Supplementary Information

Key Role of Cycloalkyne Nature in Alkyne-Dye Reagents for Enhanced Specificity of Intracellular Imaging by Bioorthogonal Bioconjugation†

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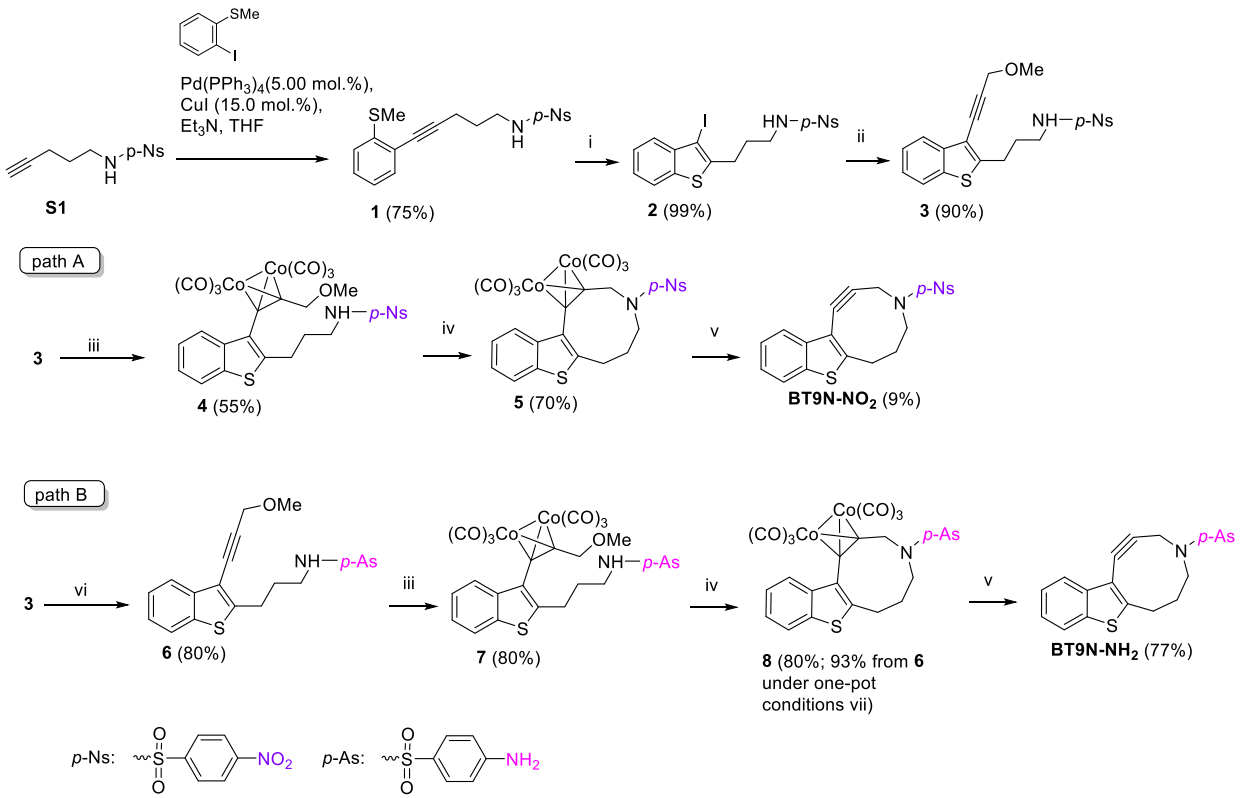
1. General Information and Methods

Solvents, reagents, and chemicals used for reactions were purchased from commercial suppliers. The chemicals were used without further purification. Catalyst $Pd(PPh_3)_4$ and $Co_2(CO)_8$ were purchased from Sigma-Aldrich. BDP FL carboxylic acid, BDP FL NHS-ester, DBCO-F, for DBCO-sulfo-Cyan5 were purchased from Lumiprobe, BCN-amine was purchased from Sigma-Aldrich, 4-nitro-*N*-(pent-4-yn-1-yl)benzenesulfonamide (**S1**)[1], methyl propargyl ether[2] and Tiazole-acid[3] were synthesized according to known procedures without any modifications. The photophysical properties of the aforementioned BDP FL derivatives are provided by Lumiprobe at https://ru.lumiprobe.com. Photophysical properties of Tiazole-acid, a starting material for BT9N-T, has been reported previously.[3]

Solvents were dried under standard conditions. Purification and drying of DCM was carried out in accordance with the literature procedure using CaH_2.[4] The Sonogashira coupling, the synthesis of Co-complexes, and the Nicholas cyclization was carried out under argon in oven-dried glassware. Other reactions were carried out under air unless stated otherwise. Evaporation of solvents and concentration of reaction mixtures were performed under vacuum at 25 °C (for cycloalkynes) and at 30 °C (for other compounds) on a rotary evaporator. TLC was carried out on silica gel plates (silica gel 60, UV 254) with detection by UV (254 nm or 365 nm for cycloalkyne-dye conjugates) or staining with a basic aqueous solution of $KMnO_4$. A normal-phase silica gel (Silica gel 60, 230–400 mesh) was used for preparative column chromatography. ^1H and $^{13}C\{^1H\}$, DEPT and HSQC NMR spectra were recorded at 400 (or 500) MHz and 101 (or 125) MHz, respectively, at 25 °C in $CDCl_3$, acetone-d_6 CD_3CN or DMSO-d_6 without an internal standard. ^{19}F NMR spectra were recorded at 376 MHz. The ^1H NMR and ^{19}F data are reported as chemical shifts (δ), multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; m, multiplet; br, broad), coupling constants (*J*, given in Hz), and number of protons (for ^1H NMR). The $^{13}C\{^1H\}$ NMR data are reported as chemical shifts (δ). Chemical shifts for ^1H and ^{13}C are reported as δ values (ppm) and referenced to residual solvents (δ = 7.26 ppm for ^1H; δ = 77.16 ppm for ^{13}C for spectra recorded in $CDCl_3$, δ = 2.05 ppm for ^1H; δ = 29.84 ppm for ^{13}C for spectra recorded in acetone-d_6, δ = 1.94 ppm for ^1H; δ = 1.32 ppm for ^{13}C for spectra in CD_3CN and δ = 2.50 ppm for ^1H; δ = 39.52 ppm for ^{13}C for spectra recorded in DMSO-d_6). Chemical shifts in ^{19}F NMR are given relative to $CFCl_3$. To obtain resolved ^1H NMR spectra for Co-complexes **5**, **7**, **8** and to avoid line broadening due to trace decomposition of Co complexes, it is advisable to rapidly remove the column chromatography eluent from the sample and measure the ^1H NMR spectrum for the freshly prepared sample as soon as possible. These spectra usually contain signals from the residual solvents. Significant line broadening was observed after further vacuum drying of the sample. For complexes **5**, **7**, **8**, copies of the resolved ^1H NMR spectra are included. These contain signals from residual solvent (ethyl acetate). High-resolution mass spectra were determined for solutions of all compounds in MeOH or acetonitrile using ESI in the mode of positive ion registration with a TOF mass analyser. For the details of MALDI MS experiments see Section 5.

2. Experimental Details

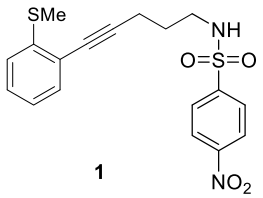
2.1 Synthesis of cycloalkynes



Reagents and conditions: (i) I_2, DCM, r.t., 1h; (ii) Methyl propargyl ether, $Pd(PPh_3)_4$ (5.00 mol.%), CuI (15.0 mol.%), KF, DMF, 50 °C, 3 h; (iii) $Co_2(CO)_8$, benzene, c = 0.01 M, r.t., 2-3 h; (iv) $BF_3{\cdot}OEt_2$, DCM, c = 0.001 M, 0 °C to r.t., 2 h; (v) TBAF·H_2O acetone/H_2O (15:1), c = 0.006 M, r.t., 4–5 h; (vi) Fe powder, NH_4Cl, acetone / H_2O (1:1), 40 °C, 48 h; (vii) Co-complexation / Nicholas reaction in one-pot: $Co_2(CO)_8$ DCM = 0.01 M, r.t., 2 h, then $BF_3{\cdot}OEt_2$, DCM, c = 0.0035 M, 0 °C to r.t., 15 min

Scheme S1. Synthesis of **BT9N-NH$_2$.**

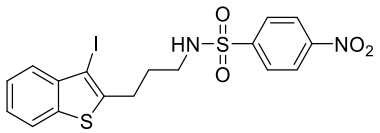
N-(5-(2-(Methylthio)phenyl)pent-4-yn-1-yl)-4-nitrobenzenesulfonamide (1)



In a three-necked round bottom flask to a stirred solution of 4-nitro-*N*-(pent-4-yn-1-yl)benzenesulfonamide (**S1**) (795 mg, 2.96 mmol, 1.00 equiv) in the mixture of triethylamine (10.0 mL) and tetrahydrofuran (3.00 mL) were added $Pd(PPh_3)_4$ (103 mg, 0.0889 mmol, 3.00 mol%) and CuI (56.4 mg, 0.296 mmol, 10.0 mol%). The reaction flask was degassed using a freeze-pump-thaw technique over three degassing cycles. After that, 2-iodothioanisol (763 mg, 3.05 mmol, 1.03 equiv) was added with a syringe. The reaction mixture was stirred at 40 °C for 5 hours (TLC control). After completion of the reaction, the reaction mixture was cooled, poured into a saturated aqueous solution of NH_4Cl (200 mL), and extracted with ethyl acetate three times (3 × 75.0 mL). The combined organic layers were washed

with a saturated solution of NH_4Cl (200 mL) and two times with brine (200 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to give the crude product, which was purified by column chromatography on silica gel (eluent: hexane / ethyl acetate = 3:1) that gave alkyne **1** (870 mg, 75 %) as a light yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25 – 8.21 (m, 2H), 8.04 – 7.99 (m, 2H), 7.30 – 7.22 (m, 2H), 7.13 – 7.00 (m, 2H), 5.21 (t, *J* = 6.0 Hz, 1H), 3.29 (q, *J* = 6.4 Hz, 2H), 2.51 (t, *J* = 6.5 Hz, 2H), 2.44 (s, 3H), 1.80 (quint, *J* = 6.5 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 150.0, 146.0, 141.2, 132.2, 128.8, 128.4, 124.42 (two overlapping signals), 123.9, 121.2, 94.8, 79.9, 42.6, 27.9, 17.1, 15.0. HRMS (ESI) m/z: $[M+Na]^+$ Calcd for $C_{18}H_{18}N_2O_4S_2Na^+$: 413.0603; Found: 413.0600.

***N*-(3-(3-Iodobenzo[*b*]thiophen-2-yl)propyl)-4-nitrobenzenesulfonamide (2)**

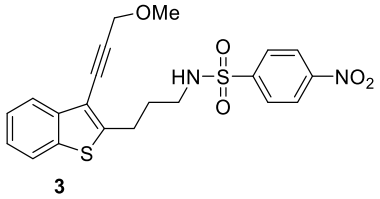


2

To a solution of alkyne **1** (2.10 g, 5.38 mmol, 1.00 equiv) in DCM (45.0 mL) was added a solution of iodine (1.64 g, 6.45 mmol, 1.20 equiv) in DCM (80.0 mL) dropwise. The reaction mixture was stirred at room temperature for 1 hour (TLC control). Then, the reaction mixture was diluted with a 5% aqueous solution of $Na_2S_2O_3$ (100 mL), the organic layer was separated and the aqueous layer was extracted with DCM (50.0 mL). The combined organic layers were washed with brine (100 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to yield the crude product, which was purified by column chromatography on silica gel (eluent: hexane / ethyl acetate = 3:1) that gave iodobenzothiophene **2** (2.70 g, 99 %) as a yellow viscous oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.27 (d, *J* = 8.8 Hz, 2H), 8.00 (d, *J* = 8.8 Hz, 2H), 7.69 (d, *J* = 7.9 Hz, 1H), 7.64 (d, *J* = 8.0 Hz, 1H), 7.44 – 7.38 (m, 1H), 7.37 – 7.31 (m, 1H), 4.96 (t, *J* = 6.0 Hz, 1H), 3.11 (q, *J* = 6.7 Hz, 2H), 2.97 (t, *J* = 7.4 Hz, 2H), 1.94 (quint, *J* = 7.1 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 150.2, 145.8, 142.1, 141.0, 138.0, 128.4, 125.6, 125.5, 125.3, 124.5, 122.4, 81.3, 42.4, 30.4, 29.9. HRMS (ESI) m/z: $[M+Na]^+$ Calcd for $C_{17}H_{15}IN_2O_4S_2Na^+$: 524.9410; Found: 524.9411.

***N*-(3-(3-(3-Methoxyprop-1-yn-1-yl)benzo[*b*]thiophen-2-yl)propyl)-4-nitrobenzenesulfonamide (3)**



3

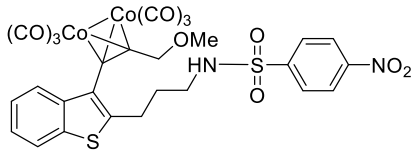
To a stirred solution of the iodobenzothiophene **2** (770 mg, 1.53 mmol, 1.00 equiv) in DMF (8.00 mL) were added $Pd(PPh_3)_4$ (88.6 mg, 0.0766 mmol, 5.00 mol %), CuI (43.8 mg, 0.229 mmol, 15.0 mol %) and KF (712

mg, 12.3 mmol, 8.00 equiv). The reaction vial was degassed using a freeze-pump-thaw technique over three degassing cycles. After that, methyl propargyl ether (161 mg, 178 µL, 2.30 mmol, 1.50 equiv) was added with a syringe. The reaction mixture was stirred at 50 °C for 3 hours (TLC control). After completion of the reaction, the reaction mixture was cooled, poured into a saturated aqueous solution of NH_4Cl (150 mL), and extracted with ethyl acetate (3 × 75 mL). The combined organic layers were washed with a saturated solution of NH_4Cl (200 mL) and two times with brine (3 × 200 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to give the crude product, which was purified by column chromatography on silica gel (eluent: hexane / ethyl acetate = 3:1) that gave alkyne **3** (615 mg, 90 %) as a yellow-orange oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.30 – 8.25 (m, 2H), 8.02 – 7.97 (m, 2H), 7.78 (d, *J* = 7.9 Hz, 1H), 7.72 (d, *J* = 7.9 Hz, 1H), 7.43 – 7.37 (m, 1H), 7.36 – 7.30 (m, 1H), 5.29 (t, *J* = 6.1 Hz, 1H), 4.45 (s, 2H), 3.53 (s, 3H), 3.10 (t, *J* = 6.8 Hz, 2H), 3.03 (q, *J* = 6.3 Hz, 2H), 1.96 (quint, *J* = 6.6 Hz, 2H). ^{13}C NMR (101 MHz, Acetone-d_6) δ 150.9, 150.3, 147.5, 140.6, 138.2, 129.2, 125.8, 125.75, 125.3, 123.2, 123.0, 115.8, 92.3, 79.4, 60.7, 57.6, 43.3, 31.7, 27.4. HRMS (ESI) m/z: $[M+Na]^+$ Calcd for $C_{21}H_{20}N_2O_5S_2Na^+$: 467.0706; Found: 467.0710.

Synthesis of Co-complex (General procedure (1))

To a solution of an alkynylbenzotiophene (1.00 equiv) in benzene (c = 0.01 M) was added octacarbonyldicobalt (1.50 – 1.70 equiv) and the mixture was stirred under a flow of argon at room temperature for 2 – 3 hours (TLC control). After completion of the reaction, the solvent was evaporated under reduced pressure, and the residue was purified by column chromatography on silica gel.

Hexacarbonyl(*N*-[3-{3-(3-methoxyprop-1-µ$_2$-[1,2-η^2,η^2]yn-1-yl)benzo[*b*]thiophen-2-yl}propyl]-4-nitrobenzenesulfonamide)dicobalt (4)



4

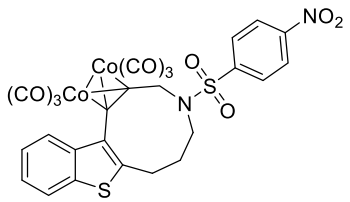
Acyclic Co-complex **4** was synthesized in accordance with the ***General procedure (1)*** from alkynylbenzotiophene **3** (1.40 g, 3.15 mmol, 1.00 equiv) in benzene (c = 0.01 M, 315 mL) and octacarbonyldicobalt (1.83 g, 5.35 mmol, 1.70 equiv) at room temperature for 3 hours (TLC control). Purification of the crude product by column chromatography (eluent: hexane / ethyl acetate = 2:1) gave acyclic Co-complex **4** (1.27 g, 55%) as a dark-brown powder. ^{1}H NMR (400 MHz, Acetone-d_6) δ 8.45 (d, *J* = 8.5 Hz, 2H), 8.16 (d, *J* = 8.5 Hz, 2H), 8.06 (d, *J* = 7.8 Hz, 1H), 7.90 (d, *J* = 7.8 Hz, 1H), 7.49 (t, *J* = 7.4 Hz, 1H), 7.39 (t, *J* = 7.4 Hz, 1H), 7.07 (m, 1H), 5.06 (s, 2H), 3.62 (s, 3H), 3.37 – 3.12 (m, 4H), 2.09 (br. s, 2H, overlaps with the solvent signal). ^{13}C NMR (101 MHz, Acetone-d_6) δ 200.9 (CO signals), 151.0, 147.6, 146.9, 140.9,

138.5, 129.3, 126.4, 125.4, 125.3, 125.1, 123.3, 123.2, 97.2, 78.8, 74.6, 59.2, 43.7, 32.1, 27.7. HRMS (ESI) m/z: $[M+Na]^+$ Calcd for $C_{27}H_{20}Co_2N_2O_{11}S_2Na^+$: 752.9065; Found: 752.9068.

