Original article

Fluorine-thiol displacement probes for acetaminophen's hepatotoxicity

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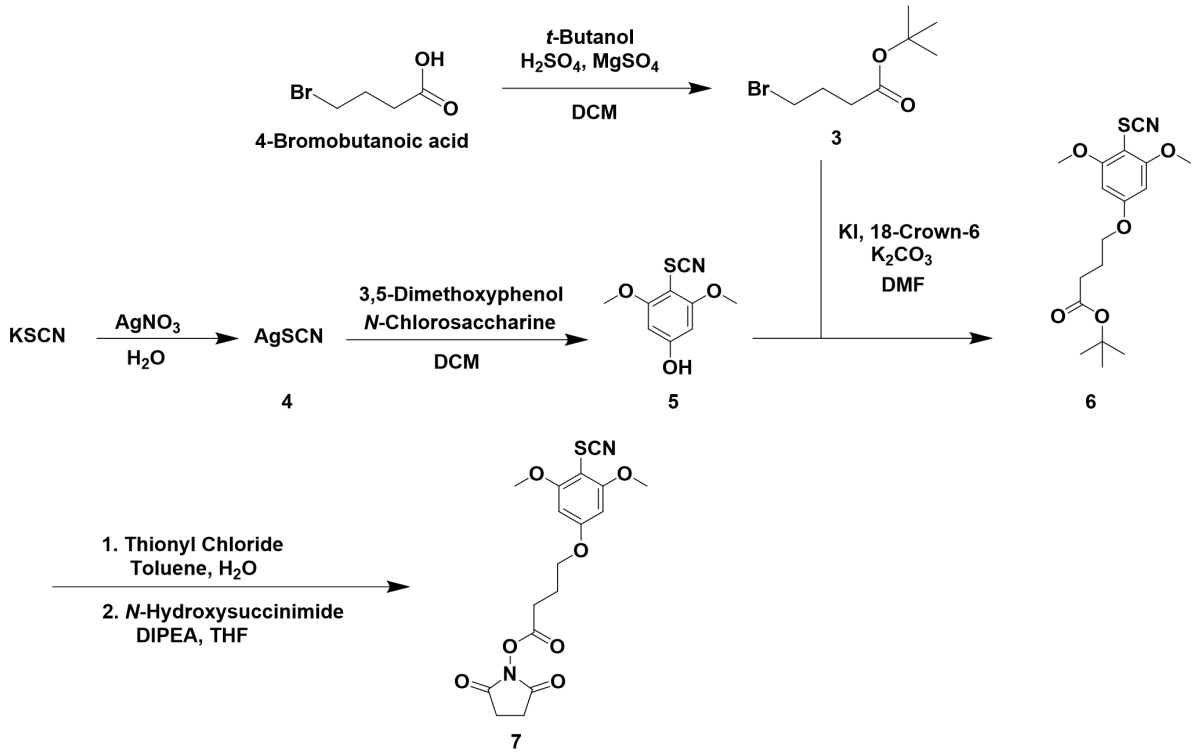
Chemical Probe Synthesis

General Information:

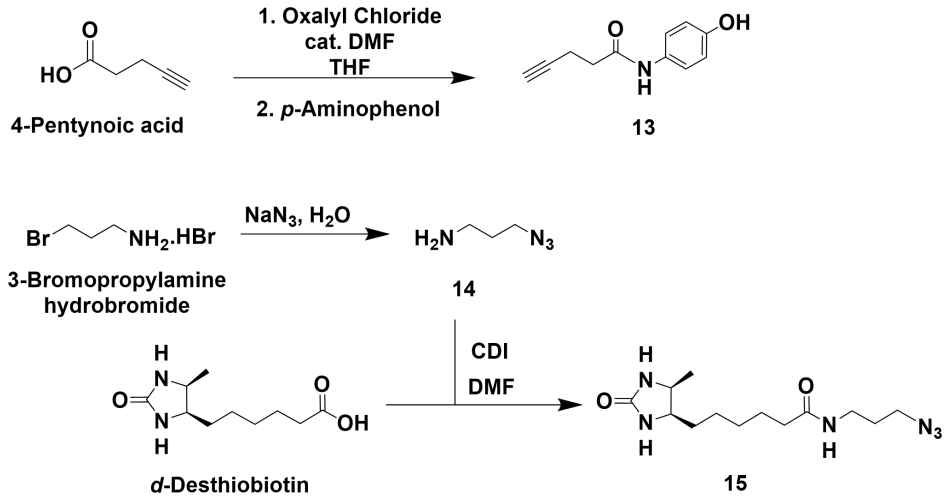
Chemical reagents and solvents were purchased from commercial resources such as VWR, Thermo Fisher, Sigma Aldrich, and Broadpharm. The chemical reagents and solvents were used directly without further purification. Analytical TLC was carried out with Silica Gel 60 F254 plates (Sigma). The chemicals on TLC were either visualized by UV 254 nm (UV lamp, Chemglass Life Sciences) or stained by phosphomolybdic acid, Ninhydrin, *p*-Anisaldehyde, or $KMnO_4$ oxidation. Compound purification was performed by normal-phase flash column chromatography on columns manually loaded with silica gel grade 60 (230–400 mesh, Fisher Scientific) or by HPLC as specified below. Routine mass spectrometry analysis was done using electrospray ionization (ESI) Advion CMS. NMR analysis results were recorded on 500 MHz Bruker Advance. The raw data were processed with MestReNova, and the chemical shifts were reported in parts per million (ppm) downfield from the internal standard tetramethylsilane (TMS).

Supplementary Synthetic Schemes:

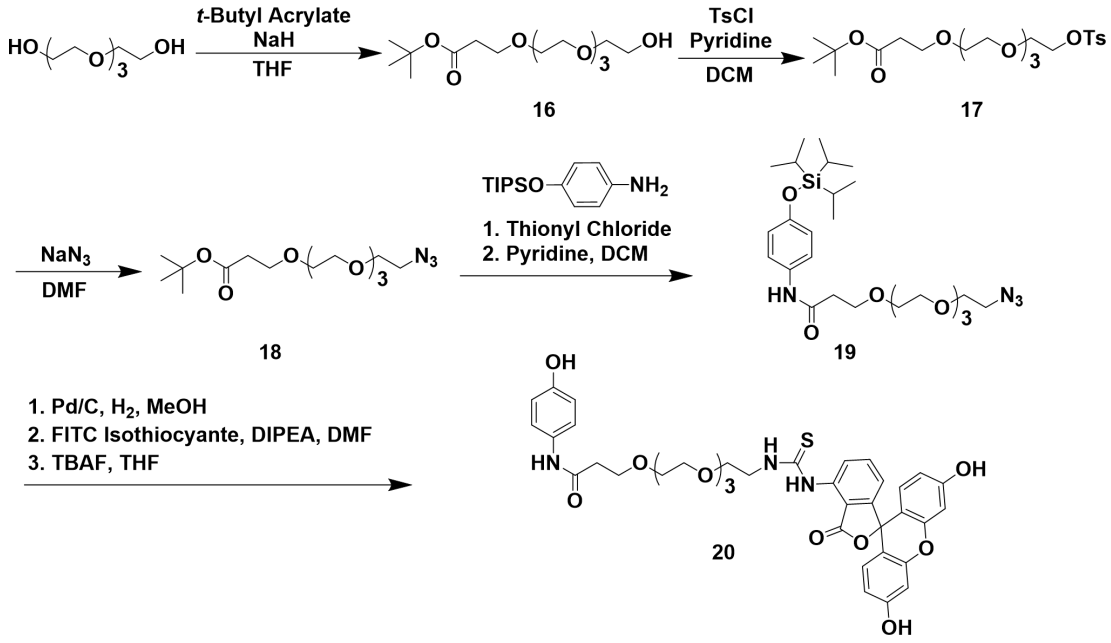
Scheme 1. Synthesis of phenyl thiocyanate NHS intermediate **7**.



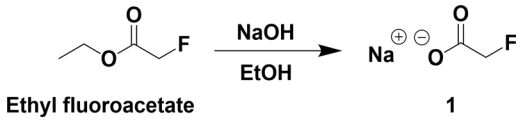
Scheme 2. Synthesis of APAP-Alkyne probe **13** and Biotin-Azide **15**.



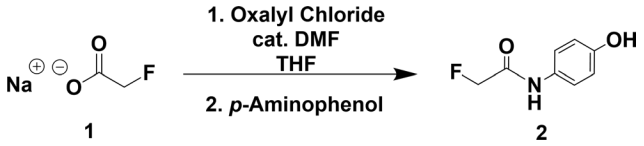
Scheme 3. Synthesis of APAP-FITC probe **20**.



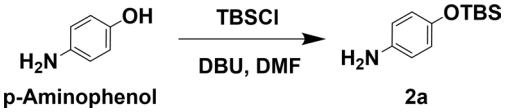
Detailed Synthesis Steps:



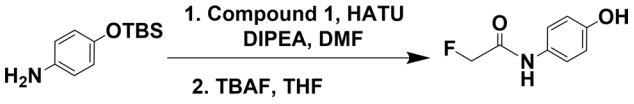
Following the reported procedure,[1] sodium hydroxide (0.8 g, 20.8 mmol, 1.0 equiv), ethyl fluoroacetate (2.0 mL, 20.8 mmol, 1.0 equiv), and ethanol (45 mL) were added into a 100 mL round-bottom flask. The resulting mixture was stirred overnight for 12 h at room temperature. The following day, the precipitate was collected via filtration, washed with cold ethanol, and dried under reduced pressure to provide sodium fluoroacetate **1** as a white solid (1.8 g, 86%). ^{1}H NMR (500 MHz, Deuterium Oxide): δ 4.68 (d, J = 48.2 Hz, 2H).



Sodium fluoroacetate **1** (0.4 g, 4.0 mmol, 2.0 equiv), THF (5 mL), 2M oxalyl chloride (1.0 mL, 2.0 mmol, 1.0 equiv),[3] and DMF (0.1 mL) were sequentially added into a 25 mL round-bottom flask. The heterogenous mixture was stirred for 30 min at room temperature, and then an additional amount of sodium fluoroacetate **1** (1.0 g, 10.0 mmol, 5.0 equiv) was added. After 1 h the reaction flask was charged with *p*-aminophenol (0.2 g, 2.0 mmol, 1.0 equiv) and left to stir for an additional 3 h. The solvent was removed under reduced pressure and the crude material was purified via flash chromatography with an eluent of 2%-8% methanol in dichloromethane to provide compound **2** as a white solid (0.16 g, 49%). ^{1}H NMR (500 MHz, Acetone-d_6): δ 9.04 (s, 1H), 8.25 (s, 1H), 7.57 (d, J = 8.9 Hz, 2H), 6.82 (d, J = 8.9 Hz, 2H), 4.91 (d, J = 47.3 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 165.8, 165.6, 154.3, 130.0, 122.3, 115.5, 81.1, 79.7. ESI-TOF *m/z* calcd for $C_8H_8FNO_2$ [M-H]$^-$, 168.0; found, 167.9.

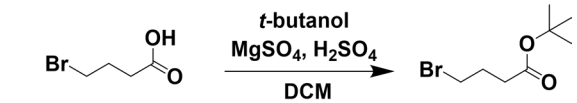


p-Aminophenol (1.0 g, 9.2 mmol, 1.0 equiv), DBU (0.2 g, 1.4 mmol, 0.15 equiv), and 30 mL of DMF were added into a 250 mL round-bottom flask and then cooled to 0°C. Afterwards, TMSCl (1.7 g, 11.0 mmol, 1.2 equiv) was charged to the reaction mixture. The reaction mixture was stirred for 3 h and then quenched with water (50 mL) and a saturated solution of $NaHCO_3$ (10 mL). The aqueous layer was washed with ethyl acetate (3 x 30 mL), and the combined organic extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude material was purified by flash chromatography with an eluent of 25% ethyl acetate and 1% triethylamine in hexane to afford compound **2a** as an opaque oil. (1.64 g, 80%).



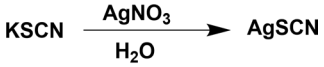
2a 2

Sodium fluoroacetate **1** (0.1 g, 1.0 mmol, 1.0 equiv), HATU (0.5 g, 1.2 mmol, 1.2 equiv), DIPEA (0.2 mL, 1.2 mmol, 1.2 equiv), and DMF (5 mL) were added into a 50 mL round-bottom flask. After the mixture was stirred for 20 min, compound **2a** (0.5 g, 2.0 mmol, 2.0 equiv) was added. The reaction continued for an additional 12 h and then was quenched with water (25 mL). Subsequently, the aqueous solution was washed with ethyl acetate (2 x 30 mL). The combined organic extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The resulting compounds were then redissolved in THF (5 mL), transferred to a 20 mL vial, and cooled to 0°C. A solution of TBAF in THF (1M, 2 mL) was then added dropwise and the reaction mixture was stirred for 2 h at 0°C. Upon completion, the reaction mixture was diluted with DCM (5 mL), quenched with water (1 mL), and washed with brine (20 mL). The organic layer was collected and concentrated under reduced pressure. Dual solvent recrystallization was performed with acetonitrile/toluene to produce compound **2** as a white solid (0.13 g, 75%).



4-bromobutanoic acid 3

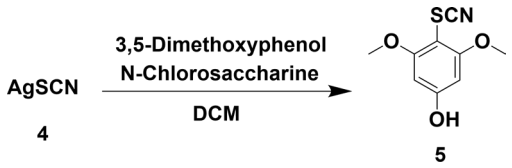
Following the reported procedure,[2] 4-bromobutanoic acid (3.4 g, 20.0 mmol, 1.0 equiv), 45 mL of DCM, magnesium sulfate (1.0 g, 80.0 mmol, 4.0 equiv), sulfuric acid (1.0 mL, 18.5 mmol, 1.0 equiv), and *t*-butyl alcohol (10.0 mL, 100 mmol, 5.0 equiv) were sequentially added to a 250 mL round-bottom flask. The reaction mixture was stirred overnight at room temperature. Subsequently, the mixture was diluted with DCM (60 mL), cooled to 0°C, and neutralized with a saturated $NaHCO_3$ solution (5 mL). The resulting solution was washed with water (40 mL) and then phase separated. The organic layer was collected, concentrated under reduced pressure, and purified by flash chromatography with a gradient of 5%-35% ethyl acetate in hexane to render compound **3** as a yellow oil (1.5 g, 33%). ^{1}H NMR (500 MHz, Chloroform-d): δ 3.45 (t, J = 6.5 Hz, 2H), 2.40 (t, J = 7.2 Hz, 2H), 2.13 (p, J = 6.8 Hz, 2H), 1.45 (s, 9H).



