Electronic Supplementary Information

A Thiol-Activated Fluorogenic Probe for Detection of a Target Protein

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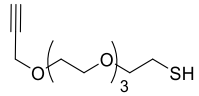
Experimental details

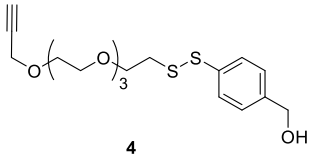
1. Synthesis

General. Analytical thin-layer chromatography (TLC) was conducted on silica gel 60 F254 glass plates (Merck, Darmstadt). Compound spots were visualized by using a handheld UV lamp (254 nm) and/or by staining with 10 wt% phosphomolybdic acid in ethanol. Flash column chromatography was carried out by using silica gel 60 (230–400 Mesh, Merck, Millipore). NMR spectra were recorded on a Bruker Avance II 400 instrument. Coupling constants were reported in Hertz (Hz). High resolution mass spectra were obtained using an Agilent 6530 Accurate-Mass Q-TOF. Chemical reagents used in this study were purchased from Sigma-Aldrich, TCI, Acros Organics and Alfa Aesar.



3

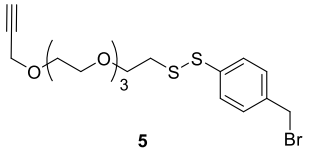
Compound 3. To a stirred solution of **2** (ref 1, 1.45 g, 5 mmol) in MeOH (50 mL) was added 0.1 N NaOMe (270 mg, 5 mmol) in MeOH (50 mL) at room temperature. After stirring for 15 min, the mixture was neutranized by using 0.1 N NH_4Cl, extracted with CH_2Cl_2 (50 mL x 3), dried over sodium sulfate, filtered and concentration under the reduced pressure. The residue was subjected to flash column chromatography (CH_2Cl_2 : MeOH = 25:1) to give **3** as a colorless liquid. Yield: 1.1 g (89%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.19 (d, 2 H, *J* = 2.4 Hz), 3.71-3.60 (m, 14 H), 2.73-2.63 (m, 2 H), 2.42 (t, 1 H, *J* = 2.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 79.8, 74.6, 73.0, 70.7, 70.6, 70.5, 70.3, 69.2, 58.5, 24.4; HR ESI-MS calcd for $C_{11}H_{20}O_5S$ $[M + H]^+$ 249.1155, found 249.1149.



4

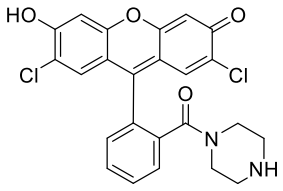
Compound 4. To a stirred solution of **3** (1.45 g, 4.4 mmol) in 20 mL CH_2Cl_2 was added 2,2′-dipyridyl disulfide (2.2 g, 9.7 mmol) in CH_2Cl_2 (20 mL) at room temperature. After stirring for 4 h, the mixture was washed with water (30 mL x 3) and brine (30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was subjected to flash column chromatography (hexane : EtOAc = 4:1 to 1:1) to give an activated compound as a colorless liquid. Yield: 1.4 g (88%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.45 (d, 1 H, *J* = 4.6 Hz), 7.79 (d, 1 H, *J* = 8.1 Hz), 7.67 (t, 1 H, *J* = 8.1 Hz), 7.09 (t, 1 H, *J* = 4.6 Hz), 4.19 (d, 2 H, *J* = 2.4 Hz), 3.74-3.56 (m, 14 H), 2.99 (t, 2 H, *J* = 6.4 Hz), 2.42 (t, 1 H, *J* = 2.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 160.5, 149.5, 137.3, 120.7, 119.8, 79.8, 74.6, 70.8, 70.7, 70.6, 70.5, 69.2, 69.0, 58.5, 24.4; HR ESI-MS calcd for $C_{16}H_{23}NO_4S_2$ $[M + H]^+$ 358.1141, found 358.1135.

To a stirred solution of above compound (1.4 g, 3.9 mmol) in CH_2Cl_2 (20 mL) was added 4-mercaptobenzyl alcohol (604 mg, 4.3 mmol) in CH_2Cl_2 (20 mL) at room temperature. After stirring for 4 h, the mixture was washed with water (30 mL x 3) and brine (30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was subjected to flash column chromatography (CH_2Cl_2 : EtOAc = 8:1 to 2:1) to give **4** as a colorless liquid. Yield: 1.07 g (71%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.53 (d, 1 H, *J* = 8.4 Hz), 7.33 (d, 1 H, *J* = 8.4 Hz), 4.67 (s, 2 H), 4.19 (d, 2 H, *J* = 2.4 Hz), 3.70-3.51 (m, 14 H), 2.92 (t, 2 H, *J* = 6.4 Hz), 2.42 (t, 1 H, *J* = 2.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 139.9, 136.8, 127.8, 127.7, 79.6, 74.6, 70.6, 70.5, 70.4, 70.3, 69.1, 69.0, 64.7, 58.4, 38.5; HR ESI-MS calcd for $C_{18}H_{26}NaO_5S_2$ $[M + Na]^+$ 409.1113, found 409.1111.



5

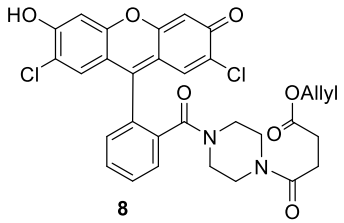
Compound 5. To a stirred solution of **4** (772 mg, 2 mmol) in 20 mL of anhydrous CH_2Cl_2 was added phosphorus tribromide (270 mg, 1 mmol) in CH_2Cl_2 (20 mL) at 0 °C under nitrogen atmosphere. After stirring for 30 min at the same temperature, the reaction was quenched by addition of 5% sodium bicarbonate. The mixture was diluted with CH_2Cl_2 (100 mL), washed with water (25 mL x 3) and brine (25 mL), dried over anhydrous Na2SO4, filtered and concentrated under reduced pressure. The residue was subjected to flash column chromatography (hexane : EtOAc = 4:1) to give **5** as a colorless liquid. Yield: 403 mg (45%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.50 (d, 1 H, *J* = 8.1 Hz), 7.34 (t, 1 H, *J* = 8.1 Hz), 4.47 (s, 2 H), 4.19 (d, 2 H, *J* = 2.4 Hz), 3.72-3.51 (m, 14 H), 2.92 (t, 2 H, *J* = 6.4 Hz), 2.42 (t, 1 H, *J* = 2.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 138.2, 136.5, 129.8, 127.5, 79.8, 74.6, 70.9, 70.8, 70.7, 70.5, 69.3, 69.1, 58.5, 38.6, 33.1: HR ESI-MS calcd for $C_{18}H_{26}BrO_4S_2$ $[M + H]^+$ 449.0450 and 451.0435, found 449.0444 and 451.0435 (1:1).



7

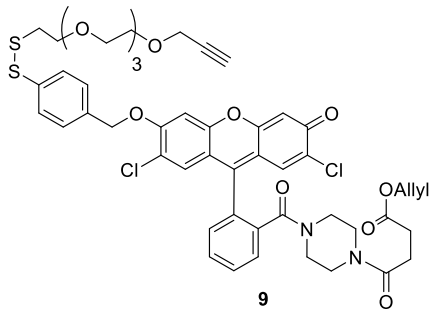
Compound 7. To a strirred solution of 2′,7′-dichlorofluorescein (**6**, 2 g, 5 mmol) and NHS (1.44 g, 12.5 mmol) in DMF (10 mL) was added EDC-HCl (2.39 g, 12.5 mmol) in DMF (10 mL) at room temperature. After sirring for 12 h, the mixture was diluted with brine (200 mL), extracted with 1:2 of CH_2Cl_2 and isopropanol (10 mL x 2), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to to give a *N*-succinimidyl ester product as an orange-red solid. The crude compound was used for the next reaction without further purification. Yield: 1.91 g (78%).

To a stirred solution of the above compound (1.79 g, mmol) and piperazine (619 mg, 7.2 mmol) in DMF (30 mL) was added TEA (1.86 mL, 10.8 mmol) at 0 °C. After stirring for 12 h at the room temperature, the mixture was diluted with brine (200 mL), extracted with 1:2 of CH_2Cl_2 and isopropanol (10 mL x 2), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give a piperazine-coupled product as an orange solid. The residue was subjected to flash column chromatography (CH_2Cl_2 : MeOH = 20:1 to 10:1, then CH_2Cl_2 : MeOH : TEA = 20:1:0.04) to give **7** as an orange-red solid. Yield: 1.09 g (65%). ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.78-7.73 (m, 3 H), 7.59-7.53 (m, 1 H), 7.08 (s, 2 H), 6.81 (s, 2 H), 3.65-3.45 (m, 2 H), 2.98 (s, 4 H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 166.6, 158.8, 158.4, 158.1, 157.7, 148.4, 134.5, 130.5, 130.3, 130.2, 130.0, 128.4, 127.7, 118.1, 115.2, 103.9, 43.7, 42.6, 38.0; HRMS calcd for $C_{24}H_{17}Cl_2N_2O_4$ $[M - H]^-$ 467.0572, found 467.0577.



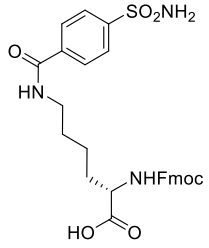
8

Compound 8. To a stirred solution of allyl succinic acid monoester (411 mg, 2.6 mmol), HBTU (986 mg, 2.6 mmol) and HOBt (405 mg, 3 mmol) in DMF (10 mL) was added DIEA (1 mL, 6 mmol) at 0 °C. After stirring for 10 min at the same temperature, **7** (936 mg, 2 mmol) in DMF (10 mL) was added to the mixture. After sirring for 12 h at room temperature, the mixture was diluted with CH_2Cl_2 (100 mL), washed with water (30 mL x 3) and brine (30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was subjected to flash column chromatography (CH_2Cl_2 : MeOH = 20:1 to 7:1) to give **8** as an orange-red solid. Yield: 865 mg (71%); ^{1}H NMR (CD_3OD, 400 MHz) δ 7.86-7.74 (m, 2 H), 7.74-7.66 (m, 1 H), 7.58-7.48 (m, 1 H), 7.20 (s, 2 H), 6.77 (s, 2 H), 5.97-5.85 (m, 1 H), 5.28 (dd, J = 17.1, 1.3 Hz, 1 H), 5.17 (dd, J = 10.5, 1.3 Hz, 1 H), 4.55 (d, J = 5.3 Hz, 2 H), 3.58-3.37 (m, 8 H), 2.68-2.58 (m, 4 H); ^{13}C NMR (CD_3OD, 100 MHz) δ 174.2, 172.4, 171.0, 169.4, 157.4, 152.2, 136.4, 133.7, 132.2, 131.9, 131.5, 131.3, 130.2, 129.0, 128.2, 118.2, 116.6, 105.0, 66.2, 46.5, 45.9, 43.0, 42.2, 30.0, 28.6; HRMS calcd for $C_{31}H_{27}Cl_2N_2O_7$ $[M + H]^+$ 609.1190, found 609.1181.



9

Compound 9. To a stirred solution of **8** (244 mg, 0.4 mmol), potassium carbonate (82 mg, 0.6 mmol) and 18-crown-6 (158 mg, 0.6 mmol) in DMF (2 mL) was added **5** (180 mg, 0.4 mmol) in DMF (2 mL) at room temperature under nitrogen atmosphere. After stirring for 4 h, the residue was diluted with CH_2Cl_2 (50 mL), washed with water (20 mL x 2) and brine (20 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was subjected to flash column chromatography (CH_2Cl_2 : MeOH = 40:1 to 20:1) to give **9** as an orange solid. Yield: 246 mg (63%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.73-7.65 (m, 2 H), 7.58 (d, 2 H, *J* = 8.1 Hz), 7.55-7.50 (m, 1 H), 7.42 (d, 2 H, *J* = 8.1 Hz), 7.39-7.34 (m, 1 H), 7.39-7.34 (m, 1 H), 7.16-7.05 (m, 3 H), 6.58 (s, 1 H), 5.98-5.81 (m, 1 H), 5.35-5.16 (m, 4 H), 4.56 (d, 2 H, *J* = 5.6 Hz), 4.17 (d, 2 H, *J* = 2.4 Hz), 3.71-3.34 (m, 22 H), 2.92 (t, 2 H, *J* = 6.4 Hz), 2.74-2.53 (m, 4 H), 2.41 (t, 1 H, *J* = 2.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 177.4; 172.7, 169.9, 167.3, 158.6, 157.6, 152.6, 147.8, 138.3, 135.2, 133.3, 132.1, 131.4, 130.7, 130.5, 129.9, 129.0, 128.0, 127.7, 127.5, 120.8, 118.3, 115.2, 105.9, 101.4, 79.7, 74.6, 71.2, 70.7, 70.6, 70.4, 69.1, 65.4, 58.4, 47.4, 45.3, 44.9, 41.8, 38.5, 29.1, 27.8; ESI-MS calcd for $C_{49}H_{51}Cl_2N_2O_{11}S_2$ $[M + H]^+$ 977.2305, found 977.2305.