The Nicholas cyclization (General procedure (2))

A stirred solution of acyclic Co-complex in dry DCM (c = 0.001 M) was cooled to 0 °C under an argon atmosphere. Then boron trifluoride diethyl etherate complex (1.50 equiv) was added in one portion. A cooling bath was immediately removed, and the resulting solution was allowed to warm to room temperature and then stirred at this temperature for 2 hours (TLC control). Then the reaction mixture was quenched with a 5% aqueous solution of $NaHCO_3$. The organic layer was separated, washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to yield a crude product, which was purified by column chromatography on silica gel.

Hexacarbonyl(1,2-μ$_2$-[1,2-η^2,η^2]didehydro-4-[{4-nitrophenyl}sulfonyl]-4,5,6,7-tetrahydro-3*H*-benzo[4,5]thieno[2,3-*e*]azonine)dicobalt (5)



5

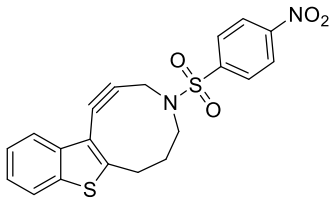
Co-complex **5** was synthesized in accordance with the ***General procedure (2)*** from acyclic Co-complex **4** (210 mg, 0.288 mmol, 1.00 equiv), boron trifluoride etherate (61.2 mg, 0.0542 mL, 0.431 mmol, 1.50 equiv) in DCM (c = 0.001M, 288 mL). The reaction time was 2 hours. Purification of the crude product by column chromatography (eluent: hexane / ethyl acetate = 4:1) gave cyclic Co-complex **5** (140 mg, 70 %) as a viscous black oil. ^{1}H NMR (400 MHz, acetone-d_6) δ 8.50 (d, *J* = 8.5 Hz, 2H), 8.25 (d, *J* = 8.5 Hz, 2H), 8.17 (d, *J* = 7.9 Hz, 1H), 7.95 (d, *J* = 7.9 Hz, 1H), 7.53 (t, *J* = 7.5 Hz, 1H), 7.43 (t, *J* = 7.5 Hz, 1H), 5.30 (s, 2H), 3.48 (br. s, 2H), 3.20 (t, *J* = 6.5 Hz, 2H), 2.76 (br. s, overlaps with the solvent signal). ^{13}C NMR (126 MHz, Acetone-d_6) δ 200.4 (CO signals), 151.2, 146.8, 144.1, 140.9, 139.9, 129.4, 127.8, 125.60, 125.62, 125.3, 123.7, 122.4, 93.1, 80.9, 51.6, 43.9, 29.9 (overlaps with the solvent signal, appears in DEPT), 28.6 HRMS (ESI) m/z: $[M+Na]^+$ Calcd for $C_{26}H_{16}Co_2N_2O_{10}S_2Na^+$: 720.8803; Found: 720.8804.

Deprotection of cycloalkynes from Co (General procedure (3))

To a stirred solution of cyclic Co-complex in a mixture of acetone/water (15:1, v/v, c = 0.006 M), tetrabutylammonium fluoride hydrate (TBAF hydrate) (18.0 – 20.0 equiv) was added in four portions with the interval of 45 min. After completion of the reaction, the reaction mixture was filtered through a pad

of Celite, the sorbent was washed with acetone, and the resulting solution was concentrated under reduced pressure at 25 °C up to ~ 1/5 of the original volume; the resulting mixture was mixed with ethyl acetate and brine. The organic layer was separated, and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed three times with brine, dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure at 25 °C. The crude product was purified by column chromatography on silica gel.

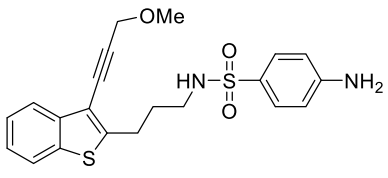
1,2-Didehydro-4-([4-nitrophenyl]sulfonyl)-4,5,6,7-tetrahydro-3*H*-benzo[4,5]thieno[2,3-*e*]azonine (BT9N-NO_2)



BT9N-NO_2

Cycloalkyne **BT9N-NO_2** was obtained according to the ***General procedure (3)*** from cyclic Co-complex **5** (115 mg, 0.165 mmol, 1.00 equiv), TBAF·H_2O (828 mg, 2.96 mmol, 18.0 equiv, added in 4 portions) in the mixture acetone / H_2O (15:1, 27.4 mL). The reaction time was 4 hours. Purification of the crude product by column chromatography (eluent: hexane / ethyl acetate = 4:1) gave **BT9N-NO_2** (6.00 mg, 9 %) as a white powder. ^{1}H NMR (400 MHz, CD_3CN) δ 8.39 – 8.34 (m, 2H), 8.09 – 8.04 (m, 2H), 7.86 – 7.82 (m, 1H), 7.67 – 7.64 (m, 1H), 7.44 – 7.33 (m, 2H), 4.36 (s, 2H), 3.47 (t, *J* = 6.1 Hz, 2H), 3.10 – 3.03 (m, 2H), 2.22 – 2.16 (m, 2H). ^{13}C NMR was not measured due to the instability of cycloalkyne **BT9N-NO_2**. DEPT NMR (101 MHz, CD_3CN) δ 129.5, 125.9, 125.6, 125.5, 123.4, 122.6, 48.9, 42.6, 30.7, 27.3. HRMS (ESI) m/z: $[M+Na]^+$ Calcd for $C_{20}H_{16}N_2O_4S_2Na^+$: 435.0444; Found: 435.0443.

4-Amino-*N*-[3-{3-(3-methoxyprop-1-yn-1-yl)benzo[*b*]thiophen-2-yl}propyl]benzenesulfonamide (6)

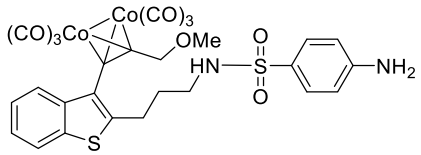


6

In a two-necked round bottom flask to a stirred solution of nitro compound **3** (230 mg, 0.571 mmol, 1.00 equiv) in the mixture of acetone / H_2O (1:1, c = 0.01 M, 52.0 mL) were added iron powder (289 mg, 5.17 mmol, 10.0 equiv) and NH_4Cl (277 mg, 5.17 mmol, 10.0 equiv). The reaction mixture was stirred at 40 °C for 48 hours (TLC control). After completion of the reaction, the reaction mixture was cooled, poured into a saturated aqueous solution of NaCl (100 mL), and extracted with ethyl acetate three times (3 × 75.0 mL). The combined organic layers were dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to give the crude product, which was purified by column chromatography on silica gel (eluent:

benzene / ethyl acetate = 3:1) that gave aminobenzenesulfonamide **6** (172 mg, 80 %) as a yellow oil. ^{1}H NMR (400 MHz, acetone-d_6) δ 7.86 (d, *J* = 7.9 Hz, 1H), 7.79 (d, *J* = 7.9 Hz, 1H), 7.56 – 7.50 (m, 2H), 7.46 – 7.40 (m, 1H), 7.39 – 7.33 (m, 1H), 6.75 – 6.71 (m, 2H), 6.11 (t, *J* = 6.0 Hz, 1H), 5.39 (br. s, 2H), 4.44 (s, 2H), 3.45 (s, 3H), 3.10 (t, *J* = 7.6 Hz, 2H), 3.02 – 2.94 (m, 2H), 1.99 – 1.89 (m, 2H). ^{13}C NMR (101 MHz, acetone-d_6) δ 153.2, 150.8, 140.6, 138.3, 129.7, 128.1, 125.8, 125.6, 123.2, 123.0, 115.7, 114.1, 92.2, 79.5, 60.7, 57.6, 43.3, 31.7, 27.7. HRMS (ESI) m/z: $[M+Na]^+$ Calcd for $C_{21}H_{22}N_2O_3S_2Na^+$: 437.0964; Found: 437.0958.

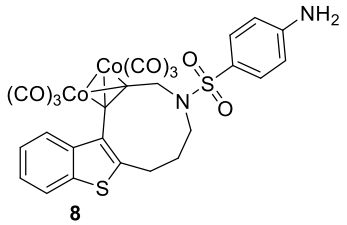
Hexacarbonyl(4-amino-*N*-[3-{3-(3-methoxyprop-1-μ$_2$-[1,2-η^2,η^2]yn-1-yl)benzo[*b*]thiophen-2-yl}propyl]benzenesulfonamide)dicobalt (7)



7

Acyclic Co-complex **7** was synthesized in accordance with the ***General procedure (1)*** from alkynylbenzotiophene **6** (500 mg, 1.21 mmol, 1.00 equiv) in benzene (c = 0.01 M, 121 mL) and octacarbonyldicobalt (619 mg, 1.81 mmol, 1.50 equiv). Reaction time was 2 hours (TLC control). Purification of the crude product by column chromatography (eluent: hexane / ethyl acetate = 1:2) gave acyclic Co-complex **7** (677 mg, 80%) as dark-brown powder. ^{1}H NMR (400 MHz, acetone-d_6) δ 8.07 (d, *J* = 7.5 Hz, 1H), 7.90 (d, *J* = 7.5 Hz, 1H), 7.55 (d, *J* = 7.7 Hz, 2H), 7.51 – 7.45 (m, 1H), 7.42 – 7.35 (m, 1H), 6.75 (d, *J* = 7.7 Hz, 2H), 6.24 (br. s, 1H), 5.42 (br. s, 2H), 5.05 (s, 2H), 3.62 (s, 3H), 3.32 – 3.16 (m, 2H), 3.14 – 3.02 (m, 2H), 2.00 (m, 2H, overlaps with the solvent signal). ^{13}C NMR (101 MHz, acetone-d_6) δ 200.9 (CO signals), 153.3, 147.4, 141.0, 138.7, 129.8, 128.1, 126.1, 125.3, 125.0, 123.3, 123.1, 114.1, 97.3, 78.9, 74.6, 59.2, 43.6, 32.1, 28.0. HRMS (ESI) m/z: $[M+Na]^+$ Calcd for $C_{27}H_{22}Co_2N_2O_9S_2Na^+$: 722.9323; Found: 722.9330.

Hexacarbonyl-4-[{1,2-μ$_2$-(1,2-η^2)didehydro-3,5,6,7-tetrahydro-4*H*-benzo[4,5]thieno[2,3-*e*]azonin-4-yl} sulfonyl]aniline)dicobalt (8)



8

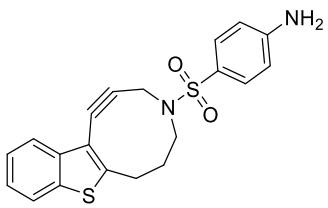
Co-complex **8** was synthesized in accordance with the ***General procedure (2)*** from acyclic Co-complex **7** (170 mg, 0.243 mmol, 1.00 equiv), boron trifluoride etherate (51.7 mg, 0.0457 mL, 0.364 mmol, 1.50 equiv) in DCM (c = 0.001 M, 243 mL). Reaction time was 2 h (TLC control). Purification of the crude product

by column chromatography (eluent: hexane / ethyl acetate = 2:1) gave cyclic Co-complex **8** (136 mg, 80%) as a viscous black oil.

Co-complex **8** was also obtained from alkyne **6** by Co complexation / Nicholas cyclization in one-pot.

In a three necked round bottom flask to a solution of alkynylbenzotiophene **6** (930 mg, 2.24 mmol, 1.00 equiv) in dry DCM (c = 0.01 M, 224 mL) was added octacarbonyldicobalt (1.15 g, 3.37 mmol, 1.50 equiv), and the mixture was stirred under a flow of Ar at room temperature for 2 hours (TLC control). After the reaction was completed, the mixture was diluted with more dry DCM (c = 0.0035 M, 416 mL). Then the reaction mixture was cooled to 0 °C under an argon atmosphere, and boron trifluoride diethyl etherate complex (414 mg, 0.366 mL, 2.91 mmol, 1.30 equiv) was added in one portion. The cooling bath was immediately removed, and the resulting solution was allowed to warm to room temperature and then stirred at this temperature for 15 min (TLC control). Then the reaction mixture was quenched with a 5% aqueous solution of $NaHCO_3$ (300 mL). The organic layer was separated, washed with brine (300 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to yield a crude product, which was purified by column chromatography on silica gel (eluent: hexane / ethyl acetate = 2:1) that gave cyclic Co-complex **8** (1.40 g, 93 %) as a viscous black oil. ^{1}H NMR (400 MHz, acetone-d_6) δ 8.18 (d, *J* = 8.1 Hz, 1H), 7.94 (d, *J* = 8.1 Hz, 1H), 7.60 (d, *J* = 8.7 Hz, 2H), 7.56 – 7.49 (m, 1H), 7.46 – 7.39 (m, 1H), 6.80 (d, *J* = 8.7 Hz, 2H), 5.55 (br. s, 2H), 5.09 (s, 2H), 3.44 – 3.27 (m, 2H), 3.20 (t, *J* = 6.7 Hz, 2H), 2.03-1.98 (m, 2H). ^{13}C NMR (101 MHz, acetone-d_6) δ 200.6 (CO signals), 153.8, 144.6, 141.1, 139.8, 130.0, 127.6, 127.0, 125.5, 125.2, 123.7, 122.5, 114.3, 94.7, 81.0, 51.8, 44.2, 30.0 (overlaps with solvent signal, appears in DEPT), 28.4. HRMS (ESI) m/z: $[M+Na]^+$ Calcd for $C_{26}H_{18}Co_2N_2O_8S_2Na^+$: 690.9061; Found: 690.9068.

4-([1,2-Didehydro-3,5,6,7-tetrahydro-4*H*-benzo[4,5]thieno[2,3-*e*]azonin-4-yl]sulfonyl)aniline (BT9N-NH$_2$)



BT9N-NH$_2$

Cycloalkyne **BT9N-NH$_2$** was obtained according to the ***General procedure (3)*** from cyclic Co-complex **8** (500 mg, 0.748 mmol, 1.00 equiv), TBAF×H_2O (4.18 g, 14.9 mmol, 20.0 equiv added in 4 portions) in the mixture acetone/H_2O (15:1, 125 mL). The reaction time was 5 hours. Purificsation of the crude product by column chromatography (eluent: hexane / ethyl acetate = 2:1) gave **BT9N-NH$_2$** (220 mg, 77 %) as a yellowish powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (d, *J* = 7.9 Hz, 1H), 7.66 (d, *J* = 7.9 Hz, 1H), 7.64 – 7.60 (m, 2H), 7.39 – 7.34 (m, 1H), 7.33 – 7.27 (m, 1H), 6.75 – 6.63 (m, 2H), 4.21 (s, 2H), 4.11 (br. s, 2H), 3.37 (t,

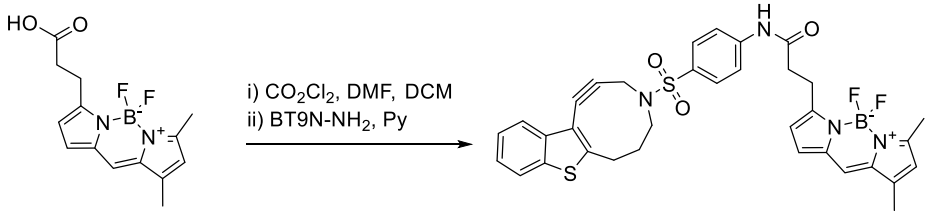
J = 6.1 Hz, 2H), 3.11 – 3.07 (m, 2H), 2.23 – 2.14 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 153.9, 150.8, 137.6, 136.8, 129.6, 126.5, 125.0, 124.6, 122.4, 122.3, 115.4, 114.3, 99.4, 90.8, 47.4, 42.1, 30.9, 26.6. HRMS (ESI) m/z: $[M+Na]^+$ Calcd for $C_{20}H_{18}N_2O_2S_2Na^+$: 405.0702; Found: 405.0708.

2.2 Synthesis of cycloalkyne-dye conjugates

Acylation of BT9N-NH_2 with fluorescent dyes acid chlorides *(General procedure (4))*

A **dye carboxylic acid** was dissolved in absolute DCM (c = 0.02 M) and the mixture was cooled to 0 °C under an argon atmosphere. Oxalyl chloride (3.00 equiv) and DMF (0.25 equiv) were added in one portion. A cooling bath was immediately removed, and the resulting solution was allowed to warm to room temperature and then stirred at this temperature for 1 hour (TLC control). After completion of the reaction, the excess of oxalyl chloride and the solvent was removed under vacuum. To the resulting dye acid chloride was added a mixture of **BT9N-NH_2** (0.80 equiv) and absolute pyridine (1.00 equiv) in absolute DCM (c = 0.02 M) under argon atmosphere. The resulting solution was stirred at room temperature for 30 minutes (TLC control). After completion of the reaction, the resulting mixture was diluted with DCM and quenched with brine. The organic layer was separated, and the aqueous layer was extracted with DCM. The combined organic layers were washed three times with brine, dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure at 25 °C. The crude product was purified by column chromatography on silica gel.

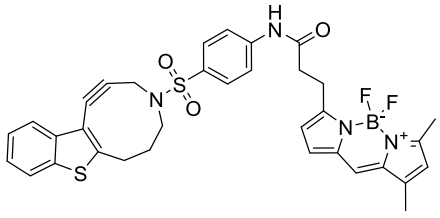
2.2.1 Synthesis of BT9N-BDP FL



BDP FL-acid **BT9N-BDP FL**

Scheme S2 Synthesis of **BT9N-BDP FL.**

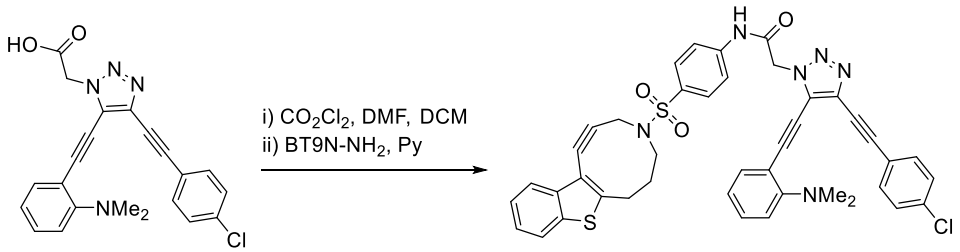
3-(5,5-difluoro-7,9-dimethyl-5*H*-5λ^4,6λ^4-dipyrrolo[1,2-c:2',1'-*f*][1,3,2]diazaborinin-3-yl)-*N*-(4-[{1,2-didehydro-3,5,6,7-tetrahydro-4*H*-benzo[4,5]thieno[2,3-*e*]azonin-4-yl}sulfonyl]phenyl)propanamide (BT9N-BDP FL)



BT9N-BDP FL

Cycloalkyne **BT9N-BDP FL** was obtained according to the ***General procedure (4)*** from **BDP-FL acid** (Lumiprobe) (5.75 mg, 0.0197 mmol, 1.00 equiv), oxalyl chloride (7.50 mg, 5.06 µL, 0.0591 mmol, 3.00 equiv), DMF (0.360 mg, 0.381 µL, 0.00492 mmol, 0.25 equiv) in absolute DCM (c = 0.02 M, V = 0.985 mL) and **BT9N-NH$_2$** (5.80 mg, 0.0157 mmol, 0.70 equiv), pyridine (1.20 mg, 1.22 µL, 0.0156 mmol, 1.00 equiv) in absolute DCM (c = 0.02 M, V = 0.785 mL). Purification of the crude product by column chromatography (eluent: hexane / ethyl acetate = 2:1) gave **BT9N-BDP FL** (8.10 mg, 81 %) as a dark red powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.44 (s, 1H), 7.97 – 7.92 (m, 1H), 7.88 – 7.75 (m, 4H), 7.70 (s, 1H), 7.65 – 7.60 (m, 1H), 7.46 – 7.32 (m, 2H), 7.08 (d, *J* = 3.9 Hz, 1H), 6.37 (d, *J* = 3.9 Hz, 1H), 6.31 (s, 1H), 4.29 (s, 2H), 3.30 – 3.27 (m, 2H, overlaps with water signal, visible in HSQC), 3.18 (t, *J* = 7.4 Hz, 2H), 3.08 – 3.01 (m, 2H), 2.80 (t, *J* = 7.4 Hz, 2H), 2.47 (s, 3H), 2.26 (s, 3H), 2.14 – 2.02 (m, 2H). ^{19}F NMR (376 MHz, DMSO-d_6) δ −143.2 (q, J(^{11}B-^{19}F) = 33.5 Hz, 2F). ^{13}C NMR (126 MHz, DMSO-d_6) δ 170.7, 159.5, 157.1, 153.7, 144.4, 143.3, 136.7, 136.0, 134.6, 133.0, 131.6, 128.9, 128.3, 125.5, 125.2, 124.7, 122.8, 121.6, 120.4, 119.0, 116.5, 114.4, 100.1, 89.2, 47.6, 41.5, 34.9, 29.6, 26.1, 23.5, 14.6, 11.0. HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{34}H_{32}BF_2N_4O_3S_2^+$: 657.1977; Found: 657.1970.

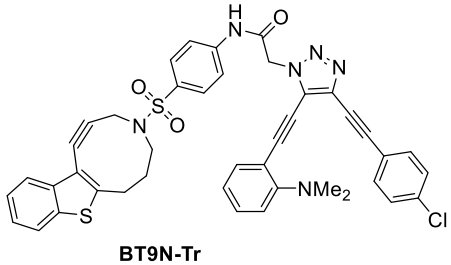
2.2.2 Synthesis BT9N-Tr



Triazole acid **BT9N-Tr**

Scheme S3 Synthesis of **BT9N-Tr.**

2-(4-[{4-Chlorophenyl}ethynyl]-5-[{2-(dimethylamino)phenyl}ethynyl]-1*H*-1,2,3-triazol-1-yl)-*N*-(4-[{1,2-didehydro-3,5,6,7-tetrahydro-4*H*-benzo[4,5]thieno[2,3-*e*]azonin-4-yl}sulfonyl]phenyl)acetamide (BT9N-Tr)

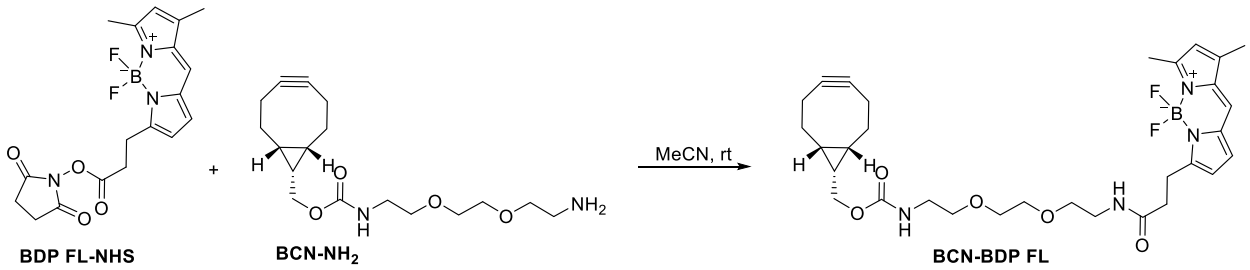


BT9N-Tr

Cycloalkyne **BT9N-Tr** was obtained according to the ***General procedure (4)*** from **Triazole acid**[3] (4.00 mg, 1.00 equiv, 0.00988 mmol), oxalyl chloride (3.76 mg, 2.54 µL, 3.00 equiv, 0.0296 mmol), DMF (0.18 mg, 0.191 µL, 0.25 equiv, 0.00247 mmol) in absolute DCM (c = 0.02 M, V = 0.494 mL) and **BT9N-NH$_2$** (2.57 mg, 0.00672 mmol, 0.50 equiv), pyridine (0.532 mg, 0.541 µL, 1.00 equiv, 0.00672 mmol) in absolute DCM (c

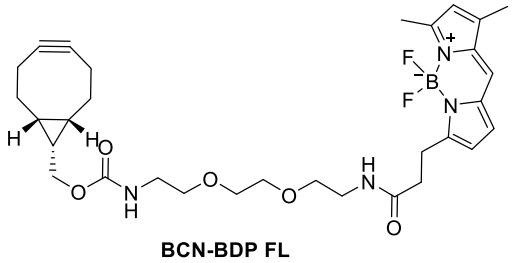
= 0.02 M, V = 0.494 mL). Purification of the crude product by column chromatography (eluent: hexane/ethyl acetate = 2:1) gave **BT9N-Tr** (4.00 mg, 77 %) as a yellow powder. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.06 (s, 1H), 7.94 (d, *J* = 7.9 Hz, 1H), 7.85 (d, *J* = 9.0 Hz, 2H), 7.81 (d, *J* = 9.0 Hz, 2H), 7.64 – 7.60 (m, 3H), 7.58 – 7.53 (m, 2H), 7.44 – 7.39 (m, 1H), 7.38 – 7.34 (m, 2H), 7.29 – 7.22 (m, 1H), 6.84 (d, *J* = 8.3 Hz, 1H), 6.79 (t, *J* = 7.4 Hz, 1H), 5.52 (s, 2H), 4.30 (s, 2H), 3.06 – 2.98 (m, 2H), 2.78 (s, 6H), 2.11 – 2.03 (m, 2H). Signal of one CH_2 group completely overlaps with water signal, visible in HSQC. ^{13}C NMR (126 MHz, DMSO-d_6) δ 164.2, 154.8, 153.7, 142.4, 136.8, 136.1, 134.5, 134.48, 133.2, 132.7, 131.4, 131.35, 129.3, 128.5, 125.5, 125.3, 124.8, 122.9, 121.6, 120.0, 119.9, 119.3, 117.1, 114.3, 110.6, 103.5, 99.9, 93.8, 89.2, 79.4, 77.6, 52.0, 47.7, 43.0, 41.5, 29.4, 26.3. HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{42}H_{34}ClN_6O_3S_2^+$: 769.1817; Found: 769.1810.

2.2.3 Synthesis BCN-BDP FL



Scheme S4 Synthesis of **BCN-BDP FL.**

([1*R*,8*S*,9*s*]-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2-[2-{2-(3-[5,5-difluoro-7,9-dimethyl-5*H*-5λ^4,6λ^4-dipyrrolo[1,2-*c*:2',1'-*f*][1,3,2]diazaborinin-3-yl]propanamido)ethoxy}ethoxy]ethyl)carbamate (BCN-BDP FL)



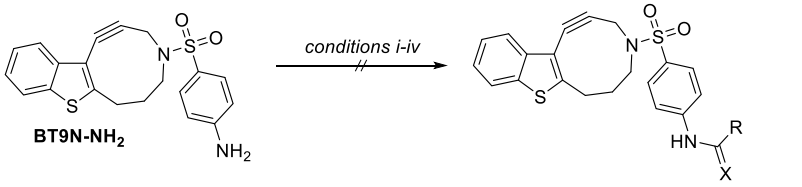
BCN-amine (Sigma-Aldrich) (3.00 mg, 0.00925 mmol, 1.00 equiv) was dissolved in MeCN (1.00 mL). **BDP FL NHS ester** (Lumiprobe) (3.96 mg. 0.0102 mmol, 1.10 equiv) was added to the reaction mixture, and the resulting solution was stirred at room temperature for 3 hours (TLC control). After completion of the reaction, the resulting mixture was mixed with ethyl acetate (15.0 mL) and brine (15.0 mL). The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (10.0 mL). The combined organic layers were washed three times with brine (20.0 mL), dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure at 25 °C. Purification of the crude product by column

chromatography (eluent: hexane / ethyl acetate = 1:1) gave **BCN-BDP FL** (3.80 mg, 69 %) as a red powder. ^{1}H NMR (400 MHz, acetone-d_6) δ 7.53 (s, 1H), 7.16 (br. s, 1H), 7.06 (d, *J* = 3.9 Hz, 1H), 6.40 (d, *J* = 3.9 Hz, 1H), 6.27 (s, 1H), 6.16 (br. s, 1H), 4.12 (d, *J* = 8.0 Hz, 2H), 3.58 (br. s, 4H), 3.56 – 3.50 (m, 4H), 3.41 – 3.37 (m, 2H), 3.33 – 3.20 (m, 4H), 2.62 (t, *J* = 7.7 Hz, 2H), 2.53 (s, 3H), 2.31 (s, 3H), 2.24 – 2.12 (m, 6H), 1.67 – 1.52 (m, 2H), 1.39 – 1.26 (m, 1H), 0.98 – 0.83 (m, 2H). ^{19}F NMR (376 MHz, acetone-d_6) δ -145.1 (q, $J(^{11}B\text{-}^{19}F)$ = 32.8 Hz, 2F). ^{13}C NMR (101 MHz, Acetone-d_6) δ 171.8, 160.3, 159.6, 157.5, 144.9, 135.8, 134.5, 129.6, 125.6, 121.0, 117.8, 99.4, 71.03, 70.98, 70.64, 70.56, 62.6, 41.5, 40.0, 35.4, 25.2, 21.7, 20.9, 18.8, 14.9, 11.2 (one CH_2 signal overlaps with the solvent signal, appears in DEPT and HSQC). HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{31}H_{42}BF_2N_4O_5^+$: 599.3216; Found: 599.3219.

2.3 Test reactions for functionalization of BT9N-NH$_2$

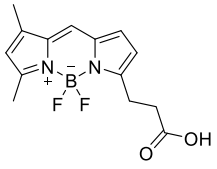
2.3.1 Reactions with acid / HATU, FITC, anhydrides and NHS-ester

Several types of reactions were tested: acylation with **BDP FL acid** in the presence of peptide coupling reagents (HATU, Scheme S2, conditions i),[5,6] interaction of **BT9N-NH$_2$** with the isothiocyanate (fluorescein isothiocyanate (5-FITC), Scheme S2, conditions ii),[7,8] acylation with cyclic anhydrides (succinic and glutaric anhydrides, Scheme S2, conditions iii)[9,10] and acylation using NHS-ester (Scheme S2, conditions iv). All these reactions either gave only unreacted staring **BT9N-NH$_2$** or led to the decomposition of **BT9N-NH$_2$**.



Conditions i
(recovering of starting BT9N-NH$_2$)

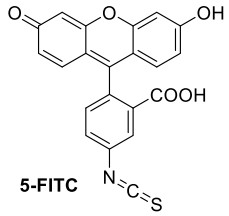
BDP FL-COOH, HATU, DIPEA,
DMF, 40 °C, up to 4 days



BDP FL-COOH

Conditions ii
(recovering of starting BT9N-NH$_2$)

a) 5-FITC, DIPEA, MeCN or THF or DCM, 40 °C, up to 4 days
b) 5-FITC, Et_3N, MeCN or THF or DCM, 40 °C, up to 4 days



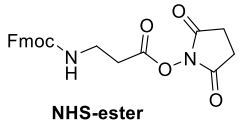
5-FITC

Conditions iii
(recovering of starting BT9N-NH$_2$ with partly decomposition of BT9N-NH$_2$)

a) Succinic anhydride, DMAP, Et_3N,
MeCN, 60 °C, up to 2 days
b) Glutaric anhydride, DMAP, Et_3N,
MeCN, 85 °C, up to 12 days

Conditions iv
(recovering of starting BT9N-NH$_2$ with partly decomposition of BT9N-NH$_2$)

NHS-ester, TEA, MeCN, 60 °C, up to 4 days

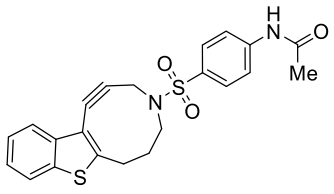


NHS-ester

Scheme S5 Test conditions for the modification of amino group in **BT9N-NH$_2$.**

2.3.2 Acylation of BT9N-NH_2 by AcCl

***N*-(4-[{1,2-didehydro-3,5,6,7-tetrahydro-4*H*-benzo[4,5]thieno[2,3-*e*]azonin-4-yl}sulfonyl]phenyl)acetamide (S2)**



S2

BT9N-NH_2 (2.00 mg, 0.00523 mmol, 1.00 equiv) and DMAP (1.28 mg, 0.0105 mmol, 2.00 equiv) were dissolved in absolute DCM (c = 0.02 M, 0.261 mL). The mixture was cooled to 0 °C under an argon atmosphere. Then acetyl chloride (0.452 mg, 0.448 µL, 0.00627 mmol, 1.20 equiv) was added in one portion. The cooling bath was immediately removed, and the resulting solution was allowed to warm to room temperature, and then stirred at this temperature for 20 minutes (TLC control: hexane / ethyl acetate 5:1). After completion of the reaction, the resulting mixture was mixed DCM (10.0 mL) and brine (10.0 mL). The organic layer was separated, and the aqueous layer was extracted with DCM (10.0 mL). The combined organic layers were washed three times with brine (20.0 mL), dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure at 25 °C. The crude product **S2** (yellow powder, 2.00 mg, 90%) gave pure ^{1}H NMR without any purification. ^{1}H NMR (400 MHz, acetone-d_6) δ 9.59 (br. s, 1H), 7.92 – 7.85 (m, 3H), 7.85 – 7.76 (m, 2H), 7.65 (d, *J* = 8.0 Hz, 1H), 7.45 – 7.39 (m, 1H), 7.38 – 7.33 (m, 1H), 4.32 (s, 2H), 3.43 (t, *J* = 6.1 Hz, 2H), 3.16 – 3.06 (m, 2H), 2.24 – 2.16 (m, 2H), 2.12 (s, 3H). HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{22}H_{21}N_2O_3S_2^+$: 425.0988; Found: 425.0989.

3. MTT Assay

HeLa and HEK293 cells were kindly provided by the cell culture collection of the Institute of Cytology RAS. The day before the assay, HeLa and HEK293 cells were seeded at a concentration of 1 × 10^4 cells per well into the inner wells of 96-well flat-bottom plates, with 200 µL of PBS added to the outer wells. The cells were incubated overnight in a humidified incubator at 37 °C with 5% CO2. The next day, the test compounds were dissolved in growth media at the appropriate concentrations and added to the wells, followed by a 24-hour incubation. After incubation, the medium was replaced with 200 µL fresh media, and 10 µL of MTT solution (5 mg/mL, PanEko, Russia) was added to each well. The cells were then incubated for an additional 2 hours in the humidified incubator to allow for formazan crystal formation. The medium was subsequently removed, and the formazan crystals were dissolved in 150 µL of DMSO (Helicon, Russia). Absorbance was measured at 570 nm using a SPECTROstar Nano microplate reader (BMG LABTECH, Ortenberg, Germany).

The HEK293 cell line exhibited a greater antiproliferative effect at high concentrations of the tested compounds compared to the HeLa cell line (Fig. S1). A compound concentration of 1 µM resulted in 100% cell viability, thus this concentration was used in subsequent experiments.

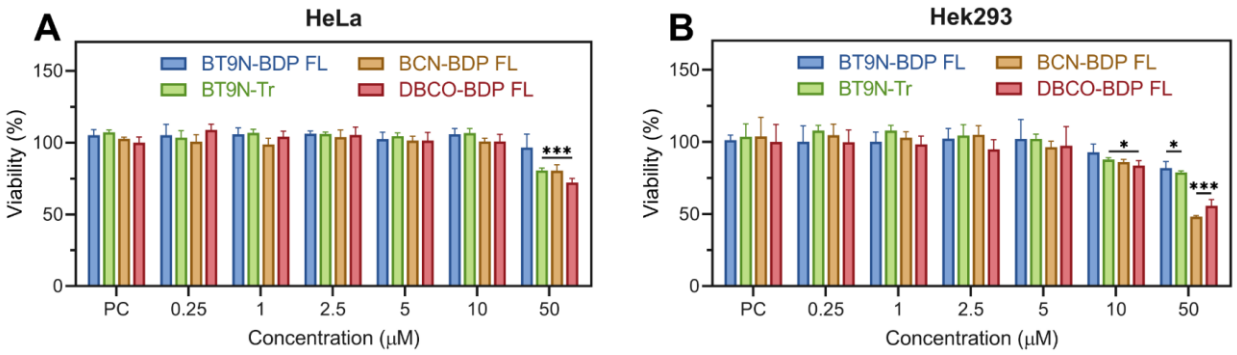


Fig. S1 MTT assay of compounds on the HeLa (A) and HEK293 (B) cell lines. The error bars represent the standard error of the mean (SEM) for the values obtained from three independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's multiple comparisons test; *$p < 0.05$, ***$p < 0.001$.