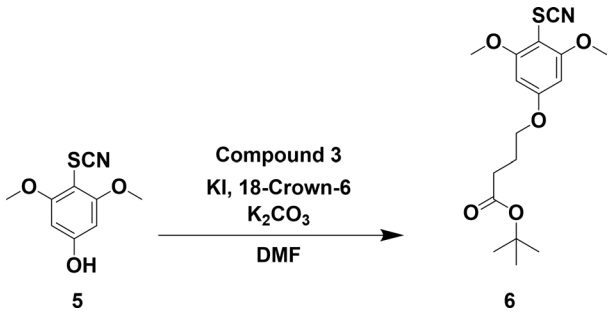
4

Following the reported procedure,[1] silver nitrate (1.8 g, 10.3 mmol, 1.0 equiv) was added into a 100 mL round-bottom flask and dissolved with water (20 mL). Subsequently, a 20 mL aqueous solution of potassium thiocyanate (1.0 g, 10.3 mmol, 1.0 equiv) was charged to the reaction flask. The reaction mixture was stirred for 1 h at room temperature. The precipitate was then collected

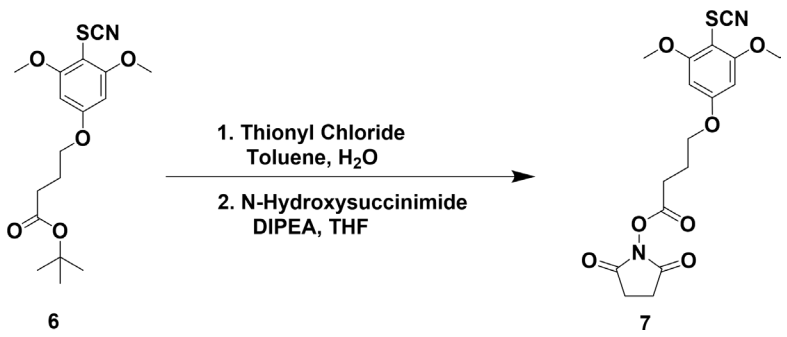
via filtration and dried under reduced pressure to produce compound **4** as a white solid (1.65 g, 97%).



Following the reported procedure,[1] *N*-chlorosaccharin (2.6 g, 12.0 mmol, 1.1 equiv), silver thiocyanate **4** (2.0 g, 12.0 mmol, 1.1 equiv), and 50 mL of DCM were added to a 250 mL round-bottom flask. The subsequent reaction mixture was stirred for 1 h at room temperature. 3,5-dimethoxyphenol (1.7 g, 11.0 mmol, 1.0 equiv) dissolved in 50 mL of DCM was then charged to the reaction mixture. The reaction was monitored by TLC. Upon completion, the solution was filtered, and the filtrate was collected. The filtrate was concentrated under reduced pressure and purified via flash chromatography with a gradient of 25% - 40% ethyl acetate in hexane to afford compound **5** as a white solid (0.7 g, 30%). ^{1}H NMR (500 MHz, Acetone-d_6): δ 9.16 (s, 1H), 6.29 (s, 2H), 3.91 (s, 6H).

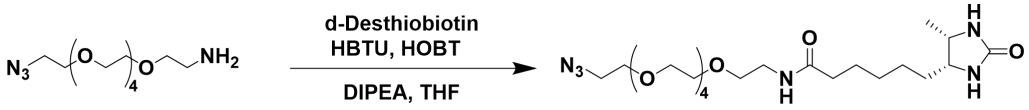


A DMF solution (~ 5 mL) containing compound **3** (0.4 g, 1.9 mmol, 2.0 equiv), potassium iodide (0.3 g, 1.9 mmol, 2.0 equiv), and 18-crown-6 (0.5 g, 1.9 mmol, 2.0 equiv) was pre-stirred for 5 min in a 20 mL vial. Concurrently, another DMF solution (~ 5 mL) containing compound **5** (0.2 g, 1.0 mmol, 1.0 equiv), potassium carbonate (0.2 g, 1.4 mmol, 1.5 equiv), and 18-Crown-6 (0.8 g, 3.0 mmol, 2.9 equiv) was pre-stirred for 5 min in a 20 mL vial. The two reaction mixtures were then pooled together, and the combined mixture was stirred overnight at room temperature. The following day, ethyl acetate (50 mL) and water (50 mL) were added. After phase separation, the organic layer was washed with 1M HCl (1 x 20 mL), a saturated solution of $NaHCO_3$ (1 x 20 mL), and water (2 x 20 mL). The organic layer was concentrated under reduced pressure and then purified by flash chromatography with a gradient of 25% - 35% ethyl acetate in hexane to provide compound **6** as a yellow oil (0.33g, 99%). ^{1}H NMR (500 MHz, Chloroform-*d*): δ 6.16 (s, 2H), 4.03 (t, J = 6.3 Hz, 2H), 3.91 (s, 6H), 2.43 (t, J = 7.2 Hz, 2H), 2.08 (p, J = 6.7 Hz, 2H), 1.46 (s, 9H). ^{13}C NMR (126 MHz, Acetone-d_6): δ 171.8, 164.0, 161.5, 111.1, 92.1, 88.9, 79.6, 67.3, 56.1, 31.3, 27.4, 24.4. ESI-TOF *m/z* calcd for $C_{17}H_{23}NO_5S$ $[M+Na]^+$, 376.1; found, 376.2.



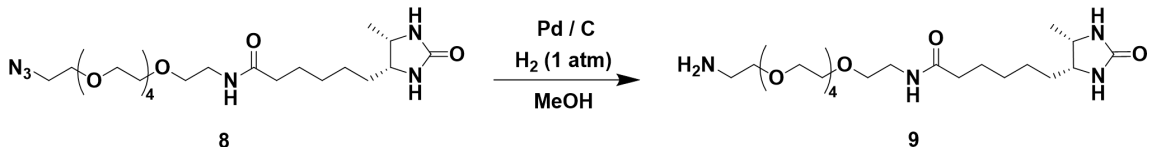
6 7

Following the reported procedure,[2] compound **6** (0.2 g, 0.5 mmol, 1.0 equiv) was dissolved in toluene (5 mL) and added into a 20 mL vial. Next, thionyl chloride (1.1 mL, 15.0 mmol, 28.0 equiv) and water (40.0 µL, 2.2 mmol, 4.1 equiv) were added into the reaction flask. The reaction mixture was stirred for 16 h at room temperature and then diluted with additional toluene (5 mL). The thionyl chloride – toluene azeotrope solution was removed under reduced pressure. The resulting acyl chloride was then redissolved in anhydrous THF (5 mL) and cooled to 0°C. *N*-hydroxysuccinimide (0.1 g, 0.8 mmol, 1.5 equiv) and DIPEA (0.2 g, 1.6 mmol, 3.0 equiv) were added, and the reaction mixture was stirred for 1 h at room temperature. Upon completion, the solvent was removed under reduced pressure and the crude material was purified via flash chromatography with an eluent of 5% - 10% acetone in dichloromethane to afford compound **7** as a crystalline solid (0.09 g, 43%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 6.42 (s, 2H), 4.16 (t, J = 6.2 Hz, 2H), 3.89 (s, 6H), 2.87 (t, J = 7.2 Hz, 2H), 2.82 (s, 4H), 2.10 (p, J = 6.4 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 170.2, 168.6, 163.1, 160.8, 111.8, 92.2, 88.2, 66.4, 56.5, 26.9, 25.3, 23.7. ESI-TOF *m/z* calcd for $C_{17}H_{18}N_2O_7S$ $[M+H]^+$, 395.1; found, 395.2.



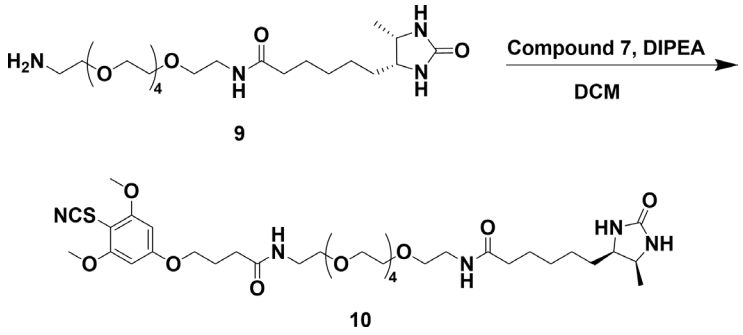
Azido-Peg5-amine 8

d-Desthiobiotin (0.2 g, 0.8 mmol, 1.0 equiv) and azido-PEG_5-amine (0.3 g, 1.0 mmol, 1.2 equiv) in 25 mL of THF were mixed in the presence of HBTU (0.4 g, 1.0 mmol, 1.2 equiv), HOBT (0.2 g, 1.0 mmol, 1.2 equiv), and DIPEA (1.4 mL, 8.0 mmol, 8.0 equiv). The reaction mixture was stirred overnight at room temperature. The following day, the solvent was removed under reduced pressure and the crude oil was purified via flash chromatography with an eluent of 8% - 11% methanol in dichloromethane to provide compound **8** as a yellow oil (0.35 g, 85%). ^{1}H NMR (500 MHz, Acetone-d_6): δ 7.09 (s, 1H), 5.67 (s, 1H), 5.43 (s, 1H), 3.82 – 3.75 (m, 1H), 3.71 (t, 2H), 3.67 – 3.55 (m, 17H), 3.51 (t, J = 5.6 Hz, 2H), 3.41 (t, J = 4.9 Hz, 2H), 3.35 (q, J =5.6 Hz, 2H), 2.18 (t, J = 7.4 Hz, 2H), 1.63 (p, J = 7.4 Hz, 2H), 1.53 – 1.45 (m, 3H), 1.42 – 1.30 (m, 3H), 1.11 (d, J = 6.4 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 172.6, 163.3, 70.4, 70.3, 70.2, 70.1, 70.0, 69.8, 69.7, 55.4, 50.7, 50.4, 38.9, 35.7, 30.0, 29.2, 26.1, 25.6, 15.9. ESI-TOF *m/z* calcd for $C_{22}H_{42}N_6O_7$ $[M+H]^+$, 503.3; found, 503.3.



8 9

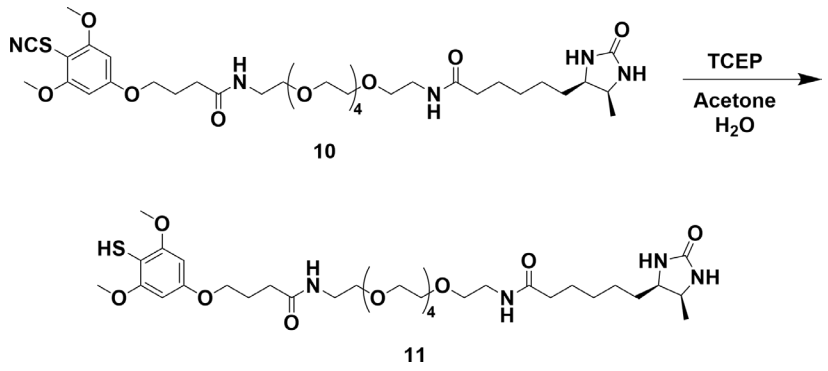
Compound **8** (0.14 g, 0.3 mmol, 1.0 equiv) was dissolved in methanol (10 mL) and transferred into a 20 mL vial. Subsequently, 10% Pd/C, 55% wet (0.07 g, 0.03 mmol, 0.1 equiv) was added under 1 atm of H_2. The reaction was stirred at room temperature and monitored by TLC. Upon completion, the Pd catalyst was removed via filtration and the filtrate was collected. The filtrate was concentrated under reduced pressure to provide compound **9** as a colorless oil (0.11 g, 81%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.93 – 7.76 (m, 1H), 6.32 (s, 1H), 6.14 (s, 1H), 3.59 – 3.54 (m, 5H), 3.51 – 3.47 (m, 16H), 3.40 – 3.34 (m, 4H), 3.17 (q, J = 5.9 Hz, 2H), 2.68 (s, 1H), 2.04 (t, J = 7.4 Hz, 2H), 1.46 (p, J = 7.3 Hz, 2H), 1.36 – 1.13 (m, 6H), 0.94 (d, J = 6.4 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 172.7, 163.3, 70.3, 70.2, 70.2, 70.0, 69.6, 55.5, 50.7, 38.9, 35.7, 30.0, 29.2, 26.1, 25.6, 16.0. ESI-TOF *m/z* calcd for $C_{22}H_{44}N_4O_7$ $[M+H]^+$, 477.3; found, 477.4.



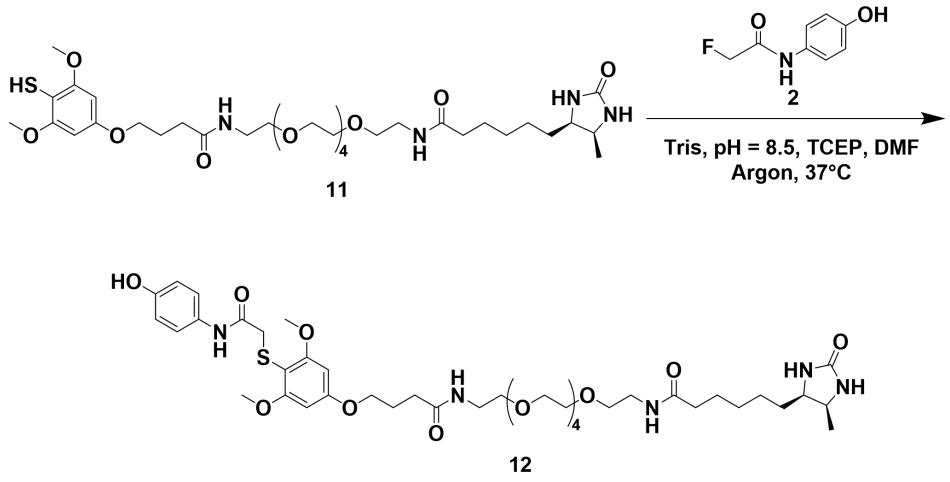
9

10

Compound **9** (0.07 g, 0.15 mmol, 1.5 equiv) was dissolved in DCM (2 mL) and transferred into a 20 mL vial. Subsequently, compound **7** (0.04 g, 0.1 mmol, 1.0 equiv) in DCM (2 mL) was added to the reaction flask along with DIPEA (0.04 g, 0.3 mmol, 3.0 equiv). The reaction mixture was left stirring for 3 h at room temperature. The solvent was removed under reduced pressure and the crude material was purified via flash chromatography with a gradient of 5% - 10% methanol in dichloromethane to afford compound **10** as a yellowish oil (0.72 g, 92%). ^{1}H NMR (500 MHz, Acetone-d_6): δ 7.28 (s, 1H), 7.11 (s, 1H), 6.43 (s, 2H), 5.68 (s, 1H), 5.45 (s, 1H), 4.15 (t, J = 6.5 Hz, 2H), 3.97 (s, 6H), 3.80 – 3.75 (m, 1H), 3.69 – 3.64 (m, 1H), 3.63 – 3.59 (m, 8H), 3.59 – 3.55 (m, 8H), 3.52 (dt, J = 7.6, 5.6 Hz, 4H), 3.40 – 3.32 (m, 4H), 2.39 (t, J = 7.1 Hz, 2H), 2.18 (t, J = 7.4 Hz, 2H), 2.10 (s, 2H), 1.62 (p, J = 7.3 Hz, 2H), 1.53 – 1.44 (m, 3H), 1.40 – 1.30 (m, 3H), 1.10 (d, J = 6.4 Hz, 3H). ^{13}C NMR (126 MHz, Acetone-d_6): δ 172.1, 171.6, 164.1, 162.8, 161.5, 111.1, 92.2, 88.8, 70.4, 70.3, 70.2, 70.1, 70.0, 69.7, 69.6, 67.5, 56.1, 55.6, 50.9, 39.0, 38.9, 35.6, 31.6, 29.8, 25.9, 25.4, 24.8, 15.1. ESI-TOF *m/z* calcd for $C_{35}H_{57}N_5O_{11}S$ $[M+H]^+$, 756.4; found, 756.5.

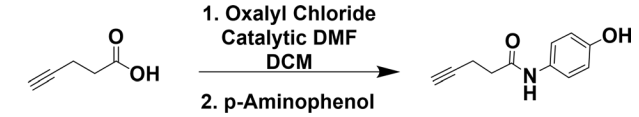


Compound **10** (0.07 g, 0.09 mmol, 1.0 equiv) was dissolved in a solution of acetone (5 mL) and water (1 mL) in a 20 mL vial. The reaction mixture was purged with argon, and left stirring overnight at room temperature, in the presence of TCEP (0.08 g, 0.28 mmol, 3.0 equiv). The next day, the solvent was removed under reduced pressure and the resultant crude material was purified via flash chromatography with an eluent of 10% methanol in dichloromethane to provide compound **11** as a yellowish oil (0.06 g, 86%). ^{1}H NMR (500 MHz, Acetone-d_6): δ 7.28 (s, 1H), 7.15 (s, 1H), 6.33 (s, 2H), 5.73 (s, 1H), 5.48 (s, 1H), 4.02 (t, J = 6.5 Hz, 2H), 3.87 (s, 6H), 3.83 (s, 1H), 3.79 – 3.74 (m, 1H), 3.67 – 3.63 (m, 1H), 3.60 – 3.54 (m, 16H), 3.50 (q, J = 5.9 Hz, 4H), 3.34 (dq, J = 11.4, 5.6 Hz, 4H), 2.36 (t, J = 7.2 Hz, 2H), 2.17 (t, J = 7.3 Hz, 2H), 2.04 – 2.00 (m, 2H), 1.61 (p, J = 7.3 Hz, 2H), 1.49 – 1.43 (m, 3H), 1.35 – 1.27 (m, 3H), 1.08 (d, J = 6.4 Hz, 3H). ^{13}C NMR (126 MHz, Acetone-d_6): δ 172.3, 171.8, 162.9, 158.2, 155.9, 99.7, 92.0, 70.3, 70.2, 70.1, 70.1, 70.0, 69.8, 69.7, 67.3, 55.7, 39.0, 38.9, 35.6, 31.8, 29.8, 25.9, 25.4, 25.1, 15.1. ESI-TOF *m/z* calcd for $C_{34}H_{58}N_4O_{11}S$ $[M+H]^+$, 731.4; found, 731.5.



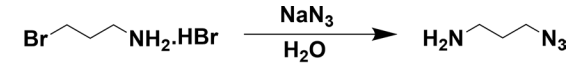
APAP-F analogue **2** (4.0 mg, 20.0 μmol, 1.1 equiv) dissolved in DMF (0.1 mL) was added to a 10 mL round-bottom reaction flask, along with a DMF solution (0.4 mL) of 100 mM TCEP and 100 mM Tris Base (pH 8.5). The mixture was heated up to 37°C, followed by the addition of probe **11** (13.0 mg, 18.0 μmol, 1.0 equiv) in DMF (0.4 mL). The reaction proceeded for 20 h at 37°C, before the removal of solvent under reduced pressure. The resulting crude oil was purified via flash chromatography with a gradient of 5% - 15% methanol in dichloromethane to afford

compound **12** as a yellow oil (9.0 mg, 57%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.63 (s, 1H), 9.25 (s, 1H), 7.98 (t, J = 5.7 Hz, 1H), 7.87 (t, J = 5.8 Hz, 1H), 7.31 (d, J = 8.9 Hz, 2H), 6.70 (d, J = 8.9 Hz, 2H), 6.32 (s, 1H), 6.25 (s, 2H), 6.14 (s, 1H), 4.01 (t, J = 6.4 Hz, 2H), 3.76 (s, 6H), 3.61 (p, J = 6.7 Hz, 1H), 3.54 – 3.52 (m, 1H), 3.52 – 3.46 (m, 16H), 3.44 – 3.37 (m, 6H), 3.24 – 3.16 (m, 4H), 2.26 (t, J = 7.4 Hz, 2H), 2.07 (t, J = 7.4 Hz, 2H), 1.93 (p, J = 6.8 Hz, 2H), 1.48 (p, J = 7.4 Hz, 2H), 1.39 – 1.29 (m, 3H), 1.25 – 1.18 (m, 3H), 0.97 (d, J = 6.4 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 172.7, 172.1, 167.1, 163.3, 161.9, 161.5, 153.8, 131.1, 121.2, 115.5, 100.6, 92.2, 70.3, 70.2, 70.0, 69.6, 69.6, 67.6, 56.6, 55.5, 50.7, 39.0, 38.9, 35.7, 32.0, 30.0, 29.2, 26.1, 25.6, 25.2, 16.0 ESI-TOF *m/z* calcd for $C_{42}H_{65}N_5O_{13}S$ $[M+Na]^+$, 902.4; found, 902.4.



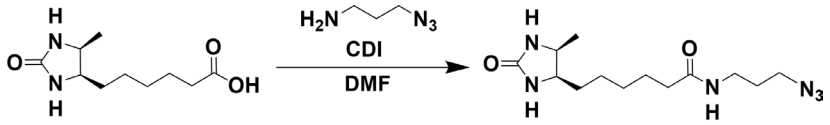
4-Pentynoic Acid 13

Following the reported oxalyl chloride experiment procedure,[3] an anhydrous solution of 4-pentynoic acid (0.1 g, 1.0 mmol, 1.0 equiv) in DCM (10 mL) was added to a 25 mL round- bottom flask. The reaction flask was then charged with 2 M oxalyl chloride (0.5 mL, 1.0 mmol, 1.0 equiv) and DMF (0.1 mL). The mixture was stirred for 30 min at room temperature, followed by the addition of *p*-Aminophenol (0.6 g, 5.1 mmol, 5.0 equiv). Upon completion of the reaction as monitored by TLC, the solvent was removed under reduced pressure. The crude product was purified with flash chromatography and dual solvent recrystallization in acetonitrile and dichloromethane to furnish compound **13** as a white solid (0.11 g, 60%). ^{1}H NMR (500 MHz, Acetonitrile-d_3): δ 8.35 (s, 1H), 7.41 (s, 1H), 7.37 – 7.31 (m, 2H), 6.78 – 6.70 (m, 2H), 2.51 – 2.47 (m, 4H). ESI-TOF *m/z* calcd for $C_{11}H_{11}NO_2$ $[M-H]^+$, 190.1; found, 190.0.



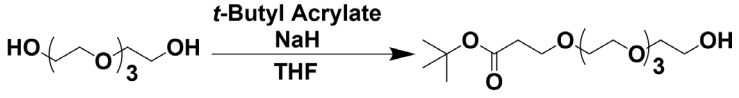
3-bromopropylamine hydrobromide 14

Following the reported procedure,[4] 3-bromopropylamine hydrobromide (3.3 g, 15.0 mmol, 1.0 equiv) was added into a 250 mL round-bottom flask and dissolved with water (10 mL). Afterwards, 15 mL of a sodium azide solution (3.3 g, 50.0 mmol, 3.3 equiv) was charged to the reaction mixture, which was then refluxed with stirring for 16 h. Upon completion, diethyl ether (50 mL) was added, and the mixture was cooled to 0°C. To the resulting solution, potassium hydroxide (4 g) was slowly added and then the crude mixture was phase-separated. The aqueous phase was washed with diethyl ether (2 x 30 mL), and the combined organic layers were collected, dried over K_2CO_3, and concentrated to provide compound **14** as a faint yellowish oil (1.3 g, 87.2%). ^{1}H NMR (500 MHz, Chloroform-d): δ 3.37 (t, J = 6.7 Hz, 2H), 2.81 (t, J = 6.8 Hz, 2H), 1.73 (p, J = 6.8 Hz, 2H), 1.27 – 1.13 (m, 2H). ^{13}C NMR (126 MHz, CDCl3): δ 49.3, 39.5, 32.6.



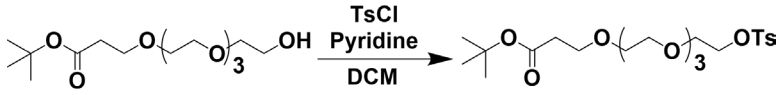
d-Desthiobiotin 15

Carbonyldiimidazole (0.15 g, 0.9 mmol, 2.0 equiv) was added into a 20 mL vial and dissolved with DMF (1 mL). Then, *d*-Desthiobiotin (0.10 g, 0.47 mmol, 1.00 equiv) in DMF (2 mL) was added to the reaction mixture and subsequently stirred for 3 h at room temperature. Compound **14** in 1 mL of DMF was finally dripped dropwise to the reaction flask. The reaction mixture was stirred for 12 h and then quenched with 5 mL of water. Ethyl Acetate (10 mL) was added to the crude mixture, and the organic layer was collected after extraction. The organic phase was washed with 5 mL of brine, dried over magnesium sulfate, and concentrated under reduced pressure. The resultant crude oil was subjected to flash chromatography with an eluent of 6% methanol in dichloromethane to provide compound **15** as a colorless oil (90 mg, 65%). ^{1}H NMR (500 MHz, Methanol-d^4): δ 7.69 (s, 1H), 7.06 (s, 2H), 3.86 – 3.77 (m, 1H), 3.69 (q, J = 7.5 Hz, 1H), 3.35 (t, J = 6.7 Hz, 2H), 3.25 (t, J = 6.8 Hz, 2H), 2.19 (t, J = 7.4 Hz, 2H), 1.75 (p, J = 6.8 Hz, 2H), 1.63 (p, J = 7.4 Hz, 2H), 1.53 – 1.47 (m, 2H), 1.47 – 1.40 (m, 1H), 1.40 – 1.28 (m, 4H), 1.10 (d, J = 6.4 Hz, 3H). ^{13}C NMR (126 MHz, Methanol-d^4): δ 176.3, 166.2, 57.4, 52.7, 50.1, 37.7, 37.0, 30.7, 30.2, 29.7, 27.2, 26.8, 15.6. ESI-TOF m/z calcd for $C_{13}H_{24}N_6O_2$ $[M+H]^+$, 297.2; found, 297.1.



Tetraethylene glycol 16

Tetraethylene glycol (21.6 g, 111 mmol, 2.7 equiv) was added into a round bottom flask and dissolved with 60 mL of THF. Then *t*-butyl acetate (6 mL, 41 mmol, 1.0 equiv) and sodium hydride in 60% dispersion oil (0.06 g, 1.5 mmol, 0.04 equiv) were charged to the flask. The reaction mixture was subsequently stirred overnight at room temperature. The following morning, 1.5 mL of 1 M HCl was added to acidify the crude mixture. Afterward, H_2O (150 mL) and chloroform (100 mL) were added to the crude mixture for phase separation. The organic layer was washed with H_2O (3 x 40 mL), then collected, concentrated, and subjected to column chromatography with an eluent of 1% to 3% MeOH in DCM to furnish compound **16** as a clear oil (4.23 g, 32%). ^{1}H NMR (500 MHz, Benzene-*d*) δ 3.57 (td, J = 6.5, 1.8 Hz, 4H), 3.47 – 3.37 (m, 8H), 3.38 – 3.34 (m, 4H), 3.34 – 3.29 (m, 2H), 2.73 (s, 1H), 2.38 (t, J = 6.4 Hz, 2H), 1.37 (s, 9H).