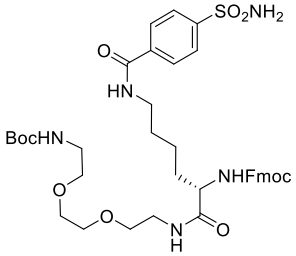


11

Compound 11. To a stirred solution of 4-carboxybenzenesulfonamide (**10**, 1.5 g, 7.46 mmol) and N-hydroxysuccinimide (NHS, 1.28 g, 11.19 mmol) in DMF (20 mL) was added EDC-HCl (1.70 g, 8.95 mmol) at room temperature. After stirring for 8 h, the mixture was diluted with EtOAc (100 mL), washed with water (30 mL x 3) and brine (30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give a N-succinimidyl ester product as a white solid. The crude compound was used for the next reaction without further purification. Yield: 1.91 g (86%).

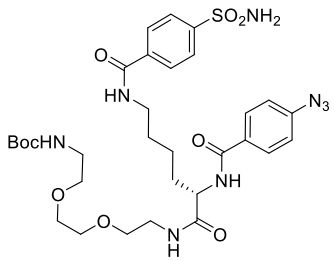
To a stirred solution of Fmoc-Lys (2.36 g, 6.4 mmol) and above compound (1.91 g, 6.4 mmol) in dioxane and H_2O (30 mL, 2:1) was added DIEA (2.9 mL, 16 mmol) at 0 °C. After

stirring for 4 h at room temperature, the volatile materials were removed under reduced pressure. The residue was diluted with EtOAc (100 mL), washed with water (30 mL x 3) and brine (30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was subjected to flash column chromatography (CH_2Cl_2 : MeOH = 20:1 to 5:1) to give **11** as a white solid. Yield: 2.3 g (65%); ^{1}H NMR (CD_3OD, 400 MHz) δ 8.00–7.91 (m, 4 H), 7.78 (d, 2 H, *J* = 7.6 Hz), 7.66 (t, 2 H, *J* = 7.6 Hz), 7.37 (t, 2 H, *J* = 7.4 Hz), 7.28 (t, 2 H, *J* = 7.4 Hz), 4.43-4.29 (m, 2 H), 4.25-4.12 (m, 2 H), 3.40 (t, 2 H, *J* = 6.9 Hz), 1.95-1.85 (m, 1 H), 1.80-1.72 (m, 1 H), 1.71-1.61 (m, 2 H), 1.57-1.41 (m, 2 H); ^{13}C NMR (CD_3OD, 100 MHz) δ 176.0, 168.8, 158.7, 147.6, 145.3, 145.1, 142.6, 139.1, 129.0, 128.8, 128.2, 127.3, 126.2, 120.9, 67.9, 55.2, 40.9, 32.3, 29.9, 24.4; HR ESI-MS calcd for $C_{28}H_{30}N_3O_7S$ $[M + H]^+$ 552.1799, found 552.1799.



12

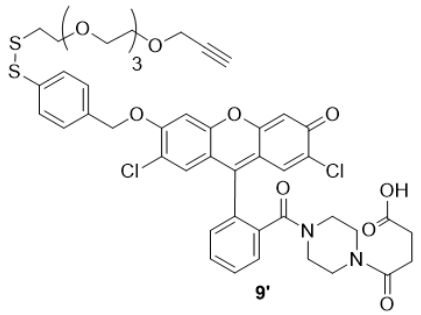
Compound 12. To a stirred solution of **11** (2.3 g, 4.2 mmol) and *N, N, N′, N′*-tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU, 1.58 g, 4.2 mmol) and 1-hydroxybenzotriazole (HOBt, 563 mg, 4.2 mmol) in DMF (30 mL) was added DIEA (1.70 g, 8.95 mmol) at 0 ^{o}C. After stirring for 10 min at the same temperature, *tert*-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (1.04 g, 4.2 mmol) in DMF (10 mL) was added to the mixture. After stirring for 4 h at room temperature, the mixture was diluted with EtOAc (100 mL), washed with water (30 mL x 3) and brine (30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was subjected to flash column chromatography (CH_2Cl_2 : MeOH = 20:1 to 5:1) to give **12** as a white solid. Yield: 2.3 g (73%); ^{1}H NMR (CD_3OD, 400 MHz) δ 8.00–7.91 (m, 4 H), 7.78 (d, 2 H, *J* = 7.6 Hz), 7.64 (t, 2 H, *J* = 7.6 Hz), 7.37 (t, 2 H, *J* = 7.4 Hz), 7.28 (t, 2 H, *J* = 7.4 Hz), 4.45-4.30 (m, 2 H), 4.24-4.16 (m, 1 H), 4.14-4.04 (m, 1 H), 3.56-3.43 (m, 8 H), 3.41-3.33 (m, 4 H), 3.19 (t, 2 H, *J* = 5.5 Hz), 1.85-1.56 (m, 4 H), 1.46-1.36 (m, 11 H); ^{13}C NMR (CD_3OD, 100 MHz) δ 174.9, 168.7, 158.4, 147.6, 145.3, 145.1, 142.6, 139.1, 129.0, 128.8, 128.2, 127.3, 126.2, 120.9, 80.1, 71.2, 71.0, 70.4, 67.8, 56.5, 41.2, 40.8, 40.3, 32.9, 30.0, 28.8, 24.3; HR ESI-MS calcd for $C_{39}H_{52}N_5O_{10}S$ $[M + H]^+$ 782.3429, found 782.3420.



13

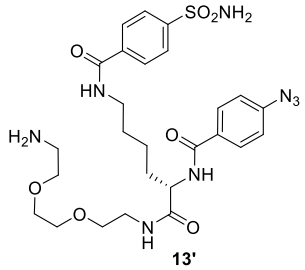
Compound 13. To a stirred solution of **12** (2.3 g, 2.9 mmol) in DMF (10 mL) was added 5 mL of 40% piperidine in DMF (10 mL) at room temperature. After stirring for 30 min, the mixture was diluted with EtOAc (200 mL), washed with water (50 mL x 3) and brine (50 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was subjected to flash column chromatography (CH_2Cl_2 : MeOH = 20:1 to 5:1) to give a Fmoc-deprotected compound as a colorless solid. Yield: 1.5 g (91%); ^{1}H NMR (CD_3OD, 400 MHz) δ 8.00-7.93 (m, 4 H), 3.62-3.33 (m, 13 H), 3.24-3.18 (m, 2 H), 1.88-1.55 (m, 4 H), 1.54-1.39 (m, 11 H); ^{13}C NMR (CD_3OD, 100 MHz) δ 175.4, 168.7, 158.4, 147.6, 139.1, 129.0, 127.3, 80.1, 71.2, 71.0, 70.4, 55.5, 41.2, 40.8, 40.2, 34.9, 30.1, 28.8, 23.8; ESI-MS calcd for $C_{24}H_{42}N_5O_8S$ $[M + H]^+$ 560.3, found 560.7.

To a stirred solution of 4-azidobenzoic acid (437 mg, 2.7 mmol) and HBTU (1.02 g, 2.7 mmol) and HOBt (362 mg, 2.7 mmol) in DMF (20 mL) was added DIEA (1.2 mL, 6.75 mmol) at 0 °C. After stirring for 10 min at the same temperature, a Fmoc-deprotected compound (1.5 g, 2.7 mmol) in DMF (3 mL) was added to the above solution. After sirring for 4 h at room temperature, the mixture was diluted with EtOAc (100 mL), washed with water (30 mL x 3) and brine (30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was subjected to flash column chromatography (CH_2Cl_2 : MeOH = 20:1 to 10:1) to give **13** as a white solid. Yield: 1.36 g (73%); ^{1}H NMR (CD_3OD, 400 MHz) δ 7.97-7.85 (m, 6 H), 7.11 (d, 2 H, J = 8.8 Hz), 4.60-4.51 (m, 1 H), 3.62-3.51 (m, 6 H), 3.50-3.45 (m, 2 H), 3.44-3.35 (m, 4 H), 3.19 (t, 2 H, J = 5.5 Hz), 1.99-1.80 (m, 2 H), 1.76-1.64 (m, 2 H), 1.61-1.46 (m, 2 H), 1.42 (s, 9 H); ^{13}C NMR (CD_3OD, 100 MHz) δ 175.4, 169.0, 168.7, 158.4, 147.6, 145.1, 139.1, 131.6, 130.5, 128.9, 127.3, 119.9, 80.1, 71.2, 71.0, 70.5, 55.3, 41.2, 40.7, 40.3, 32.7, 30.0, 28.8, 24.4; HRMS calcd for $C_{31}H_{45}N_8O_9S$ $[M + H]^+$ 705.3025, found 705.3020.



9'

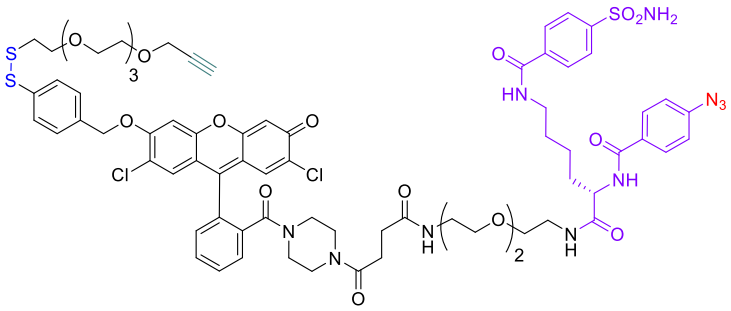
Compound 9'. To a stirred solution of **9** (244 mg, 0.25 mmol) and tetrakis(triphenylphosphine)palladium (28.9 mg, 0.025 mmol) in THF and MeOH (10 mL, 1:1) was added 1,3-dimethylbarbituric acid (78 mg, 0.5 mmol) in THF and MeOH (10 mL, 1:1) at room temperature. After stirring for 1 h, the volatile materials were removed under reduced pressure. The residue was diluted with CH_2Cl_2 (100 mL), washed with water (30 mL x 3), and concentrated under reduced pressure. The crude compound was used for the next reaction without further purification. Yield: 166 mg (71%). However, for NMR analysis, a small amount of crude compound was purified by flash column chromatography (CH_2Cl_2 : MeOH = 20:1 to 5:1) to give **9'** as an orange solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.73-7.65 (m, 2 H), 7.61-7.52 (m, 2 H), 7.47-7.35 (m, 4 H), 7.19-7.05 (m, 3 H), 6.60 (s, 1 H), 5.27-5.15 (m, 2 H), 4.19 (d, 2 H, *J* = 2.4 Hz), 3.71-3.40 (m, 22 H), 2.94 (t, 2 H, *J* = 6.4 Hz), 2.73-2.53 (m, 4 H), 2.41 (t, 1 H, *J* = 2.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 177.9, 175.7, 170.7, 167.5, 157.8, 152.7, 138.3, 135.4, 133.4, 131.5, 130.7, 130.5, 130.0, 129.7, 129.0, 128.0, 127.8, 127.6, 120.9, 115.2, 106.0, 101.4, 79.7, 74.7, 71.2, 70.7, 70.6, 70.5, 69.2, 69.1, 58.5, 47.4, 45.7, 45.2, 41.9, 38.5, 29.4, 27.7; ESI-MS calcd for $C_{46}H_{47}Cl_2N_2O_{11}S_2$ $[M + H]^+$ 937.1993, found 937.1997.



13'

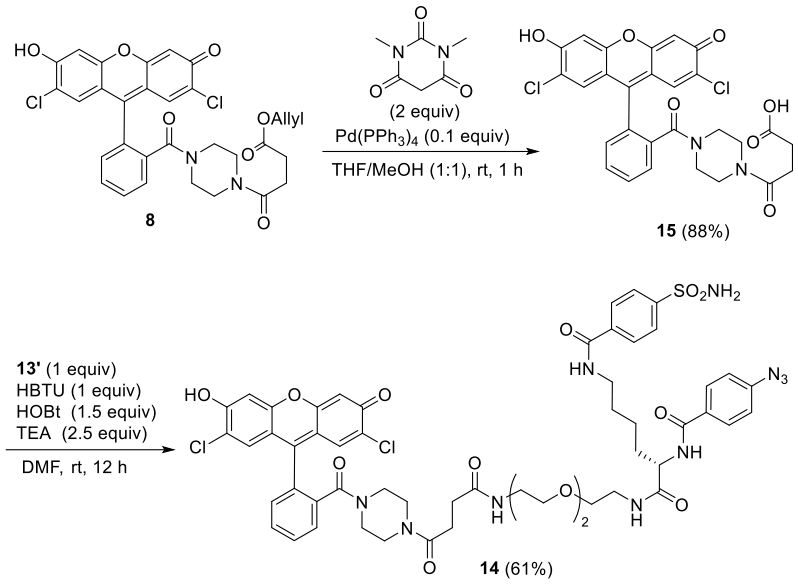
Compound 13'. Compound **13** (704 mg, 1 mmol) dissolved in TFA (5 mL) and CH_2Cl_2 (5 mL) was stirred for 30 min at room temperature. The volatile materials were removed under reduced pressure. The residue was diluted with EtOAc (50 mL), added with 5% $NaHCO_3$ (20 mL), water (20 mL x 2) and brine (20 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude product was used for the next reaction without further purification. Yield: 764 mg (89%); ^{1}H NMR (CD_3OD, 400 MHz) δ 7.97-7.86 (m, 6 H), 7.12 (d, 2 H, *J* = 8.2 Hz), 4.55-4.47 (m, 1 H), 3.70-3.37 (m, 12 H), 3.11 (t, 2 H, *J* = 4.9 Hz), 2.00-1.80 (m, 2 H), 1.76-1.64 (m, 2 H), 1.59-1.44 (m, 2 H); ^{13}C NMR (CD_3OD, 100 MHz) δ 174.8, 169.2, 168.8, 147.6, 145.2, 139.1, 131.6, 130.5, 128.9, 127.3,

120.0, 80.1, 71.3, 70.5, 67.4, 55.5, 40.7, 40.6, 40.3, 32.6, 30.0, 24.4; HR ESI-MS calcd for $C_{26}H_{37}N_8O_7S$ $[M + H]^+$ 605.2500, found 605.2494.