4. Cell culture and confocal microscopy

4.1 Single labelling experiment

HeLa cells were cultured in DMEM medium (Biolot, Saint-Petersburg, Russia) supplemented with 10% FBS (Cytiva, Marlborough, MA, USA), L-glutamine (Biolot, Saint-Petersburg, Russia), and gentamicin (Biolot, Saint-Petersburg, Russia) at 37 °C with 5% CO2 in a humidified incubator. Cell imaging was performed by plating cells on 35 mm glass Ibidi dishes. After 72 h treatment with the corresponding sugars (50 µM), cells were incubated with the respective CA-dye conjugate (1 µM) for 4 hours. After incubation, the cells were washed twice with DPBS (pH 7.4) and then fixed with formaldehyde fixative for 15 min at room temperature. Following fixation, the cells were washed three times with DPBS. The cells were visualized by using confocal fluorescence inverted Nikon Eclipse Ti2 microscope (Nikon Corporation, Tokyo, Japan). Image analysis was performed using ImageJ software v.1.53c (National Institutes of Health, Bethesda, MY, USA). Graphs were generated and analyzed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA).

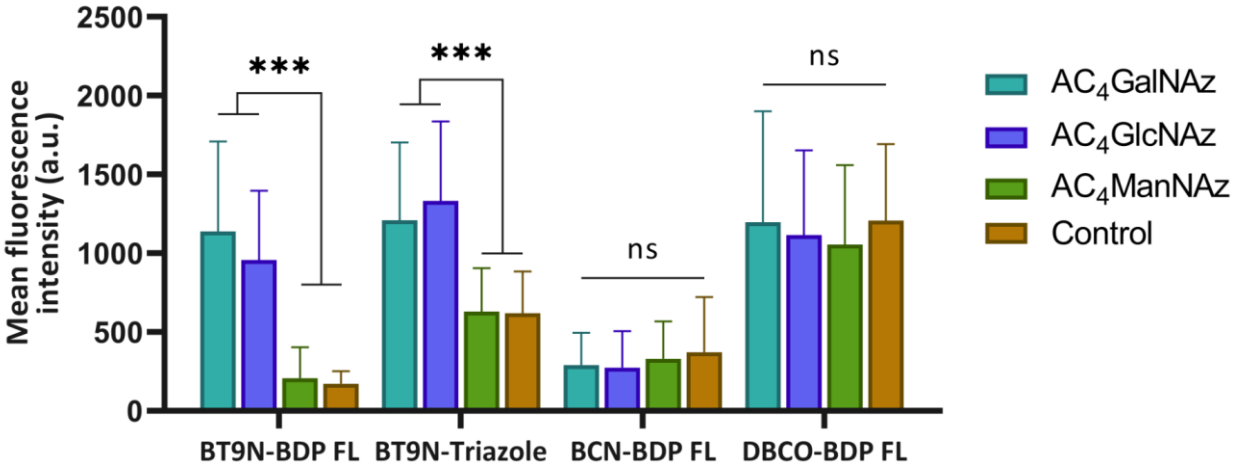


Fig. S2 Mean fluorescence intensity of cycloalkyne-dye conjugates in HeLa cells treated with the corresponding sugars (50 µM, 72 h) and then incubated with the respective conjugate (1 µM) for 4 hours. Graphs represent means ± SD. Data compared by one-way ANOVA with Tukey's multiple comparisons test. ***p-value < 0.001, ns = not significant; n = 5 per group.

4.2 Double labelling experiment

HeLa and HEK293 cells were cultured in DMEM medium (Biolot, Saint-Petersburg, Russia) supplemented with 10% FBS (Cytiva, Marlborough, MA, USA), L-glutamine (Biolot, Saint-Petersburg, Russia), and gentamicin (Biolot, Saint-Petersburg, Russia) at 37 °C with 5% CO2 in a humidified incubator. Cell imaging was performed by plating cells on 35 mm glass Ibidi dishes. After 72 h treatment with the corresponding

sugars (50 μM), cells were incubated first with **BT9N-BDP FL** (1 μM) for 3 hour, followed by two washes with PBS, and subsequent addition of **DBCO-SCy5** in complete growth medium for 30 minutes. After incubation, the cells were washed twice with DPBS (pH 7.4) and then fixed with formaldehyde fixative for 15 min at room temperature. Following fixation, the cells were washed three times with DPBS. The cells were visualized by using confocal fluorescence inverted Nikon Eclipse Ti2 microscope (Nikon Corporation, Tokyo, Japan). Image analysis was performed using ImageJ software v.1.53c (National Institutes of Health, Bethesda, MY, USA). Graphs were generated and analyzed using GraphPad Prism 7 (GraphPad Software, San Diego, CA,

5. Study of the interaction of cycloalkyne dye reagents with glutathione and human globin

MALDI-TOF-MS and MALDI-TOF-MS/MS experiments were performed with a Ultraflextreme mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with a Nd:YAG laser (λ=355 nm). 1 µL of the solution (Table S1) was transferred from each sample to the spot, and 1 µL of matrix solution (CHCA 5 mg/ml in 50% aqueous acetonitrile with 0.1% TFA or DHB 10 mg/ml in 50% aqueous acetonitrile with 0.1% TFA) was added. In the case of **DBCO-BDP FL**, the matrix solution was added twice. The target was then dried at room temperature before MALDI-MS analysis.

Mass spectra of GSH adducts with each alkyne dye reagent were recorded in the reflector positive ion mode under the following parameters: m/z range – 150-800; Ion source voltage 1 – 19.0 kV; Ion source voltage 2 – 16.8 kV; Lens voltage – 7.0 kV; Reflector voltage 1 – 20.5 kV; Reflector voltage 2 – 10.5 kV; Laser repetition rate – 2000 Hz; Pulsed ion extraction delay – 90 ns; Number of accumulated laser shots – 8000. Peptide Calibration Standard II was used for calibration of the mass spectrometer. The following signals were used for calibration of mass spectrometer prior to analysis: m/z 155.034 (DHB $[M+H]^+_{mono}$); m/z 309.061 (DHB $[2M+H]^+_{mono}$); m/z 757.399 (Bradykinin (1-7) $[M+H]^+_{mono}$); m/z 1046.542 (Angiotensin II $[M+H]^+_{mono}$); m/z 1296.685 (Angiotensin I $[M+H]^+_{mono}$).

Mass spectra of Hb adducts with each alkyne dye reagent were acquired in the linear positive ion mode under the following parameters: m/z range – 5000-18000; Ion source voltage 1 – 20.1 kV; Ion source voltage 2 – 18.9 kV; Lens voltage – 5.6 kV; Laser repetition rate – 2000 Hz; Pulsed ion extraction delay – 90 ns; Number of accumulated laser shots – 10000. Protein Calibration Standard I was used for calibration of the mass spectrometer. The following signals were used for calibration of mass spectrometer prior to analysis: m/z 5734.52 (Insulin $[M+H]^+\__{avg}$); m/z 6181.05 (Cytochrome C $[M+2H]^{2+}\__{avg}$); m/z 8565.76 (Ubiquitin I $[M+H]^+\__{avg}$); m/z 12360.97 (Cytochrome C $[M+H]^+\__{avg}$); m/z 16952.31 (Myoglobin $[M+H]^+\__{avg}$).

MALDI-TOF/TOF experiments were carried out in reflector positive ion mode under laser-induced dissociation conditions. The following settings were used: Ion source voltage 1 – 7.6 kV; Ion source voltage 2 – 6.9 kV; Lens voltage – 3.5 kV; Reflector voltage 1 – 29.4 kV; Reflector voltage 2 – 14.1 kV; LIFT 1 – 19.0 kV; LIFT 2 – 3.2 kV; Pulsed ion extraction delay – 90 ns; Precursor ion selector window – 2 m/z; Laser repetition rate – 2000 Hz; Number of accumulated laser shots – 10000.

Table S1. Preparation of samples for MALDI-TOF-MS experiments

Sample	C_1, mM[a]	C_2, mM[b]	C_{DMSO}, %	V_1, µL[c]	V_2, µL[d]	t, h[e]	SI Figure
BT9N-BDP FL	GSH (glutathione)						
	3.3×10^{-3}	3.3×10^{-3}	≤ 1 %	10	90	3	S3, S4, S11
				50	50	3	
				90	10	3	
	HHb (human globin)						
	5×10^{-5}	5×10^{-5}	≤ 0,01%	10	90	3	S12
				50	50	3	
				90	10	3	
BCN-BDP FL	GSH (glutathione)						
	3.3×10^{-3}	3.3×10^{-3}	≤ 1 %	10	90	3	S5, S6, S11
				50	50	3	
				90	10	3	
	HHb (human globin)						
	5×10^{-5}	5×10^{-5}	≤ 0,01%	10	90	3	S13
				50	50	3	
				90	10	3	
BT9N-Tr	GSH (glutathione)						
	3.3×10^{-3}	3.3×10^{-3}	≤ 1 %	10	90	3	S7, S8, S11
				50	50	3	
				90	10	3	
	HHb (human globin)						
	5×10^{-5}	5×10^{-5}	≤ 0,01%	10	90	3	S14
				50	50	3	
				90	10	3	
DBCO-BDP FL	GSH (glutathione)						
	1×10^{-4}	1×10^{-4}	≤ 1 %	10	90	3	S9, S10, S11
				50	50	3	
				90	10	3	
	HHb (human globin)						
	5×10^{-5}	5×10^{-5}	≤ 0,01%	10	90	3	S15
				50	50	3	
				90	10	3	

[a] C_1 – concentration of cycloalkyne-dye solution in 10mM NH_4HCO_3, pH 7.2
[b] C_2 – concentration of the solution of trapping agent (GSH or human globin) in 10mM NH_4HCO_3, pH 7.2
[c] V_1 – volume of cycloalkyne-dye solution
[d] V_2 – volume of trapping agent solution
[e] t – incubation time

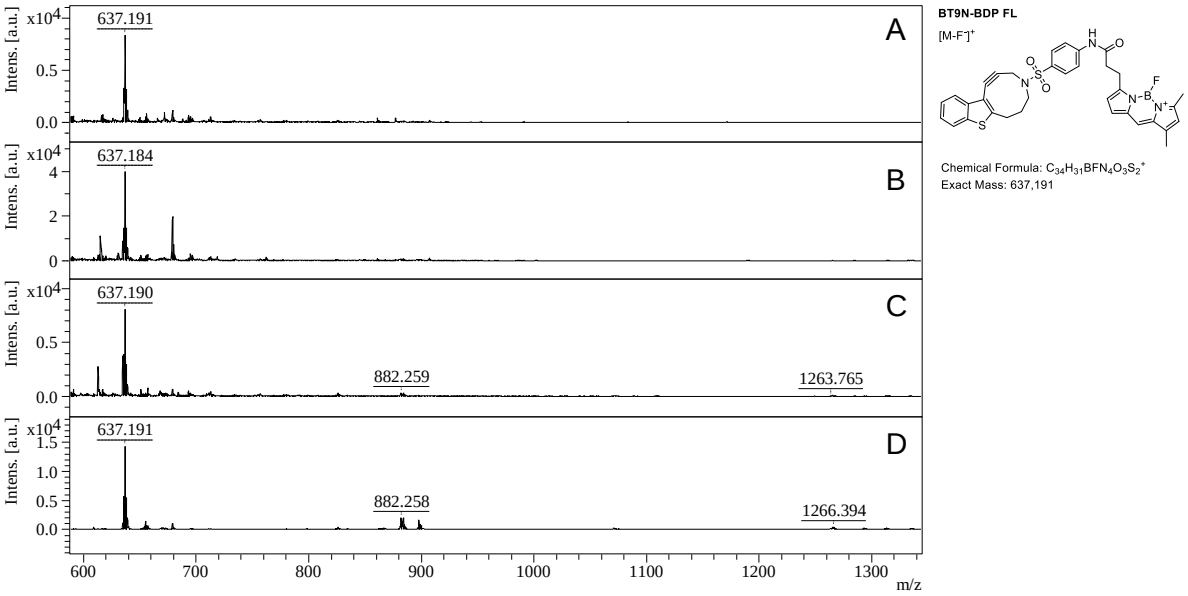


Fig. S3. MALDI TOF mass spectrum of **BT9N-BDP FL** after 3 h incubation with GSH; matrix - alpha-cyano-4-hydroxycinnamic acid (CHCA); A - **BT9N-BDP FL** without any additives, B - **BT9N-BDP FL** / GSH (1:9); C - **BT9N-BDP FL** / GSH (1:1); D - **BT9N-BDP FL** / GSH (9:1).

Adducts with either one GSH $[M+GSH+H]^+$ Exact Mass: 964.281 // $[M+GSH-F^-]^+$ Exact Mass: 944.275 or two GSHs $[M+2GSH+H]^+$ Exact Mass: 1271.365 or $[M+2GSH-F^-]^+$ Exact Mass: 1251.359 were not observed.

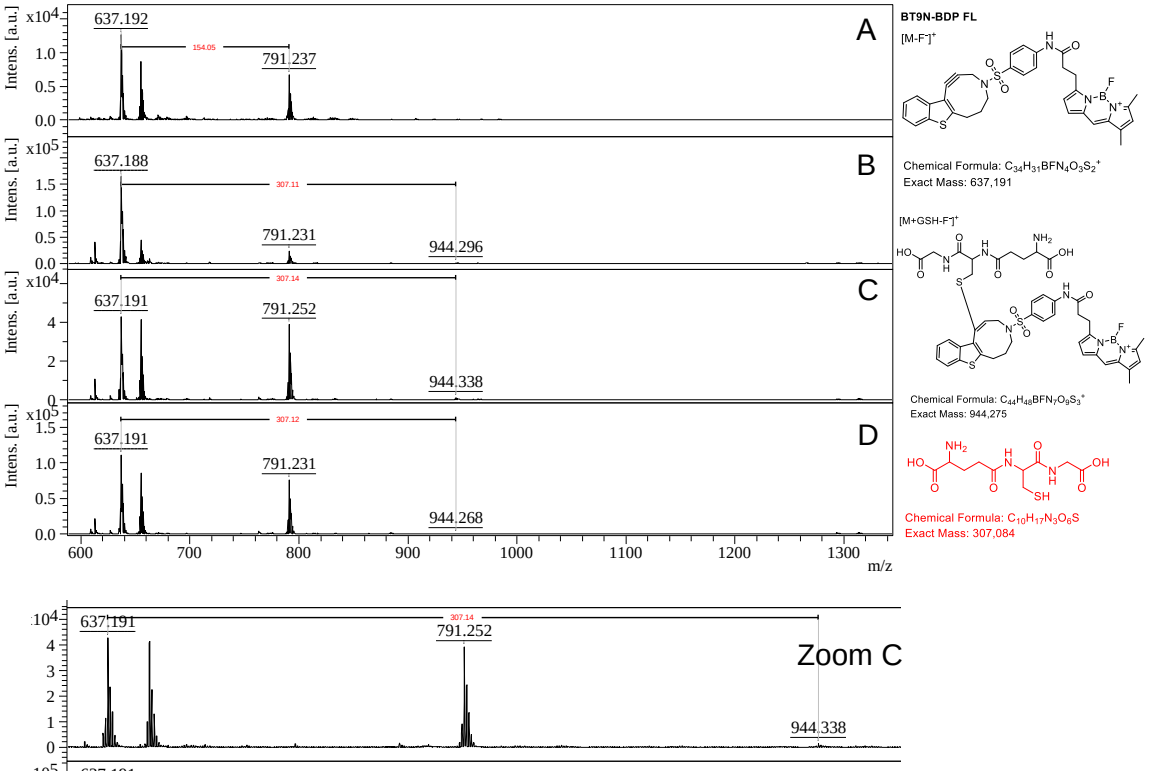


Fig. S4. MALDI TOF mass spectrum of **BT9N-BDP FL** after 3 h incubation with GSH; matrix - 2,5-dihydroxy benzoic acid (DHB) (Δ_{DHB} = 154.03 Da); A - **BT9N-BDP FL** without any additives, B - **BT9N-BDP FL** / GSH (1:9); C - **BT9N-BDP FL** / GSH (1:1); D - **BT9N-BDP FL** / GSH (9:1). 791.237 – adduct of **BT9N-BDP FL** with matrix;

Only weak signals of monoadducts with GSH are observed (Δ_{GSH} = 307.09 Da is marked in red).

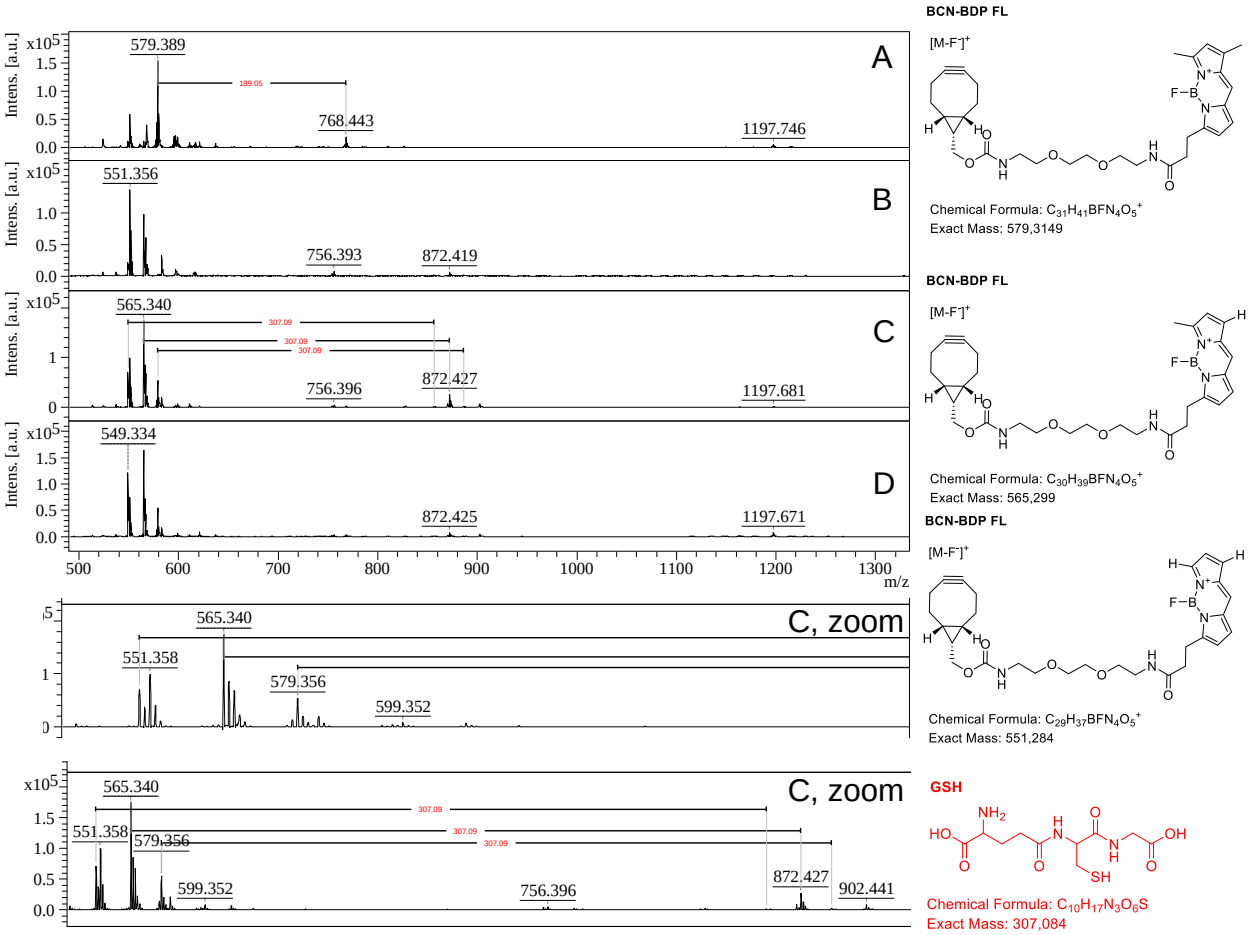


Fig. S5. MALDI TOF mass spectrum of **BCN-BDP FL** after 3 h incubation with GSH; matrix - alpha-cyano-4-hydroxycinnamic acid (CHCA) (Δ_{CHCA} = 189.04 Da); A - **BCN -BDP FL** without any additives, B - **BCN -BDP FL** / GSH (1:9); C - **BCN-BDP FL** / GSH (1:1); D - **BCN-BDP FL** / GSH (9:1).

BCN-BDP FL susceptible to degradation, probably by loss of CH_2; both **BCN-BDP FL** and its demethylated degradants give monoadducts with GSH (Δ_{GSH} = 307.09 Da is marked in red).

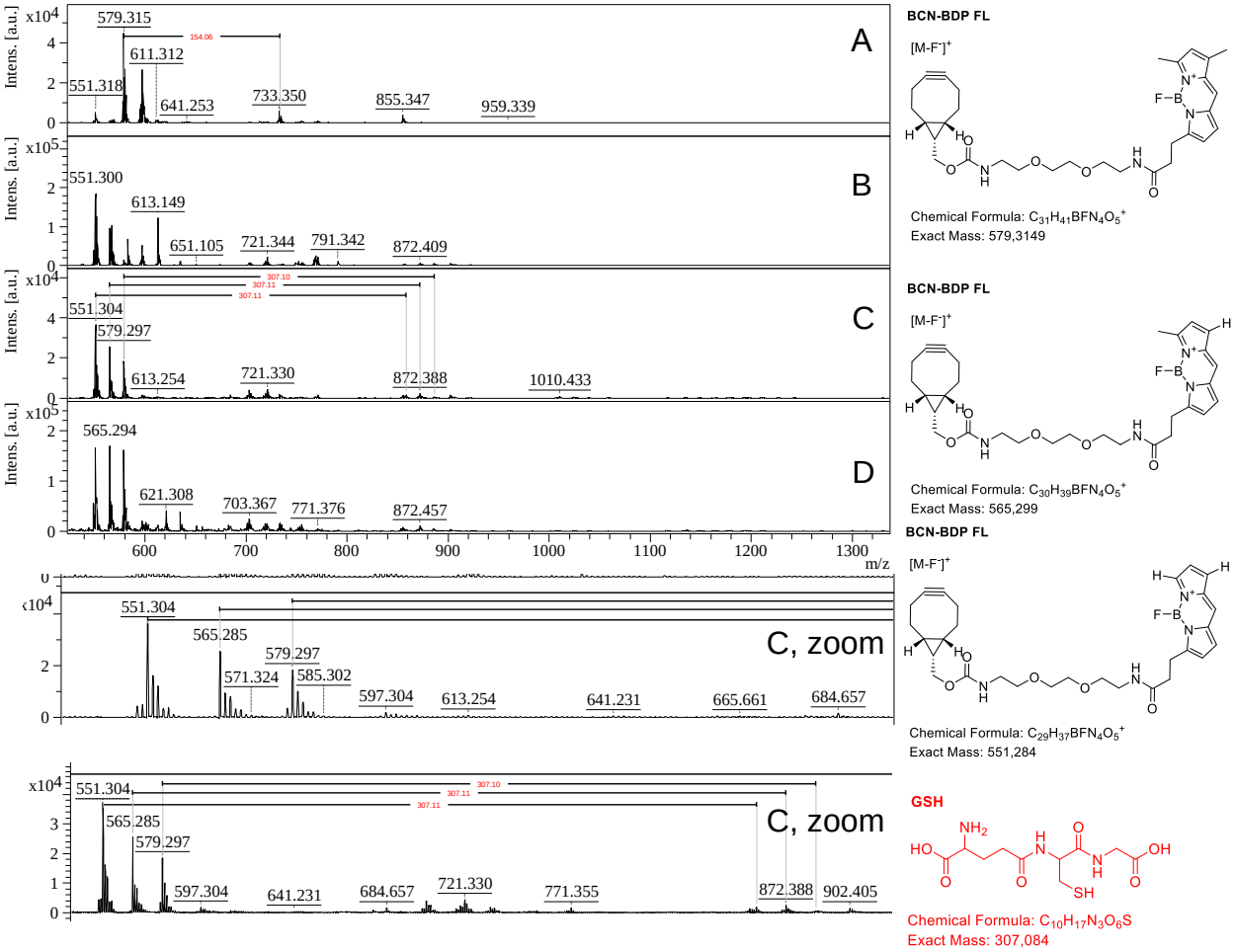


Fig. S6. MALDI TOF mass spectrum of **BCN-BDP FL** after 3 h incubation with GSH; matrix - 2,5-dihydroxy benzoic acid (DHB) (Δ_{DHB} = 154.03 Da); A - **BCN-BDP FL** without any additives, B - **BCN -BDP FL** / GSH (1:9); C - **BCN-BDP FL** / GSH (1:1); D - **BCN-BDP FL** / GSH (9:1).

BCN-BDP FL susceptible to degradation, probably by loss of CH_2; both BCN-BDP FL and its demethylated degradants give monoadducts with GSH (Δ_{GSH} = 307.09 Da is marked in red).

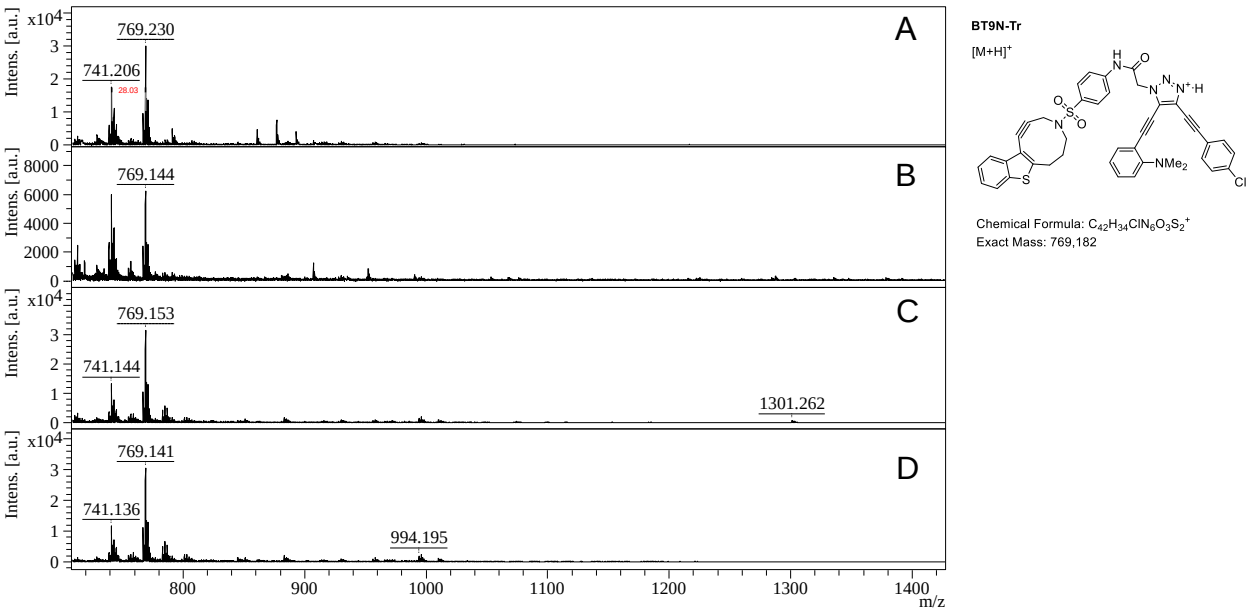


Fig. S7. MALDI TOF mass spectrum of **BT9N-Tr** after 3 h incubation with GSH; matrix - alpha-cyano-4-hydroxycinnamic acid (CHCA); A - **BT9N-Tr** without any additives, B - **BT9N-Tr** / GSH (1:9); C - **BT9N-Tr**/ GSH (1:1); D - **BT9N-Tr** / GSH (9:1).

Adducts with either one GSH $[M+GSH+H]^+$ Exact Mass: 1076.265 or two GSHs $[M+2GSH+H]^+$ Exact Mass: 1383.349 were not observed.

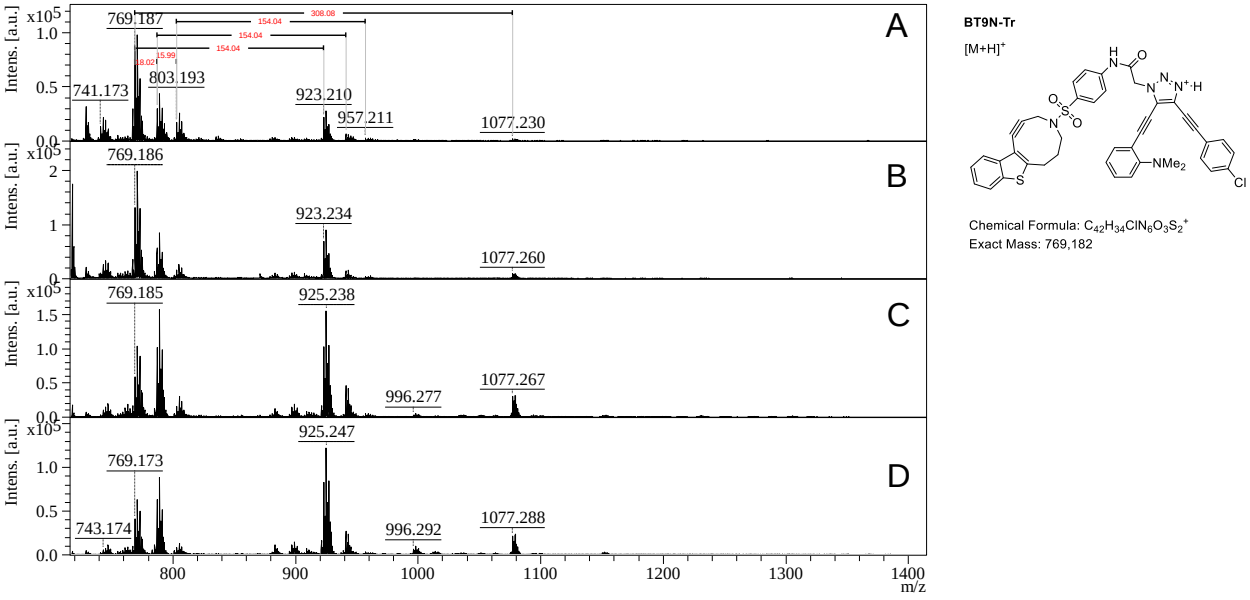


Fig. S8. MALDI TOF mass spectrum of **BT9N-Tr** after 3 h incubation with GSH; matrix – 2,5-dihydroxy benzoic acid (DHB) (Δ_{DHB} = 154.03 Da, 308.6 Da); A - **BT9N-Tr** without any additives, B - **BT9N-Tr** / GSH (1:9); C - **BT9N-Tr**/ GSH (1:1); D - **BT9N-Tr** / GSH (9:1).

BT9N-Tr is unstable under the measurement and gives adducts with water molecule and oxygen (Δ_{H2O} 18.02 Da and Δ_O 15.99 Da are marked in red). Adducts of **BT9N-Tr** and its degradants with either one GSH or two GSHs were not observed.

Adducts with either one GSH $[M+GSH+H]^+$ Exact Mass: 1076.265 or two GSHs $[M+2GSH+H]^+$ Exact Mass: 1383.349 were not observed.

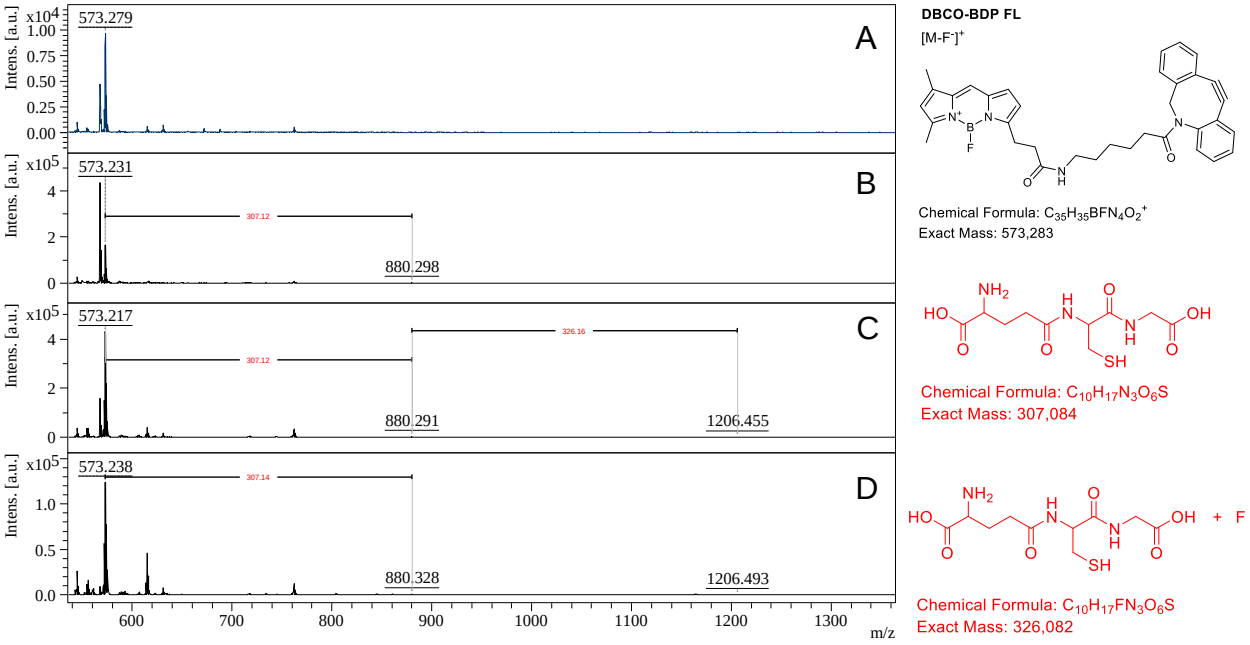


Fig. S9. MALDI TOF mass spectrum of **DBCO-BDP FL** after 3 h incubation with GSH; matrix - alpha-cyano-4-hydroxycinnamic acid (CHCA); A - **DBCO-BDP FL** without any additives, B - **DBCO -BDP FL** / GSH (1:9); C - **DBCO-BDP FL** / GSH (1:1); D - **DBCO-BDP FL** / GSH (9:1).

Monoadducts of **DBCO-BDP FL** with one GSH (Δ_{GSH} = 307.09 Da is marked in red) $[M+GSH+H]^+$ Exact Mass: 880.367 and bisadducts with two GSHs (Δ_{GSH+F} = 326.16 Da is marked in red) $[M+2GSH]^+$ Exact Mass 1206.450 are observed.

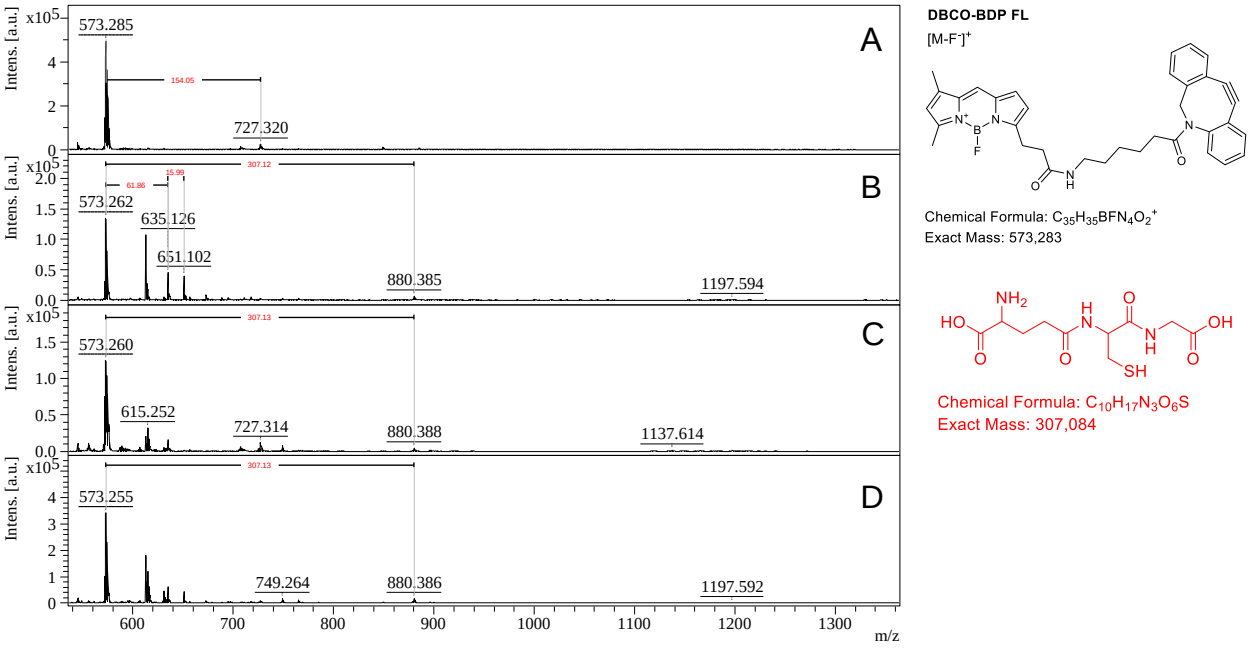


Fig. S10. MALDI TOF mass spectrum of **DBCO-BDP FL** after 3 h incubation with GSH; matrix – 2,5-dihydroxy benzoic acid (DHB) (Δ_{DHB} = 154.03 Da); A - **DBCO-BDP FL** without any additives, B - **DBCO -BDP FL** / GSH (1:9); C - **DBCO-BDP FL** / GSH (1:1); D - **DBCO-BDP FL** / GSH (9:1).

Monoadducts of **DBCO-BDP FL** with one GSH (Δ_{GSH} = 307.09 Da is marked in red) $[M+GSH+H]^+$ Exact Mass: 880.367 are observed.

DHB matrix provides stronger signals of monoadducts, than CHCA matrix.

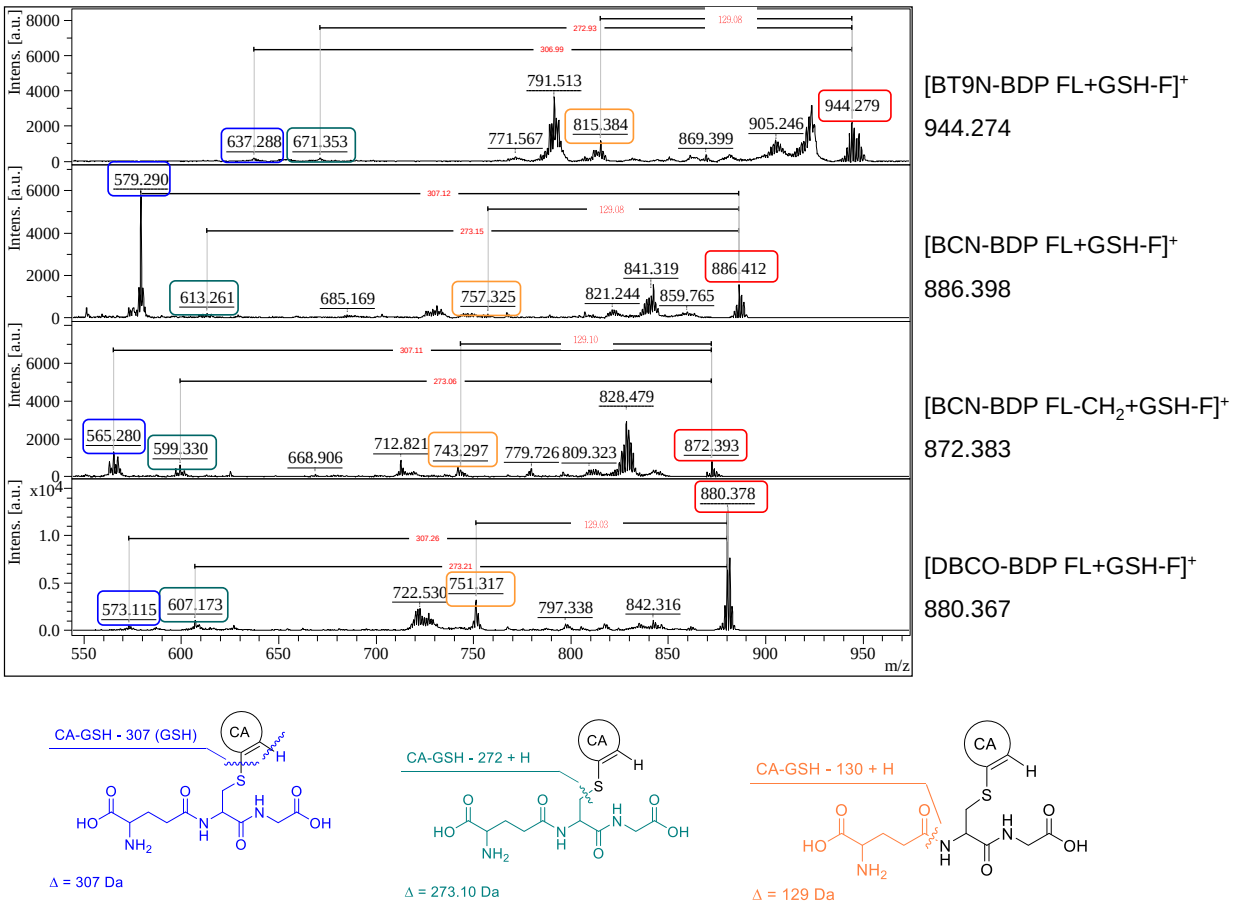


Fig. S11. MALDI TOF-TOF mass spectrum of **BT9N-BDP FL**, **BCN-BDP FL** and **DBCO-BDP FL**; known fragmentation of glutathione conjugates[11] is marked in colors; matrix – 2,5-dihydroxy benzoic acid (DHB)

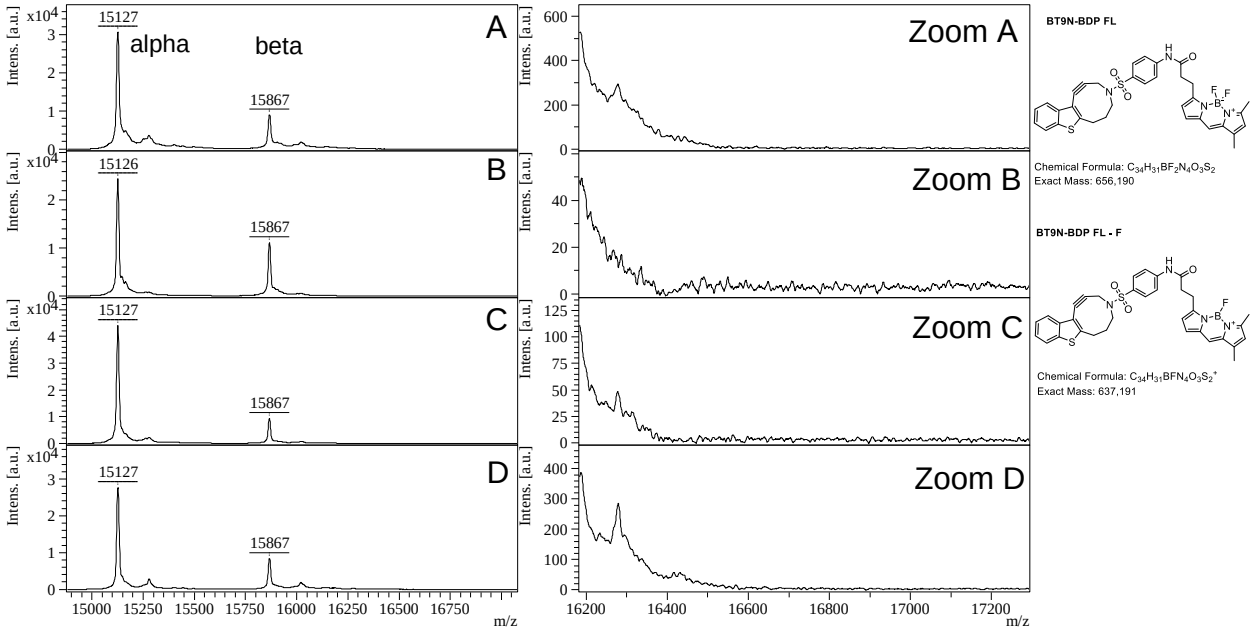


Fig. S12 MALDI TOF mass spectrum of **BT9N-BDP FL** after 3 h incubation with human globin (HHb); matrix – 2,5-dihydroxy benzoic acid (DHB); A - **BT9N -BDP FL** without any additives, B - **BT9N -BDP FL** / HHb (1:9); C - **BT9N -BDP FL** / HHb (1:1); D - **BT9N -BDP FL** / HHb (9:1).
BT9N-BDP FL - HHb adducts were not observed.

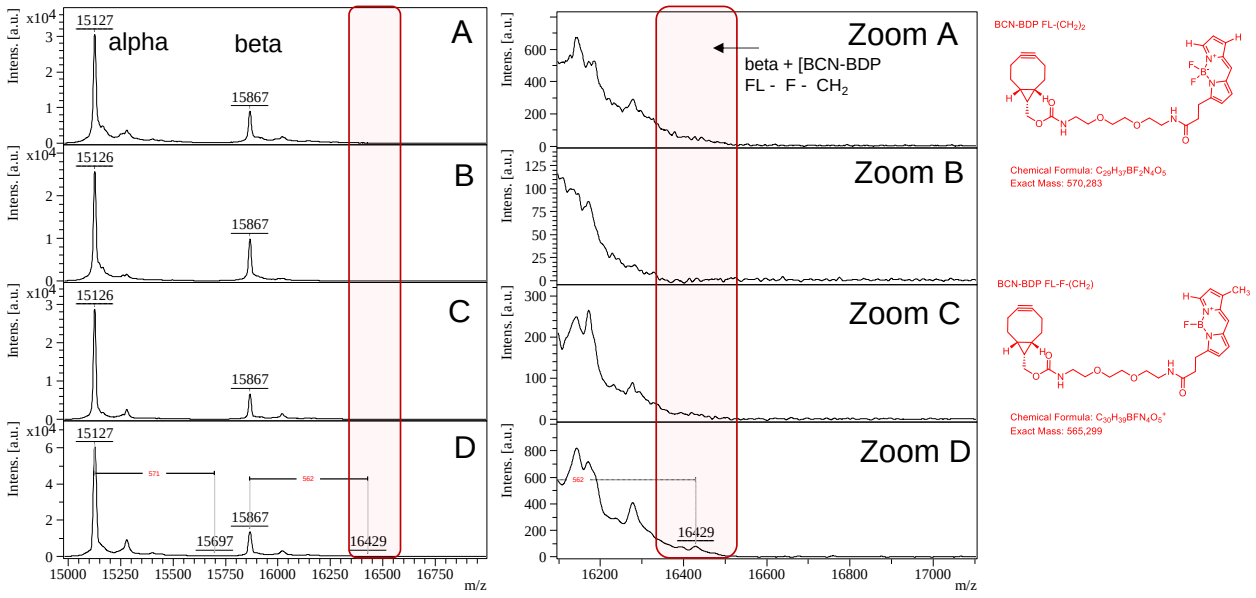


Fig. S13 MALDI TOF mass spectrum of **BCN-BDP FL** after 3 h incubation with human globin (HHb); matrix – 2,5-dihydroxy benzoic acid (DHB); A - **BCN -BDP FL** without any additives, B - **BCN -BDP FL** / HHb (1:9); C - **BCN -BDP FL** / HHb (1:1); D - **BCN -BDP FL** / HHb (9:1).
It is possible to propose **BCN -BDP FL** - HHb adducts through the cysteine residues C-112 or C-93 of the beta globin subunit (15867 Da) and through cysteine C-104 of the alpha subunit (15126 Da). $\Delta_{\text{BCN-BDP FL-2CH2}}$ = 570 Da and $\Delta_{\text{BCN-BDP FL - F - CH2}}$ = 565 Da are marked in red.

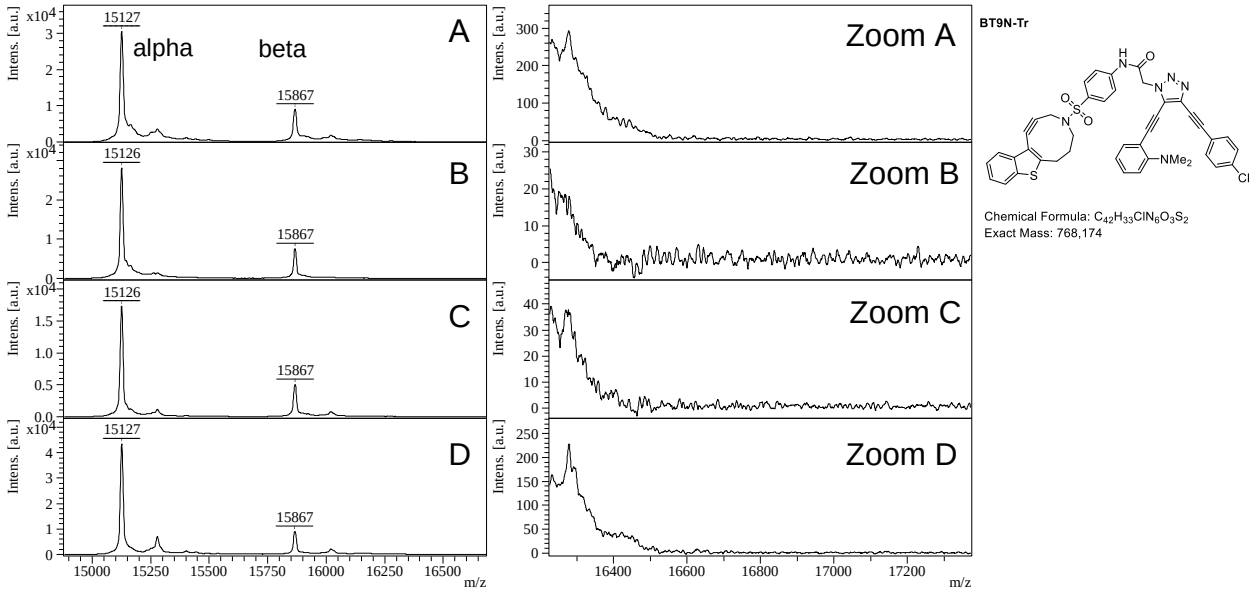


Fig. S14 MALDI TOF mass spectrum of **BT9N-Tr** after 3 h incubation with human globin (HHb); matrix – 2,5-dihydroxy benzoic acid (DHB); A - **BT9N-Tr** without any additives, B - **BT9N-Tr** / HHb (1:9); C - **BT9N-Tr** / HHb (1:1); D - **BT9N-Tr** / HHb (9:1).
BT9N-Tr - HHb adducts were not observed.

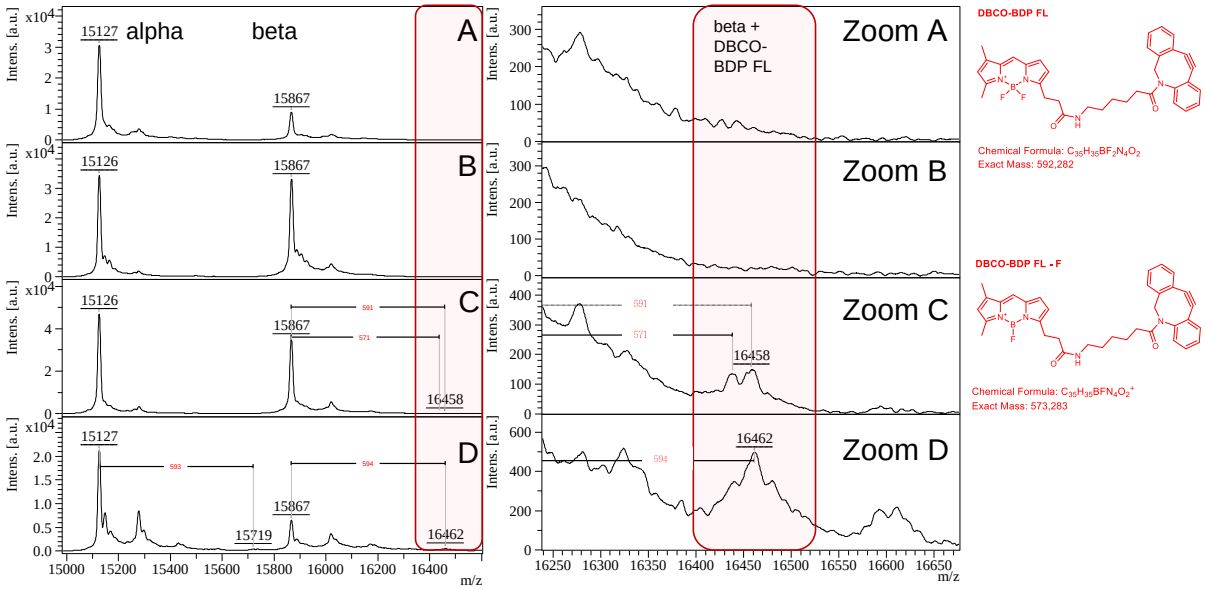


Fig. S15 MALDI TOF mass spectrum of **DBCO-BDP FL** after 3 h incubation with human globin (HHb); matrix – 2,5-dihydroxy benzoic acid (DHB); A - **DBCO-BDP FL** without any additives, B - **DBCO -BDP FL** / HHb (1:9); C - **DBCO-BDP FL** / HHb (1:1); D - **DBCO-BDP FL** / HHb (9:1).
DBCO-BDP FL - HHb adducts were identified through the cysteine residues C-112 or C-93 of the beta globin subunit (15867 Da) and through cysteine C-104 of the alpha subunit (15126 Da). $\Delta_{\text{DBCO-BDP FL}}$ = 592 Da and $\Delta_{\text{DBCO-BDP FL - F}}$ = 573 Da are marked in red.

