Supporting Information

Tuning the Physicochemical Properties of BODIPY for Bioimaging via *meso*-Amino Acylation

Wei Shi,[†] Jie Li,[†] Xinyu He, Siming Zhou, Hongbao Sun,* and Haoxing Wu*

Huaxi MR Research Center, Department of Radiology, Functional and Molecular Imaging Key Laboratory of Sichuan Province, Frontiers Science Center for Disease-related Molecular Network, West China Hospital, Sichuan University, Chengdu 610041, China.

[†]W.S. and J.L. contributed equally.

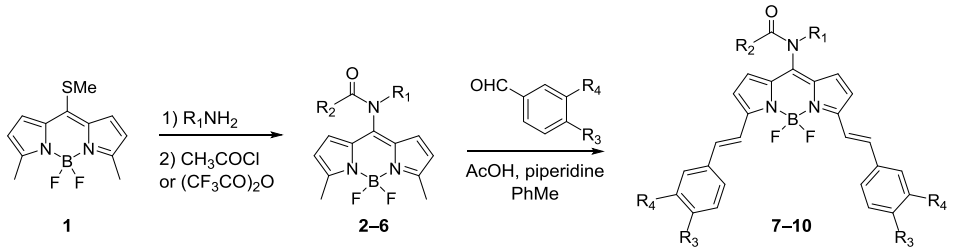
E-mail: hbsun7@sina.com; haoxingwu@scu.edu.cn

Table of contents

1. General methods

All reagents were purchased from Adamas-Beta, TCI, Energy Chemical, and Bide Pharm and used without further purification, unless otherwise noted. TLC plates (silica, 200×200 mm, pH 6.2–6.8, MF = 254, glass-backed) were purchased from Yan Tai Technologies. Column chromatography was performed on silica gel (300–400 mesh). ^{1}H and ^{13}C NMR spectra were recorded on a Varian spectrometer at 400 MHz (^{1}H) and 101 MHz (^{13}C), or a JEOL spectrometer at 600 MHz (^{1}H) and 151 MHz (^{13}C). All ^{13}C NMR spectra were ^{1}H-decoupled. HRMS spectra were recorded on a Bruker Daltonics Data analysis 3.4 mass spectrometer or a Bruker micro-TOF-QII time of flight mass spectrometer with electrospray ionization. LC-MS spectrometry was performed on an Agilent 1260 Infinity HPLC system equipped with a G6125B Single Quadrupole MS system. Fluorescence spectra were recorded on a wavelength-calibrated FluoroMax-4 fluorometer (Horiba Jobin Yvon), and the absolute quantum yield was measured using an integrating sphere (K-Sphere). UV-Vis absorption data were collected on a Quawell scientific Q6000+ microvolume spectrophotometer. Live-cell confocal fluorescence imaging was conducted on a LSM 880 system (Carl ZEISS). Spiro[2.3]hex-1-ene (**Sph**)[1], *trans*-cyclooctene–triphenylphosphonium (**TCO–TPP**)[2], compound **1**[3] and compound **11**[4] were synthesized according to previous studies.

2. Synthesis of *meso*-*N*-acyl BODIPYs



Scheme S1. Synthesis of *meso*-*N*-acyl BODIPYs**.**

General procedure A: A solution of *N*-substituted methylamine (2.0 eq.) in ethanol was added to a solution of compound **1** (1.0 eq.) in DCM (0.18 M) and stirred at room temperature for 1 h under argon. After reaction completion, the mixture was evaporated *in vacuo* and the obtained residue was dissolved in anhydrous DCM (0.04 M) and cooled to 0 ℃. DIPEA (3.0 eq.) was then added dropwise, followed by the addition of acetyl chloride (2.0 eq.) or trifluoroacetic anhydride (1.2 eq.) at room temperature. After reaction completion, the mixture was evaporated *in vacuo* and the residue was purified by silica gel column chromatography to afford analogue **2** or **3**.

General procedure B:

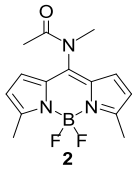
In a flask, compound **1** (1.0 eq.), amino derivative (1.2–2.0 eq.), and TEA (1.2–3.0 eq.) were dissolved in MeCN (0.1 M) or DCM (0.1 M) under argon atmosphere. The reaction solution was stirred at room temperature for 2.5 h. The reaction mixture was then evaporated under reduced pressure and purified by silica column chromatography to afford analogue **S-3**, **S-6**, **S-10**, **S-14**, **S-17** or **S-21**.

General procedure C:

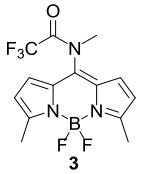
N-Substituted methylamine (1.0 eq.) was dissolved in dry DCM (0.1 M) and cooled to 0 ℃. DIPEA (3.0 eq.) was then added dropwise, followed by the addition of acetyl chloride (2.0 eq.). After stirring at 40 ℃ for 24 h, the reaction mixture was evaporated *in vacuo* and the residue was purified by silica gel column chromatography to afford analogue **S-4**, **S-7**, **S-11**, **13** or **14**.

General procedure D:

A mixture of the appropriate *meso*-*N*-acyl BODIPY (1.0 eq.) and benzaldehyde (3.7 eq.) was dissolved in toluene (0.1 M) under argon. Piperidine (4.0 eq.) and glacial acetic acid (4.0 eq.) were then added at 0 ℃ and the resulting mixture was stirred at 110 ℃ for 1 h. After reaction completion, the solvent was removed *in vacuo* and the obtained residue was purified by silica gel column chromatography to afford BODIPYs **7**–**10**.

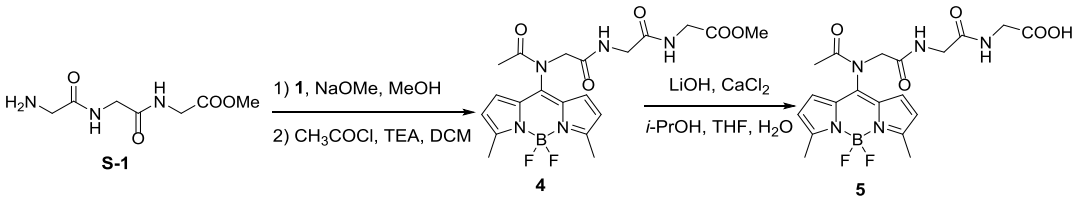

2

Synthesis of BODIPY 2: Following the general procedure A, starting material **1** (625 mg, 2.3 mmol, 1.0 eq.), methylamine (33 wt.% in EtOH, 587 μL, 4.60 mmol, 2.0 eq.), DIPEA (797 μL, 4.83 mmol, 3.0 eq.), acetyl chloride (229 μL, 3.21 mmol, 2.0 eq.) and DCM (13 mL) were used. Compound **2** was purified by silica column chromatography (PE:EA = 2:1) as a red solid (440 mg), yield: 60%. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 6.92 (d, J = 4.1 Hz, 2H), 6.32 (d, J = 4.2 Hz, 2H), 3.34 (s, 3H), 2.63 (s, 6H), 2.02 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 170.3, 159.9, 140.7, 133.3, 127.0, 120.7, 39.1, 22.2, 15.2. **HRMS** $[M+Na]^+$ *m/z* calcd. for $[C_{14}H_{16}BF_2N_3NaO]^+$ 314.1247, found 314.1245.


3

Synthesis of BODIPY 3: Following the general procedure A, starting material **1** (106 mg, 0.4 mmol, 1.0 eq.), methylamine (33 wt.% in EtOH, 100 μL, 0.8 mmol, 2.0 eq.), DIPEA (209 μL, 1.2 mmol, 3.0 eq.), trifluoroacetic anhydride (111 μL, 0.8mmol, 2.0 eq.) and DCM (2.3 mL) were used. Compound **3** was purified by silica column chromatography (PE:DCM = 2:1) as a red solid (53 mg), yield: 33%. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 6.90 (d, J = 4.2 Hz, 2H), 6.34 (d, J = 4.2 Hz, 2H), 3.47 (s, 3H), 2.64 (s, 6H). **^{13}C NMR** (101 MHz,

$CDCl_3$): δ 161.0, 157.8, 157.4, 136.4, 132.9, 126.9, 121.11, 121.09, 121.07, 121.04, 117.5 (q, J = 288.9 Hz, C-F), 41.5, 15.3. **HRMS** $[M+Na]^+$ *m/z* calcd. for $[C_{14}H_{13}BF_5N_3NaO]^+$ 368.0964, found 368.0965.

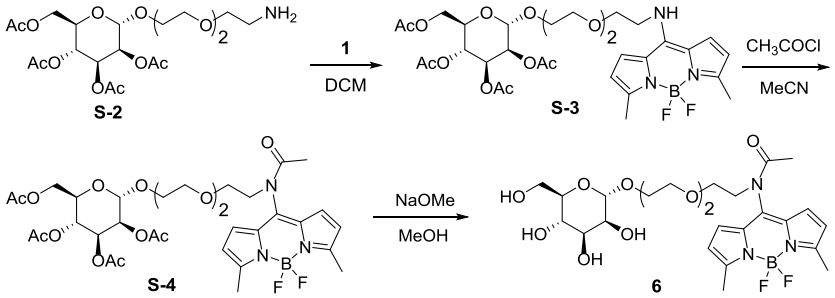


Scheme S2. Synthesis of BODIPY **4** and **5**.

Synthesis of BODIPY 4: Compound **S-1** was synthesized according to a previous study.[5] Sodium methoxide (32.4 mg, 0.6 mmol, 3.0 eq.) was added to a mixture of compound **1** (53.2 mg, 0.2 mmol, 1.0 eq.) and compound **S-1** (121.8 mg, 0.6 mmol, 3.0 eq.) in methanol (2.0 mL). The resulting mixture was stirred at room temperature for 1 h under argon atmosphere. After reaction completion, as determined by TLC, the reaction was quenched with saturated ammonium chloride solution, extracted three times with DCM (20 mL), and washed with brine (20 mL). The organic phase was dried with anhydrous Na_2SO_4, filtered, and concentrated. Subsequently, the residue was dissolved in dry DCM and cooled to 0 ℃, followed by the dropwise addition of TEA (83.7 μL, 0.6 mmol, 3.0 eq.) and then addition of acetyl chloride (28.2 μL, 0.4 mmol, 2.0 eq.) at the same temperature. After the reaction completion, the reaction mixture was evaporated *in vacuo* and the obtained residue was purified by silica gel column chromatography using ethyl acetate as the eluent to afford **4** as a red solid (18.5 mg) in 20% yield. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.26 (d, J = 4.0 Hz, 2H), 7.19 (t, J = 5.1 Hz, 1H), 7.14 (t, J = 5.6 Hz, 1H), 6.31 (d, J = 4.2 Hz, 2H), 4.23 (s, 2H), 4.03 (t, J = 5.4 Hz, 4H), 3.73 (s, 3H), 2.61 (s, 6H), 2.05 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 171.6, 170.5, 169.4, 168.2, 160.6, 138.7, 133.3, 128.2, 120.9, 54.7, 52.5, 43.1, 41.3, 22.1, 15.2. **HRMS** $[M–H]^-$ *m/z* calcd. for $[C_{20}H_{23}BF_2N_5O_5]^-$ 462.1766, found 462.1776.

Synthesis of BODIPY 5: Compound **4** (2.4 mg, 0.005 mmol, 1.0 eq.) and calcium chloride (8.6 mg, 0.075 mmol, 15.0 eq.) were dissolved in isopropanol (200 μL) and tetrahydrofuran (50 μL). Subsequently, the lithium hydroxide (0.9 mg, 0.021 mmol, 4.2 eq.) in water (110 μL) was dropped into the reaction solution. Then the reaction was stirred at room temperature for 12 h. After reaction completion, hydrochloric acid (1 M) was slowly added to the reaction solution until the pH value was 3–4. The aqueous phase was extracted with EA (30 mL×6), organic phase was dried with anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by HPLC preparation column to afford compound **5** as a red solid (1.5 mg), yield: 68%. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.41 (s, 2H), 6.38 (s,

2H), 4.23 (s, 2H), 3.95 (s, 2H), 3.76 (s, 2H), 2.55 (s, 6H), 1.95 (s, 3H). **^{13}C NMR** (101 MHz, MeOD): δ 171.0, 171.0, 170.9, 169.6, 161.7, 139.1, 134.4, 129.8, 121.7, 55.1, 42.7, 22.1, 15.0. **HRMS** $[M–H]^-$ *m/z* calcd. for $[C_{19}H_{21}BF_2N_5O_5]^-$ 448.1609, found 448.1611.



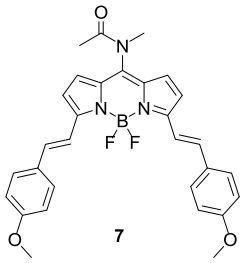
Scheme S3. Synthesis of BODIPY **6**.

Synthesis of compound S-3: Compound **S-2** was synthesized according to reference[6]. Following the general procedure B, starting material **1** (61.5 mg, 0.2 mmol, 1.0 eq.), compound **S-2** (121.8 mg, 0.6 mmol, 3.0 eq.), sodium methoxide (32.4 mg, 0.6 mmol, 3.0 eq.), and MeCN (2.0 mL) were used. Compound **S-3** was purified by silica column chromatography (DCM:MeOH = 20:1) as a yellow solid (38 mg), yield: 24%. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.00 (d, *J* = 2.7 Hz, 2H), 6.18 (d, *J* = 3.3 Hz, 2H), 5.30 – 5.19 (m, 3H), 4.85 (s, 1H), 4.22 (dd, *J* = 12.2, 5.1 Hz, 1H), 4.08 (dd, *J* = 12.4, 1.9 Hz, 1H), 4.03 – 3.94 (m, 1H), 3.91 (d, *J* = 4.3 Hz, 2H), 3.90 – 3.83 (m, 3H), 3.76 – 3.67 (m, 6H), 2.56 (s, 6H), 2.14 (s, 3H), 2.08 (s, 3H), 1.97 (d, *J* = 4.3 Hz, 6H). **HRMS** $[M–H]^-$ *m/z* calcd. for $[C_{31}H_{41}BF_2N_3O_{12}]^-$ 696.2757, found 696.2752.

Synthesis of compound S-4: Following the general procedure C, starting material **S-3** (38.0 mg, 0.05 mmol, 1.0 eq.), DIPEA (26 μL, 0.15 mmol, 3.0 eq.), acetyl chloride (8 μL, 0.11 mmol, 2.0 eq.) and DCM (0.5 mL) were used. Compound **S-4** was purified by silica column chromatography (DCM:MeOH = 20:1) as a red solid (5 mg), yield: 12%. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.02 (s, 2H), 6.32 (d, *J* = 4.0 Hz, 2H), 5.32 – 5.22 (m, 3H), 4.85 (d, *J* = 1.0 Hz, 1H), 4.28 (dd, *J* = 12.2, 4.9 Hz, 1H), 4.10 (d, *J* = 2.3 Hz, 1H), 4.00 (d, *J* = 1.7 Hz, 1H), 3.77 – 3.73 (m, 1H), 3.66 – 3.55 (m, 6H), 3.50 (d, *J* = 3.3 Hz, 3H), 2.63 (s, 6H), 2.15 (s, 3H), 2.09 (s, 3H), 2.03 (s, 3H), 2.01 (s, 3H), 1.98 (s, 3H). **^{13}C NMR** (151 MHz, $CDCl_3$): δ 170.8, 170.4, 170.2, 170.0, 169.9, 159.8, 135.8, 130.1, 127.6, 120.5, 97.9, 70.7, 70.3, 70.2, 69.7, 69.2, 68.6, 67.5, 66.3, 62.6, 50.1, 21.0, 20.9, 20.9, 20.8, 14.3. **HRMS** $[M–H]^-$ *m/z* calcd. for $[C_{33}H_{43}BF_2N_3O_{13}]^-$ 738.2862, found 738.2862.

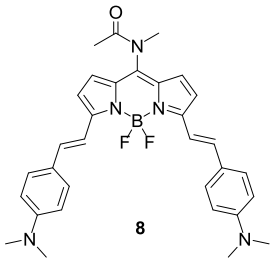
Synthesis of BODIPY 6: Compound **S-4** (4.0 mg, 0.005 mmol, 1.0 eq.) was added to a 10 mL reaction bottle, and a methanol solution of sodium methanol (0.05 M, 2 mL) was added. The reaction solution

was stirred at room temperature for 0.5 h. After reaction completion, Amberlite IR-120 (H^+ type) was added until the reaction liquid became neutral, filtered to remove Amberlite IR-120 (H^+ type), washed by methanol (5 mL×3), and organic phase was concentrated. The residue was purified by HPLC preparation column to afford compound **6** as a red solid (2 mg), yield: 70%. **^{1}H NMR** (400 MHz, D_2O): δ 7.30 (d, J = 4.2 Hz, 2H), 6.58 (d, J = 4.3 Hz, 2H), 4.16 – 4.10 (m, 2H), 3.94 (d, J = 14.8 Hz, 2H), 3.82 (dd, J = 10.0, 6.8 Hz, 3H), 3.79 – 3.72 (m, 3H), 3.67 (dd, J = 16.5, 10.5 Hz, 5H), 3.52 (s, 4H), 2.66 (s, 6H), 2.11 (s, 3H). **^{13}C NMR** (151 MHz, D_2O): δ 173.7, 160.4, 138.3, 133.3, 128.6, 121.0, 99.9, 72.7, 70.5, 69.9, 69.6, 69.6, 69.2, 68.5, 66.6, 66.3, 60.8, 50.0, 21.6, 14.2. **HRMS** $[M+H]^+$ *m/z* calcd. for $[C_{25}H_{37}BF_2N_3O_9]^+$ 572.2585, found 572.2582.



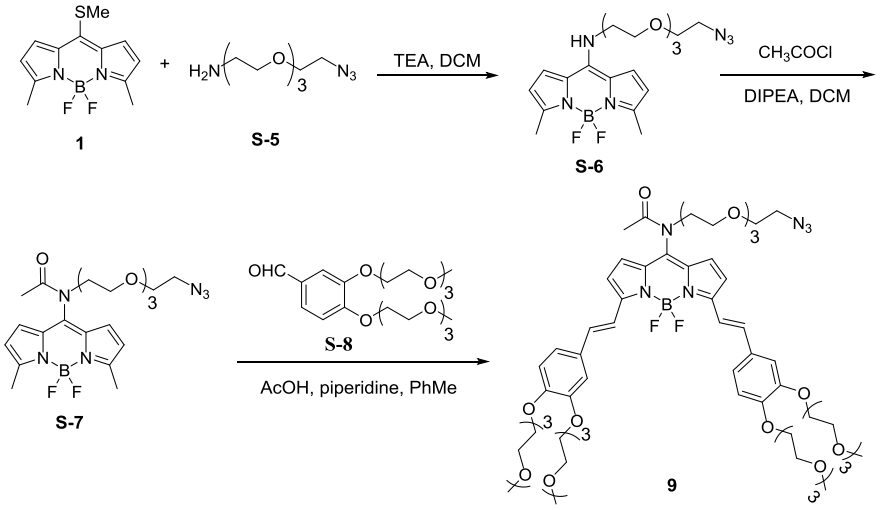
7

Synthesis of BODIPY 7: Following the general procedure D, starting material **2** (25.0 mg, 0.08 mmol, 1.0 eq.), 4-methoxybenzaldehyde (16 μL, 0.13 mmol, 3.7 eq.), piperidine (20 μL, 0.22 mmol, 4.0 eq.), glacial acetic acid (13 μL, 0.22 mmol, 4.0 eq.) and toluene (0.4 mL) were used. Compound **7** was purified by silica column chromatography (PE:EA = 2:1) as a green solid (25 mg) yield: 56%. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.65 – 7.56 (m, 6H), 7.34 (d, J = 16.2 Hz, 2H), 6.95 (t, J = 4.3 Hz, 8H), 3.86 (s, 6H), 3.36 (s, 3H), 2.04 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 170.7, 161.1, 156.5, 138.0, 136.3, 134.8, 129.6, 129.2, 125.7, 117.2, 117.2, 117.0, 114.6, 55.6, 55.5, 38.7, 22.2. **HRMS** $[M+Na]^+$ *m/z* calcd. for $[C_{30}H_{28}BF_2N_3NaO_3]^+$ 550.2084, found 550.2083.



8

Synthesis of BODIPY 8: Following the general procedure D, starting material **2** (29.0 mg, 0.1 mmol, 1.0 eq.), 4-dimethylaminobenzaldehyde (45.0 mg, 0.3 mmol, 3.0 eq.), piperidine (46 μL, 0.5 mmol, 5.0 eq.), glacial acetic acid (29 μL, 0.5 mmol, 5.0 eq.) and toluene (1.0 mL) were used. Compound **8** was purified by silica column chromatography (DCM:EA = 15:1) as a green solid (56 mg) yield: 95%. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.60 – 7.48 (m, 6H), 7.30 (d, J = 16.1 Hz, 2H), 6.90 (dd, J = 17.2, 4.5 Hz, 4H), 6.70 (d, J = 8.8 Hz, 4H), 3.34 (s, 3H), 3.03 (s, 12H), 2.03 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 171.0, 156.3, 151.3, 138.5, 134.6, 134.0, 129.6, 124.7, 124.6, 116.8, 114.5, 112.1, 40.3, 38.5, 22.0. **HRMS** $[M+Na]^+$ *m/z* calcd. for $[C_{32}H_{34}BF_2N_5NaO]^+$ 576.2717, found 576.2721.



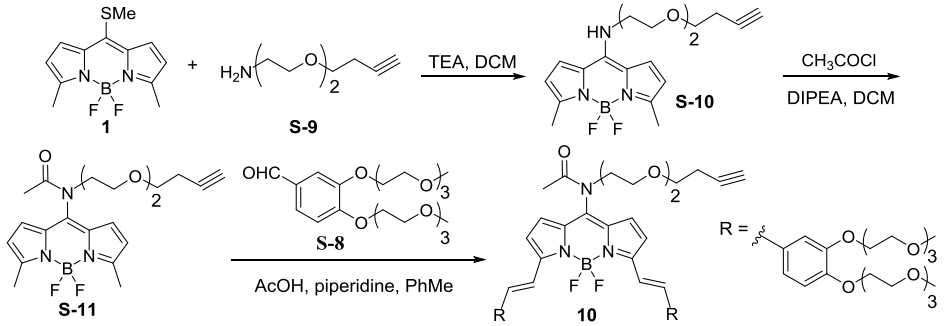
Scheme S4. Synthesis of BODIPY **9**.