1

Compound 1. To a stirred solution of **9'** (166 mg, 0.18 mmol), *N*, *N*, *N'*, *N'*-tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU, 101 mg, 0.27 mmol) and 1-hydroxybenzotriazole (HOBt, 563 mg, 0.27 mmol) in DMF (1 mL) was added TEA (64 uL, 0.45 mmol) at 0 °C. After stirring for 10 min at the same temperature, **13'** (107 mg, 0.18 mmol) in DMF (11 mL) was added to the mixture. After stirring for 12 h, the residue was diluted with CH_2Cl_2 (50 mL), washed with water (20 mL x 2) and brine (20 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was subjected to flash column chromatography (CH_2Cl_2 : MeOH = 20:1 to 7:1) to give **1** as an orange solid. Yield: 159 mg (59%); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.97-7.91 (m, 4 H), 7.88-7.83 (m, 3 H), 7.78-7.73 (m, 2 H), 7.70-7.66 (m, 1 H), 7.63-7.50 (m, 6 H), 7.18 (m, 3 H), 6.47 (s, 1 H), 5.43 (s, 2 H), 4.43-4.36 (m, 1 H), 4.21 (d, 2 H, J = 2.4 Hz) 3.60 (t, 2 H, J = 6.2 Hz), 3.54-3.38 (m, 21 H), 3.31-3.11 (m, 13 H), 2.97 (t, 2 H, J = 6.2 Hz), 2.49-2.42 (m, 2 H), 2.31-2.26 (m, 2 H), 1.80-1.70 (m, 2 H), 1.57-1.50 (m, 2 H), 1.42-1.32 (m, 2 H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 172.7, 172.0, 171.5, 170.0, 166.8, 165.4, 165.1, 156.0, 148.4, 146.1, 142.4, 137.6, 135.2, 131.3, 130.7, 130.4, 129.6, 129.5, 129.4, 127.8, 127.5, 127.4, 127.2, 125.6, 118.8, 109.2, 103.5, 80.4, 77.1, 69.8, 69.7, 69.6, 69.5, 69.1, 69.0, 68.5, 68.2, 57.5, 53.5, 46.7, 44.5, 41.0, 38.6, 38.2, 31.4, 30.3, 28.7, 27.8, 23.3; ESI-MS calcd for $C_{72}H_{81}Cl_2N_{10}O_{17}S_3$ $[M + H]^+$ 1523.4, found 1525.5.



Scheme S1. Synthesis of a control 14.

Compound 15. To a stirred solution of **8** (608 mg, 1 mmol) and tetrakis(triphenylphosphine)palladium (115 mg, 0.1 mmol) in THF and MeOH (10 mL, 1:1) was added 1,3-dimethylbarbituric acid (312 mg, 2 mmol) in THF and MeOH (10 mL, 1:1) at room temperature. After stirring for 1 h, the volatile materials were removed under reduced pressure. The residue was diluted with water (100 mL), washed with CH_2Cl_2 (30 mL x 3) and concentrated under reduced pressure. The residue was subjected to flash column chromatography (CH_2Cl_2 : MeOH = 10:1 to CH_2Cl_2 : MeOH : AcOH = 6:1:0.1) to give **15** as an orange-red solid. Yield: 499 mg (88%); ^{1}H NMR (CD_3OD, 400 MHz) δ 7.84-7.76 (m, 2 H), 7.74-7.69 (m, 1 H), 7.57-7.52 (m, 1 H), 7.26 (s, 2 H), 6.87 (s, 2 H), 3.60-3.37 (m, 8 H), 2.68-2.55 (m, 4 H); ^{13}C NMR (CD_3OD, 100 MHz) δ 176.4, 172.7, 170.2, 169.4, 157.4, 152.9, 136.4, 132.2, 131.8, 131.5, 131.4, 130.4, 129.1, 128.0, 117.2, 105.0, 43.0, 29.9, 28.7; HR ESI-MS calcd for $C_{28}H_{22}Cl_2N_2O_7$ $[M + H]^+$ 569.0877, found 569.0867.

Compound 14. To a stirred solution of **15** (341 mg, 0.6 mmol), HBTU (228 mg, 0.6 mmol) and HOBt (122 mg, 0.9 mmol) in DMF (5 mL) was added DIEA (257 uL, 1.5 mmol) at 0 ^{o}C. After stirring for 10 min at the same temperature, **13'** (363 mg, 0.6 mmol) in DMF (1 mL) was added to the above solution. After sirring for 4 h at room temperature, the volatile materials were removed under reduced pressure. The residue was subjected to flash column chromatography (CH_2Cl_2 : MeOH = 10:1 to CH_2Cl_2 : MeOH : AcOH = 7:1:0.1) to give **14** as a orange-red solid. Yield: 424 mg (61%); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.00-7.92 (m, 4 H), 7.89-7.85 (m, 3 H), 7.73-7.66 (m, 2 H), 7.65-7.57 (m, 1 H), 7.19 (d, 2 H, J = 8.6 Hz), 6.82 (s, 2 H), 6.27 (s, 2 H), 4.47-4.36 (m, 1 H), 3.41-3.13 (m, 22 H), 2.49-2.42 (m, 2 H), 2.31-2.26 (m, 2 H), 1.80-1.69 (m, 2 H), 1.61-1.50 (m, 2 H), 1.44-1.29 (m, 2 H); ^{13}C NMR

(DMSO-d_6, 100 MHz) δ 172.7, 172.0, 171.5, 170.0, 166.8, 165.4, 165.1, 156.0, 148.4, 146.1, 142.2, 137.6, 135.2, 131.3, 130.7, 130.3, 129.6, 129.4, 127.8, 127.5, 127.3, 127.2, 125.6, 118.8, 109.2, 103.5, 69.6, 69.1, 69.0, 53.5, 46.7, 44.5, 42.1, 41.0, 38.6, 31.0, 30.3, 28.7, 27.8, 23.3; HR ESI-MS calcd for $C_{54}H_{57}Cl_2N_{10}O_{13}S$ $[M + H]^+$ 1155.3199, found 1154.3193.

2. Biochemical studies

Photolabeling of hCA2 by probe 1. The hCA2 (1 μM, Sino Biological), pre-incubated with or without 1 μM acetazolamide, were exposed to **1** for 1 h. The mixture was irradiated for 1 min using a hand-held UV lamp (365 nm) in the dark. The mixture was subjected to click reaction with N_3-biotin (10 μM), a premixed BTTAA-$CuSO_4$ complex ([BTTAA] = 200 μM, [$CuSO_4$] = 50 μM), freshly prepared sodium ascorbate (45 μM) at room temperature for 5 min and then treated with 10 mM DTT for 0.5 h. The protein was separated by 12% SDS-PAGE. Wet slab gel was scanned on fluorescence using the Typhoon laser scanner platform (GE Healthcare). The protein gel was also subjected to silver staining.

Selective photolabeling of hCA2 by probe 1. A mixture of five proteins (Hsp70 (purified according to the known procedure),[2] BSA (Sigma-Aldrich), β-Hex (purified according to the known procedure),[3] CPK (Sigma-Aldrich), hCA2), pre-incubated with or without 1 μM acetazolamide, were treated with 2 μM **1** for 1 h. The mixture was irradiated for 1 min using a hand-held UV lamp (365 nm) in the dark. The mixture was then subjected to click reaction with N_3-biotin (10 μM), a premixed BTTAA-$CuSO_4$ ([BTTAA] = 200 μM, [$CuSO_4$] = 50 μM), freshly prepared sodium ascorbate (45 μM) at room temperature for 5 min. Residual N_3-biotin was removed by using an Amicon membrane with a 10-kDa cutoff. The mixture was incubated with 20 μL streptavidin-agarose resin for 1 h at room temperature. After washing the resin, proteins were eluted from the streptavidin resin by using 10 mM DTT. The eluted proteins were separated by 12% SDS-PAGE. Wet slab gel was scanned on fluorescence using the Typhoon laser scanner platform. The protein gel was also subjected to silver staining.

Cell Culture. HEK293 cells (human embryonic kidney cell line) and NIH3T3 cells (mouse embryonic fibroblast cell line) were cultured in DMEM(Gibco) with 10% (v/v) fetal bovine serum (FBS), 50 units/mL penicillin and 50 units/mL streptomycin. Cells were maintained in a humidified incubator containing 5% CO_2 at 37 ^oC.

Detection of cellular hCA2 using 1. HEK293 and NIH3T3 cells were lysed with RIPA buffer (50 mM Tris-HCL, 150 mM NaCl, 1% NP-40, 1% CHAPS, 10% glycine, and one tablet of protease inhibitor cocktail (Roche), pH 7.4) for 20 min at 4 ^oC. After centrifugation at 13,000 rpm for 5 min, the streptavidin resin was added to lysates for preclearing. The pre-cleared cell lysates were incubated with 2 μM **1** in the absence and presence of 1 μM acetazolamide. The treated cell lysates were irradiated for 1 min using a hand-held UV lamp

(365 nm) in the dark. The mixture was then subjected to click reaction with N_3-biotin (10 μM), a premixed BTTAA-$CuSO_4$ ([BTTAA] = 200 μM, [$CuSO_4$] = 50 μM), freshly prepared sodium ascorbate (45 μM) at room temperature for 5 min. Residual N_3-biotin was removed by using an Amicon membrane with a 10-kDa cutoff. The mixture was incubated with 20 μL streptavidin-agarose resin for 1 h at room temperature. After washing the resin, proteins were eluted from the streptavidin resin with 10 mM DTT. The eluted proteins were separated by 12% SDS-PAGE. Wet slab gel was scanned on fluorescence using the Typhoon laser scanner platform. The protein gel was also subjected to silver staining.

MS analysis of hCA2 labeled by 1. The target protein band on the SDS-PAGE gel was cut and subjected to in-gel tryptic digestion. The fraction was washed with 100 μL water. To the fraction was added 50 μL of 50 mM NH_4HCO_3 (pH 7.8) and acetonitrile (6:4) and shaken for 10 min. This process was repeated three times. The supernatant was decanted, and the fraction was dried in a speed vacuum concentrator (LaBoGeneAps, Lynge, Denmark) for 10 min. One hundred nanogram proteins per fraction were digested with trypsin (Promega, Southhampton, U.K.) in 50 mM ammonium bicarbonate and left on ice for 45 min. The fraction was incubated at 37 ºC for 12 h.

A nano chip column (Agilent, 150 mm × 0.075 mm) was used for peptide separation. The mobile phases A and B for LC separation were 0.1% formic acid in deionized water and 0.1% formic acid in acetonitrile (ACN), respectively. The chromatography gradient was designed for a linear increase from 5% B to 8% B for 1 min, 8% B to 35% B for 19 min, 85% B for 10 min, and 5% B for 10 min. The flow rate was maintained at 400 nL/min. Product ion spectra were collected in the information-dependent acquisition (IDA) mode and analyzed by Agilent 6530 Accurate-Mass Q-TOF using continuous cycles of one full scan from 300−1500 m/z (four spectra/s) plus three product ion scans from 100−1700 m/z (two spectra/s). Precursor m/z values were selected starting with the most intense ion using a selection quadruple resolution of 4 Da.

3. Supplementary References

1. L. N. Goswami, Z. H. Houston, S. J. Sarma, S. S. Jalisatgi and M. F. Hawthorne, *Org. Biomol. Chem.*, 2013, **11**, 1116-1126.

2. S. -K. Ko, J. Kim, D. C. Na, S. Park, S. -H. Park, J. Y. Hyun, K. -H. Baek, N. D. Kim, N. -K. Kim, Y. N. Park, K. Song and I. Shin, *Chem. Biol.*, 2015, **22**, 391-403.

3. J. Y. Hyun, S. Kim, H. S. Lee and I. Shin, *Cell Chem. Biol.*, 2018, **25**, 1255-1267.

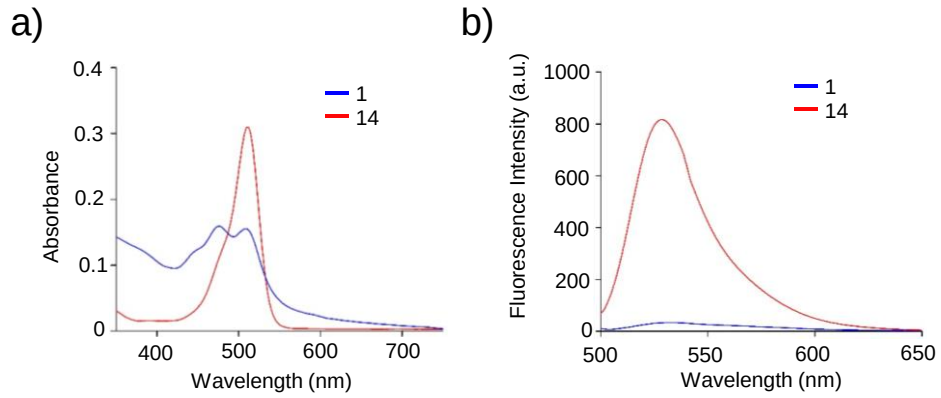


Figure S1. (a) UV-VIS absorption and (b) fluorescence spectra of **1** and **14** (λ_{ex} = 490 nm) in PBS (1% DMSO, pH 7.4).