4. References

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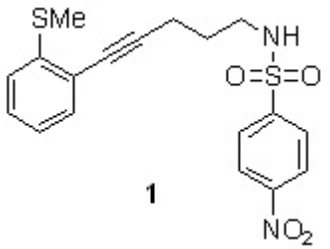
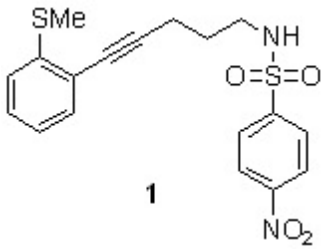
5. Copies of NMR spectra

^{1}H NMR, $CDCl_3$, 400 MHz



1

$^{13}C\{^{1}H\}$ NMR, $CDCl_3$, 101 MHz



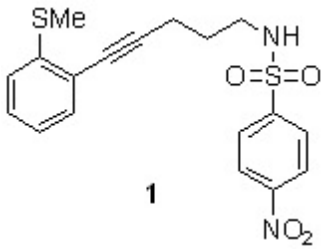
1

150.0
146.0
141.2
132.2
128.8
128.4
124.4
124.4
123.9
121.2
94.7
79.9
77.2 Chloroform-d
42.6
27.9
17.1
15.0

190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10

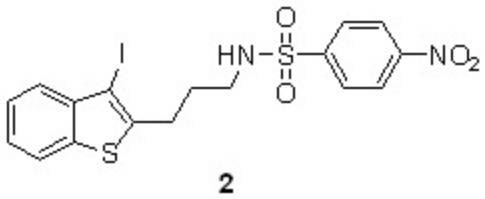
ppm

DEPT NMR, $CDCl_3$, 101 MHz



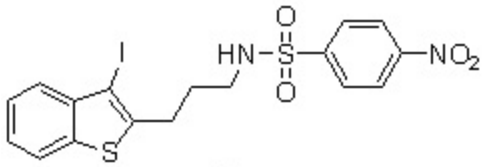
1

^{1}H NMR, $CDCl_3$, 400 MHz



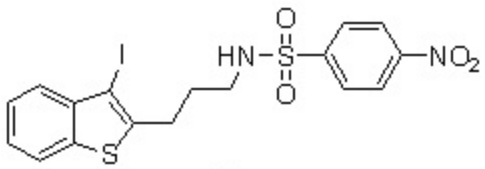
2

$^{13}C\{^{1}H\}$ NMR, $CDCl_3$, 101 MHz



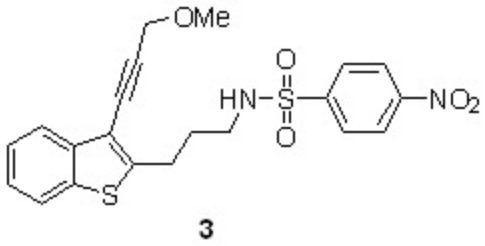
2

DEPT NMR, $CDCl_3$, 101 MHz



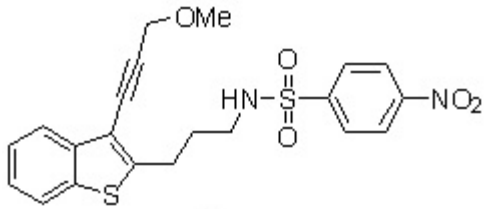
2

^{1}H NMR, $CDCl_3$, 400 MHz



3

$^{13}C\{^{1}H\}$ NMR, Acetone-d_6, 101 MHz

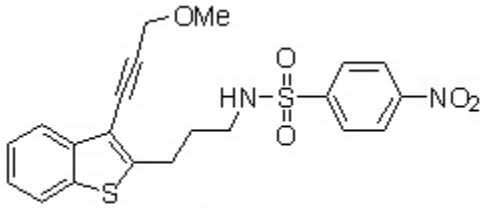


3

150.9
150.3
147.5
140.6
138.2
129.2
125.8
125.8
125.2
123.2
123.0
115.8
92.3
79.4
60.7
57.6
43.3
31.6
29.8 Acetone-d6
27.4

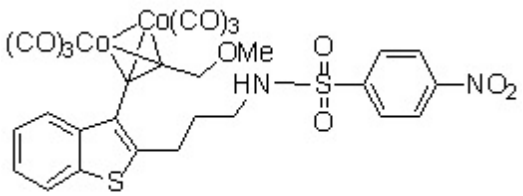
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
ppm

DEPT NMR, Acetone-d_6, 101 MHz



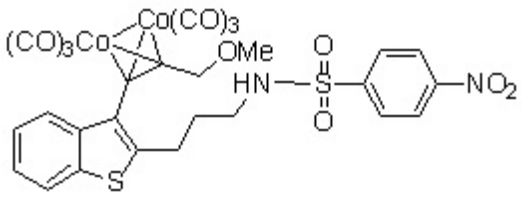
3

^{1}H NMR, Acetone-d_6, 400 MHz



4

$^{13}C\{^{1}H\}$ NMR, Acetone-d_6, 101 MHz



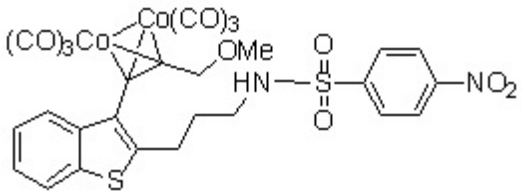
4

200.9
151.0
147.6
146.9
140.9
138.5
129.3
126.4
125.4
125.3
125.1
123.3
123.2
97.2
78.8
74.6
59.2
43.7
32.1
29.8 Acetone-d6
27.7

210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10

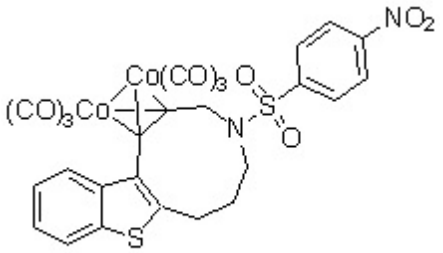
ppm

DEPT NMR, Acetone-d_6, 101 MHz



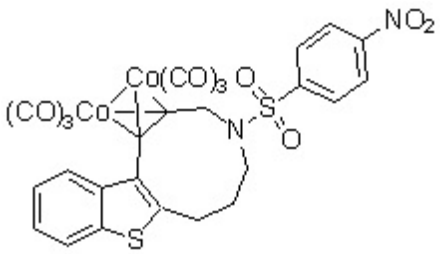
4

^{1}H NMR, Acetone-d_6, 400 MHz



5

$^{13}C\{^{1}H\}$ NMR, Acetone-d_6, 126 MHz

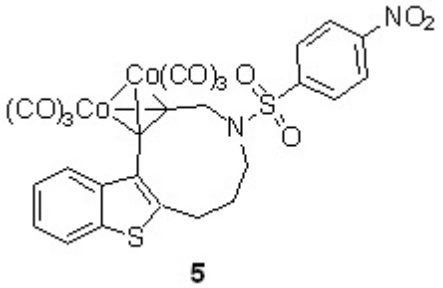


5

200.4
151.2
146.8
144.1
140.9
139.9
129.4
127.8
125.6
125.6
125.3
123.7
122.4
93.1
80.9
51.6
43.9
29.8 Acetone-d6
28.6
125.62
125.60
125.6 ppm

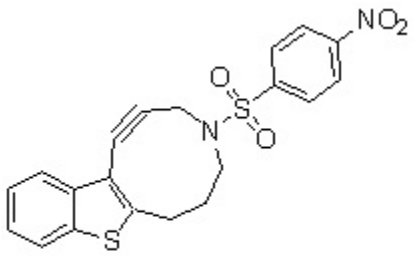
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
ppm

DEPT NMR, Acetone-d_6, 126 MHz



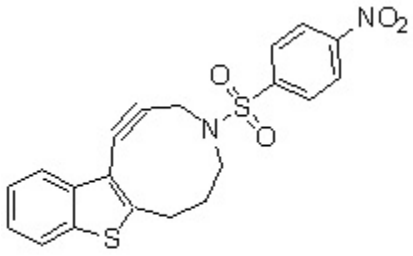
5

^{1}H NMR, CD_3CN, 400 MHz



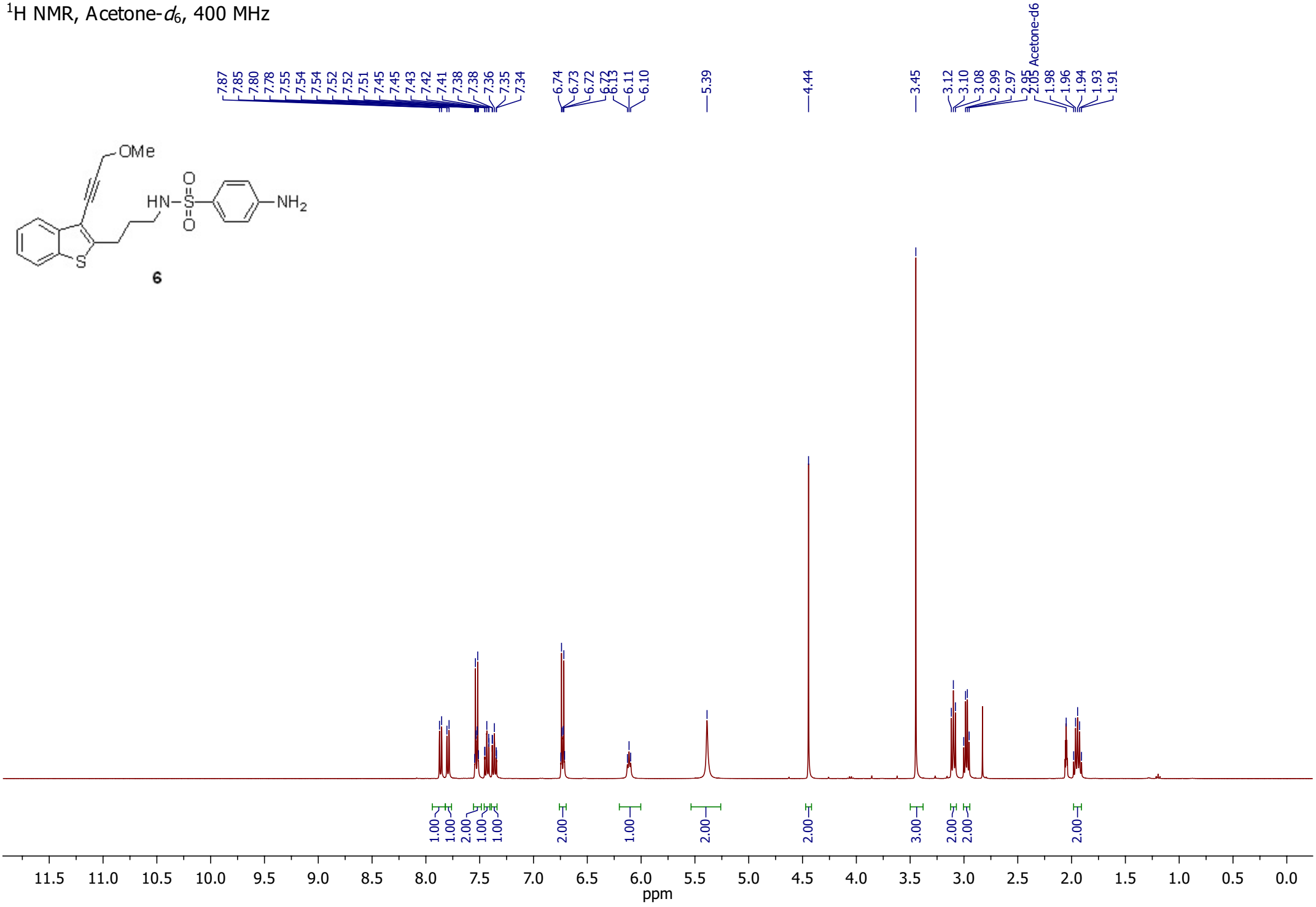
BT9N-NO_2

DEPT NMR, CD_3CN, 101 MHz

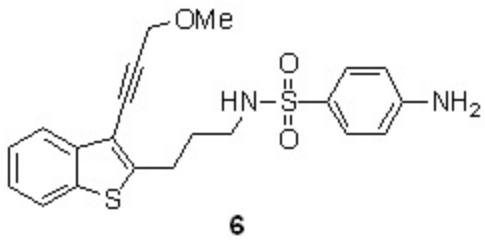


BT9N-NO_2

^{1}H NMR, Acetone-d_6, 400 MHz



$^{13}C\{^{1}H\}$ NMR, Acetone-d_6, 101 MHz



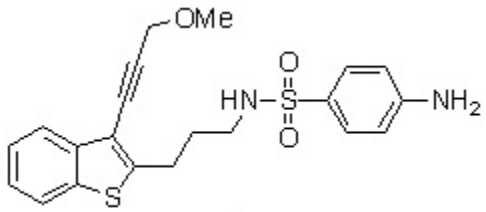
6

153.2
150.8
140.6
138.3
129.7
128.1
125.8
125.6
123.2
123.0
115.7
114.1
92.2
79.5
60.7
57.6
43.3
31.7
29.8 Acetone-d6
27.7

210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0

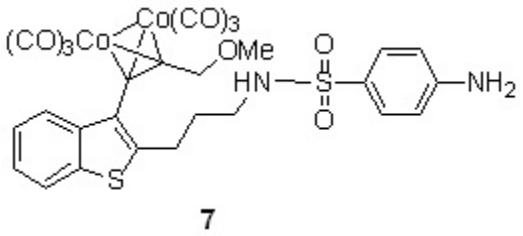
ppm

DEPT NMR, Acetone-d_6, 101 MHz



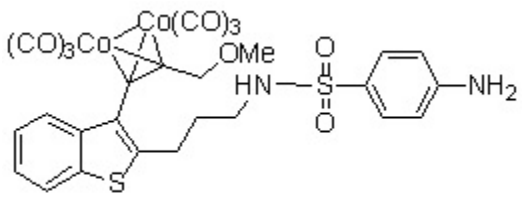
6

^{1}H NMR, Acetone-d_6, 400 MHz



7

$^{13}C\{^{1}H\}$ NMR, Acetone-d_6, 101 MHz

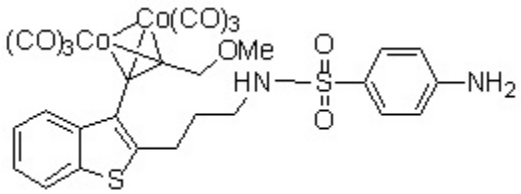


7

200.9
153.3
147.3
141.0
138.7
129.7
128.1
126.1
125.3
125.0
123.3
123.1
114.1
97.3
78.9
74.6
59.2
43.6
32.1
29.8 Acetone-d6
28.0

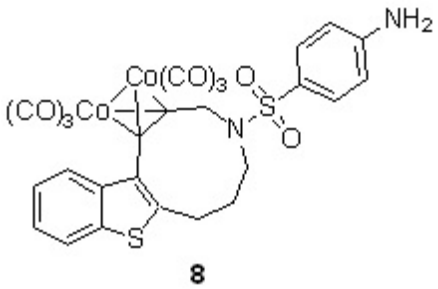
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
ppm

DEPT NMR, Acetone-d_6, 101 MHz



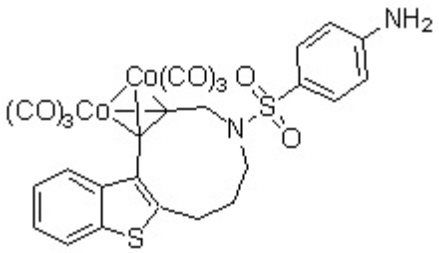
7

^{1}H NMR, Acetone-d_6, 400 MHz



8

$^{13}C\{^{1}H\}$ NMR, Acetone-d_6, 101 MHz



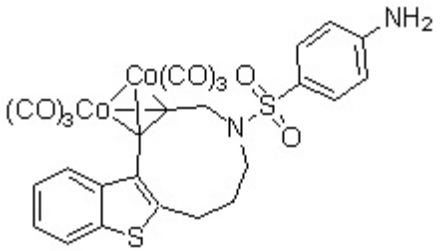
8

200.6
153.8
144.5
141.1
139.8
130.0
127.6
127.0
125.5
125.2
123.7
122.5
114.3
94.7
81.0
51.8
44.2
29.8 Acetone-d6
28.4

* Ethyl acetate
* Ethyl acetate
* Ethyl acetate

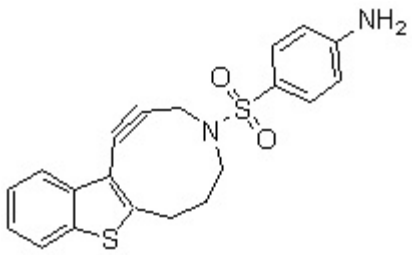
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
ppm

