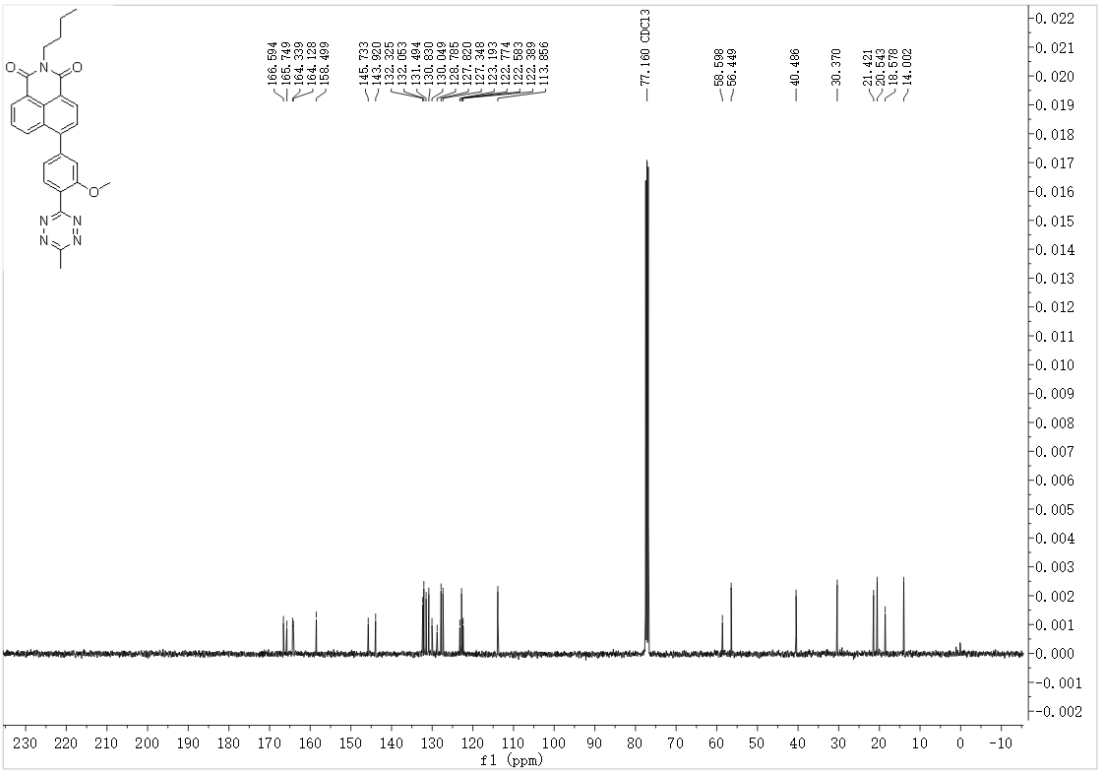
^{13}C NMR of compound **NP-Tz6** (101 MHz in $CDCl_3$)



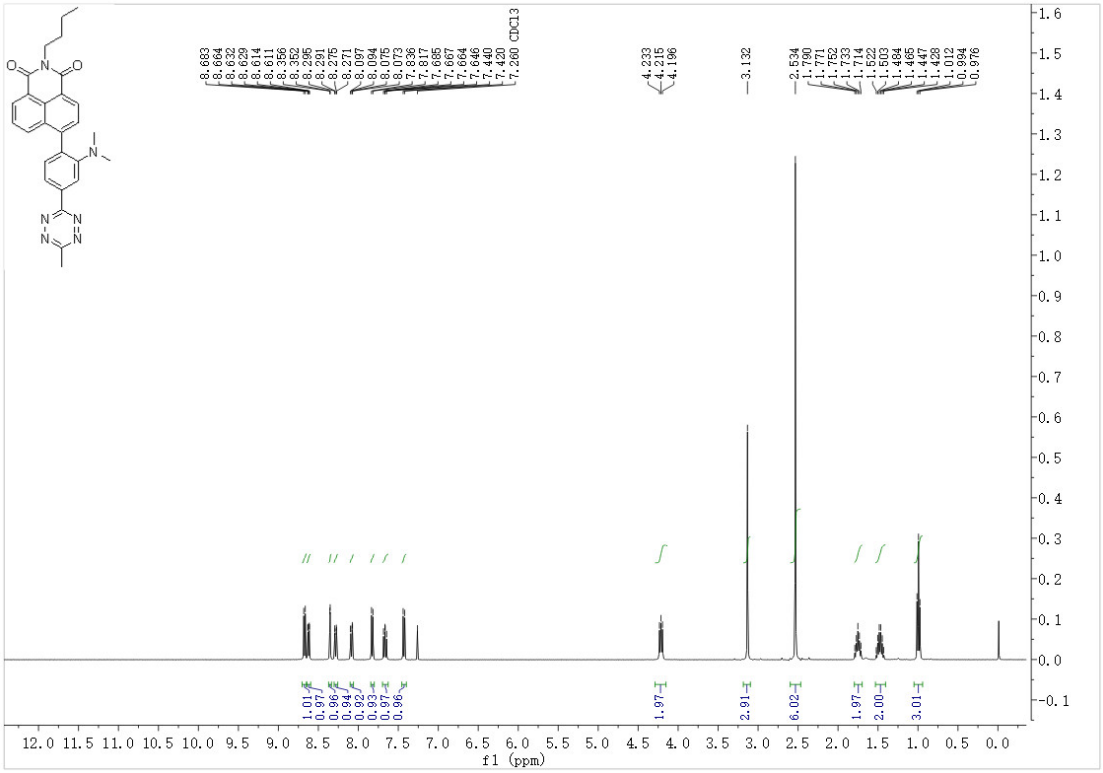
^{1}H NMR of compound **NP-Tz7** (400 MHz in $CDCl_3$)



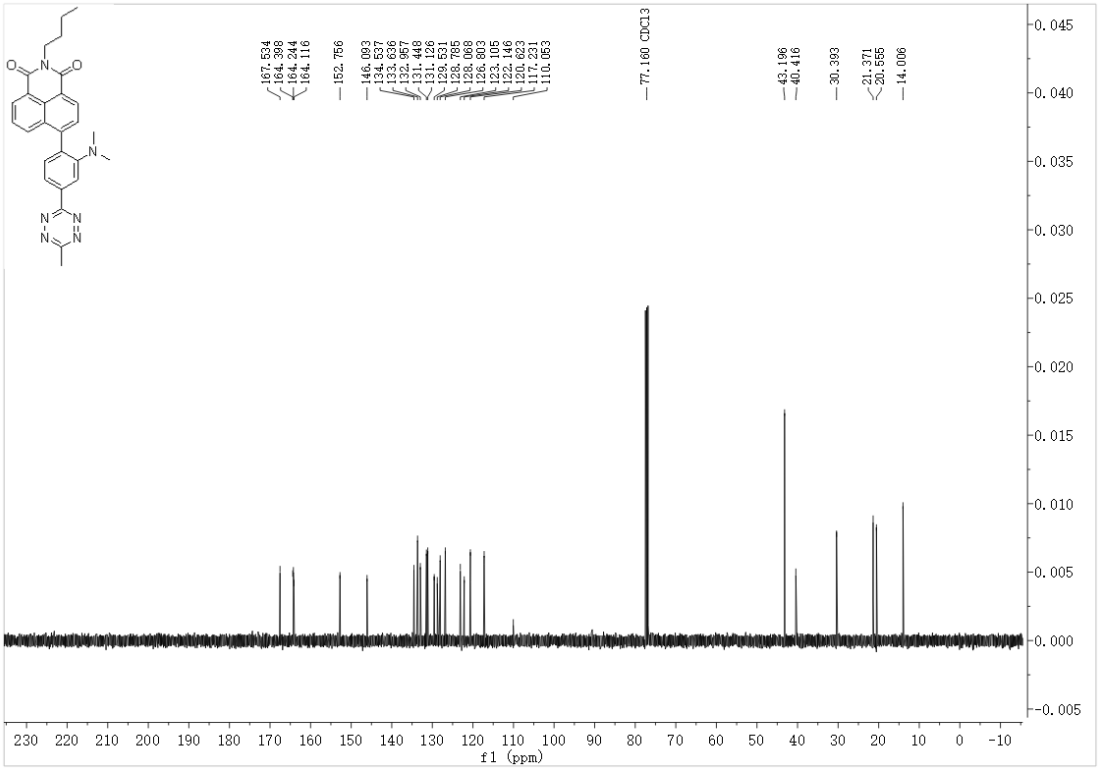
^{13}C NMR of compound **NP-Tz7** (101 MHz in $CDCl_3$)



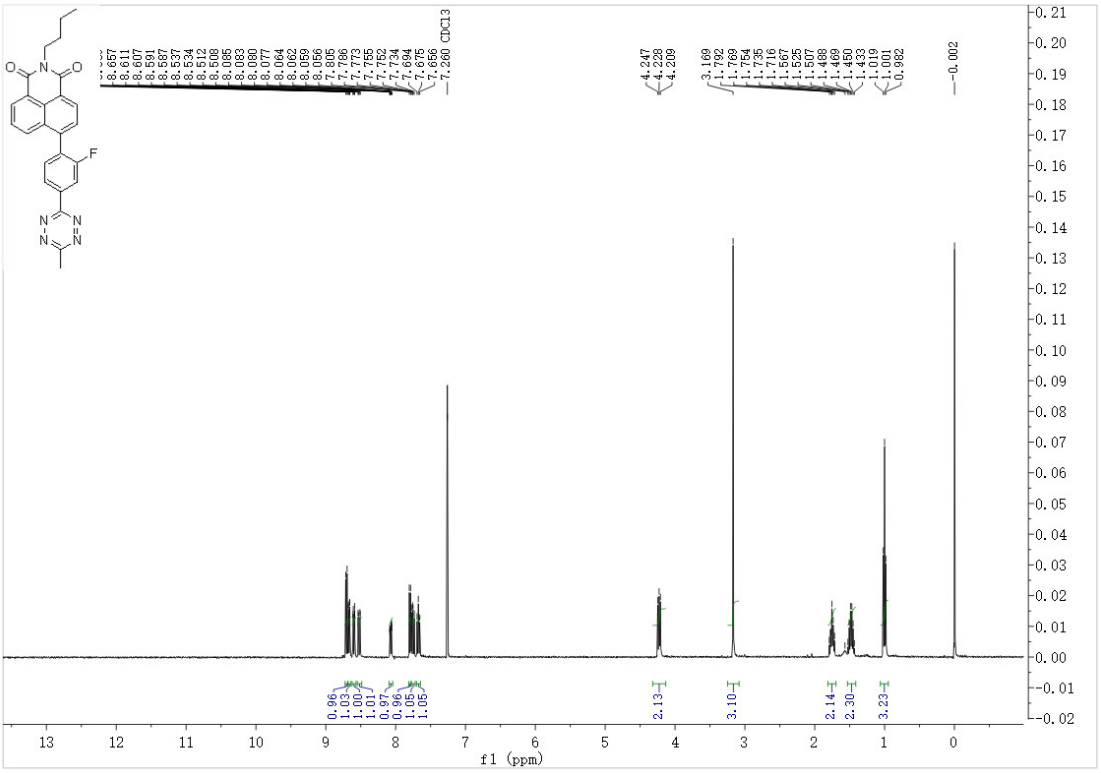
^{1}H NMR of compound **NP-Tz8** (400 MHz in $CDCl_3$)



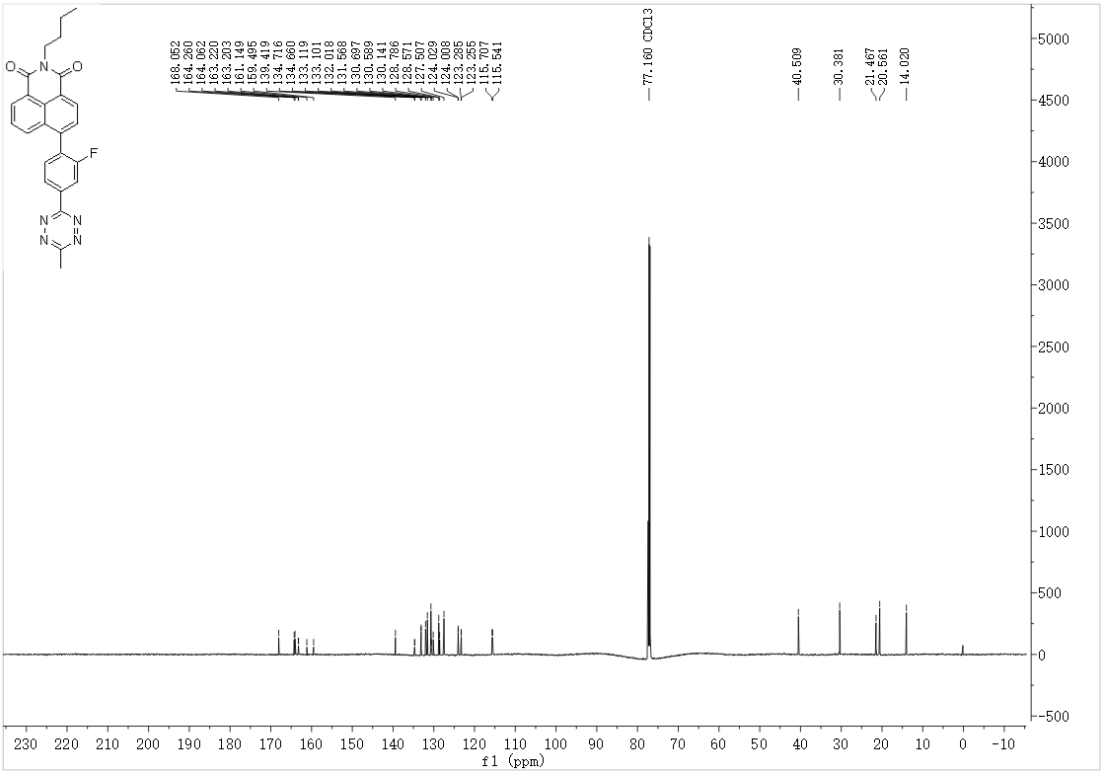
^{13}C NMR of compound **NP-Tz8** (101 MHz in $CDCl_3$)