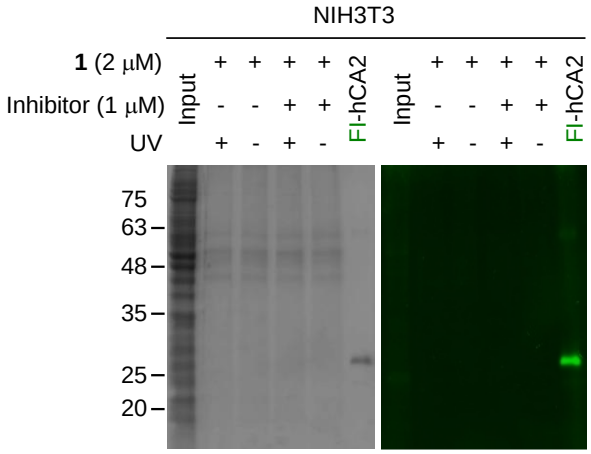


Figure S2. NIH3T3 cell lysates, incubated for 1 h with 2 μM 1 in the absence and presence of 1 μM acetazolamide, were irradiated with UV light for 1 min and then subjected to click reaction with N_3-biotin. The mixtures were then subjected to streptavidin-based affinity chromatography followed by separation of proteins by SDS-PAGE (left: silver staining, right: fluorescence). The FITC-labeled hCA2 protein (Fl-hCA2) was used as a control.

Table S1. Identification of a target protein of 1 by LC-MS/MS.

No.	accession no.	protein name	Score	MW	PI	sequence coverage	matched peptide
1	gi|115456	Full Carbonic anhydrase 2	808	29285	6.87	45%	23
2	gi|212375003	Chain A, X-Ray Crystal Structure Of Mutant N62d Of Human Carbonic Anhydrase II	807	29155	6.63	45%	22
3	gi|109156957	Chain A, Carbonic Anhydrase II	763	29154	6.86	45%	22
4	gi|146386955	Chain A, Carbonic Anhydrase 2	760	29284	6.87	45%	21
5	gi|14636961	Chain A, Carbonic Anhydrase 2	757	29284	6.87	45%	21
6	gi|835020720	Chain A, Carbonic Anhydrase 2	722	29231	6.87	39%	21

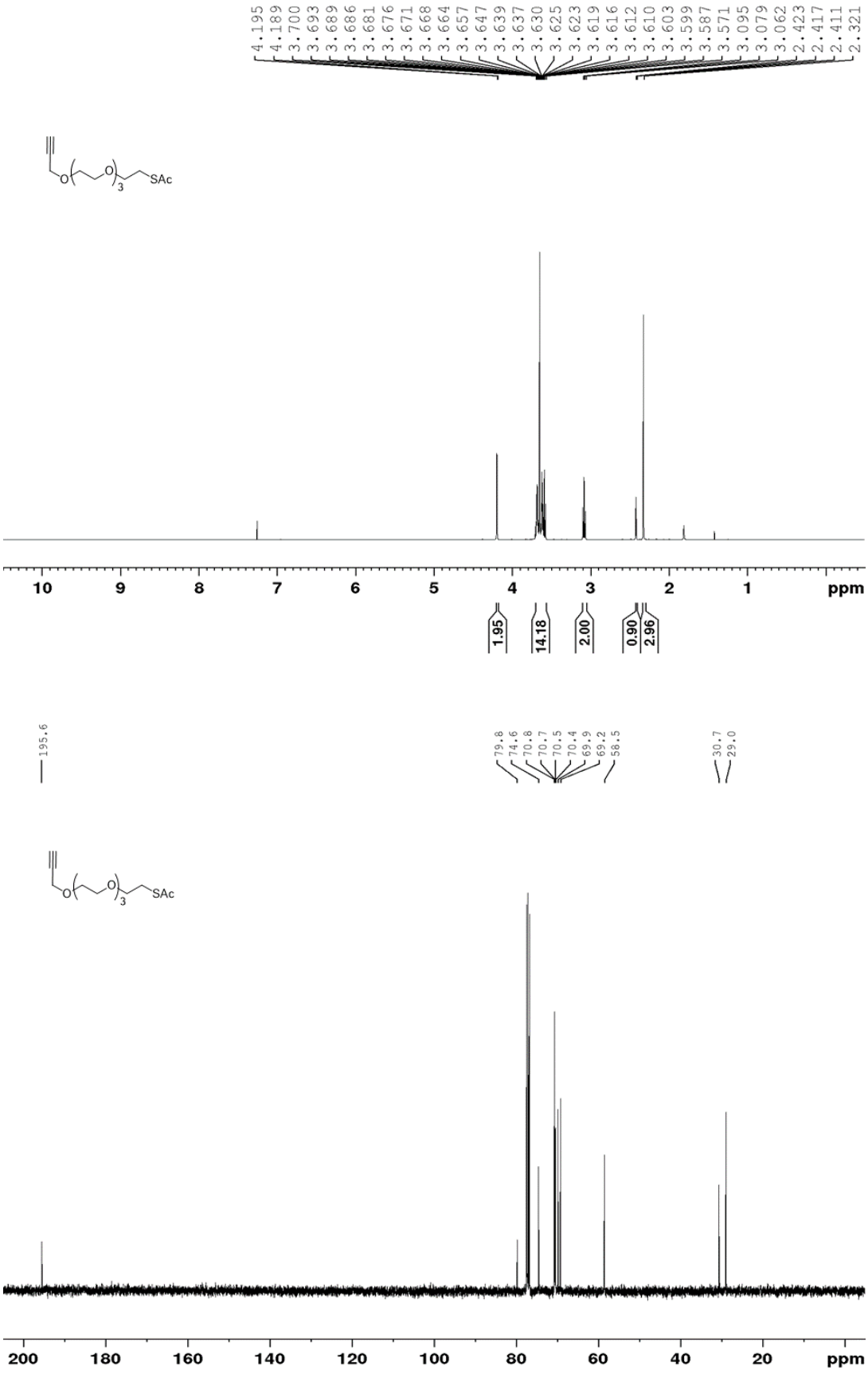
Table S2. Mascot report of all matched peptides of hCA2.

Swiss Prot Code	Accession No.	Protein name	MW (Da)	Seq. Coverage	Number on hit	Protein Score	PI
CAH2_Human	sp|P00918	Carbonic Anhydrase II	29285	45%	23	808	6.87

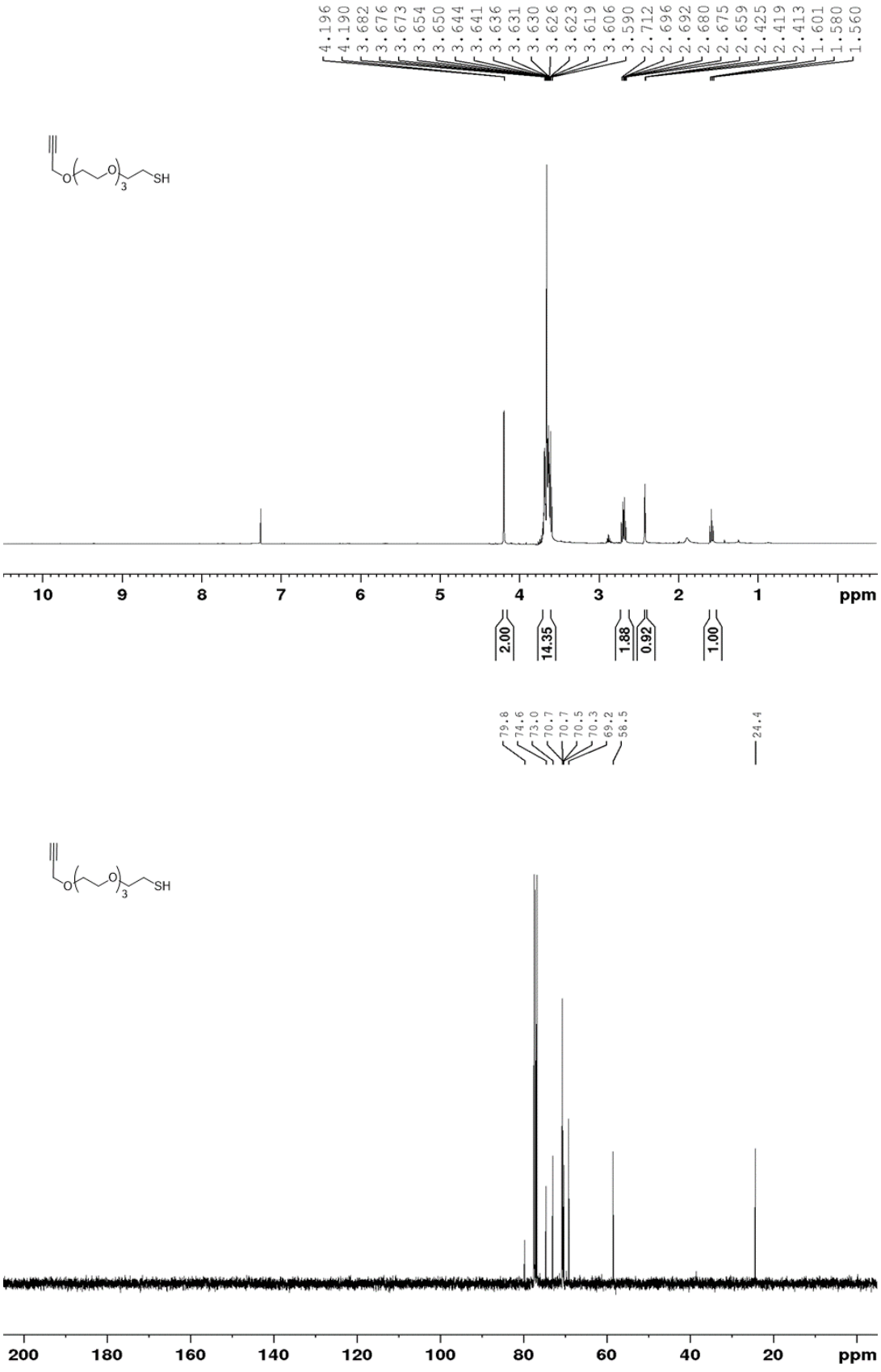
Start-End	Observed(mz)	Expected (mr)	z	Calculated (mr)	Pep delta	Pep_score	Pep_sequence	Pep_modification
28 - 39	437.8901	1310.6486	3	1310.6467	0.014	48	R.QSPVDIDTHTAK.Y	
59 - 76	1032.4716	2062.9286	2	2062.9232	0.0065	50	R.ILNNGHAFNVEFDDSQDK.A	Deamidated (NQ)
59 - 80	825.4163	2473.2271	3	2473.2237	0.0055	54	R.ILNNGHAFNVEFDDSQDKAVLK.G	
59 - 80	825.7442	2474.2108	3	2474.2077	0.027	47	R.ILNNGHAFNVEFDDSQDKAVLK.G	Deamidated (NQ)
59 - 80	825.7445	2474.2117	3	2474.2077	0.0072	53	R.ILNNGHAFNVEFDDSQDKAVLK.G	Deamidated (NQ)
59 - 80	619.5612	2474.2155	4	2474.2077	0.01	51	R.ILNNGHAFNVEFDDSQDKAVLK.G	Deamidated (NQ)
59 - 89	848.6742	3390.6677	4	3390.648	0.014	49	R.ILNNGHAFNVEFDDSQDKAVLKGGPLDGTYR.L	Deamidated (NQ)
81 - 89	468.2323	934.45	2	934.4509	0.0018	56	K.GGPLDGTYR.L	
133 - 148	834.9905	1667.9664	2	1667.961	0.032	39	K.AVQQPDGLAVLGIFLK.V	
133 - 148	834.9916	1667.9687	2	1667.961	6.80E-07	86	K.AVQQPDGLAVLGIFLK.V	
149 - 158	492.7959	983.5773	2	983.5764	0.0014	52	K.VGSAKPGLQK.V	
159 - 167	494.2895	986.5644	2	986.5648	2.30E-05	73	K.VVDVLDSIK.T	
159 - 167	494.2899	986.5653	2	986.5648	0.0019	54	K.VVDVLDSIK.T	
159 - 169	608.8616	1215.7086	2	1215.7075	2.20E-05	72	K.VVDVLDSIKTK.G	
159 - 169	608.8618	1215.7091	2	1215.7075	0.00071	56	K.VVDVLDSIKTK.G	
159 - 171	701.4193	1400.8239	2	1400.8239	2.20E-05	69	K.VVDVLDSIKTKGK.S	
170 - 181	452.2178	1353.6316	3	1353.6313	0.012	47	K.GKSADFTNFDPR.G	
172 - 181	585.2657	1168.5169	2	1168.5149	0.0018	53	K.SADFTNFDPR.G	
172 - 181	585.2663	1168.518	2	1168.5149	0.00043	61	K.SADFTNFDPR.G	
172 - 181	585.2668	1168.5191	2	1168.5149	0.028	42	K.SADFTNFDPR.G	
227 - 251	976.8177	2927.4314	3	2927.4123	0.044	46	R.KLNFNGEGEPEELMVDNWRPAQPLK.N	Deamidated (NQ); Oxidation (M)
227 - 251	977.1465	2928.4178	3	2928.3963	0.028	47	R.KLNFNGEGEPEELMVDNWRPAQPLK.N	2 Deamidated (NQ); Oxidation (M)
227 - 253	800.4024	3197.5805	4	3197.5563	0.031	46	R.KLNFNGEGEPEELMVDNWRPAQPLKNR.Q	Deamidated (NQ); Oxidation (M)