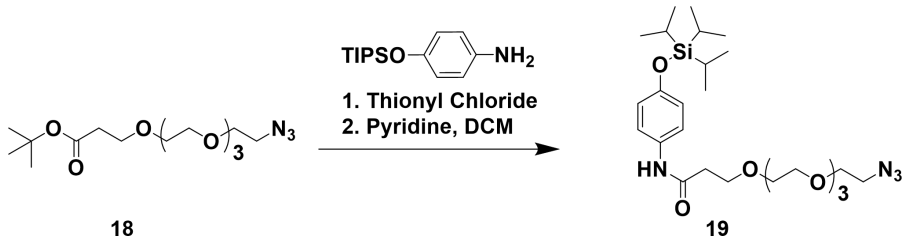


16 17

Compound **16** (3.0 g, 9.3 mmol, 1 equiv) was added to a round bottom flask and dissolved with 100 mL of DCM. Pyridine (3 mL, 37 mmol, 4 equiv) was charged to the flask which was then placed under an ice bath and cooled to 0°C. Subsequently, tosyl chloride (3.5 g, 18.6 mmol, 2 equiv) was added to the flask and the reaction mixture was stirred for 24 h at room temperature. The following day, 100 mL of water was added to quench the reaction. After phase separation, the organic layer was washed with 1 M HCl (2 x 25 mL) and then concentrated. The crude material was purified via column chromatography with an eluent of 2% - 6% MeOH in DCM to yield compound **2** as a clear oil (2.13 g, 48%). ^{1}H NMR (500 MHz, Benzene-*d*) δ 7.76 (d, J = 8.3 Hz, 2H), 6.72 (d, J = 7.9 Hz, 2H), 3.93 (t, 2H), 3.58 (t, J = 6.4 Hz, 2H), 3.43 – 3.38 (m, 8H), 3.32 – 3.29 (m, 2H), 3.26 – 3.23 (m, 4H), 2.37 (t, J = 6.4 Hz, 2H), 1.85 (s, 3H), 1.38 (s, 9H).

17 18

Compound **17** (1.5 g, 3.15 mmol, 1 equiv) was dissolved in 30 mL of DMF, and subsequently mixed with sodium azide (0.6 g, 9.5 mmol, 3 equiv) in a round bottom flask. The reaction mixture was heated to 65 °C. The solution was stirred for 12 h and then slowly cooled down to room temperature. Afterward, H_2O (80 mL) and ethyl acetate (50 mL) were added to the crude mixture. After phase separation, the organic layer was washed with water (3 x 30 mL), concentrated, and subjected to column chromatography with an eluent of 5% MeOH in DCM to yield compound **3** as a clear oil (1.05 g, 95%). ^{1}H NMR (500 MHz, Benzene-*d*) δ 3.58 (t, *J* = 6.4 Hz, 2H), 3.46 – 3.39 (m, 10H), 3.32 (dd, *J* = 5.8, 3.9 Hz, 2H), 3.18 (t, 2H), 2.78 (t, *J* = 5.1 Hz, 2H), 2.37 (t, *J* = 6.4 Hz, 2H), 1.38 (s, 9H).

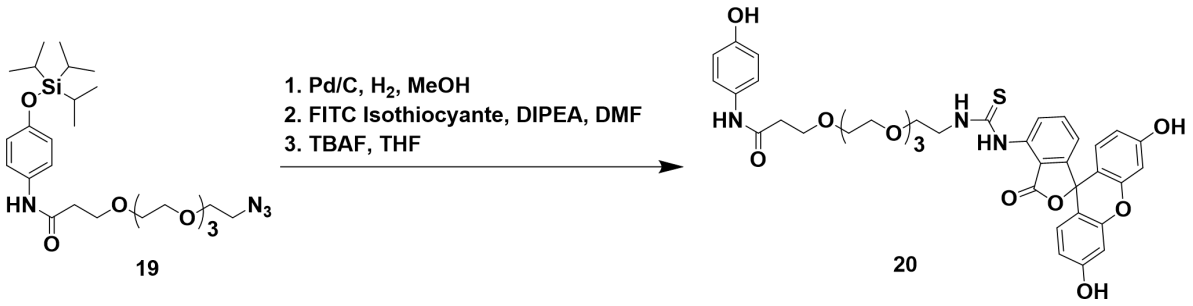


18 19

To a round bottom flask, *p*-aminophenol (1.8 g, 16.5 mmol, 1.0 equiv), DCM (200 mL), and imidazole (2.2 g, 33 mmol, 2.0 equiv) were added. Subsequently, TIPS-Cl (3.5 mL, 16.5 mmol, 1 equiv) was charged to the reaction flask and the solution was stirred overnight at room temperature. The following day, water was added to quench the reaction and the organic layer was washed with 1M NaOH (2 x 60 mL). The organic phase was collected, concentrated, and

subjected to column chromatography with an eluent of 20% to 50% ethyl acetate in hexane to provide TIPS protected APAP as a brown oil. (3.6 g, 82%). ^{1}H NMR (500 MHz, Benzene-*d*) δ 6.80 (dd, 2H), 6.29 (dd, 2H), 2.63 (s, 2H), 1.22 – 1.16 (m, 3H), 1.14 – 1.10 (m, 18H).

Following this with the reported procedure,[2] compound **18** (0.10 g, 0.28 mmol, 1.0 equiv) was dissolved in 0.21 mL of thionyl chloride in a reaction vial. The mixture was stirred for 16 h at room temperature and then diluted with toluene (5 mL). The thionyl chloride – toluene azeotrope solution was removed under reduced pressure. The resulting acyl chloride was then redissolved in anhydrous DCM (8 mL) and cooled to 0°C. Pyridine (0.09 mL, 1.12 mmol, 4.0 equiv) was added, followed by the dropwise addition of TIPS protected APAP (0.09 g, 0.34 mmol, 1.2 equiv). The reaction mixture was stirred for 30 min at 0°C before the addition of H_2O (8 mL). The organic phase was then washed with H_2O (2 x 5 mL) and 1M HCl (1 x 5 mL), dried with anhydrous sodium sulfate. The organic layer was finally concentrated under vacuum, and subjected to column chromatography with an eluent of 60% - 95% ethyl acetate in hexane to furnish compound **19** as a brown oil (0.09 g, 60%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.49 (s, 1H), 7.39 (dd, 2H), 6.81 (dd, 2H), 3.81 (t, 2H), 3.70 (s, 4H), 3.68 – 3.61 (m, 6H), 3.61 (s, 4H), 3.36 (t, J = 5.1 Hz, 2H), 2.63 (t, 2H), 1.23 (ddt, J = 14.0, 10.1, 6.7 Hz, 3H), 1.09 (d, J = 7.4 Hz, 18H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 169.7, 152.4, 131.8, 121.4, 119.9, 70.7, 70.6, 70.6, 70.6, 70.3, 70.3, 70.0, 67.2, 50.7, 37.8, 17.9, 12.3. ESI-MS *m/z* calcd for $C_{26}H_{46}N_4O_6Si$ $[M+H]^+$, 539.3; found, 539.4.



Compound **19** (0.09 g, 0.17 mmol, 1.0 equiv) was dissolved in methanol (5 mL) and transferred into a three-neck round bottom flask. After purging with H_2 gas, the flask was added 10% Pd/C, 55% wet (0.04 g, 0.017 mmol, 0.1 equiv). The reaction mixture was stirred at room temperature and monitored by TLC. Upon reaction completion, the Pd catalyst was removed via filtration and the filtrate was collected and concentrated under reduced pressure. After dissolving in DMF (2 mL), the filtrate was transferred into a vial and mixed with DIPEA (50 µL, 0.28 mmol, 1.6 equiv) and FITC Isothiocyanate (73 mg, 0.18 mmol, 1.1 equiv). The mixture was stirred overnight at room temperature. The following day, water (7 mL) and ethyl acetate (5 mL) were added to quench the reaction. The organic layer after phase separation was washed with additional water (2 x 4 mL) and 1M HCl (1 x 2 mL). After drying with anhydrous sodium sulfate, the organic layer was concentrated and subjected to column chromatography with a gradient eluent of 5% - 15% MeOH in DCM. Fluorescent fractions were pooled together and concentrated under reduced pressure. The resultant material was then resuspended in THF (1 mL), transferred to a round bottom flask, and cooled to 0°C. Successively, 1M TBAF in THF (0.17 mL, 0.17 mmol, 1 equiv)

was added dropwise to the crude reaction mixture which was then stirred for 30 min at 0°C. Finally, the mixture was added a saturated aqueous solution of ammonium chloride (0.2 mL), water (2 mL), and then chloroform (2 mL) in a sequential order. After phase separation, the aqueous layer was extracted with chloroform (3 x 1 mL). The organic phase was collected, concentrated, and then subjected to column chromatography with an eluent of 10% - 20% MeOH in DCM to afford the APAP-FITC probe as a red solid (24 mg, 19%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.24 (s, 1H), 10.12 (s, 1H), 9.68 (s, 1H), 9.17 (s, 1H), 8.30 (s, 1H), 8.20 (s, 1H), 7.76 (d, J = 8.4 Hz, 1H), 7.35 (dd, 2H), 7.17 (d, J = 8.3 Hz, 1H), 6.70 – 6.63 (m, 4H), 6.62 – 6.53 (m, 4H), 3.71 – 3.62 (m, 4H), 3.61 – 3.57 (m, 2H), 3.57 – 3.52 (m, 4H), 3.51 – 3.46 (m, 8H), 2.48 – 2.46 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 180.6, 168.6, 168.3, 153.1, 152.3, 141.4, 130.9, 129.1, 124.4, 120.7, 116.8, 115.0, 113.4, 110.0, 102.3, 69.8, 69.7, 69.7, 69.6, 68.4, 66.7, 43.6, 37.0. ESI-MS *m/z* calcd for $C_{38}H_{39}N_3O_{11}S$ $[M+H]^+$, 746.2; found, 746.4. This final probe **20** was also analyzed by LC-MS and showed >95% purity.

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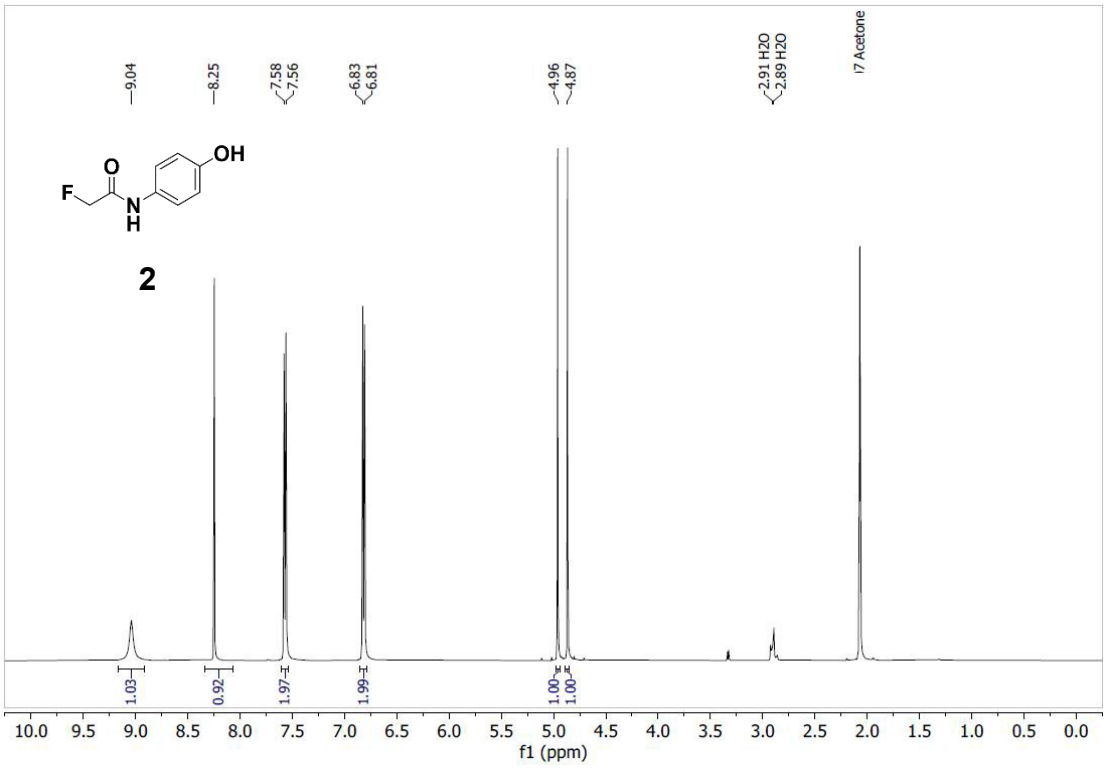
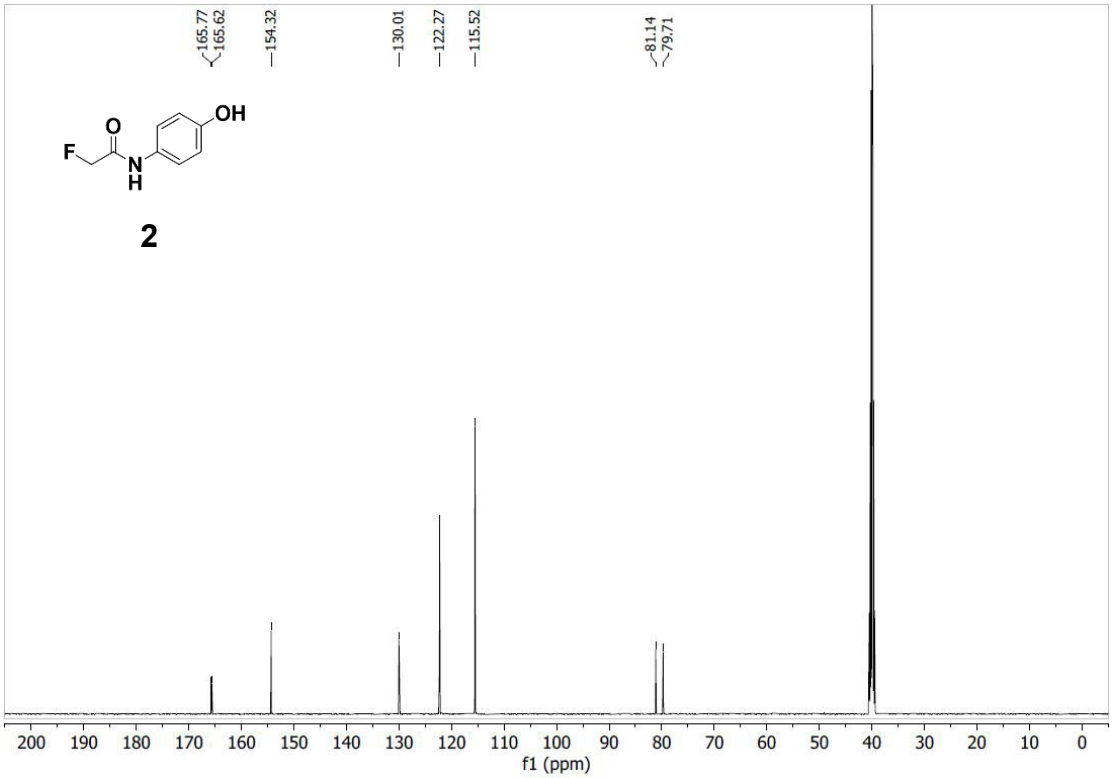
^{1}H- and ^{13}C- NMR Spectra