Synthesis of compound S-6: Following the general procedure B, starting material **1** (26.6 mg, 0.10 mmol, 1.0 eq.), 11-azido-3,6,9-trioxaundecan-1-amine **S-5** (43.6 mg, 0.20 mmol, 2.0 eq.), TEA (17 μL, 0.12 mmol, 1.2 eq.) and DCM (1.0 mL) were used. Compound **S-6** was purified by silica column chromatography (PE:EA = 1:1) as a yellow solid (39 mg), yield: 83%. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 6.97 (d, J = 3.8 Hz, 2H), 6.18 (d, J = 3.6 Hz, 2H), 3.91 – 3.79 (m, 4H), 3.73 (s, 4H), 3.70 (dd, J = 5.9, 2.9 Hz, 2H), 3.65 (dd, J = 6.0, 3.0 Hz, 2H), 3.63 – 3.56 (m, 2H), 3.35 – 3.29 (m, 2H), 2.57 (s, 6H). **HRMS** $[M+Na]^+$ *m/z* calcd. for $[C_{19}H_{27}BF_2N_6NaO_3]^+$ 459.2098, found 459.2101.

Synthesis of compound S-7: Following the general procedure C, starting material **S-6** (35.0 mg, 0.08 mmol, 1.0 eq.), DIPEA (37 μL, 0.24 mmol, 3.0 eq.), acetyl chloride (11 μL, 0.15 mmol, 2.0 eq.) and DCM (0.8 mL) were used. Compound **S-7** was purified by silica column chromatography (PE:EA = 1:1) as a red solid (12 mg), yield: 32%. **^{1}H NMR** (400 MHz, CDCl3): δ 7.01 (d, J = 4.0 Hz, 2H), 6.31 (d, J = 4.1 Hz, 2H), 3.99 (t, J = 5.5 Hz, 2H), 3.66 – 3.56 (m, 8H), 3.52 (d, J = 4.0 Hz, 4H), 3.37 (t, J = 5.0 Hz, 2H), 2.63 (s, 6H), 2.01 (s, 3H). **^{13}C NMR** (101 MHz, CDCl3): δ 170.4, 159.8, 139.7, 133.9, 127.7, 120.5, 70.8, 70.7, 70.3, 70.1, 68.5, 50.8, 50.2, 22.6, 15.2. **HRMS** $[M+Na]^+$ *m/z* calcd. for $[C_{21}H_{29}BF_2N_6NaO_4]^+$ 501.2204, found 501.2204.

Synthesis of BODIPY 9: Following the general procedure D, starting material **S-7** (12.0 mg, 0.03 mmol, 1.0 eq.), **S-8** (33.0 mg, 0.08 mmol, 2.5 eq.), piperidine (12 μL, 0.13 mmol, 4.0 eq.), glacial acetic acid (8 μL, 0.13 mmol, 4.0 eq.) and toluene (0.3 mL) were used. Compound **9** was purified by silica column chromatography (DCM:MeOH = 20:1) as a green oil (16 mg), yield: 48%. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 7.54 (d, J = 16.1 Hz, 2H), 7.31 (d, J = 4.6 Hz, 2H), 7.28 (s, 2H), 7.15 (d, J = 1.7 Hz, 2H), 7.05 (d, J = 4.4 Hz, 2H), 6.98 – 6.90 (m, 4H), 4.29 – 4.20 (m, 8H), 4.01 (t, J = 5.3 Hz, 2H), 3.90

(dd, *J* = 7.1, 5.3 Hz, 8H), 3.77 (td, *J* = 6.0, 3.7 Hz, 8H), 3.69 – 3.59 (m, 24H), 3.54 (ddd, *J* = 9.3, 5.9, 3.7 Hz, 12H), 3.39 – 3.34 (m, 14H), 2.04 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): *δ* 170.8, 156.3, 150.8, 149.1, 138.1, 135.6, 135.3, 130.0, 126.4, 122.0, 117.4, 117.2, 114.6, 114.3, 72.1, 71.0, 70.8, 70.7, 70.3, 70.1, 69.9, 69.8, 69.4, 68.8, 68.5, 59.2, 59.1, 50.8, 49.9, 22.6. **HRMS** $[M+Na]^+$ *m/z* calcd. for $[C_{63}H_{93}BF_2N_6NaO_{20}]^+$ 1325.6398, found 1325.6396.



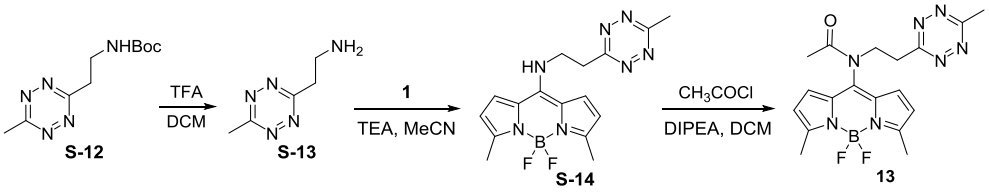
Scheme S5. Synthesis of BODIPY **10**.

Synthesis of compound S-10: Following the general procedure B, starting material **1** (26.6 mg, 0.10 mmol, 1.0 eq.), 2-[2-(2-propynyloxy)ethoxy] ethylamine **S-9** (28.6 mg, 0.20 mmol, 2.0 eq.), TEA (17 μL, 0.12 mmol, 1.2 eq.) and DCM (1.0 mL) were used. Compound **S-10** was purified by silica column chromatography (PE:EA = 2:1) as a yellow solid (35 mg), yield: 97%. **^{1}H NMR** (400 MHz, $CDCl_3$): *δ* 6.96 (d, *J* = 3.8 Hz, 2H), 6.81 (s, 1H), 6.19 (d, *J* = 3.7 Hz, 2H), 4.23 (d, *J* = 2.4 Hz, 2H), 3.93 – 3.79 (m, 4H), 3.76 (s, 4H), 2.57 (s, 6H), 2.46 (t, *J* = 2.4 Hz, 1H). **HRMS** $[M+Na]^+$ *m/z* calcd. for $[C_{18}H_{22}BF_2N_3NaO_2]^+$ 384.1665, found 384.1672.

Synthesis of compound S-11: Following the general procedure C, starting material **S-10** (35.0 mg, 0.1 mmol, 1.0 eq.), DIPEA (48 μL, 0.29 mmol, 3.0 eq.), acetyl chloride (14 μL, 0.19 mmol, 2.0 eq.) and DCM (1.0 mL) were used. Compound **S-11** was purified by silica column chromatography (PE:EA = 1:1) as a red solid (29 mg), yield: 74%. **^{1}H NMR** (400 MHz, $CDCl_3$): *δ* 7.02 (d, *J* = 3.9 Hz, 2H), 6.31 (d, *J* = 4.1 Hz, 2H), 4.18 – 4.07 (m, 4H), 3.63 (t, *J* = 5.5 Hz, 2H), 3.55 (dd, *J* = 13.5, 5.2 Hz, 4H), 2.62 (s, 6H), 2.41 (t, *J* = 2.0 Hz, 1H), 2.01 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): *δ* 170.5, 159.8, 139.6, 133.9, 127.7, 120.5, 79.8, 74.6, 70.0, 69.1, 68.5, 58.5, 50.1, 22.6, 15.2. **HRMS** $[M+Na]^+$ *m/z* calcd. for $[C_{20}H_{24}BF_2N_3NaO_3]^+$ 426.1771, found 426.1776.

Synthesis of BODIPY 10: Following the general procedure D, starting material **S-11** (15.0 mg, 0.04 mmol, 1.0 eq.), **S-8** (48.0 mg, 0.11 mmol, 2.8 eq.), piperidine (17 μL, 0.19 mmol, 5.0 eq.), glacial acetic acid (11 μL, 0.19 mmol, 5.0 eq.) and toluene (0.4 mL) were used. Compound **10** was purified by silica column chromatography (DCM:MeOH = 10:1) as a green oil (38 mg), yield: 83%. **^{1}H NMR** (400 MHz, $CDCl_3$): *δ* 7.55 (d, *J* = 16.2 Hz, 2H), 7.35 – 7.29 (m, 2H), 7.28 (s, 2H), 7.16 (s, 2H), 7.07 (d, *J* =

4.4 Hz, 2H), 6.95 (t, *J* = 7.1 Hz, 4H), 4.30 – 4.18 (m, 8H), 4.15 (d, *J* = 2.3 Hz, 2H), 4.02 (t, *J* = 5.3 Hz, 2H), 3.91 (t, *J* = 6.1 Hz, 8H), 3.80 – 3.74 (m, 8H), 3.72 – 3.63 (m, 18H), 3.62 – 3.51 (m, 12H), 3.37 (d, *J* = 9.4 Hz, 12H), 2.42 (t, *J* = 2.3 Hz, 1H), 2.05 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): *δ* 170.8, 156.3, 150.8, 149.0, 138.1, 135.6, 135.2, 130.0, 126.4, 122.0, 117.3, 117.2, 114.6, 114.3, 79.8, 74.6, 72.0, 71.0, 70.8, 70.6, 70.0, 69.9, 69.7, 69.3, 69.1, 68.8, 68.4, 59.1, 59.1, 58.5, 49.8, 22.5. **HRMS** $[M+Na]^+$ *m/z* calcd. for $[C_{62}H_{88}BF_2N_3NaO_{19}]^+$ 1250.5965, found 1250.5973.



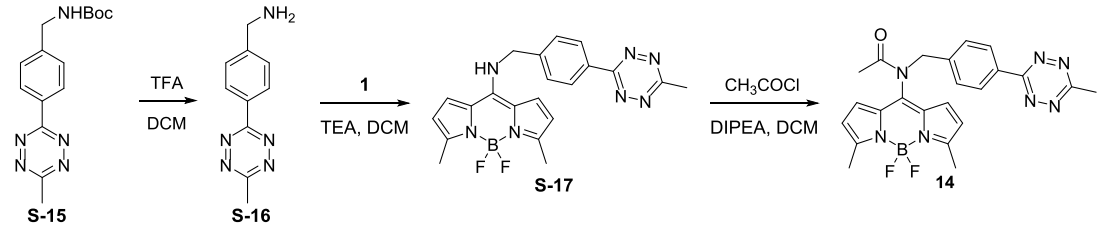
Scheme S6. Synthesis of BODIPY **13**.

Synthesis of compound S-13: Compound **S-12** was synthesized according to reference[7]. To a solution of compound **S-12** (480 mg, 2.0 mmol, 1.0 eq.) in DCM (2.6 mL) was added trifluoroacetic acid (2.6 mL) in an ice bath. The reaction mixture was stirred for 16 h at room temperature. After reaction completion, the reaction was quenched by adding a saturated sodium bicarbonate solution. The aqueous phase was extracted with DCM (20 mL×3). The organic phase was washed with saturated brine, dried anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by silica column chromatography (DCM:MeOH = 20:1) to afford **S-13** as a red liquid (200 mg), yield: 72%. NMR data of this material agreed with the reported one [7]. **^{1}H NMR** (400 MHz, MeOD): *δ* 3.62 (t, *J* = 6.3 Hz, 2H), 3.54 (t, *J* = 6.4 Hz, 2H), 2.96 (s, 3H).

Synthesis of compound S-14: Following the general procedure B, starting material **1** (53.2 mg, 0.2 mmol, 1.0 eq.), tetrazine **S-13** (56.0 mg, 0.4 mmol, 2.0 eq.), TEA (56 μL, 0.4 mmol, 2.0 eq.) and DCM (2.0 mL) were used. Compound **S-14** was purified by silica column chromatography (DCM:EA = 20:1) as a yellow solid (20.0 mg), yield: 28%. **^{1}H NMR** (400 MHz, $CDCl_3$): *δ* 7.07 (t, *J* = 4.6 Hz, 1H), 6.92 (d, *J* = 3.9 Hz, 2H), 6.13 (d, *J* = 3.9 Hz, 2H), 4.04 (dd, *J* = 11.8, 6.0 Hz, 2H), 3.67 (t, *J* = 6.3 Hz, 2H), 3.07 (s, 3H), 2.53 (s, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$): *δ* 168.6, 167.2, 147.8, 145.4, 123.3, 119.5, 115.4, 44.5, 33.3, 21.3, 14.3. **HRMS** $[M–H]^-$ *m/z* calcd. $[C_{16}H_{17}BF_2N_7]^-$ 356.1612, found 356.1622.

Synthesis of BODIPY 13: Following the general procedure C, starting material **S-14** (9.0 mg, 0.02 mmol, 1.0 eq.), DIPEA (12.5 μL, 0.08 mmol, 3.0 eq.), acetyl chloride (4 μL, 0.05 mmol, 2.0 eq.) and DCM (0.2 mL) were used. Compound **13** was purified by silica column chromatography (PE:EA = 20:1) as a red solid (4.0 mg), yield: 40%. **^{1}H NMR** (400 MHz, $CDCl_3$): *δ* 7.17 (d, *J* = 2.5 Hz, 2H), 6.36 (d, *J* = 3.3 Hz, 2H), 4.34 (t, *J* = 6.3 Hz, 2H), 3.57 (t, *J* = 5.7 Hz, 2H), 3.04 (s, 3H), 2.64 (s, 6H), 1.92 (s,

3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 170.4, 167.7, 167.7, 160.4, 138.9, 133.7, 127.9, 121.0, 49.6, 34.5, 22.2, 21.3, 15.2. **HRMS** $[M+H]^+$ *m/z* calcd. for $[C_{18}H_{21}BF_2N_7O]^+$ 400.1863, found 400.1863.

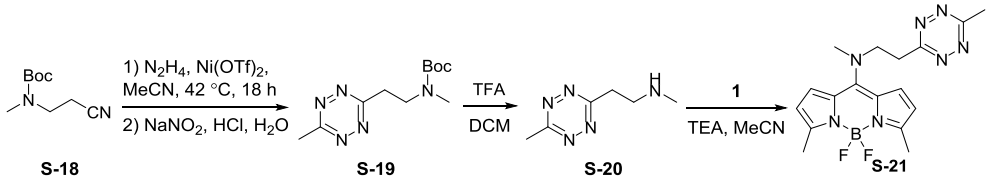


Scheme S7. Synthesis of BODIPY **14**.

Synthesis of compound S-16: Compound **S-15** was synthesized according to reference[7]. To a solution of compound **S-15** (180 mg, 0.6 mmol) in DCM (8.0 mL) was added trifluoroacetic acid (8.0 mL) in an ice bath. The reaction mixture was stirred for 0.5 h at room temperature. After reaction completion, the reaction was quenched by adding a saturated sodium bicarbonate solution. The aqueous phase was extracted with DCM (30 mL×1). The organic phase was washed with brine, dried with anhydrous Na_2SO_4, filtered and concentrated. The residues were purified by silica column chromatography (DCM:MeOH = 10:1) to afford **S-16** as a red liquid (89.0 mg), yield: 74%.

Synthesis of compound S-17: Following the general procedure B, starting material **1** (144.0 mg, 0.5 mmol, 1.0 eq.), tetrazine **S-16** (120.0 mg, 0.6 mmol, 1.2 eq.), TEA (90 μL, 0.65 mmol, 1.3 eq.) and DCM (5.0 mL) were used. Compound **S-17** was purified by silica column chromatography (PE:EA = 2:1) as a yellow solid (108.0 mg), yield: 47%. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 8.65 (d, *J* = 8.3 Hz, 2H), 7.59 (d, *J* = 8.3 Hz, 2H), 6.92 (d, *J* = 3.9 Hz, 2H), 6.32 (t, *J* = 5.3 Hz, 1H), 6.19 (d, *J* = 3.9 Hz, 2H), 4.93 (d, *J* = 5.6 Hz, 2H), 3.12 (s, 3H), 2.58 (s, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 167.7, 163.7, 148.6, 145.8, 140.1, 132.5, 129.1, 128.5, 123.3, 119.5, 115.6, 51.1, 21.4, 14.3. **HRMS** $[M+Na]^+$ *m/z* calcd. for $[C_{21}H_{20}BF_2N_7Na]^+$ 442.1734, found 442.1739.

Synthesis of BODIPY 14: Following the general procedure C, starting material **S-17** (100.0 mg, 0.24 mmol, 1.0 eq.), DIPEA (119 μL, 0.72 mmol, 3.0 eq.), acetyl chloride (34 μL, 0.48 mmol, 2.0 eq.) and DCM (0.24 mL) were used. Compound **14** was purified by silica column chromatography (PE:EA = 2:1) as a red solid (101.0 mg), yield: 87%. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 8.49 (d, *J* = 7.9 Hz, 2H), 7.53 (d, *J* = 8.0 Hz, 2H), 6.57 (s, 2H), 6.19 (s, 2H), 5.00 (s, 2H), 3.09 (s, 3H), 2.60 (s, 6H), 2.04 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 170.2, 167.5, 164.0, 160.2, 141.4, 138.8, 133.7, 131.7, 130.8, 128.2, 127.6, 120.8, 54.3, 22.5, 21.3, 15.2. **HRMS** $[M+Na]^+$ *m/z* calcd. for $[C_{23}H_{22}BF_2N_7NaO]^+$ 484.1839, found 484.1842.



Scheme S8. Synthesis of BODIPY **S-21**.

Synthesis of compound S-19: A solution of *tert*-butyl (2-cyanoethyl)(methyl) carbamate **S-18** (800.0 mg, 4.4 mmol, 1.0 eq.), anhydrous acetonitrile (1.6 g, 30.0 mmol, 6.8 eq.) and nickel trifluoromethane sulfonate (776.0 mg, 2.2 mmol, 0.5 eq.) was cooled to 0 °C under argon atmosphere. To this mixture was added hydrazine hydrate (5.0 mL, 157.0 mmol, 35.6 eq.) dropwise at the same temperature. Upon completion of addition, the reaction mixture was stirred at 42 °C for 18 h before being cooled to room temperature. The reaction solution was poured into sodium nitrite (6.1 g, 87.0 mmol, 20.0 eq.) ice-water solution, stirred at 0 °C. Addition of 1 M HCl continued until gas evolution ceased and the pH value was 3–4, and then continued stirring for 30 min. The aqueous phase was extracted with DCM (50 mL×3). The organic phase was washed with brine, dried with anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (PE:EA = 2:1) to afford **S-19** as a red liquid (150.0 mg), yield: 14.0%. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 3.76 (d, J = 21.4 Hz, 2H), 3.50 (t, J = 6.6 Hz, 2H), 3.03 (s, 3H), 2.91 (s, 3H), 1.45 (s, 9H). **HRMS** $[M+H]^+$ *m/z* calcd. for $[C_{11}H_{20}N_5O_2]^+$ 254.1612, found 254.1610.

Synthesis of compound S-20: To a solution of compound **S-19** (130.0 mg, 0.5 mmol, 1.0 eq.) in DCM (6.5 mL) was added trifluoroacetic acid (6.5 mL) in an ice bath. The reaction mixture was stirred for 0.5 h at room temperature. After reaction completion, the reaction was quenched with saturated sodium bicarbonate solution. The aqueous phase was extracted with DCM (30 mL×1). The organic phase was washed with brine, dried with anhydrous Na_2SO_4, filtered and concentrated. The residues were purified by silica gel column chromatography (DCM:MeOH = 10:1) to afford **S-20** as a red liquid (50.0 mg), yield: 64%. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 3.84 (t, J = 6.5 Hz, 2H), 3.64 (q, J = 6.9 Hz, 2H), 3.05 (s, 3H), 2.75 (s, 3H).

Synthesis of BODIPY S-21: Following the general procedure B, starting material **1** (10.4 mg, 0.04 mmol, 1.0 eq.), tetrazine **S-20** (5.0 mg, 0.03 mmol, 0.75 eq.), TEA (7 μL, 0.05 mmol, 1.2 eq.) and DCM (0.4 mL) were used. Compound **S-21** was purified by silica column chromatography (PE:EA = 2:1) as a yellow solid (5.5 mg), yield: 45%. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 6.98 (d, J = 3.8 Hz, 2H), 6.20 (d, J = 3.8 Hz, 2H), 4.52 (t, J = 7.1 Hz, 2H), 3.78 (t, J = 7.0 Hz, 2H), 3.62 (s, 3H), 3.00 (s, 3H),

2.54 (*s*, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$): δ 168.2, 167.1, 151.4, 148.7, 126.1, 124.1, 115.9, 56.8, 45.0, 33.3, 21.3, 14.5. **HRMS** $[M+Na]^+$ *m/z* calcd. for $[C_{17}H_{20}BF_2N_7Na]^+$ 394.1734, found 394.1732.

3. Photophysical Properties of BODIPYs

Table S1. Photophysical properties of probes **2-11** in different solvents.

Probes	λ_{ab} (nm)	λ_{em} (nm)	Quantum yield (Φ_F)	ε ($\times 10^4$ M^{-1} cm^{-1})	Stokes shift ($\Delta\lambda$/nm)	Solvent[a]
2	521	530	0.99	7.4	9	PBS/DMSO
	521	531	0.96	8.5	10	DMSO
	520	529	0.93	8.9	9	EtOH
3	525	536	0.71	4.4	11	PBS/DMSO
	525	538	0.51	7.7	13	DMSO
	523	534	0.97	7.9	11	EtOH
4	521	532	0.92	4.8	11	PBS
	522	532	0.95	6.4	10	DMSO
5	523	534	0.84	5.5	11	PBS
	523	533	0.59	5.8	10	DMSO
	521	530	0.81	6.9	9	EtOH
7	680	705	0.28	9.8	25	PBS/DMSO
	678	711	0.50	12.4	33	DMSO
	673	693	0.39	16.2	20	EtOH
8	773	902	ND	7.2	129	PBS/DMSO
	765	873	ND	7.7	108	DMSO
	752	851	ND	8.7	99	EtOH
9	689	722	0.19	8.2	33	PBS/DMSO
	689	719	0.33	7.8	20	DMSO
	679	710	0.32	7.8	31	EtOH
10	691	721	0.17	7.3	30	PBS/DMSO
	688	721	0.34	8.5	33	DMSO
	677	711	0.35	8.7	34	EtOH
11	504	512	0.99	7.65	8	PBS/DMSO

[a] "PBS/DMSO" refers to 1:1 (v/v) mixture; ND: not detected.

Stock solutions of probes **2**–**11** were prepared in DMSO and diluted to the corresponding concentration with the appropriate solvents. For UV-Vis spectrophotometric measurements, probe solutions were prepared by directly diluting the stock solution in PBS, PBS/DMSO (1:1 v/v), DMSO, or EtOH to a final concentration of 10 μM. UV absorption was then measured using a Quawell DNA/Protein analyzer 6000+ equipped with a cuvette. For the fluorescence measurements, each stock solution was diluted in PBS, PBS/DMSO (1:1 v/v), DMSO or EtOH to a final concentration of 1 μM.

4. Computational Data for the fluorescent dyes S-22 and 7

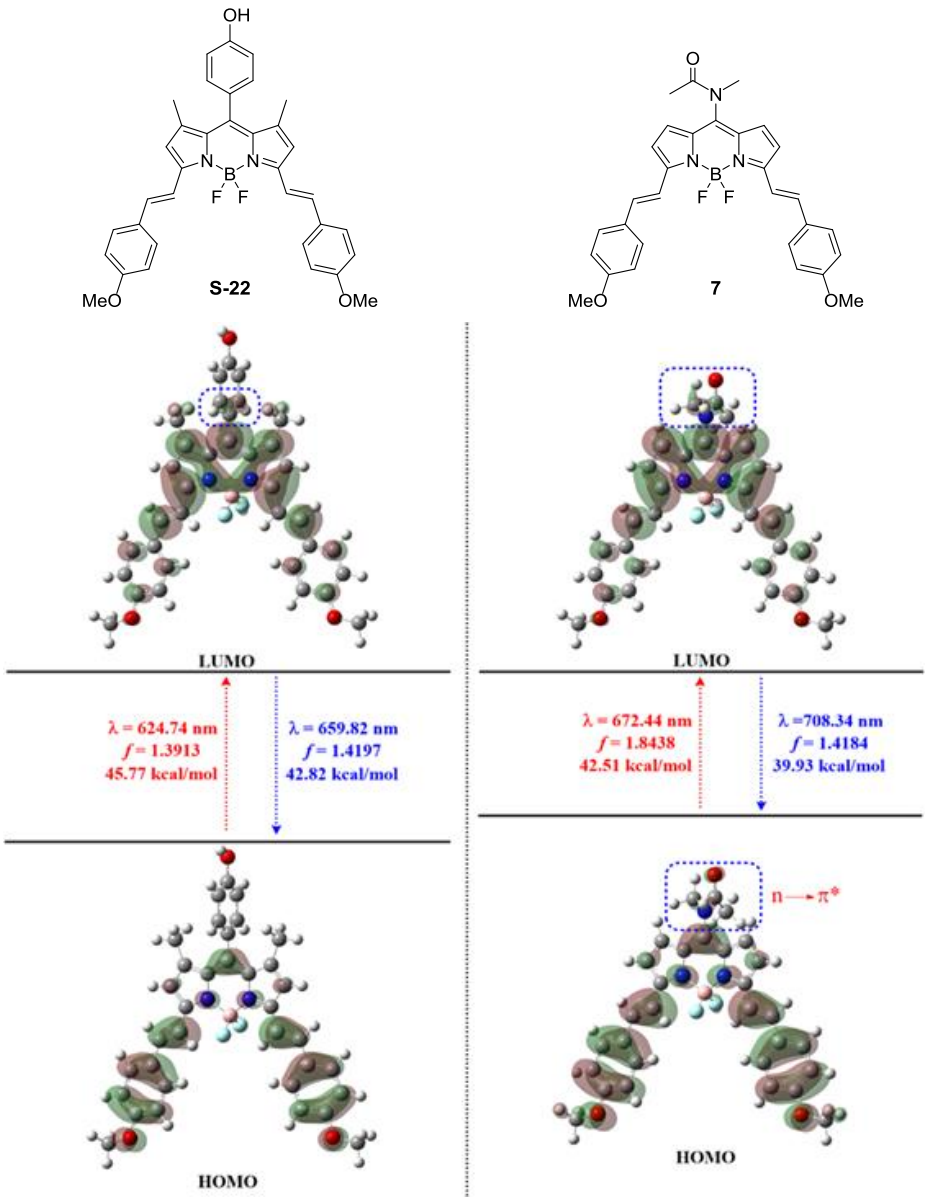


Figure S1. The HOMO/LUMO transition diagram of the fluorescent dyes **S-22** and **7**.

Computational Method

All the calculations were carried out in Gaussian 16 package[8] by employing the density-functional theory (DFT) method and time-dependent density-functional theory (TD-DFT) method[9]. For geometry optimization, all the structures including ground states and first excited states were performed at CAM-B3LYP method[10] with 6-31G (d, p) basis set[11]. For vibration frequency calculation, the same computational method with 6-31G (d, p) basis set[11] was carried out to make sure that the optimized structures were true energy minima. All the calculations considered the solvation effect (CH_3CN solvent) provided by the SMD solvent continuum models[12].

All geometry optimization and vibration frequency calculations were performed using the Gaussian09 software package.[8] The LUMOs of the fluorescent dyes **S-22** and **7** were similar, whereas significant differences were observed between the HOMOs of the two BODIPYs (Figure S1), indicating that the lone-pair electrons on the N and O atoms in the *N*-acetyl group of BODIPY **7** participate in the formation of the HOMO and increase its energy. Moreover, the energy difference between the ground and first excited states of **S-22** and **7** (45.77 *vs* 42.51 kcal/mol and 42.82 *vs* 39.93 kcal/mol) indicated that the energy difference between HOMO and LUMO was smaller in **7** than in **S-22**. This smaller energy difference was reflected as a red shift.

Table S2. HOMO/LUMO Orbits energies (unit: hartree, 1 hartree = 627.5 kcal/mol) of **S-22** and **7**.

Orbits	**S-22**	**7**
LUMO	-0.06116	-0.06134
HOMO	-0.21694	-0.21004

Table S3. Electron energies (unit: hartree, 1 hartree = 627.5 kcal/mol) of ground states and first excited states of **S-22**.

Orbits	**Ground State**	**First excited state**	**Difference (hartree)**	**Difference (kcal/mol)**
Vertical absorption	-1911.39087986	-1911.31794833	0.07293153	45.77
Vertical emission	-1911.38824459	-1911.32001109	0.06823350	42.82

Table S4. Electron energies (unit: hartree, 1 hartree = 627.5 kcal/mol) of ground states and first excited states of **7**.

Orbits	**Ground State**	**First excited state**	**Difference (hartree)**	**Difference (kcal/mol)**
Vertical absorption	-1773.88166556	-1773.81390716	0.06775840	42.51
Vertical emission	-1773.87963147	-1773.81599602	0.06363545	39.93

Molecular Coordinates (Cartesian coordinates)

S-22 (first excited state)

C	-2.584172	1.214082	0.036122
C	-3.301502	0.000002	0.021180
C	-2.584173	-1.214078	0.036129
N	-1.203974	-1.231877	0.014935
B	-0.325171	0.000000	-0.241983
F	0.779070	-0.000001	0.626554
F	0.153835	-0.000001	-1.559456
N	-1.203972	1.231879	0.014933
C	-0.772013	-2.538648	0.050500
C	0.594470	-2.903435	0.052276
C	1.038019	-4.196082	0.013933
C	-1.923796	-3.371503	0.100814
C	-3.047090	-2.585267	0.094167
C	-4.450211	-3.096680	0.155009
C	-0.772010	2.538650	0.050488
C	0.594473	2.903435	0.052267
C	1.038025	4.196081	0.013920
C	-1.923792	3.371507	0.100793
C	-3.047087	2.585272	0.094148
C	-4.450208	3.096689	0.154979
C	-7.596388	0.000003	-0.039887
O	-8.951515	0.000002	-0.119819
C	-6.878670	-0.000015	-1.235482
C	-5.491433	-0.000016	-1.205077
C	-4.791847	0.000002	0.005137
C	-5.527616	0.000019	1.190971
C	-6.917252	0.000019	1.177381
C	5.058489	5.600942	0.030675
O	6.357713	5.962246	0.037495
C	6.673705	7.348465	0.000681
C	4.806360	4.220035	0.065630
C	3.514523	3.746064	0.061437
C	2.411535	4.629710	0.021840
C	2.691186	6.007193	-0.013571
C	3.987247	6.497383	-0.008751
C	5.058482	-5.600947	0.030666
O	6.357706	-5.962253	0.037478
C	6.673695	-7.348473	0.000667
C	4.806355	-4.220041	0.065618
C	3.514519	-3.746068	0.061433
C	2.411530	-4.629712	0.021846
C	2.691178	-6.007196	-0.013561
C	3.987239	-6.497388	-0.008748
H	1.310516	-2.091812	0.098375
H	0.302539	-4.996340	-0.030330
H	-1.909458	-4.451592	0.156587
H	-5.044116	-2.774577	-0.704590
H	-4.975139	-2.752140	1.050314
H	-4.439003	-4.189184	0.169219
H	1.310519	2.091812	0.098375
H	0.302546	4.996340	-0.030351
H	-1.909453	4.451596	0.156558
H	-4.975131	2.752179	1.050299
H	-5.044117	2.774557	-0.704606
H	-4.438999	4.189193	0.169154
H	-9.326879	0.000013	0.773919

H	-7.418584	-0.000028	-2.176418
H	-4.939328	-0.000029	-2.139945
H	-5.006562	0.000033	2.143460
H	-7.476520	0.000032	2.109065
H	7.761732	7.408957	0.011911
H	6.272862	7.871524	0.874822
H	6.294137	7.819274	-0.911783
H	5.651546	3.540560	0.094883
H	3.351157	2.674137	0.087143
H	1.862912	6.709279	-0.044952
H	4.156008	7.566387	-0.035576
H	7.761723	-7.408967	0.011890
H	6.294121	-7.819285	-0.911794
H	6.272857	-7.871529	0.874812
H	5.651542	-3.540566	0.094863
H	3.351154	-2.674140	0.087137
H	1.862903	-6.709281	-0.044934
H	4.155997	-7.566391	-0.035571

7 (ground state)

C	1.210160	3.239343	-0.225463
C	-0.000670	3.926260	-0.232310
C	-1.211240	3.238895	-0.225483
N	-1.247779	1.851897	-0.181727
B	-0.000117	0.959805	0.058568
F	-0.000023	0.481580	1.366257
F	0.000078	-0.115256	-0.831457
N	1.247212	1.852359	-0.181736
C	-2.544425	1.463383	-0.214454
C	-2.935496	0.072688	-0.192595
C	-4.212969	-0.329970	-0.059009
C	-3.366158	2.619139	-0.286083
C	-2.538671	3.718454	-0.290416
C	2.544005	1.464328	-0.214410
C	2.935593	0.073776	-0.192547
C	4.213212	-0.328403	-0.058918
C	3.365309	2.620389	-0.285988
C	2.537411	3.719398	-0.290333
C	5.746403	-4.315348	0.030583
O	6.148259	-5.605914	0.049532
C	7.533514	-5.875969	0.215040
C	4.369089	-4.106022	-0.128038
C	3.856813	-2.826293	-0.159535
C	4.696978	-1.704541	-0.034585
C	6.066943	-1.935774	0.122290
C	6.599255	-3.219084	0.156029
C	-5.744621	-4.317503	0.030513
O	-6.145980	-5.608224	0.049474
C	-7.531128	-5.878812	0.215005
C	-4.367386	-4.107647	-0.128086
C	-3.855605	-2.827721	-0.159591
C	-4.696208	-1.706292	-0.034673
C	-6.066087	-1.938053	0.122177
C	-6.597902	-3.221569	0.155924
N	-0.000923	5.342609	-0.334072
C	-0.000894	5.900685	-1.684047
C	-0.001539	6.176171	0.761519
C	-0.002044	5.516232	2.117496
O	-0.002322	7.392722	0.631276

H	-2.135575	-0.650498	-0.298100
H	-4.989673	0.424187	0.049083
H	-4.444781	2.615211	-0.346599
H	-2.822079	4.760020	-0.349276
H	2.135944	-0.649705	-0.298088
H	4.989628	0.426046	0.049212
H	4.443936	2.616867	-0.346456
H	2.820431	4.761071	-0.349155
H	7.632463	-6.961371	0.202322
H	7.904240	-5.490544	1.170466
H	8.124446	-5.450163	-0.602444
H	3.720708	-4.970450	-0.223870
H	2.786986	-2.694715	-0.282531
H	6.736945	-1.086452	0.221011
H	7.666420	-3.352835	0.279138
H	-7.629655	-6.964252	0.202324
H	-8.122234	-5.453263	-0.602487
H	-7.901992	-5.493499	1.170422
H	-3.718669	-4.971826	-0.223894
H	-2.785828	-2.695726	-0.282566
H	-6.736420	-1.088988	0.220871
H	-7.665017	-3.355734	0.279011
H	-0.890879	5.579325	-2.231162
H	0.888911	5.578938	-2.231213
H	-0.000667	6.984765	-1.591438
H	-0.002357	6.298591	2.875028
H	-0.884982	4.885755	2.247042
H	0.880775	4.885744	2.247734

7 (first excited state)

C	1.210103	3.231327	-0.223200
C	-0.000273	3.940563	-0.230255
C	-1.210547	3.231150	-0.223225
N	-1.239080	1.857905	-0.142768
B	-0.000060	0.984475	0.147780
F	-0.000033	0.563865	1.481900
F	0.000027	-0.148222	-0.676952
N	1.238835	1.858085	-0.142759
C	-2.555966	1.449396	-0.172240
C	-2.939694	0.089682	-0.127217
C	-4.239004	-0.334739	-0.083702
C	-3.373896	2.615701	-0.277550
C	-2.551999	3.711597	-0.309129
C	2.555783	1.449770	-0.172203
C	2.939711	0.090113	-0.127179
C	4.239084	-0.334112	-0.083653
C	3.373543	2.616196	-0.277482
C	2.551486	3.711972	-0.309069
C	5.702729	-4.328339	0.029880
O	6.083255	-5.619901	0.063319
C	7.474400	-5.915864	0.108541
C	4.317392	-4.099145	-0.013057
C	3.824049	-2.815848	-0.050177
C	4.691852	-1.699230	-0.046259
C	6.074033	-1.955681	-0.001647
C	6.583640	-3.242888	0.035657
C	-5.702014	-4.329199	0.029843
O	-6.082331	-5.620823	0.063292
C	-7.473426	-5.917007	0.108618

C	-4.316716	-4.099784	-0.013180
C	-3.823579	-2.816410	-0.050310
C	-4.691558	-1.699928	-0.046316
C	-6.073696	-1.956599	-0.001630
C	-6.583097	-3.243888	0.035687
N	-0.000366	5.352864	-0.370577
C	-0.000377	5.887207	-1.728286
C	-0.000684	6.208753	0.702776
C	-0.000661	5.574263	2.072745
O	-0.000927	7.425471	0.555532
H	-2.136225	-0.637521	-0.139494
H	-5.028853	0.413021	-0.071550
H	-4.453106	2.611281	-0.341200
H	-2.832704	4.751482	-0.397138
H	2.136350	-0.637211	-0.139472
H	5.028820	0.413767	-0.071508
H	4.452756	2.611938	-0.341104
H	2.832037	4.751900	-0.397055
H	7.549092	-7.002806	0.127529
H	7.939070	-5.504483	1.010110
H	7.991316	-5.532228	-0.776688
H	3.651567	-4.955509	-0.015900
H	2.749943	-2.669184	-0.081887
H	6.762612	-1.115693	0.003322
H	7.654833	-3.395237	0.069157
H	-7.547944	-7.003960	0.127616
H	-7.990469	-5.533456	-0.776575
H	-7.938094	-5.505696	1.010220
H	-3.650754	-4.956042	-0.016081
H	-2.749498	-2.669574	-0.082093
H	-6.762409	-1.116721	0.003393
H	-7.654264	-3.396406	0.069251
H	-0.889378	5.552348	-2.270401
H	0.888306	5.551796	-2.270568
H	-0.000048	6.973720	-1.667096
H	-0.000656	6.371831	2.814736
H	-0.883128	4.945691	2.215977
H	0.881780	4.945658	2.215971

5. Water solubility test

5.1 Calculation of water partition coefficient [9]

5.2 Solubility test

DMSO stock solutions of BODIPY **12** (10 μL, 10 mM) and probes **11** and **5** (2 μL, 50 mM) were diluted in 0.5 mL PBS (pH 7.4) in 1.5-mL Eppendorf tubes. The resulting solutions were centrifuged at 20000 g for 10 min to ensure that no free particles were suspended in the supernatant.

5.3 Solubility of probe 13 in PBS

Solution of probe **13** in DMSO (5 mM) was diluted in PBS (final concentration: 10, 20, 40, 50 and 60 μM). The solution was vortexed for 5 min, then centrifuged at 20000 g for 10 min. The supernate of **13** at separate concentrations was collected and the absorption of each collection at 525 nm was measured in three replicates.

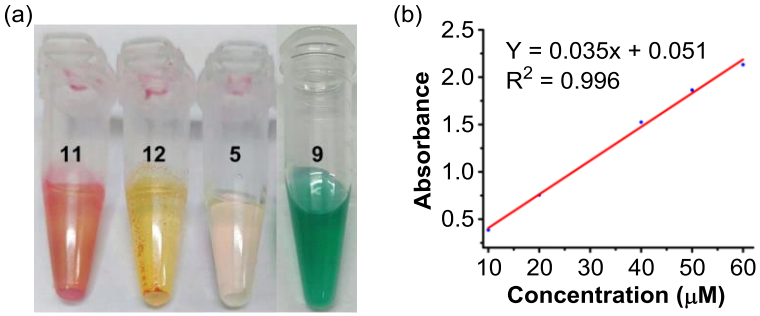


Figure S2. Solubility test. (**a**) From left to right, solutions of BODIPYs **11** (0.2 mM), **12** (0.2 mM), **5** (0.2 mM), and **9** (0.1 mM) in PBS (pH 7.4) under normal light. (**b**) The absorption of **13** in phosphate-buffered saline (PBS), as determined by the peak intensity at 525 nm over different concentrations. Probe **13** is completely dissolved in PBS with a concentration of upto 60 μM.

6. Bioorthogonal reactions of BODIPY-tetrazine probes

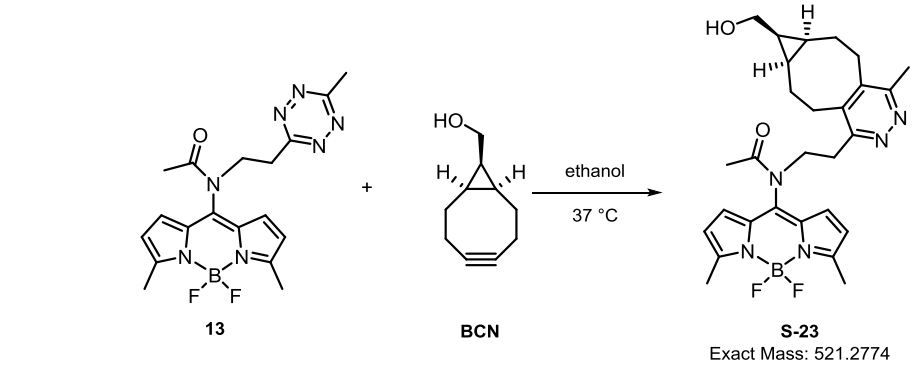
Stock solutions of BODIPY-tetrazine probes and dienophiles were prepared in DMSO (5 mM). The IEDDA reactions between BODIPY-tetrazine probes (1 mM) and dienophiles (5 mM) were performed in ethanol at 37 °C for 6 h. For UV-Vis spectrophotometric measurements, the reaction solutions were diluted in ethanol to a final concentration of 10 μM. For fluorescence measurements, the reaction solutions were diluted in ethanol to a final concentration of 1 μM.

Table S5. Photophysical properties of BODIPY-tetrazine probes before and after IEDDA reactions with different dienophiles in ethanol.

probes	dienophile	$\lambda_{ab}/\lambda_{em}$(nm)	stokes shift (nm)	turn-on ratio	ε ($M^{-1}cm^{-1}$)	Φ_F (%)
13	-	521/531	10	-	35500	0.6
	-[a]	525/532	7	-	44000	< 0.1
	BCN	521/529	8	62	30200	85
	TCO	520/530	10	65	28400	87
	TCO[a]	525/536	11	115	27700	82
	Sph	510/522	12	128	25200	84
14	-	521/532	11	-	45400	0.8
	-[a]	523/534	11	-	23400	< 0.1
	BCN	521/531	10	59	44100	91
	TCO	521/531	10	10	49400	28
	TCO[a]	523/534	11	12	19000	9.5
	Sph	522/531	9	28	39400	74
S-21	-	441/507	66	-	15400	0.5
	TCO	432/463	31	4	12400	1

[a] Photophysical properties in PBS

6.1 Bioorthogonal reaction of probe 13 with bicyclononyne (BCN)



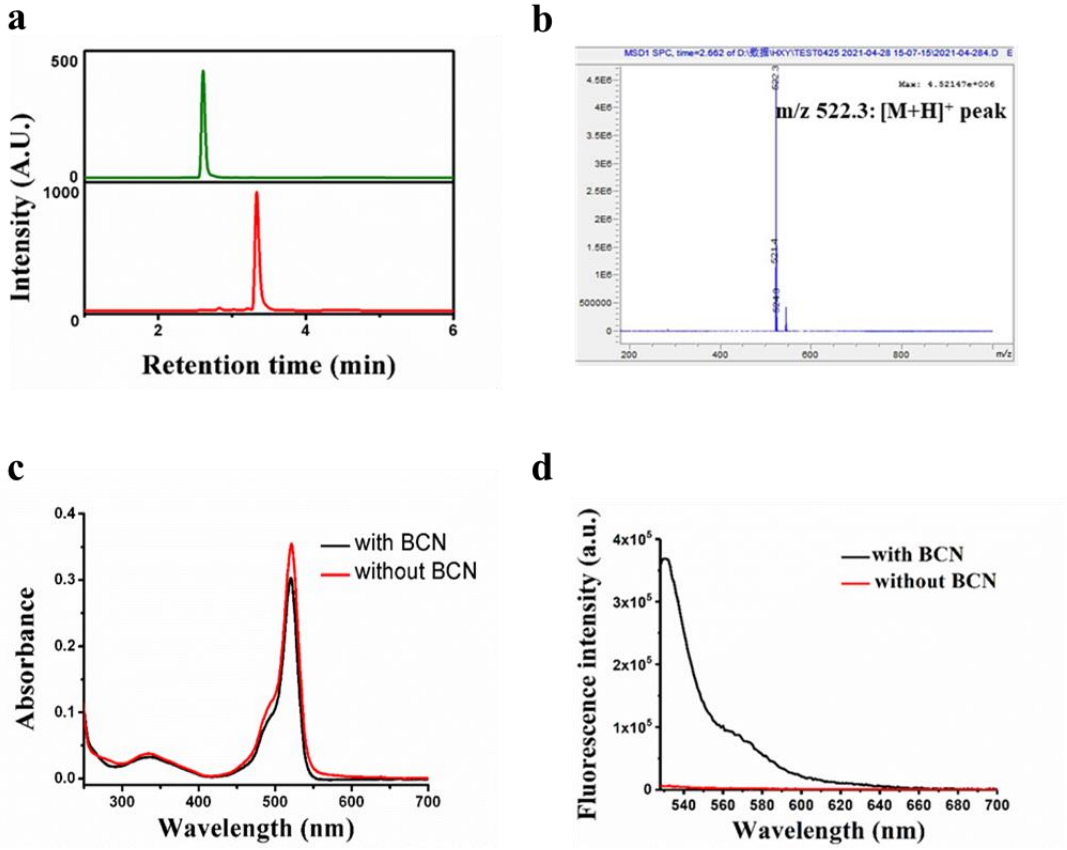
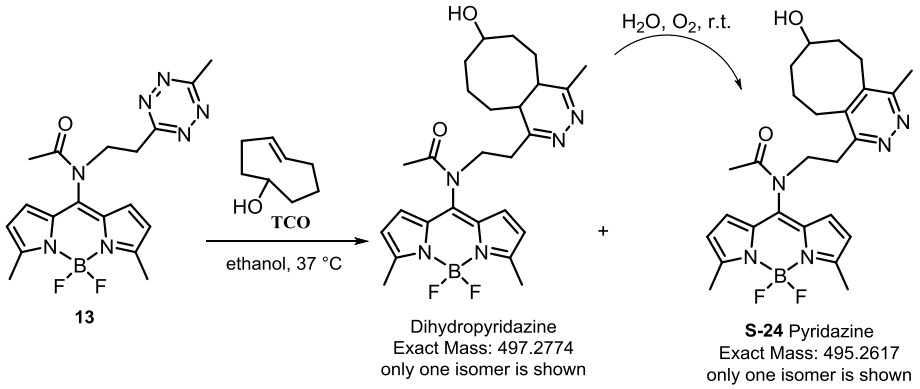
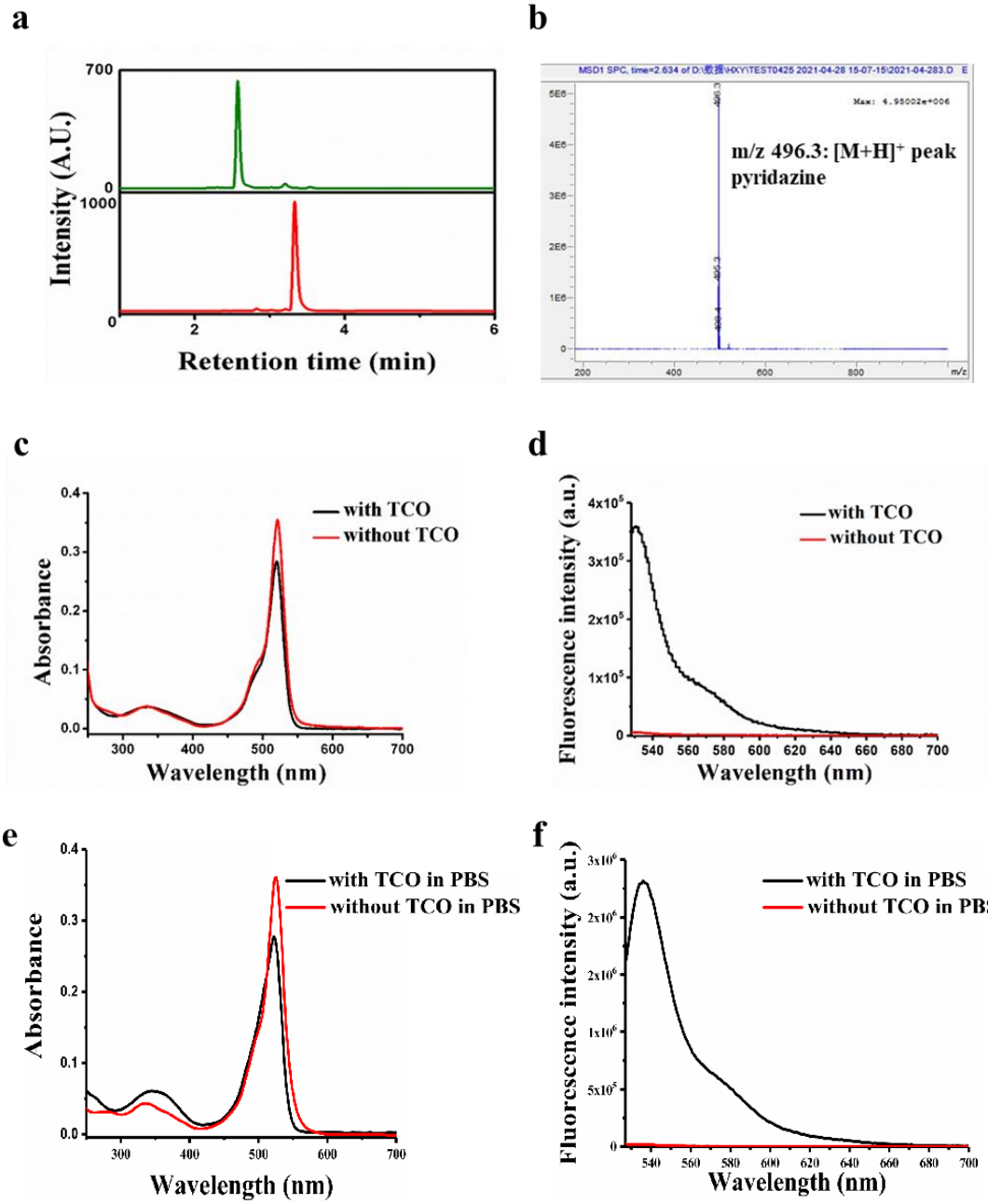


Figure S3. (a) HPLC traces of probe **13** (red) and the bioorthogonal product **S-23** (green) at 520 nm. (**b**) Mass traces of the reaction solution between **13** (1 mM) and BCN (5 mM) in EtOH after 6 h at 37 ℃. (**c**) Absorption spectra of **13** (10 μM) before (red) and after (black) reaction with BCN. (**d**) Fluorescence emission spectrum of **13** before (red) and after (black) reaction with BCN. Excitation wavelength, 521 nm; emission wavelength, 518–700 nm; slit: 1 nm.

6.2 Bioorthogonal reaction of probe 13 with trans-cyclooctene (TCO)





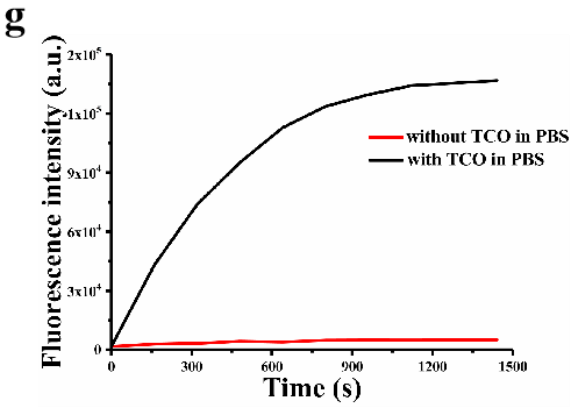
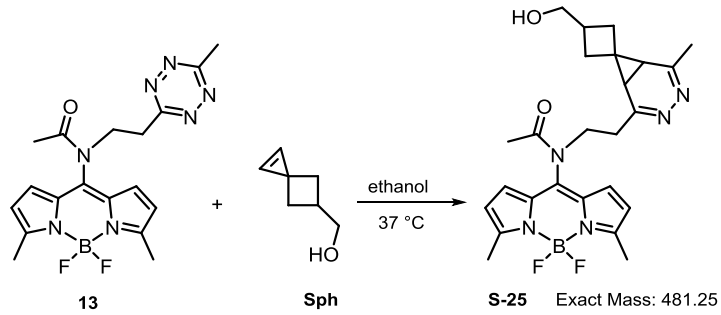


Figure S4. (**a**) HPLC traces of probe **13** (red) and the bioorthogonal product **S-24** (green) at 520 nm. (**b**) Mass traces of the reaction solution between **13** (1 mM) and TCO (5 mM) in EtOH after 6 h at 37 ℃. (**c**) Absorption spectra of **13** (10 μM) before (red) and after (black) reaction with TCO. (**d**) Fluorescence emission spectrum of **13** before (red) and after (black) reactih TCO. Excitation wavelength, 520 nm; emission wavelength, 528–700 nm; slit 1 nm. (**e**) Absorption spectra of **13** (10 μM) before (red) and after (black) reaction with TCO in PBS. (**f**) Fluorescence emission spectrum of **13** before (red) and after (black) reaction with TCO in PBS. Excitation wavelength, 525 nm; emission wavelength, 527–700 nm; slit 1.8 nm. (**g**) Time dependence of fluorescence intensity of probe **13** (red) and probe **13** with TCO (black) in PBS. Probe **13** (1 μM) mixed with TCO (20 μM) at 25 °C in PBS/DMSO (95:5, v/v). The reaction solution was measured by fluorometer. The fluorescence signal was collected at 25 °C by kinetics program over time (λ_{ex} = 525 nm, λ_{em} = 536 nm, slit 1.8 nm).

6.3 Bioorthogonal reaction of probe 13 with spiro[2.3]hex-1-ene (Sph)



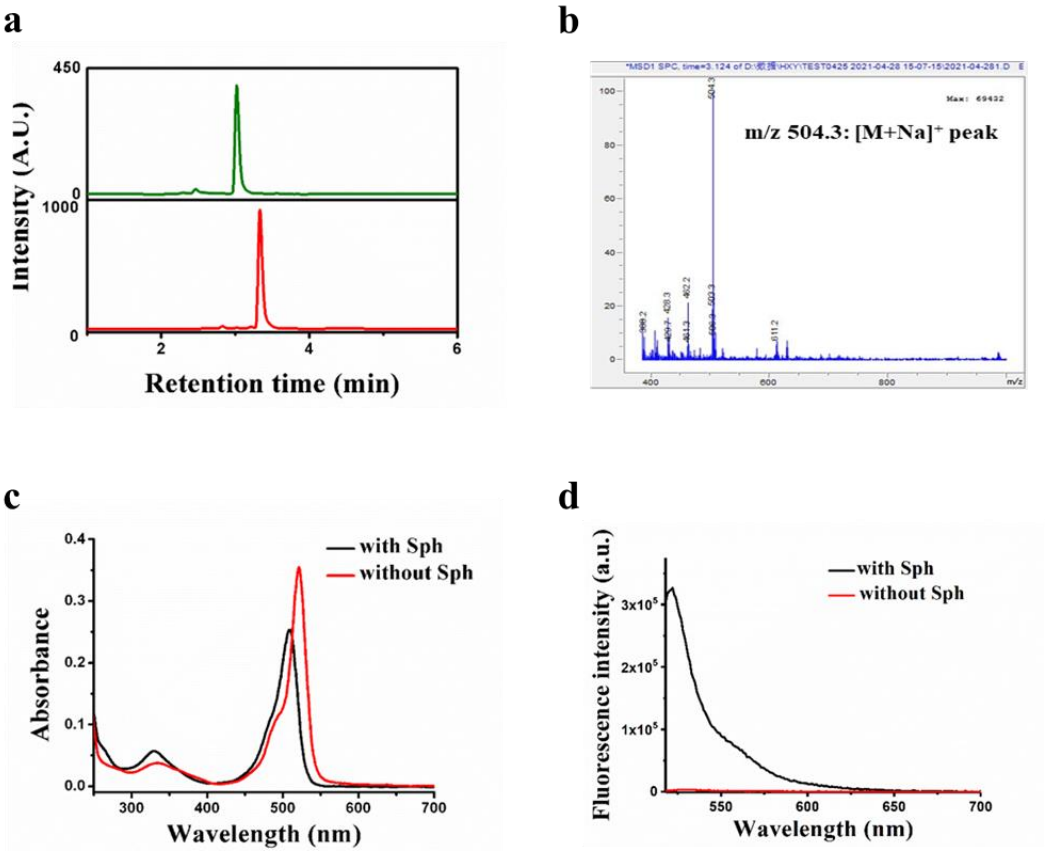
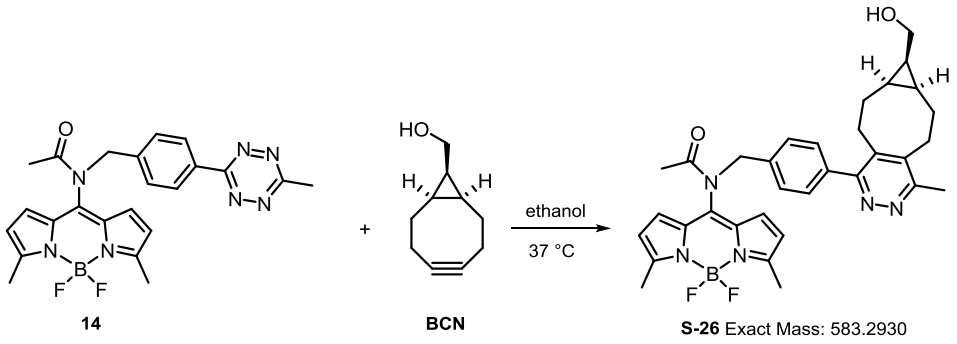


Figure S5. (**a**) HPLC traces of probe **13** (red) and the bioorthogonal product **S-25** (green) at 520 nm. (**b**) Mass traces of the reaction solution between **13** (1 mM) and Sph (5 mM) in EtOH after 6 h at 37 ℃. (**c**) Absorption spectra of **13** (10 μM) before (red) and after (black) reaction with Sph. (**d**) Fluorescence emission spectrum of **13** before (red) and after (black) reaction with Sph. Excitation wavelength, 510 nm; emission wavelength, 518–700 nm; slit: 1 nm.

6.4 Bioorthogonal reaction of probe 14 with bicyclononyne (BCN)



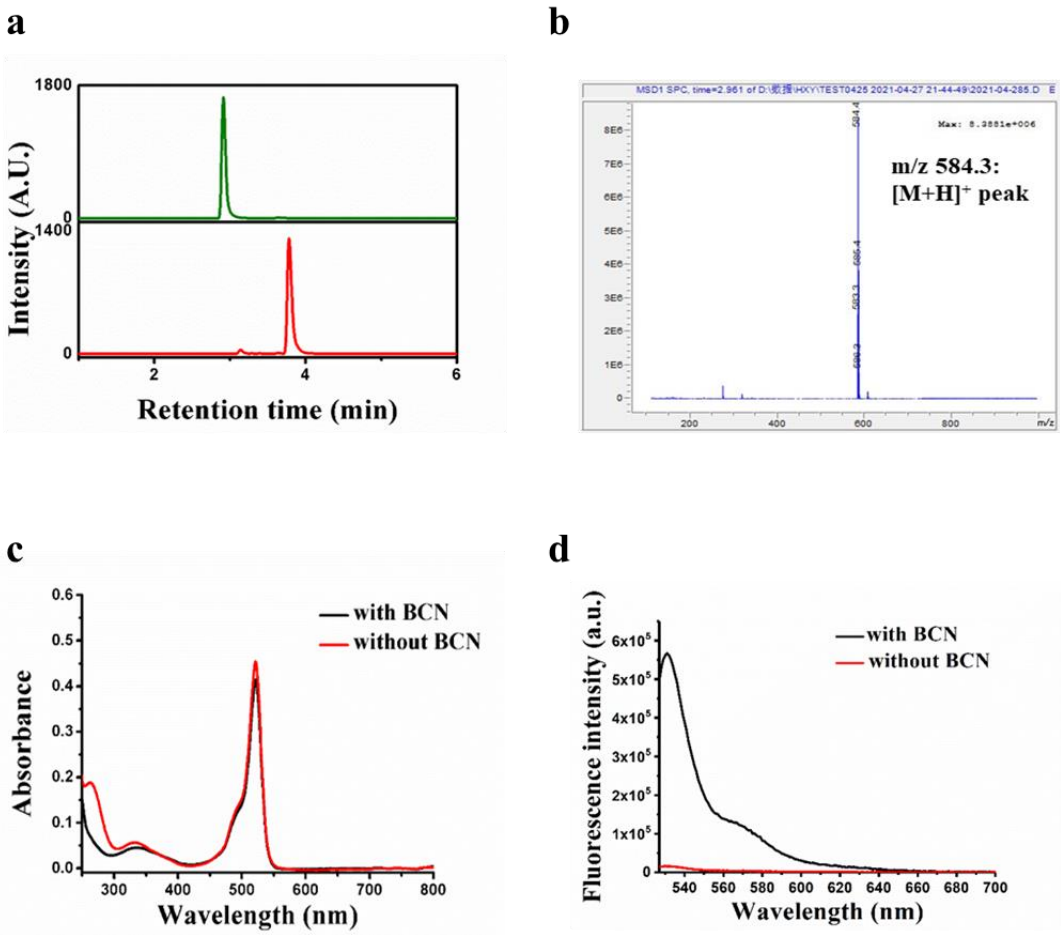
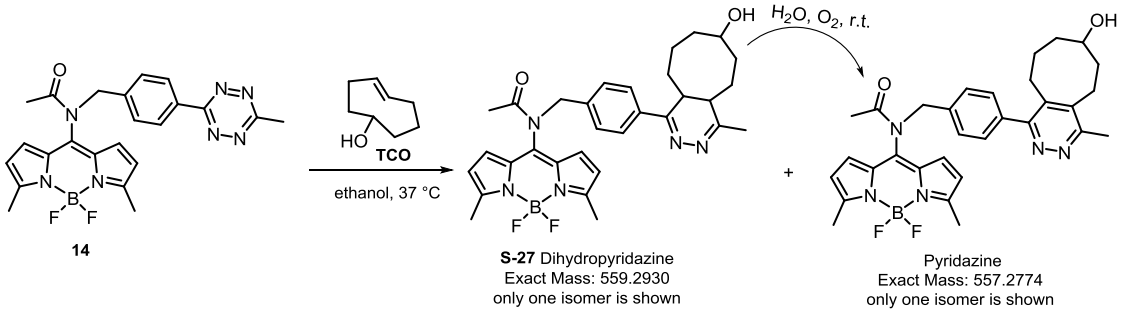


Figure S6. (**a**) HPLC traces of probe **14** (red) and the bioorthogonal product **S-26** (green) at 520 nm. (**b**) Mass traces of the reaction solution between **14** (1 mM) and BCN (5 mM) in EtOH after 6 h at 37 ℃. (**c**) Absorption spectra of **14** (10 μM) before (red) and after (black) reaction with BCN. (**d**) Fluorescence emission spectrum of **14** before (red) and after (black) reaction with BCN. Excitation wavelength, 521 nm; emission wavelength, 527–700 nm; slit: 1 nm.