<NMR spectra>

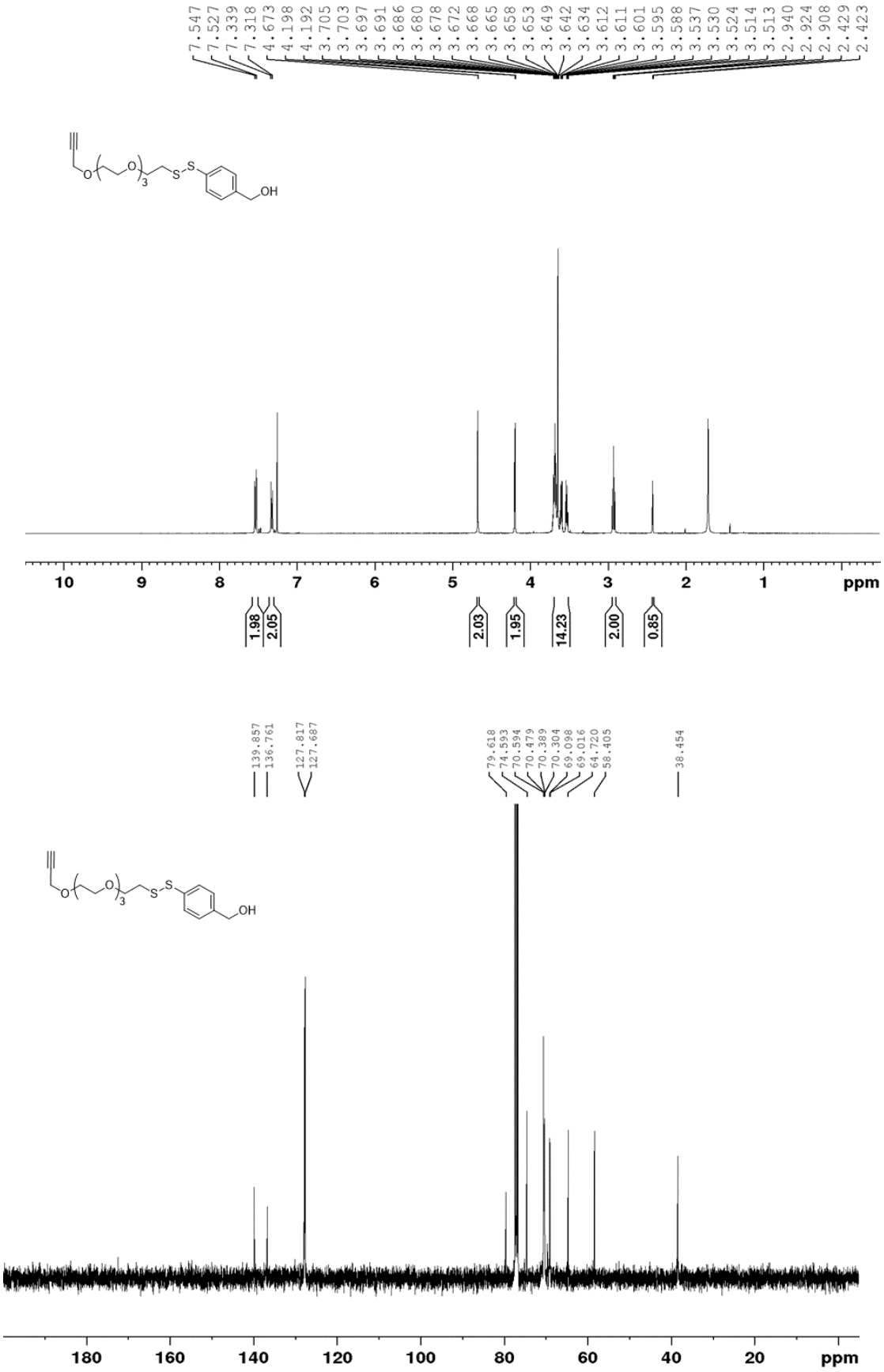
Compound 2 ($CDCl_3$, 400 MHz ^1H NMR, 100 MHz ^{13}C NMR)



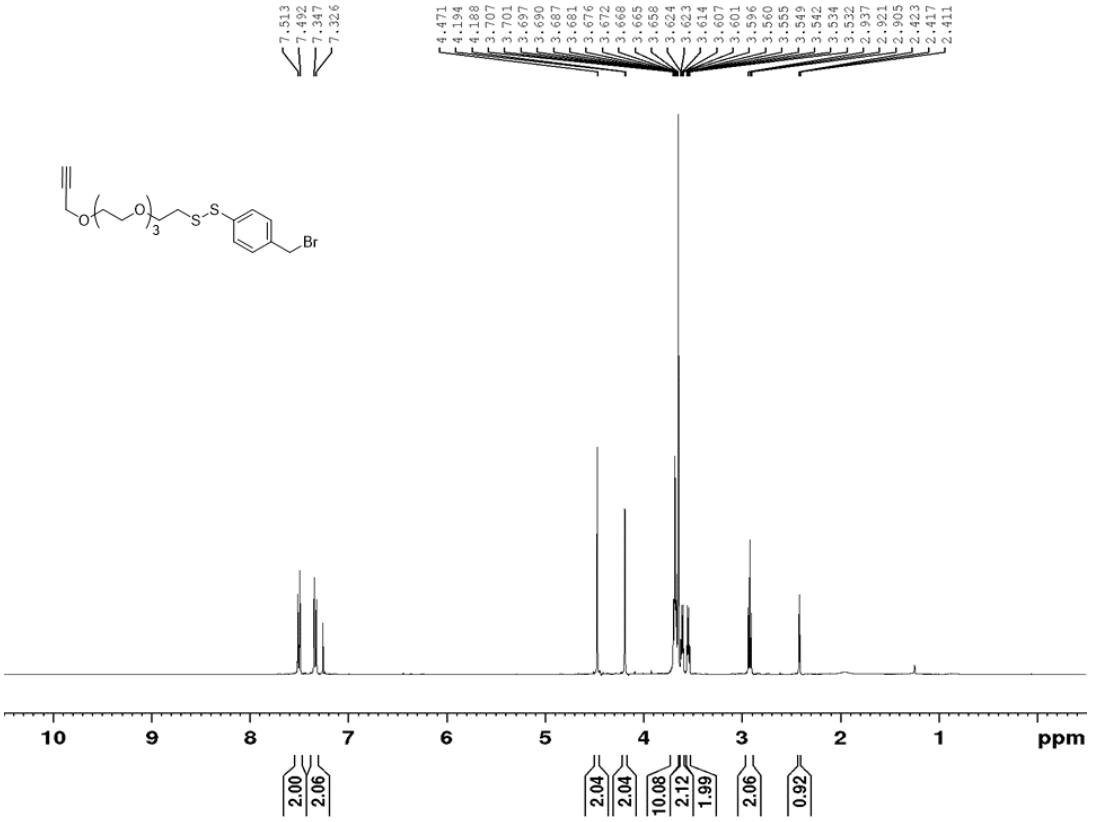
Compound 3 ($CDCl_3$, 400 MHz ^1H NMR, 100 MHz ^{13}C NMR)

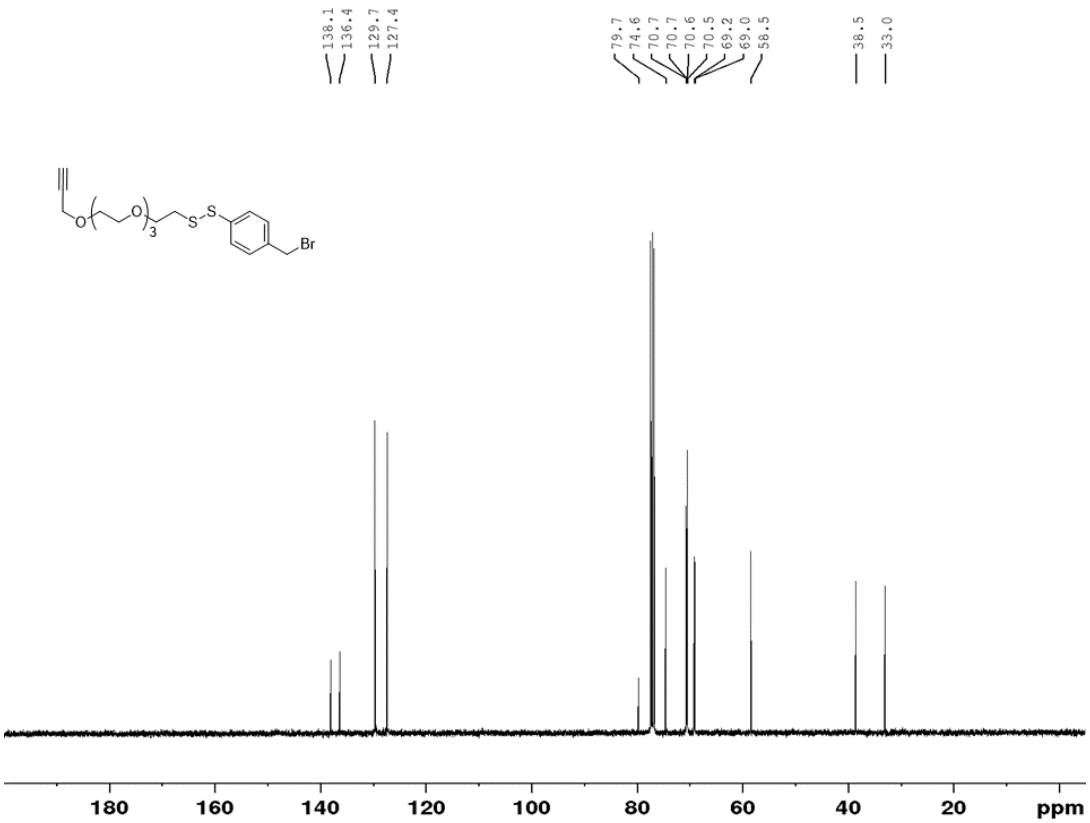


Compound 4 ($CDCl_3$, 400 MHz ^1H NMR, 100 MHz ^{13}C NMR)

