Electronic Supplementary Material (ESI) for Materials Chemistry Frontiers.

Supporting Information for

Tetrazine-Derived Chromones as Conditionally Activated Solvatochromic Fluorescent Probes for Dual Imaging of Droplets and Mitochondria

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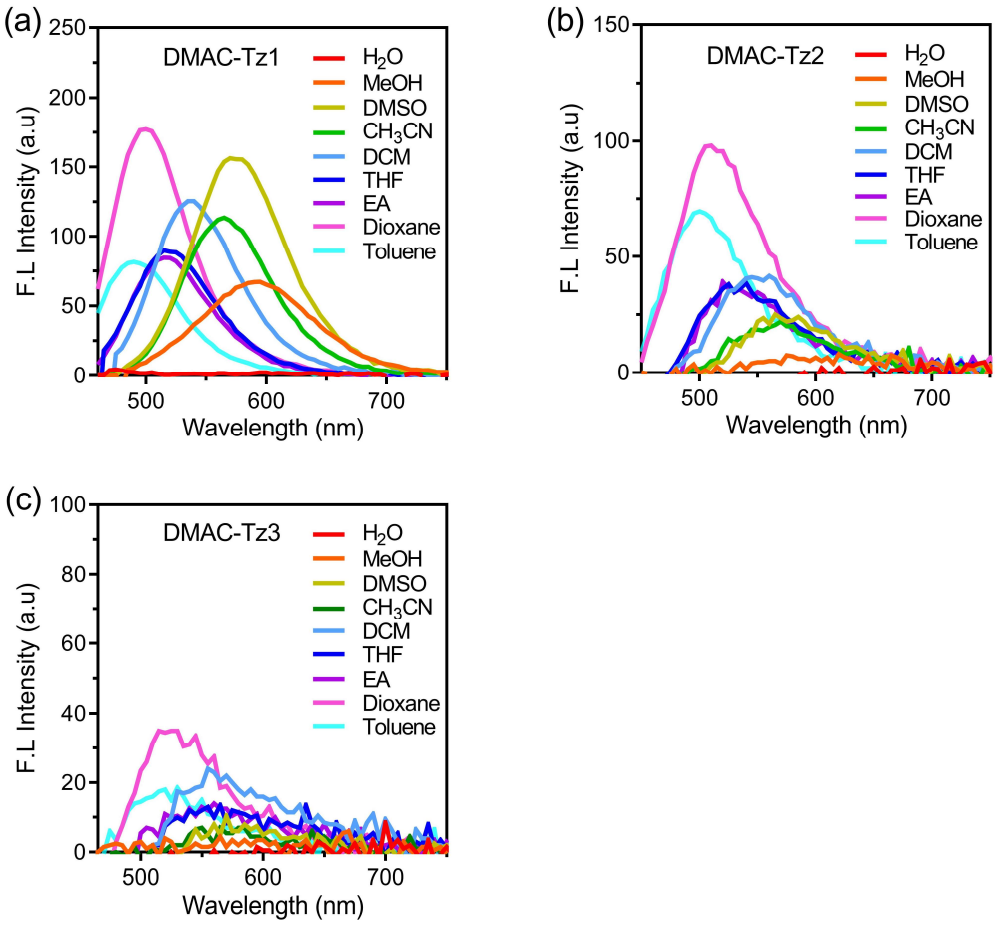
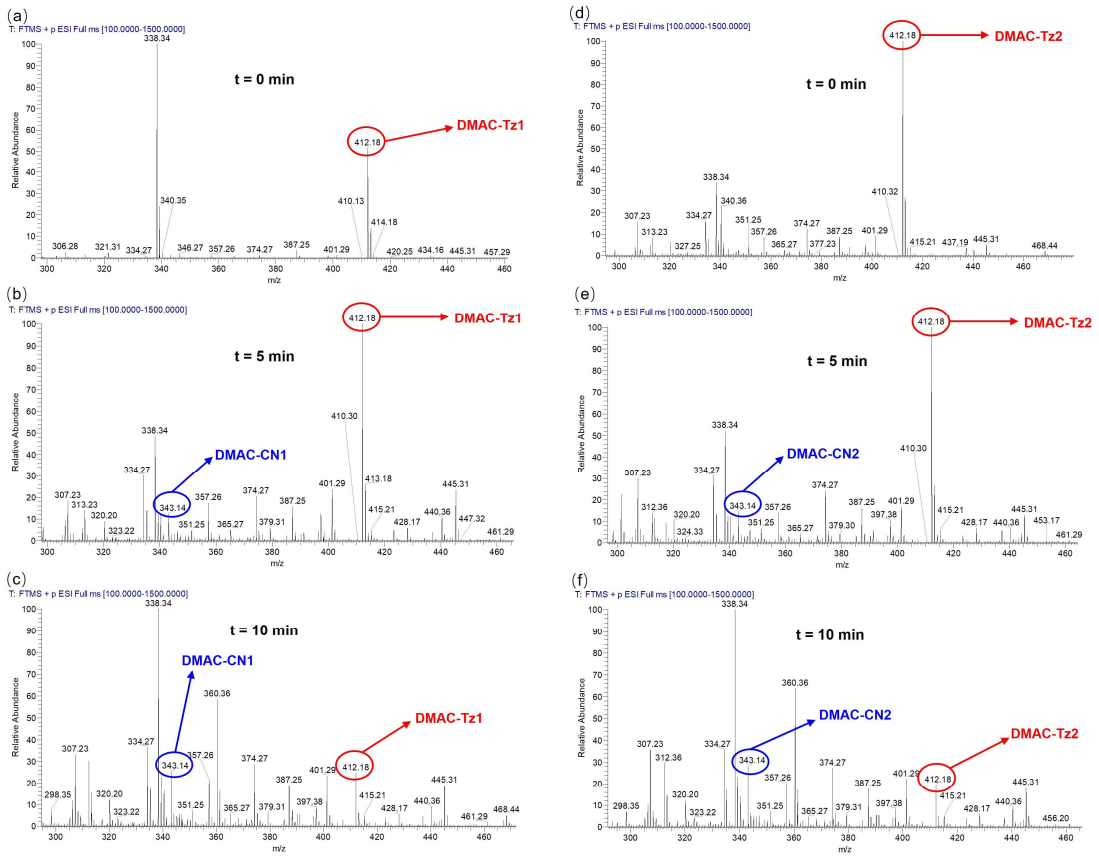


Fig. S1 Fluorescence emission spectra of DMAC-Tz1 (a), DMAC-Tz2 (b), and DMAC-Tz3 (c) in various solvents (10 μM) (λ_{exc} = 405 nm).



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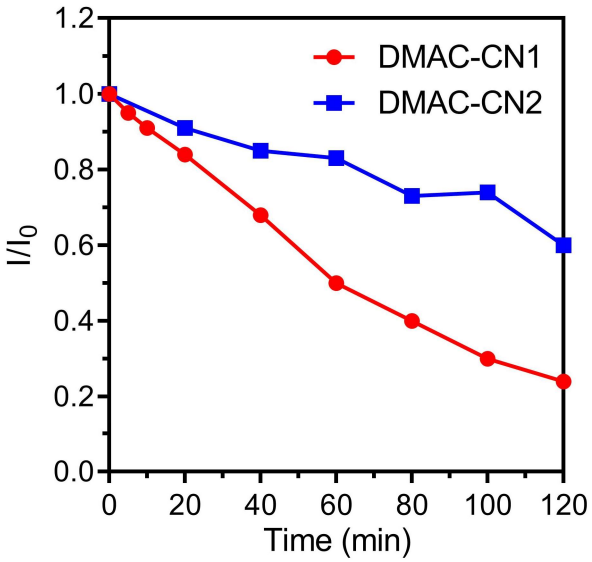
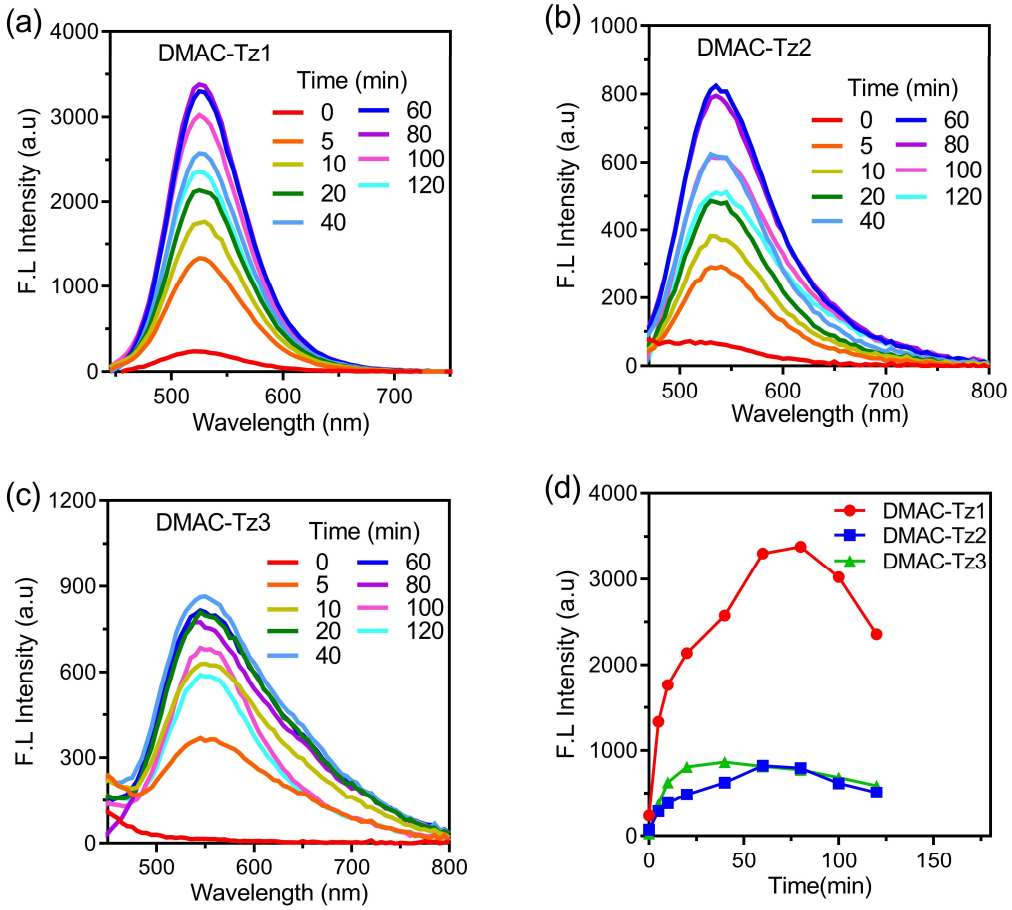


Fig. S3 Plot of relative emission intensity (I/I_0) at maximum emission of DMAC-CN1 and DMAC-CN2 versus the irradiation time (254 nm UV light irradiation).



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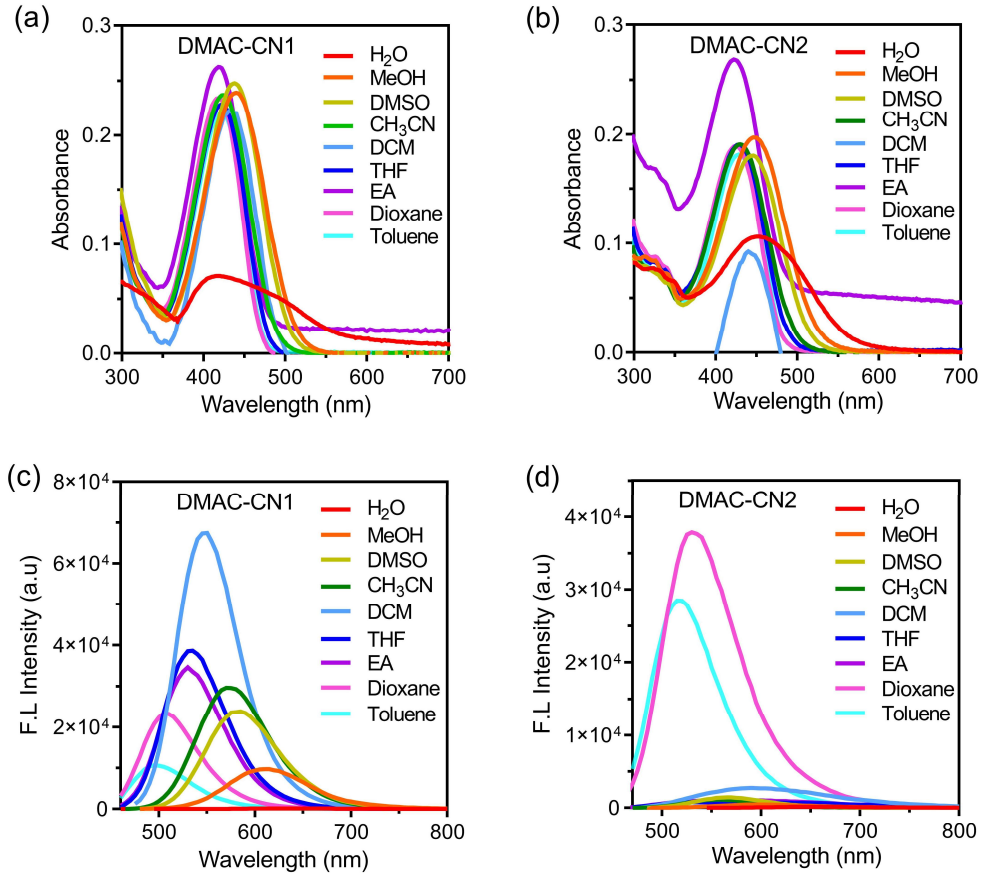

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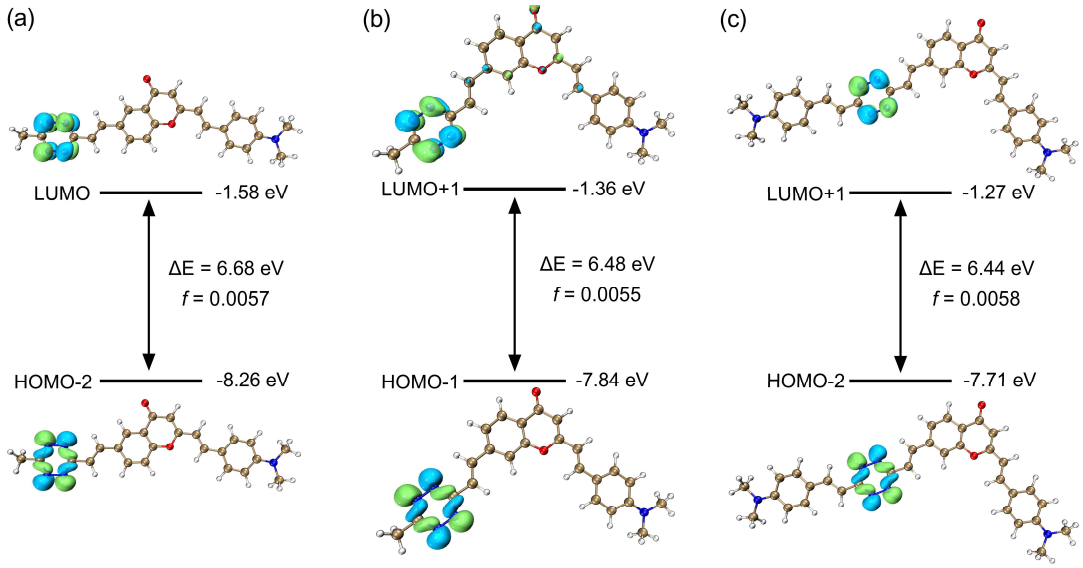


Fig. S6 Molecular orbital distribution, HOMO-LUMO energy gap, and oscillator strength values (*f*) of DMAC-Tz1 (a), DMAC-Tz2 (b), and DMAC-Tz3 (c) obtained by TD-DFT calculation (CAM-B3LYP/6-311G*) of the corresponding first excited-state optimized structures. The main contributing orbital of S_0−S_1 for each compound is illustrated.

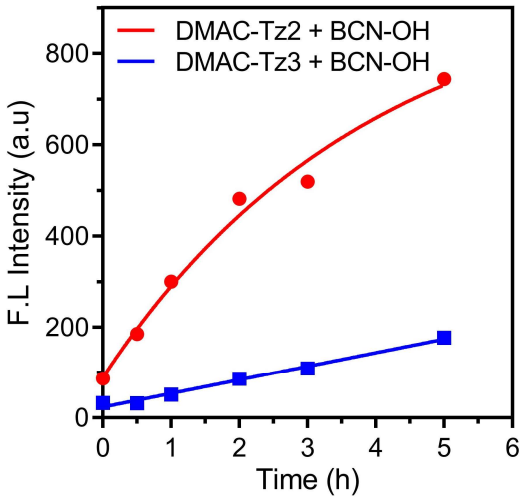
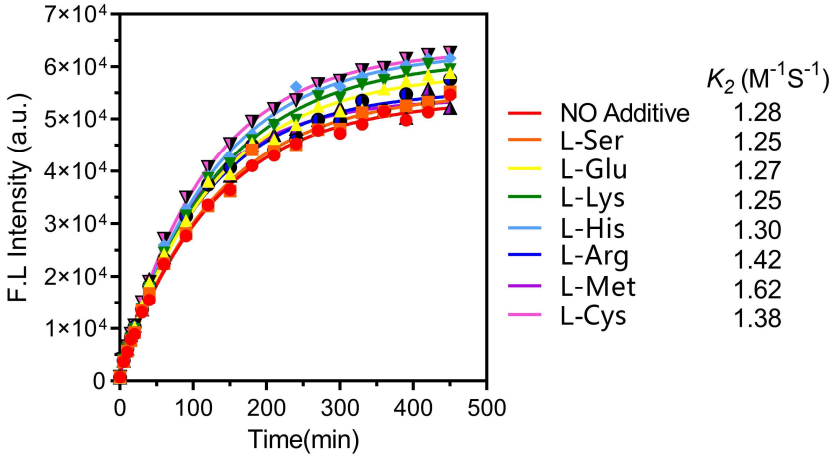


Fig. S7 Analysis of reaction kinetics for the reaction between DMAC-Tz2 (0.1 mM) or DMAC-Tz3 (0.1 mM) and BCN-OH (1 mM) in dioxane at 37 ℃.



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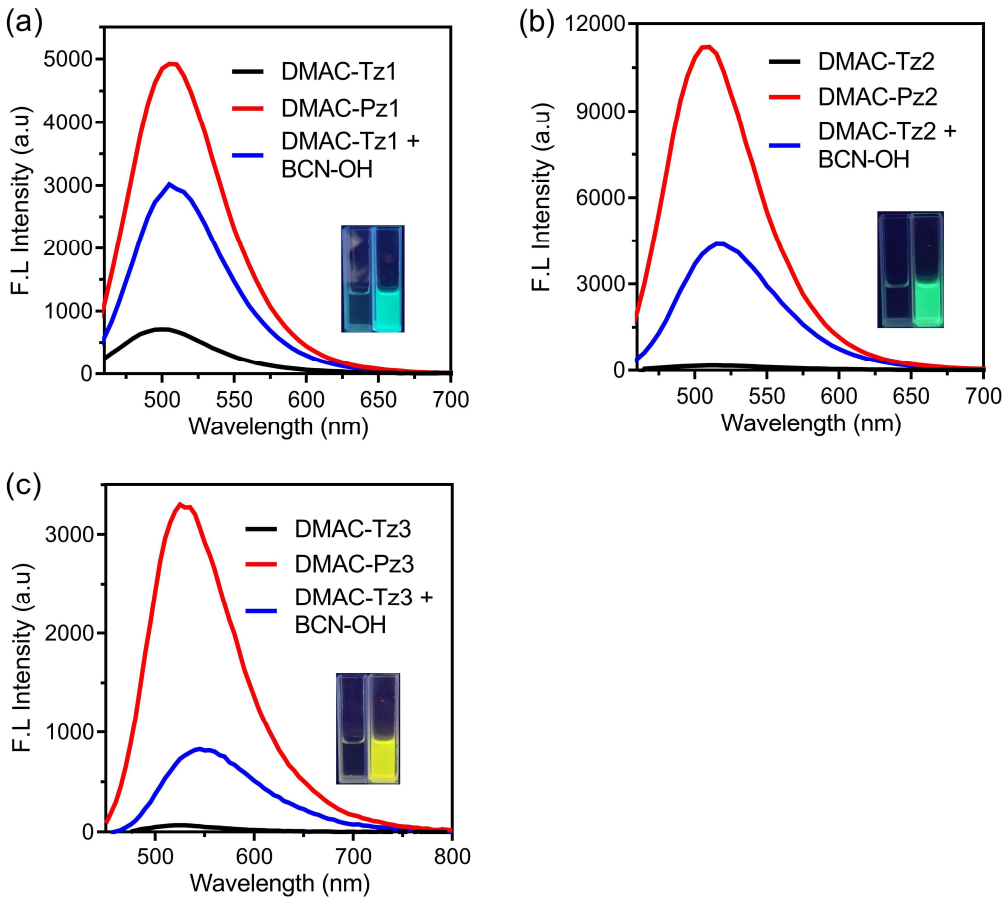

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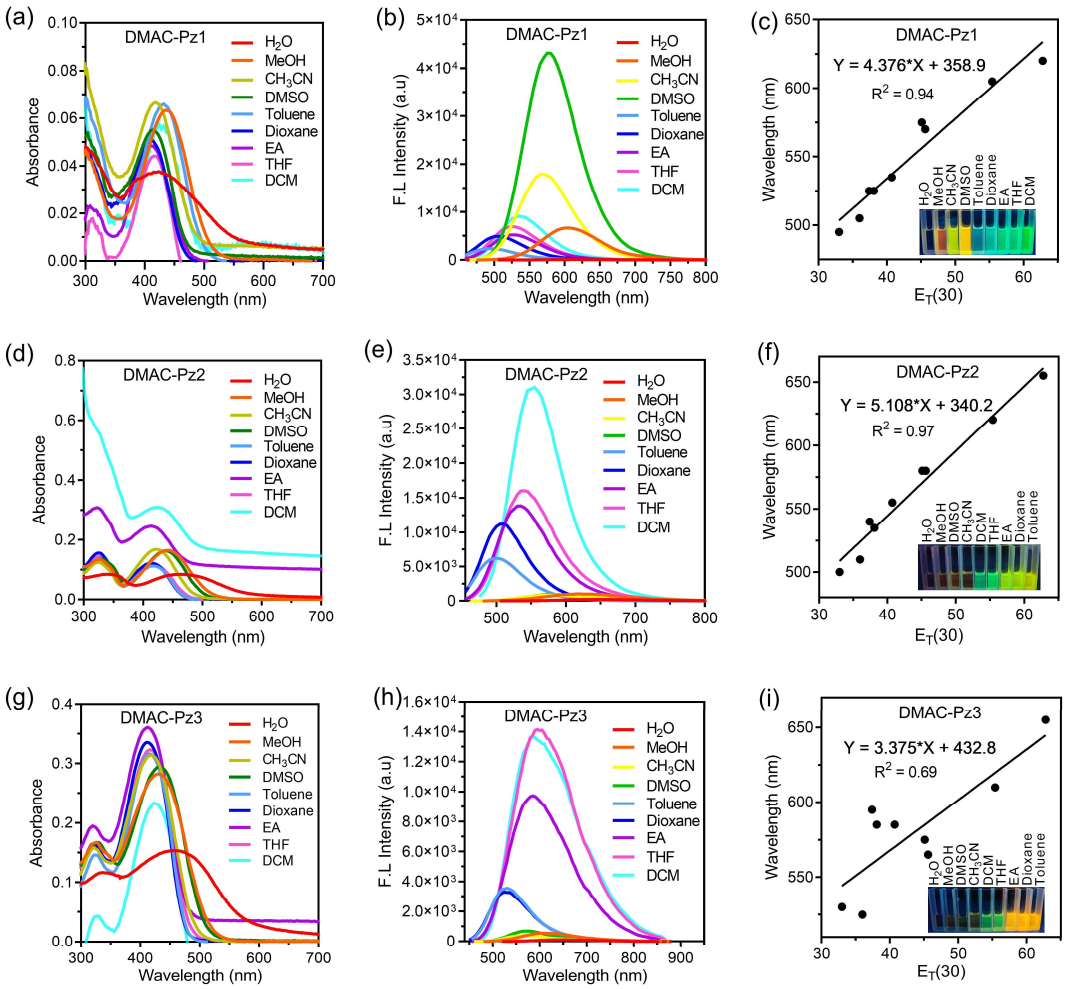


Fig. S10 Absorption spectra of DMAC-Pz1 (a), DMAC-Pz2 (d), and DMAC-Pz3 (g) in various solvents (10 μM). Fluorescence emission spectra of DMAC-Pz1 (b), DMAC-Pz2 (e), and DMAC-Pz3 (h) in various solvents (10 μM) (λ_{exc} = 405 nm). Fluorescent images and plot of the emission maximum wavelength of DMAC-Pz1 (c), DMAC-Pz2 (f), and DMAC-Pz3 (i) in different solvents with $E_T(30)$.

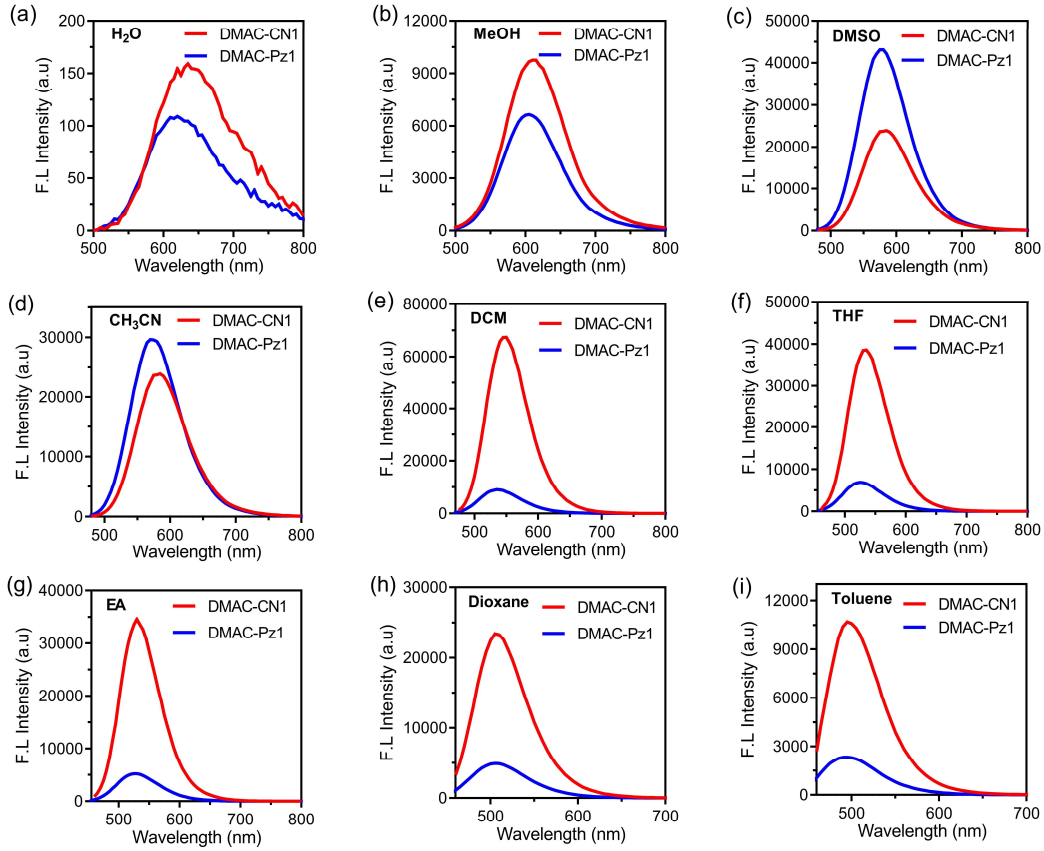


Fig. S11 Fluorescence emission spectra of DMAC-CN1 and DMAC-Pz1 in H_2O (a), MeOH (b), DMSO (c), CH_3CN (d), DCM (e), THF (f), EA (g), dioxane (h), and toluene (i) (λ_{exc} = 405 nm).

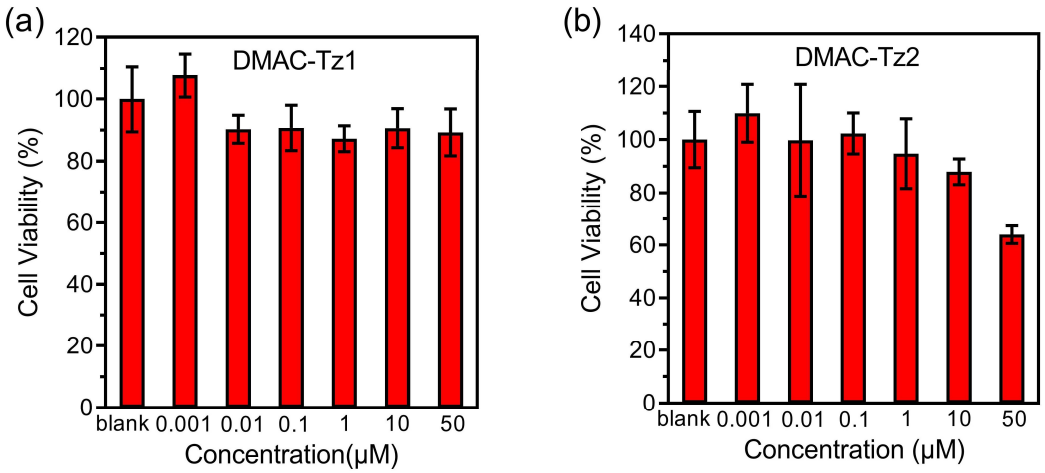


Fig. S12 Cytotoxicity of DMAC-Tz1 (a), and DMAC-Tz2 (b) against HeLa cells based on MTT assay. The cells were incubated with each probe for 48 h.

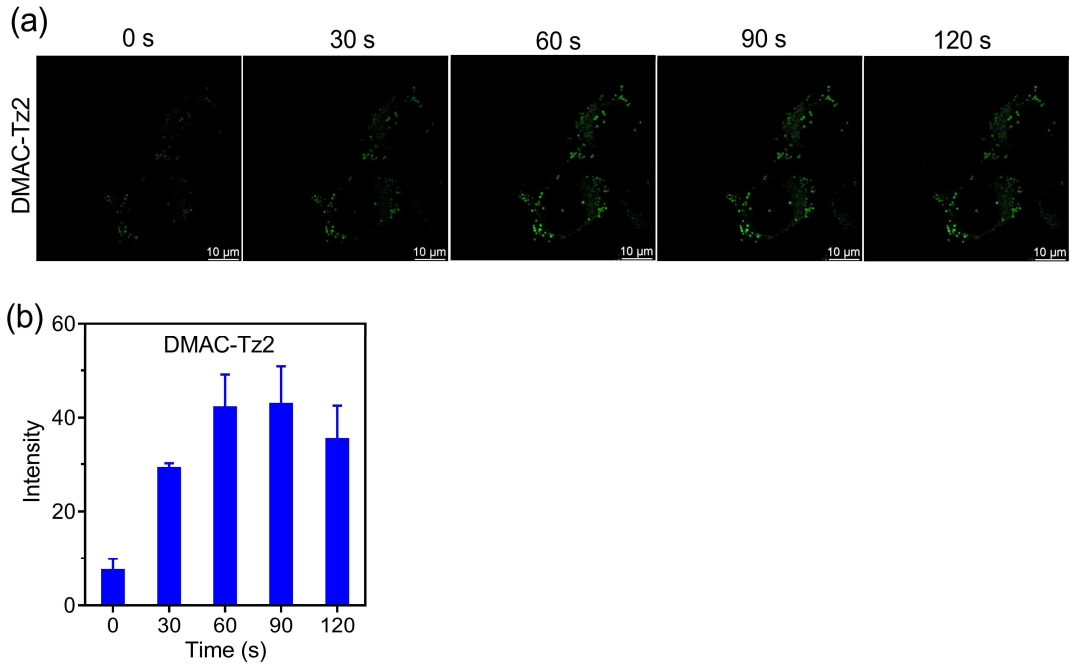


Fig. S13 (a) CLSM images of HeLa cells stained with DMAC-Tz2 (1 μM) under 405 nm laser scanning for 0-120 s. λ_{ex} = 405 nm, λ_{em} = 480–540 nm. Scale bar: 10 μm. (b) The quantification of fluorescence intensity of HeLa cells with the increasing irradiation time under 405 nm laser processed by ImageJ.

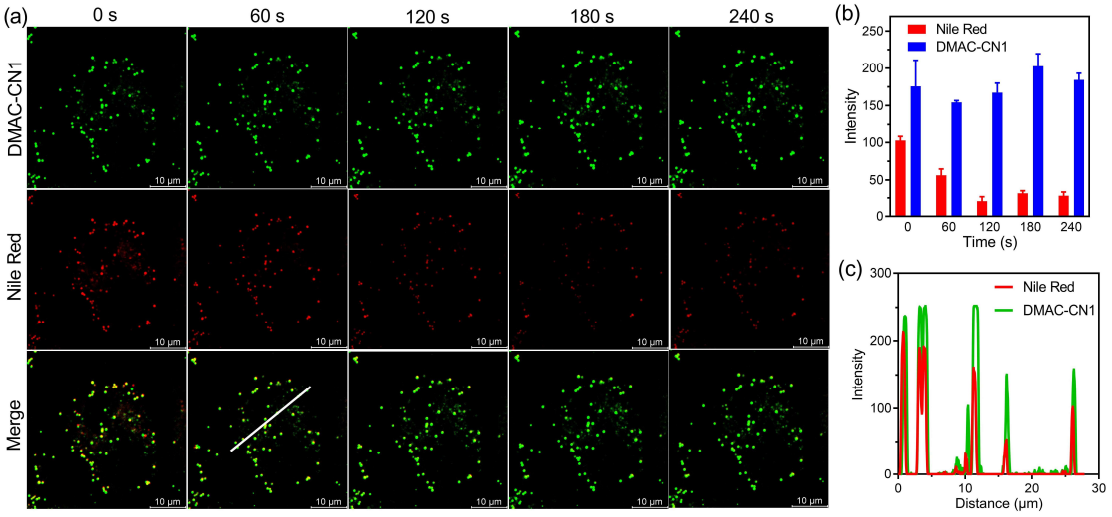


Fig. S14 (a) CLSM images of HeLa cells co-stained with DMAC-CN1 and Nile Red under 405 nm laser scanning for 0-240 s. For green channel, λ_{ex} = 405 nm, λ_{em} = 480–540 nm; For red channel, λ_{ex} = 552 nm, λ_{em} = 560–660 nm. Scale bar: 10 μm. DMAC-CN2: 1 μM; Nile Red: 10 μM. (b) The quantification of fluorescence intensity of HeLa cells with the increasing irradiation time under 405 nm laser processed by ImageJ. (c) Plot of intensities along the white line in (a).

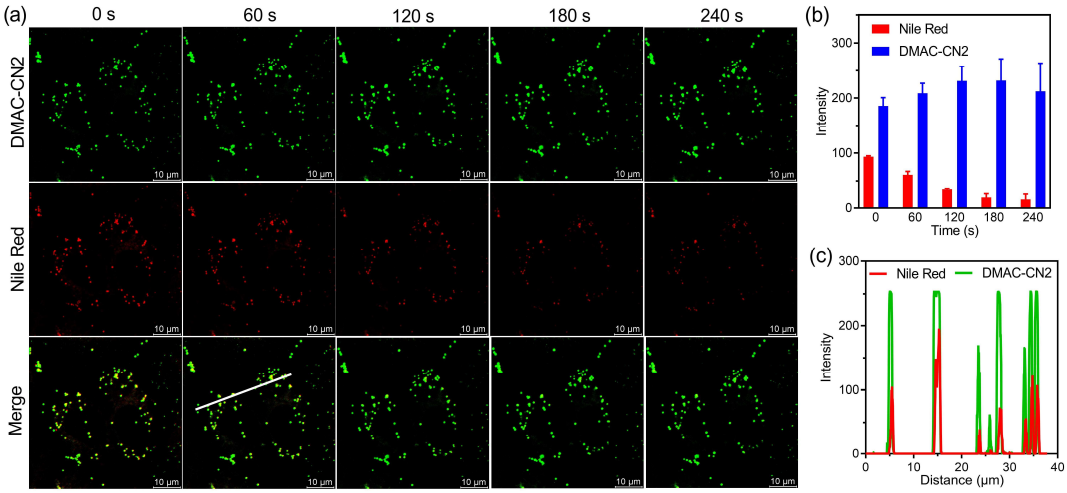


Fig. S15 (a) CLSM images of HeLa cells co-stained with DMAC-CN2 and Nile Red under 405 nm laser scanning for 0-240 s. For green channel, λ_{ex} = 405 nm, λ_{em} = 480–540 nm; For red channel, λ_{ex} = 552 nm, λ_{em} = 560–660 nm. Scale bar: 10 μm. DMAC-CN2: 1 μM; Nile Red: 10 μM. (b) The quantification of fluorescence intensity of HeLa cells with the increasing irradiation time under 405 nm laser processed by ImageJ. (c) Plot of intensities along the white line in (a).

Table S1 Photophysical properties of DMAC-CN1/2 in various solvents [a]

Solvent	DMAC-CN1				DMAC-CN2			
	$\lambda_{abs}/\lambda_{em}$ (nm)	Stokes shift (nm)	ε [b] ($M^{-1}cm^{-1}$)	Φ_F [c]	$\lambda_{abs}/\lambda_{em}$ (nm)	Stokes shift (nm)	ε [b] ($M^{-1}cm^{-1}$)	Φ_F [c]
H_2O	418/635	217	13000	0.008	450/652	202	13083	0.002
MeOH	440/616	176	41593	0.08	446/593	147	35600	0.005
DMSO	438/587	149	45447	0.17	446/566	120	33700	0.011
CH_3CN	424/571	147	42600	0.28	430/558	128	36110	0.008
DCM	434/549	115	36850	0.24	440/592	152	31077	0.18
THF	422/534	112	39503	0.27	430/544	114	36000	0.015
EA	418/530	112	39130	0.14	422/571	149	37757	0.014
Dioxane	418/505	87	34773	0.16	424/528	104	31597	0.39
Toluene	422/496	74	31510	0.07	430/515	85	29683	0.22

[a] The samples were prepared at the concentration of 10 μM. [b] Extinction coefficient. [c] Fluorescence quantum yield, 4-(dicyanomethylene)-2-methyl-6-(4-dimethylaminostyryl)-4*H*-pyran in methanol (Φ_F = 43%) as standard.

Table S2 The TD-DFT calculation data for DMAC-Tz1/2/3 and DMAC-CN1/2 at the first excited state optimized structures (CAM-B3LYP/6-311G*)

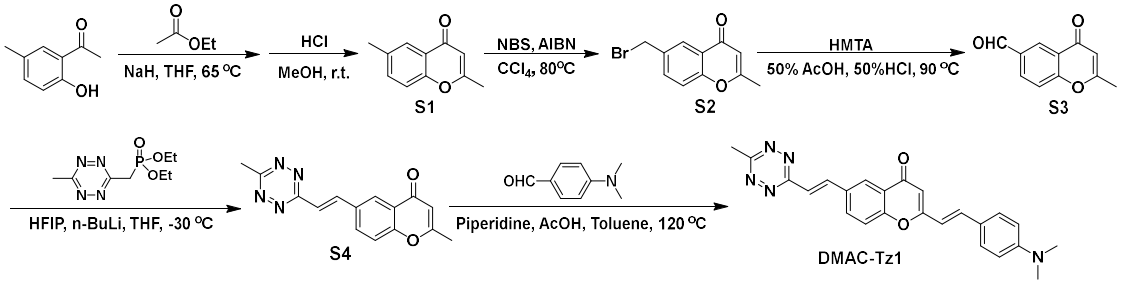
Probe	*f* [a]	*ΔE* (eV) [b]	Transitions [c]	CI expansion coefficient [d]
DMAC-Tz1	0.0057	6.68	HOMO-2 → LUMO	-0.59818
DMAC-Tz2	0.0055	6.48	HOMO-1 → LUMO+1	-0.54160
DMAC-Tz3	0.0058	6.44	HOMO-2 → LUMO+1	0.66701
DMAC-CN1	1.9195	4.93	HOMO → LUMO	0.68027
DMAC-CN2	1.7070	4.77	HOMO → LUMO	-0.62333

[a] Oscillator strength values for S_0–S_1 transition. [b] HOMO-LUMO energy gap. [c] Orbital transitions involved in S_0–S_1. [d] CI expansion coefficients (orbital contribution).

General Information

Compound **BCN-TPP** was prepared by the same procedure according to the reported literature[1]. Mito Tracker Red (MTR) was purchased from Beyotime. All the other 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 700 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 were recorded on Tecan Spark™ 10M Multimode Microplate Reader. Confocal laser scanning microscope imaging was conducted using a Leica TCS SP8 X Confocal Microscope.

Experimental Procedures and Characterization Data



Scheme S1 Synthesis of **DMAC-Tz1**

Synthesis of 2,6-dimethyl-4*H*-chromen-4-one (S1)

To a suspension of sodium hydride in THF (40 mL) was added a mixed solution of 1-(2-hydroxy-5-methylphenyl)ethan-1-one (2.43 g, 16.2 mmol) and ethyl acetate (4 mL, 40.5 mmol) in THF (20 mL) dropwise at room temperature. The reaction mixture was stirred at 65 ℃ for 4 h under argon protection, then poured into ice water. The pH of the reaction solution was adjusted to 6 with 6 N HCl, and the mixture was extracted with EtOAc (3×30 mL). The combined organic layers were washed with brine, then concentrated. The residue was redissolved in MeOH (20 mL), then added 2 mL conc. HCl. The mixture solution was stirred at room temperature for 12 h, then concentrated. The residue was purified by silica gel flash column chromatography (PE: EtOAc = 4:1) to afford the titled compound as white solid (2.12 g, 75.2% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.96 (s, 1H), 7.44 (dd, J = 8.5, 2.2 Hz, 1H), 7.31 (d, J = 8.5 Hz, 1H), 6.16 (s, 1H), 2.43 (s, 3H), 2.37 (s, 3H).

Synthesis of 6-(bromomethyl)-2-methyl-4*H*-chromen-4-one (S2)

To a solution of **S1** (1.0 g, 5.74 mmol) in CCl_4 (30 mL) was added *N*-Bromosuccinimide (1.23 g, 6.89 mmol) and 2,2'-Azobis(2-methylpropionitrile) (94 mg, 0.57 mmol). The reaction mixture was stirred at 80 ℃ for 8 h under argon protection,

then concentrated. The crude product was directly used in the next step without further purification.

Synthesis of 2-methyl-4-oxo-4*H*-chromene-6-carbaldehyde (S3)

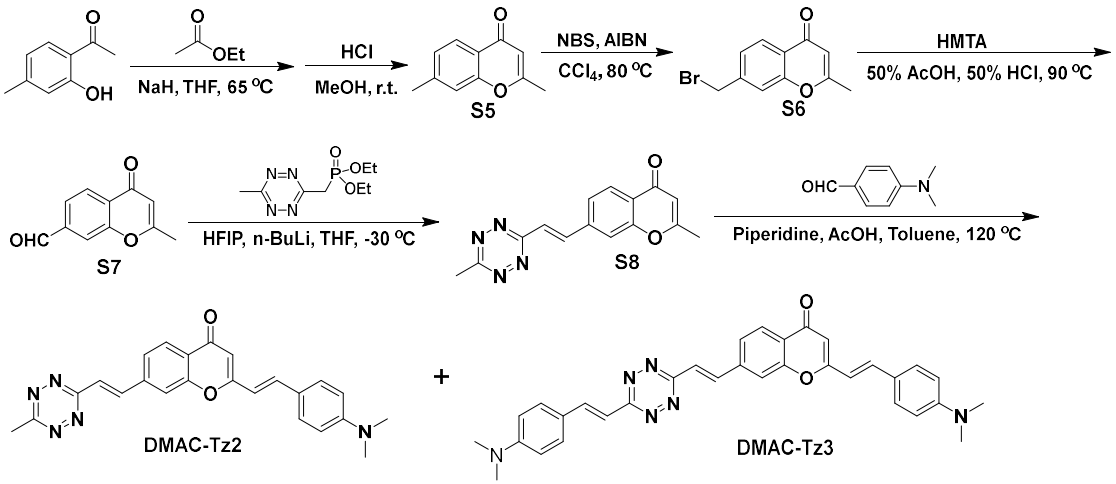
To a solution of **S2** (288 mg, 1.14 mmol) in 50% AcOH (6 mL) was added HMTA (1.15 g, 8.19 mmol). The mixture solution was stirred at 90 ℃ under argon protection for 2 h. Then 50% HCl (3 mL) was added, and the mixture solution was stirred at 90 ℃ under argon protection for another 0.5 h. Then the reaction mixture was extracted with EtOAc and the combined layers were washed with brine, then concentrated. The residue was purified by silica gel flash column chromatography (DCM: EtOAc = 5:1) to afford the titled compound as white solid (113 mg, 52.7% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.09 (s, 1H), 8.67 (d, *J* = 2.1 Hz, 1H), 8.19 (dd, *J* = 8.7, 2.1 Hz, 1H), 7.55 (d, *J* = 8.7 Hz, 1H), 6.24 (s, 1H), 2.43 (s, 3H).

Synthesis of (*E*)-2-methyl-6-(2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)-4*H*-chromen-4-one (S4)

To a solution of 1,1,1,3,3,3-hexafluoro-2-propanol (333 μL, 3.17 mmol) in dry THF (10 mL) was added 1.6 M *n*-BuLi (1.98 mL, 3.17 mmol) at -30 °C under argon protection. The reaction was stirred at -30 °C for 1 h, then a solution of **S3** (298 mg, 1.58 mmol) and diethyl ((6-methyl-1,2,4,5-tetrazin-3-yl)methyl)phosphonate[2] (584.6 mg, 2.38 mmol) in THF was added dropwise. The reaction mixture was stirred at -30 °C for 8 h, then quenched by H_2O (20 mL) and extracted with EtOAc (3×20 mL). 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 to afford the titled compound as purple solid (306 mg, 69% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.45 (s, 1H), 8.35 (d, *J* = 16.3 Hz, 1H), 7.95 (d, *J* = 8.7 Hz, 1H), 7.58 – 7.45 (m, 2H), 6.21 (s, 1H), 3.06 (s, 3H), 2.42 (s, 3H).

Synthesis of 2-((*E*)-4-(dimethylamino)styryl)-6-((*E*)-2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)-4H-chromen-4-one (DMAC-Tz1)

To a solution of **S4** (150 mg, 0.54 mmol) in toluene (5 mL) was added 4-(dimethylamino)benzaldehyde (160 mg, 1.07 mmol), piperidine (162 μL, 1.6mmol) and AcOH (162 μL, 2.8 mmol). The mixture solution was stirred at 120 ℃ under argon protection for 5 h, then concentrated. The residue was purified by silica gel flash column chromatography to afford the titled compound as purple solid (107 mg, 48.6% yield). ^{1}H NMR (700 MHz, DMSO-d_6) δ 8.39 (dd, *J* = 8.8, 2.2 Hz, 1H), 8.37 – 8.34 (m, 2H), 7.79 (d, *J* = 8.6 Hz, 1H), 7.72 (d, *J* = 16.3 Hz, 1H), 7.64 (d, *J* = 15.9 Hz, 1H), 7.57 (d, *J* = 8.8 Hz, 2H), 6.92 (d, *J* = 15.9 Hz, 1H), 6.77 (d, *J* = 8.9 Hz, 2H), 6.39 (s, 1H), 3.00 (s, 6H), 2.97 (s, 3H). ^{13}C NMR (600MHz, DMSO) δ 176.33, 166.24, 164.08, 162.95, 156.29, 151.47, 138.30, 137.72, 132.65, 131.93, 129.56, 125.21, 123.87, 122.38, 121.98, 119.06, 114.26, 112.00, 108.26, 22.21, 20.90. HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{24}H_{22}O_2N_5$ 412.1768; Found 412.1759.



Scheme S2 Synthesis of **DMAC-Tz2** and **DMAC-Tz3**

Synthesis of 2,7-dimethyl-4*H*-chromen-4-one (S5)

To a suspension of sodium hydride in THF (40 mL) was added a mixed solution of 1-(2-hydroxy-4-methylphenyl)ethan-1-one (3.0 g, 20.0 mmol) and ethyl acetate (4.88 mL, 50.0 mmol) in THF (20 mL) dropwise at room temperature. The reaction mixture was stirred at 65 ℃ for 4 h under argon protection, then poured into ice water. The pH of the reaction solution was adjusted to 6 with 6 N HCl, and the mixture was extracted with EtOAc (3×30 mL). The combined organic layers were washed with brine, then concentrated. The residue was redissolved in MeOH (20 mL), then added 2 mL conc. HCl. The mixture solution was stirred at room temperature for 12 h, then concentrated. The residue was purified by silica gel flash column chromatography (PE: EtOAc = 4:1) to afford the titled compound as white solid (3.3 g, 94.8% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.88 (d, J = 8.1 Hz, 1H), 7.41 (s, 1H), 7.27 (d, J = 8.1 Hz, 1H), 6.19 (s, 1H), 2.44 (s, 3H), 2.37 (s, 3H).

Synthesis of 7-(bromomethyl)-2-methyl-4*H*-chromen-4-one (S6)

To a solution of **S5** (500 mg, 2.87 mmol) in CCl_4 (10 mL) was added *N*-Bromosuccinimide (664 mg, 3.73 mmol) and 2,2'-Azobis(2-methylpropionitrile) (48 mg, 0.29 mmol). The reaction mixture was stirred at 80 ℃ for 8 h under argon protection, then concentrated. The crude product was directly used in the next step without further purification.

Synthesis of 2-methyl-4-oxo-4*H*-chromene-7-carbaldehyde (S7)

To a solution of **S6** (366 mg, 1.45 mmol) in 50% AcOH (8 mL) was added HMTA (1.42 mg, 10.12 mmol). The mixture solution was stirred at 90 ℃ under argon protection for 2 h. Then 50% HCl (4 mL) was added, and the mixture solution was stirred at 90 ℃ under argon protection for another 0.5 h. Then the reaction mixture was extracted with EtOAc and the combined layers were washed with brine, then concentrated. The residue was purified by silica gel flash column chromatography (DCM: EtOAc = 2:1) to afford the titled compound as white solid (107 mg, 39.3% yield). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 10.27 – 9.90 (m, 1H), 8.34 (d, J = 8.0 Hz,

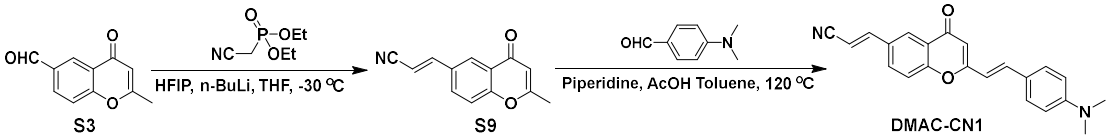
1H), 7.93 (s, 1H), 7.86 (s, 1H), 6.25 (s, 1H), 2.44 (s, 3H).

Synthesis of (*E*)-2-methyl-7-(2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)-4*H*-chromen-4-one (S8)

To a solution of 1,1,1,3,3,3-hexafluoro-2-propanol (29.4 μL, 0.28 mmol) in dry THF (10 mL) was added 1.6 M *n*-BuLi (166 μL, 0.27 mmol) at -30 °C under argon protection. The reaction was stirred at -30 °C for 1 h, then a solution of **S7** (50 mg, 0.27 mmol) and diethyl ((6-methyl-1,2,4,5-tetrazin-3-yl)methyl)phosphonate[2] (98 mg, 0.40 mmol) in THF was added dropwise. The reaction mixture was stirred at -30 °C for 8 h, then quenched by H_2O (20 mL) and extracted with EtOAc (3×20 mL). 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 to afford the titled compound as purple solid (38.9 mg, 52.2% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.35 (d, *J* = 16.3 Hz, 1H), 8.25 (d, *J* = 8.2 Hz, 1H), 7.71 (dd, *J* = 8.2, 1.4 Hz, 1H), 7.69 (s, 1H), 7.61 (d, *J* = 16.3 Hz, 1H), 6.24 (s, 1H), 3.09 (s, 3H), 2.43 (s, 3H).

Synthesis of 2-((*E*)-4-(dimethylamino)styryl)-7-((*E*)-2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)-4*H*-chromen-4-one (DMAC-Tz2) and 2-((*E*)-4-(dimethylamino)styryl)-7-((*E*)-2-(6-((*E*)-4-(dimethylamino)styryl)-1,2,4,5-tetrazin-3-yl)vinyl)-4*H*-chromen-4-one (DMAC-Tz3)

To a solution of **S8** (280 mg, 1.0 mmol) in toluene (5 mL) was added 4-(dimethylamino)benzaldehyde (298 mg, 2.0 mmol), piperidine (1.48 mL, 15.0 mmol) and AcOH (1.48 mL, 25.8 mmol). The mixture solution was stirred at 120 °C under argon protection for 4 h, then concentrated. The residue was purified by silica gel flash column chromatography to afford **DMAC-Tz2** as yellow solid (160 mg, 29.5% yield), and **DMAC-Tz3** as brown solid (30 mg, 5.5% yield). **DMAC-Tz2**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (d, *J* = 16.4 Hz, 1H), 7.79 (s, 1H), 7.59 (d, *J* = 8.2 Hz, 1H), 7.52 (d, *J* = 8.3 Hz, 1H), 7.44 (d, *J* = 16.4 Hz, 1H), 7.18 (d, *J* = 16.2 Hz, 1H), 7.12 (d, *J* = 8.9 Hz, 2H), 6.48 (d, *J* = 16.0 Hz, 1H), 6.33 (d, *J* = 9.0 Hz, 2H), 5.93 (s, 1H), 2.56 (s, 6H), 2.54 (s, 3H). ^{13}C NMR (700 MHz, $CDCl_3$) δ 212.75, 196.09, 190.77, 177.80, 166.86, 164.54, 163.28, 156.39, 152.78, 140.21, 139.11, 137.78, 137.26, 129.65, 126.58, 125.11, 123.98, 117.42, 109.68, 29.85, 21.43. HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{24}H_{22}O_2N_5$ 412.1768; Found 412.1765. **DMAC-Tz3**: ^{1}H NMR (700 MHz, Methylene Chloride-d_2) δ 8.32 (dd, *J* = 28.1, 16.1 Hz, 2H), 8.18 (d, *J* = 8.0 Hz, 1H), 7.84 (d, *J* = 1.6 Hz, 1H), 7.74 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.70 – 7.62 (m, 4H), 7.58 (d, *J* = 8.2 Hz, 2H), 7.30 (d, *J* = 15.9 Hz, 1H), 6.91 (s, 4H), 6.69 (d, *J* = 15.8 Hz, 1H), 6.26 (s, 1H), 3.08 (s, 6H), 3.07 (s, 6H). ^{13}C NMR (700 MHz, DMSO) δ 176.63, 163.66, 163.06, 162.02, 162.00, 158.87, 155.89, 140.80, 139.54, 137.65, 135.61, 135.11, 135.03, 130.04, 129.40, 125.52, 125.48, 125.03, 124.70, 124.66, 124.09, 117.90, 110.52, 44.20, 43.74. HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{33}H_{31}O_2N_6$ 543.2464; Found 543.2513.



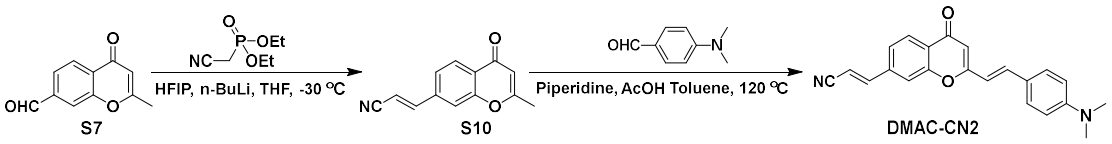
Scheme S3 Synthesis of **DMAC-CN1**

Synthesis of (*E*)-3-(2-methyl-4-oxo-4*H*-chromen-6-yl)acrylonitrile (S9)

To a solution of 1,1,1,3,3,3-hexafluoro-2-propanol (35 μL, 0.33 mmol) in dry THF (2 mL) was added 2.5 M *n*-BuLi (130 μL, 0.32 mmol) at -30 °C under argon protection. The reaction was stirred at -30 °C for 1 h, then a solution of **S3** (60 mg, 0.32 mmol) and diethyl (cyanomethyl) phosphonate (84.7 mg, 0.48 mmol) in THF was added dropwise. The reaction mixture was stirred at -30 °C for 8 h, then quenched by H_2O (10 mL) and extracted with EtOAc (3×20 mL). 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 to afford the titled compound as white solid (67 mg, 100% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.28 – 8.26 (m, 1H), 7.71 (dd, *J* = 8.7, 2.3 Hz, 1H), 7.48 (s, 1H), 7.44 (d, *J* = 8.1 Hz, 1H), 6.21 (s, 1H), 5.97 (d, *J* = 16.6 Hz, 1H), 2.41 (s, 3H).

Synthesis of (*E*)-3-(2-((*E*)-4-(dimethylamino)styryl)-4-oxo-4*H*-chromen-6-yl)acrylonitrile (DMAC-CN1)

To a solution of **S9** (30.0 mg, 0.14 mmol) in toluene was added 4-(dimethylamino) benzaldehyde (42 mg, 0.28 mmol), piperidine (210 μL, 2.13 mmol) and AcOH (210 μL, 3.67 mmol). The mixture solution was stirred at 120℃ under argon protection for 8 h, then concentrated. The residue was purified by silica gel flash column chromatography to afford the titled compound as yellow solid (22 mg, 45% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.22 (d, *J* = 2.2 Hz, 1H), 8.09 (dd, *J* = 8.8, 2.2 Hz, 1H), 7.83 – 7.72 (m, 2H), 7.60 (d, *J* = 15.9 Hz, 1H), 7.55 (d, *J* = 8.5 Hz, 2H), 6.89 (d, *J* = 16.0 Hz, 1H), 6.75 (d, *J* = 8.6 Hz, 2H), 6.59 (d, *J* = 16.7 Hz, 1H), 6.36 (s, 1H), 2.99 (s, 6H). ^{13}C NMR (400 MHz, DMSO-d_6) δ 176.19, 162.99, 156.73, 151.47, 149.19, 137.79, 132.02, 130.66, 129.55, 125.22, 123.74, 122.31, 119.08, 118.72, 114.12, 111.95, 108.24, 97.69. $[M+H]^+$ Calcd for $C_{22}H_{19}O_2N_2$ 343.1402; Found 343.1435.



Scheme S4 Synthesis of **DMAC-CN2**

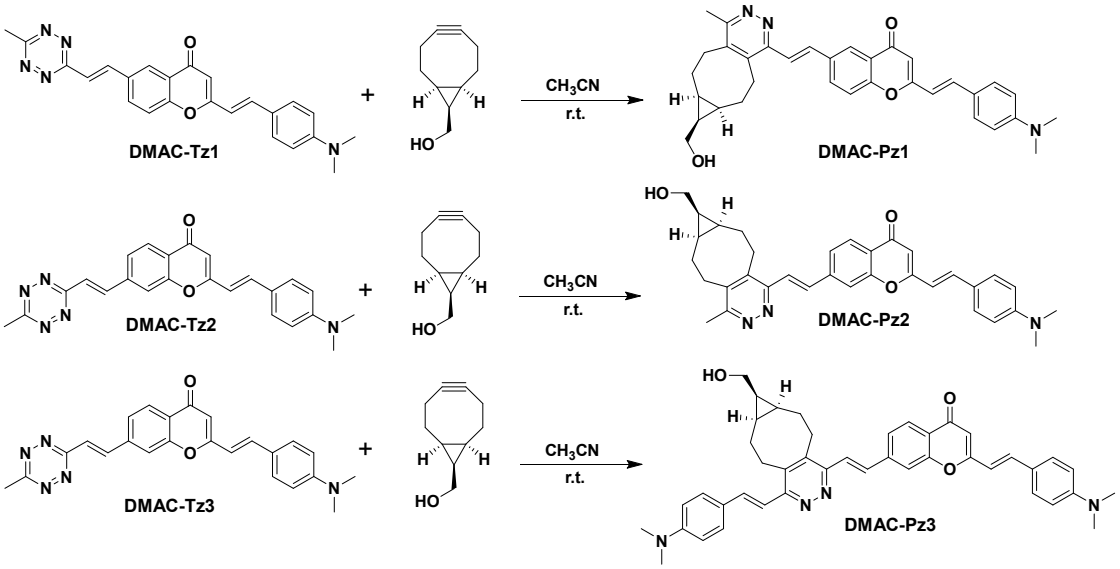
Synthesis of (*E*)-3-(2-methyl-4-oxo-4*H*-chromen-7-yl)acrylonitrile (S10)

To a solution of 1,1,1,3,3,3-hexafluoro-2-propanol (35 μL, 0.33 mmol) in dry THF (2 mL) was added 2.5 M *n*-BuLi (130 μL, 0.32 mmol) at -30 °C under argon protection. The reaction was stirred at -30 °C for 1 h, then a solution of **S7** (60 mg, 0.32 mmol) and diethyl (cyanomethyl) phosphonate (84.7 mg, 0.48 mmol) in THF was added dropwise. The reaction mixture was stirred at -30 °C for 6 h, then quenched by H_2O (10 mL) and extracted with EtOAc (3×20 mL). 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 to afford the titled compound as white solid (62

mg, 93% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.20 (d, *J* = 8.1 Hz, 1H), 7.48 (d, *J* = 1.3 Hz, 2H), 7.46 – 7.43 (m, 1H), 6.21 (s, 1H), 6.04 (d, *J* = 16.7 Hz, 1H), 2.41 (s, 3H).

Synthesis of (*E*)-3-(2-((*E*)-4-(dimethylamino)styryl)-4-oxo-4*H*-chromen-7-yl)acrylonitrile (DMAC-CN2)

To a solution of **S10** (30.0 mg, 0.14 mmol) in toluene (5 mL) was added 4-(dimethylamino) benzaldehyde (42 mg, 0.28 mmol), piperidine (210 μL, 2.13 mmol) and AcOH (210 μL, 3.67 mmol). The mixture solution was stirred at 120 ℃ under argon protection for 8 h, then concentrated. The residue was purified by silica gel flash column chromatography to afford the titled compound as yellow solid (28 mg, 58% yield). ^{1}H NMR (400 MHz, Acetone-d_6) δ 8.11 (d, *J* = 8.2 Hz, 1H), 8.07 – 8.01 (m, 2H), 7.89 – 7.81 (m, 2H), 7.73 – 7.57 (m, 4H), 7.56 – 7.50 (m, 5H), 6.82 (dd, *J* = 15.9, 4.1 Hz, 2H), 6.77 – 6.72 (m, 4H), 6.54 (d, *J* = 16.7 Hz, 1H), 6.24 (d, *J* = 9.4 Hz, 2H), 5.97 (d, *J* = 12.1 Hz, 1H), 3.00 (d, *J* = 0.7 Hz, 12H). ^{13}C NMR (400 MHz, DMSO-d_6) δ 176.02, 163.22, 155.49, 151.47, 148.92, 138.73, 137.60, 129.52, 124.75, 123.71, 122.28, 118.33, 117.51, 114.28, 111.98, 108.49, 100.43. $[M+H]^+$ Calcd for $C_{22}H_{19}O_2N_2$ 343.1402; Found 343.1441.



Scheme S5 Synthesis of **DMAC-Pz1**, **DMAC-Pz2**, and **DMAC-Pz3**

Synthesis of 2-((*E*)-4-(dimethylamino)styryl)-6-((*E*)-2-((6aS,7S,7aR)-7-(hydroxymethyl)-4-methyl-6,6a,7,7a,8,9-hexahydro-5*H* cyclopropa[5,6]cycloocta [1,2-d]pyridazin-1-yl)vinyl)-4*H*-chromen-4-one (DMAC-Pz1)

To a solution of **DMAC-Tz1** (15 mg, 0.036 mmol) in CH_3CN (3 mL) was added BCN-OH (14.0 mg, 0.092 mmol). The reaction mixture was stirred at room temperature for 15 min, then concentrated. The residue was purified by silica gel flash column chromatography (DCM : MeOH = 10 : 1) to afford the titled compound as orange solid (19.5 mg, 100% yield).^{1}H NMR (700 MHz, DMSO-d_6) δ 8.26 (dd, *J* = 8.7, 2.3 Hz, 1H), 8.21 (d, *J* = 2.3 Hz, 1H), 7.85 (dd, *J* = 15.6, 3.0 Hz, 1H), 7.72 (dd, *J* = 13.9, 9.1 Hz,

2H), 7.62 (d, *J* = 15.9 Hz, 1H), 7.56 (d, *J* = 8.5 Hz, 2H), 6.90 (d, *J* = 15.7 Hz, 1H), 6.76 (d, *J* = 8.5 Hz, 2H), 6.36 (s, 1H), 4.31 (t, *J* = 5.0 Hz, 1H), 3.52-3.47 (m, 2H), 3.07 – 3.02 (m, 2H), 2.99 (s, 6H), 2.97-2.92 (m, 1H), 2.86-2.78 (m, 1H), 2.65 (s, 3H), 2.29-2.18 (m, 2H), 1.22 (s, 1H), 0.91-0.86 (m 1H), 0.60 (s, 2H). ^{13}C NMR (700 MHz, DMSO-d_6) δ 176.53, 162.80, 155.39, 151.42, 137.49, 133.53, 132.52, 132.43, 131.51, 129.50, 123.78, 123.65, 123.48, 122.43, 118.68, 114.39, 111.98, 111.11, 108.20, 57.08, 26.40, 26.40, 20.53. HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{34}H_{36}O_3N_3$ 534.2751; Found 534.2753.

Synthesis of 2-((*E*)-4-(dimethylamino)styryl)-7-((*E*)-2-((6aR,7R,7aS)-7-(hydroxymethyl)-4-methyl-6,6a,7,7a,8,9-hexahydro-5*H*-cyclopropa[5,6]cycloocta[1,2-d]pyridazin-1-yl)vinyl)-4*H*-chromen-4-one (DMAC-Pz2)

To a solution of **DMAC-Tz2** (11 mg, 0.027 mmol) in CH_3CN (3 mL) was added BCN-OH (8.1 mg, 0.054 mmol). The reaction mixture was stirred at room temperature for 2 h, then concentrated. The residue was purified by a preparative TLC plate (DCM/MeOH = 30:1) to afford the titled compound as yellow solid (4.4 mg, 30.8% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.07 (d, *J* = 1.2 Hz, 1H), 7.98 (d, *J* = 8.2 Hz, 1H), 7.88 (s, 2H), 7.85 (dd, *J* = 8.3, 1.4 Hz, 1H), 7.61 (d, *J* = 15.9 Hz, 1H), 7.58 – 7.54 (m, 2H), 6.90 (d, *J* = 15.9 Hz, 1H), 6.76 (d, *J* = 9.0 Hz, 2H), 6.34 (s, 1H), 4.32 (t, *J* = 5.0 Hz, 1H), 3.50 (m, 2H), 3.11 – 3.03 (m, 2H), 2.99 (s, 6H), 2.97-2.91 (m, 1H), 2.88-2.79 (m, 1H), 2.66 (s, 3H), 2.35-2.16 (m, 2H), 1.22 (s, 1H), 0.96 – 0.82 (m, 1H), 0.61 (s, 2H). ^{13}C NMR (500 MHz, DMSO-d_6) δ 176.34, 162.96, 157.38, 155.91, 154.18, 151.41, 142.11, 139.21, 137.27, 132.29, 129.46, 126.22, 125.05, 124.18, 123.11, 122.41, 116.29, 114.58, 112.02, 108.44, 57.08, 26.41, 25.22, 20.58. HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{34}H_{36}O_3N_3$ 534.2712; Found 534.2750.

Synthesis of 2-((*E*)-4-(dimethylamino)styryl)-7-((*E*)-2-((6aR,7R,7aS)-4-((*E*)-4-(dimethylamino)styryl)-7-(hydroxymethyl)-6,6a,7,7a,8,9-hexahydro-5*H*-cyclopropa[5,6]cycloocta[1,2-d]pyridazin-1-yl)vinyl)-4*H*-chromen-4-one (DMAC-Pz3)

To a solution of **DMAC-Tz3** (10 mg, 0.018 mmol) in CH_3CN (2 mL) was added BCN-OH (8.3 mg, 0.055 mmol). The reaction mixture was stirred at room temperature for 24 h, then concentrated. The residue was purified by a preparative TLC plate (DCM/MeOH = 25:1) to afford the titled compound as yellow solid (3 mg, 24.6% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.09 (d, *J* = 1.5 Hz, 1H), 8.00 (d, *J* = 8.2 Hz, 1H), 7.95 (d, *J* = 2.0 Hz, 2H), 7.89 – 7.83 (m, 2H), 7.67 – 7.54 (m, 5H), 7.37 (d, *J* = 15.5 Hz, 1H), 6.92 (d, *J* = 16.0 Hz, 1H), 6.77 (dd, *J* = 8.7, 6.5 Hz, 4H), 6.35 (s, 1H), 4.30 (t, *J* = 5.0 Hz, 1H), 3.57-3.45 (m, 2H), 3.14-3.03 (m, 3H), 3.00 (s, 6H), 2.98 (s, 6H), 2.36-2.22 (m, 2H), 1.27-1.20 (m, 1H), 0.97 – 0.79 (m, 2H), 0.62 (s, 2H). $[M+H]^+$ Calcd for $C_{43}H_{45}O_3N_4$ 665.3447; Found 665.3520.

General spectra measurements

The stock solution of each probe was prepared at 10 mM in DMSO. A fresh work solution of corresponding probe was prepared by diluting the stock solution to different

solvents (MeOH, THF, dioxane, etc. 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 fluorescence signal of solvent for the same time period.

Reaction kinetics measurements

To a solution of 0.1 mM DMAC probes in dioxane was added 10-fold excess of BCN reagent (1 mM) at room temperature (DMAC-Tz1 was measured in DMSO). The fluorescence intensity of the mixture solution at different time interval was recorded at λ_{em} of each probe using TECAN fluorescence plate reader (λ_{exc}=420 nm). 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 reagent].

Photoactivated fluorescence enhancement measurements

Three milliliters of 10 μM probe in THF was placed into a glass cuvette and exposed to 254 nm UV light from a handheld UV lamp on ice for certain time, then the fluorescence spectra were recorded using the same method mentioned above.

Proteins Labeling

BSA-BCN conjugate (BCN-modified BSA protein) was prepared according to the literature procedure.[3] For fluorogenic BSA labeling, 1 μL of 10 mM DMAC-Tz1/2/3 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 analyzed by SDS-PAGE and the fluorescence images were recorded. Finally, the gel was subjected to coomassie blue staining.

MTT assay

HeLa cells were seeded at a density of 4×10^3/well into 96-well plate (100 μL/well) and incubated at 37 °C, 5% CO_2 for 24 h. Then, the cells were treated with 100 μL DMAC probes in DMEM with different concentrations (1, 1×10^1, 1×10^2, 1×10^3, 1×10^4 and 5×10^4 nM) and incubated for 48 h; MTT solution (5 mg/mL in PBS, 20 μL) was then added to each well. After 4 h, the remaining MTT solution was removed, and 150 μL of DMSO was added into each well to dissolve the formazan crystals. Absorbance was measured at 490 nm for determination of OD values. All data were carried out in triplicate.

LDs imaging

HeLa cells were seeded into 35-mm glass-bottom dishes and cultured for 24 h, followed by incubation with Nile Red (10 μM) and DMAC-Tz1/2 (1 μM) or DMAC-CN1/2 (1 μM) in DMEM at 37 °C and 5% CO_2 for 1 h. Then the medium was removed and the cells were washed twice with PBS (pH 7.4), and submitted for laser scanning confocal microscopy imaging for 0-120 s or 240 s. The confocal fluorescence images

were acquired using a Leica TCS SP8 X microscope equipped with a 63×objective and appropriate filters (DMAC probes: excitation = 405 nm, emission =480 ~ 540 nm; Nile Red: excitation = 552 nm, emission =560 ~ 660 nm).

Monitoring LDs dynamics in Ferroptosis

HeLa cells were seeded into 35-mm glass-bottom dishes and cultured for 24 h, followed by incubation with DMAC-Tz1 (1 μM) in DMEM at 37 °C and 5% CO_2 for 1 h. Then the medium was removed and the cells were washed twice with PBS (pH 7.4). After incubation with erastin (10 μM) or erastin (10 μM)/Fer-1(15 μM) in DMEM for 0, 1, 2 h, the cells were directly submitted for laser scanning confocal microscopy imaging. The confocal fluorescence images were acquired using a Leica TCS SP8 X microscope equipped with a 63×objective and appropriate filters (excitation = 405 nm, emission =480 ~ 540 nm).

Mitochondrion imaging

HeLa cells were seeded into 35-mm glass-bottom dishes and cultured for 24 h, followed by incubation with Mito-Tracker 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 twice with PBS (pH 7.4). After incubation with DMAC-Tz1 (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 a Leica TCS SP8 X microscope equipped with a 63×objective and appropriate filters (DMAC-Tz1: excitation = 405 nm, emission =480 ~ 540 nm; Mito-Tracker Red: excitation = 579 nm, emission = 585 ~ 660 nm).

Simultaneous imaging of LDs and mitochondria

HeLa cells were seeded into 35-mm glass-bottom dishes and cultured for 24 h, followed by incubation with BCN-TPP (1 μM) and Nile Red (10 μM) or Mito-Tracker Red (200 nM) in DMEM at 37 °C and 5% CO_2 for 1 h. Then the medium was removed and the cells were washed twice with PBS (pH 7.4). After incubation with DMAC-Tz1 (2 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 a Leica TCS SP8 X microscope equipped with a 63×objective and appropriate filters (DMAC-Tz1: excitation = 405 nm, emission = 480 ~ 640 nm; Nile Red: excitation = 552 nm, emission =560 ~ 660 nm; Mito-Tracker Red: excitation = 579 nm, emission = 585 ~ 660 nm).

Lambda Mode imaging

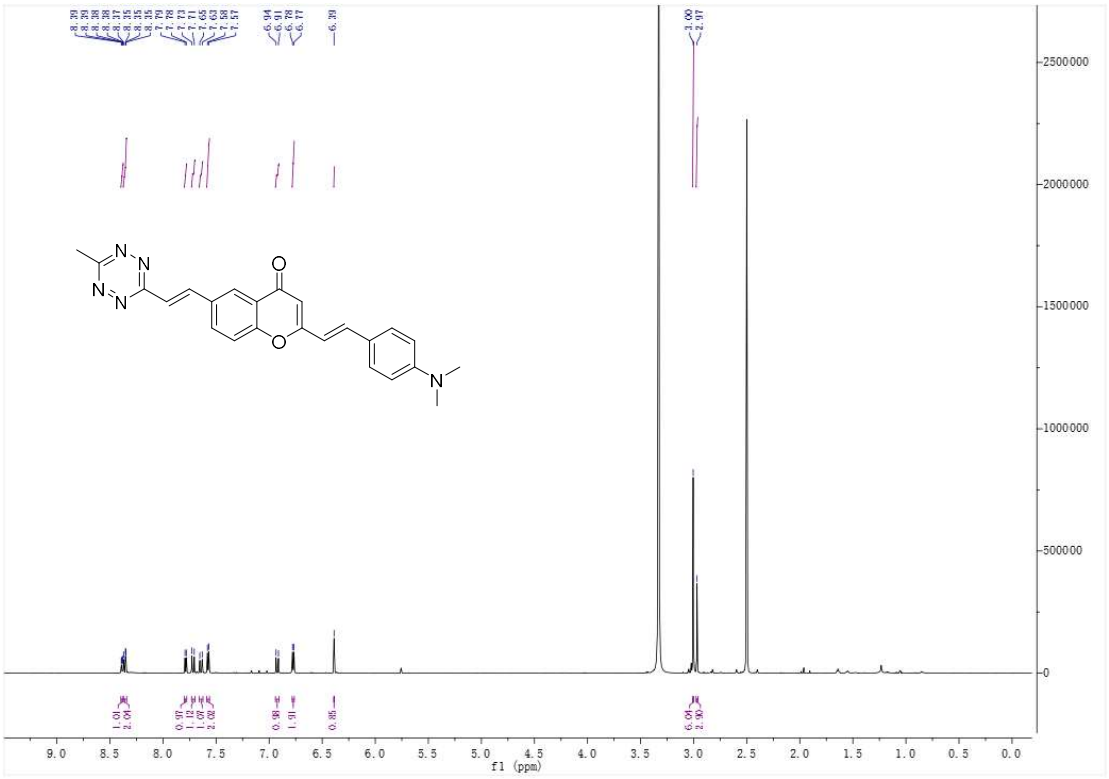
HeLa cells were seeded into 35-mm glass-bottom dishes and cultured for 24 h, followed by incubation with BCN-TPP (1 μM) in DMEM at 37 °C and 5% CO_2 for 1 h. Then the medium was removed and the cells were washed twice with PBS (pH 7.4). After incubation with DMAC-Tz1 (2 μM) in DMEM for another 1 h, the cells were submitted for laser scanning confocal microscopy imaging. The confocal fluorescence images were acquired using a Leica TCS SP8 X microscope equipped with a 63×

objective. The excitation wavelength was set to 405 nm, and the real-color images were acquired every 20 nm from 480 to 640 nm.

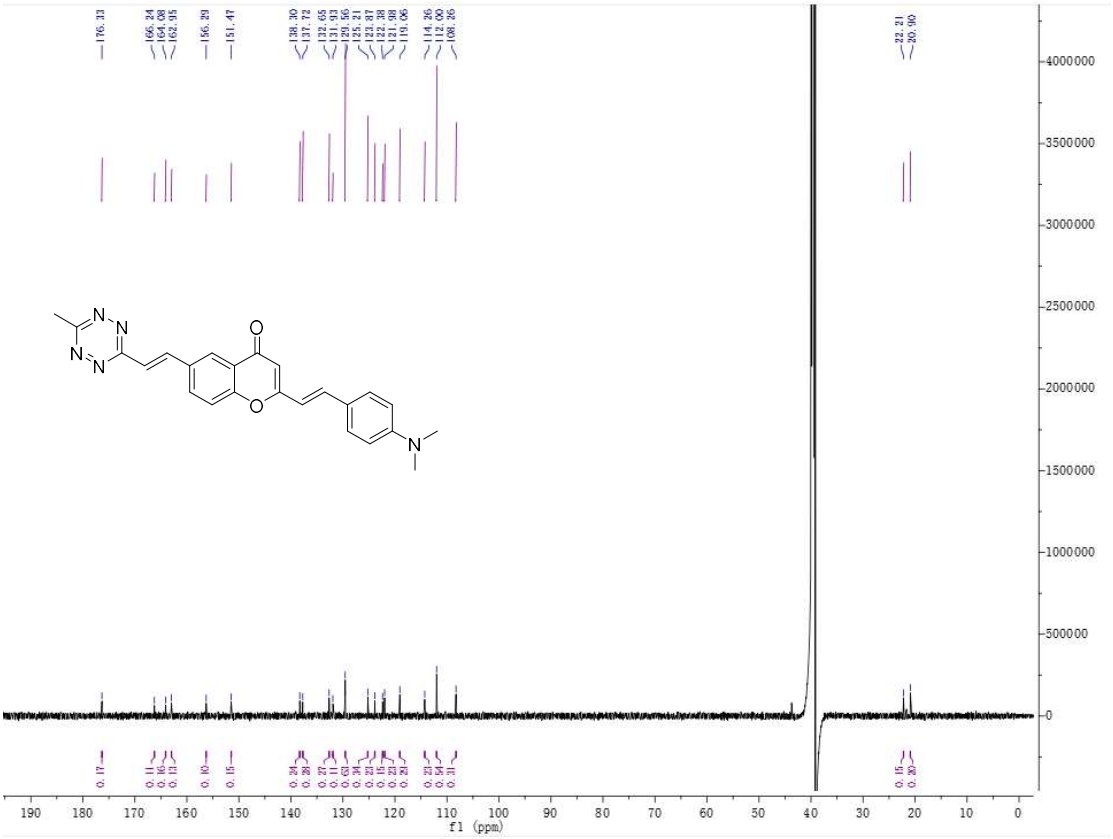
Reference

1. Teng, Y.; Yang, H.; Li, X.; Wang, Y. C.; Yin, D. L.; Tian, Y. L., *Chin. J. Chem*. **2022**, 40, 209-214.
2. Mao, W.; Shi, W.; Li, J.; Su, D.; Wang, X.; Zhang, L.; Pan, L.; Wu, X.; Wu, H., *Angew. Chem. Int. Ed*. **2019**, 58, 1106-1109.
3. Favre, C.; Friscourt, F., *Org. Lett*., **2018**, 20, 4213-4217.

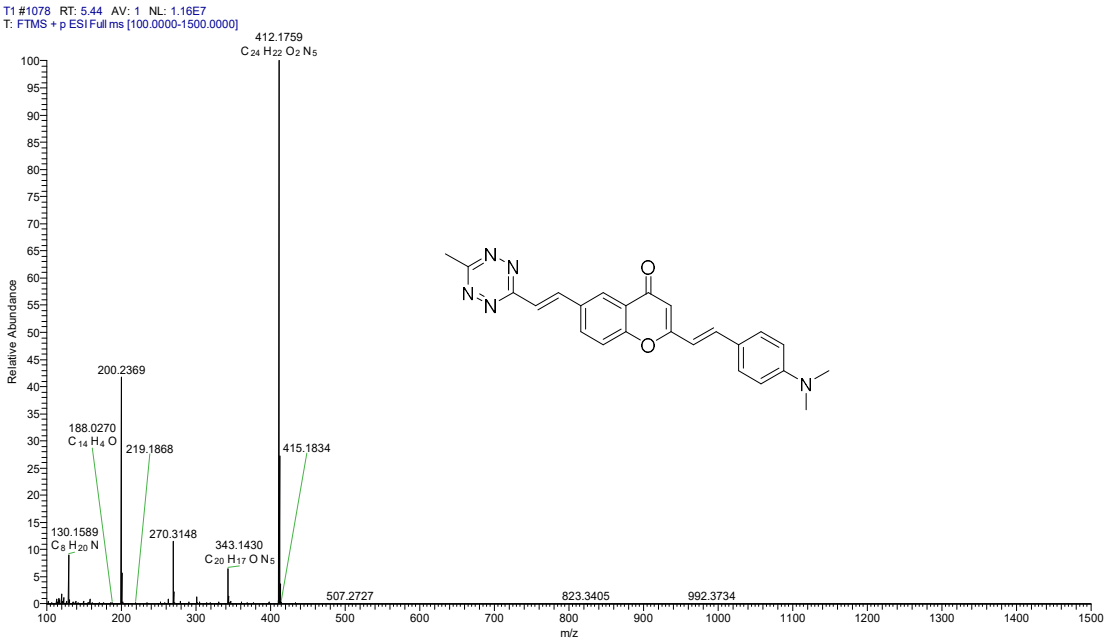
^{1}H NMR of **DMAC-Tz1** (700 MHz in DMSO)



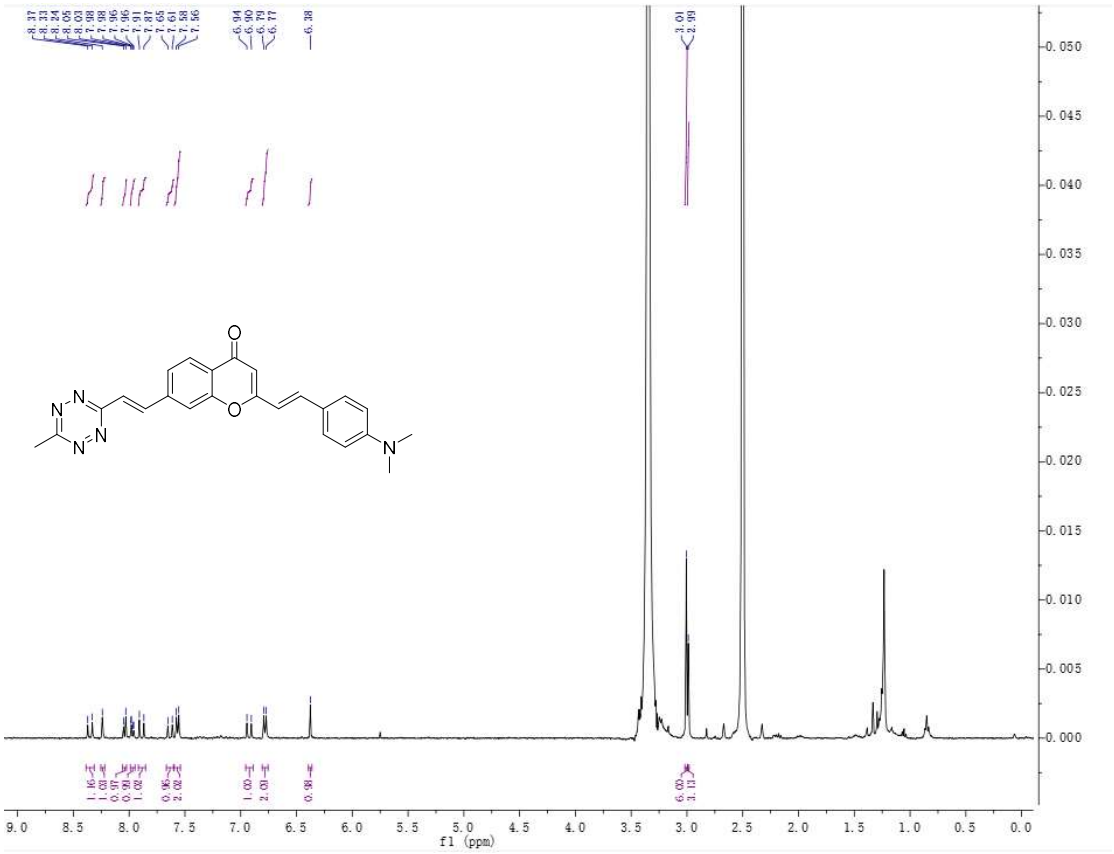
^{13}C NMR of **DMAC-Tz1** (700 MHz in DMSO)