DEPT NMR, Acetone-d_6, 101 MHz



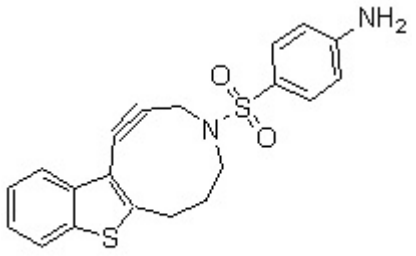
8

^{1}H NMR, $CDCl_3$, 400 MHz



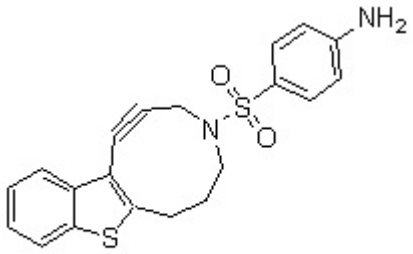
BT9N-NH_2

$^{13}C\{^{1}H\}$ NMR, $CDCl_3$, 101 MHz



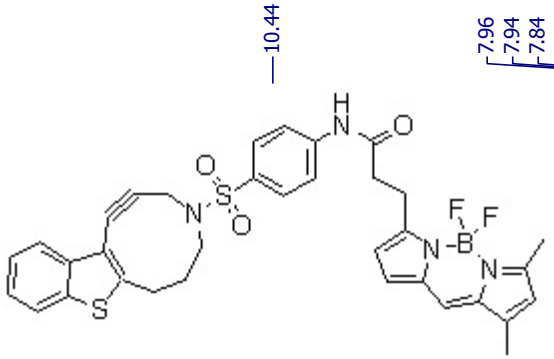
BT9N-NH_2

DEPT NMR, $CDCl_3$, 101 MHz



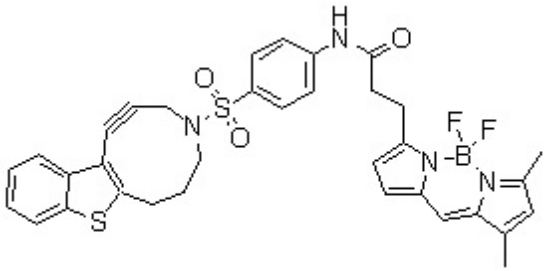
BT9N-NH_2

^{1}H NMR, DMSO-d_6, 400 MHz



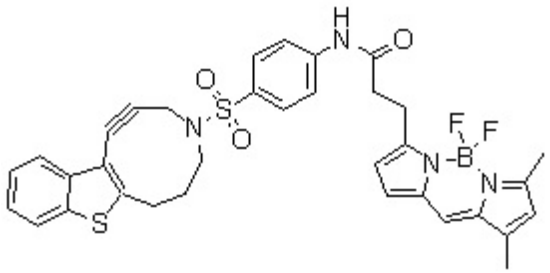
BT9N-BDP FL

$^{13}C\{^{1}H\}$ NMR, DMSO-d_6, 126 MHz



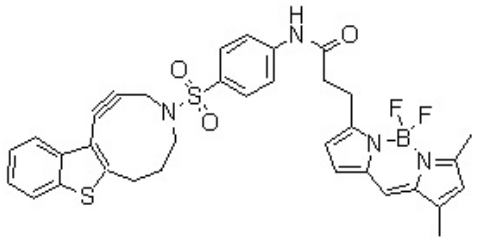
BT9N-BDP FL

DEPT NMR, DMSO-d_6, 126 MHz



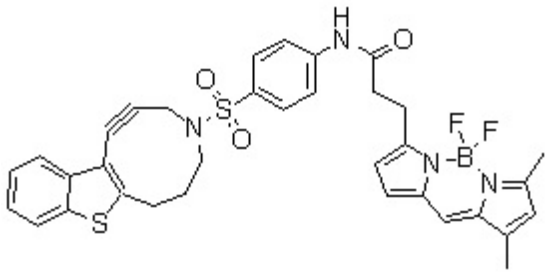
BT9N-BDP FL

HSQC NMR, DMSO-d_6, 126 MHz

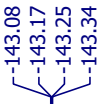


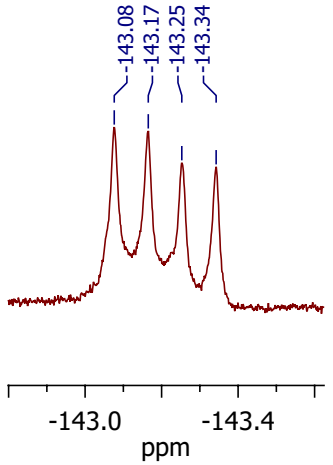
BT9N-BDP FL

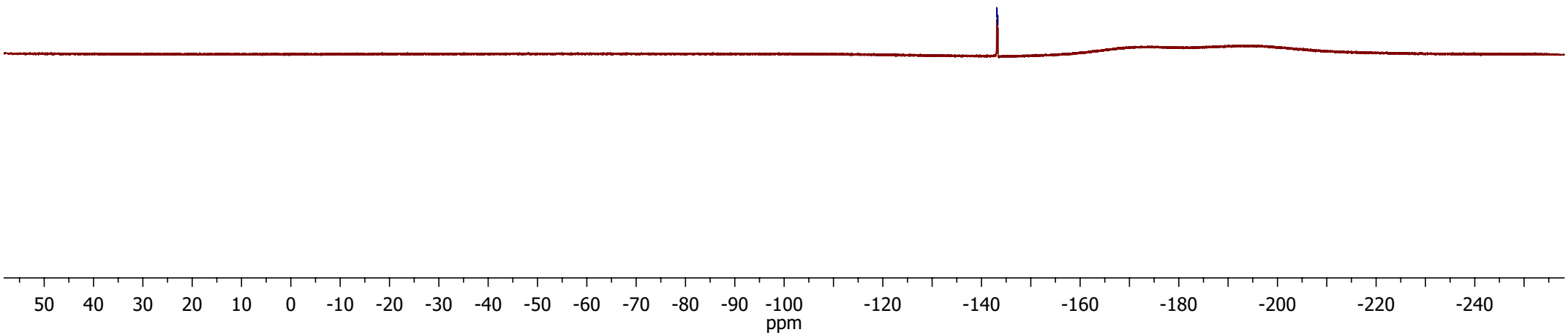
$^{19}F\{^{1}H\}$ NMR, DMSO-d_6, 376 MHz



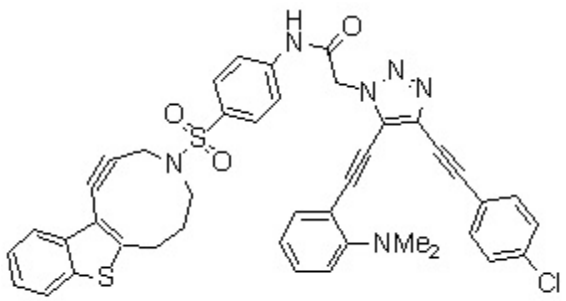
BT9N-BDP FL





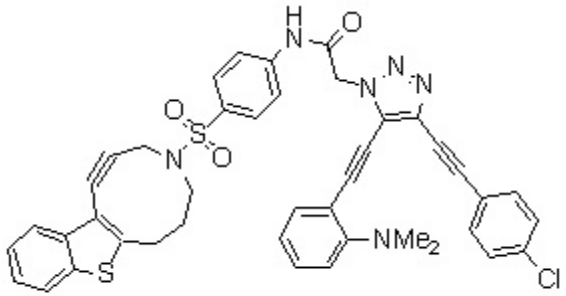


^{1}H NMR, DMSO-d_6, 500 MHz



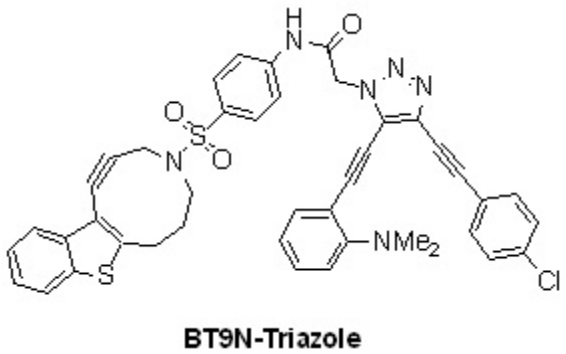
BT9N-Triazole

$^{13}C\{^{1}H\}$ NMR, DMSO-d_6, 126 MHz



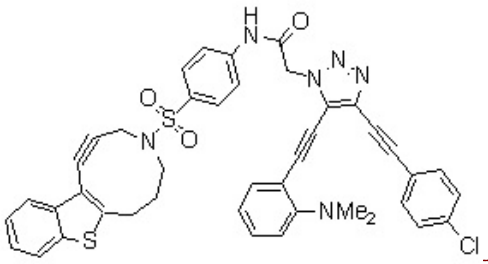
BT9N-Triazole

DEPT NMR, DMSO-d_6, 126 MHz

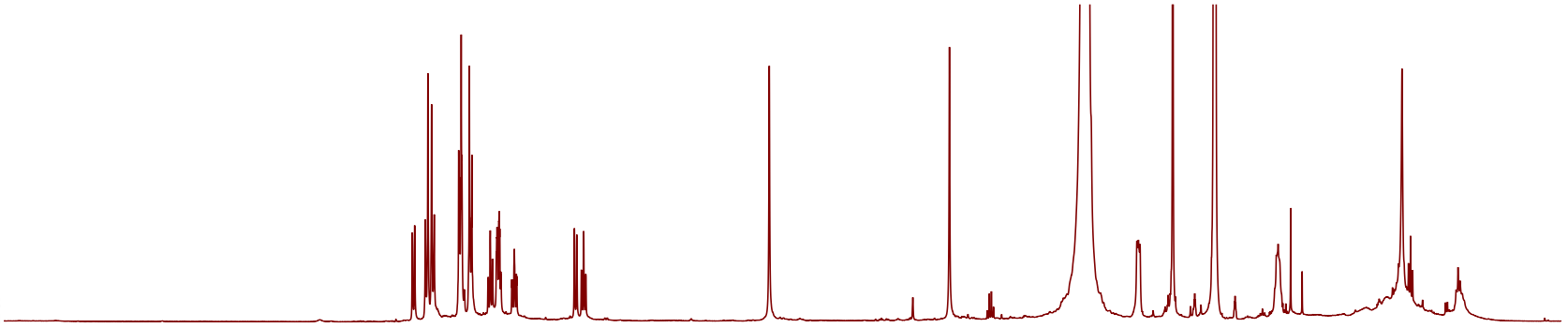


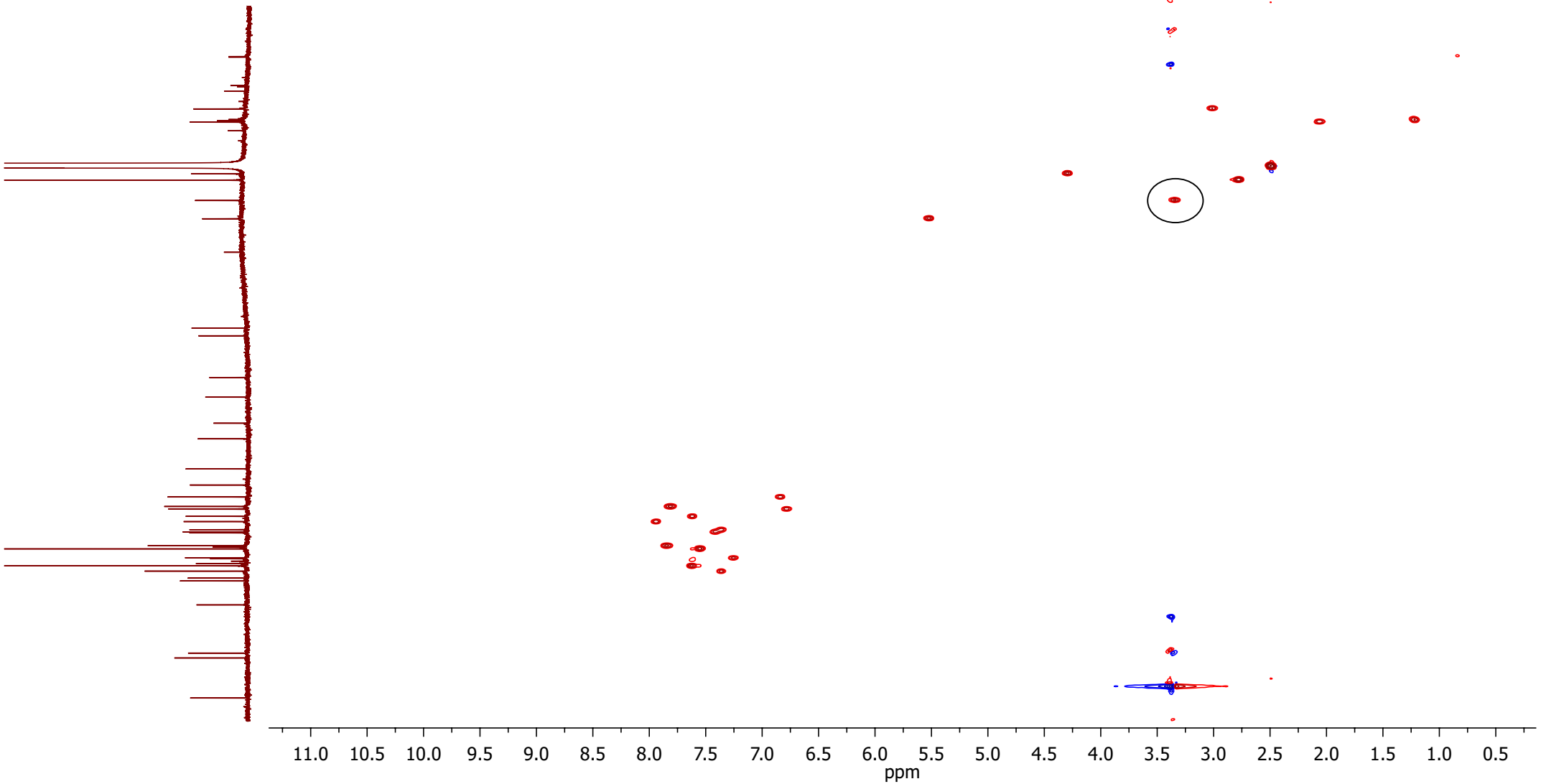
BT9N-Triazole

HSQC NMR, DMSO-d_6, 126 MHz

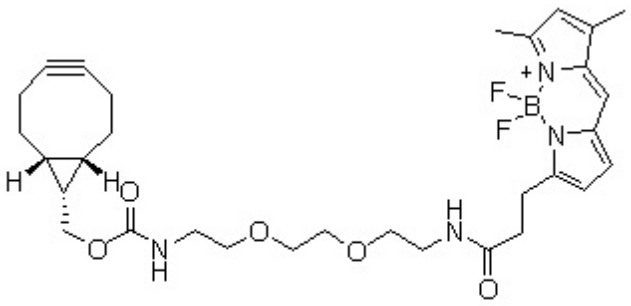


BT9N-Triazole



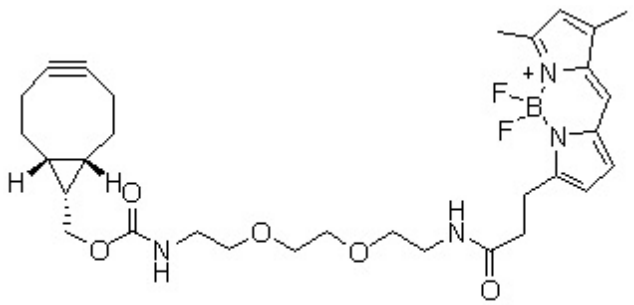


^{1}H NMR, Acetone-d_6, 400 MHz



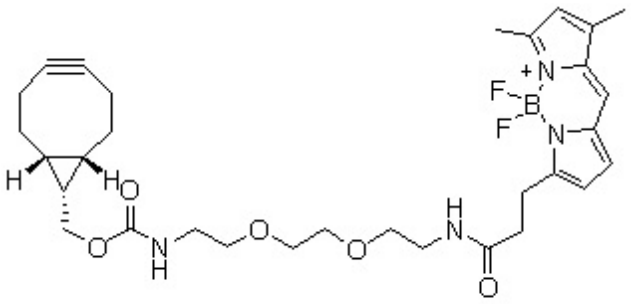
BCN-BDP FL

$^{13}C\{^{1}H\}$ NMR, Acetone-d_6, 101 MHz



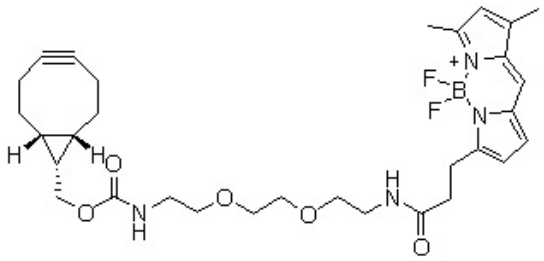
BCN-BDP FL

DEPT NMR, Acetone-d_6, 101 MHz



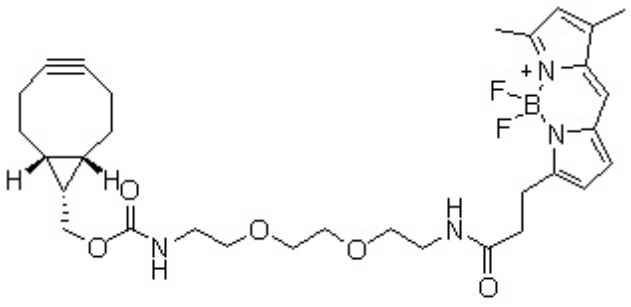
BCN-BDP FL

HSQC NMR, Acetone-d_6, 101 MHz



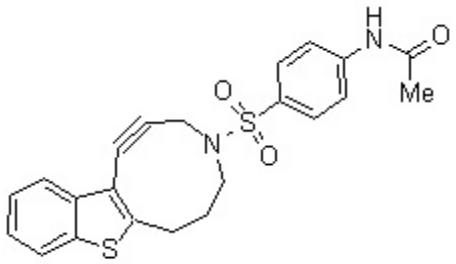
BCN-BDP FL

$^{19}F\{^{1}H\}$ NMR, Acetone-d_6, 376 MHz



BCN-BDP FL

^{1}H NMR, Acetone-d_6, 400 MHz



S2 (crude product)