6.5 Bioorthogonal reaction of probe 14 with trans-cyclooctene (TCO)



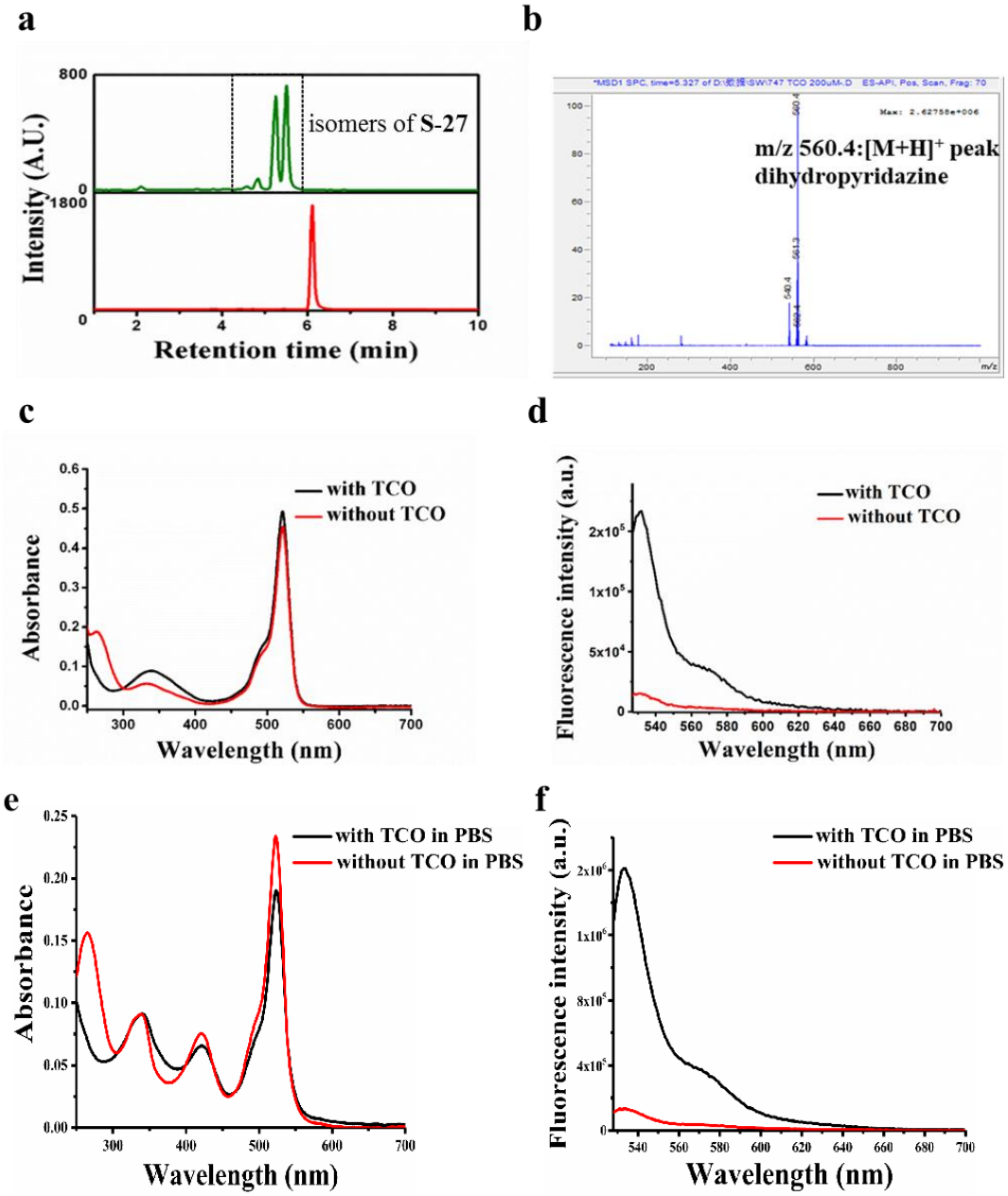
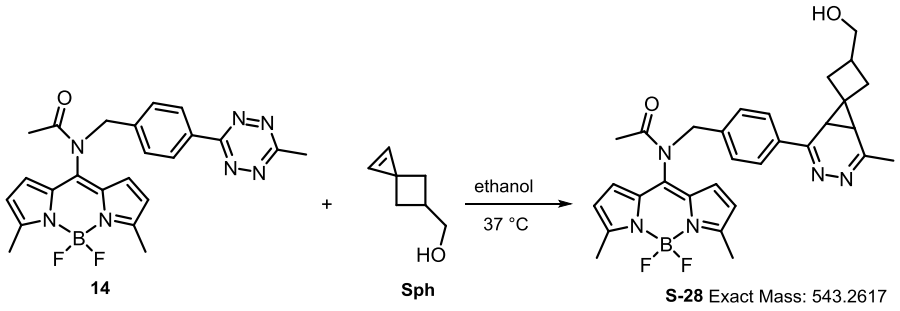
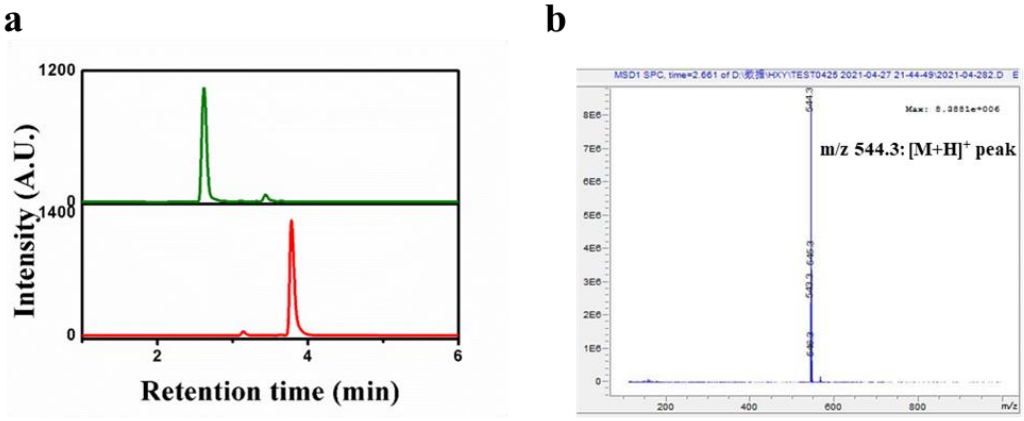


Figure S7. (**a**) HPLC traces of probe **14** (red) and the bioorthogonal product **S-27** (green) at 520 nm. (**b**) Mass traces of the reaction solution between **14** (1 mM) and TCO (5 mM) in EtOH after 6 h at 37 ℃. (**c**) Absorption spectra of **14** (10 μM) before (red) and after (black) reaction with TCO. (**d**) Fluorescence emission spectrum of **14** before (red) and after (black) reaction with TCO. Excitation

wavelength, 521 nm; emission wavelength, 527–700 nm; slit: 1 nm. (**e**) Absorption spectra of **14** (10 μM) before (red) and after (black) reaction with TCO in PBS. (**f**) Fluorescence emission spectrum of **14** before (red) and after (black) reaction with TCO in PBS. Excitation wavelength, 523 nm; emission wavelength, 528–700 nm; slit: 2.5 nm.

6.6 Bioorthogonal reaction of probe 14 with spiro[2.3]hex-1-ene (Sph)





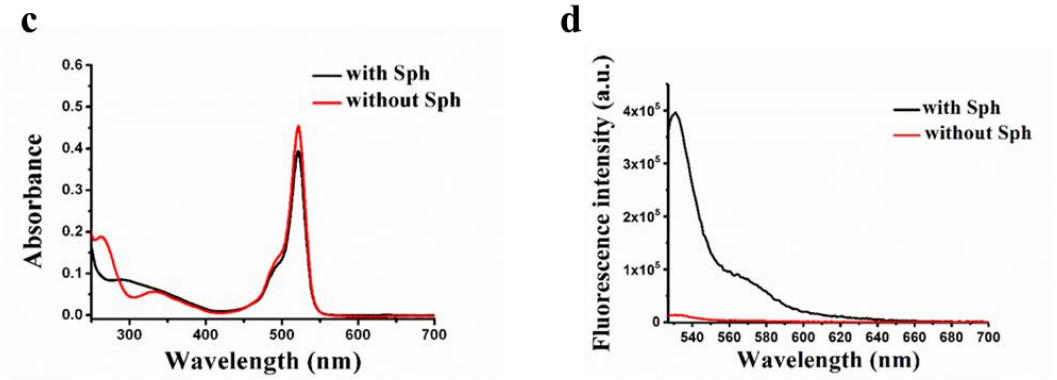


Figure S8. (**a**) HPLC traces of probe **14** (red) and the bioorthogonal product **S-28** (green) at 520 nm. (**b**) Mass traces of the reaction solution between **14** (1 mM) and Sph (5 mM) in EtOH after 6 h at 37 ℃. (**c**) Absorption spectra of **14** (10 μM) before (red) and after (black) reaction with Sph. (**d**) Fluorescence emission spectrum of **14** before (red) and after (black) reaction with Sph. Excitation wavelength, 522 nm; emission wavelength, 527–700 nm; slit 1 nm.

7. Bioorthogonal reaction kinetic measurements

The kinetics of the bioorthogonal reaction between probe **13** (final concentration: 1 μM) and TCO (final concentration: 10, 20, or 30 μM) in DMSO/PBS (1:9, v/v) at 37 ℃ were measured by evaluating the changes in the fluorescence intensity over time at 530 nm. Measurements started immediately after the addition of TCO and were performed using a FluoroMax-4 fluorometer (Horiba Jobin Yvon) equipped with a cuvette.

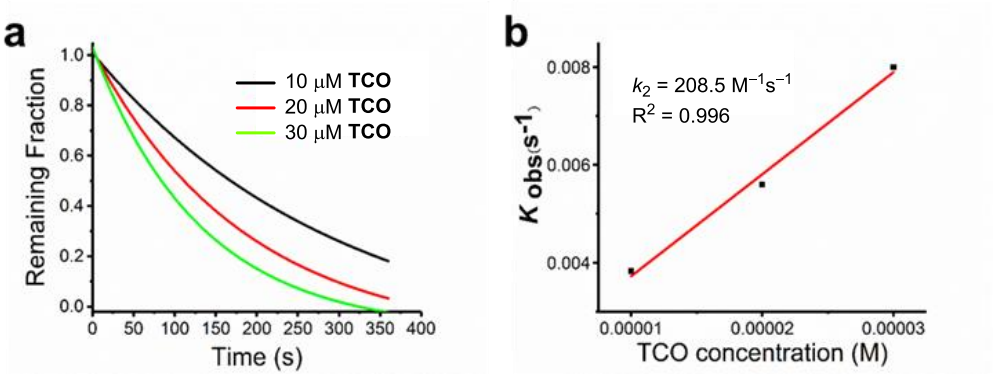


Figure S9. (**a**) Remaining fraction of probe **13** over time upon reaction with three different TCO concentrations in DMSO/PBS (1:9, v/v) at 37 ℃. (**b**) Linear plot of the pseudo-first-order kinetics used to calculate the second-order rate constant. Excitation wavelength, 520 nm; emission wavelength, 530 nm.

8. Stability test

8.1 Stability of probes (13, 14, and S-21) in cell culture medium

Solutions of probes **13**, **14,** and **S-21** in DMSO (5 mM, 2 μL) were diluted in a mixture (1 mL) of MeCN and 60% DMEM supplemented with 10% FBS and incubated at 37 °C. At selected time points, the samples (100 μL) were treated with an equal volume of cold MeCN (100 μL) to precipitate serum proteins. After centrifugation at 9000 g for 10 min, the supernatant was collected and used for further analysis. The decrease in the peak area of samples at 520 nm (probes **13** and **14**) and 455 nm (probe **S-21**) was monitored by LC-MS. Each stability test was performed in triplicate.

8.2 Stability of probe 13 in PBS

Solution of probe **13** in DMSO (5 mM, 2.4 μL) was diluted in PBS (197.6 μL) (final concentration: 60 μM) and incubated at 37 °C. The stability of probe **13** in PBS was performed and analyzed at different time points (0, 2, 6, 18 and 24 h). The decrease in the peak area of sample at 520 nm was monitored by LC-MS. Each stability test was performed in triplicate.

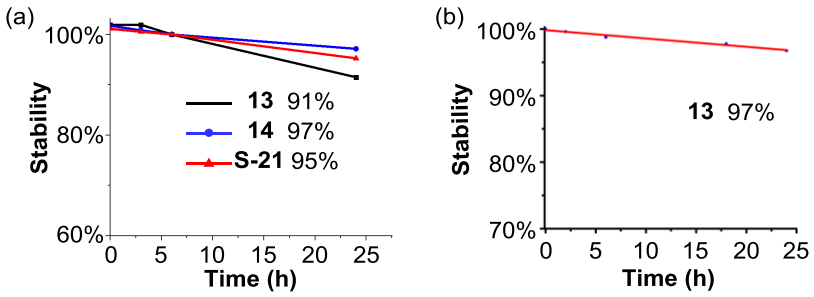


Figure S10. (**a**) Stability of probes **13**, **14**, and **S-21** in cell culture medium. (**b**) Stability of probe **13** in PBS.

9. Pretargeted cell imaging

9.1 Pretargeted cell imaging of SKOV3

SKOV3 ovarian cells were seeded in 35-mm glass-bottom dishes and cultured for 48 h. Cell nuclei were then stained with Hoechst 33342 (5 μM) in culture medium for 5 min, followed by treatment with Mito-Tracker red (100 nM) for 10 min. After washing with PBS, the cells were treated with **TCO–TPP** (10 μM) in culture medium for 30 min. After washing again with PBS, the cells were treated with probe **13** (0.5 μM) in 1X Cell Imaging Solution (Invitrogen) and imaged at 1 h post-treatment by confocal microscopy (Zeiss 880). Cells that were not treated with **TCO–TPP** served as the control.

9.2 Time-dependent fluorogenic cellular imaging

SKOV3 ovarian cells were seeded in flat-bottom 12-well plates dishes and cultured for 48 h. Cell nuclei were then stained with Hoechst 33342 (5 μM) in culture medium for 5 min, after washing cells with PBS, they were treated with TCO-TPP (10 μM) in culture medium for 30 min. After washing cells again with PBS, they were treated with probe **13** (0.5 μM) in 1X Cell Imaging Solution (Invitrogen) and imaged every 10 min by confocal microscopy (Zeiss 880) without washing.

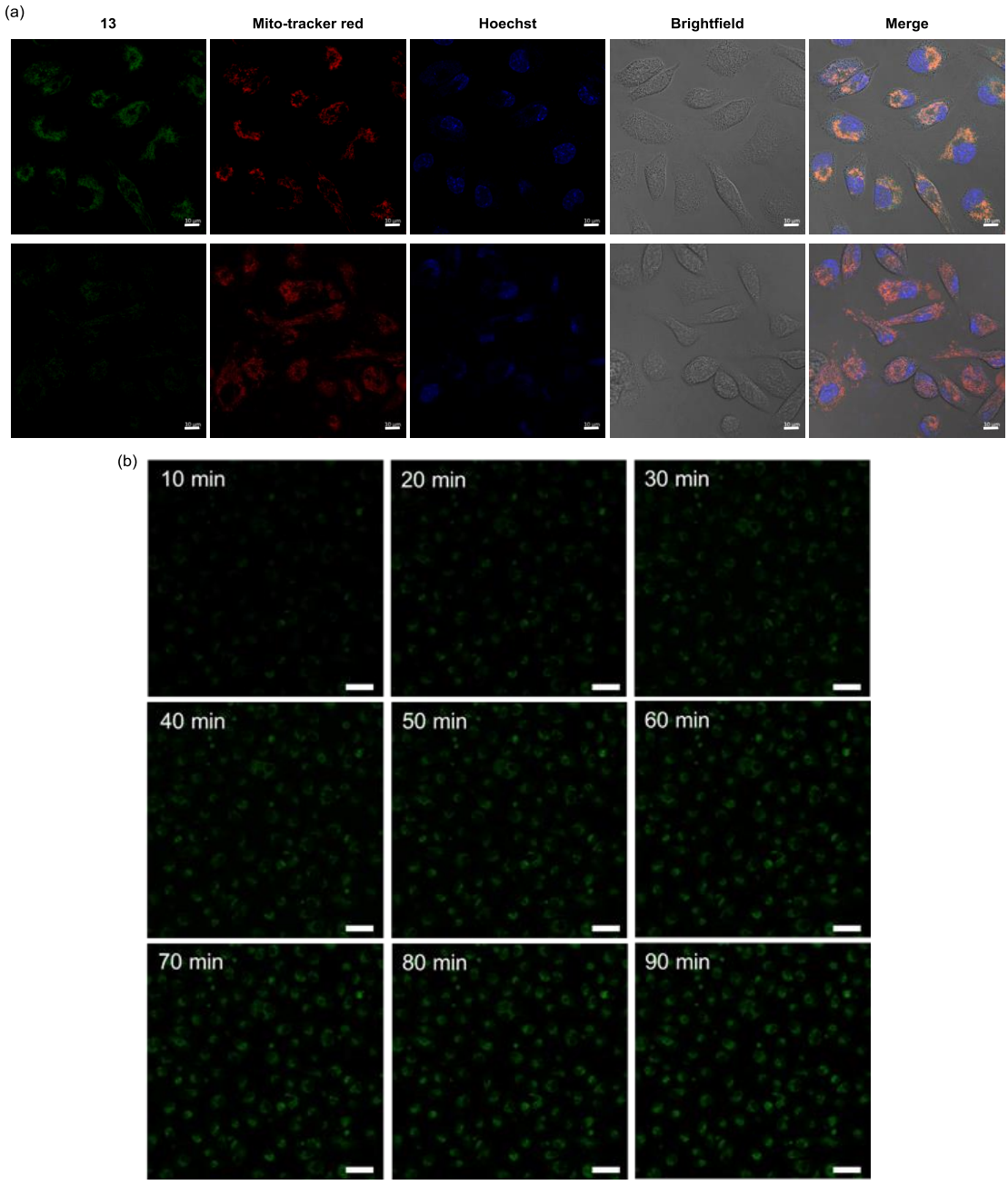


Figure S11. Pretargeted cell imaging of probe **13**. (**a**) Blue fluorescence signals were collected at 410–480 nm with excitation at 405 nm. Red fluorescence signals were collected at 600–700 nm with excitation at 594 nm. Green fluorescence signals were collected at 530–580 nm with excitation at 514 nm. Scale bar: 10 μm. (**b**) Time-dependent fluorogenic cellular imaging. Green fluorescence signals were collected at 530–600 nm with excitation at 514 nm. Scale bar: 100 μm.

10. Reference

[1] Yu Z.; Lin Q. Design of Spiro[2.3]hex-1-ene, a Genetically Encodable Double-Strained Alkene for Superfast Photoclick Chemistry. *J. Am. Chem. Soc.* **2014**, *136*, 4153–4156.

[2] Lee Y.; Cho W.; Sung J.; Kim E.; Park S. B. Monochromophoric Design Strategy for Tetrazine-Based Colorful Bioorthogonal Probes with a Single Fluorescent Core Skeleton. *J. Am. Chem. Soc.* **2018**, *140*, 974–983.

[3] Goud T. V.; Tutar A.; Biellmann J.-F. Synthesis of 8-Heteroatom-substituted 4,4-Difluoro-4-bora-3a,4a-diaza-*s*-indacene Dyes (BODIPY). *Tetrahedron* **2006**, *62*, 5084–5091.

[4] Sozmen F.; Kolemen S.; Kumada H-O.; Ono M.; Sajib H.; Akkaya E. U. Designing BODIPY-Based Probes for Fluorescence Imaging of β-Amyloid Plaques. *RSC Adv.* **2014**, *4*, 51032–51037.

[5] X. Lu, S. K. Olsen, A. D. Capili, J. S. Cisa, C. D. Lima, D. S. Tan. Designed Semisynthetic Protein Inhibitors of Ub/Ubl E1 Activating Enzymes. *J. Am. Chem. Soc.* **2010**, *132*, 1748–1749.

[6] Yu L.; Hou Y.; Cheng C.; Schlaich C.; Noeske P.-L. M.; Wei Q.; Haag R. High-Antifouling Polymer Brush Coatings on Nonpolar Surfaces via Adsorption-Cross-Linking Strategy. *ACS Appl. Mater. Interfaces* **2017**, *9*, 44281–44292.

[7] Sarris A. J. C.; Hansen T.; de Geus M. A. R.; Maurits E.; Doelman W.; Overkleeft H. S.; Codee J. D. C.; Filippov D. V.; van Kasteren S. I. Fast and pH-Independent Elimination of *trans*-Cyclooctene by Using Aminoethyl-Functionalized Tetrazines. *Chem. Eur. J.* **2018**, *24*, 18075–18081.

[8] Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Petersson, G. A.; Nakatsuji, H.; Li, X.; Caricato, M.; Marenich, A. V.; Bloino, J.; Janesko, B. G.; Gomperts, R.; Mennucci, B.; Hratchian, H. P.; Ortiz, J. V.; Izmaylov, A. F.; Sonnenberg, J. L.; Williams-Young, D.; Ding, F.; Lipparini, F.; Egidi, F.; Goings, J.; Peng, B.; Petrone, A.; Henderson, T.; Ranasinghe, D.; Zakrzewski, V. G.; Gao, J.; Rega, N.; Zheng, G.; Liang, W.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Throssell, K.; Montgomery, J. A.; Peralta, J. E.; Ogliaro, F.; Bearpark, M. J.; Heyd, J. J.; Brothers, E. N.; Kudin, K. N.; Staroverov, V. N.; Keith, T. A.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A. P.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Millam, J. M.; Klene, M.; Adamo, C.; Cammi, R.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Farkas, O.; Foresman, J. B.; Fox, D. J., *Gaussian, Inc., Wallingford CT*, Gaussian 16 Rev. A. 03, **2016**.

[9] Kohn, W. Nobel Lecture: Electronic Structure of Matter-Wave Functions and Density Functionals. *Rev. Mod. Phys.* **1999**, *71*, 1253–1266.

[10] Lee, C.; Yang, W.; Parr, R. G. Development of the Colle-Salvetti Correlation-Energy Formula into a Functional of the Electron Density. *Phys. Rev. B* **1988**, *37*, 785–789.

[11] McLean, A. D.; Chandler, G. S. Contracted Gaussian Basis Sets for Molecular Calculations. I. Second Row Atoms, Z = 11-18. *J. Chem. Phys* **1980**, *72*, 5639–5648.

[12] Marenich, A. V.; Cramer, C. J.; Truhlar, D. G. Universal Solvation Model Based on Solute Electron Density and A Continuum Model of the Solvent Defined by the Bulk Dielectric Constant and Atomic Surface Tensions. *J. Phys. Chem. B* **2009**, *113*, 6378–6396.

[13] Toulmin A.; Wood J. M.; Kenny P. W. Toward Prediction of Alkane/Water Partition Coefficients. *J. Med. Chem.* **2008**, *51*, 3720–3730.

11. NMR spectra

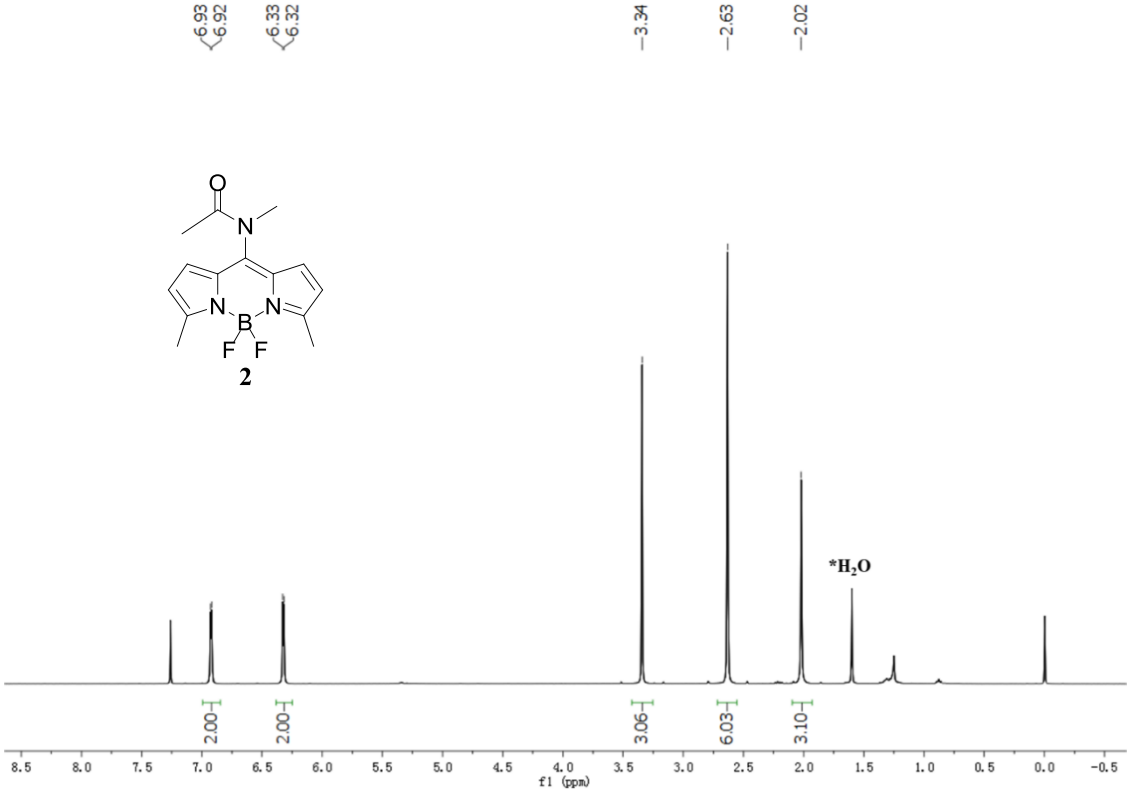


Figure S12. [1] H NMR (400 MHz, $CDCl_3$) of compound **2**

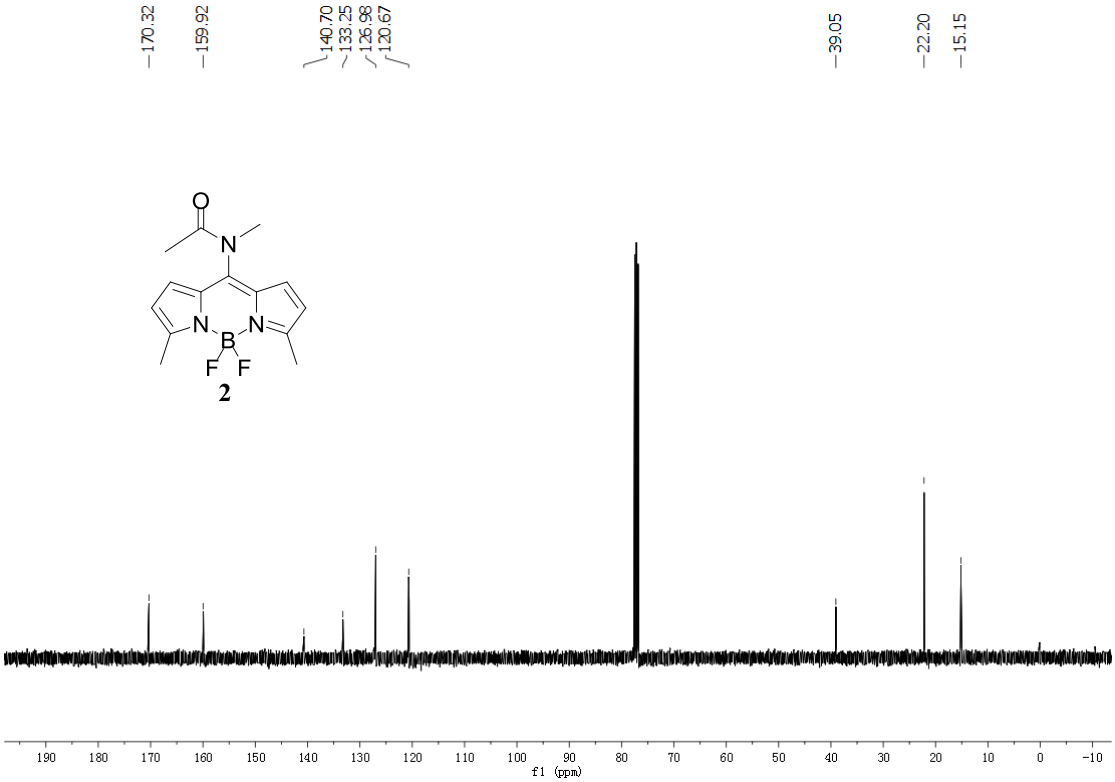


Figure S13. [13]C NMR (101 MHz, $CDCl_3$) of compound **2**

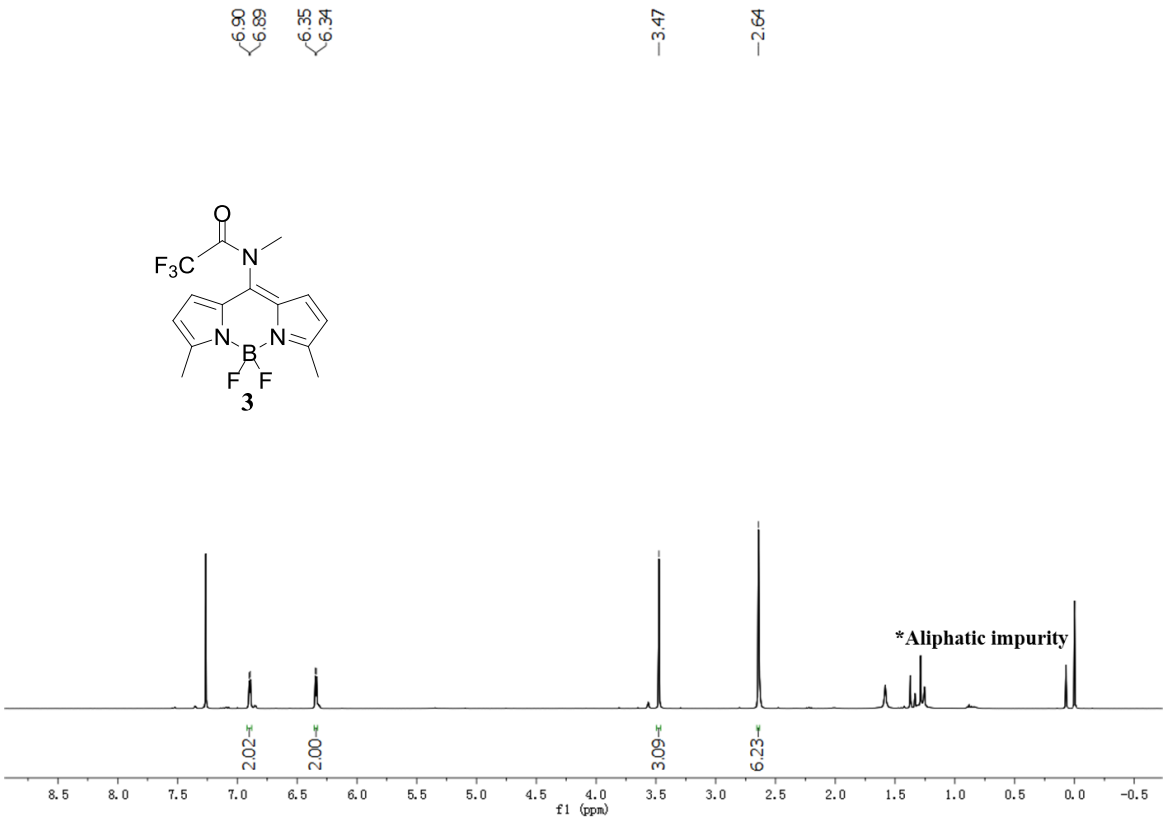


Figure S14. [1] H NMR (400 MHz, $CDCl_3$) of compound **3**

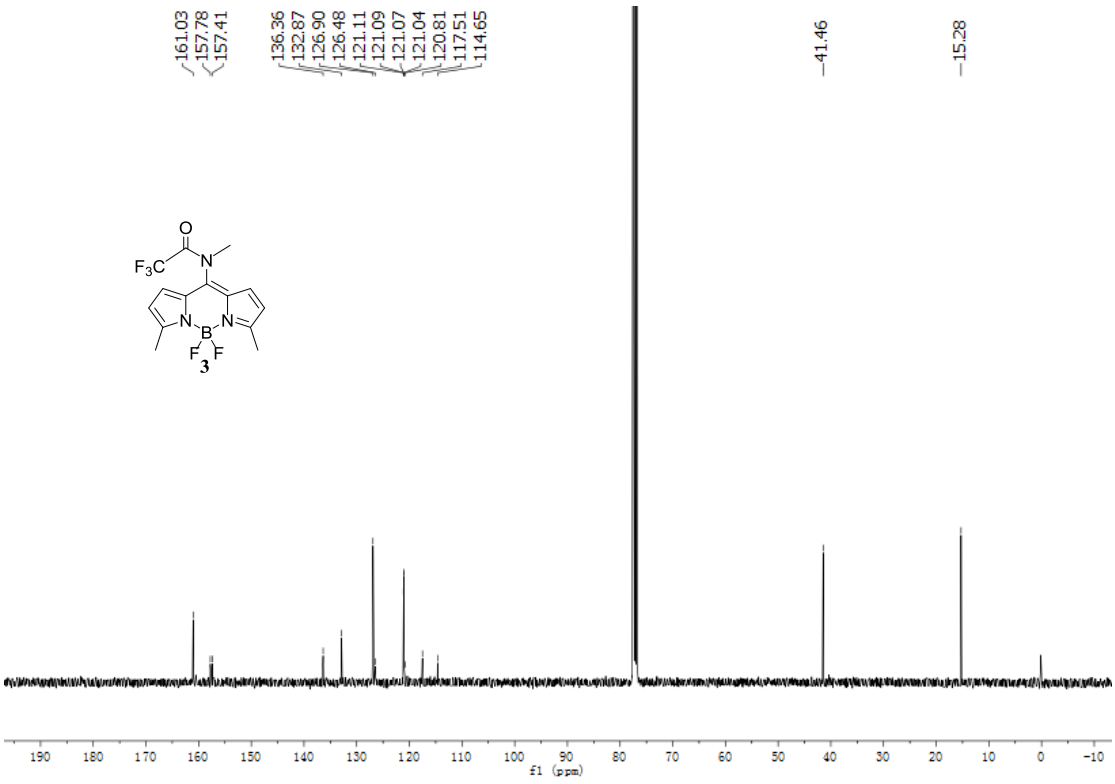


Figure S15. [13]C NMR (101 MHz, $CDCl_3$) of compound **3**

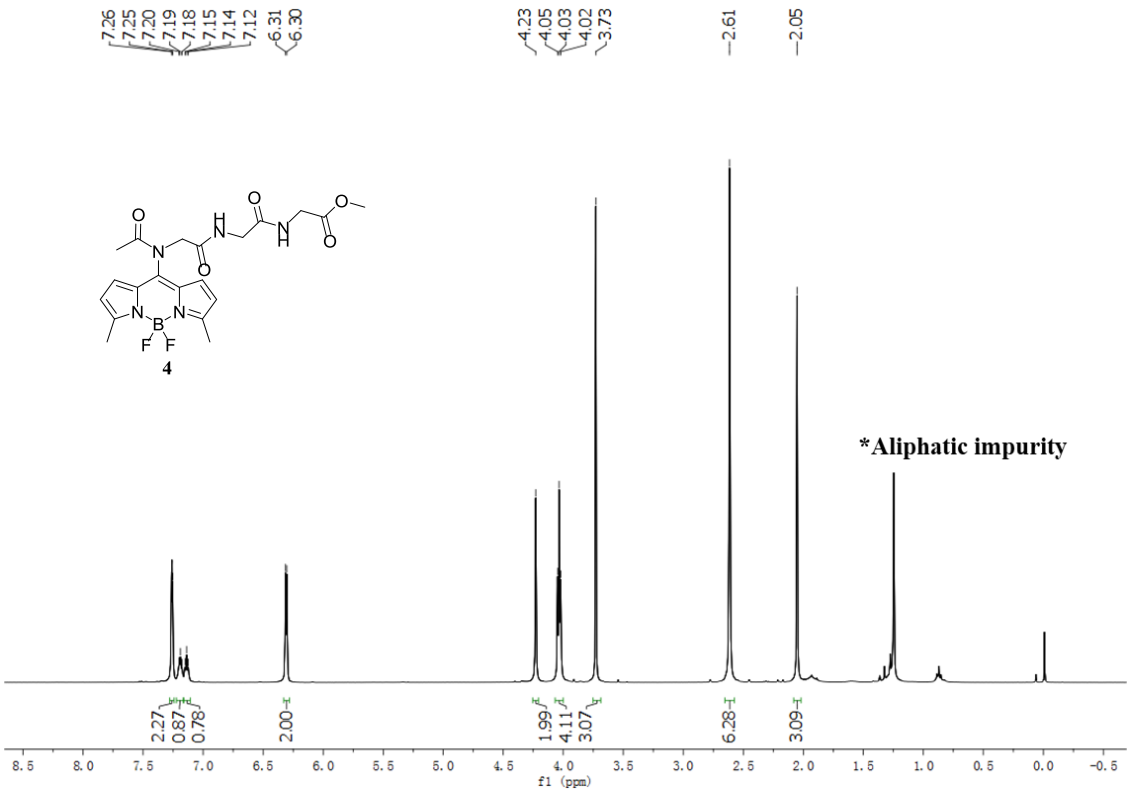


Figure S16. [1] H NMR (400 MHz, $CDCl_3$) of compound **4**

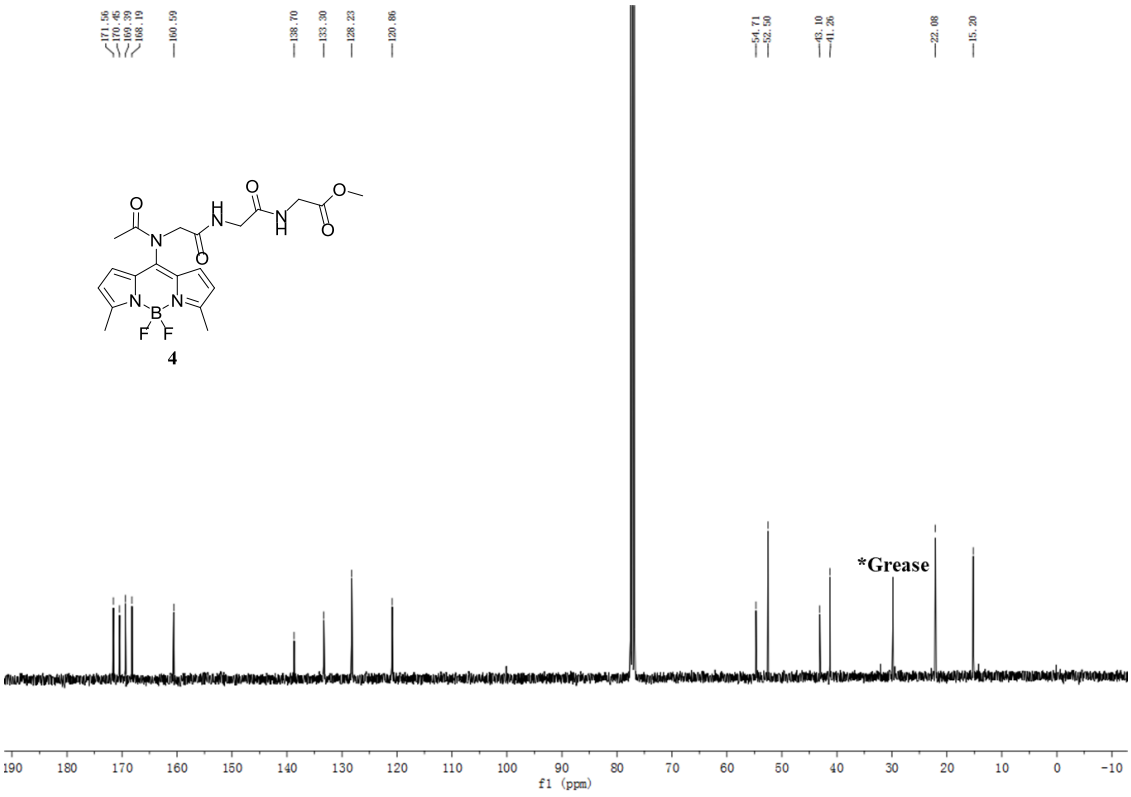


Figure S17. [13]C NMR (101 MHz, $CDCl_3$) of compound **4**

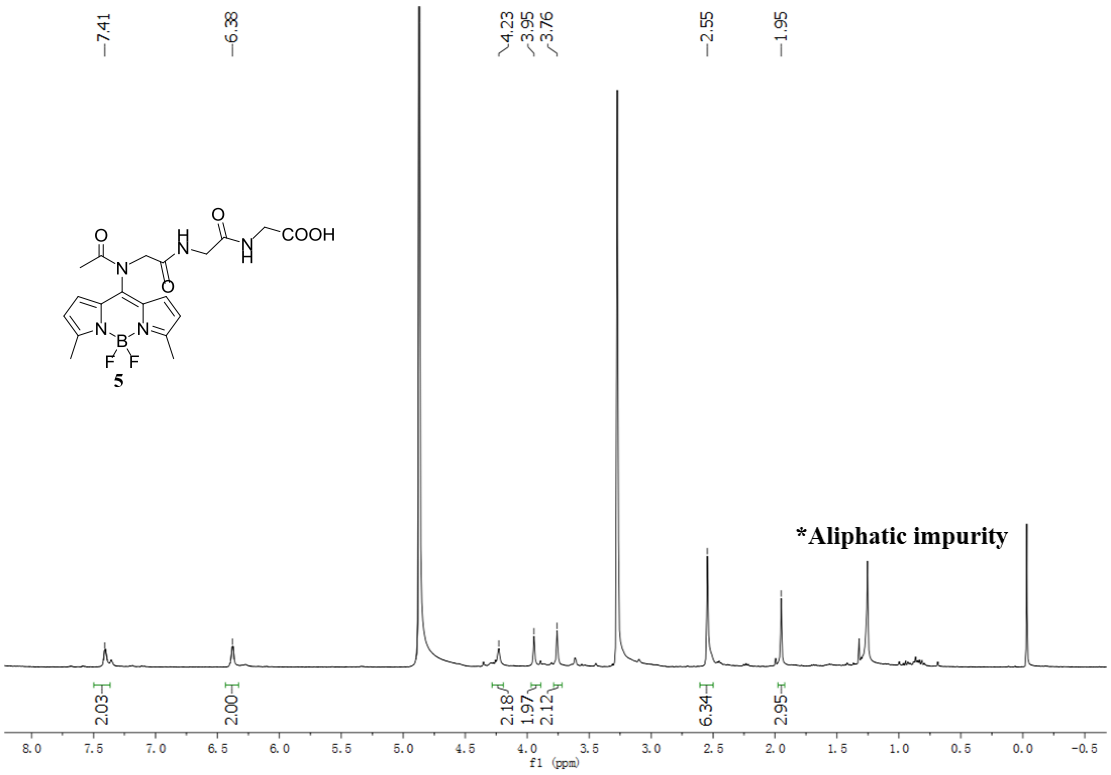


Figure S18. [1] H NMR (400 MHz, CD_3OD) of compound **5**

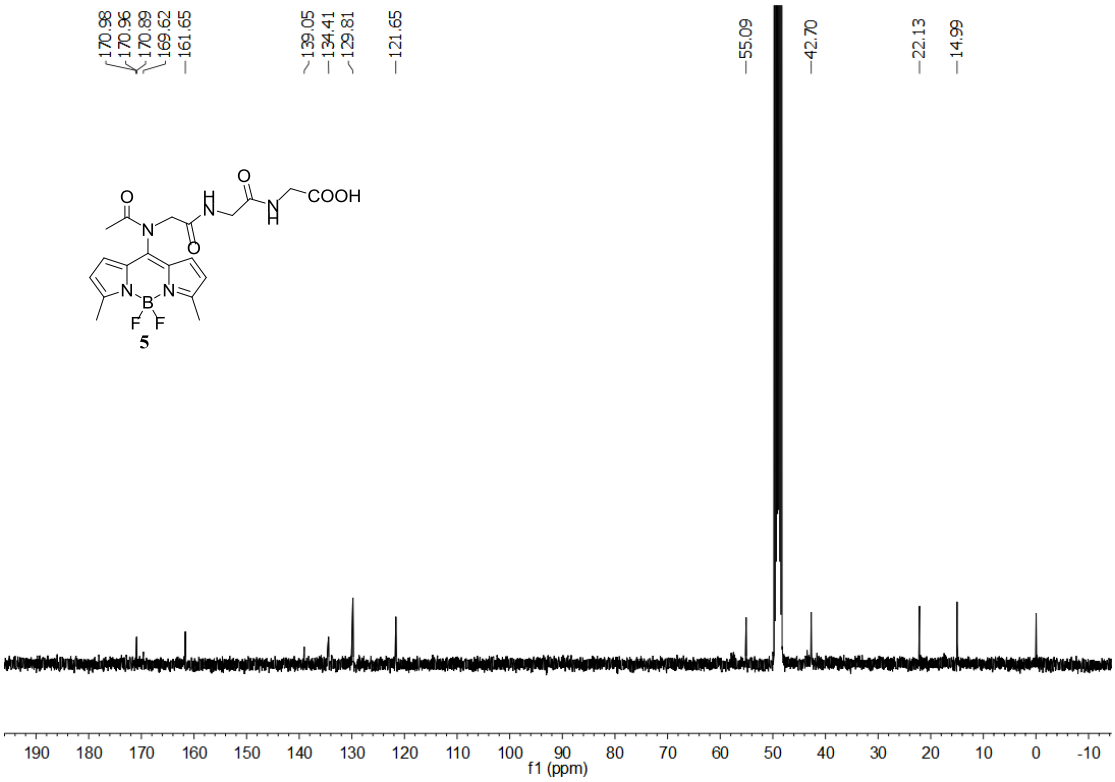


Figure S19. [13]C NMR (101 MHz, CD_3OD) of compound **5**