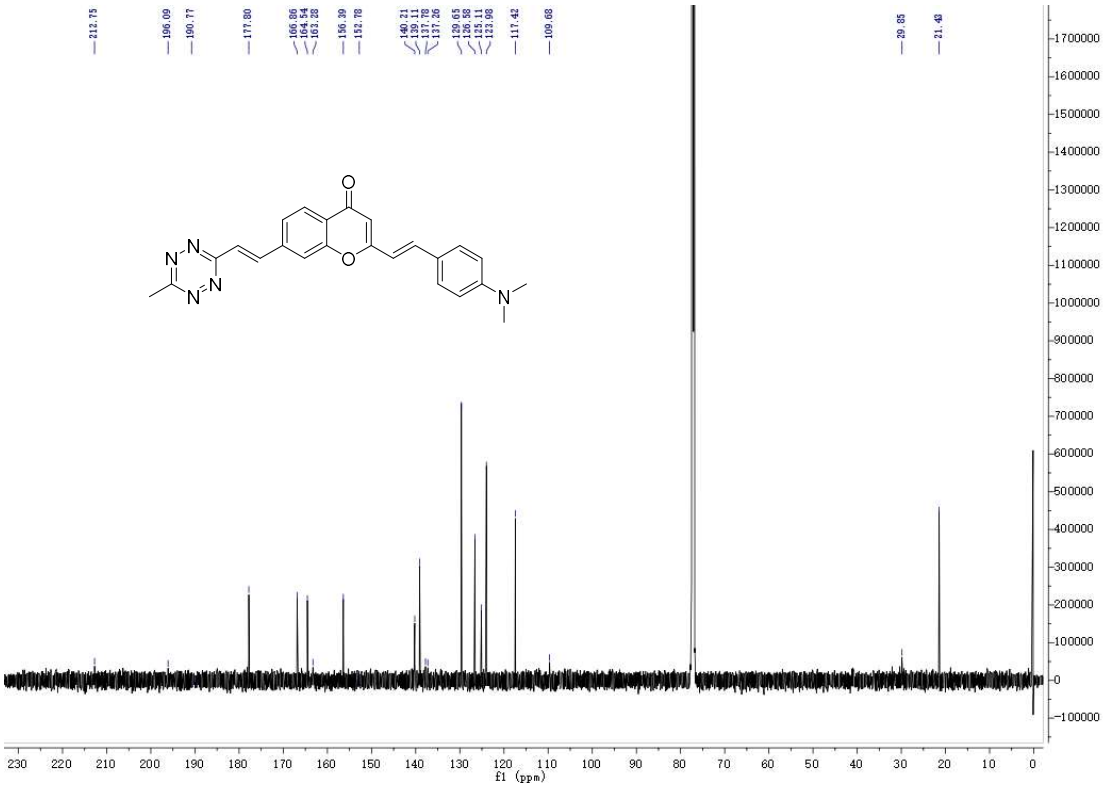
MS spectra of **DMAC-Tz1**



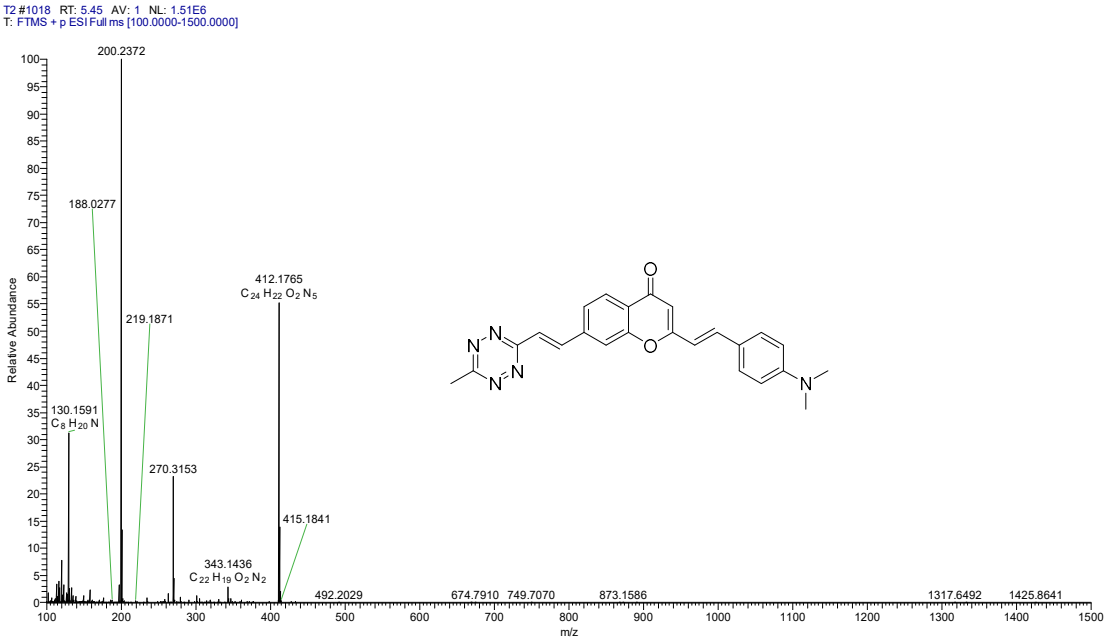
^{1}H NMR of **DMAC-Tz2** (400 MHz in DMSO)



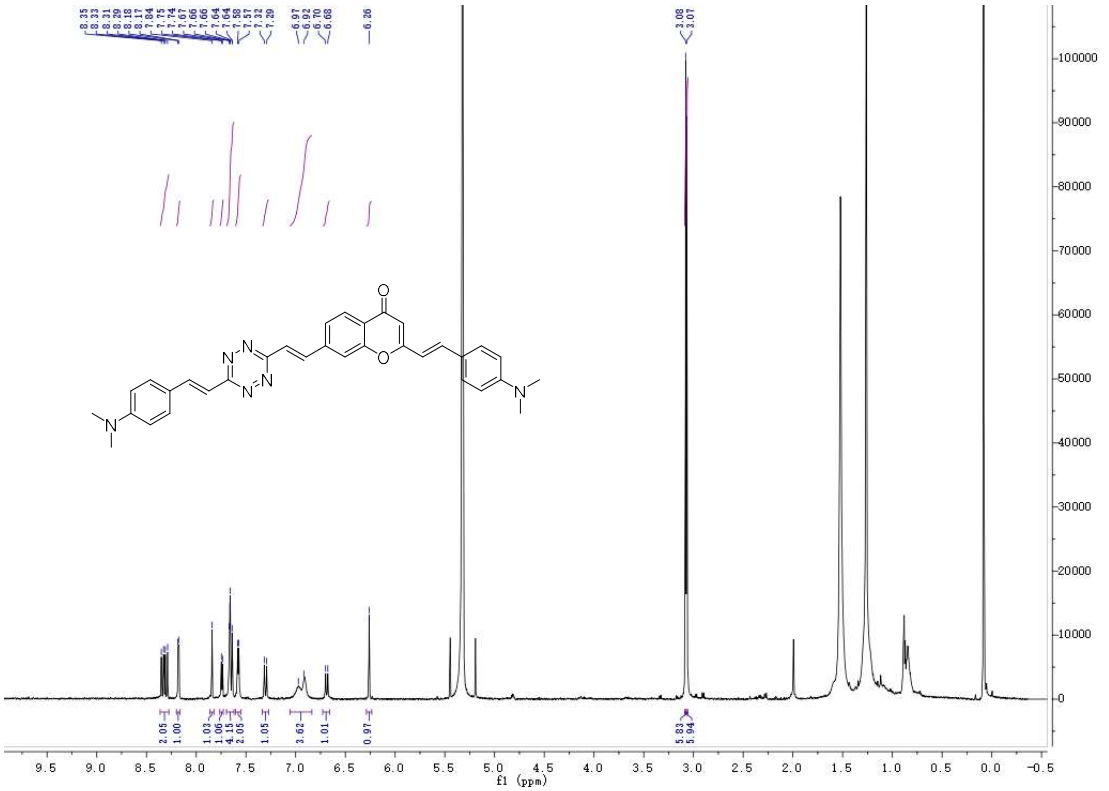
^{13}C NMR of **DMAC-Tz2** (700 MHz in $CDCl_3$)



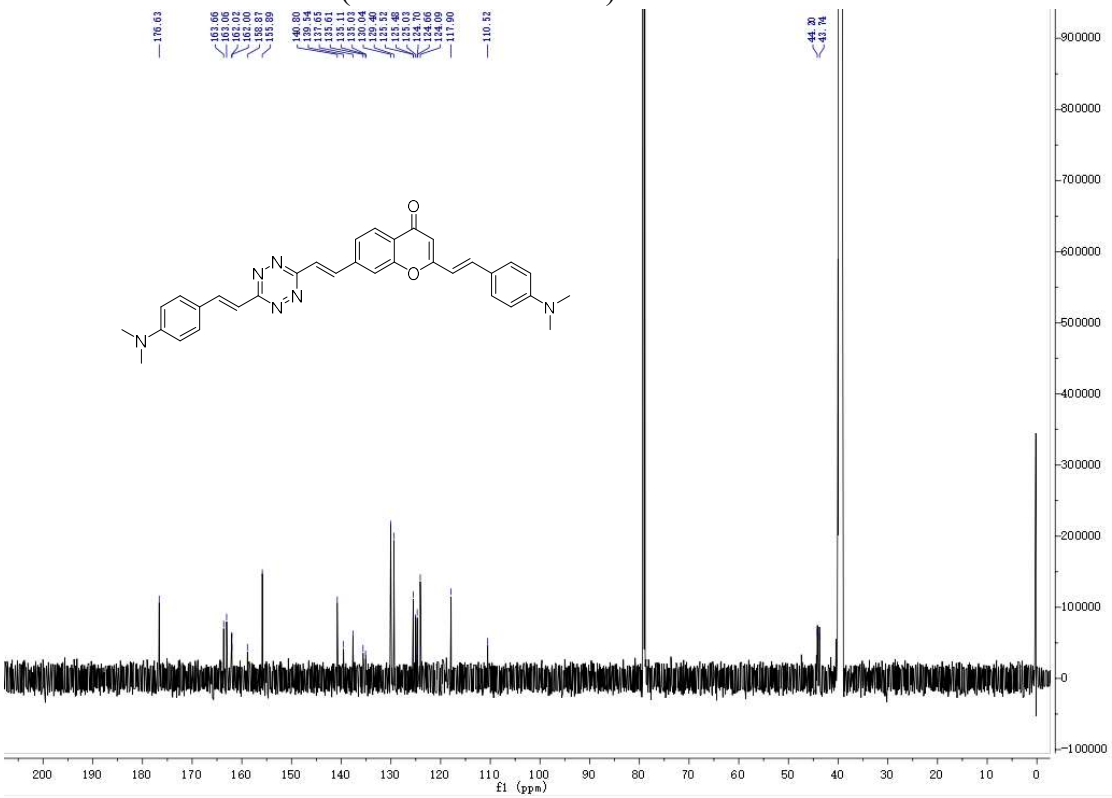
MS spectra of **DMAC-Tz2**



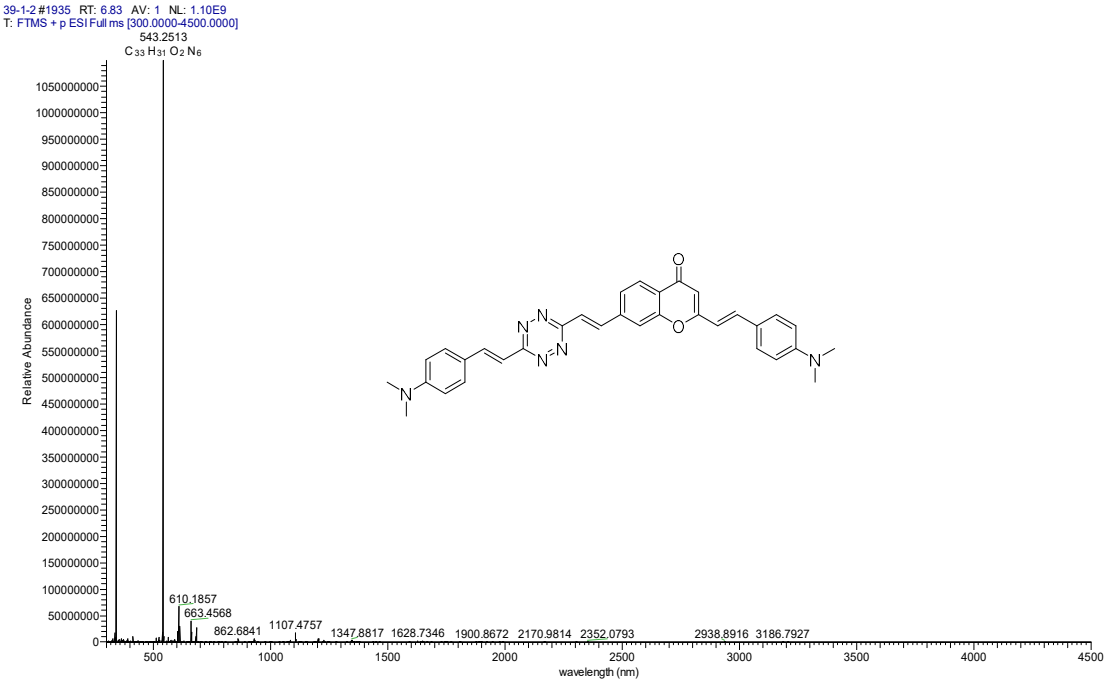
^{1}H NMR of **DMAC-Tz3** (700 MHz in Methylene Chloride-d_2)



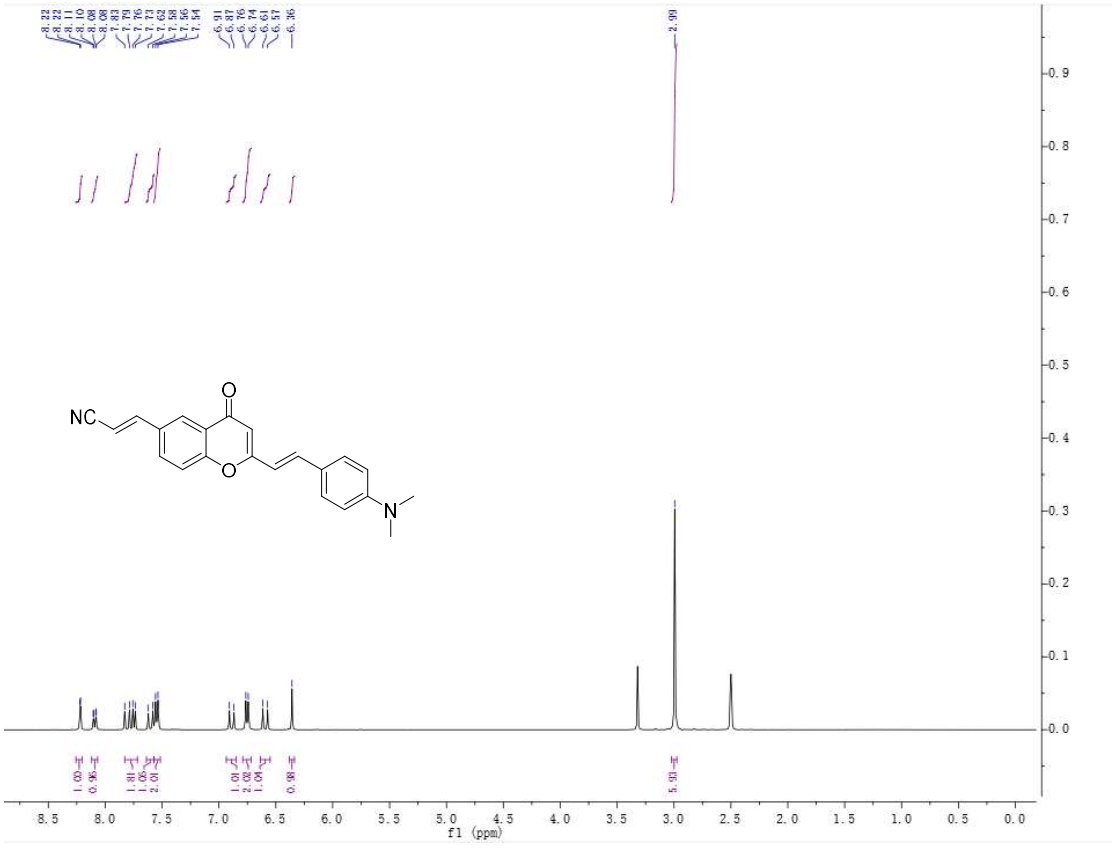
^{13}C NMR of **DMAC-Tz3** (700 MHz in DMSO)



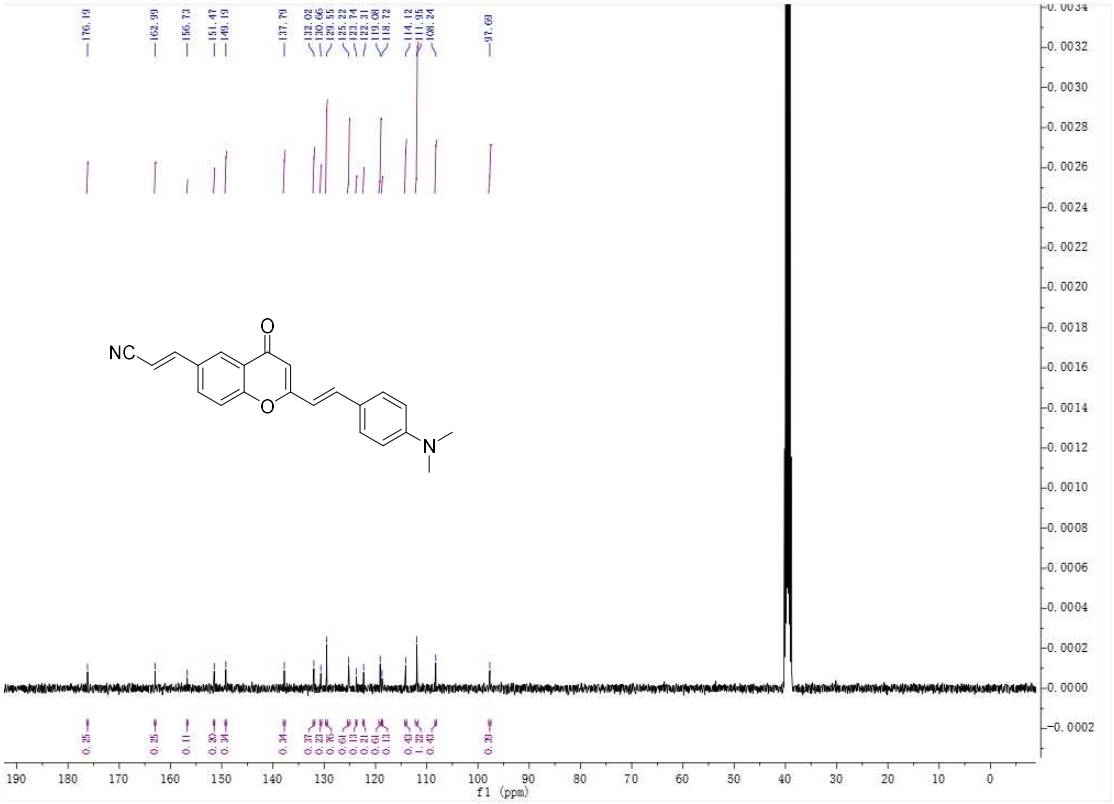
MS spectra of **DMAC-Tz3**



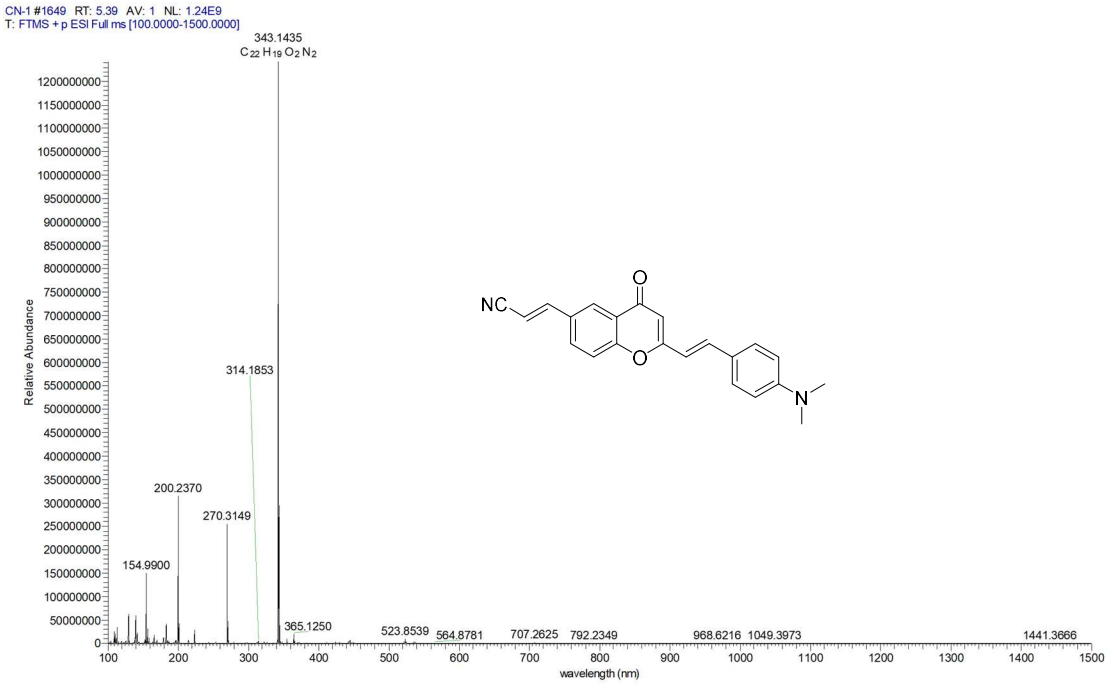
^{1}H NMR of **DMAC-CN1** (400 MHz in DMSO)



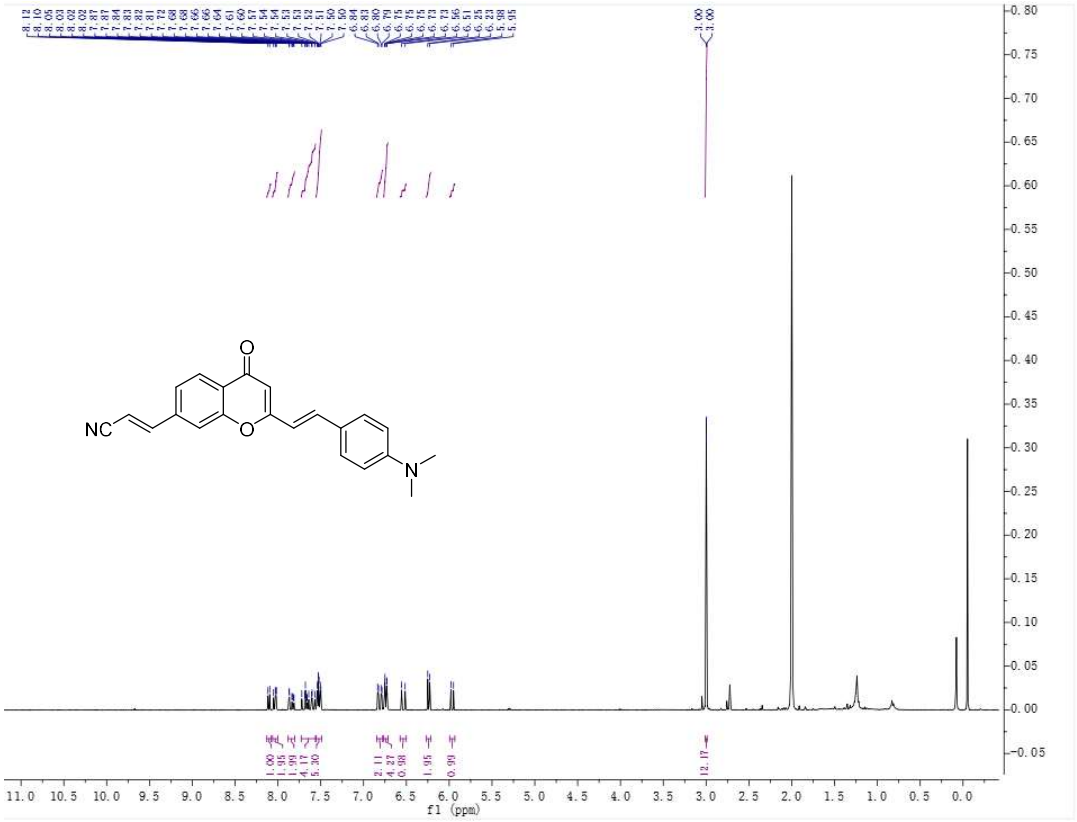
^{13}C NMR of **DMAC-CN1** (400 MHz in DMSO)



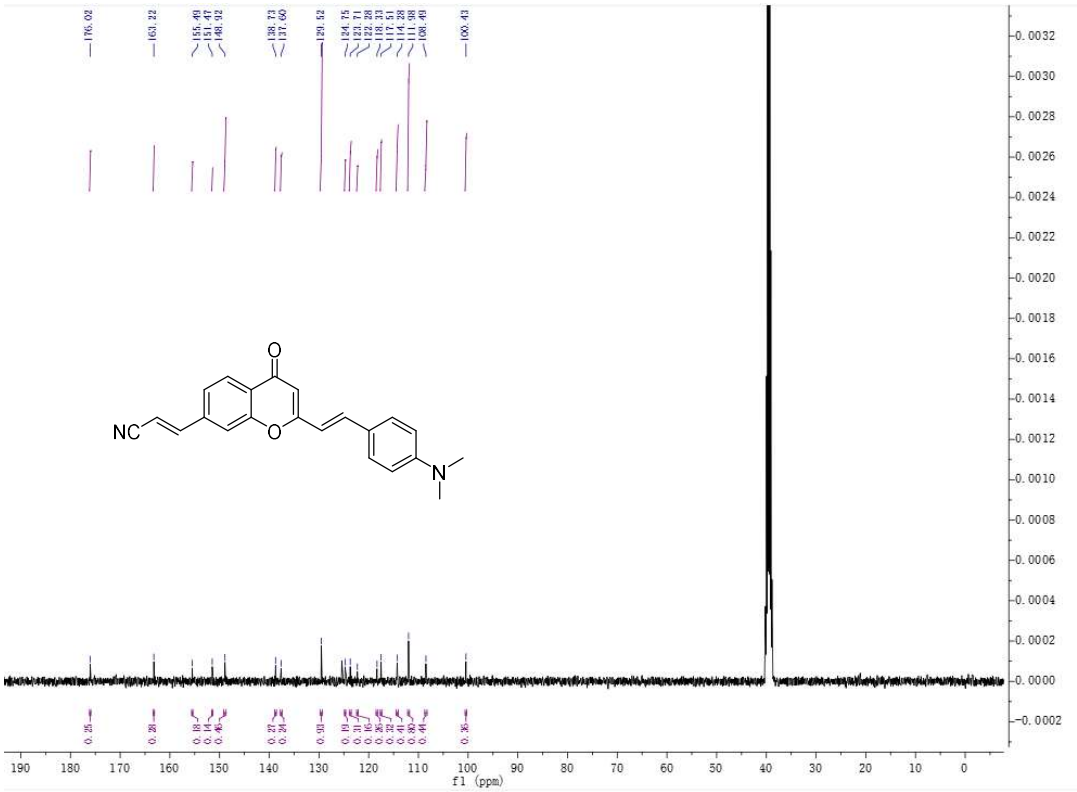
MS spectra of **DMAC-CN1**



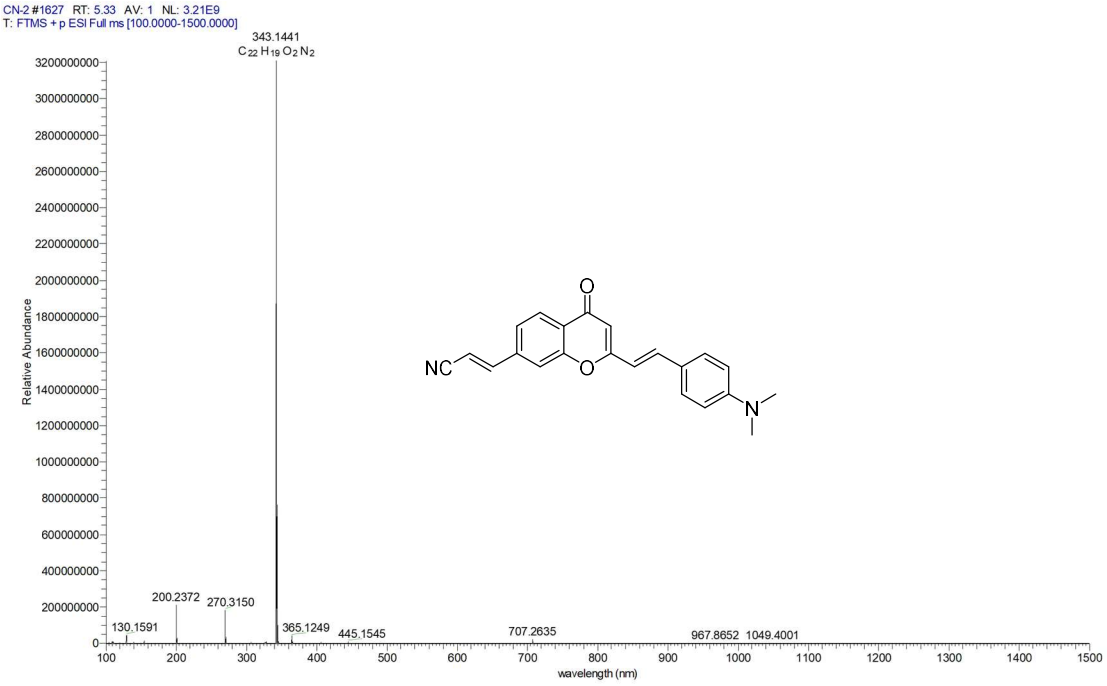
^{1}H NMR of **DMAC-CN2** (400 MHz in DMSO)



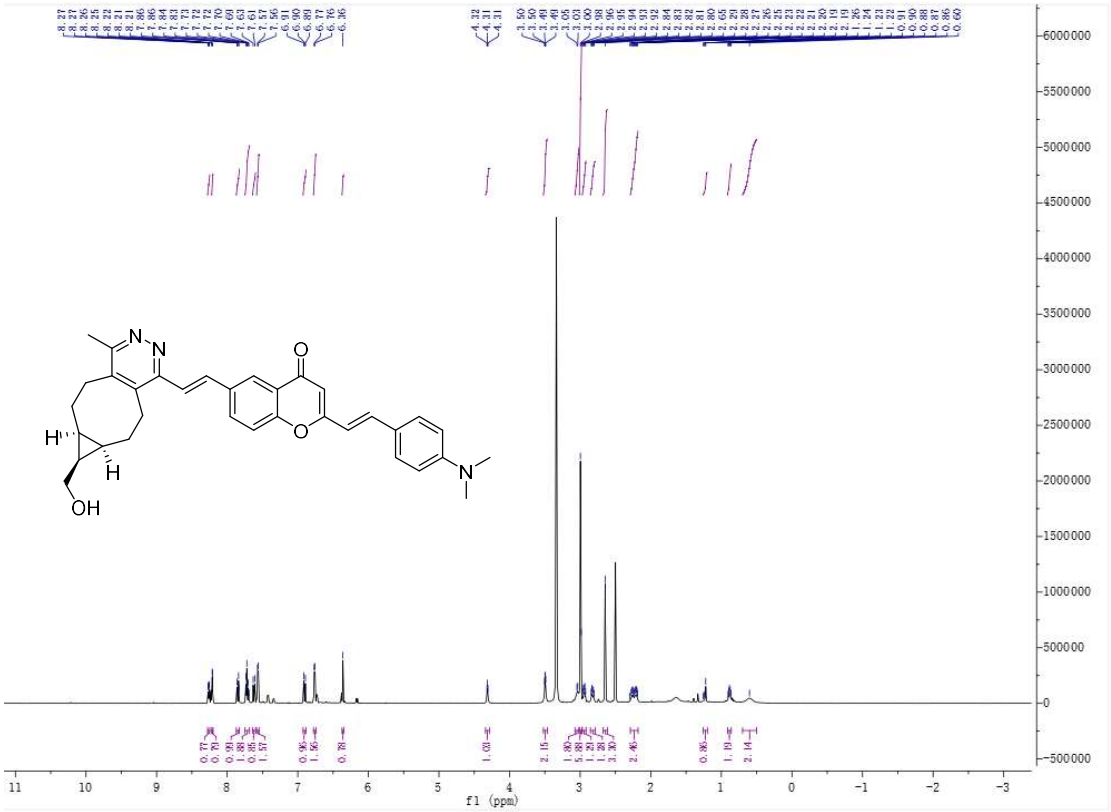
^{13}C NMR of **DMAC-CN2** (400 MHz in DMSO)



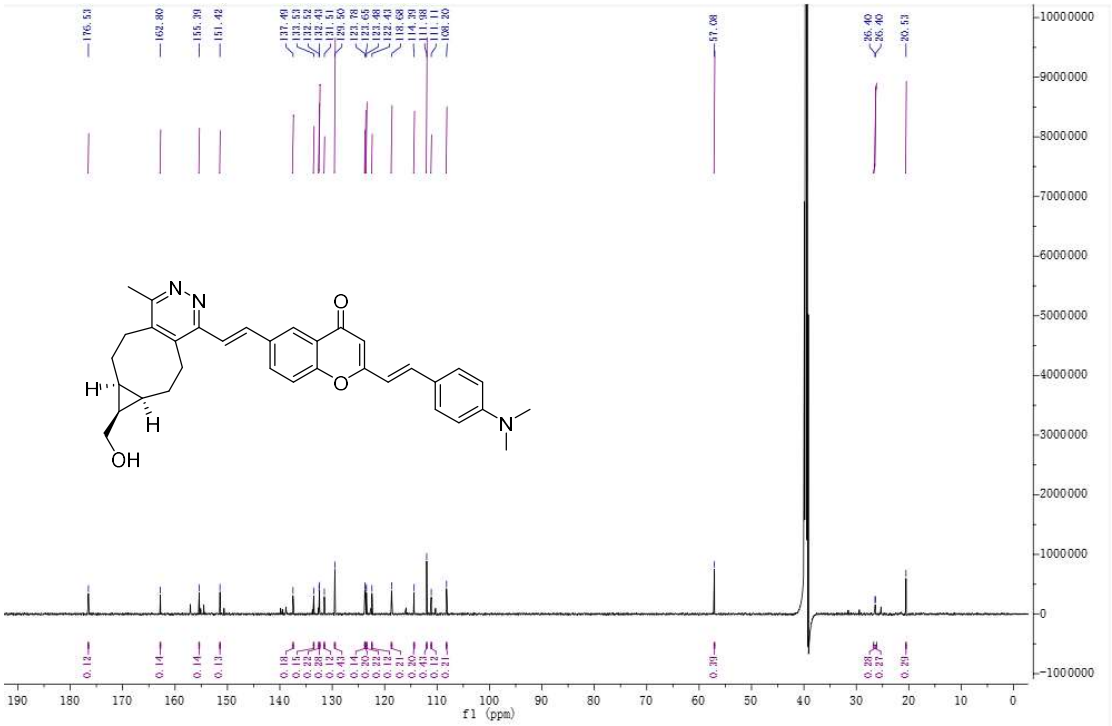
MS spectra of **DMAC-CN2**



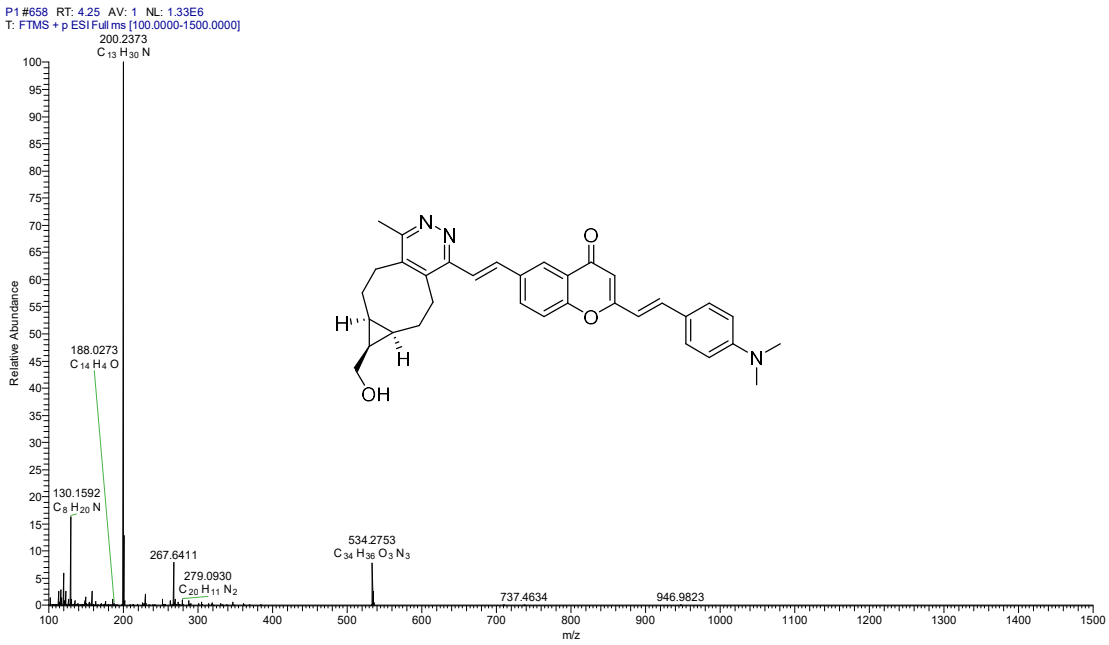
^{1}H NMR of **DMAC-Pz1** (700 MHz in DMSO)



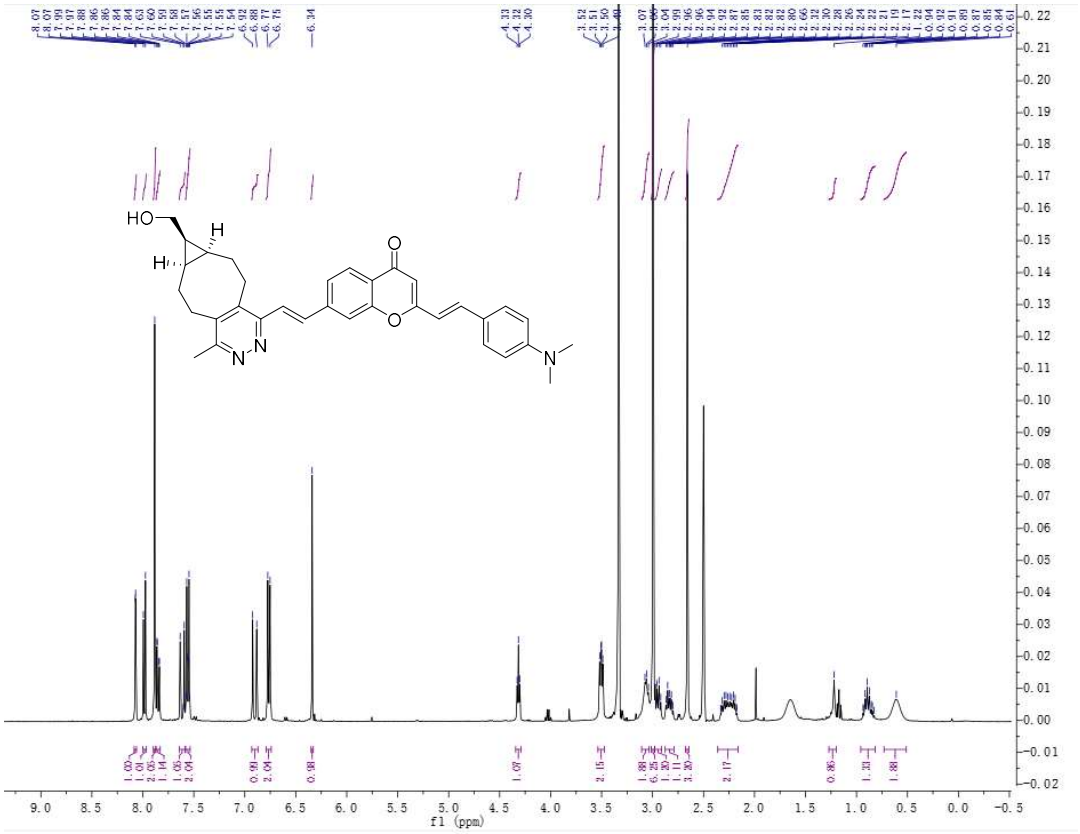
^{13}C NMR of **DMAC-Pz1** (700 MHz in DMSO)



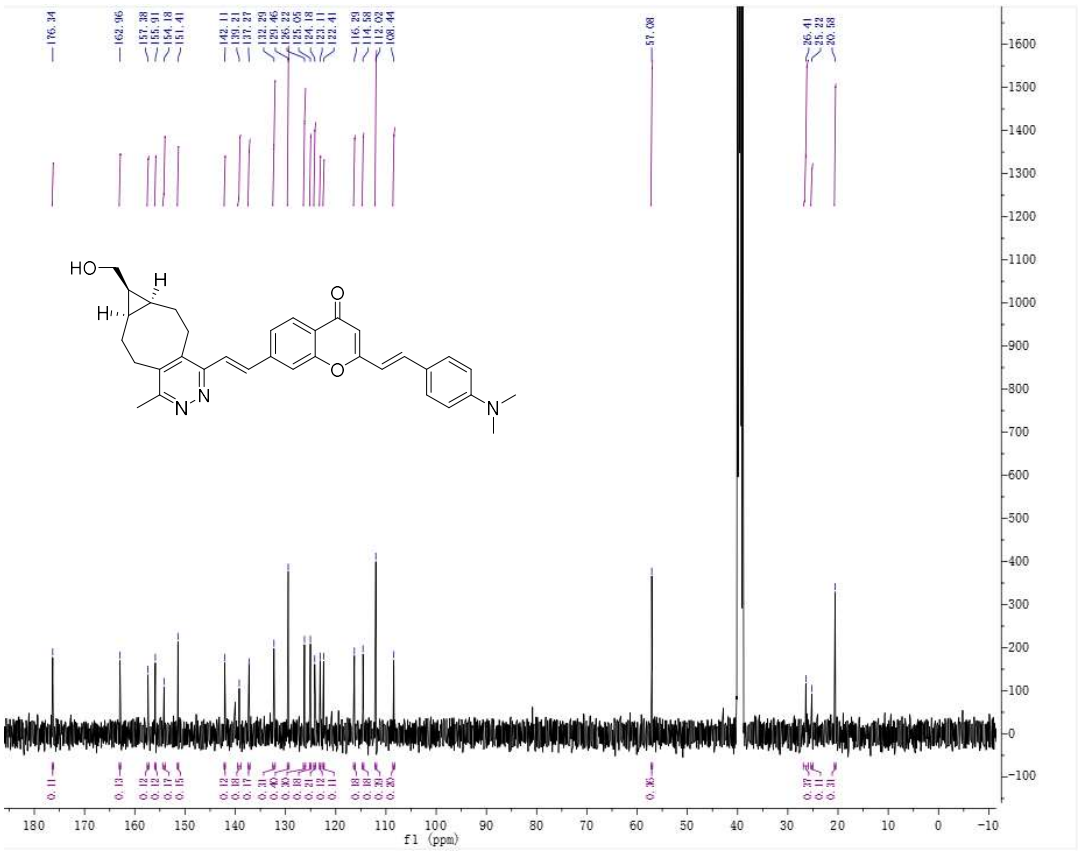
MS spectra of **DMAC-Pz1**



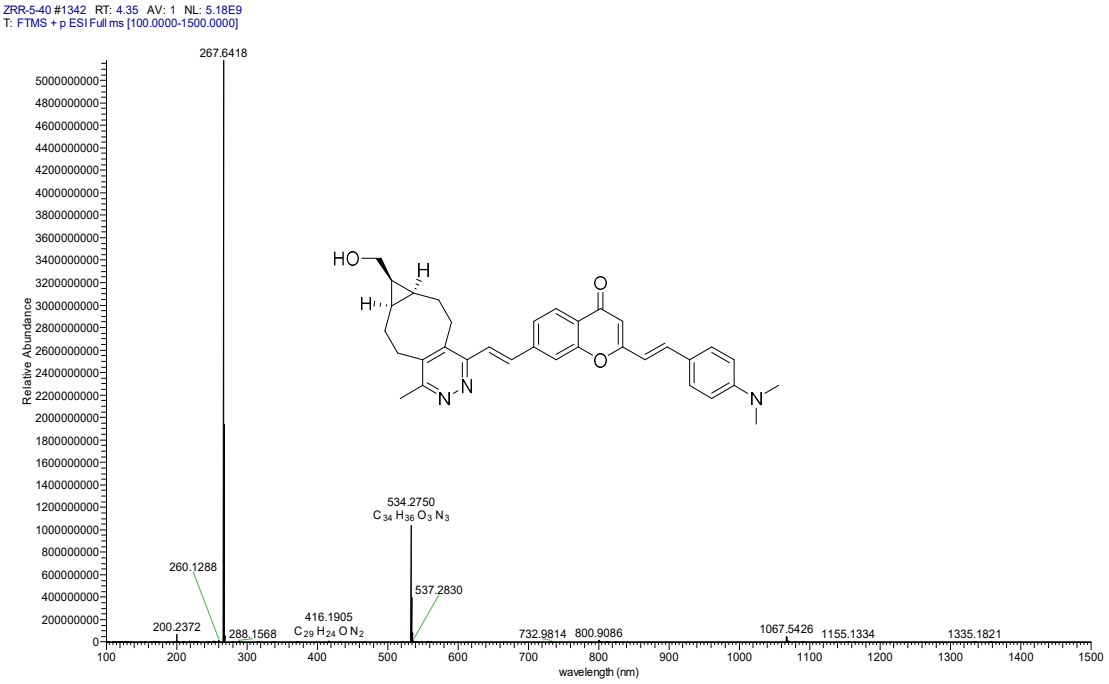
^{1}H NMR of **DMAC-Pz2** (400 MHz in DMSO)



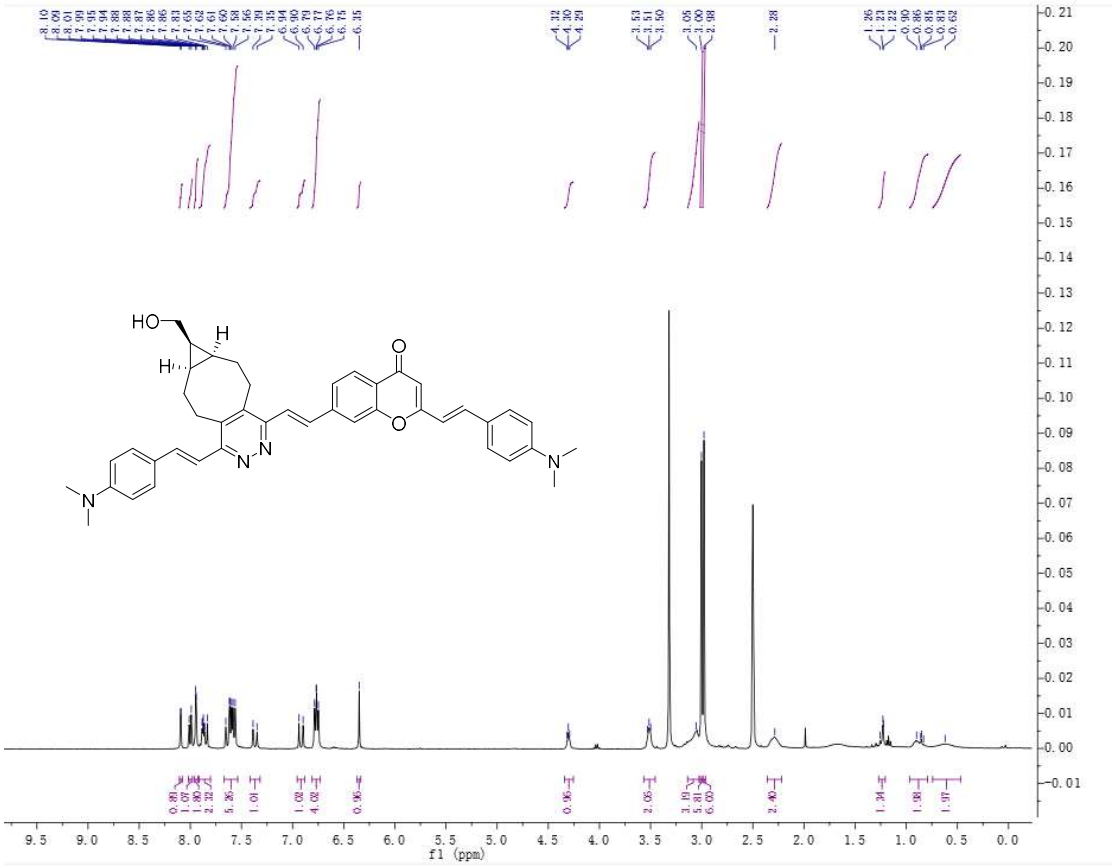
^{13}C NMR of **DMAC-Pz2** (500 MHz in DMSO)



MS spectra of **DMAC-Pz2**



^{1}H NMR of **DMAC-Pz3** (400 MHz in DMSO)



MS spectra of **DMAC-Pz3**