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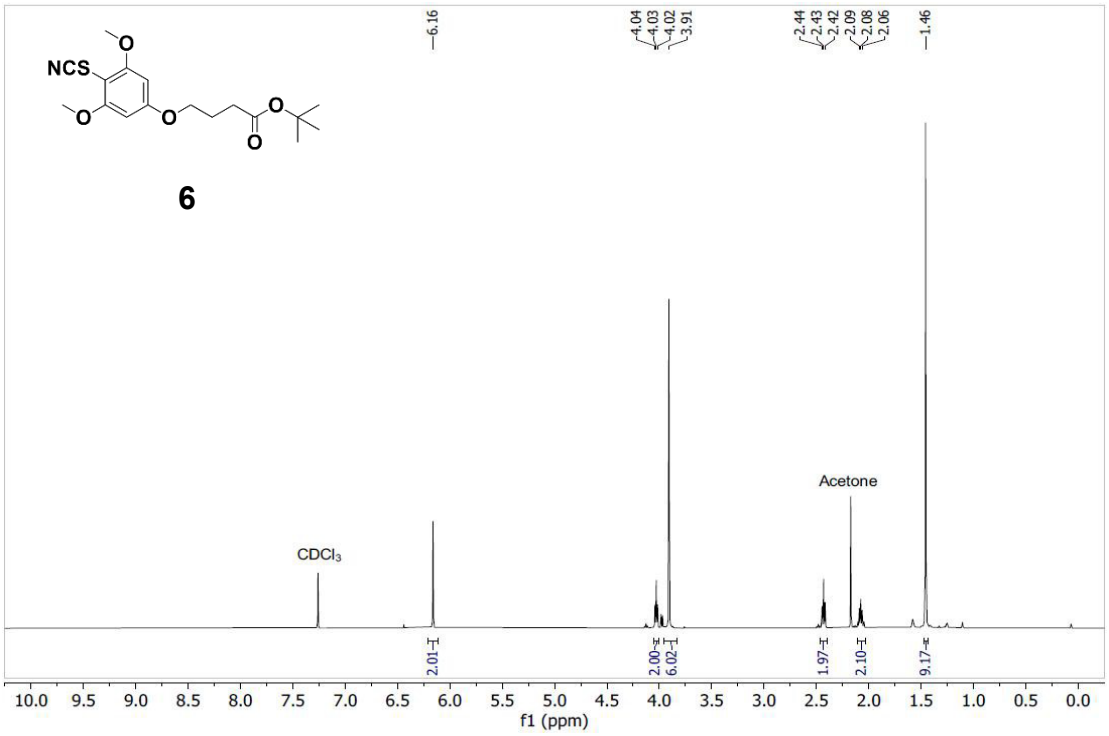
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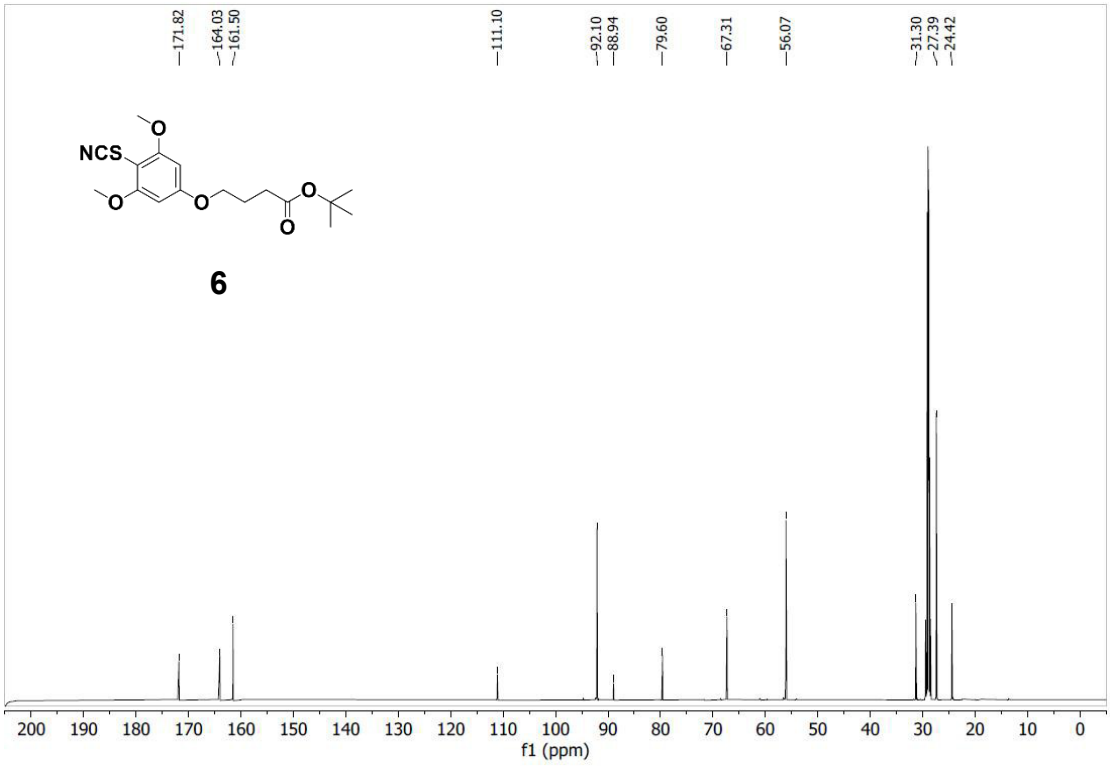
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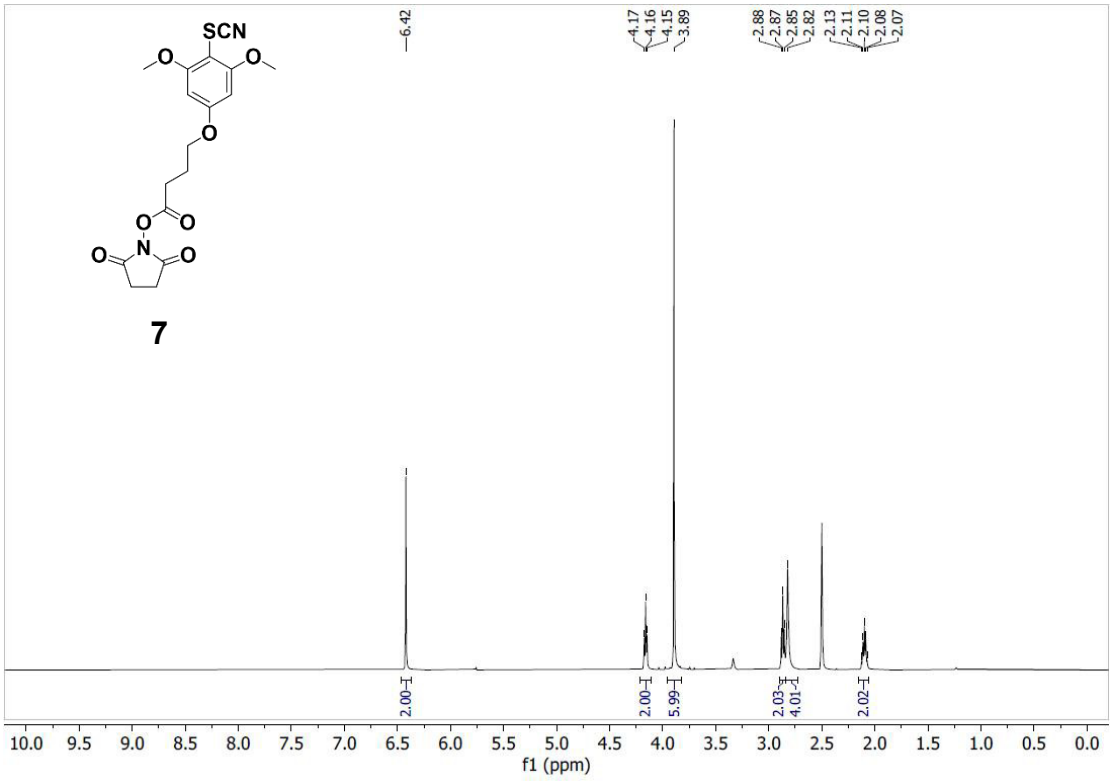
F2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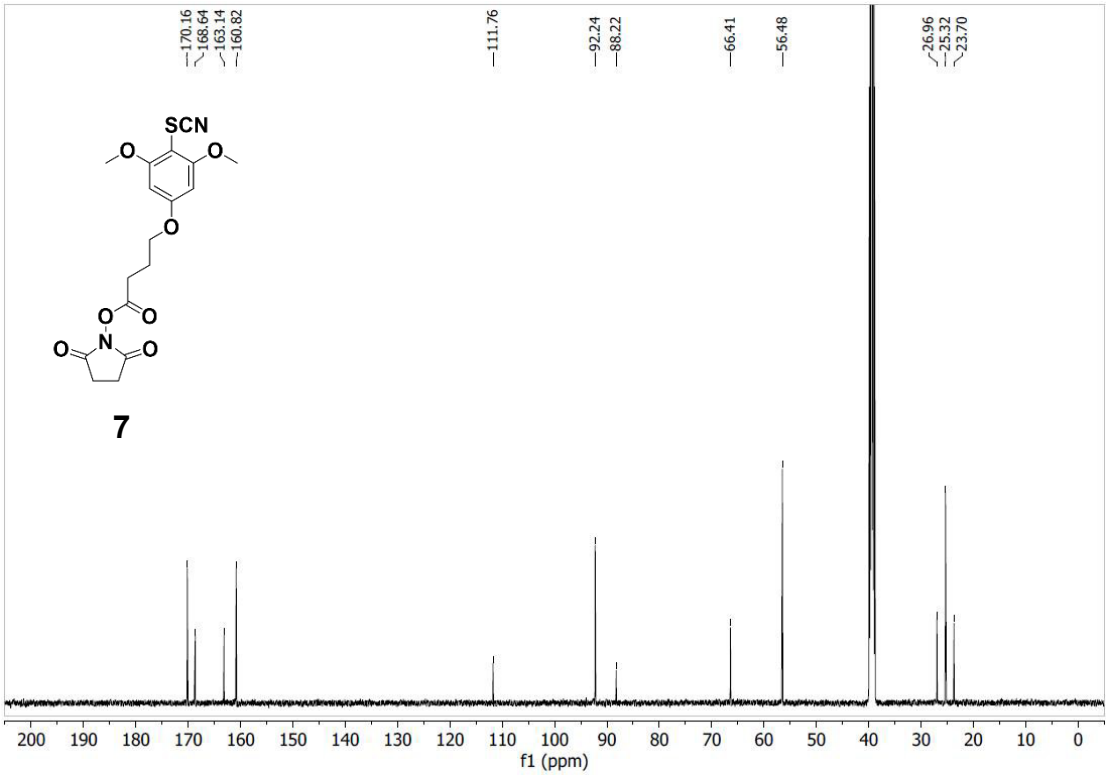
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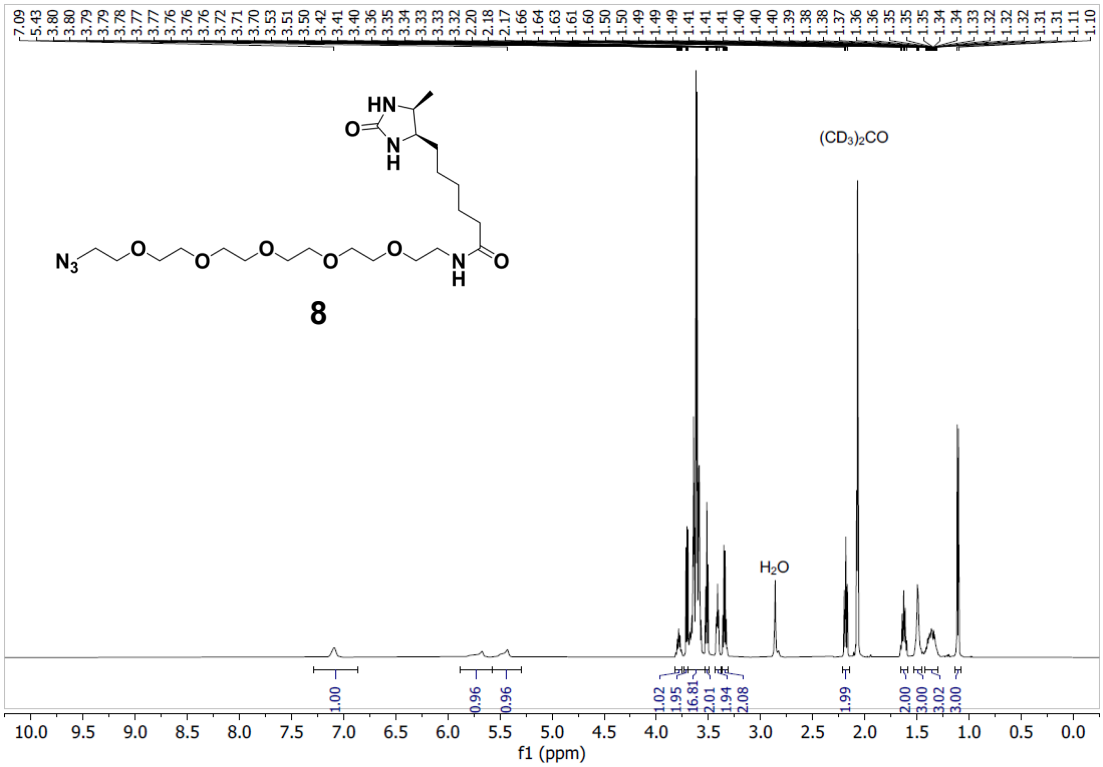
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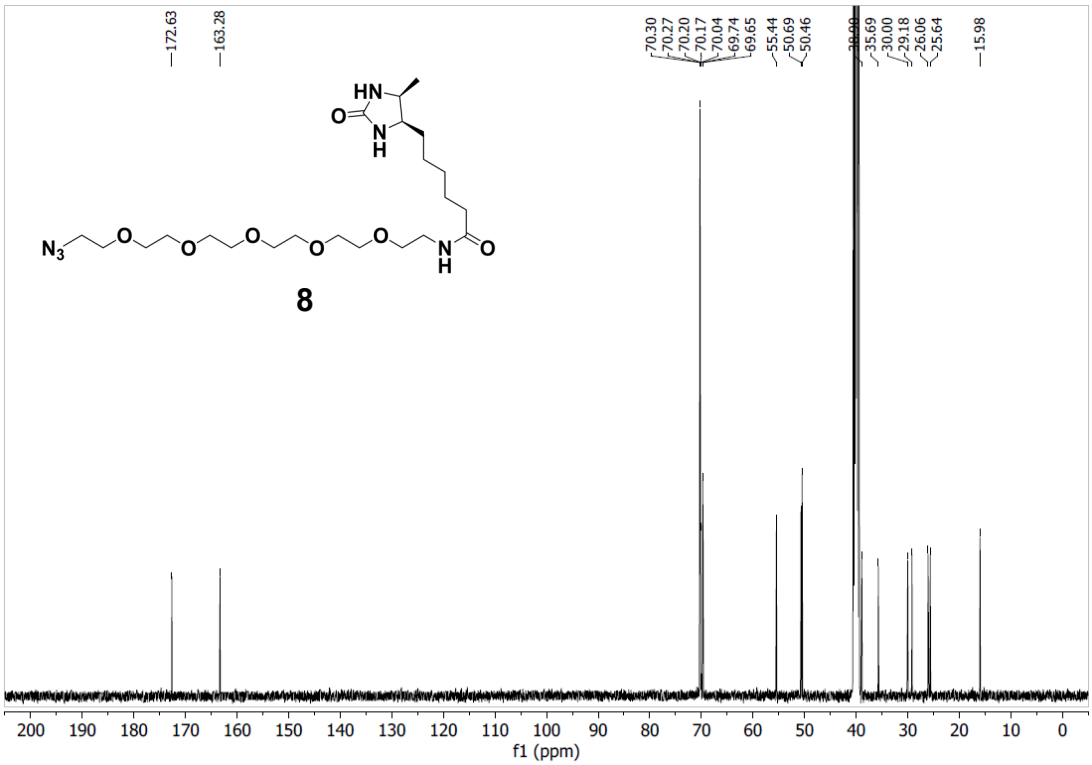
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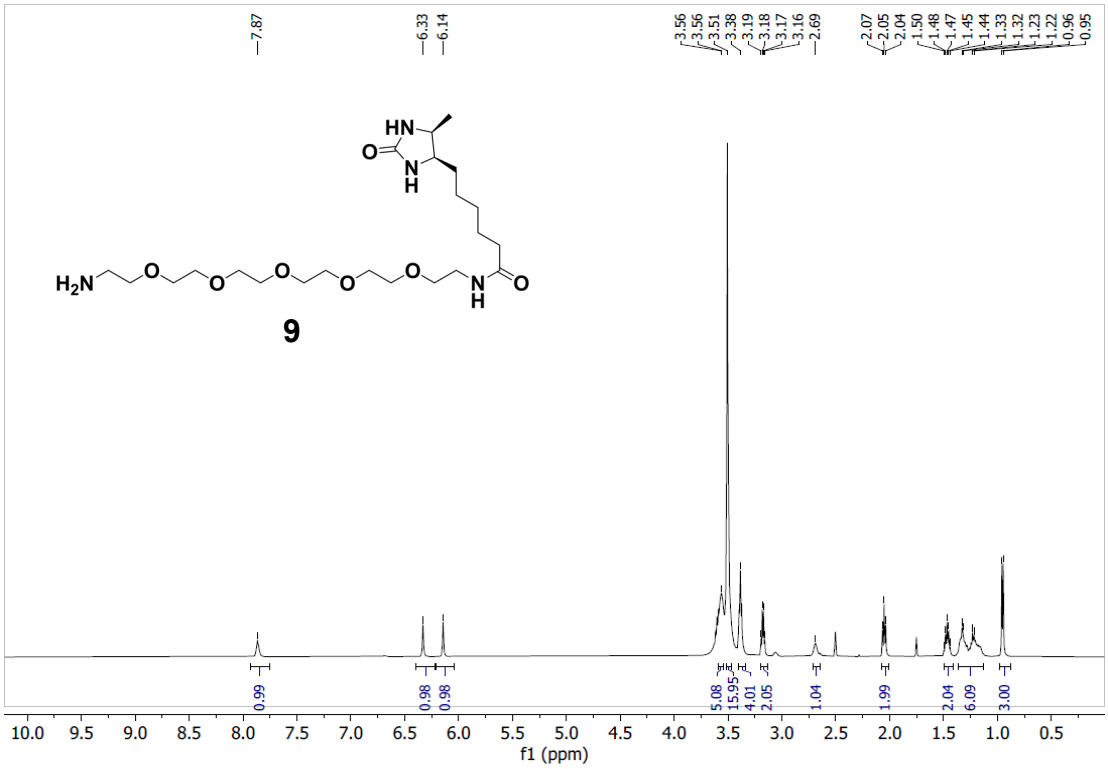
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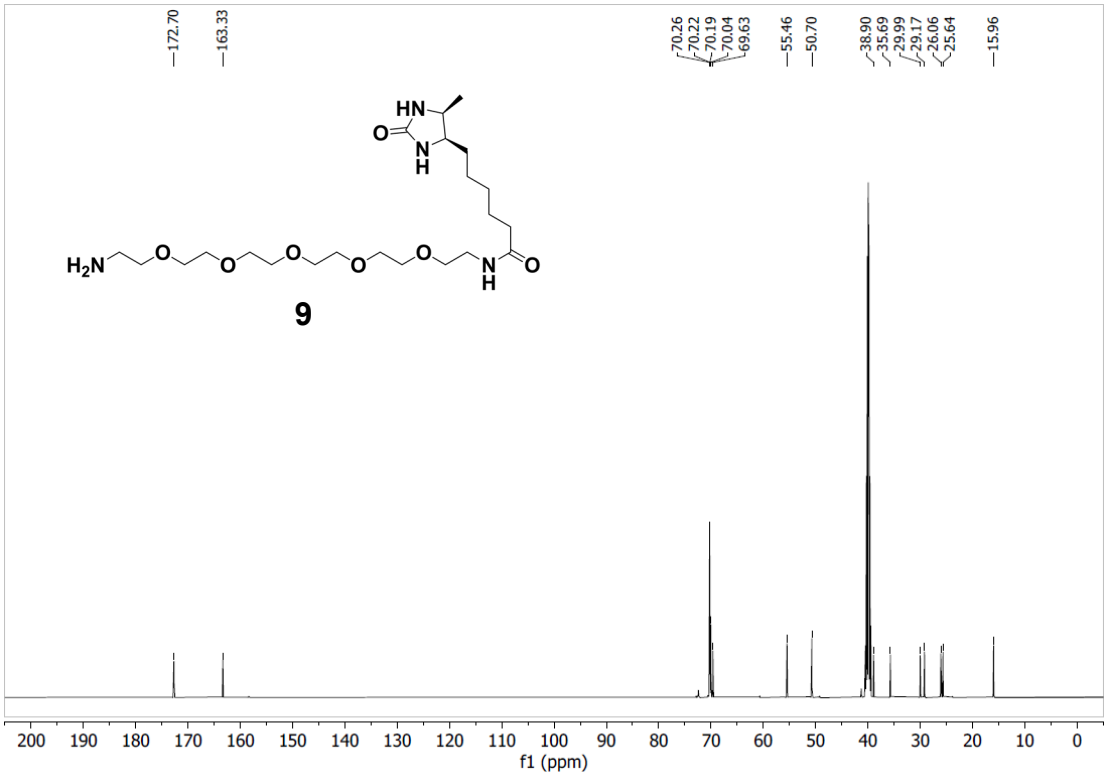
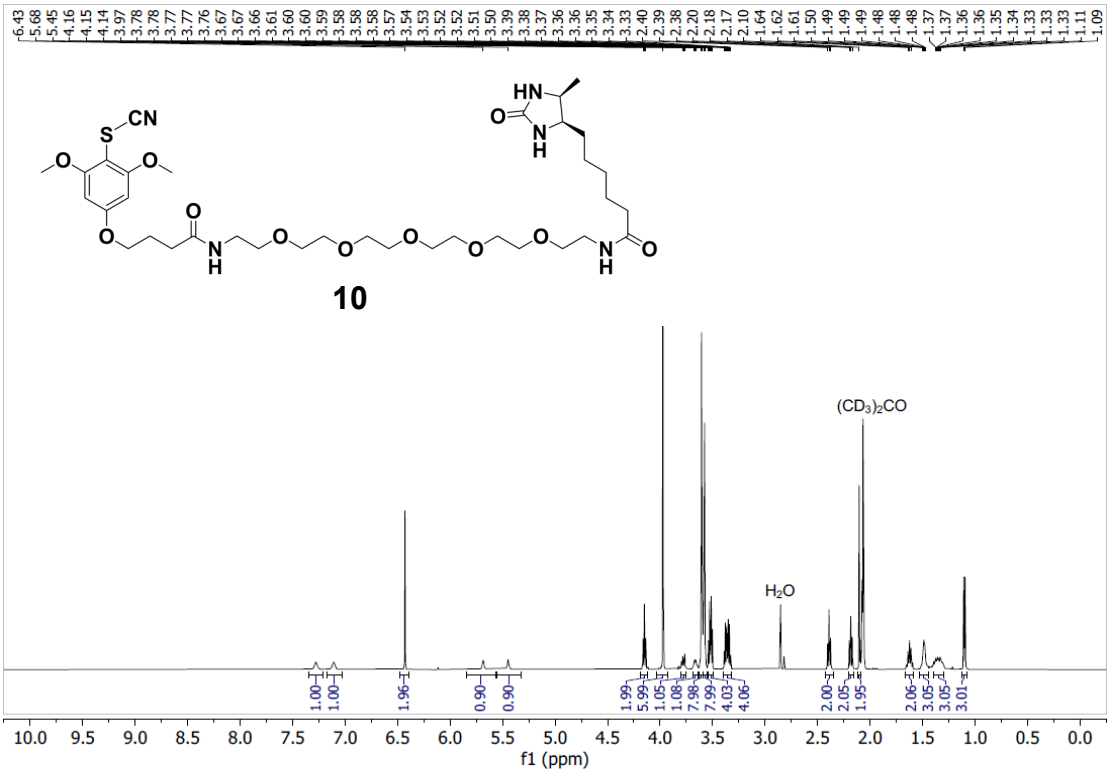
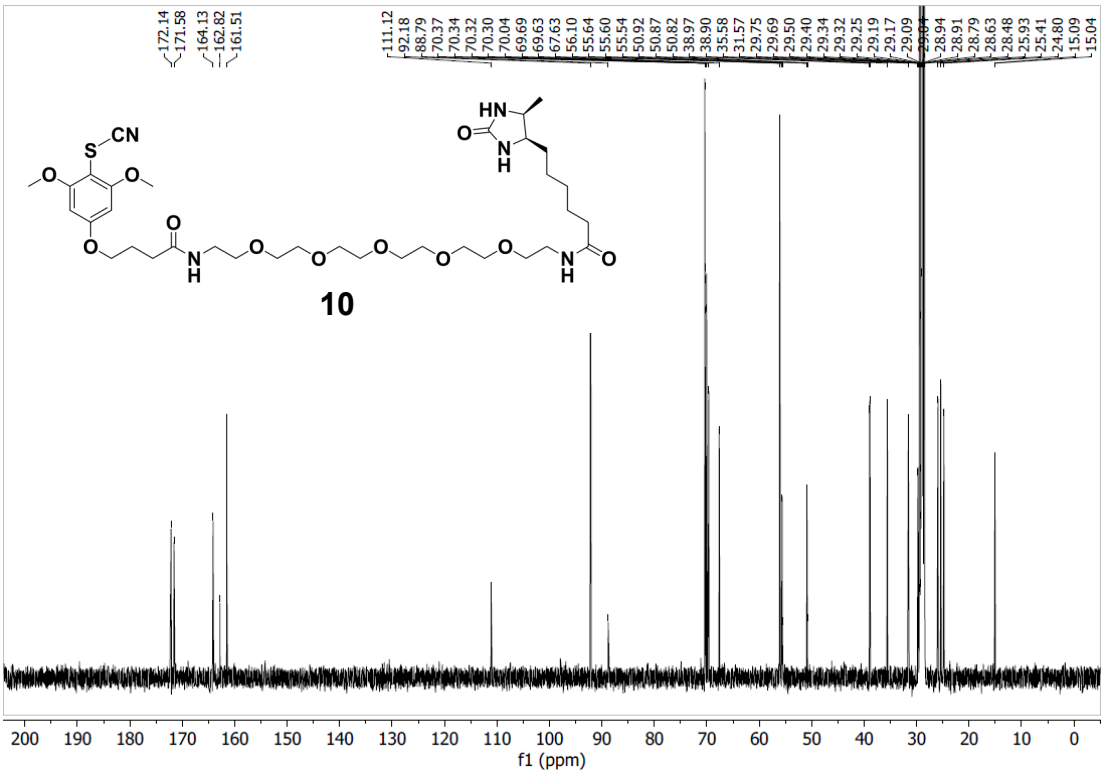
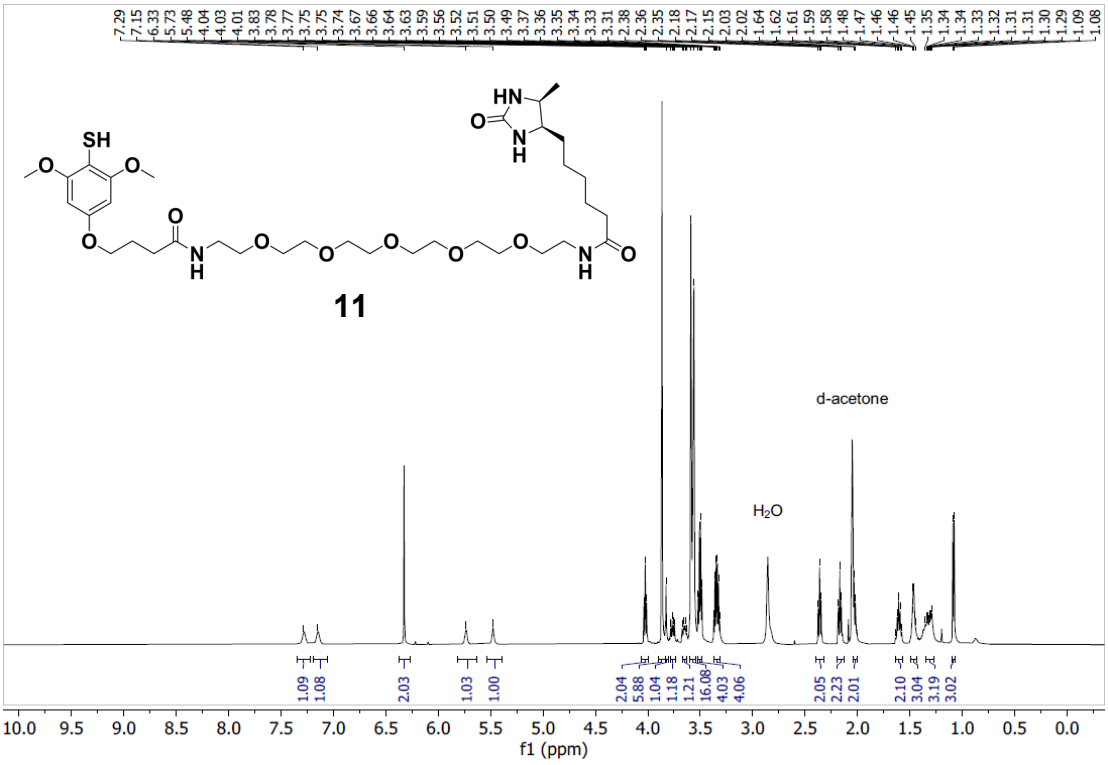
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)

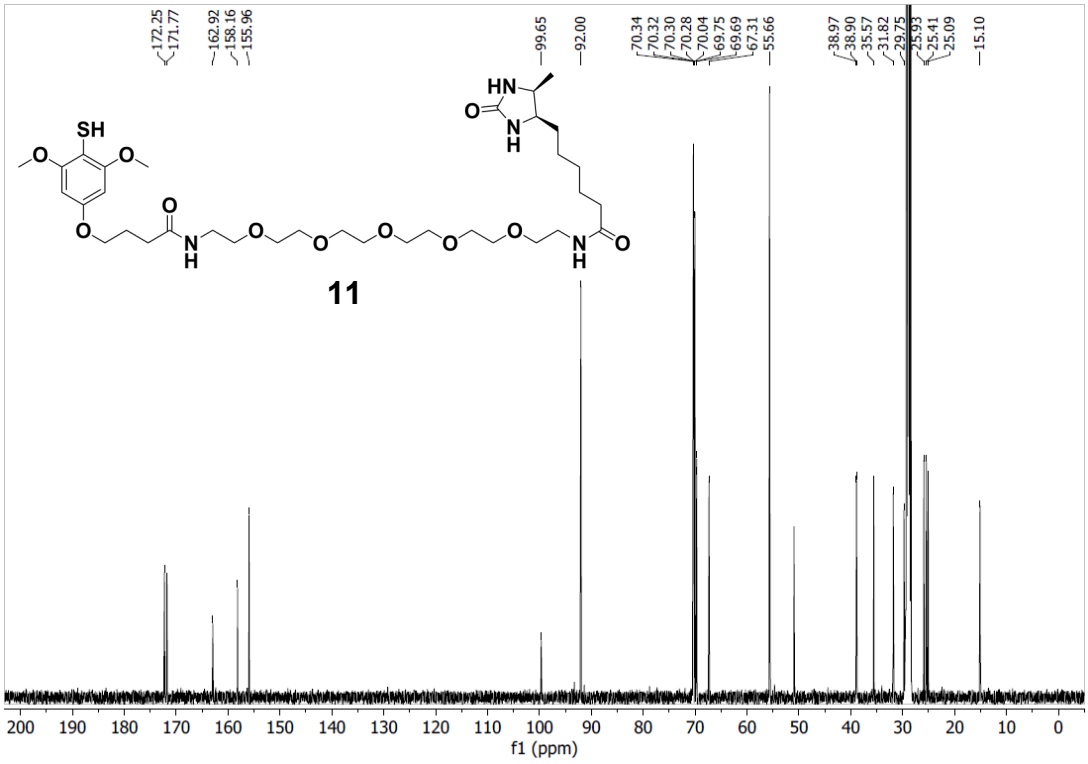

172.70
163.33
70.26
70.22
70.19
70.04
69.63
55.46
50.70
38.90
35.69
29.99
29.17
26.06
25.64
15.96
HN
O
N
H
H2N
O
N
H
O
9
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

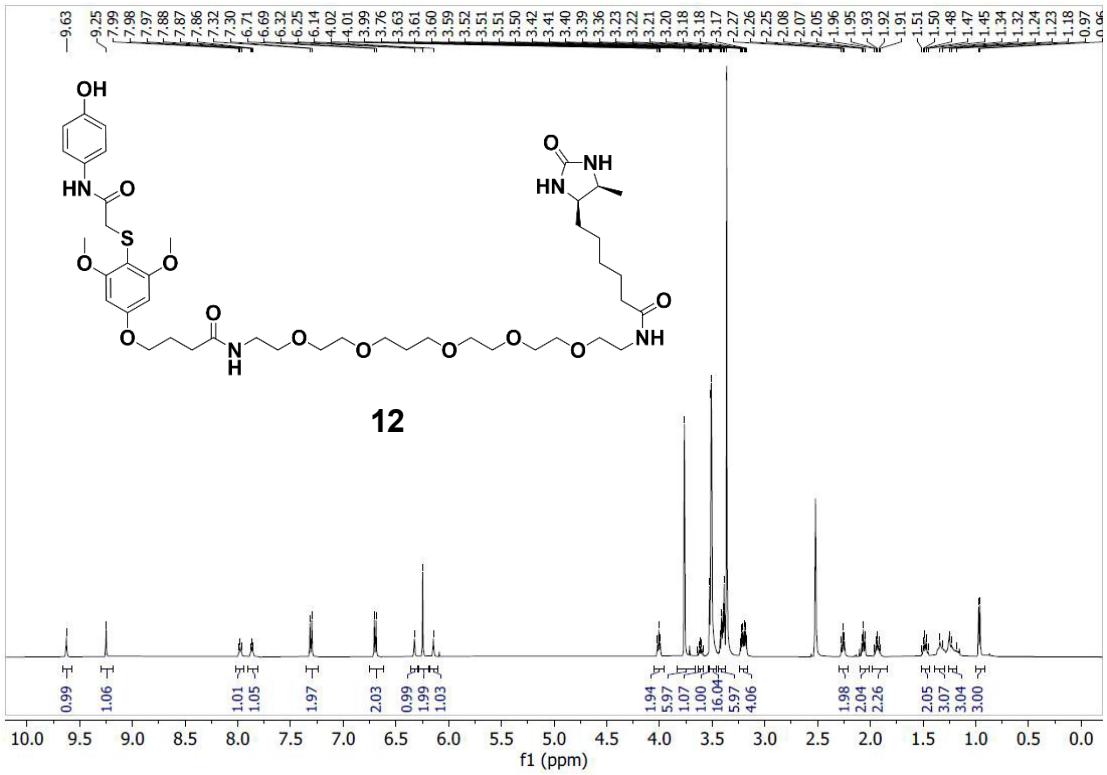


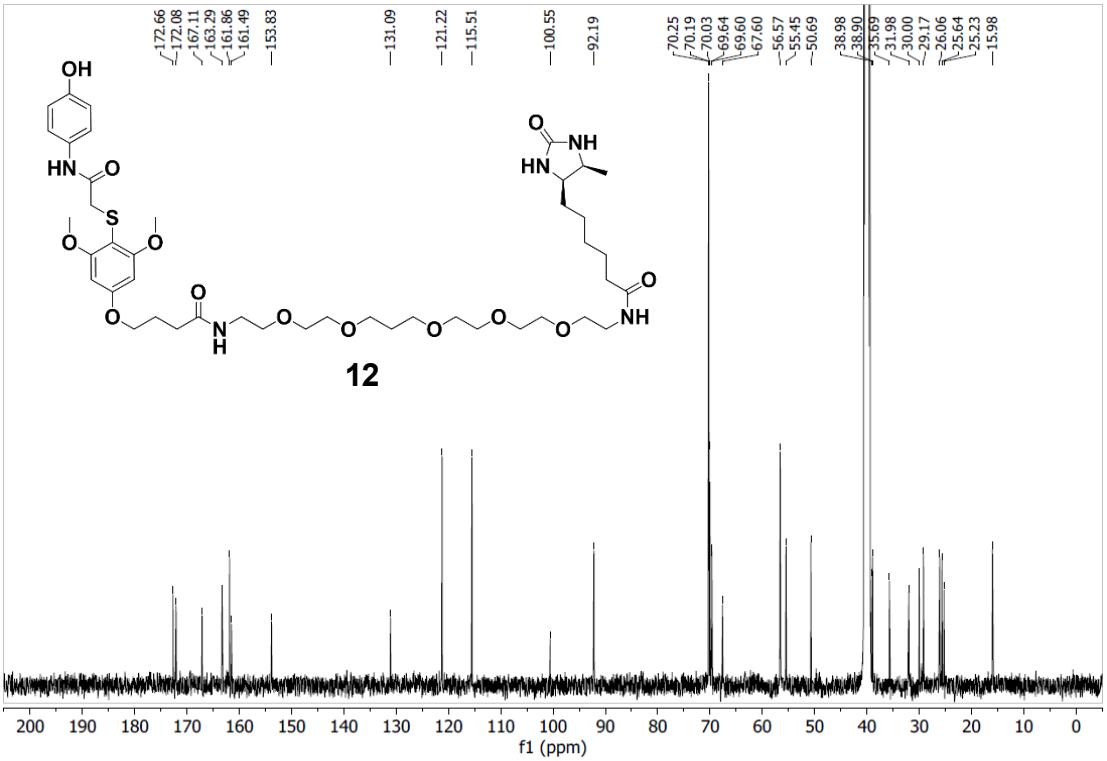


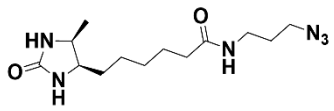
F6


11
d-acetone
H2O
f1 (ppm)

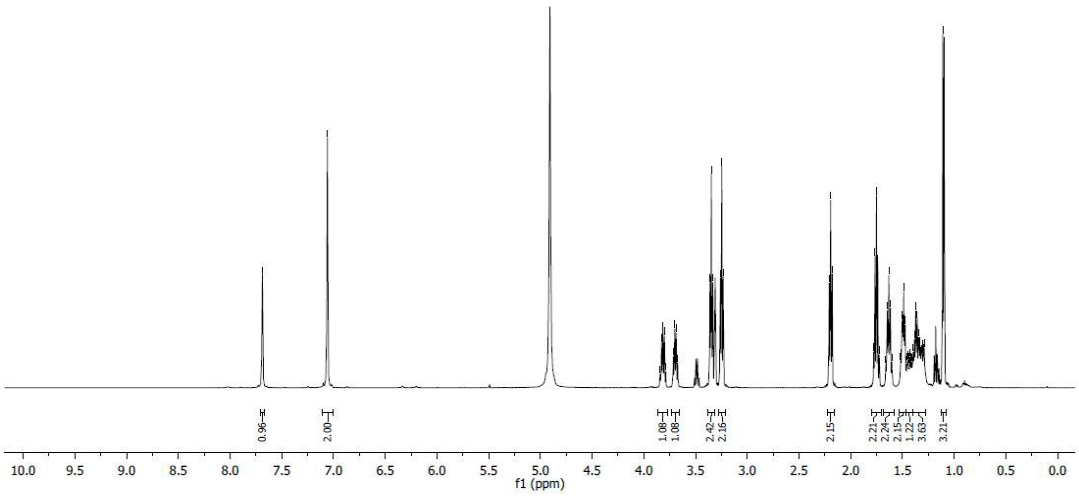

11
f1 (ppm)

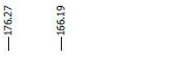

12
f1 (ppm)

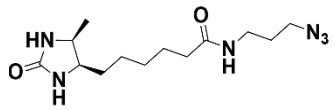

12
f1 (ppm)



15

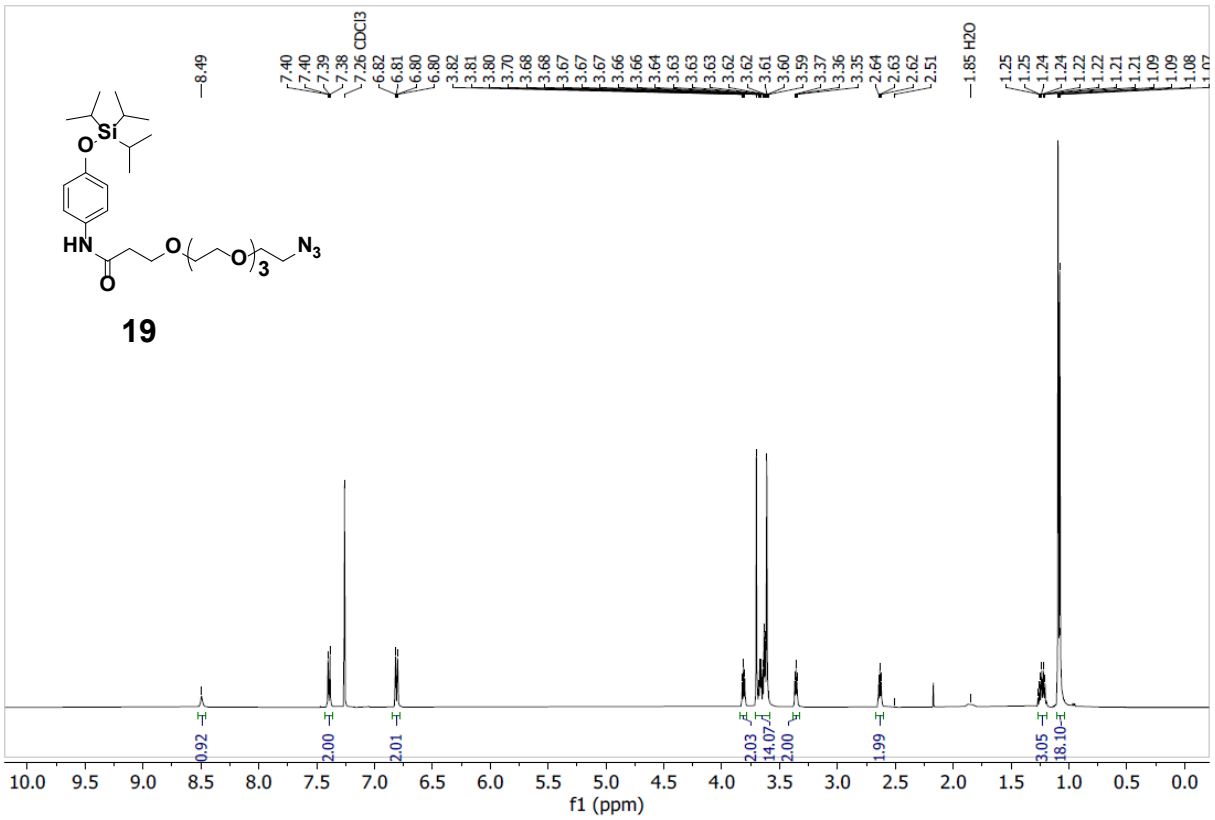

0.96
2.00
1.08
1.08
2.42
2.16
2.15
2.21
2.24
2.15
1.22
3.63
3.21
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

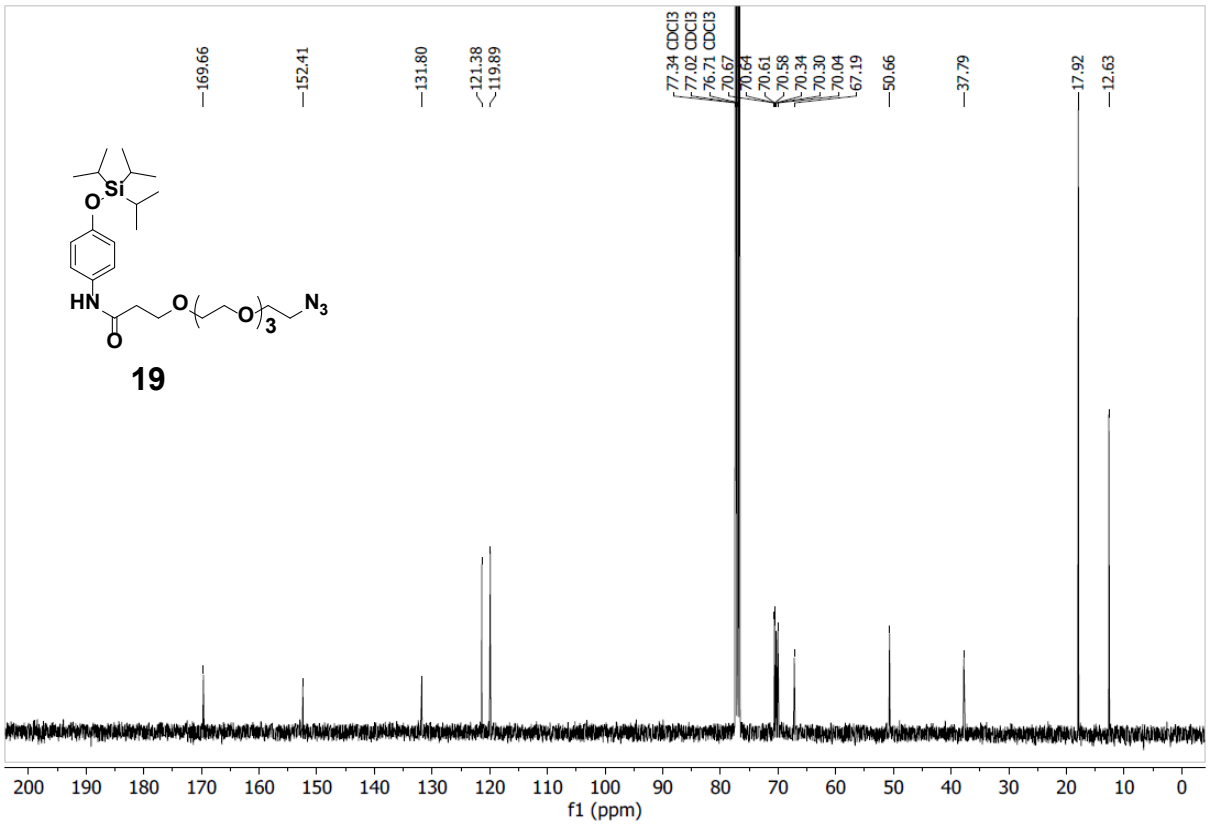


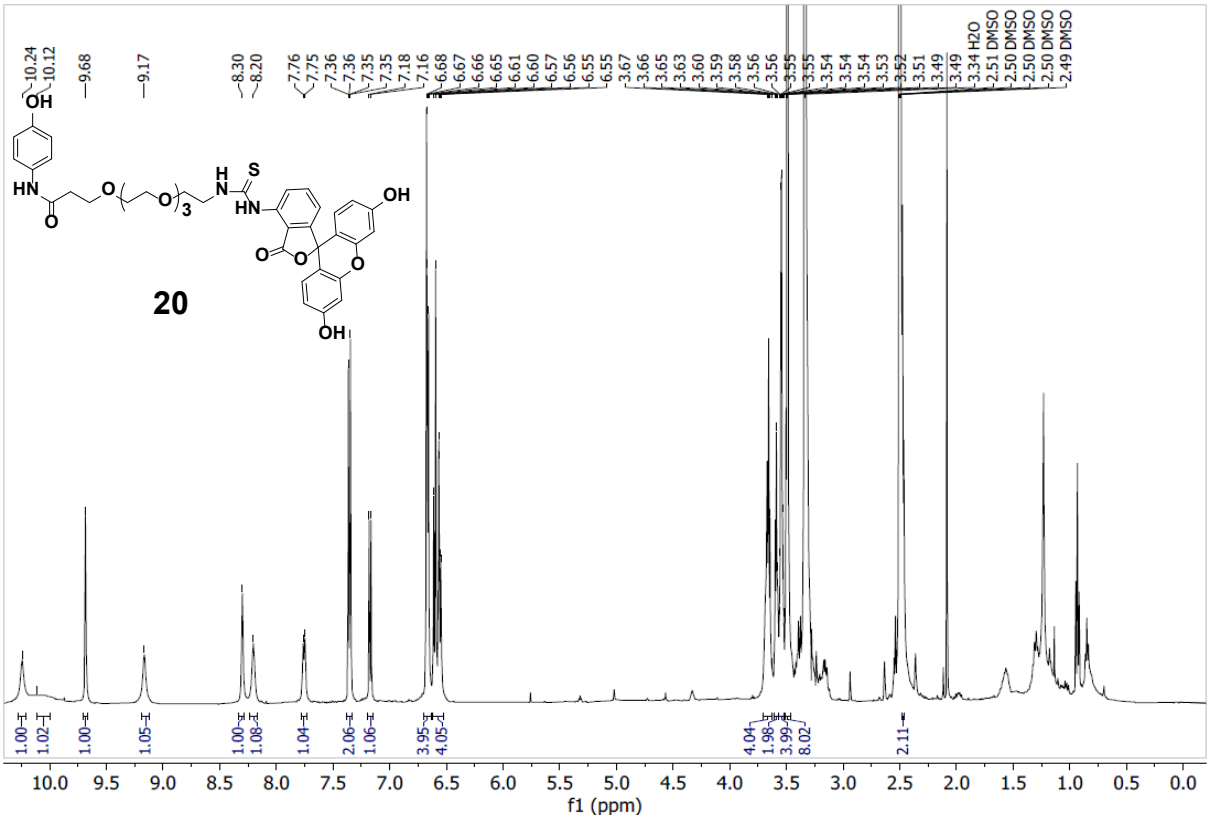


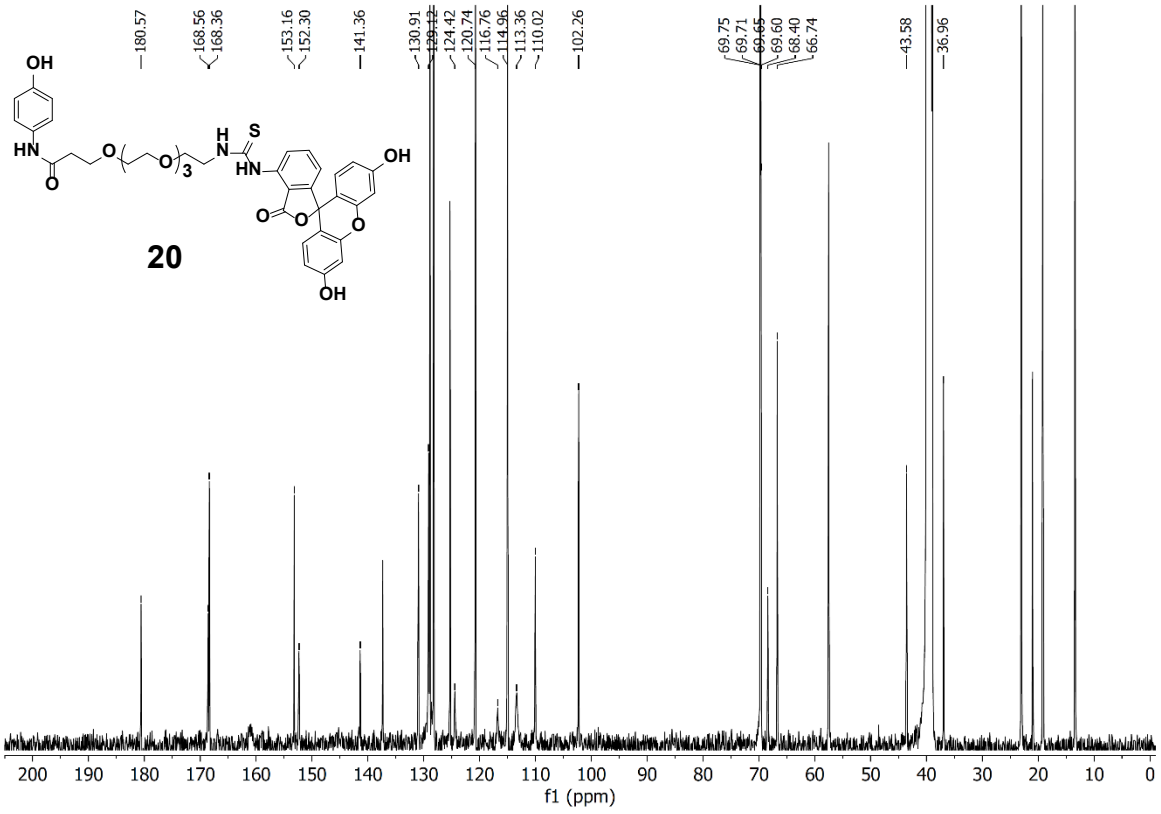
15

176.27
166.19
57.39
52.69
50.08
37.67
36.95
30.72
30.24
29.73
27.15
26.84
15.61
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)


19
f1 (ppm)

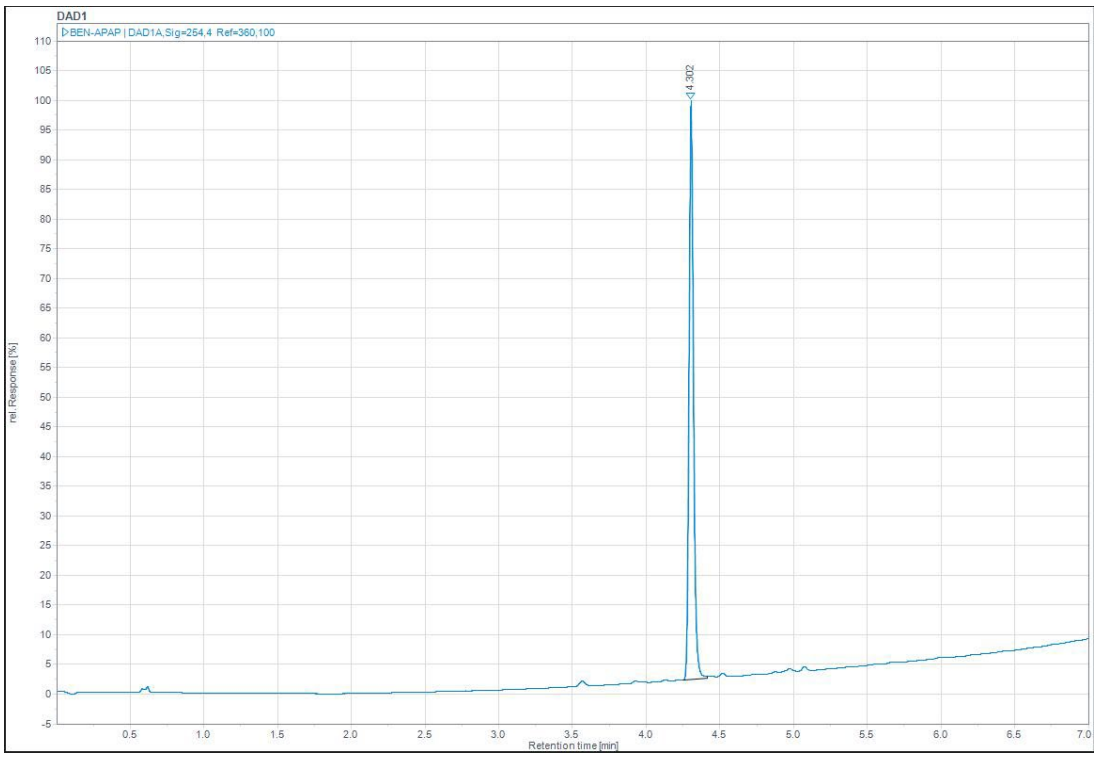

19
f1 (ppm)





F11

LC-Chromatogram (Probe 20)



ESI MS-Spectra (Probe 20)