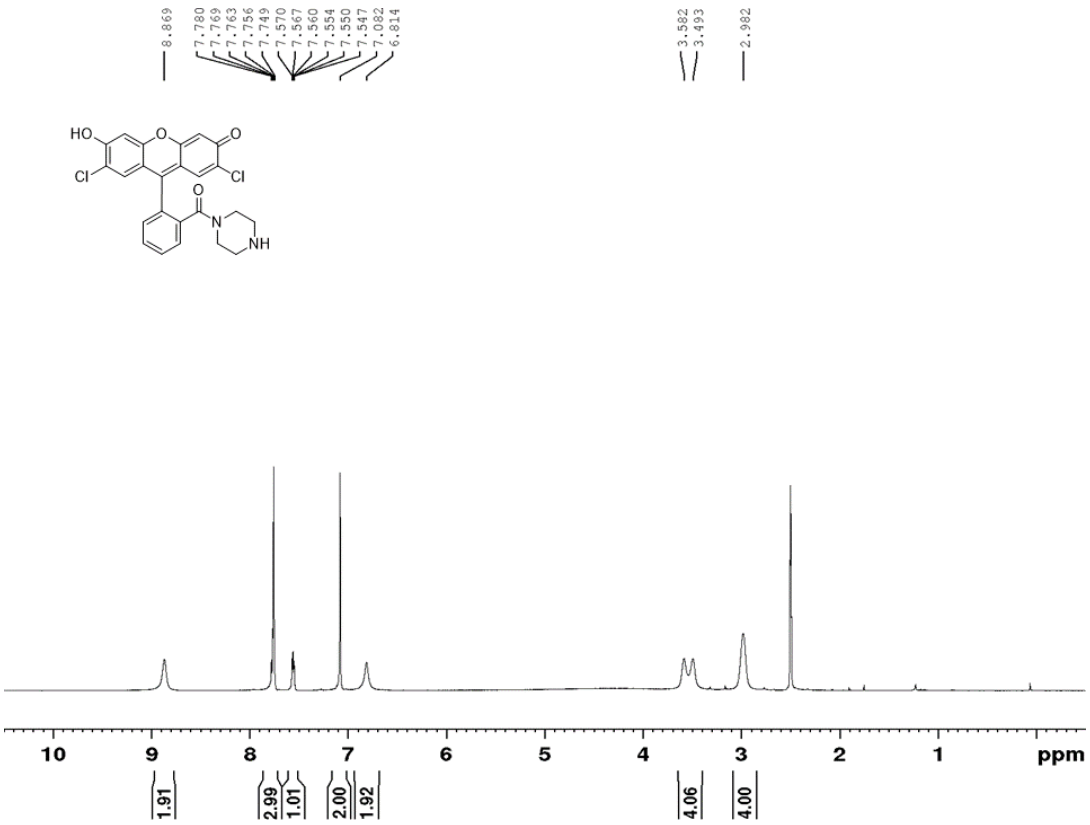


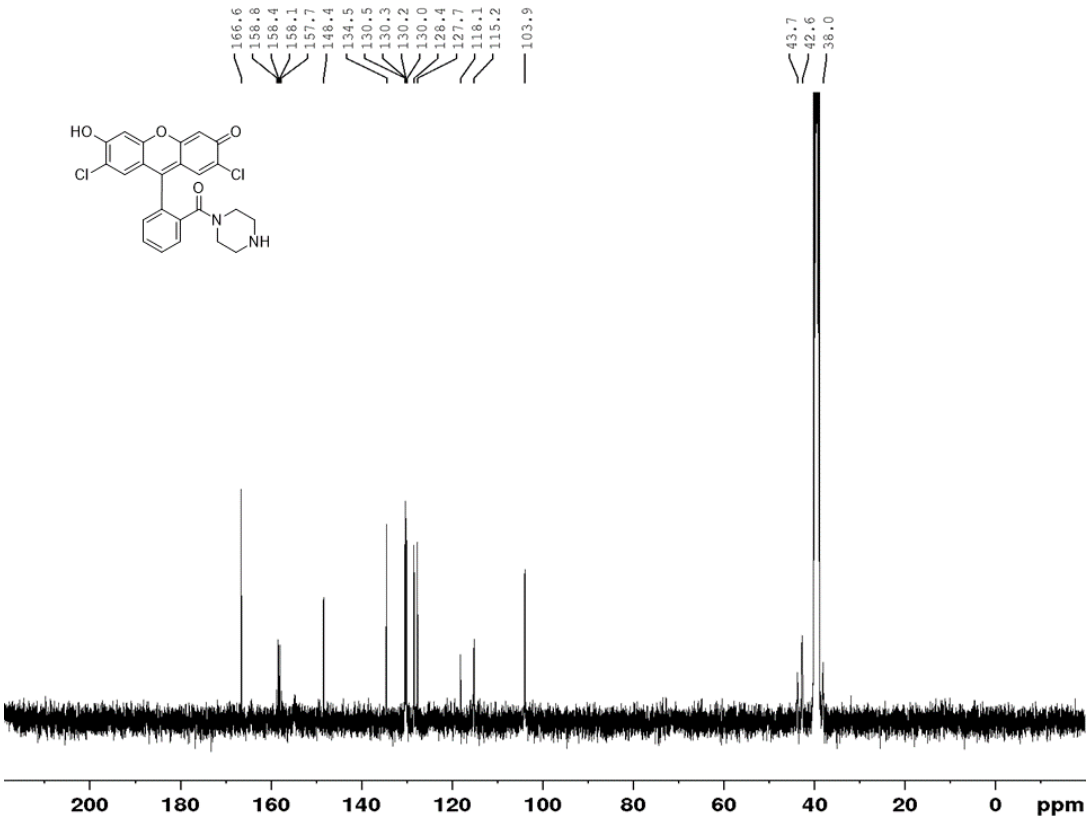
Compound 5 ($CDCl_3$, 400 MHz ^1H NMR, 100 MHz ^{13}C NMR)



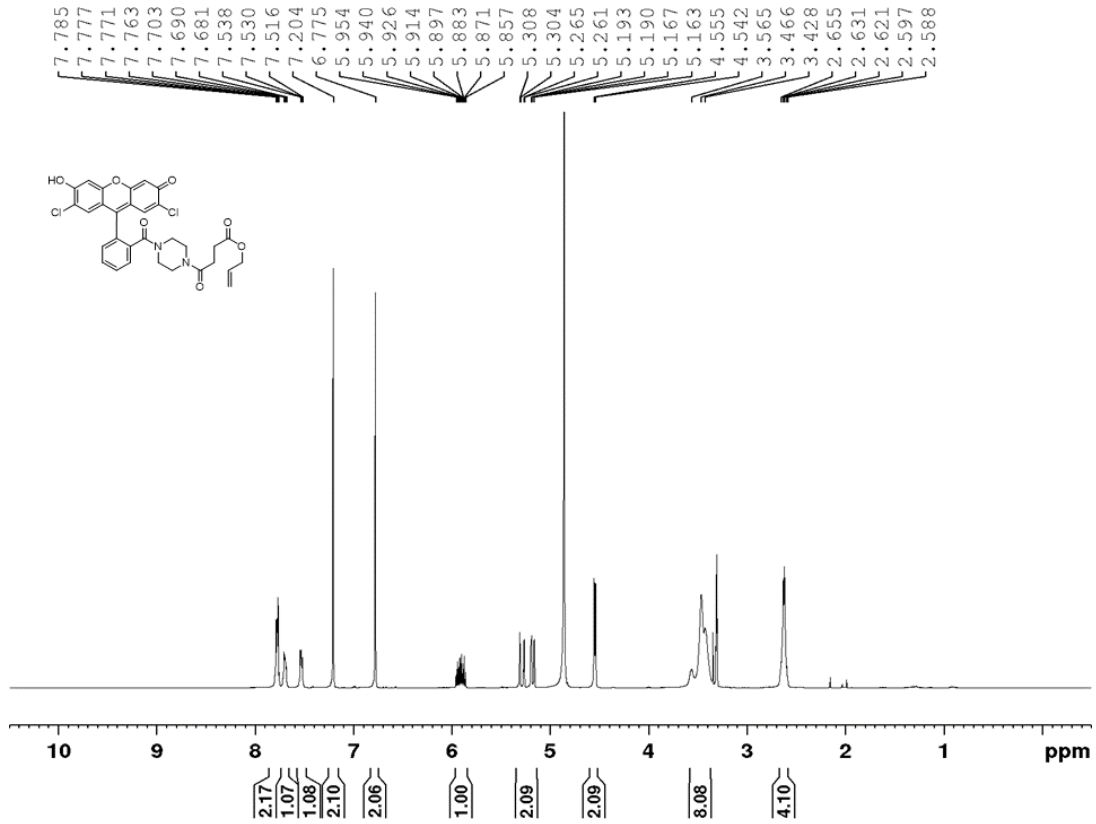


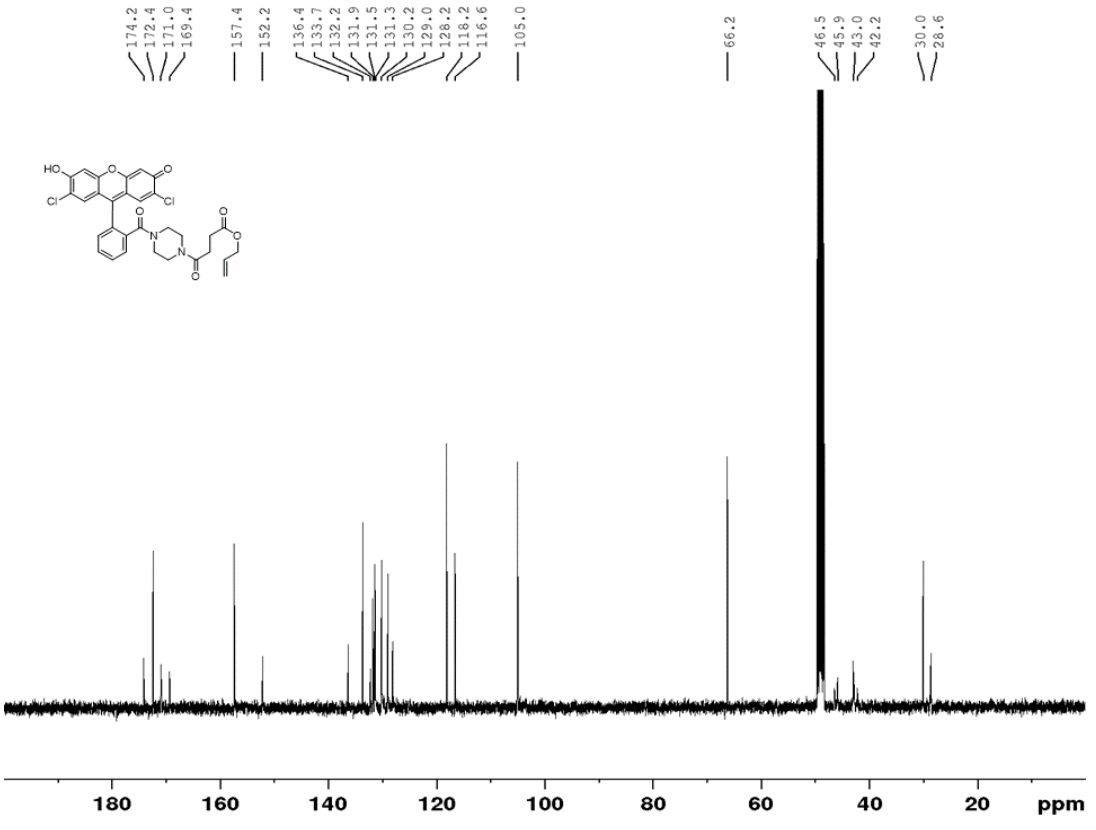
Compound 7 (DMSO-d_6, 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR)



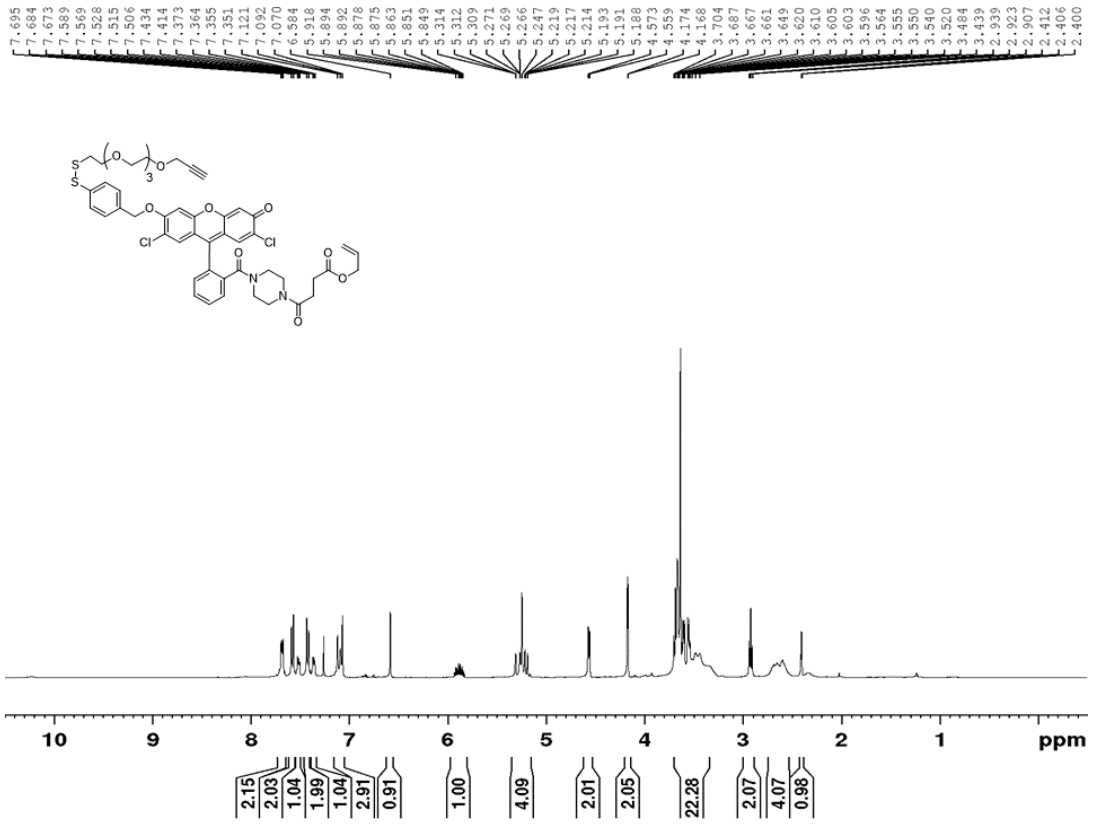


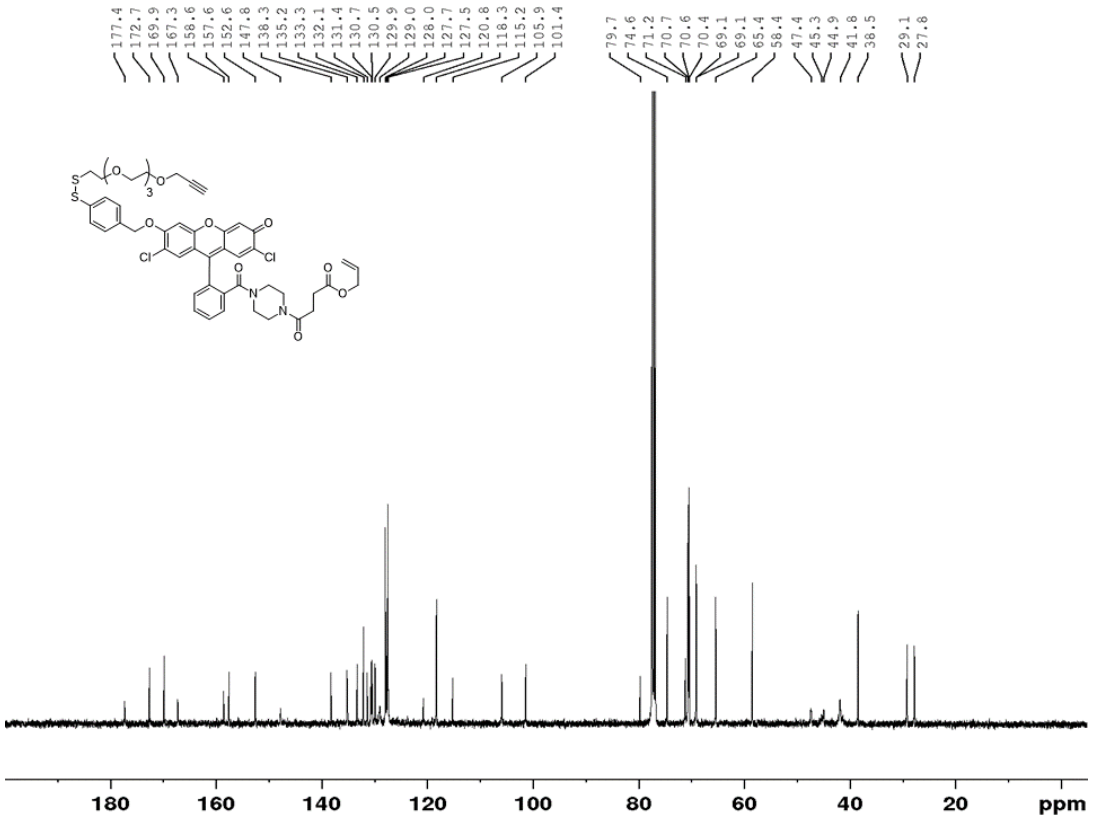
Compound 8 (CD_3OD, 400 MHz ^1H NMR, 100 MHz ^{13}C NMR)