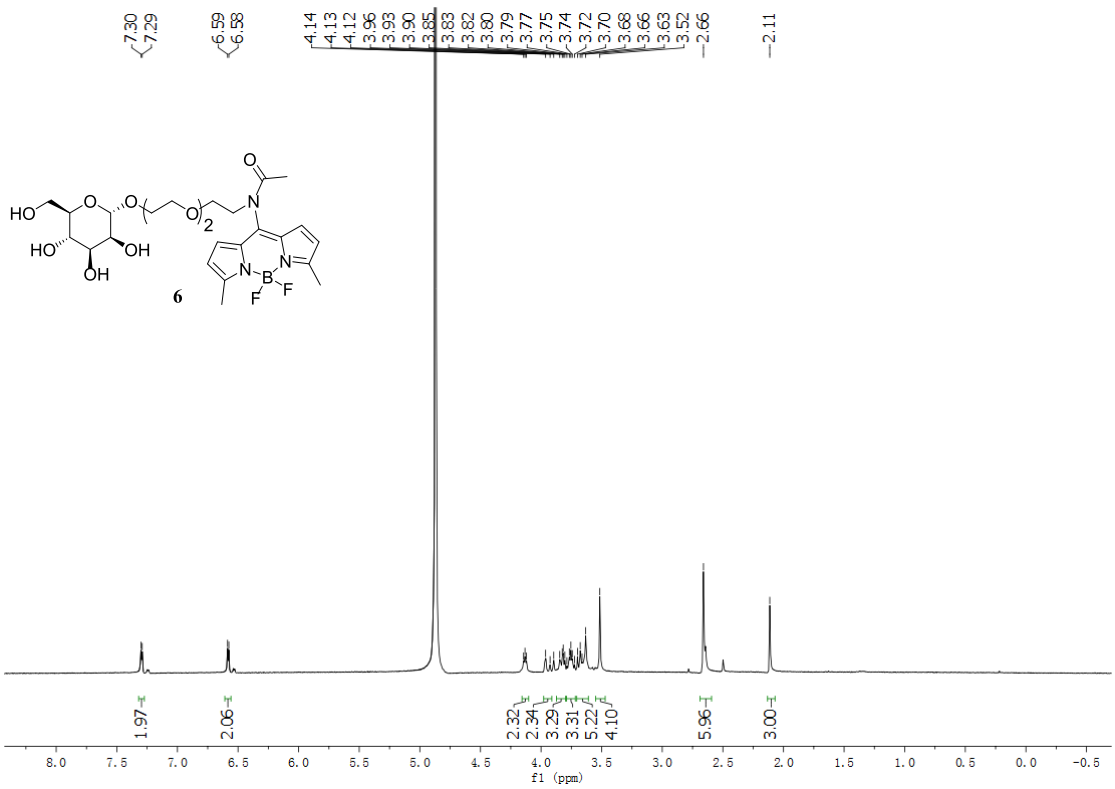


Figure S20. [1] H NMR (400 MHz, D_2O) of compound **6**

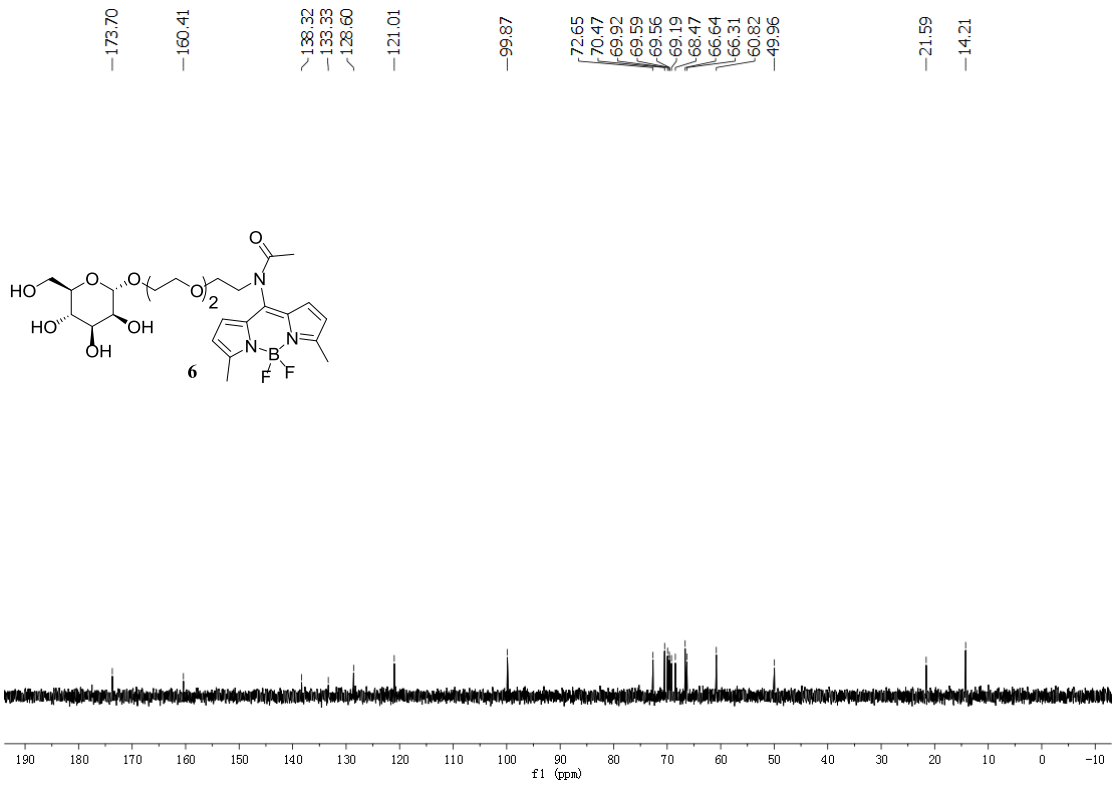


Figure S21. [13]C NMR (101 MHz, D_2O) of compound **6**

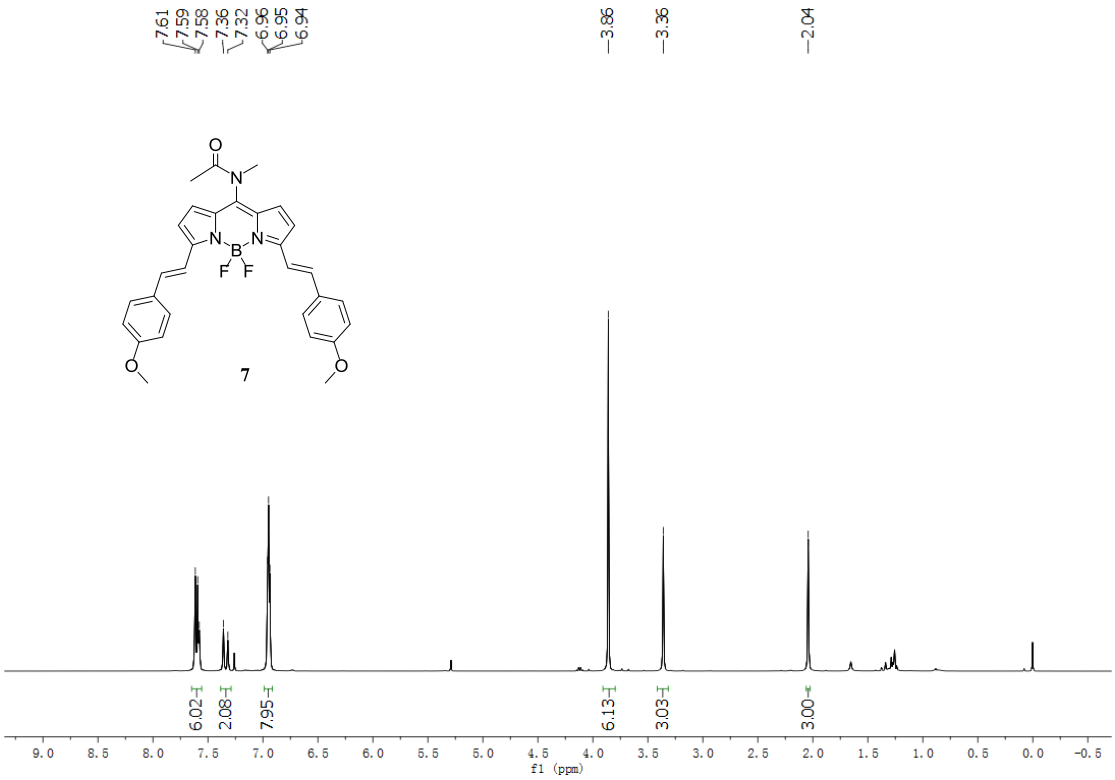


Figure S22. ^{1}H NMR (400 MHz, $CDCl_3$) of compound **7**

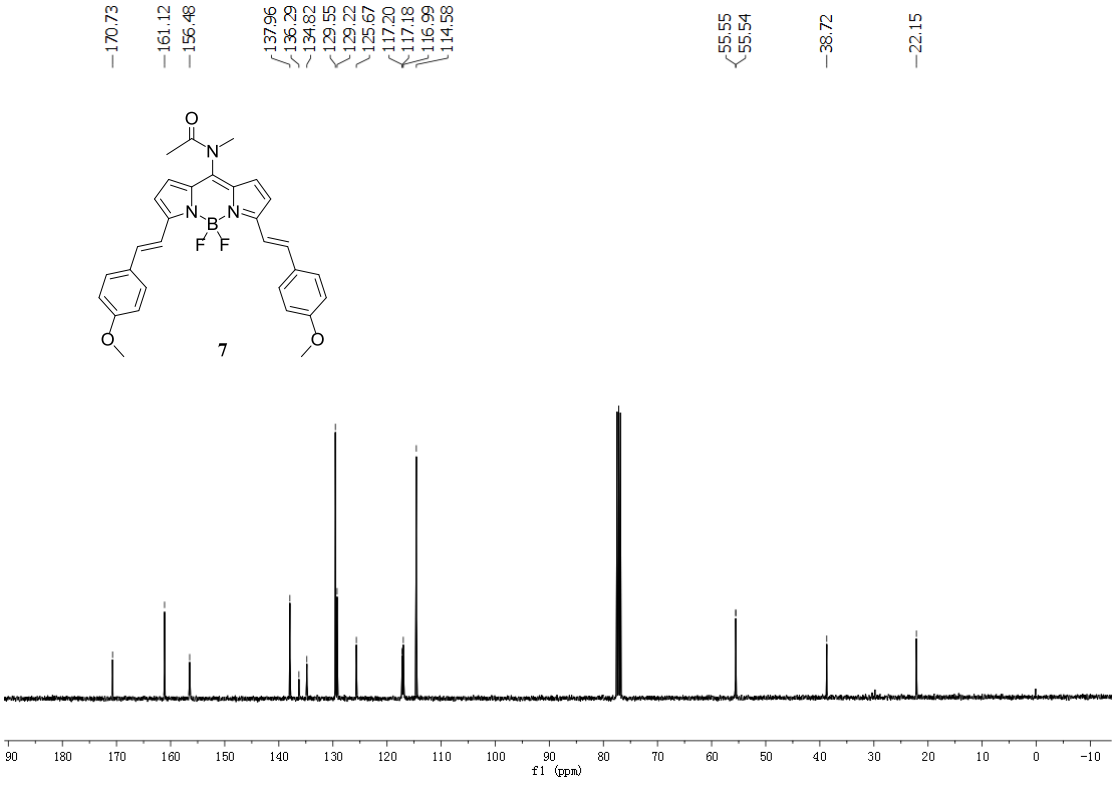


Figure S23. ^{13}C NMR (101 MHz, $CDCl_3$) of compound **7**

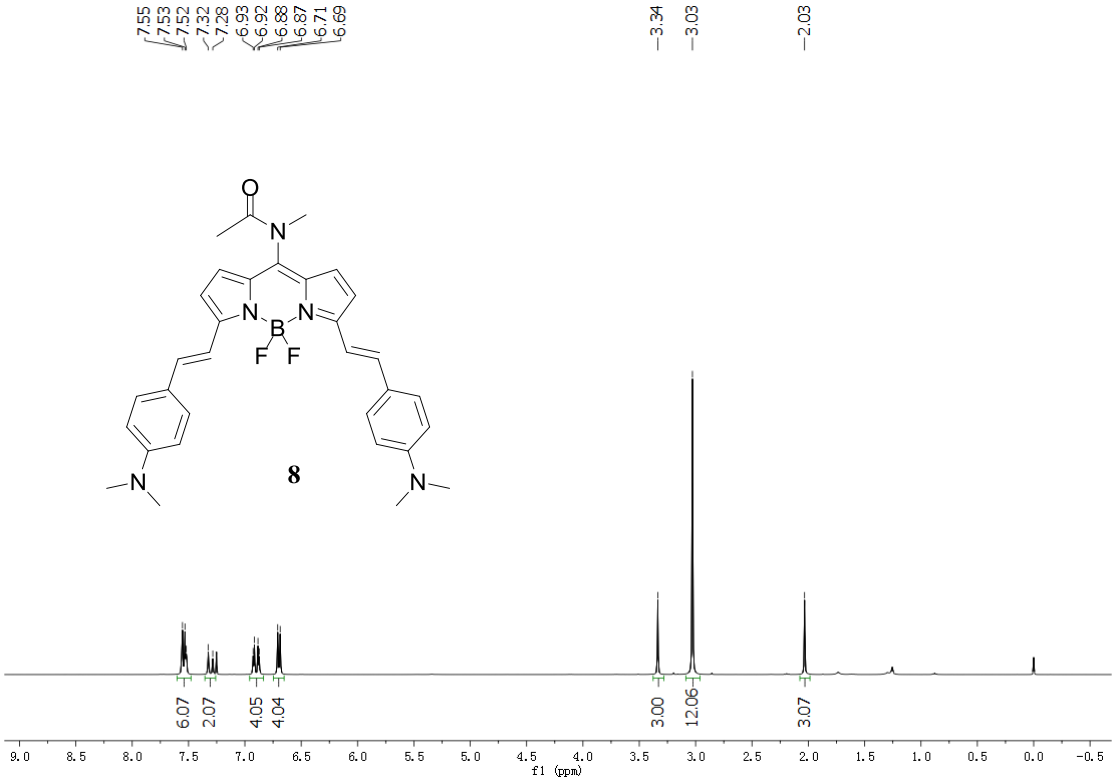


Figure S24. [1] H NMR (400 MHz, $CDCl_3$) of compound **8**

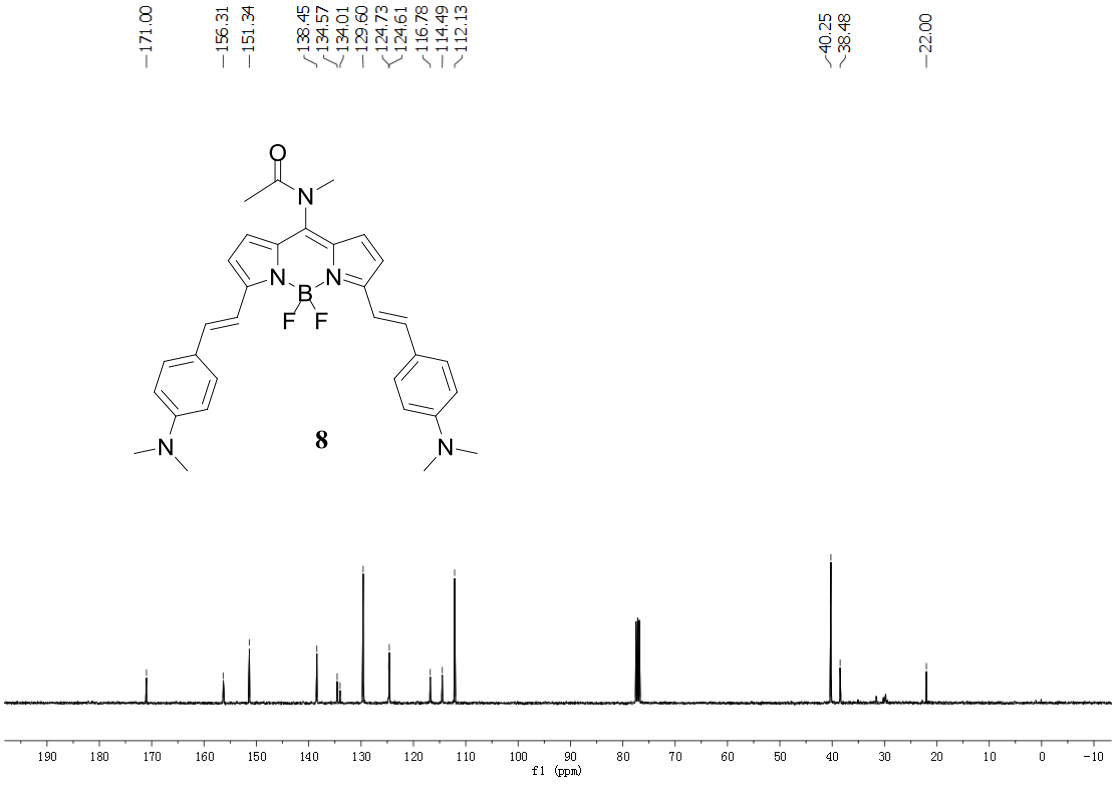


Figure S25. [13]C NMR (101 MHz, $CDCl_3$) of compound **8**

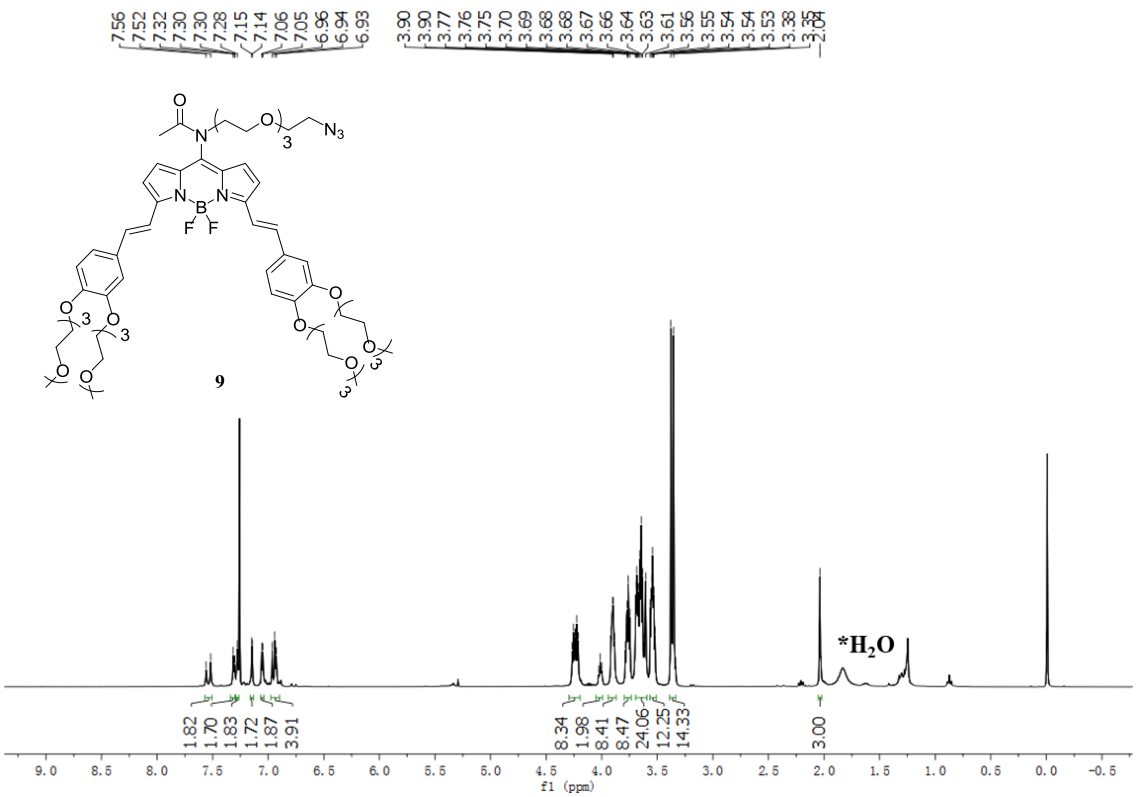


Figure S26. [1] H NMR (400 MHz, $CDCl_3$) of compound **9**

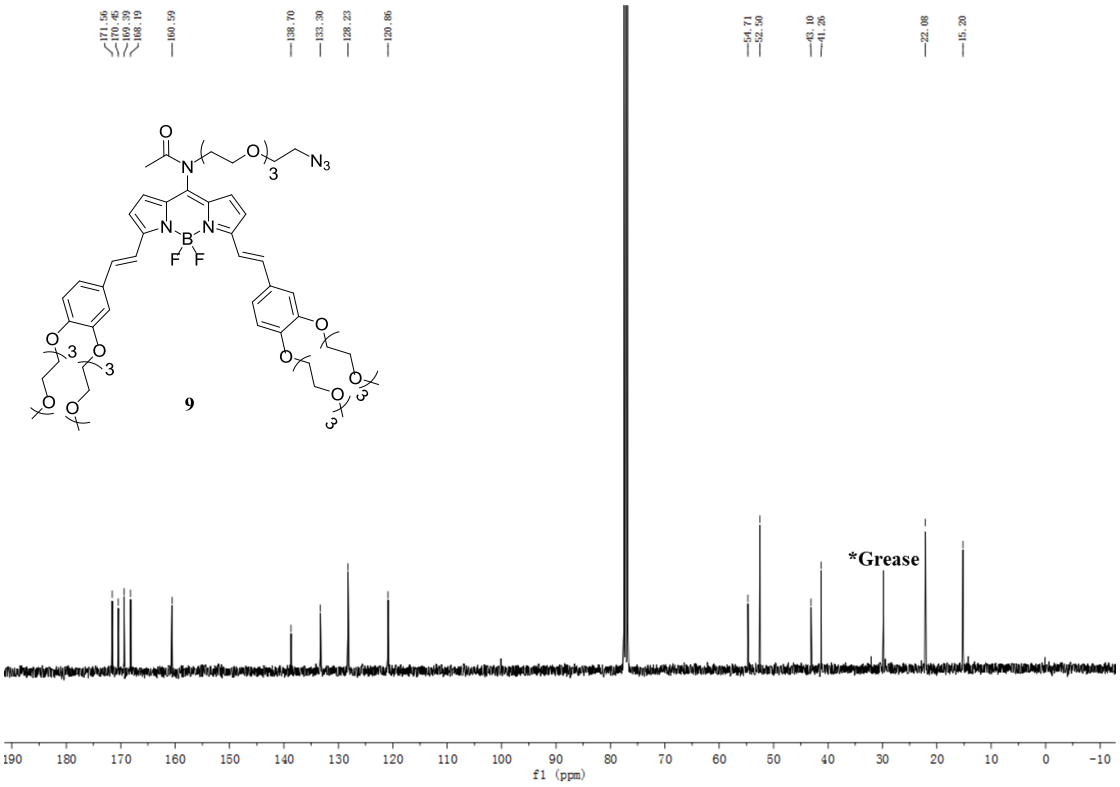


Figure S27. [13]C NMR (101 MHz, $CDCl_3$) of compound **9**

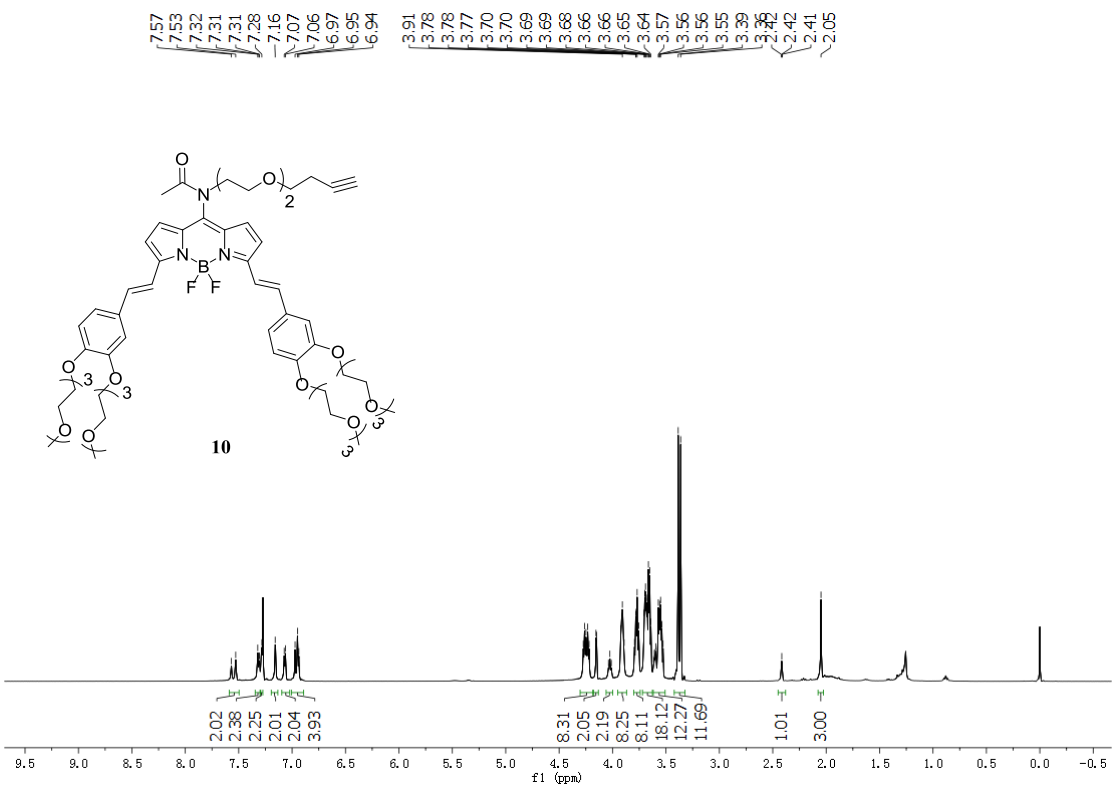


Figure S28. [1] H NMR (400 MHz, $CDCl_3$) of compound **10**

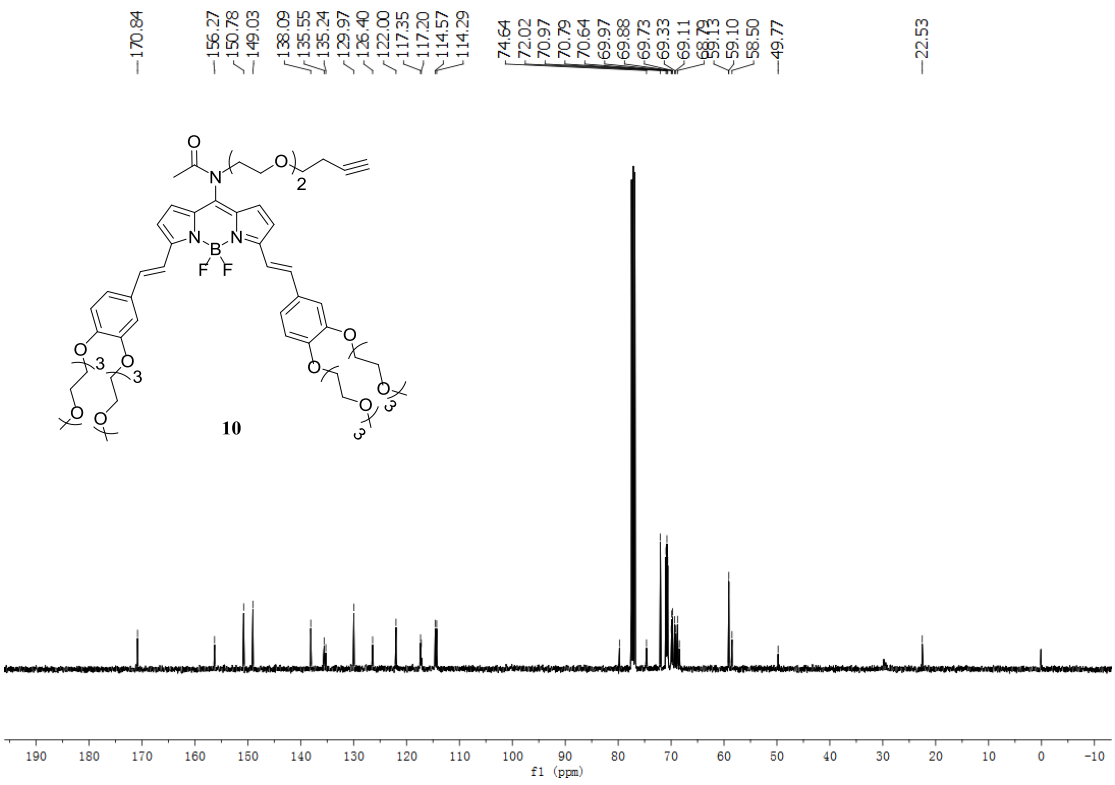


Figure S29. [13]C NMR (101 MHz, $CDCl_3$) of compound **10**

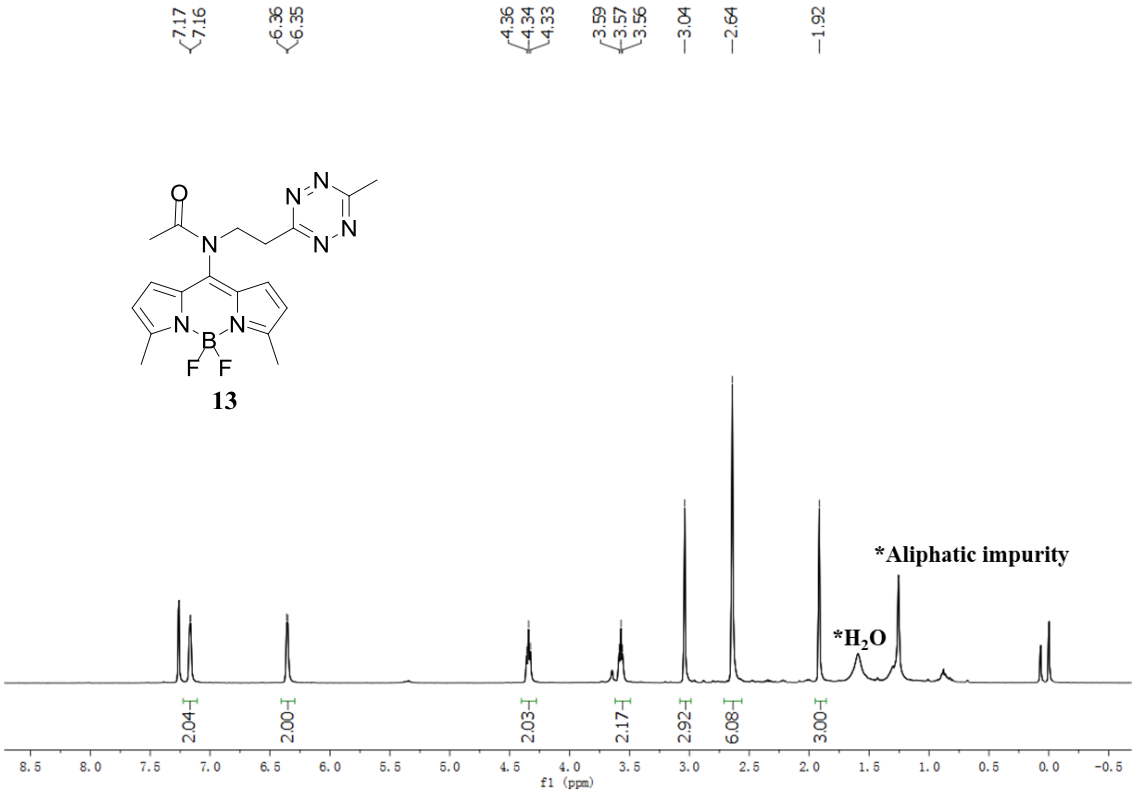


Figure S30. [1] H NMR (400 MHz, $CDCl_3$) of compound **13**

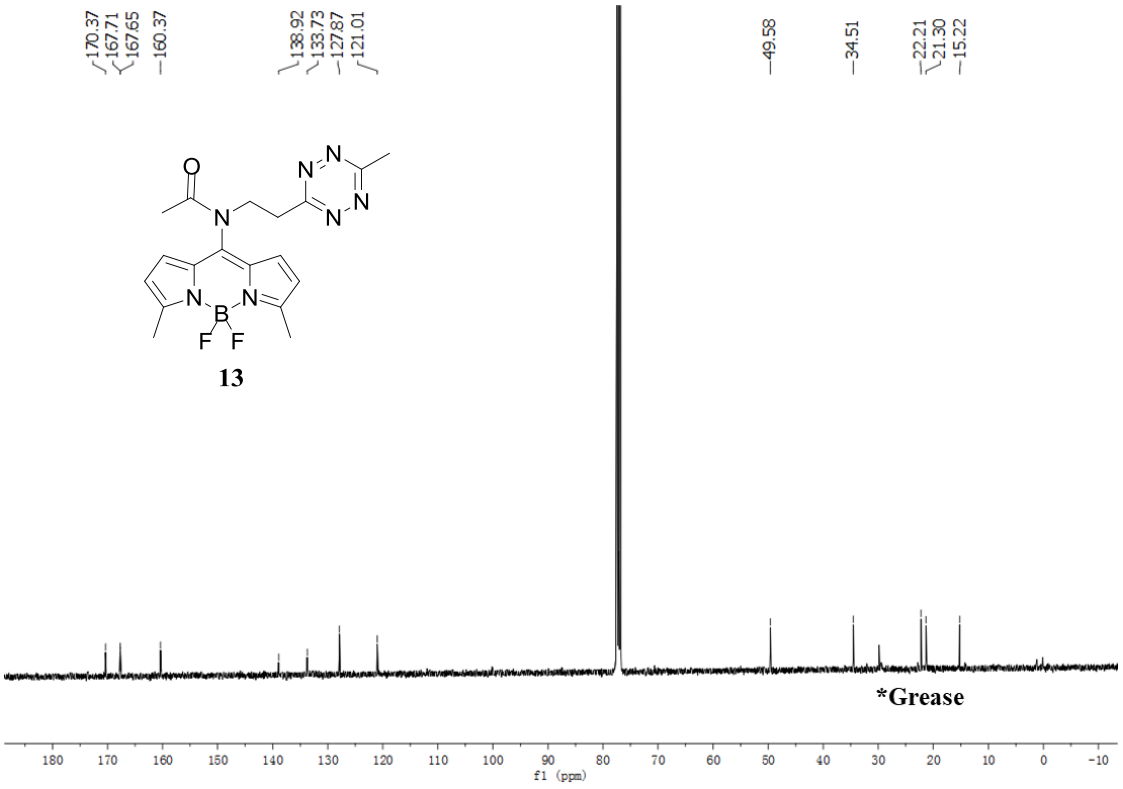


Figure S31. [13]C NMR (101 MHz, $CDCl_3$) of compound **13**

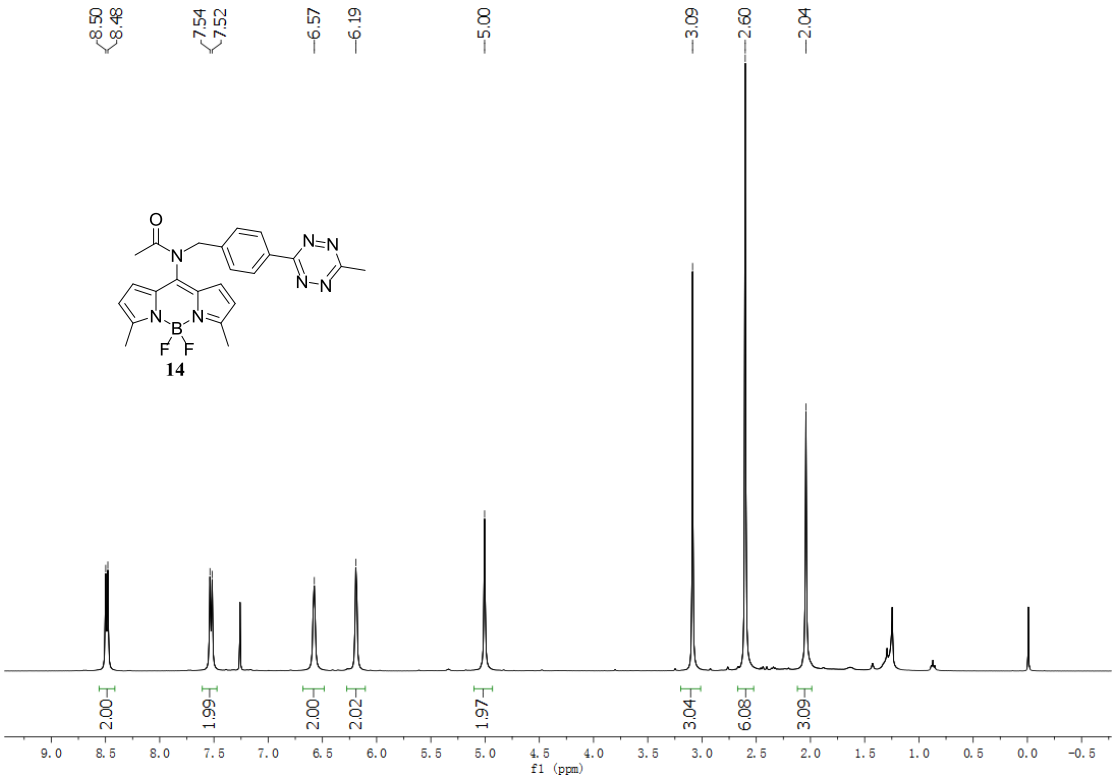


Figure S32. [1] H NMR (400 MHz, $CDCl_3$) of compound **14**

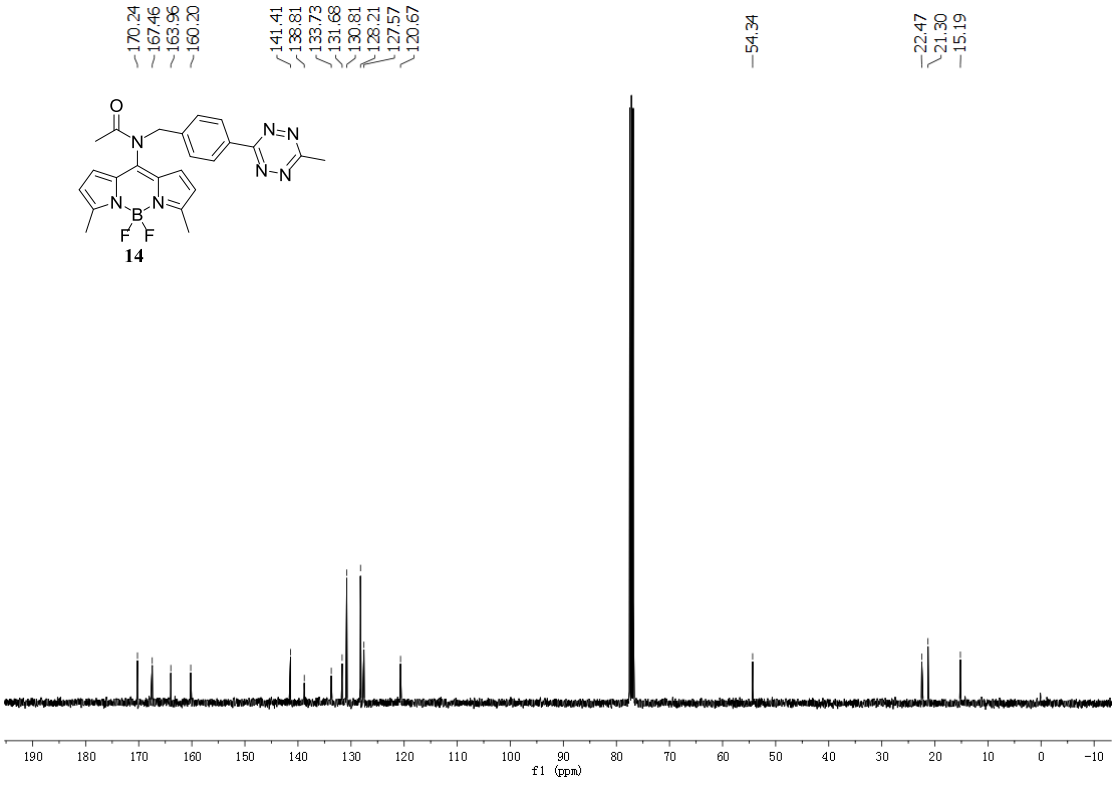


Figure S33. [13]C NMR (101 MHz, $CDCl_3$) of compound **14**

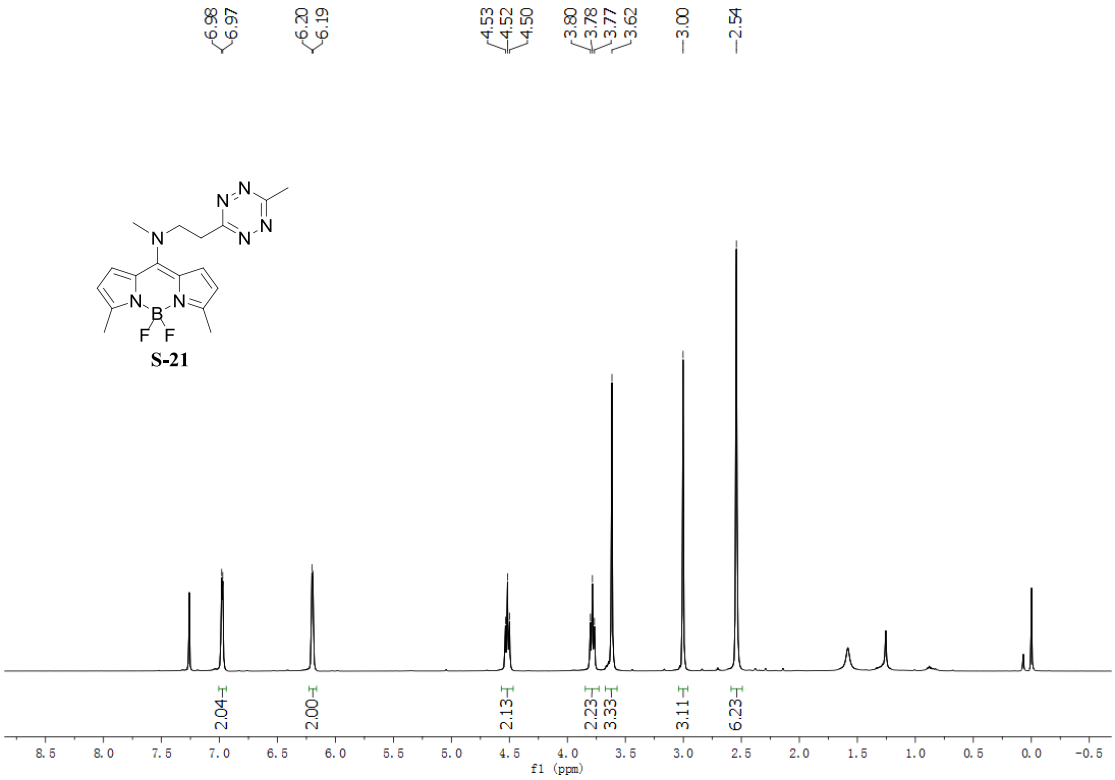


Figure S34. [1] H NMR (400 MHz, $CDCl_3$) of compound **S-21**

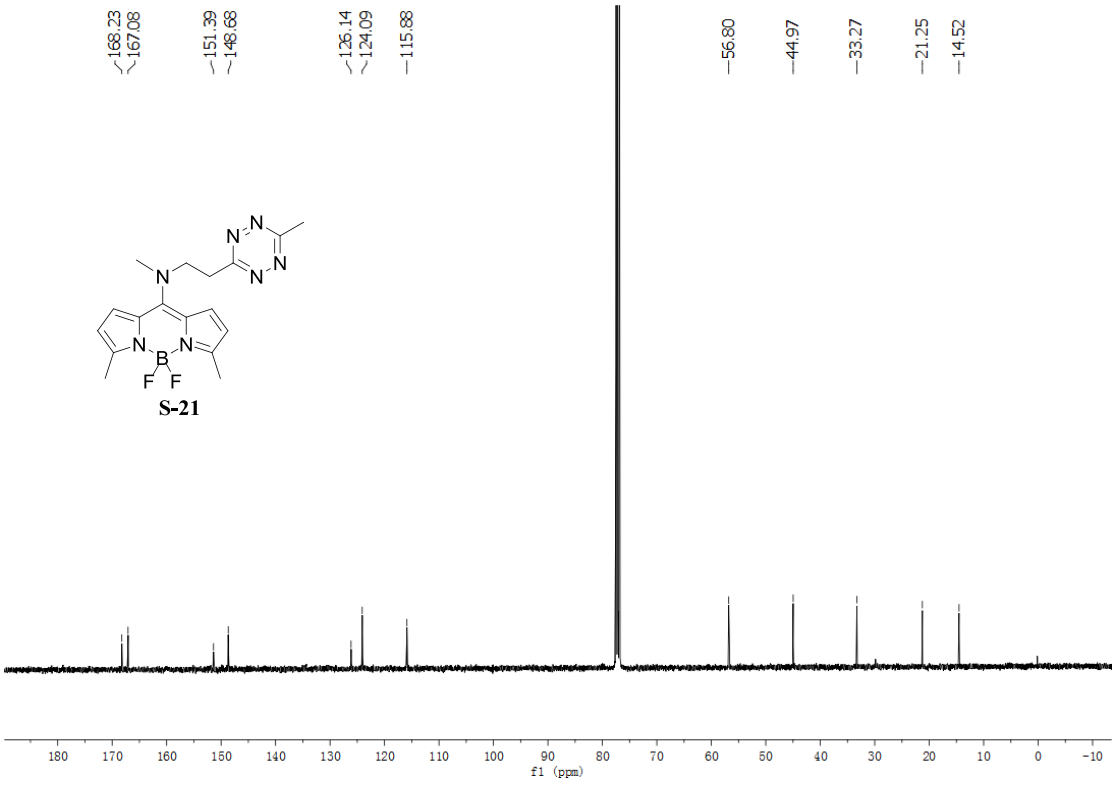


Figure S35. [13]C NMR (101 MHz, $CDCl_3$) of compound **S-21**