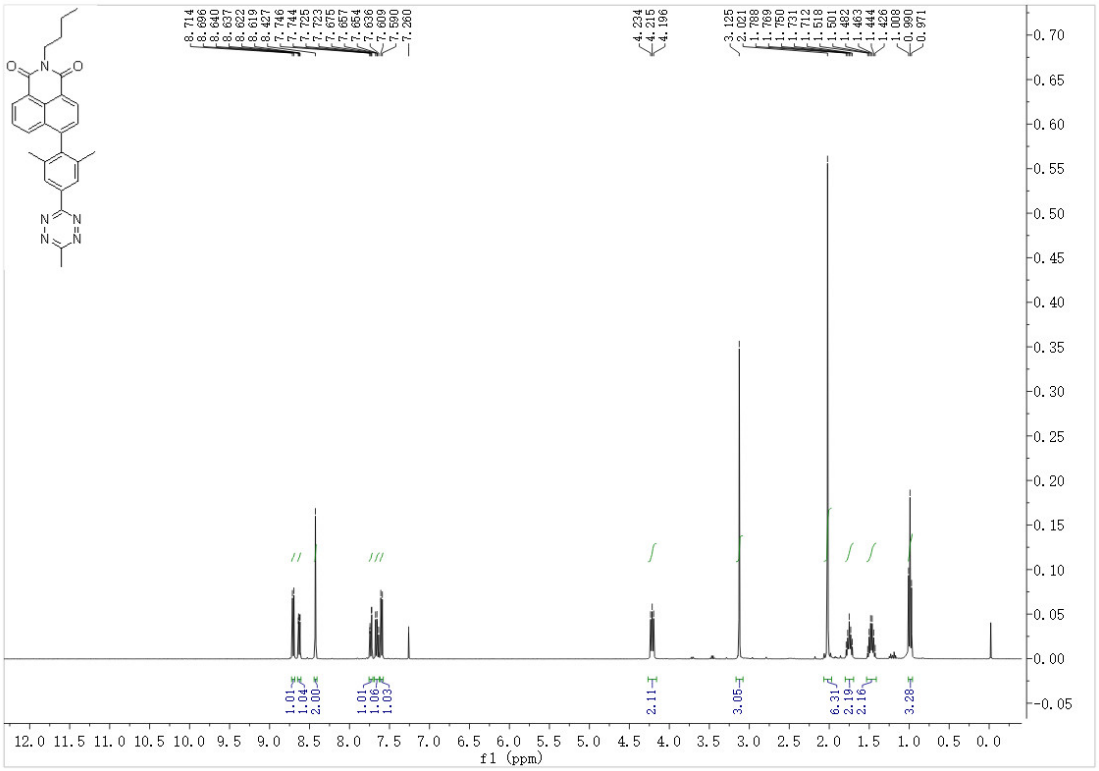
^{1}H NMR of compound **NP-Tz9** (400 MHz in $CDCl_3$)



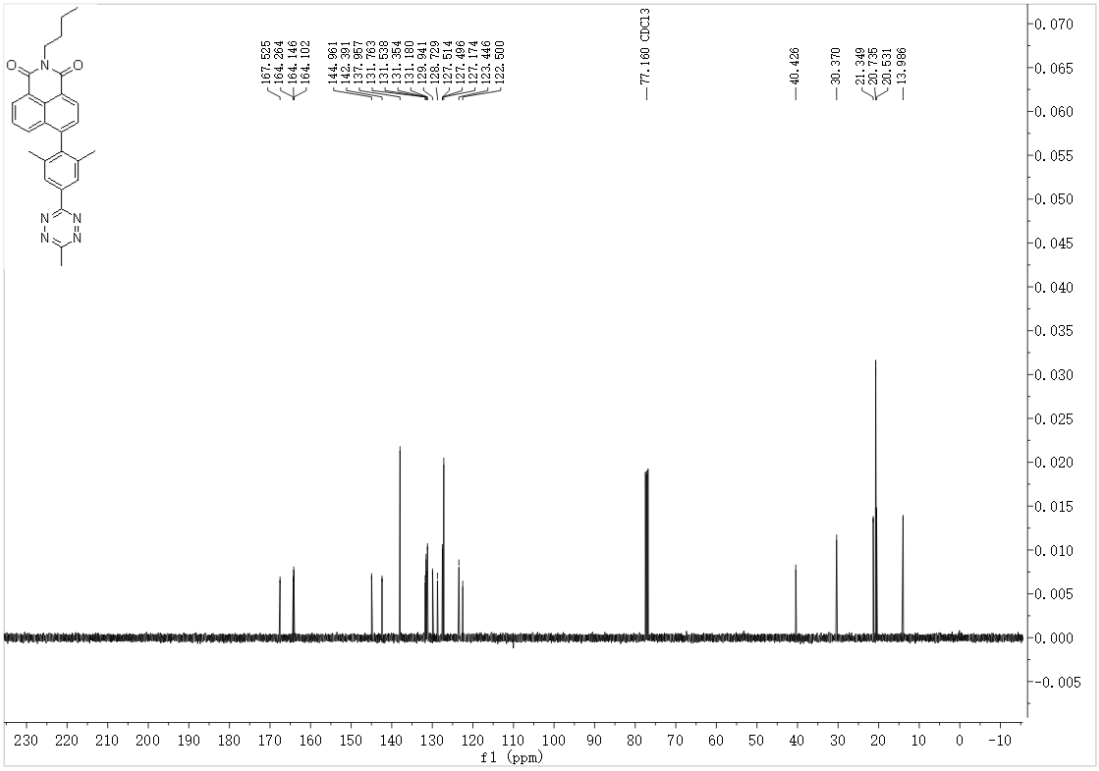
^{13}C NMR of compound **NP-Tz9** (151 MHz in $CDCl_3$)



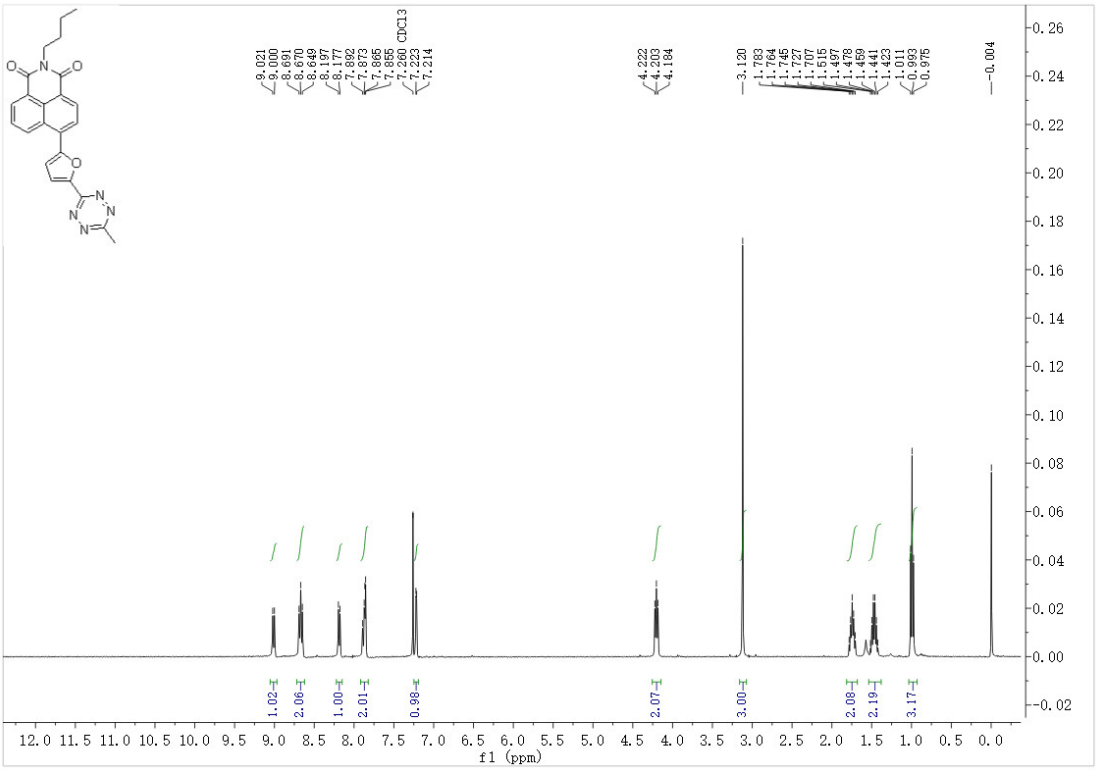
^{1}H NMR of compound **NP-Tz10** (400 MHz in $CDCl_3$)



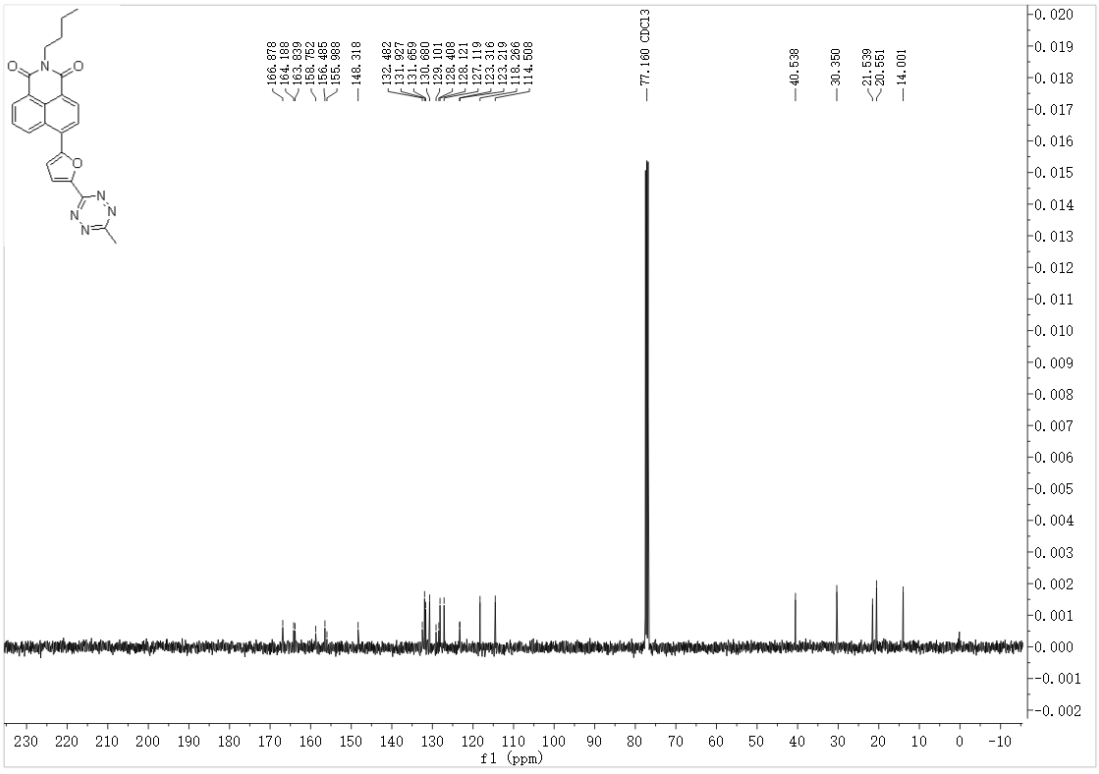
^{13}C NMR of compound **NP-Tz10** (101 MHz in $CDCl_3$)



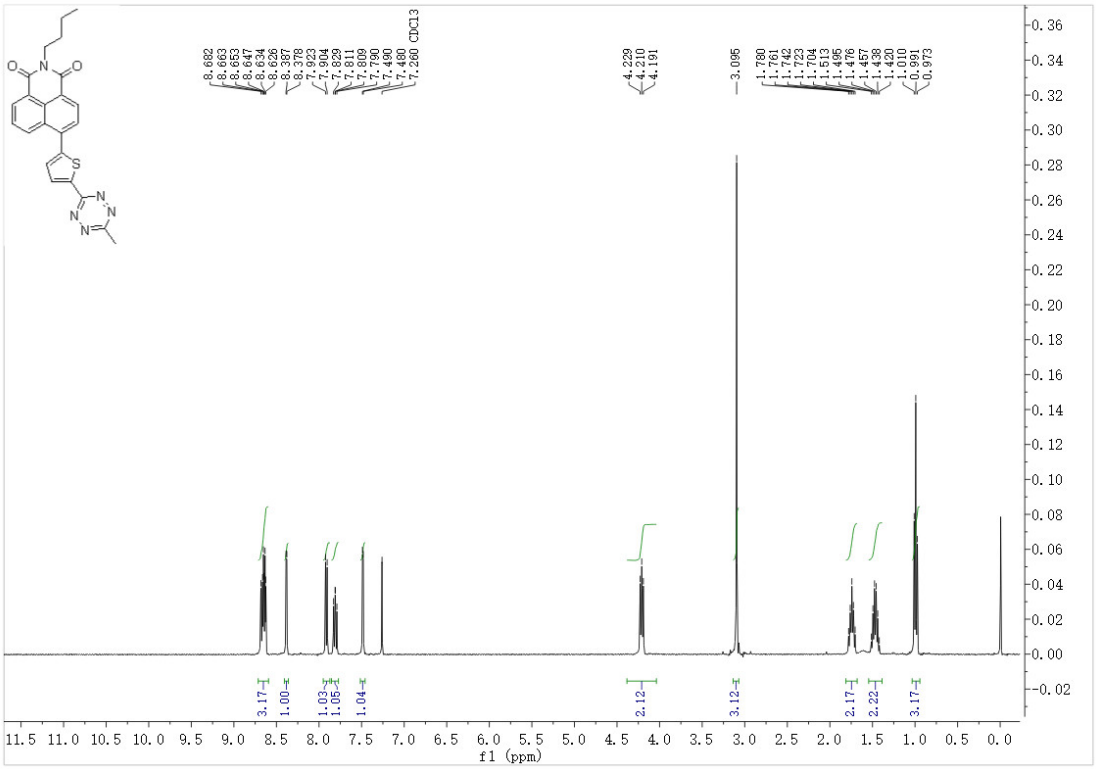
^{1}H NMR of compound **NP-Tz11** (400 MHz in $CDCl_3$)



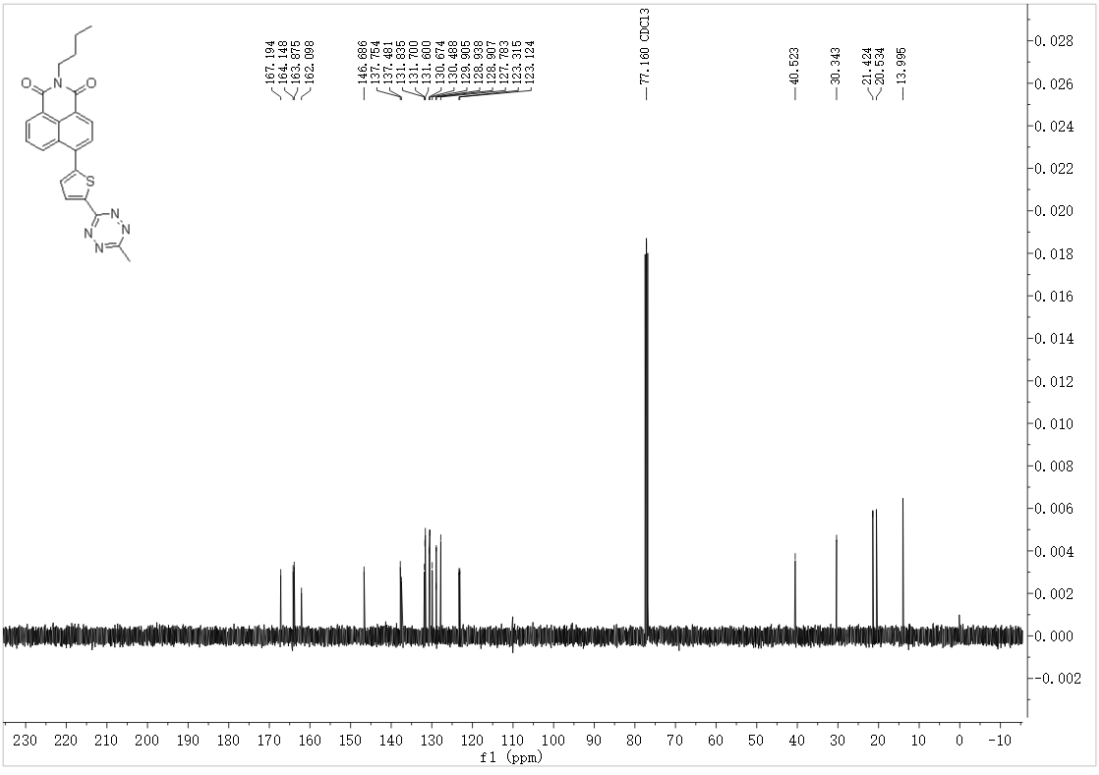
^{13}C NMR of compound **NP-Tz11** (101 MHz in $CDCl_3$)



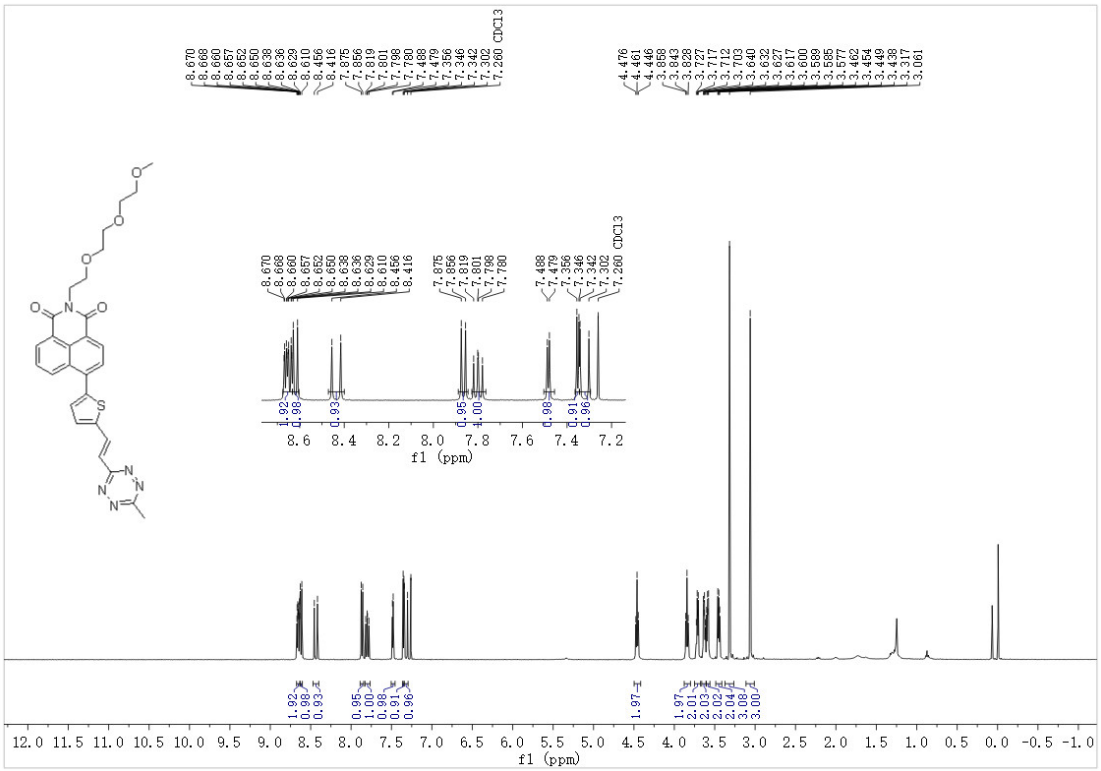
^{1}H NMR of compound **NP-Tz12** (400 MHz in $CDCl_3$)



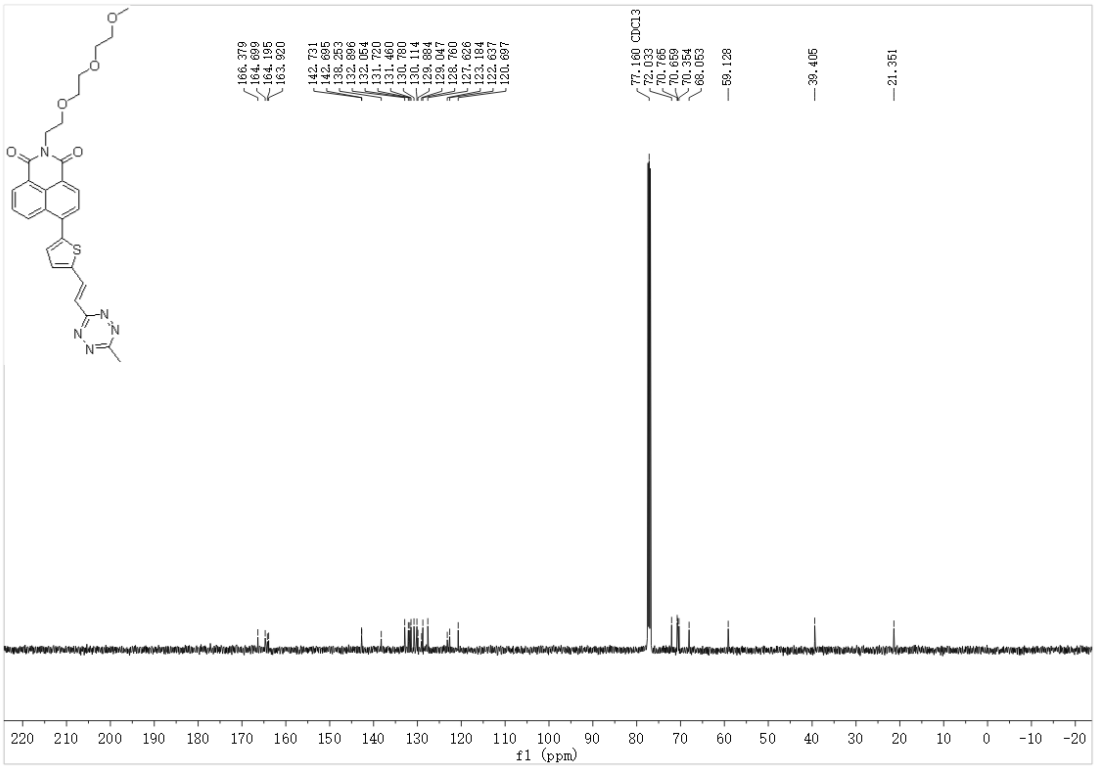
^{13}C NMR of compound **NP-Tz12** (101 MHz in $CDCl_3$)



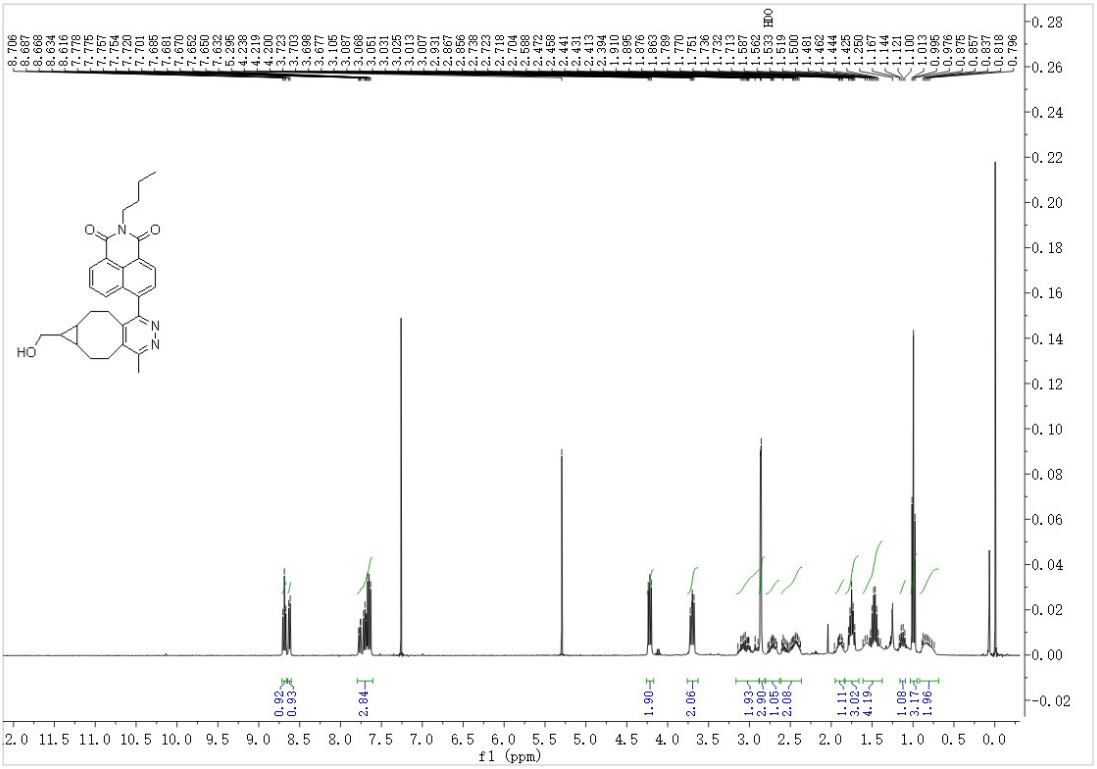
^{1}H NMR of compound **NP-Tz13** (400 MHz in $CDCl_3$)



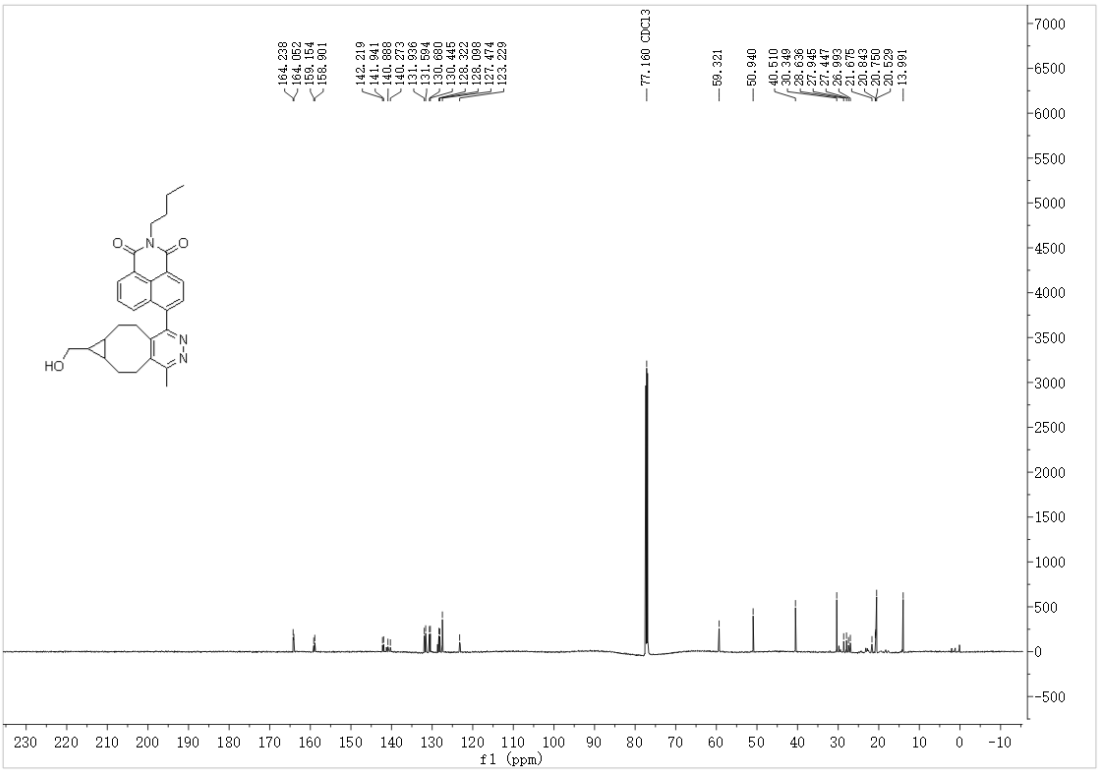
^{13}C NMR of compound **NP-Tz13** (126 MHz in $CDCl_3$)



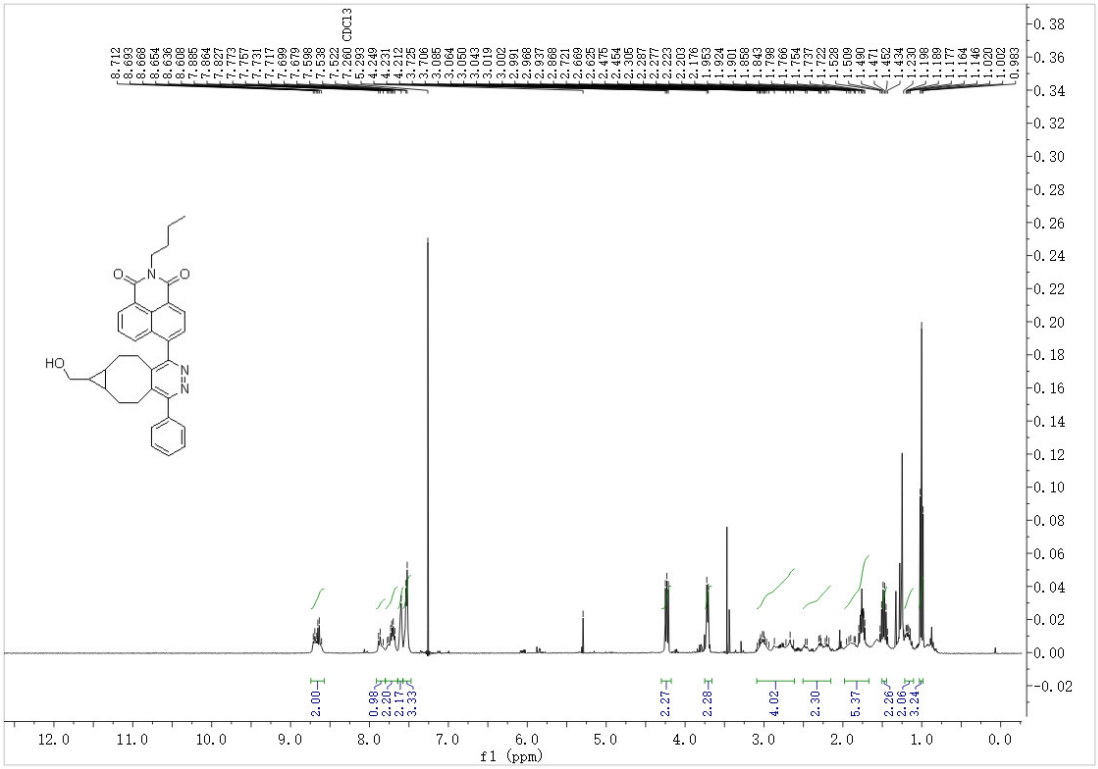
^{1}H NMR of compound **NP-Tz1BCN** (400 MHz in $CDCl_3$)



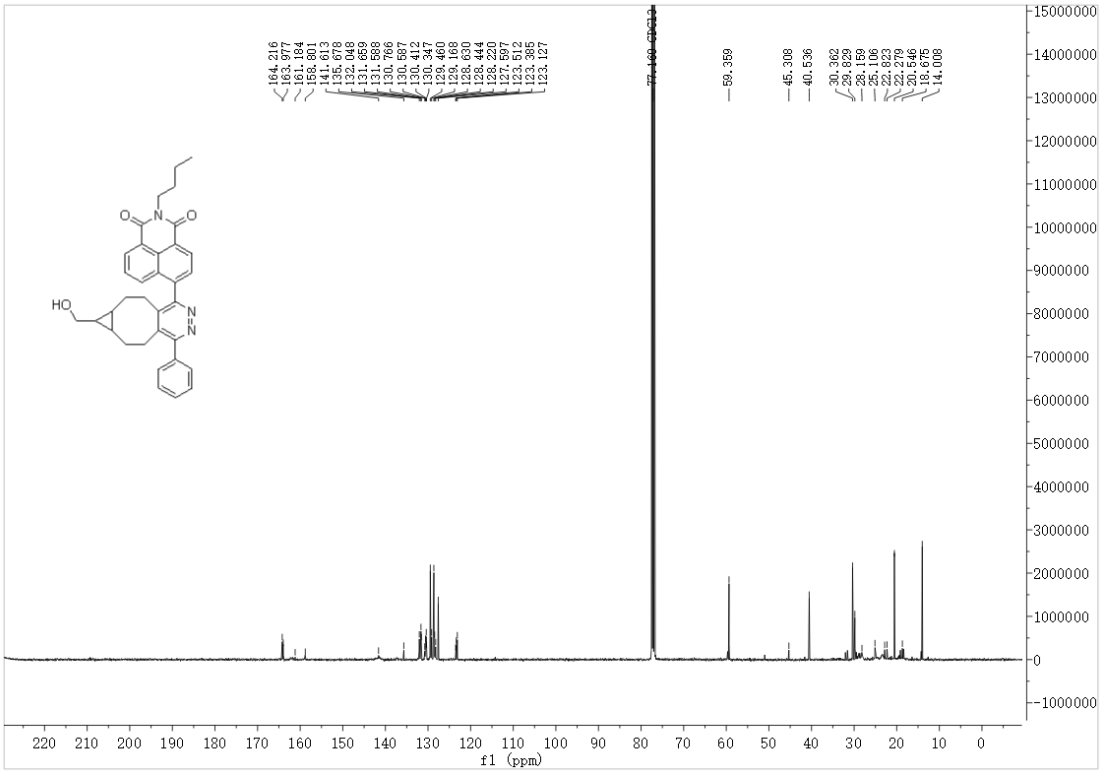
^{13}C NMR of compound **NP-Tz1BCN** (151 MHz in $CDCl_3$)



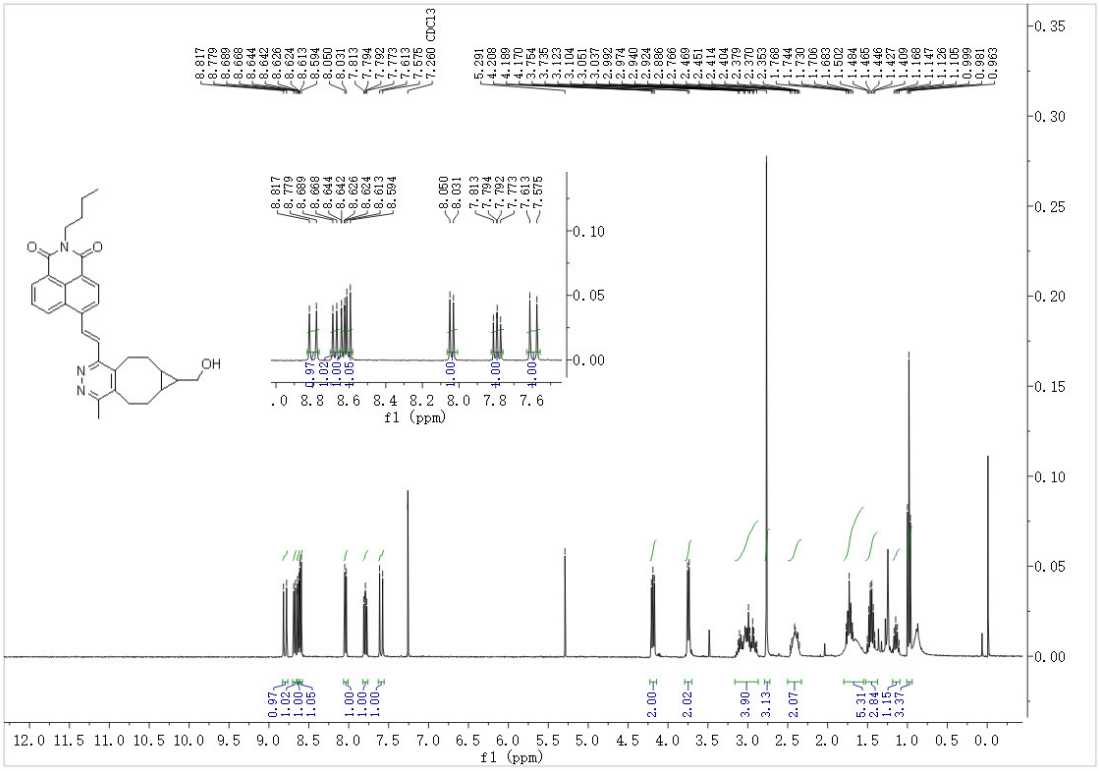
^{1}H NMR of compound **NP-Tz2BCN** (400 MHz in $CDCl_3$)



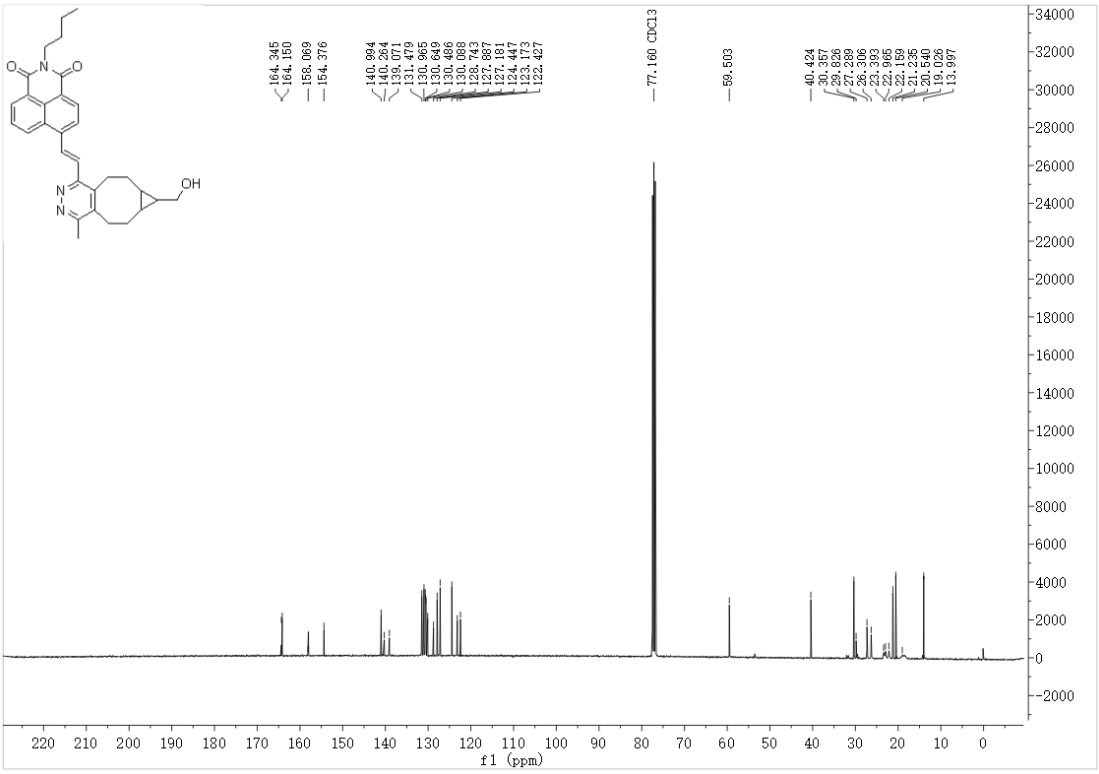
^{13}C NMR of compound **NP-Tz2BCN** (101 MHz in $CDCl_3$)



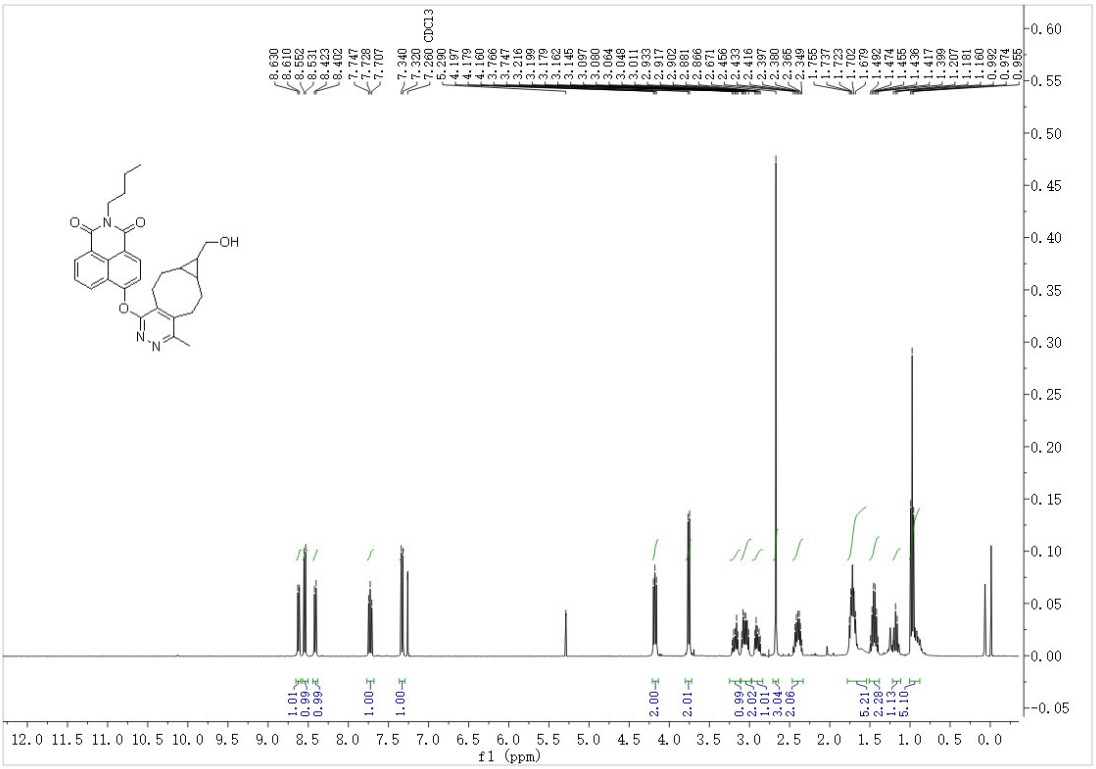
^{1}H NMR of compound **NP-Tz3BCN** (400 MHz in $CDCl_3$)



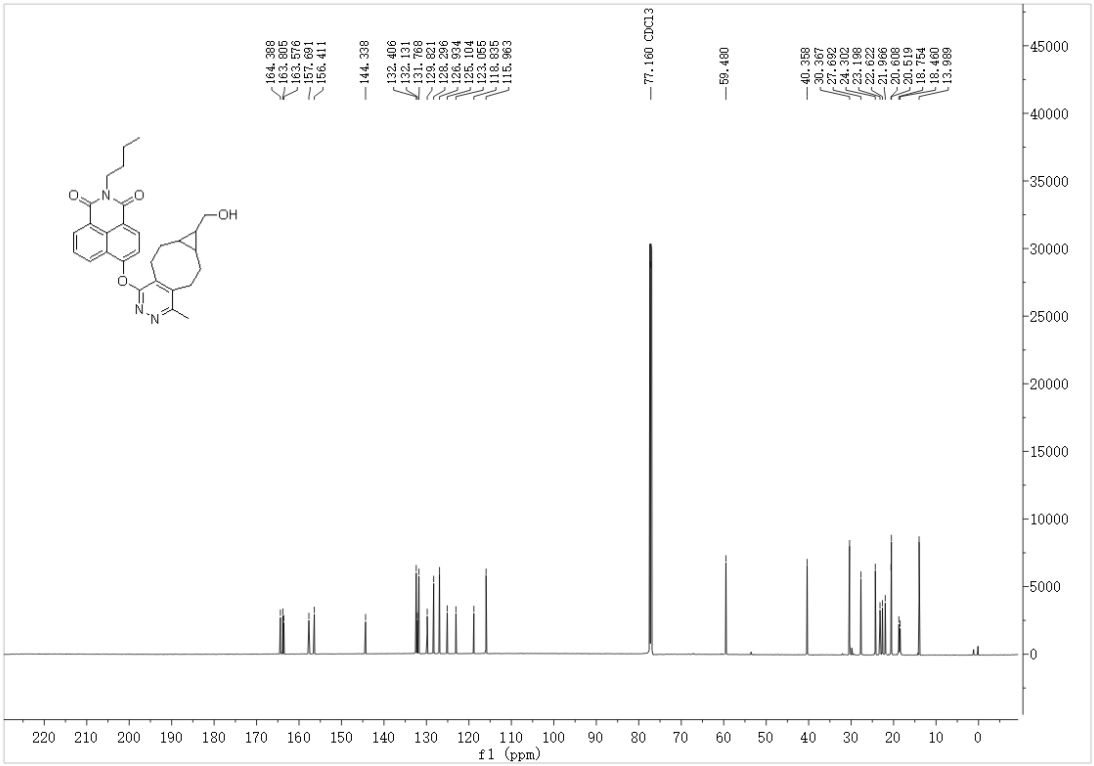
^{13}C NMR of compound **NP-Tz3BCN** (101 MHz in $CDCl_3$)



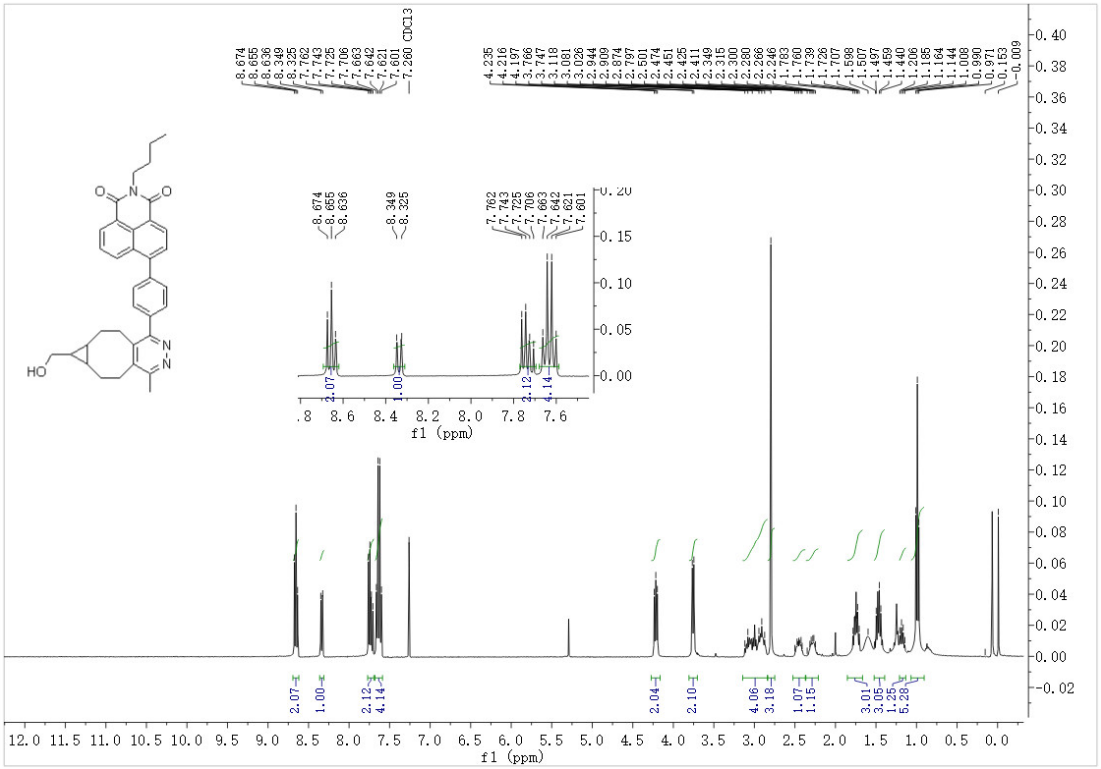
^{1}H NMR of compound **NP-Tz4BCN** (400 MHz in $CDCl_3$)



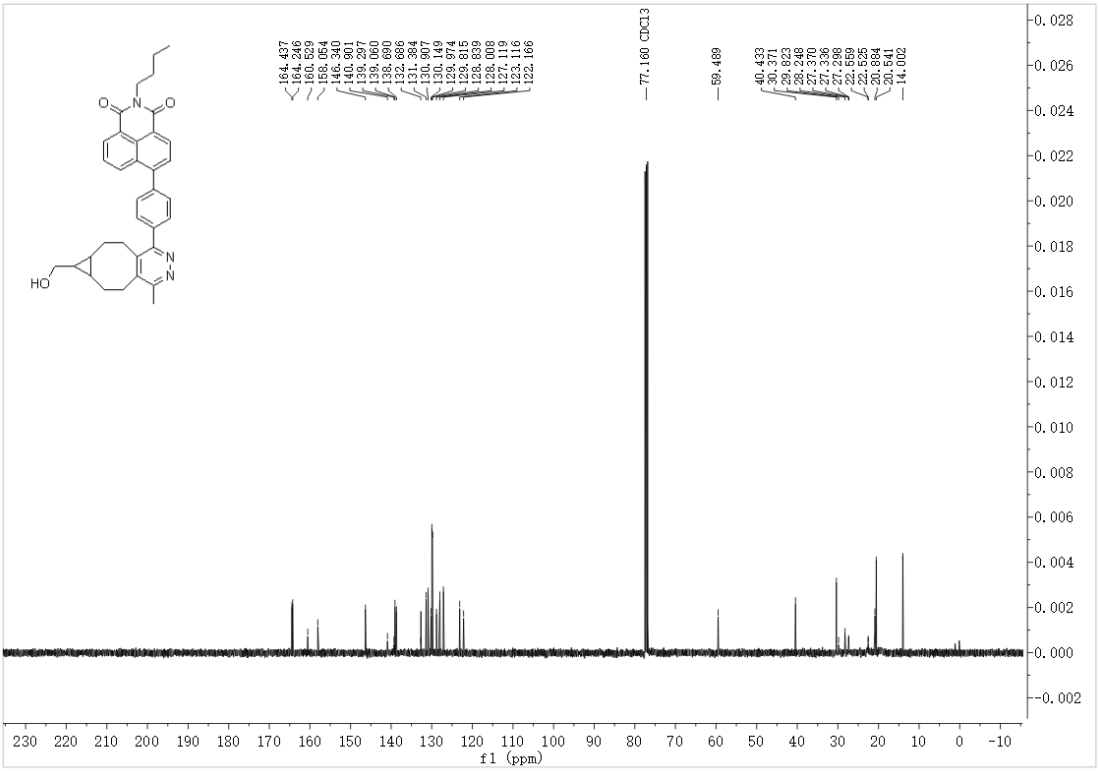
^{13}C NMR of compound **NP-Tz4BCN** (151 MHz in $CDCl_3$)



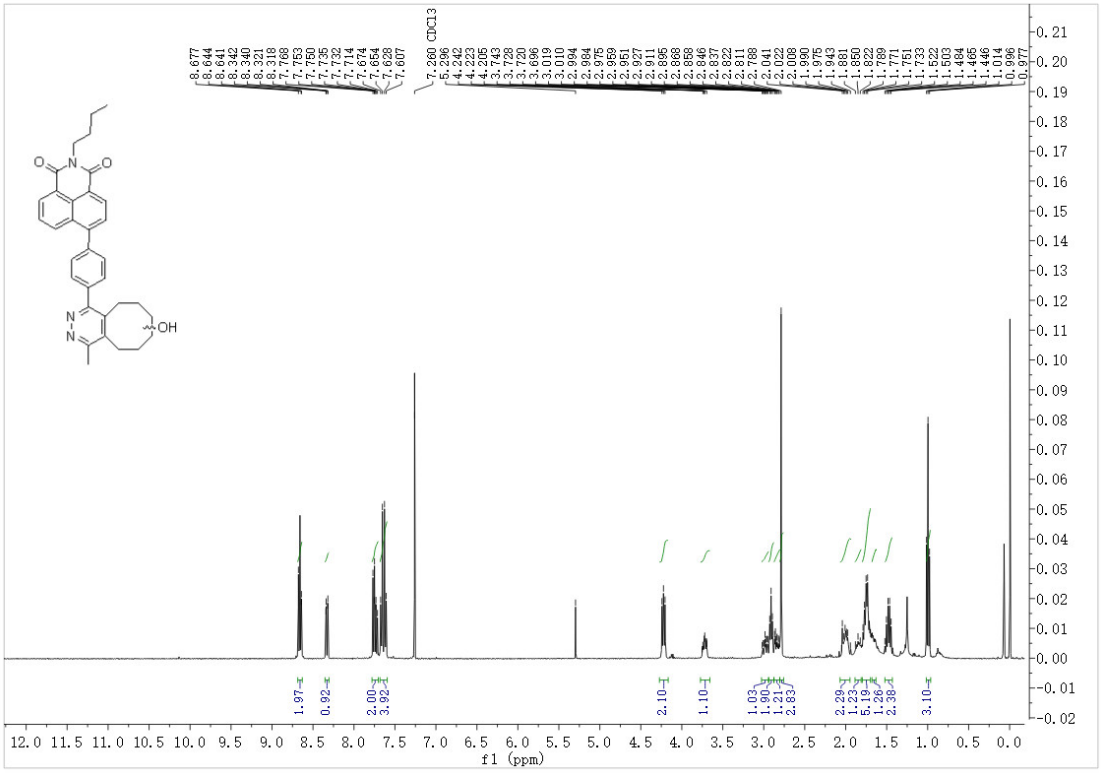
^{1}H NMR of compound **NP-Tz5BCN** (400 MHz in $CDCl_3$)



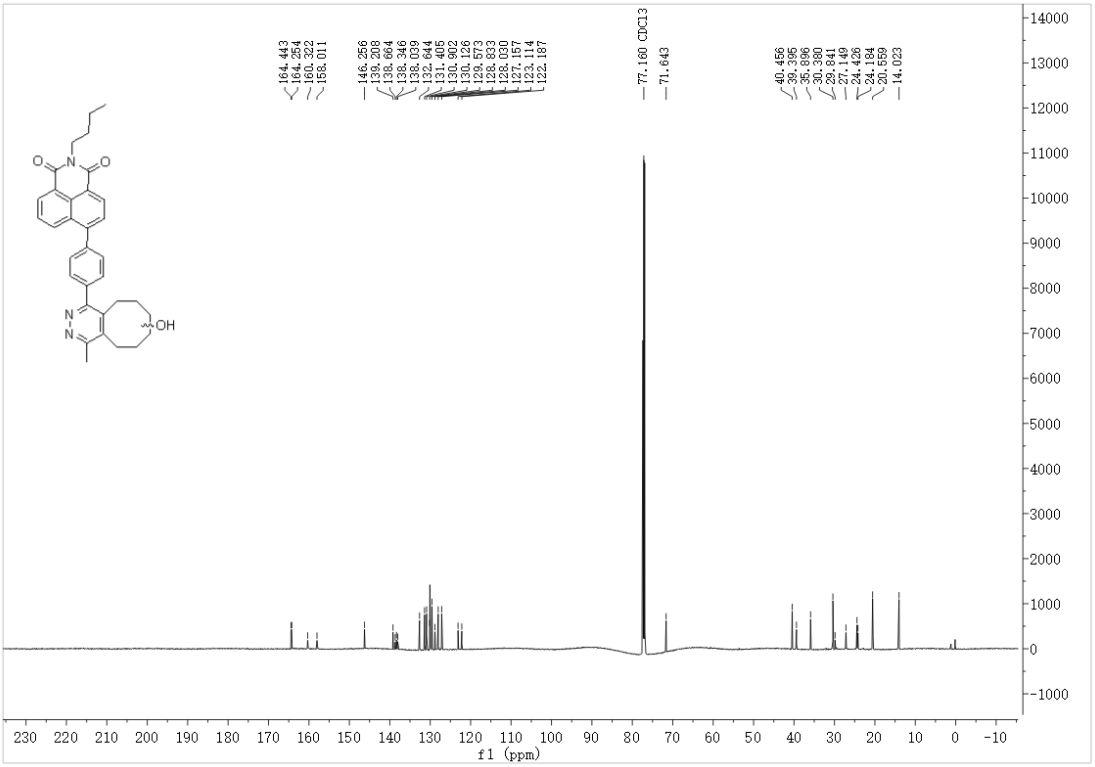
^{13}C NMR of compound **NP-Tz5BCN** (101 MHz in $CDCl_3$)



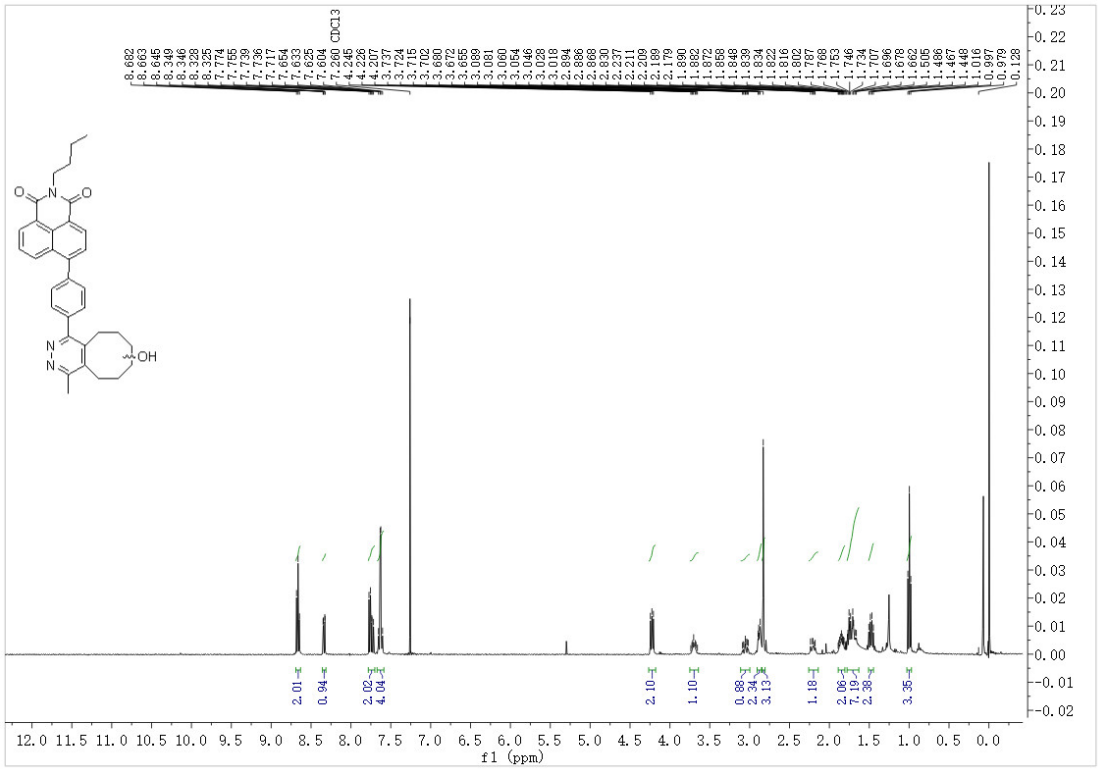
^{1}H NMR of compound **NP-Tz5TCO-1** (400 MHz in $CDCl_3$)



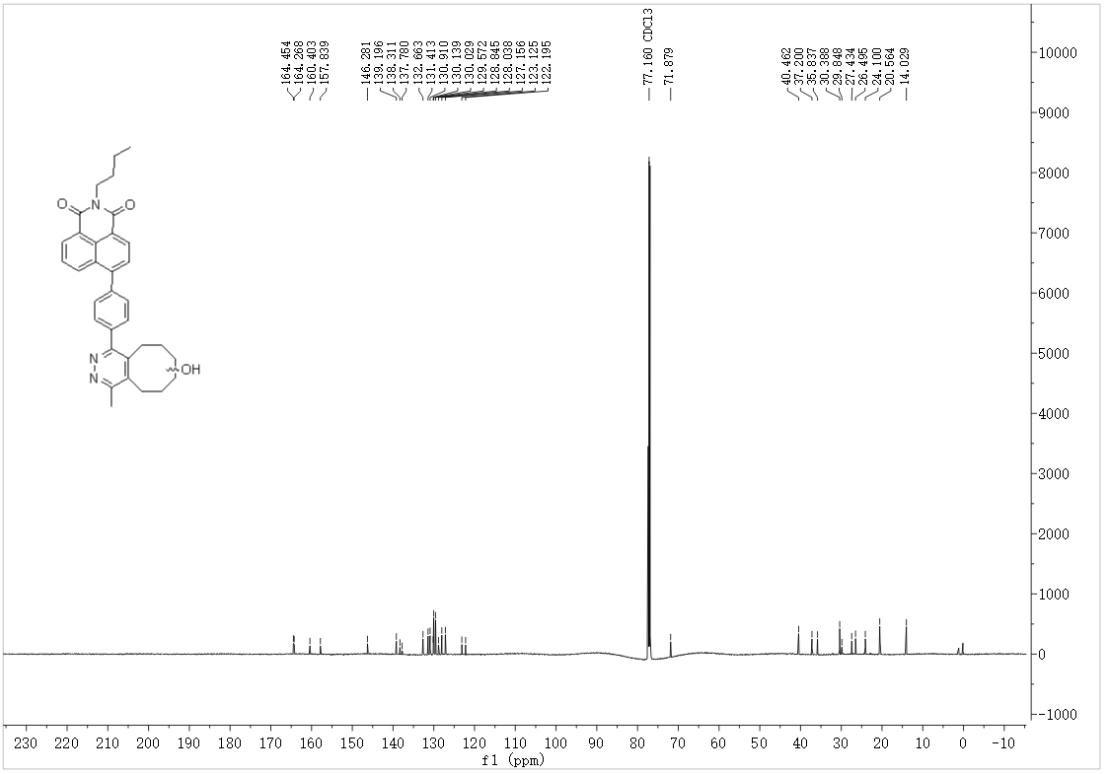
^{13}C NMR of compound **NP-Tz5TCO-1** (151 MHz in $CDCl_3$)



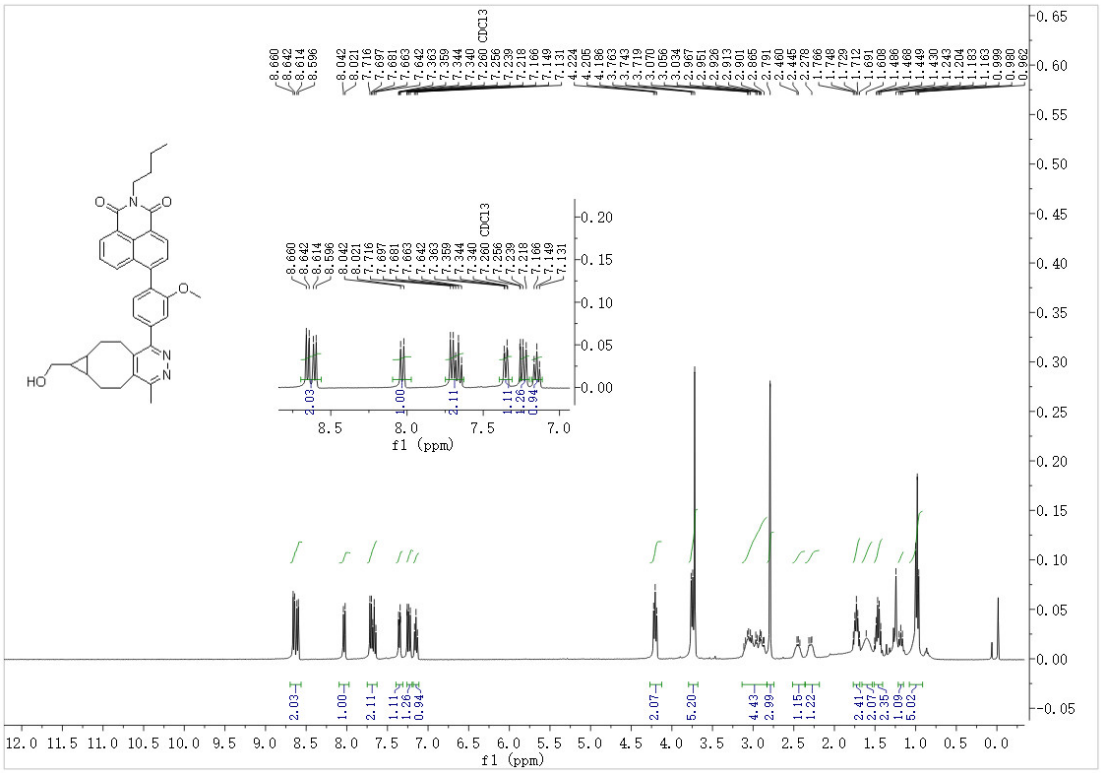
^{1}H NMR of compound **NP-Tz5TCO-2** (400 MHz in $CDCl_3$)



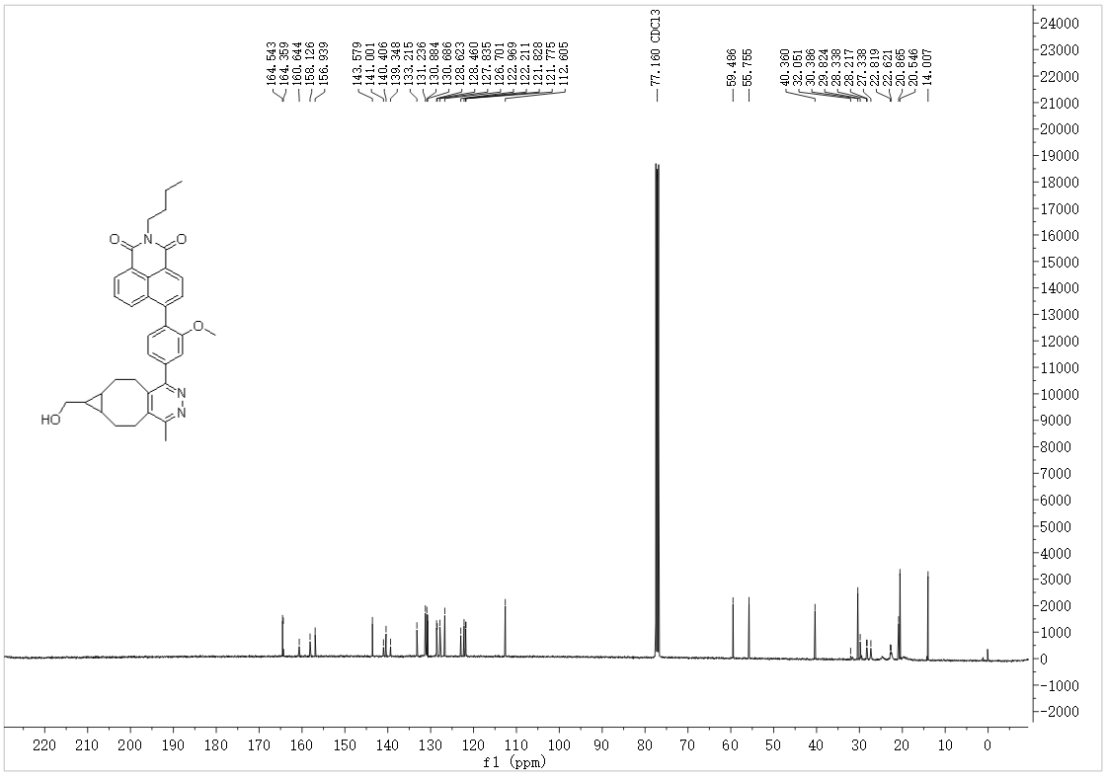
^{13}C NMR of compound **NP-Tz5TCO-2** (151 MHz in $CDCl_3$)



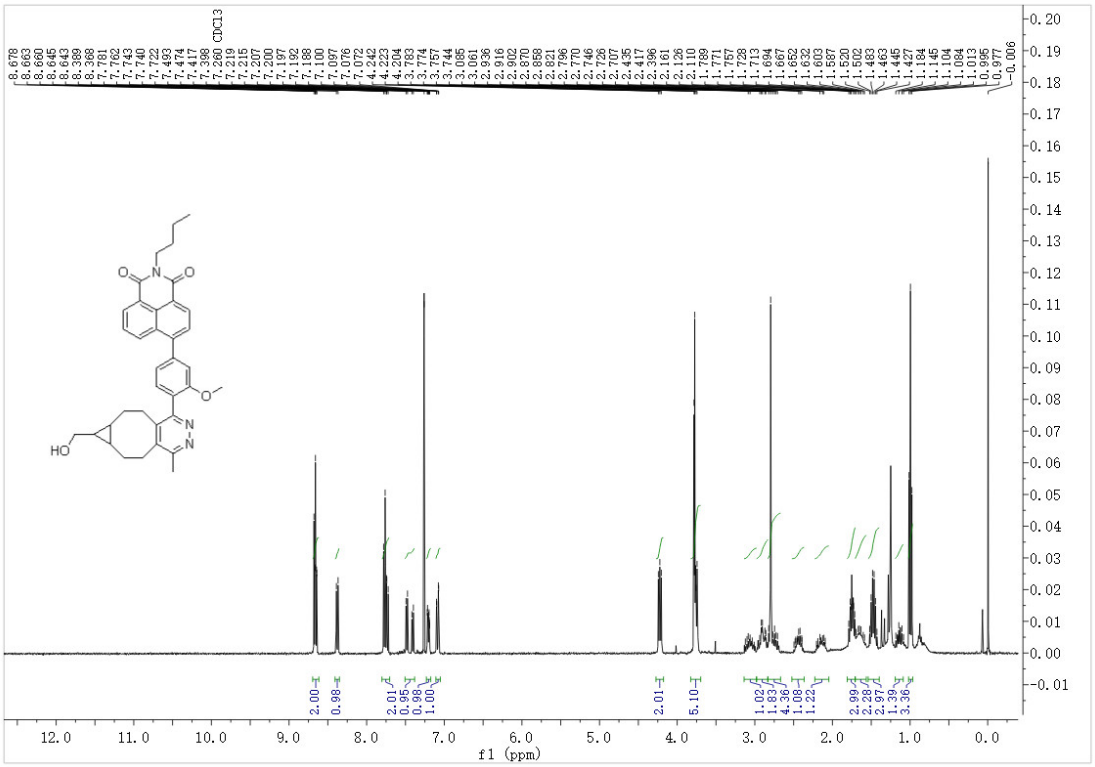
^{1}H NMR of compound **NP-Tz6BCN** (400 MHz in $CDCl_3$)



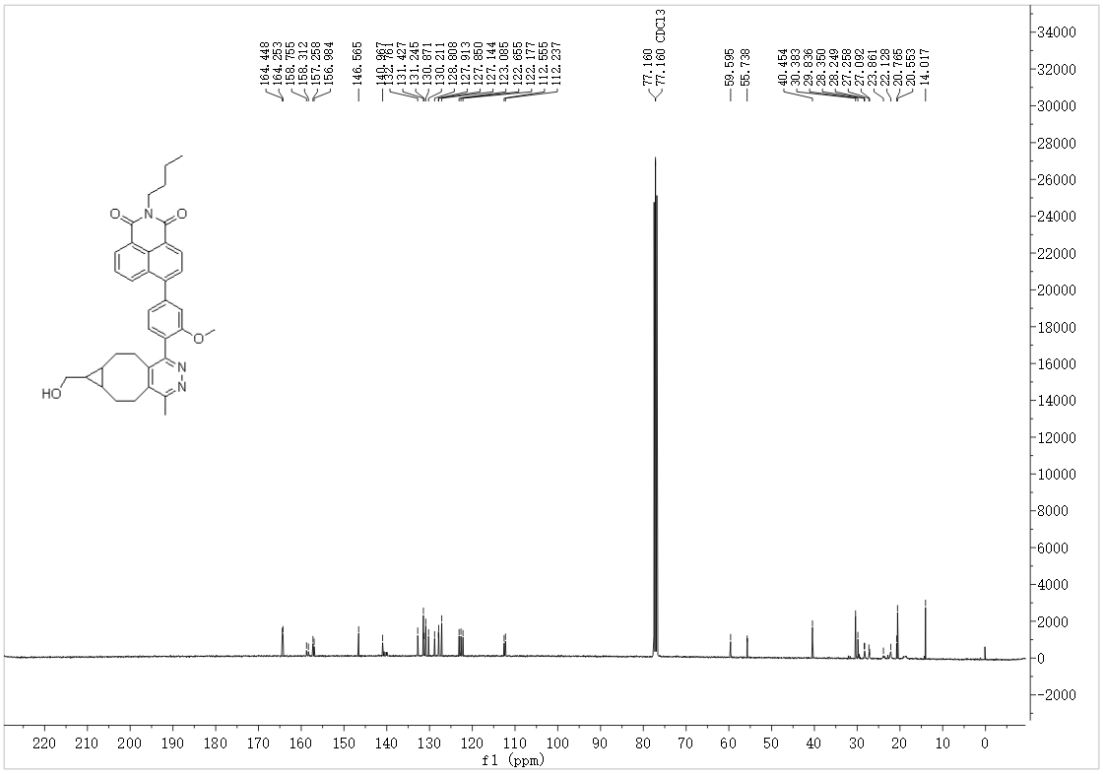
^{13}C NMR of compound **NP-Tz6BCN** (101 MHz in $CDCl_3$)



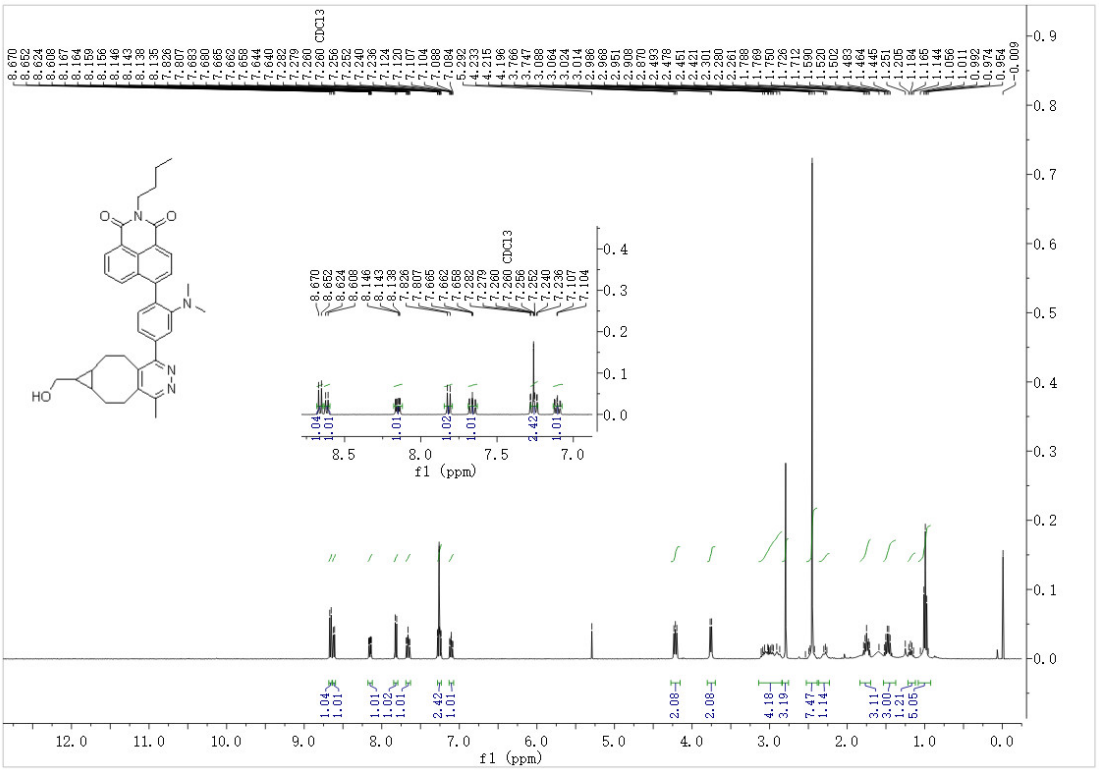
^{1}H NMR of compound **NP-Tz7BCN** (400 MHz in $CDCl_3$)



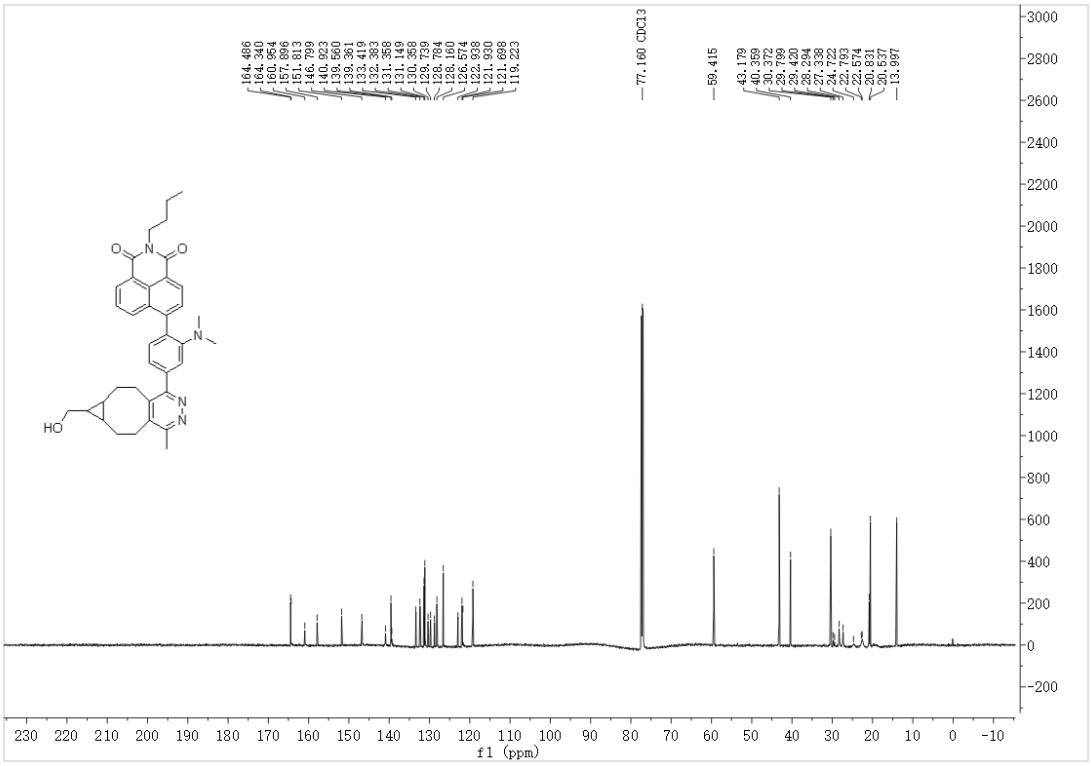
^{13}C NMR of compound **NP-Tz7BCN** (101 MHz in $CDCl_3$)



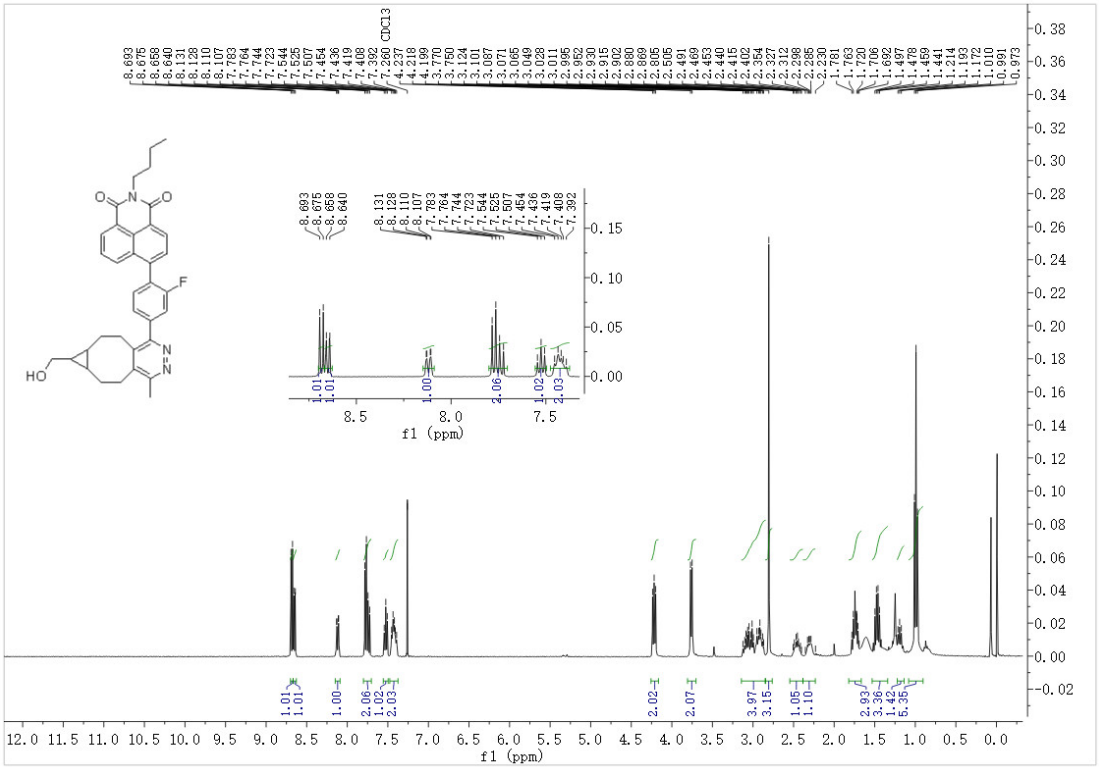
^{1}H NMR of compound **NP-Tz8BCN** (400 MHz in $CDCl_3$)



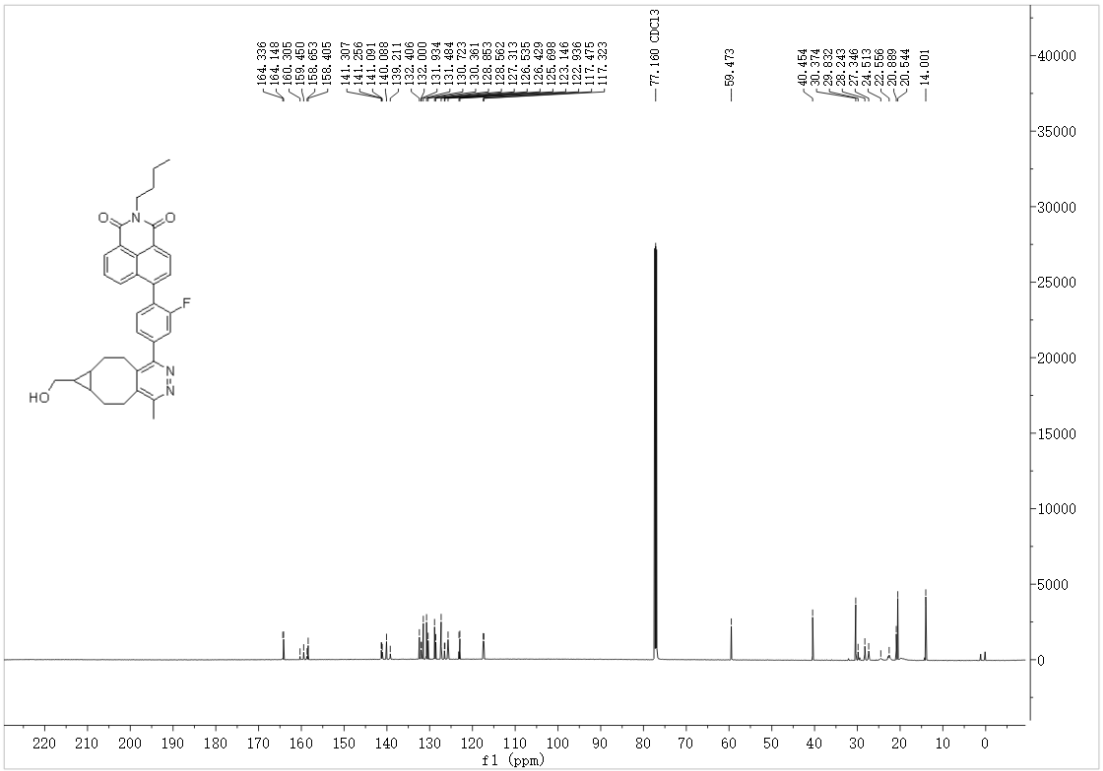
^{13}C NMR of compound **NP-Tz8BCN** (151 MHz in $CDCl_3$)



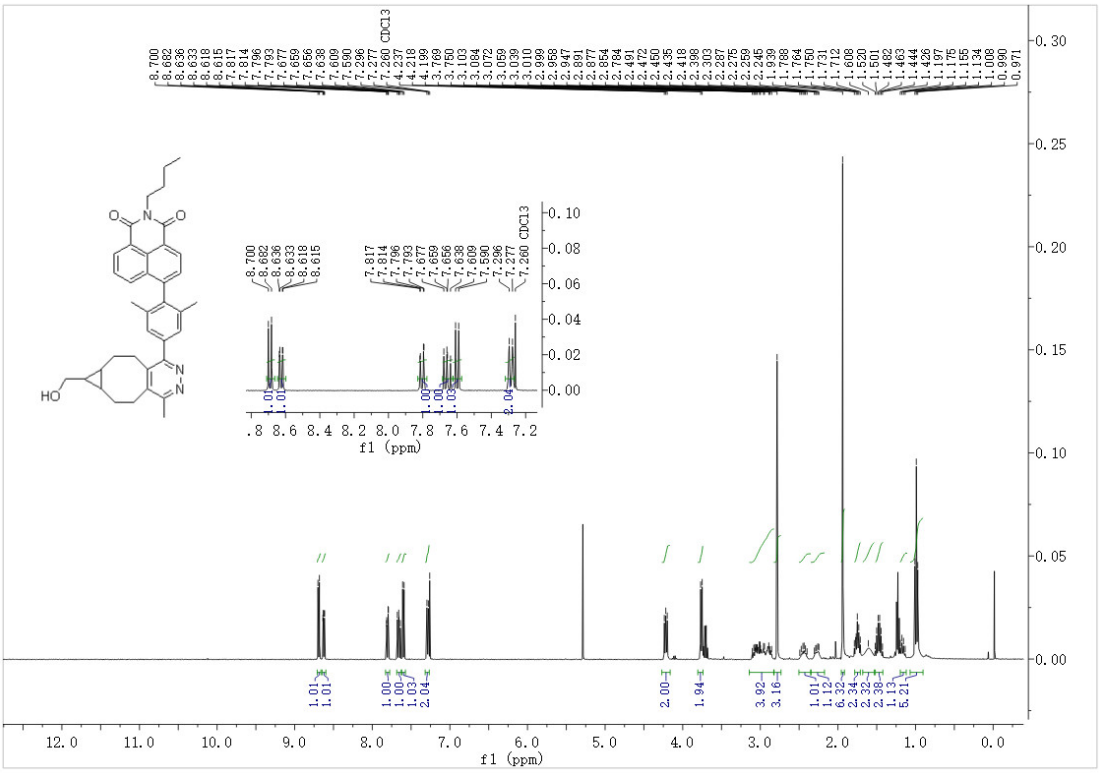
^{1}H NMR of compound **NP-Tz9BCN** (400 MHz in $CDCl_3$)



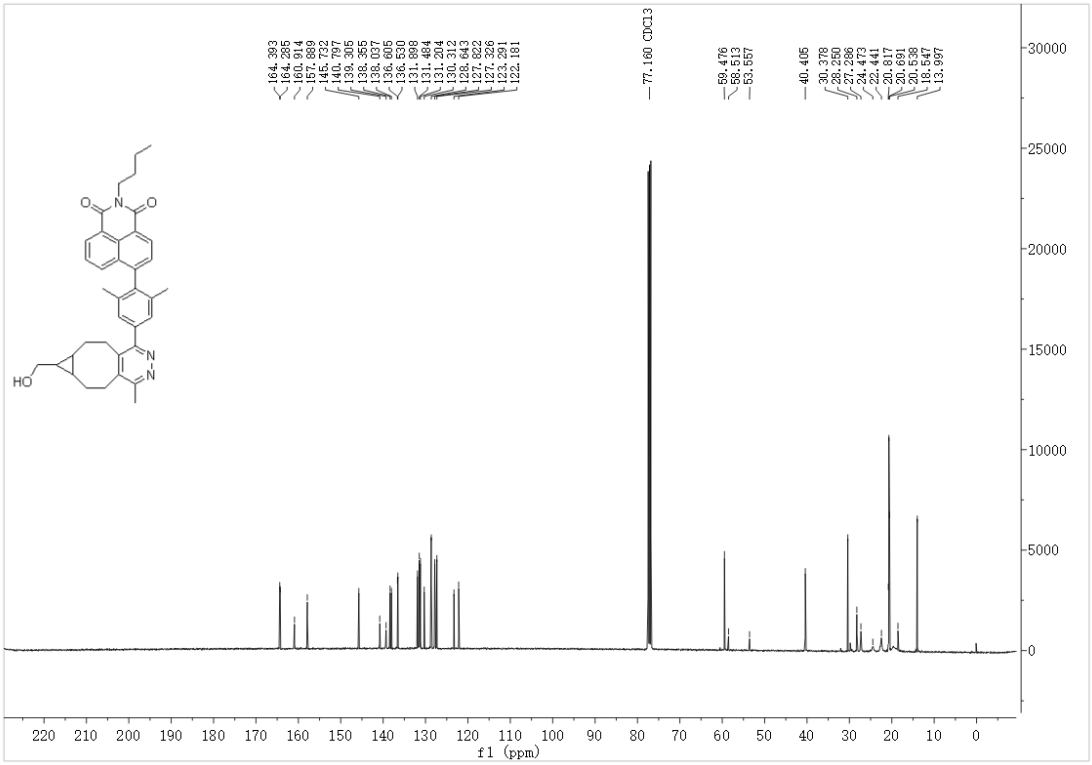
^{13}C NMR of compound **NP-Tz9BCN** (101 MHz in $CDCl_3$)



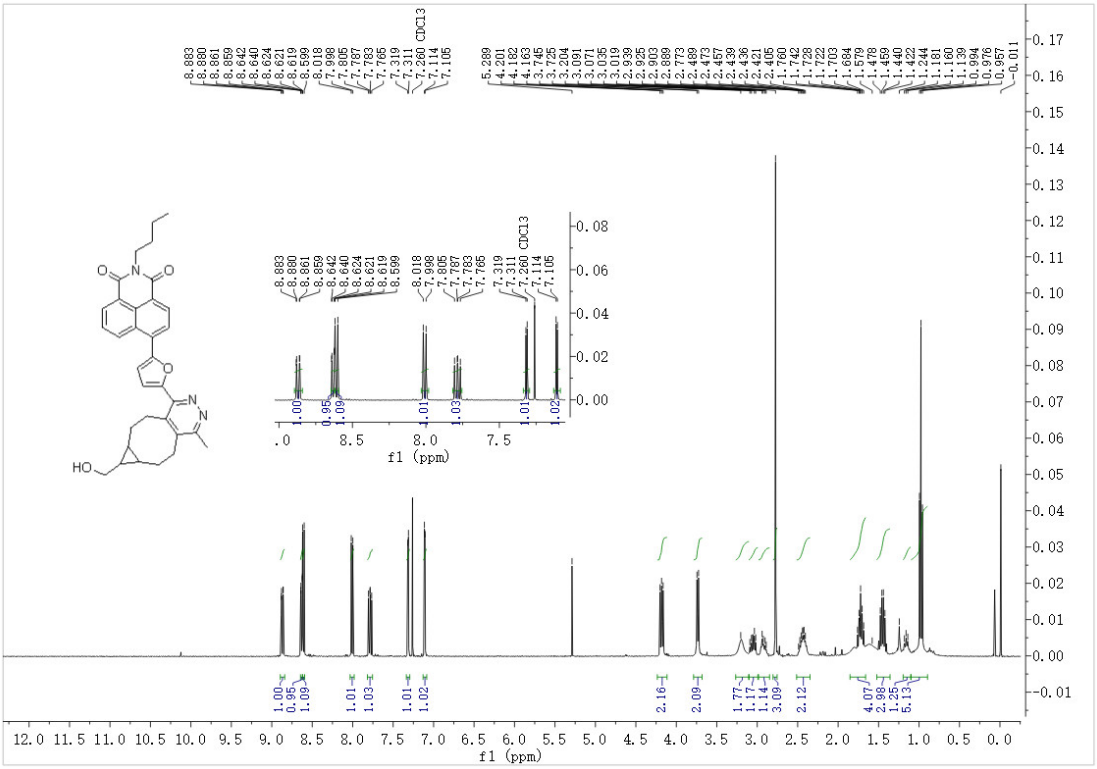
^{1}H NMR of compound **NP-Tz10BCN** (400 MHz in $CDCl_3$)



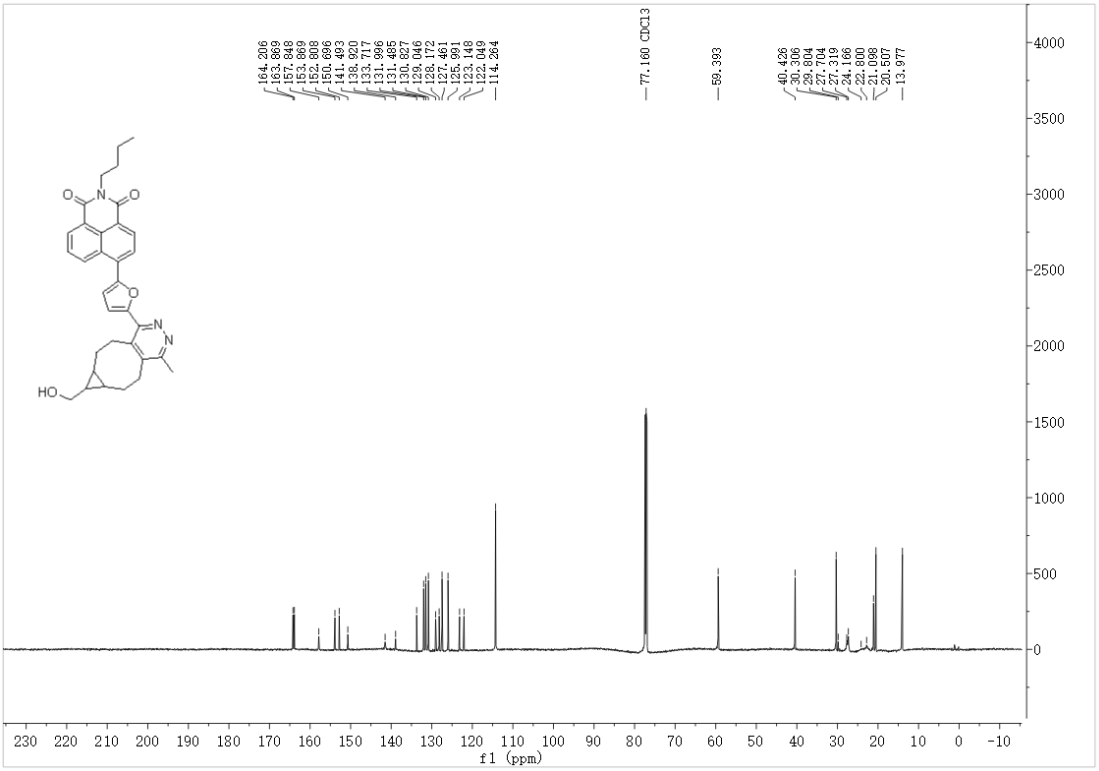
^{13}C NMR of compound **NP-Tz10BCN** (101 MHz in $CDCl_3$)



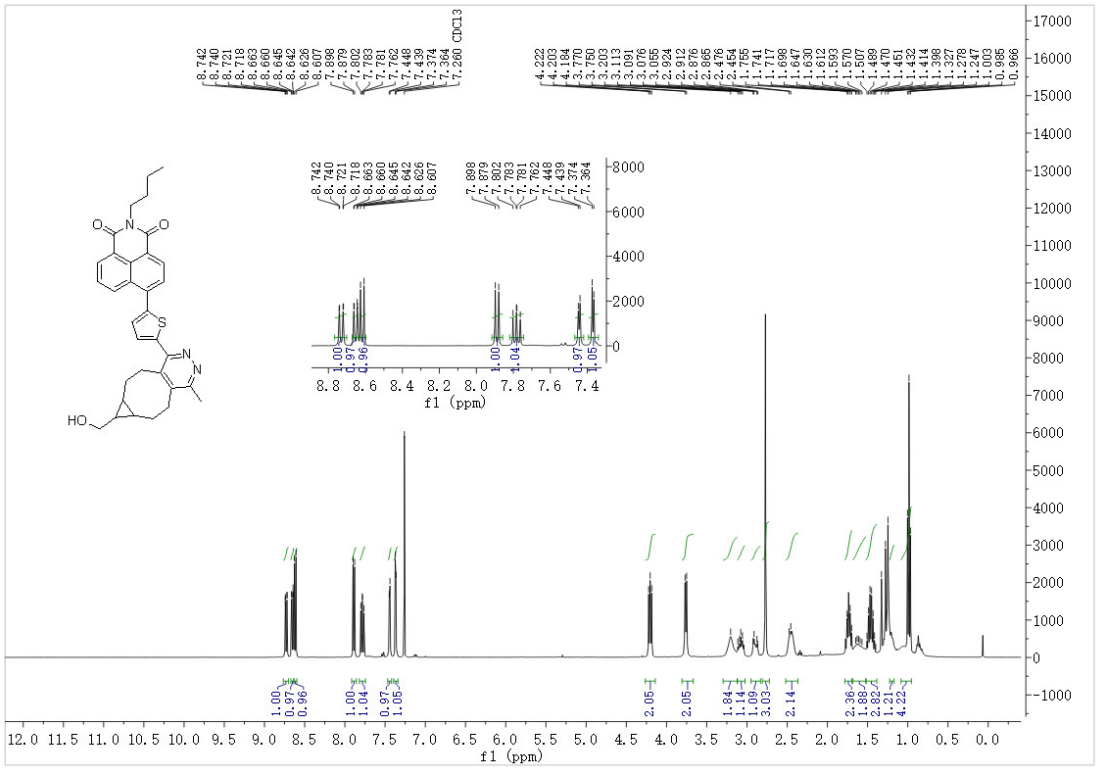
^{1}H NMR of compound **NP-Tz11BCN** (400 MHz in $CDCl_3$)



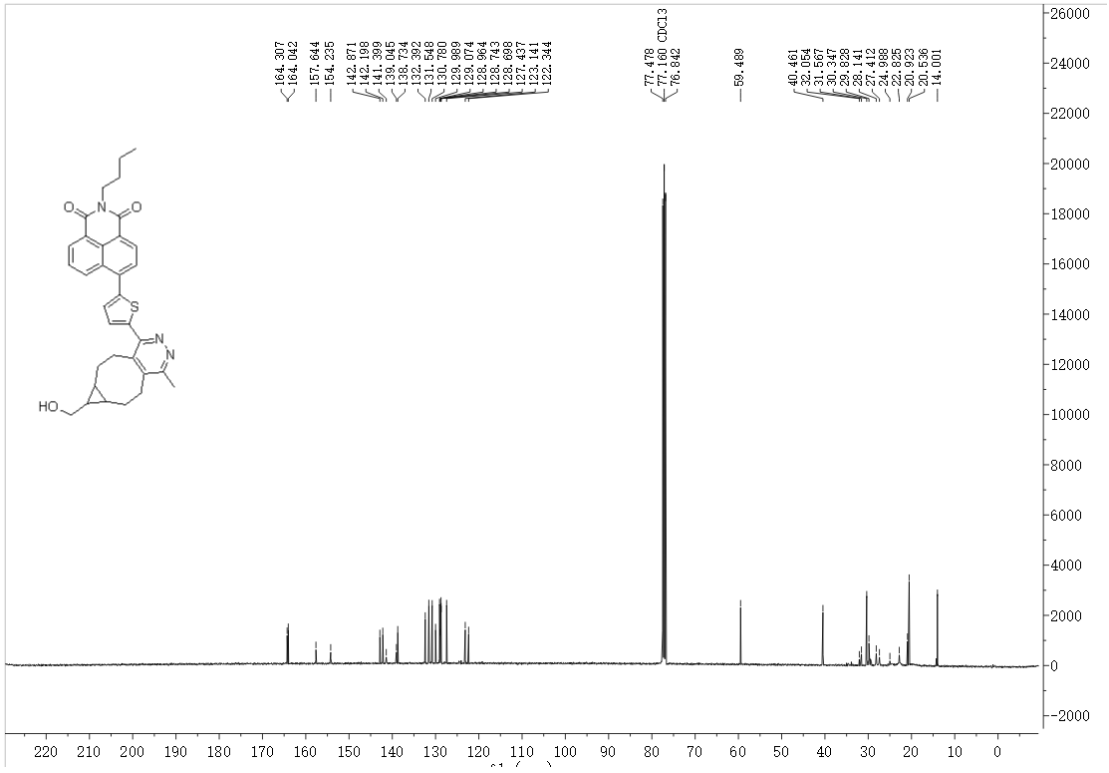
^{13}C NMR of compound **NP-Tz11BCN** (151 MHz in $CDCl_3$)



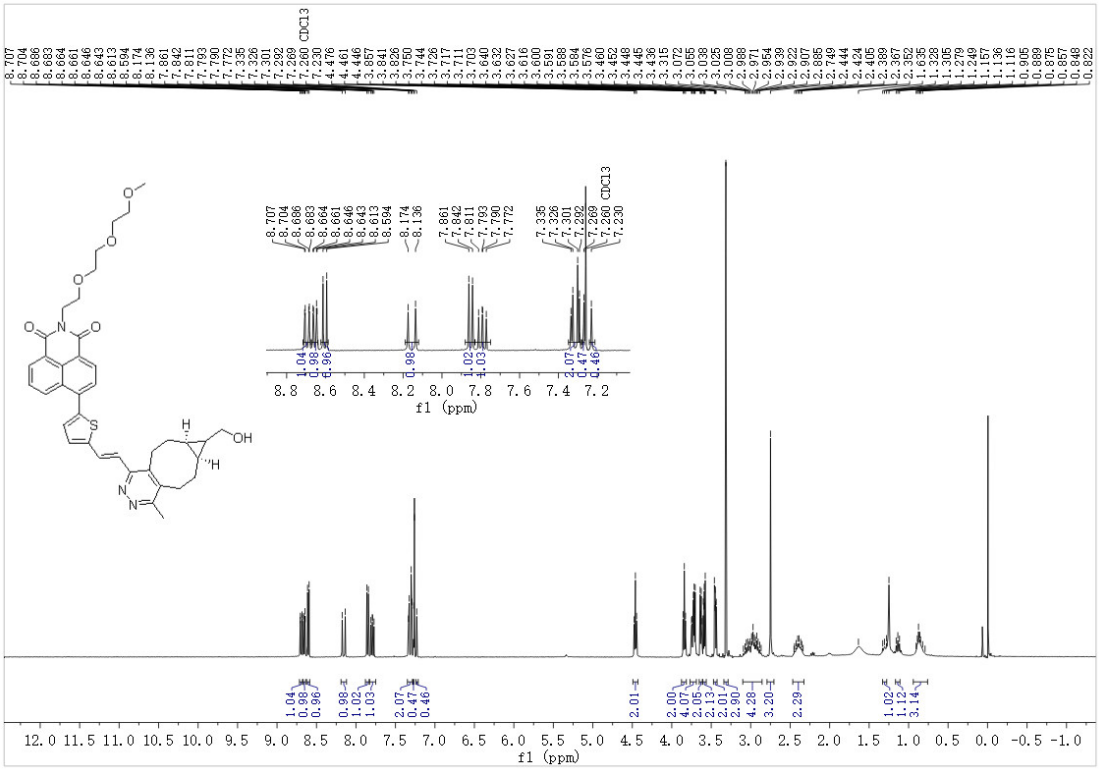
^{1}H NMR of compound **NP-Tz12BCN** (400 MHz in $CDCl_3$)



^{13}C NMR of compound **NP-Tz12BCN** (101 MHz in $CDCl_3$)



^{1}H NMR of compound **NP-Tz13BCN** (400 MHz in $CDCl_3$)



^{13}C NMR of compound **NP-Tz13BCN** (101 MHz in $CDCl_3$)