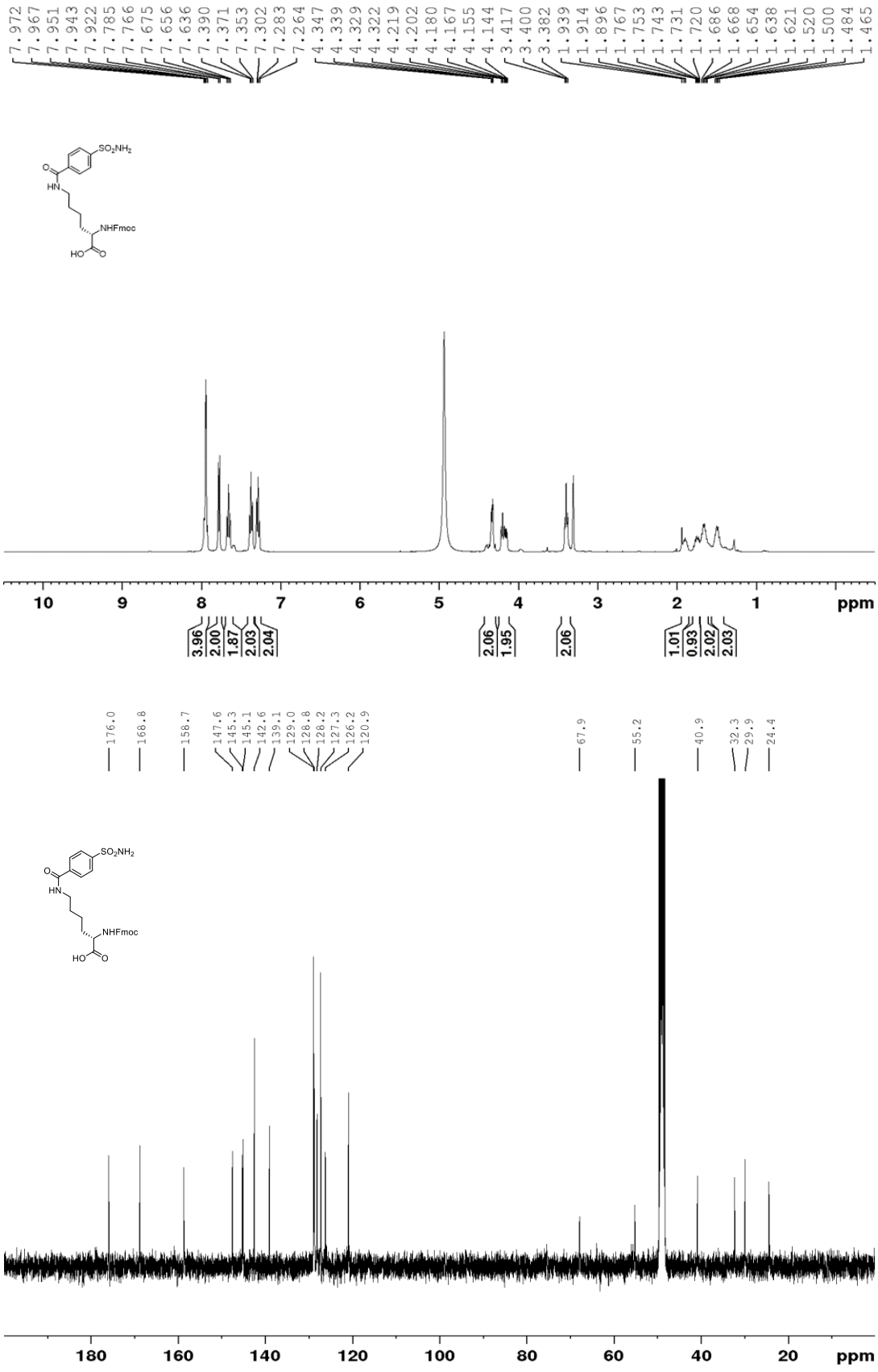


Compound 9 ($CDCl_3$, 400 MHz ^1H NMR, 100 MHz ^{13}C NMR)

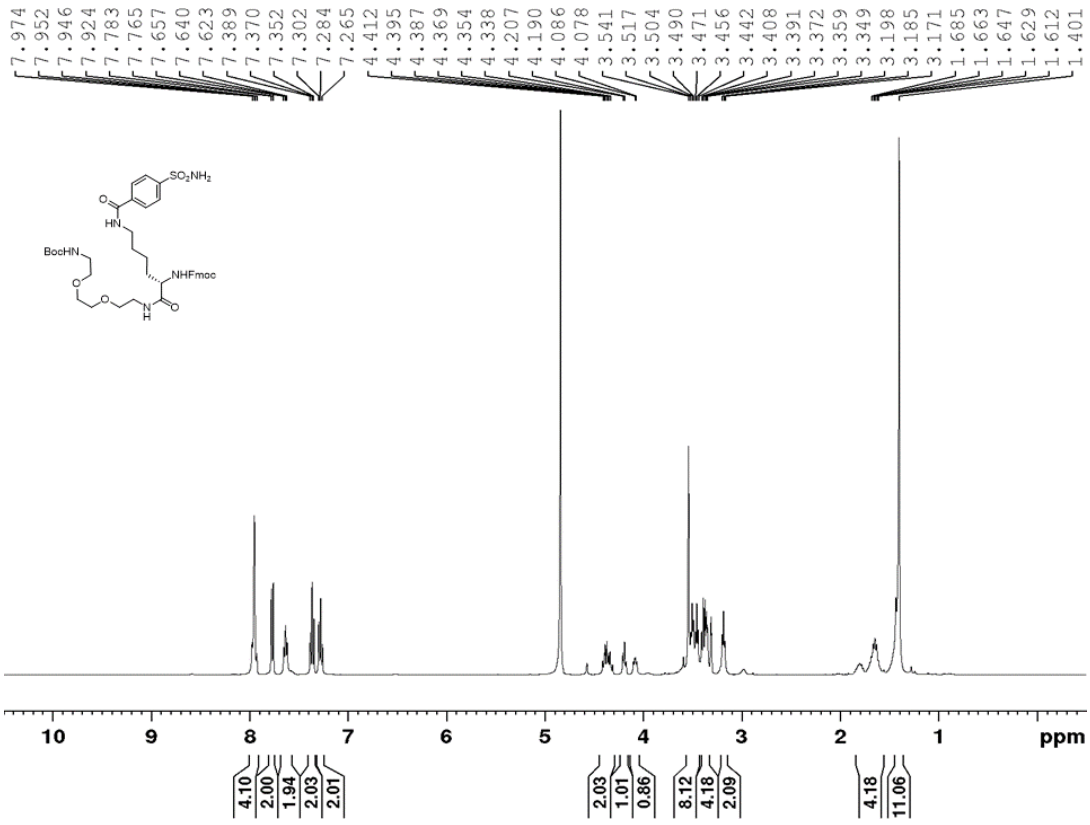


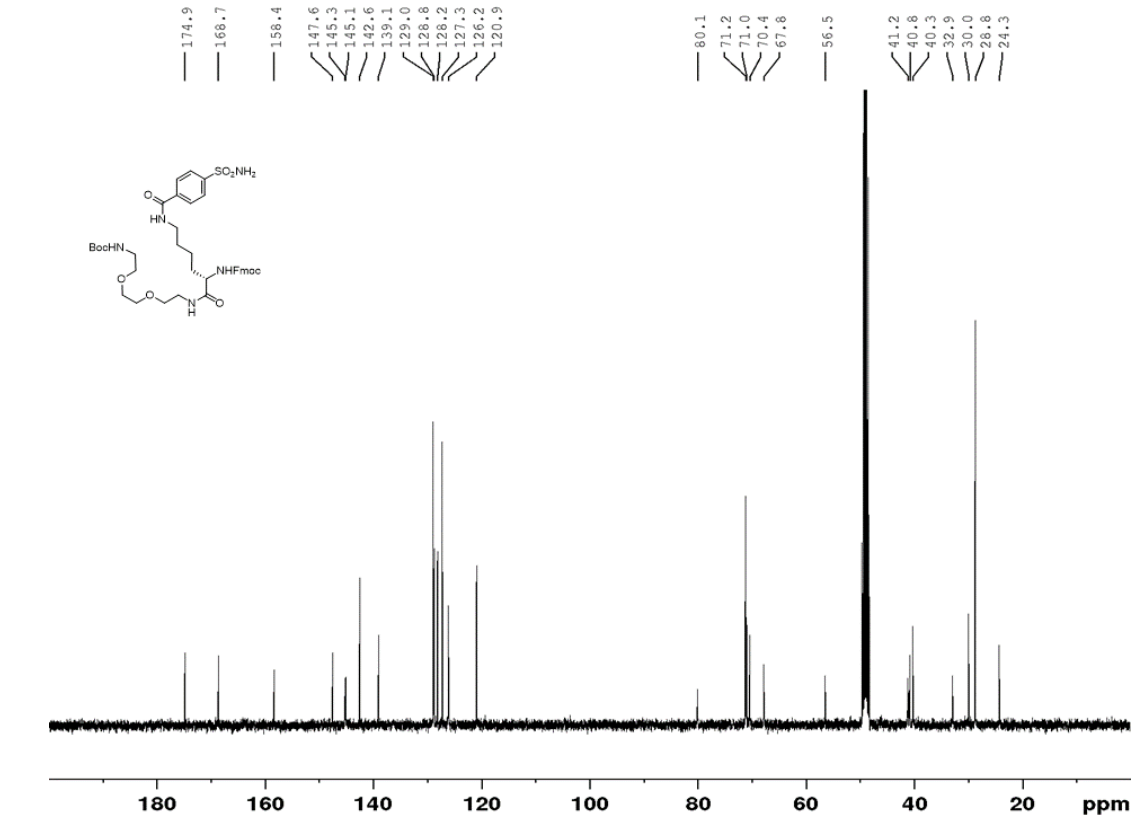


Compound 11 (CD_3OD, 400 MHz ^1H NMR, 100 MHz ^{13}C NMR)

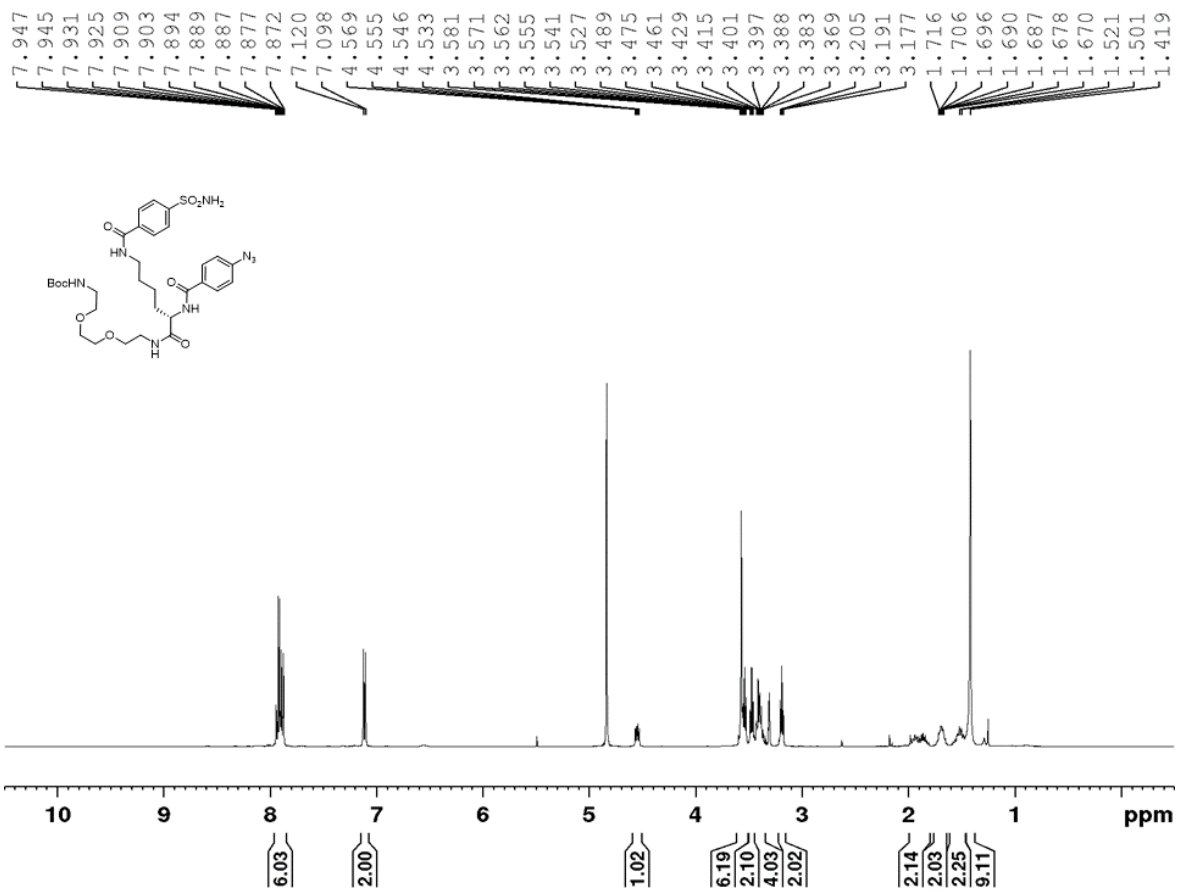


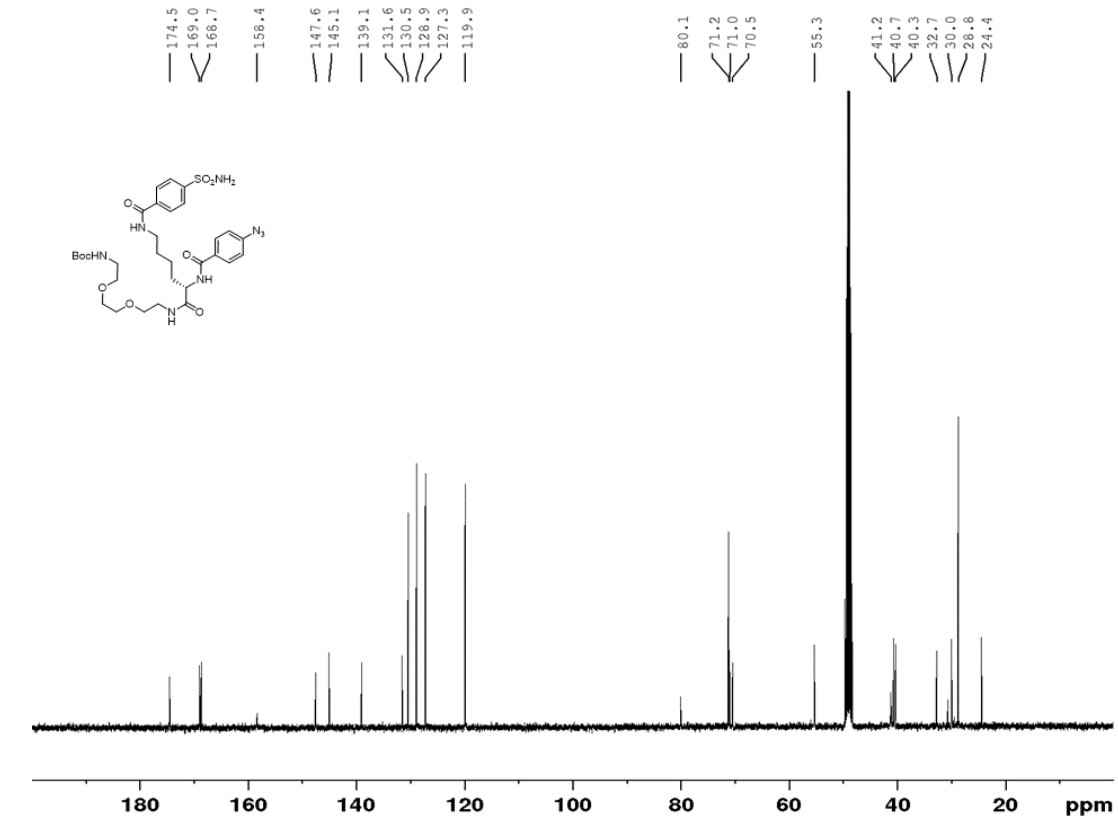
Compound 12 (CD_3OD, 400 MHz ^1H NMR, 100 MHz ^{13}C NMR)



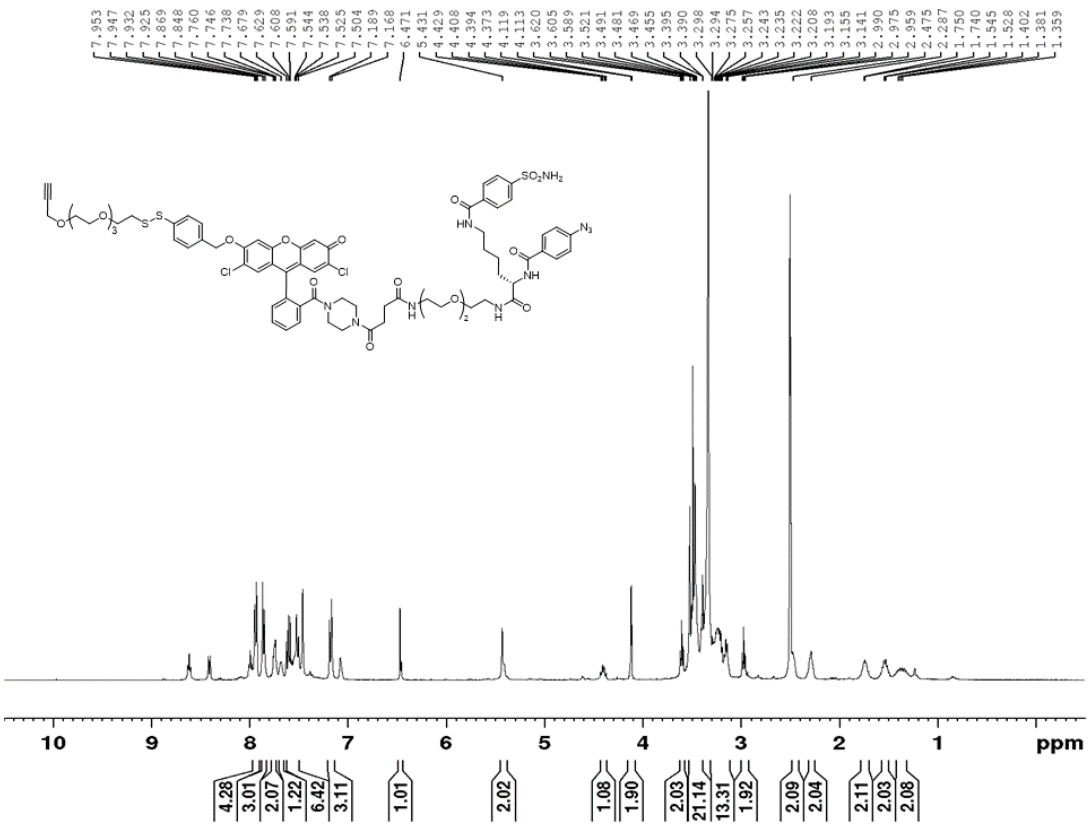


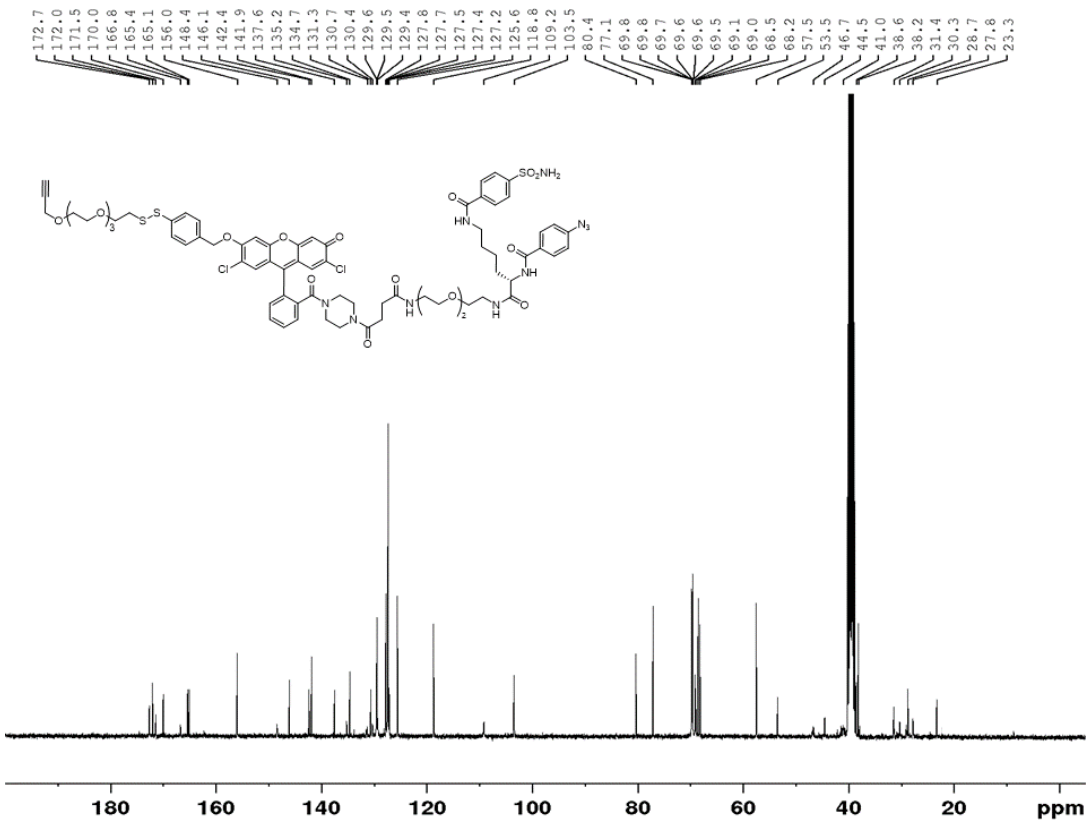
Compound 13 (CD_3OD, 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR)



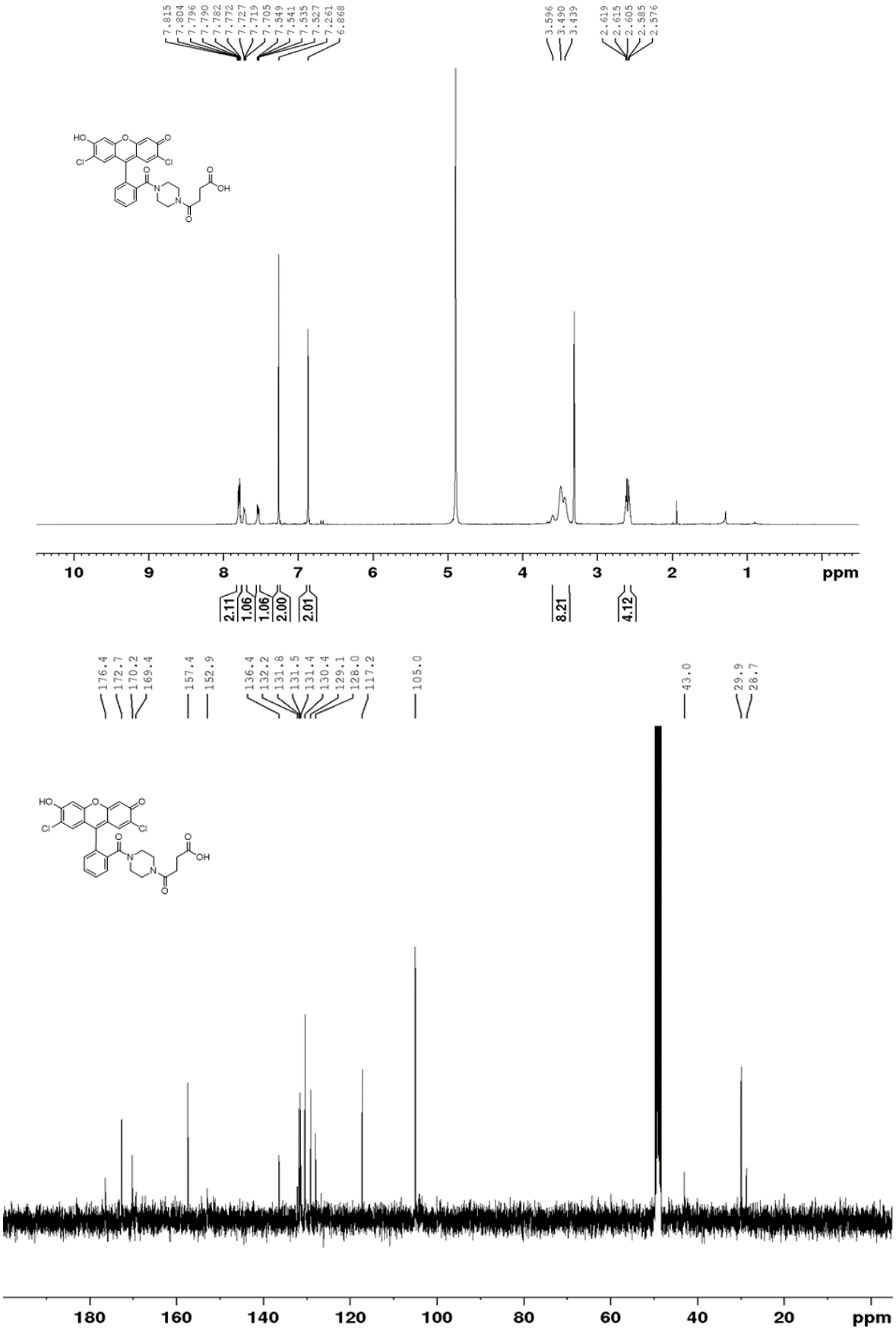


Compound 1 (DMSO-d_6, 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR)





Compound 14 (CD_3OD, 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR)



Compound 15 (DMSO-d_6, 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR)