Fluorogenic Azidofluoresceins for Biological Imaging

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Supporting Information

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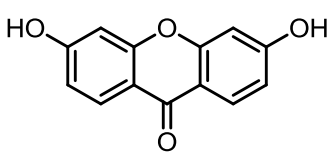
Materials and General Synthetic Procedures

All chemical reagents obtained from commercial suppliers were used without further purification. All reactions were performed in oven- or flame-dried glassware under an inert atmosphere, unless otherwise noted. Anhydrous dichloromethane, tetrahydrofuran and acetonitrile were either dried over CaH_2 and freshly distilled or passed through an activated alumina column prior to use. Anhydrous DMF was used as purchased. Water was either double distilled or passed through a Milli-Q filtration system prior to use. Tris-(benzyltriazoylmethyl)amine (TBTA)[3], DIFO[4], DIMAC[5], BTTAA[6], Ac_4ManNAl[7], and 3-azido-7-hydroxycoumarin[8] were prepared according to literature procedures. THPTA was generously provided by the M.G. Finn group (Georgia Institute of Technology).

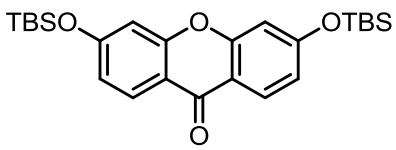
Flash chromatography was performed using Merck 60 Å 230-400 mesh silica gel or Silicycle SiliaFlash P60 silica gel. Analytical thin layer chromatography was performed using glass-backed Analtech Uniplate silica gel plates containing a fluorescent indicator. Reversed-phase HPLC was performed on a Varian Pro Star system with a Varian UV-Vis detector model 345 (210, 254 nm) on a Dynamax Microsorb C-18 preparative column (21.4 x 250 mm) at a flow rate of 20 mL/min or on a Dynamax Microsorb C-18 semi-preparative column (10.0 x 250 mm) at a flow rate of 3 mL/min.

NMR spectra were obtained on Bruker AVQ-400, AVB-400 or DRX-500 spectrometers at ambient temperature at the UC Berkeley College of Chemistry NMR Facility. ^{13}C resonances are unassigned and reported as observed. ^{1}H NMR shifts are calibrated to residual undeuterated solvent: δ 7.26 for $CHCl_3$, 2.50 for d_5-DMSO, and 3.31 for CHD_2OD. ^{13}C NMR shifts are calibrated to solvent peaks: δ 77.16 for CDCl3, 39.52 for d_6-DMSO, and 49.00 for CD_3OD. Low resolution mass spectrometry was performed using an Agilent 6120 Single Quad mass spectrometer. High resolution mass spectrometry was performed at the UC Berkeley Mass Spectrometry Laboratory. Uncorrected melting points were determined using a Thomas Hoover capillary melting point apparatus.



3,6-dihydroxy-9H-xanthen-9-one (S1). Known **S1**[10] was prepared according to literature procedure with minor modifications.[10] In brief, bis(2,4-dihydroxyphenyl)methanone (4.92 g, 20 mmol), a bright yellow solid, was suspended in 40 mL H_2O in a pressure tube behind a blast shield. The sealed pressure tube was heated to 180 °C for 72 h, during which the solid dissolved and a salmon-colored precipitate gradually began to form. The vessel was then allowed to cool to 80 °C, upon which the tube was opened and the contents immediately poured out and filtered. The solids were further washed with water, yielding analytically pure **S1** (4.49 g, 98%) as a salmon colored solid that was carried directly onto the next step.

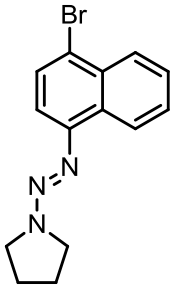
R_f = 0.30 (10:1 $CHCl_3$/MeOH); ^{1}H NMR (400 MHz, d_6-DMSO): δ 6.81, (d, 2H, *J* = 2.2 Hz), 6.85 (dd, 2H, *J* = 8.8 Hz, 2.2 Hz), 7.98 (d, 2H, *J* = 8.8 Hz), 10.82 (s, 2H); ^{13}C NMR (100 MHz, d_6-DMSO): δ 102.15, 113.69, 114.18, 127.89, 157.64, 163.37, 174.13. HRMS (ESI): Calculated for $C_{13}H_9O_4$ $[M+H]^+$: 229.0495, found 229.0497.



3,6-bis(tert-butyldimethylsilyloxy)-9H-xanthen-9-one (5). Known **5**[11] was prepared according to literature procedure with minor modifications.[11] In brief, **S1** (5.67 g, 24.85 mmol) was dissolved in 50 mL anhydrous DMF. The salmon colored solution was cooled to 0 °C in an ice bath, then *tert*-butyldimethylsilyl chloride (22.47 g, 149.1 mmol) and imidazole (16.92 g, 248.5 mmol) were added. Upon addition of the imidazole, the salmon colored solution into a thick white slurry. The slurry was allowed to warm to rt and stirred for 4 h. Then, the reaction was diluted with 300 mL toluene and the reaction washed with 5 x 200 mL H_2O, dried over $MgSO_4$, and concentrated *in vacuo*, yielding an off-white wet solid. The solid was taken up in 100 mL pentane and the undissolved solid was collected by filtration. The filtrate was concentrated *in vacuo* and this procedure was repeated twice more with 50 mL pentane to collect more product. The collected solids were combined and further dried *in vacuo* to yield **S3** (8.05 g, 71%) as a free-flowing white powder.

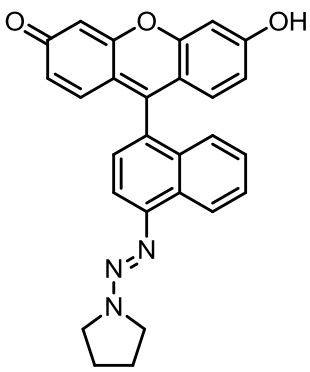
R_f = 0.70 (4:1 hexanes/EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.24 (12H, s), 0.98 (18H, s), 6.80 (2H, s), 6.81, (d, 2H, *J* = 8.6 Hz), 8.16 (d, 2H, *J* = 8.6Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ -4.08,

25.83, 107.62, 116.72, 117.84, 128.47, 158.02, 161.63, 175.99. HRMS (ESI): Calculated for $C_{25}H_{37}O_4Si_2$ $[M+H]^+$ 457.2225, found 457.2233.



(E)-1-((4-bromonaphthalen-1-yl)diazenyl)pyrrolidine (7). 1-amino-4-bromonaphthalene (4.50 g, 20.3 mmol), a grey powder, was suspended in 34 mL concentrated HCl and cooled to 0 °C. A solution of $NaNO_2$ (1.398 g, 20.3 mmol) in 2.25 mL ice cold water was added dropwise, turning the grey suspension a greenish color. The resulting solution of diazonium salt was stirred for 10 min at 0 °C, then poured into a chilled solution of pyrrolidine (1.60 g, 1.85 mL, 22.5 mmol) in 67.5 mL 1 M KOH, resulting in a considerable warming and formation of a brown solution. The reaction mixture was stirred for 1 h at 0 °C, brought to pH 12 with the careful addition of 10 M KOH, extracted with 2 x 150 mL CH_2Cl_2, dried over $MgSO_4$, and concentrated *in vacuo* to yield a red solid. The solid was purified by flash chromatography on silica gel (hexans to 10:1 hexanes/EtOAc) to yield **7** (3.93 g, 64%) as a dark red solid.

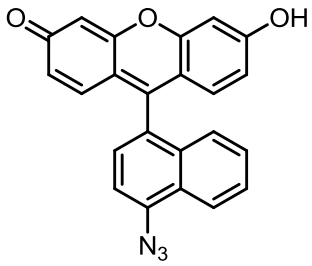
R_f = 0.20 (10:1 hexanes/EtOAc); m.p. 74-75 °C (decomp); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.95 (m, 4H), 3.85 (m, 4H), 7.46 (d, 1H, *J* = 8.4 Hz), 7.66 (m, 2H), 7.84 (d, 1H, *J* = 8.0 Hz), 8.33 (m, 1H), 8.81 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 23.64, 23.89, 46.78, 51.13, 111.92, 118.53, 124.42, 125.90, 126.81, 127.31, 130.15, 130.59, 132.41, 146.67; HRMS (ESI): Calculated for $C_{14}H_{15}N_3Br$ $[M+H]^+$ 304.0449, found 304.0454.



(E)-6-hydroxy-9-(4-(pyrrolidin-1-yldiazenyl)naphthalen-1-yl)-3H-xanthen-3-one (8). 7 (0.912 g, 3.00 mmol), a deep red solid, was dissolved in 12 mL anhydrous THF. The deep red solution was cooled to -78 °C. Then, *tert*-butyllithium (1.31 M in pentanes, 4.58 mL, 6.00 mmol) was slowly added down the side of the flask. The now red-brown solution was stirred at -78 °C for 30 min. Then, **5** (1.50 g, 3.30 mmol), dissolved in 2 mL anhydrous THF, was slowly added. The reaction was stirred at -78 °C for 2 h, then warmed to rt and quenched with 2 mL 2 M HCl. After stirring for 15 minutes, the reaction was neutralized with 1 mL 1 M NaOH. The solvent was

removed *in vacuo* and the remaining red residue was purified by flash chromatography on silica gel ($CHCl_3$ to 10:1 $CHCl_3$), yielding **8** (0.839 g, 64%) as a red-orange solid.

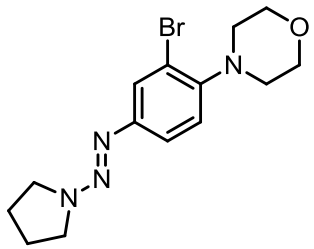
R_f = 0.40 (10:1 $CHCl_3$/MeOH); m.p. 283-285 °C; ^{1}H NMR (400 MHz, d_6-DMSO): δ 3.76 (m, 4H), 3.98 (m, 4H), 6.45 (m, 2H), 6.55 (m, 2H), 6.76 (m, 2H), 7.45 (m, 5H), 8.65 (m, 1H); ^{13}C NMR (100 MHz, d_6-DMSO): δ 23.46, 23.89, 47.33, 51.49, 102.49, 103.77, 110.88, 115.90, 122.06, 124.33, 125.49, 126.21, 126.60, 127.57, 128.36, 128.77, 130.70, 132.46, 147.72, 149.38, 156.77; HRMS (ESI): Calculated for $C_{27}H_{22}N_3O_3$ $[M+H]^+$ 436.1661, found 436.1653.



9-(4-azidonaphthalen-1-yl)-6-hydroxy-3H-xanthen-3-one (1). **8** (0.218 g, 0.50 mmol), a red-orange powder, was suspended in 50 mL water and cooled to 0 °C. NaN_3 (3.25 g, 50.00 mmol) was then added. After the NaN_3 had dissolved, 25 mL concentrated HCl was carefully added, turning the suspension a greenish-blue color. [**Caution:** Addition of NaN_3 to acid results in formation of hydrazoic acid, which is hazardous. Therefore, perform this addition in a well-ventilated hood with proper protection.] The solution was stirred at 0 °C for 90 min, upon which the red-orange color had returned. The reaction was neutralized with the slow addition of 40 mL 10M NaOH, then the ice bath removed and the reaction stirred at rt for 10 min. Then, 2M HCl was added dropwise until the pH reached ~5-6 and a red precipitate had formed. The solid was collected by filtration, washed with 2 x 30 mL H_2O, then taken up in MeOH and concentrated to yield crude product as a red-orange solid. The solid was taken up in MeOH and 0.05 mL 10M NaOH was added, turning the solution a deep red color. The solvent was removed *in vacuo* to yield the sodium salt of **1**, which as purified in batches by preparative reversed-phase HPLC with a 30 min gradient from 40% to 100% MeCN in H_2O to yield analytically pure **1** (100 mg, 50% for sodium salt) as a red solid.

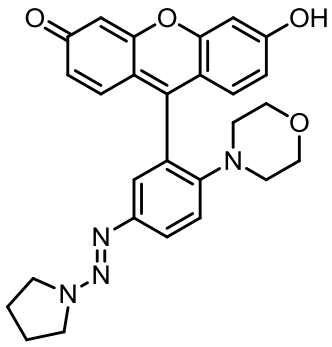
R_f = 0.45 (10:1 $CHCl_3$/MeOH); ^{1}H NMR (500 MHz, d_6-DMSO): δ 6.09 (dd, 2H, *J* = 9.3 Hz, 2.1 Hz), 6.13 (d, 2H, *J* = 2.0 Hz), 6.48 (d, *J* = 9.3 Hz, 2H), 7.50 (d, 8.4 Hz, 1H), 7.55 (t, 1H, *J* = 7.5 Hz), 7.59 (d, 1H, *J* = 7.7 Hz), 7.64 (t, 1H, *J* = 7.3 Hz), 7.66 (d, 1H, *J* = 7.5 Hz), 8.19 (d, 1H, *J* = 8.6 Hz), ^{13}C NMR (125 MHz, d_6-DMSO): δ 103.37, 110.17, 114.36, 122.56, 123.53, 125.52,

125.72, 126.96, 127.56, 128.13, 128.40, 129.65, 132.31, 136.78, 148.02, 157.49, 179.90; HRMS (ESI): Calculated for $C_{23}H_{12}N_3O_3$ $[M-H]^-$ 378.0884, found 378.0889.



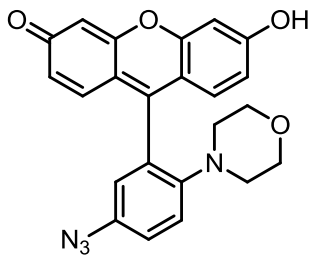
(E)-4-(2-bromo-4-(pyrrolidin-1-yldiazenyl)phenyl)morpholine (S2). 3-bromo-4-(4-morpholinyl)-benzenamine (500 mg, 1.95 mmol) was dissolved in 0.4 mL concentrated HCl and cooled to 0 °C. A solution of $NaNO_2$ (134 mg, 1.95 mmol) in 0.2 mL cold H_2O was added dropwise. The resulting solution of diazonium salt was stirred for 10 min and then added all at once to a chilled solution of pyrrolidine (0.18 mL, 2.1 mmol) in 1.67 mL 1 M KOH. The reaction mixture was stirred for 30 min at 0 °C, then diluted with 5 mL H_2O, extracted with CH_2Cl_2 and dried over $MgSO_4$. The crude product was concentrated *in vacuo* and purified by flash chromatography on silica gel (CH_2Cl_2 to 20:1 CH_2Cl_2/MeOH) to yield **S2** (456.2 mg, 69%) as dark red crystals.

m.p. 131-132 °C (decomp); ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.01 (m, 4H), 3.02 (m, 4H), 3.76 (m, 4H), 3.87 (m, 4H), 7.00 (d, 1H, *J* = 8.4 Hz), 7.32 (dd, 1H, *J* = 8.4, 2.0 Hz), 7.68 (d, 1H, *J* = 2.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 18.42, 18.42, 52.29, 67.21, 120.15, 120.62, 120.63, 124.84, 147.20, 148.12; HRMS (ESI): Calculated for $C_{14}H_{20}N_4OBr$ $[M+H]+$ 339.0820, found 339.0829.



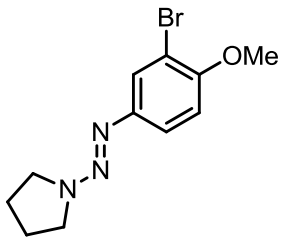
(E)-6-hydroxy-9-(2-morpholino-5-(pyrrolidin-1-yldiazenyl)phenyl)-3H-xanthen-3-one (S3). S2 (218 mg, 0.644 mmol) was dissolved in 4 mL anhydrous THF and the solution was cooled to -78 °C. *tert*-butyllithium (0.42 mL of 1.7 M solution in pentane, 0.708 mmol) was added dropwise. The resulting solution was stirred for 10 min at -78 °C and then **5** (323 mg, 0.708 mmol) predissolved in 2 mL anhydrous THF cooled to -78 °C was added dropwise. The solution was stirred for 1 h at -78 °C, then diluted with 2 mL H_2O by dropwise addition and adjusted to pH 1 with concentrated HCl. The solution was warmed to rt and stirred overnight, then neutralized with KOH. The crude product was concentrated *in vacuo* and purified by flash chromatography on silica gel (CH_2Cl_2 to 10:1 CH_2Cl_2/MeOH) to yield **S3** (155 mg, 51%) as a dark red solid.

m.p. 249-250 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.99 (m, 4H), 2.76 (m, 4H), 3.28 (m, 4H), 3.74 (m, 4H), 6.88 (m, 4H), 7.20 (m, 1H), 7.30 (m, 3H), 7.56 (d, 1H, *J* = 8.8 Hz), 8.49 (br s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 23.74, 23.74, 52.05, 66.82, 103.53, 114.24, 120.45, 121.78, 122.72, 123.17, 127.62, 131.88, 147.06, 147.97, 155.35, 158.20, 175.60; HRMS (ESI): Calculated for $C_{27}H_{27}N_4O_4$ $[M+H]^+$ 471.2032, found 471.2025.



9-(5-azido-2-morpholinophenyl)-6-hydroxy-3H-xanthen-3-one (2). **S3** (117 mg, 0.248 mmol) was dissolved in 15 mL 2:1 H_2O/MeOH and cooled to 0 °C. NaN_3 (1.61 g, 24.8 mmol) was added, followed by 5 mL concentrated HCl. [**Caution:** Addition of NaN_3 to acid results in formation of hydrazoic acid, which is hazardous. Therefore, perform this addition in a well-ventilated hood with proper protection.] The solution was stirred at 0 °C for 3 h, then the MeOH was removed *in vacuo*. The remaining solution was neutralized with sat. $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was then removed and concentrated *in vacuo* to yield **2** (87 mg, 85%) as an orange solid. For analytical experiments, a small quantity of **2** was further purified by semi-preparative reversed-phase HPLC.

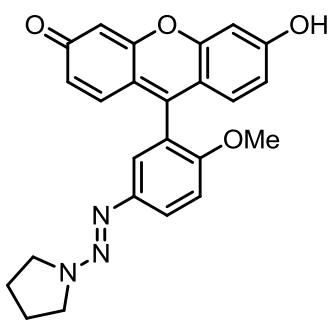
m.p. 252-253 °C; ^{1}H NMR (400 MHz, CD_3OD): δ 2.77 (m, 4H), 3.26 (m, 4H), 6.71 (m, 3H), 6.74 (m, 1H), 6.98 (m, 1H), 7.25 (m, 2H), 7.32 (m, 1H), 7.40 (m, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ 50.20, 64.88, 101.32, 112.36, 113.64, 119.68, 119.81, 120.27, 127.41, 129.72, 130.05, 134.01, 146.54, 150.96, 156.41; HRMS (ESI): Calculated for $C_{23}H_{19}N_4O_4$ $[M+H]^+$ 415.1406, found 415.1412.



(E)-1-((3-bromo-4-methoxyphenyl)diazenyl)pyrrolidine (S4). 3-bromo-4-methoxyaniline (985 mg, 4.87 mmol) was suspended in 20 mL concentrated HCl and cooled to 0 °C. A solution of $NaNO_2$ (342 mg, 4.95 mmol) in 2 mL cold H_2O was added dropwise. The resulting solution of diazonium salt was stirred for 10 min and then added all at once to a chilled solution of pyrrolidine (0.45 mL, 5.5 mmol) in 12 mL 1 M KOH. The reaction mixture was stirred for 30 min at 0 °C and then diluted with 50 mL H_2O, brought to pH 12 with

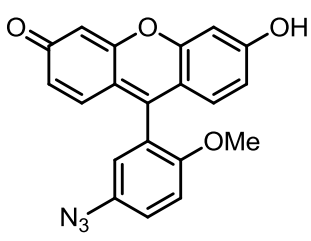
KOH, extracted with CH_2Cl_2 and dried over Na_2SO_4. The solution was filtered and concentrated *in vacuo* to yield **S4** (1.34 g, 97%) as a dark red solid.

m.p. 79-81 °C (decomp); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.88 (m, 4H), 3.65 (m, 4H), 3.77 (s, 3H), 6.78 (d, 1H, *J* = 8.8 Hz), 7.27 (dd, 1H, *J* = 8.4, 2.4 Hz), 7.64 (d, 1H, *J* = 2.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 23.73, 23.73, 46.67, 50.61, 56.35, 111.73, 111.83, 120.95, 124.12, 145.89, 153.23; HRMS (ESI): Calculated for $C_{11}H_{15}N_3OBr$ $[M+H]^+$ 284.0398, found 284.0393.



(E)-6-hydroxy-9-(2-methoxy-5-(pyrrolidin-1-yldiazenyl)phenyl)-3H-xanthen-3-one (S5). S4 (750 mg, 2.61 mmol) was dissolved in 7.5 mL anhydrous THF and cooled to -78 °C. *tert*-butyllithium (1.54 mL of 1.7 M solution in pentane, 2.61 mmol) was added dropwise. The resulting solution was stirred for 30 min at -78 °C and then **S1** (1.31 g, 2.87 mmol) predissolved in 10 mL THF cooled to -78 °C was added dropwise. The solution was stirred for 2 h at -78 °C, then diluted with 50 mL water by dropwise addition and adjusted to pH 1 with concentrated HCl. The solution was warmed to rt and stirred for 10 min, then neutralized with sat. $NaHCO_3$. The crude product was concentrated *in vacuo* and purified by flash chromatography on silica gel (CH_2Cl_2 to 10:1 CH_2Cl_2/MeOH) to yield **S4** (497 mg, 46%) as an orange solid.

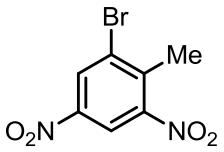
m.p. 270-272 °C; 1H NMR (400 MHz, d_6-DMSO): δ 3.48 (m, 8H), 3.66 (s, 3H), 6.52 (m, 4H), 6.93 (d, 2H, *J* = 9.6 Hz), 7.20 (m, 2H), 7.48 (d, 1H, *J* = 8.0); 13C NMR (100 MHz, d6-DMSO): δ 23.66, 23.66, 56.29, 103.64, 112.73, 114.72, 121.42, 121.76, 123.29, 130.74, 144.86, 147.91, 153.19, 154.31, 156.22, 156.68; HRMS (FAB): Calculated for $C_{24}H_{22}N_3O_4$ $[M+H]^+$ 416.1610, found 416.1612.



9-(5-azido-2-methoxyphenyl)-6-hydroxy-3H-xanthen-3-one (3). S5 (251 mg, 0.604 mmol) was suspended in 56 mL water and cooled to 0 °C. NaN_3 (3.93 g, 40 mmol) was added, followed by 28 mL concentrated HCl. [**Caution:** Addition of NaN_3 to acid results in formation of hydrazoic acid, which is hazardous. Therefore, perform

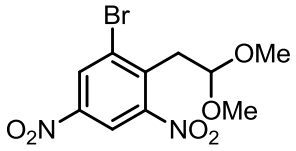
this addition in a well-ventilated hood with proper protection.] The solution was stirred at 0 °C for 2 h and as neutralized with sat. $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, filtered, and concentrated *in vacuo* to yield **3** (215 mg, 99%) as an orange solid. For analytical experiments, a small quantity of **3** was further purified by semi-preparative reversed-phase HPLC.

m.p. 254-255 °C; ^{1}H NMR (400 MHz, d_6-DMSO): δ 3.65 (s, 3H), 6.10 (m, 2H), 6.20 (m, 2H), 6.65 (m, 2H), 6.97 (m, 1H), 7.19 (m, 2H); ^{13}C NMR (100 MHz, d_6-DMSO): δ 56.42, 103.49, 109.99, 113.66, 121.40, 121.44, 123.63, 124.12, 129.94, 132.15, 146.97, 154.34, 157.66, 179.76; HRMS (FAB): Calculated for $C_{20}H_{14}N_3O_4$ [M+H]+ 360.0984, found 360.0985.



1-bromo-2-methyl-3,5-dinitrobenzene (S6). Known **S6**[12,13] was synthesized based off of literature procedure.[14] 1-methyl-2,4-dinitrobenzene (10.93 g, 66.0 mmol), an off-white crystalline solid, was added to a flask containing 30 mL H_2SO_4. The suspension was heated to 60 °C, upon which the solid had dissolved. To this clear solution was added N-bromosuccinimide (14.13 g, 79.0 mmol) in three portions over 20 min. After the first addition, the solution warmed significantly and began to take on a reddish color. Once all the N-bromosuccinimide had been added, the flask was gently capped with a Teflon cap and the reaction was stirred for 2.5 h at 60 °C. The now pink reaction mixture was cooled to ~40 °C, then poured into a beaker containing 100 g ice. The mixture immediately turned a cloudy white, with a red-orange liquid remaining at the bottom. This liquid solidified over the course of 15 min of standing at room temperature. The supernatant was poured out and this red-orange solid dissolved in 250 mL Et_2O. To this solution was added 200 mL 50% saturated $Na_2S_2O_3$, and turning the red solution a light yellow color. The aqueous layer was removed, and the organic layer was washed with 3 x 150 mL sat. $NaHCO_3$ (caution: gas evolution!), dried over $MgSO_4$, and then concentrated *in vacuo* to yield **S6** (13.71 g, 80%) as a white solid.

R_f = 0.60 (4:1 hexanes/EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.68 (s, 3H), 8.59 (d, 1H, J = 2.3 Hz), 8.65 (d, 1H, J = 2.3 Hz); ^{13}C NMR (125 MHz, $CDCl_3$): δ 20.13, 118.45, 120.08, 130.71, 139.78, 145.91, 150.89.

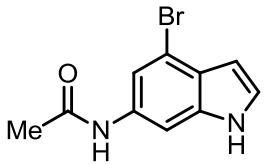


1-bromo-2-(2,2-dimethoxyethyl)-3,5-dinitrobenzene (9). Known **9** was synthesized based off modifications of literature procedure.[13,14] **S6** (13.71 g, 52.5 mmol), an off-white solid, was dissolved in 40 mL anhydrous N,N-dimethylformamide. Then, to this pale yellow solution was added N,N-dimethylformamide dimethyl acetal (7.51 g, 8.37 mL, 63.02 mmol) was added, immediately turning the solution a dark blue color. The reaction was stirred for 18 h at rt, during which it turned a deep red color. The solvent was removed *in vacuo* to yield crude enamine as a deep red solid, which was carried directly onto the next step without further purification.

R_f = 0.25 (4:1 hexanes/EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.96 (s, 6H), 5.26 (d, 1H, *J* = 13.4 Hz), 6.98 (d, 1H, *J* = 13.4 Hz), 8.29 (d, 1H, *J* = 3.4 Hz), 8.45 (d, 1H, *J* = 3.4 Hz).

The enamine was dissolved in 105 mL MeOH. Then, 8.7 mL concentrated HCl was slowly added to the deep red solution. The reaction allowed to reflux for 5 h, during which its appearance changed to a cloudy brown. The reaction was cooled and diluted with 100 mL H_2O and 100 mL EtOAc. The organic layer was collected and the product was further extracted with 2 x 150 mL EtOAc. The combined organic layers were washed with saturated NH_4Cl, dried over $MgSO_4$, and concentrated, yielding a deep brown oil. The compound was purified by flash chromatography on silica gel (hexanes to 10:1 hexanes/EtOAc) to yield **9** (11.10 g, 63%) as a red-orange solid.

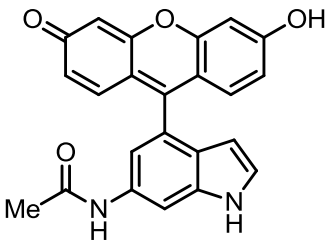
R_f = 0.20 (10:1 hexanes/EtOAc); ^{1}H NMR (400 MHz, CDCl3): δ 3.30 (s, 6H), 3.66 (d, 2H, *J* = 5.3 Hz), 4.48 (t, 1H, *J* = 5.3 Hz), 8.54 (d, 1H, *J* = 2.4 Hz), 8.64 (d, 1H, *J* = 2.4 Hz); ^{13}C NMR (125 MHz, CDCl3) δ 35.50, 54.46, 103.03, 118.91, 128.18, 130.81, 138.10 146.29, 152.08.



N-(4-bromo-1H-indol-6-yl)acetamide (10). **9** (8.07 g, 24.08 mmol) was dissolved in 200 mL glacial acetic acid. Then, iron powder (26.90 g, 481.6 mmol) was added. A bubbler was attached, and the reaction was heated to 85 °C for 1 h, turning the grey suspension to a thick yellow slurry. Then, the reaction was heated to 100 °C and stirred for 16 h. The reaction was cooled to rt and the

acetic acid removed *in vacuo*. The remaining light brown residue was diluted with 200 mL 1 M KOH and 200 mL EtOAc and the organic layer collected. The aqueous layer was extracted with another 200 mL EtOAc. The organic layer was washed with 5 x 200 mL sat. $NaHCO_3$ (caution: vigorous gas evolution!). The organic layer was dried with $MgSO_4$ and concentrated *in vacuo*, yielding a brown residue. The compound was partially purified by flash chromatography on silica gel (4:1 to 1:1 to 1:2 hexanes/EtOAc), to yield a mixture of desired product and unacetylated indole as a viscous brown oil. The solid was taken up in 30 mL Et_2O and filtered to yield product as a light brown solid. The solid was further washed with another 50 mL Et_2O. This was repeated once more to yield another crop of product, yielding **10** (3.13 g, 51%) as a light brown solid.

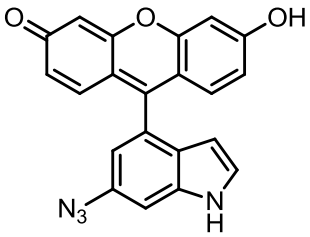
R_f = 0.20 (1:1 hexanes/EtOAc); ^{1}H NMR (500 MHz, CD_3OD): δ 2.14 (s, 3H), 6.40 (d, 1H, *J* = 3.2 Hz), 7.26 (d, 1H, *J* = 3.2 Hz) 7.28 (d, 1H, *J* = 1.7 Hz) 7.82 (s, 1H) ^{13}C NMR (125 MHz, CD_3OD): δ 23.73, 102.32, 103.67, 114.43, 116.69, 126.53, 126.97, 134.61, 137.57, 171.47. HRMS (ESI): Calculated for $C_{10}H_{10}O_1N_2Br$ $[M+H]^+$ 252.9974, 254.9953, found 252.9971, 254.9951.



N-(4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-1H-indol-6-yl)acetamide (11). 10 (0.506 g, 2.00 mmol) was dissolved in 20 mL anhydrous THF and cooled to 0 °C. Then, sodium hydride (40% dispersion in mineral oil, 0.160 g, 4.00 mmol) was added. Bubbling was observed and the clear solution turned a green color. After stirring for 15 min, the solution was cooled to -78 °C. Then, *tert*-butyllithium (1.41 M in pentanes, 2.83 mL, 4.00 mmol) was added dropwise down the side of the flask, immediately turning the solution into a viscous yellow slurry. After stirring for 30 min, a solution of **S1** (1.04 g, 2.20 mmol) in 6 mL anhydrous THF was slowly added down the side of the flask, turning the reaction mixture a deep red color. After stirring for 2 h at -78 °C, the reaction was allowed to warm to room temperature over 15 min. The reaction was quenched with the careful addition of 4 mL 2 M HCl (caution: gas evolution!), upon which the deep red color of the reaction mixture changed to a light red-orange partway through addition. As more of the HCl was added, the deep red color reappeared. After stirring for another 15 min, the solvent was removed *in vacuo* and the remaining deep red solid

was purified by flash chromatography on silica gel ($CHCl_3$ to 10:1 $CHCl_3$/MeOH to 7:1 $CHCl_3$/MeOH), yielding **11** (0.442 g, 58%) as a red solid.

R_f = 0.05 (10:1 $CHCl_3$/MeOH); ^{1}H NMR (400 MHz, CD_3OD): δ 2.18 (s, 3H), 6.01 (s, 1H), 7.08 (d, 2H, *J* = 9.5 Hz), 7.25 (s, 1H), 7.29 – 7.36 (m, 2H), 7.72 (d, 2H, *J* = 9.4 Hz), 8.09 (s, 1H), 11.14 (s, 1H); ^{13}C NMR (100 MHz, d_6-DMSO): δ 24.18, 92.58, 99.94, 103.18, 103.67, 113.57, 114.92, 116.78, 121.23, 123.15, 123.41, 126.65, 131.64, 133.86, 136.16, 157.28, 168.51. HRMS (ESI): Calculated for $C_{23}H_{15}N_2O_4$ $[M-H]^-$ 383.1026, found 383.1037.



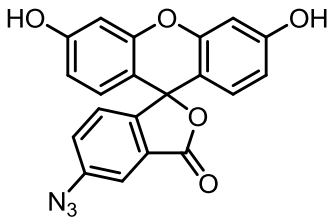
9-(6-azido-1H-indol-4-yl)-6-hydroxy-3H-xanthen-3-one (4).

11 (81.0 mg, 0.211 mmol), a red solid, was dissolved in 6 mL anhydrous MeOH. Then, boron trifluoride diethyl etherate, (0.180 g, 0.160 mL, 1.27 mmol) was added. The deep red solution was allowed to reflux for 6 h. After cooling to rt, 0.600 mL triethylamine was added dropwise to quench the reaction and the solution was concentrated *in vacuo* to yield a deep red solid. The solid was taken up in 10 mL H_2O and filtered. The solid was washed with another 10 mL H_2O, dissolved in MeOH and concentrated *in vacuo* to yield crude aminoindole as a red-brown solid, which was carried directly onto the next step without further purification.

R_f = 0.05 (10:1 $CHCl_3$/MeOH); ^{1}H NMR (400 MHz, CD_3OD): δ 5.86 (d, 1H, *J* = 3.3 Hz), 7.01 (s, 1H), 7.06 (d, 1H, *J* = 3.2 Hz), 7.11 (s, 1H), 7.32 (d, 2H, *J* = 9.4 Hz). LRMS (ESI): Calculated for $C_{21}H_{15}N_2O_3$ $[M+H]^+$ 343.1, found 343.4.

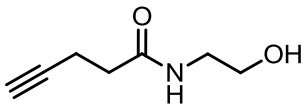
The crude aminoindole was dissolved in 6 mL 2:1 AcOH/H_2O. The deep red solution was cooled to 0 °C. Then, $NaNO_2$ (16.1 mg, 0.232 mmol) was slowly added and the reaction stirred for 10 min at 0 °C. Then, NaN_3 (15.1 mg, 0.232 mmol) was carefully added (caution: gas evolution!). The resulting red solution was stirred for 30 min at 0 °C, then warmed to rt and stirred for 14 h. The solution was concentrated *in vacuo* to yield a red-brown residue. The residue was purified by preparative reversed-phase HPLC with a 25 min gradient from 25% to 100% MeCN in H_2O to yield **4** (23.0 mg, 30%) as a red-brown solid.

R_f = 0.2 (10:1 $CHCl_3$/MeOH); ^{1}H NMR (400 MHz, d_6-DMSO): δ 6.02 (s, 1H), 6.46-6.50 (m, 4H), 6.86-6.92 (m, 3H), 7.33 (s, 1H), 7.39 (s, 1H), 8.31 (s, 1H), 11.44 (s, 1H); ^{13}C (125 MHz, d_6-DMSO): δ 100.92, 102.81, 103.69, 125.63, 126.64, 127.79, 130.59, 133.21, 136.81, 148.78, 157.44, 157.47. 159.11. HRMS (ESI): Calculated for $C_{21}H_{13}O_3N_4$ $[M+H]^+$ 369.0982, found 369.0989.



5-azidofluorescein (N_3-fluor) 5-aminofluorescein (0.174 g, 0.500 mmol), a deep-red solid, was dissolved in 10 mL 2:1 AcOH/H_2O and cooled to 0 °C. To this deep red solution was added $NaNO_2$, a white powder (52 mg, 0.75 mmol). After stirring for 15 minutes, the solution had turned to a light red color. NaN_3 (66 mg, 1.00 mmol) was then carefully added (caution: gas evolution!), turning the solution to a yellow slurry. The reaction was stirred for 1 hr at 0 °C and 1 h at room temperature. The slurry was filtered over vacuum and the solid washed with 20 mL 2M HCl and 100 mL H_2O, yielding 5-azidofluorescein[15,16] (0.156 g, 84%) as a yellow solid after further drying *in vacuo*.

R_f = 0.40 (10:1 $CHCl_3$/MeOH); ^{1}H NMR (400 MHz, d_6-DMSO): δ 6.51-6.63 (m, 4H), 6.67 (d, 2H, *J* = 2.3 Hz), 7.30 (d, 1H, *J* = 8.2 Hz), 7.51 (dd, 1H, *J* = 8.2 Hz, *J* = 2.3 Hz), 7.64, (d, 1H, *J* = 2.2 Hz), 10.13, (s, 2H); ^{13}C NMR (100 MHz, d_6-DMSO): δ 83.27, 102.23, 109.28, 112.60, 114.35, 125.61, 126.75, 127.97, 129.03, 141.74, 148.72, 151.85, 159.54, 167.75. HRMS (ESI): Calculated for $C_{20}H_{12}O_5N_3$ $[M+H]^+$ 374.0771, found 374.0773.

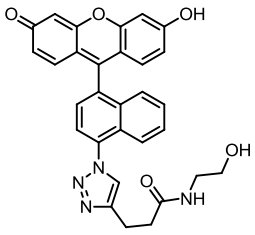


N-(2-hydroxyethyl)pent-4-ynamide (12). 4-pentynoic acid (0.196 g, 2.00 mmol), a white crystalline solid, and N-hydroxysuccinimide (0.230 g, 2.00 mmol), a white solid, were dissolved in 10 mL anhydrous THF. The clear solution was cooled to 0 °C. Then, N,N'-dicyclohexylcarbodiimide (0.454 g, 2.20 mmol), a waxy white solid, was added. The reaction was stirred at 0 °C for 5 minutes, then allowed to stir at rt for 6 h, during which it turned a cloudy white. The reaction was then filtered and the filtrate concentrated *in vacuo* to yield crude NHS ester as a pale yellow oil. This crude product was dissolved in 10 mL anhydrous THF and cooled to 0 °C. Then, 2-

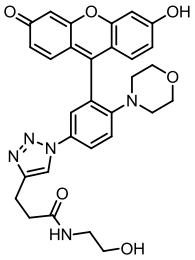
aminoethanol (0.122 g, 0.121 mL, 2.00 mmol), a clear liquid, was added and the reaction was stirred at rt for 16 h, then concentrated *in vacuo*, yielding a white residue. The residue was purified by flash chromatography on silica gel ($CHCl_3$ to 10:1 $CHCl_3$/MeOH), yielding known **S1**[9] (0.140 g, 49%) as a pale yellow oil that solidified upon storage at 4 °C to a white solid.

R_f = 0.35 (10:1 $CHCl_3$/MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.05 (s, 1H), 2.36 (s, 1H), 2.46 (t, 2H, *J* = 7.0 Hz), 2.56 (t, 2H, *J* =) 3.49 (dd, 2H, *J* = 6.2 Hz, *J* = 5.2 Hz) 3.77 (2H, s) 6.07, (1H, s) ^{13}C NMR (100 MHz, $CDCl_3$): 14.89, 35.24, 42.41, 62.16, 69.41, 82.83, 172.09. HRMS (ESI): Calculated for $C_7H_{12}O_2N_1$ $[M+H]^+$ 142.0863, found 142.0865.

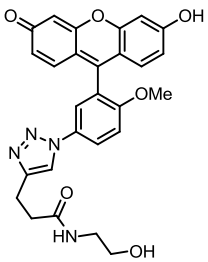
General procedure for azidofluorescein-alkyne cycloaddition product. Azidofluorescein (0.010 mmol) was dissolved in 1 mL MeOH and the solution degassed under vacuum and backfilled with N_2. The reaction was stirred with $Cu(MeCN)_4PF_6$ (1.8 mg, 0.0050 mmol), tris-(benzyltriazoylmethyl)amine (2.7 mg, 0.0050 mmol), and alkyne **12** (7.0 mg, 0.050 mmol). The mixture was stirred in the dark under N_2 for 2 days, then concentrated. The residue then purified by semi-preparative reversed-phase HPLC to yield the adduct.



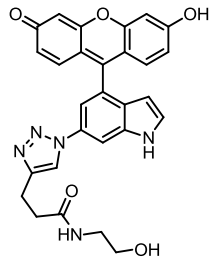
1-12. Purified by a 30 minute gradient from 10% to 95% MeCN in H_2O. HRMS (ESI): Calculated for $C_{30}H_{25}N_4O_5$ $[M+H]^+$ 521.1819, found 521.1829.



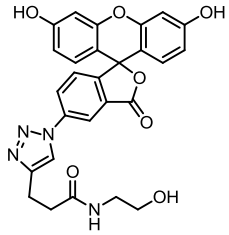
2-12. Purified by a 25 minute gradient from 10% to 30% MeCN in H_2O. HRMS (ESI): Calculated for $C_{30}H_{30}N_5O_6$ $[M+H]^+$ 556.2191, found 556.2204.



3-12. Purified by a 25 minute gradient from 10% to 30% MeCN in H_2O. HRMS (ESI): Calculated for $C_{27}H_{25}N_4O_6$ $[M+H]^+$ 501.1769, found 501.1779.

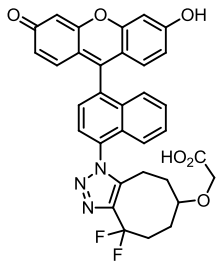


4-12. Purified by a 25 minute gradient from 10% to 30% MeCN in H_2O. HRMS (ESI): Calculated for $C_{28}H_{24}N_5O_5$ $[M+H]^+$ 510.1772, found 510.1788.

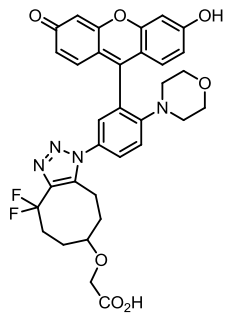


N_3-fluor-12. Purified by a 30 minute gradient from 0% to 40% MeCN in H_2O. HRMS (ESI): Calculated for $C_{27}H_{23}N_4O_7$ $[M+H]^+$ 515.1561, found 515.1580.

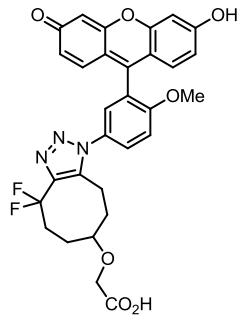
General procedure for DIFO cycloaddition products. Azidofluorescein (0.010 mmol) and DIFO (2.2 mg, 0.010 mmol) were dissolved in 1 mL 1:1 MeCN/H_2O with 5% MeOH cosolvent. The solution as stirred for 1 h at rt in the dark. The crude product was concentrated *in vacuo* and purified by semi-preparative reversed-phase HPLC. No attempt was made to separate the roughly 1:1 mixture of regioisomeric cycloaddition products.



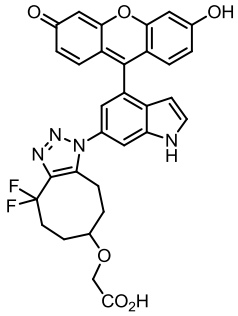
1-DIFO. Purified by a 25 minute gradient from 20% to 60% MeCN in H_2O. LRMS (ESI): Calculated for $C_{33}H_{26}F_2N_3O_6$ $[M+H]^+$ 598.2, found 598.9.



2-DIFO. Purified by a 25 minute gradient from 20% to 60% MeCN in H_2O. HRMS (ESI): Calculated for $C_{33}H_{31}F_2N_4O_7$ $[M+H]^+$ 633.2155, found 633.2172.

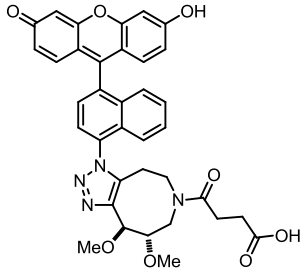


3-DIFO. Purified by a 25 minute gradient from 20% to 60% MeCN in H_2O. HRMS (ESI): Calculated for $C_{30}H_{26}F_2N_3O_7$ $[M+H]^+$ 578.1733, found 578.1748.

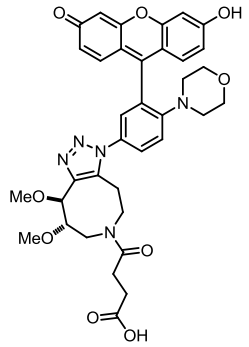


4-DIFO. Purified by a 25 minute gradient from 0% to 40% MeCN in H_2O. HRMS (ESI): Calculated for $C_{31}H_{25}F_2N_4O_6$ $[M+H]^+$ 587.1737, found 587.1750.

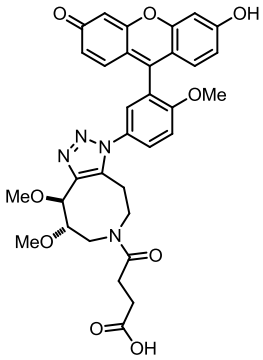
General procedure for DIMAC cycloaddition products. Azidofluorescein (0.010 mmol) and DIMAC (2.9 mg, 0.010 mmol) were dissolved in 1 mL 1:1 MeCN/H_2O. The solution as stirred for 16 h at rt in the dark. The crude product was concentrated *in vacuo* and purified by semi-preparative reversed-phase HPLC. No attempt was made to separate the regioisomeric cycloaddition products.



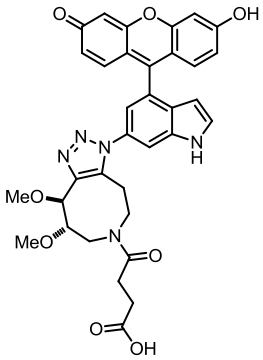
1-DIMAC. Purified by a 25 minute gradient from 10% to 60% MeCN in H_2O. HRMS (ESI): Calculated for $C_{36}H_{33}N_4O_8$ $[M+H]^+$: 649.2293, found 649.2308.



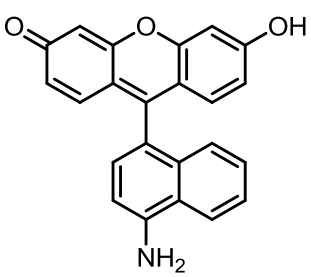
2-DIMAC. Purified by a 25 minute gradient from 0% to 60% MeCN in H_2O. HRMS (ESI): Calculated for $C_{36}H_{38}N_5O_9$ $[M+H]^+$ 684.2664, found 684.2688.



3-DIMAC. Purified by a 25 minute gradient from 0% to 60% MeCN in H_2O. HRMS (ESI): Calculated for $C_{33}H_{33}N_4O_9$ $[M+H]^+$ 629.2242, found 629.2251.



4-DIMAC. Purified by a 25 minute gradient from 0% to 40% MeCN in H_2O. LRMS (ESI): Calculated for $C_{34}H_{32}N_5O_8$ $[M+H]^+$: 638.2, found 638.3.

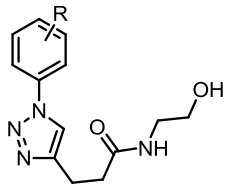
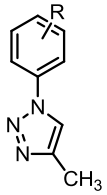


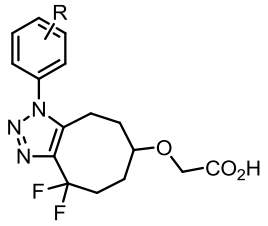
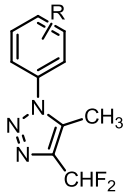
9-(4-aminonaphthalen-1-yl)-6-hydroxy-3H-xanthen-3-one (reduced-1) (S7). (3.8 mg, 0.010 mmol), a red solid, was dissolved in 5 mL 1:1 0.1 M pH 7.4 KP_i buffer/MeOH. Then, dithiothreitol (15.4 mg, 0.100 mmol), a white solid, was added. Bubbling was observed upon addition. The reaction was stirred at rt for 1 h, then concentrated. The red residue was purified by semi-preparative reversed-phase HPLC using a 25 minute gradient from 20 to 60% MeCN in H_2O to yield S7 as a red-orange solid.

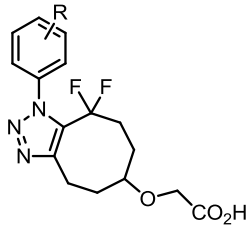
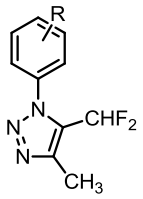
HRMS (ESI): Calculated for $C_{23}H_{16}NO_3$ $[M+H]^+$: 354.1125, found 354.1131.

Density functional theory (DFT) calculations

Geometries were optimized and HOMO energy levels were calculated using the Gaussian 09 software package at the B3LYP/6-31G(d) level of theory, as reported previously.[1] Calculations were performed at the UC Berkeley College of Chemistry Molecular Graphics and Computation Facility. Structures were truncated to simplify calculations. Azidofluoresceins were predicted to exist primarily in the anionic form at pH 7.4, so the trend derived at pH 13 was used to identify target compounds for synthesis.

Cu-click triazole:  truncated as: 

DIFO triazole:`  truncated as: 

 truncated as: 

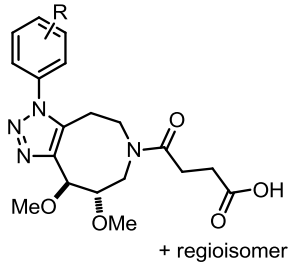
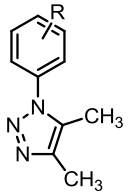
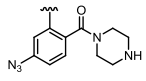
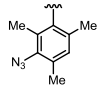
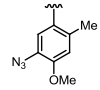
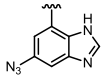
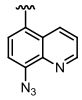
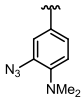
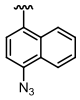
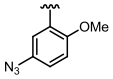
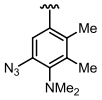
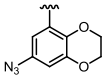
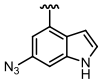
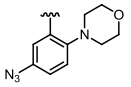
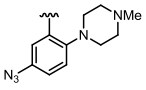
DIMAC triazole:  truncated as: 

Figure S1: Truncated structures used for modeling.

Table S1: Calculated HOMO Energies (Hartrees) of candidate azidofluoresceins and their products.

Azidofluorescein	Azide	Cu-triazole	DIFO-triazole	DIMAC-triazole
	-0.223	-0.225	-0.224/-0.230	-0.225
	-0.224	-0.232	-0.231/-0.238	-0.231
	-0.222	-0.231	-0.240/-0.248	-0.229
	-0.219	-0.240	-0.243/-0.249	-0.232
	-0.219	-0.238	-0.243/-0.250	-0.225
	-0.215	-0.238	-0.232/-0.240	-0.225
	-0.214	-0.224	-0.238/-0.245	-0.215
	-0.210	-0.212	-0.209/-0.218	-0.211
	-0.210	-0.220	-0.223/-0.234	-0.221
	-0.208	-0.218	-0.223/-0.231	-0.220
	-0.207	-0.207	-0.201/-0.210	-0.204
	-0.205	-0.214	-0.218/-0.226	-0.216
	-0.198	-0.208	-0.210/-0.218	-0.210
	-0.196	-0.203	-0-.205/-0.213	-0.204
	-0.193	-0.196	-0.197/-0.203	-0.197

Measuring fluorescence quantum yield

Fluorescence quantum yield measurements were performed following literature procedure.[2] 2 mM solutions of the compounds in MeOH were diluted in phosphate buffered saline (PBS) for measurements at pH 7.4 or 0.1 M NaOH (aq) for measurements at pH 13. Similar measurements were made for a standard of known fluorescence quantum yield (fluorescein in 0.1 M NaOH, Φ = 0.85). The reported quantum yields are the average of three measurements. Absorbance spectra were recorded on a Varian Cary 50 UV-Visible spectrophotometer. Fluorescence spectra were recorded on a Photon Technology International Quanta Master 4 L-format scanning spectrofluorometer equipped with an LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with an integrated igniter, switchable 814 photon counting/analog photomultiplier detection unit, and MD5020 motor driver. Measurements were made in 1 cm x 0.1 cm quartz cuvettes with a total sample volume of 1 mL.

Table S2: Fluorescence properties of all compounds.

Compound	λ_{ex} (pH 7.4)	λ_{em} (pH 7.4)	Φ_{500} (pH 7.4)	Φ_{500} (pH 13)
fluorescein	490	510	--	0.85
1	497	513	0.024	0.014
1-12	495	518	0.70	0.59
1-DIFO	499	517	0.81	--
1-DIMAC	499	517	0.72	--
2	501	521	0.00056	0.0012
2-12	506	517	0.0018	0.0072
2-DIFO	507	522	0.0018	--
2-DIMAC	507	521	0.00076	--
3	496	516	0.057	0.046
3-12	499	520	0.72	--
3-DIFO	499	519	0.49	--
3-DIMAC	499	518	0.72	--
4	497	518	0.0052	--
4-12	499	519	0.13	--
4-DIFO	497	520	0.019	--
4-DIMAC	497	516	0.020	--
5-N_3-fluor	492	511	0.75	0.80
5-N_3-fluor-12	494	516	0.59	--
reduced-1	495	514	0.0067	0.0061

Plate-Reader Fluorescence Measurements

Reactions were run in a 96-well black-bottom plate, and measurements were made using a Molecular Devices SpectraMax M3 Multi-mode Microplate Reader. Each well contained 200 µL of sample containing 1.5 µM **1**, 5 mM sodium ascorbate, 50 µM $CuSO_4$, 100 µM ligand, and 100 µM alkyne **12** in 95:5 0.1 M pH 7.4 potassium phosphate buffer (KP_i)/DMSO. The ligands, THPTA[17] and BTTAA[6], have been shown to efficiently catalyze the azide-alkyne [3+2] cycloaddition in the presence of micromolar concentrations of copper, and their relative efficiencies have been evaluated using a similar plate-reader assay.[6] To make these solutions, to each well was added 160.4 µL pH 7.4 KP_i, 6 µL DMSO, 0.2 µL of 50 mM $CuSO_4$ in H_2O, 10 µL of freshly-prepared 100 mM sodium ascorbate in pH 7.4 KP_i, and 0.4 µL of 50 mM ligand in H_2O. Immediately before measurements were made, 3 µL of 50 µM **1** in DMSO and 20 µL 1 mM alkyne in 95:5 pH 7.4 KPi/DMSO were added. For measurements without alkyne, 20 uL 95:5 pH 7.4 KPi/DMSO was added instead. The plate was shaken for 8 seconds, then fluorescence measurements (excitation/emission at 500/515 nm) were taken every minute for 1 h. Runs were performed in triplicate.

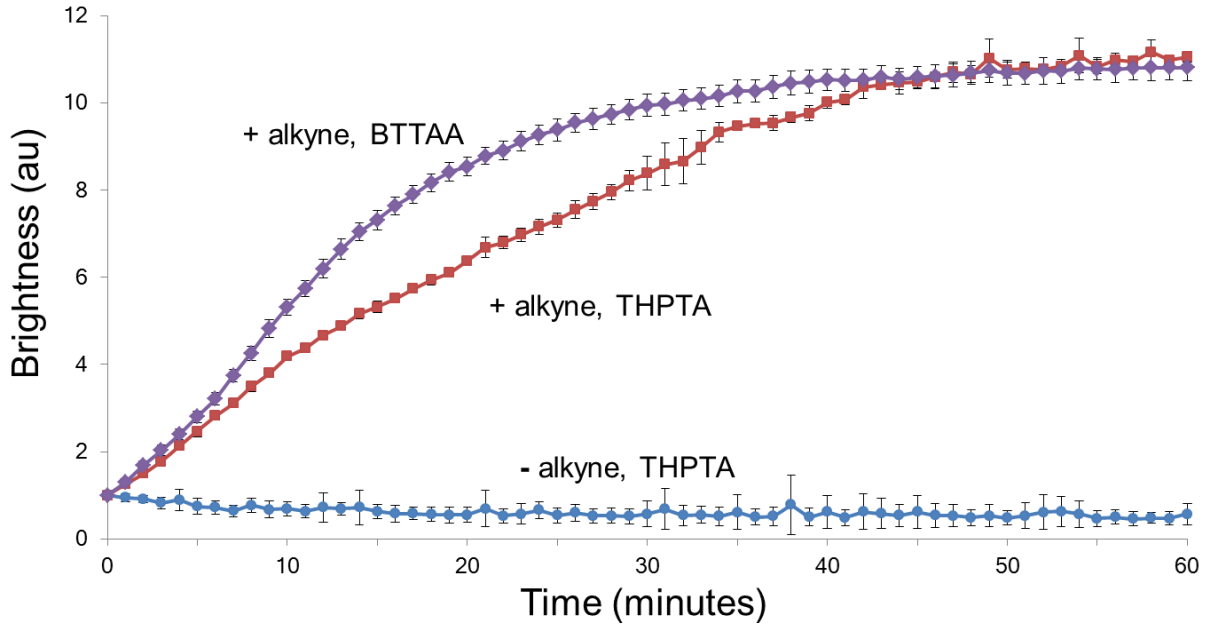


Figure S2: Alkyne- and ligand- dependent fluorescence turn-on by **1**. A significant increase in fluorescence is observed only in the presence of alkyne **12**.

Protein Labeling with 1

Bovine serum albumen (BSA) was incubated with either 4-pentynoyl or 4-pentenoyl NHS ester to generate the corresponding alkyne- and alkene- labeled BSA. 5 mg BSA was incubated with 450 µL pH 8.4 0.1 M potassium phosphate buffer and 50 µL 12 mM NHS-ester in DMSO at 4 °C overnight (18 h). The reaction was diluted w/ 10 mL pH 7.4 phosphate-buffered saline (PBS), then concentrated using Amicon Ultra-15 centrifugal filter unit with a 30K MWCO at 3750 x g for 10 minutes at 4 °C to remove any remaining NHS-ester. The dilution and filtration were repeated two more times. ESI analysis of the collected protein indicated an average of 3 modifications for 4-pentynoyl NHS ester and 10 modifications for 4-pentenoyl NHS ester. 0.5 mg/mL stocks of protein in pH 7.4 PBS were prepared based off A_{280}. For the labeling experiment, 0.44 mg/mL protein in 95:5 pH 7.4 PBS/tBuOH was incubated with 100 µM TBTA, 2 mM sodium ascorbate, 0 or 1 mM $CuSO_4$, and 0 or 50 µM **1** at rt for 1 h in the dark. Specifically, to 88.5 µL of 0.5 mg/mL protein in PBS was added 2.5 µL of 4 mM TBTA in DMSO, 2 µL of 100 mM freshly-prepared sodium ascorbate in PBS, 2 µL of 50 mM $CuSO_4$ in H_2O or 2 µL pH 7.4 PBS, and 5 µL 1 mM **1** in tBuOH or just tBuOH. 30 µL of each mixture was mixed with 10 µL 4x SDS loading dye lacking β-mercaptoethanol, and 25 uL of each was loaded onto a 10% Bis-Tris gel. The gel was run for 90 min at 180 V. The gel was imaged using the Typhoon 9410 Variable Mode Imager (excitation/emission at 488/520 nm) and stained with Coomassie.

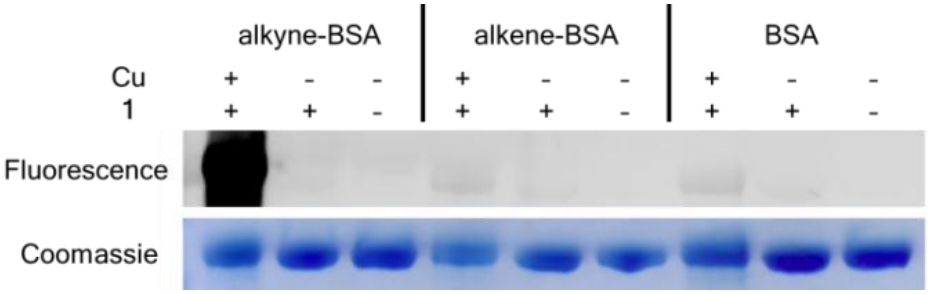


Figure S3: Protein labeling by **1** is alkyne- and copper-dependent. Significant protein labeling by 1 is only observed for alkyne-modified BSA in the presence of $CuSO_4$.

Cell Labeling Experiments

CHO K1 cells were grown in 8-well Lab-Tek Chambered Coverglass systems. Each well contained cells in 300 μL F12 media containing fetal bovine serum, penicillin/streptomycin, and either 50 μM Ac_4ManNAl or Ac_4ManNAc for 3 d at 37 °C, as described previously.[7,18] The cells were then gently washed with 3 x 300 μL pH 7.4 PBS, then fixed with 200 μL chilled 3% w/v paraformaldehyde in pH 7.4 PBS for 20 min at 4 °C. The fixed cells were washed with 3 x 300 μL PBS, then incubated with 200 μL PBS containing 25 μM dye, 10.0 μM TBTA, 1 mM $CuSO_4$, 2 mM sodium ascorbate, and 0.1 mg/mL BSA in the dark at rt for 1 h. BSA was found to help keep the reagents in solution during the labeling period. Specifically, to each well was added 195 μL of solution containing 146 μL PBS, 40 μL 0.5 mg/mL BSA in PBS, 5 μL of 4 mM TBTA in DMSO, and 4 μL of 100 mM freshly prepared sodium ascorbate in PBS. 1 μL of 5 mM dye in tBuOH or DMSO, then 4 μL of 50 mM $CuSO_4$ in H_2O were added to initiate the reaction. The cells were then taken directly on for imaging for no-wash labeling experiments. For labeling experiments with washing, the cells were first washed with 3 x 300 μL PBS, then covered in 200 μL PBS before imaging. Microscopy was performed using a Zeiss AxioVert 200M inverted microscope using a Plan-Neofluar 20x/0.75 objective. A 175W xenon lamp housed in a Sutter DG4 illuminator linked to the microscope by an optical fiber assured shuttering and illumination. Exposure time was 150 ms for all images except for no-wash imaging with 5-azidofluorescein, which required an exposure time of 10 ms to prevent saturation of the detector. Images were acquired and processed using SlideBook 5.0, and are shown as a single z-plane.

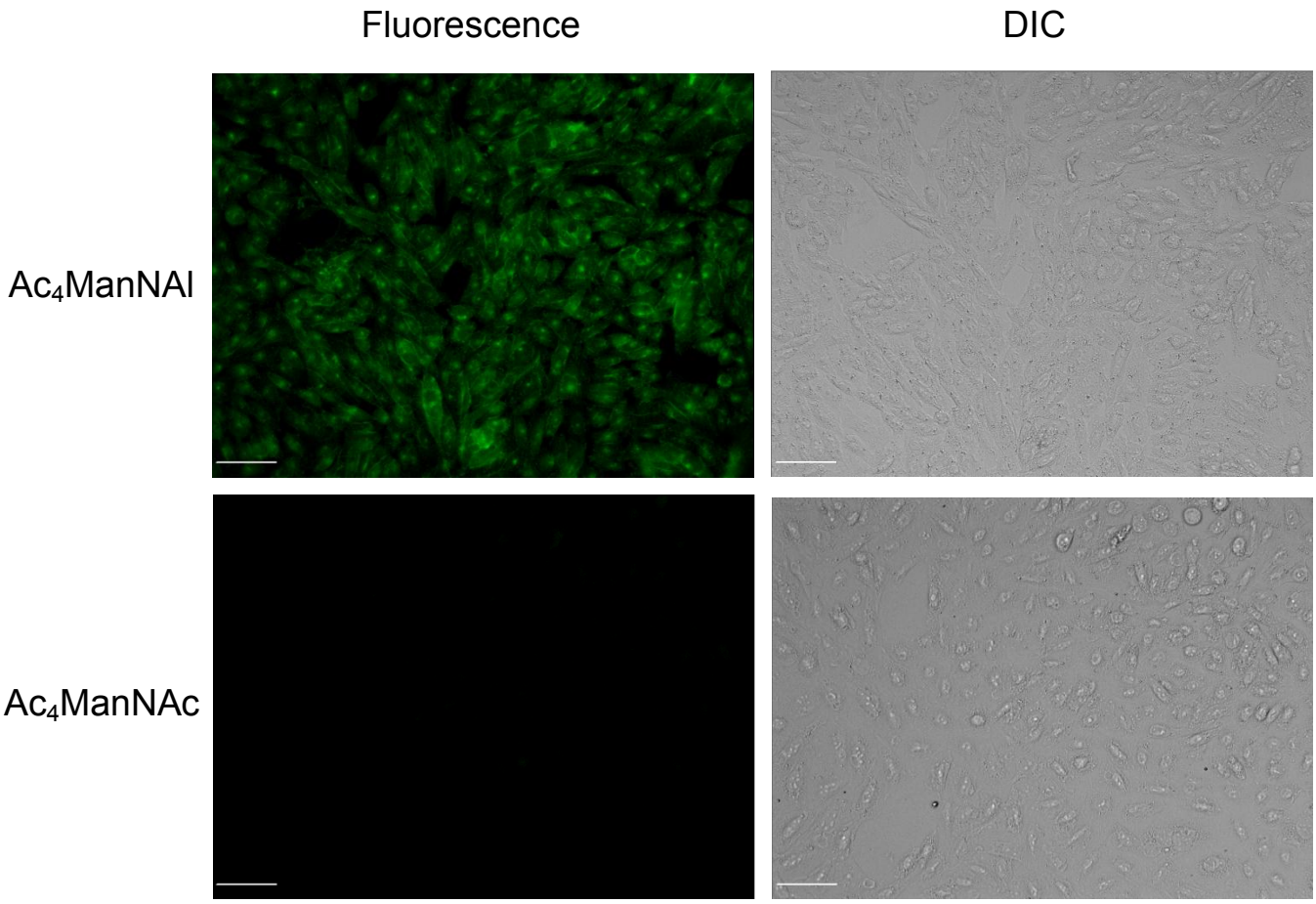


Figure S4: Cell-surface labeling by **1** (25 μM from a 5 mM stock in tBuOH) is alkyne-dependent. Scale bar = 50 μm. FITC filter, fluorescence cut-off at 210 to 600.

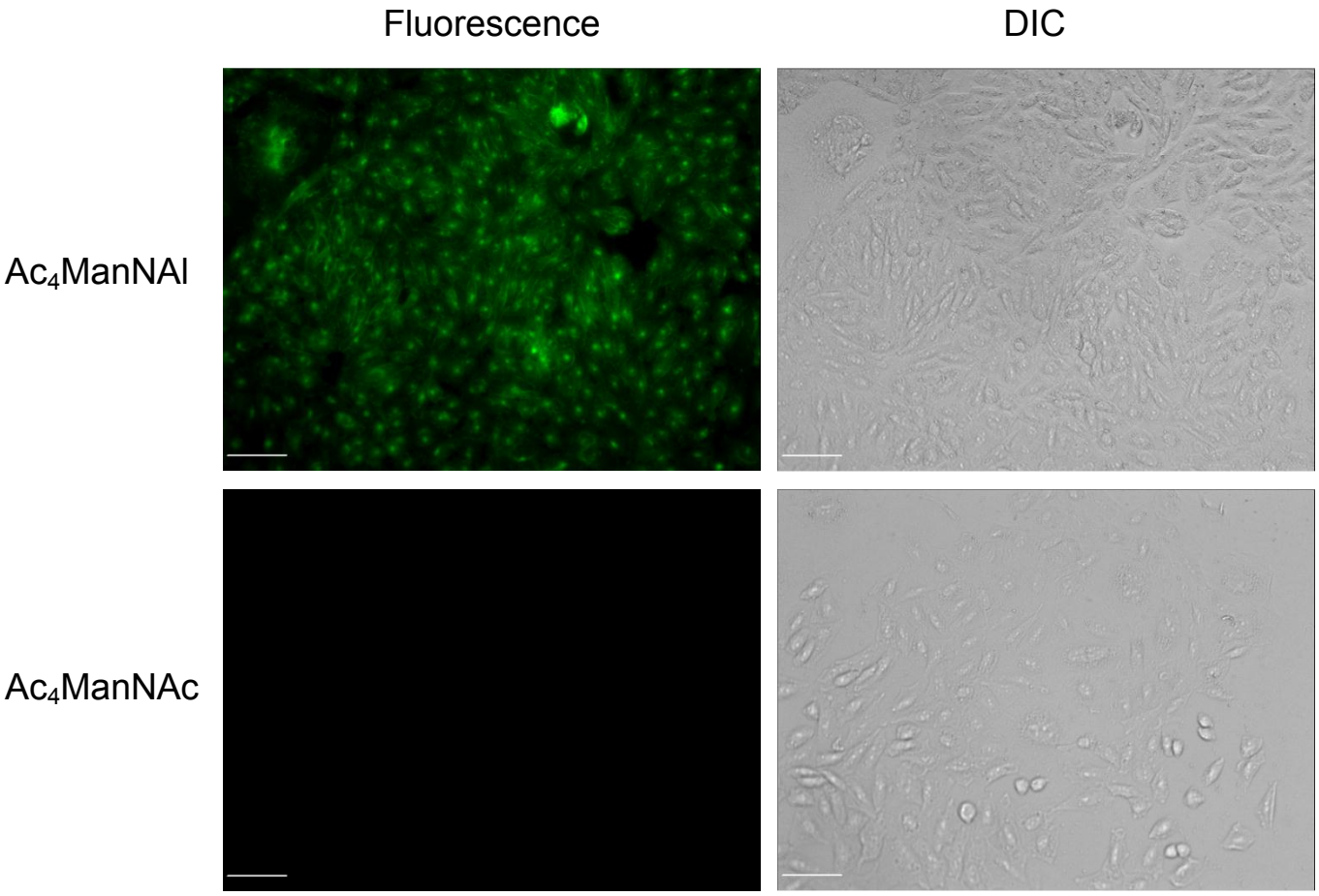


Figure S5: Cell-surface labeling by 5-azidofluorescein (25 μM from a 5 mM stock in DMSO) is alkyne-dependent. Scale bar = 50 μm. FITC filter, with fluorescence cut-off at 250 to 1500.

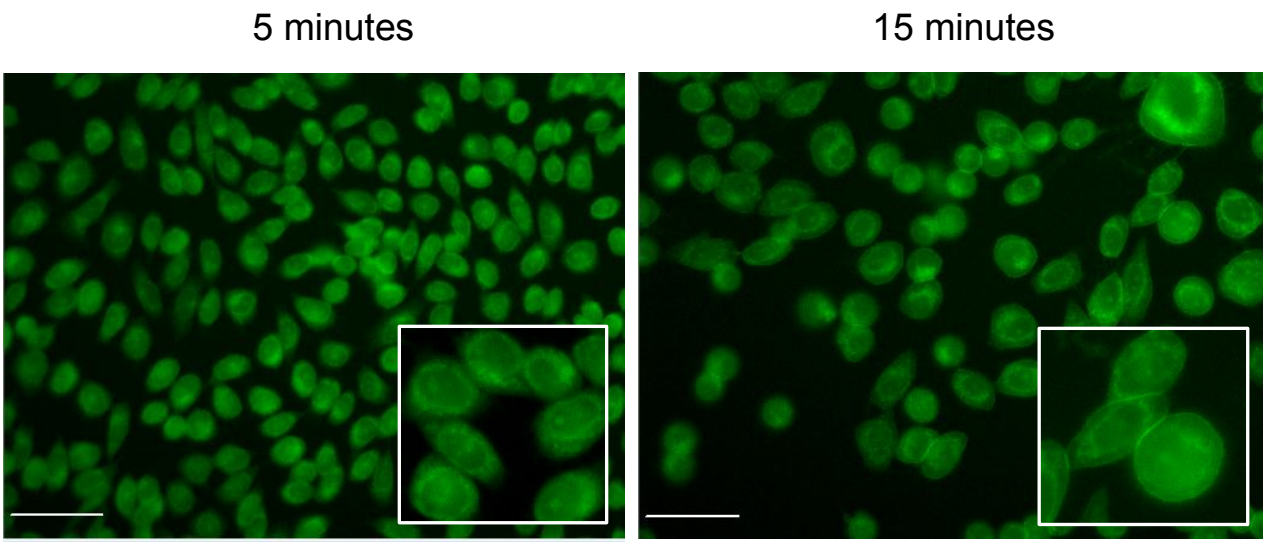
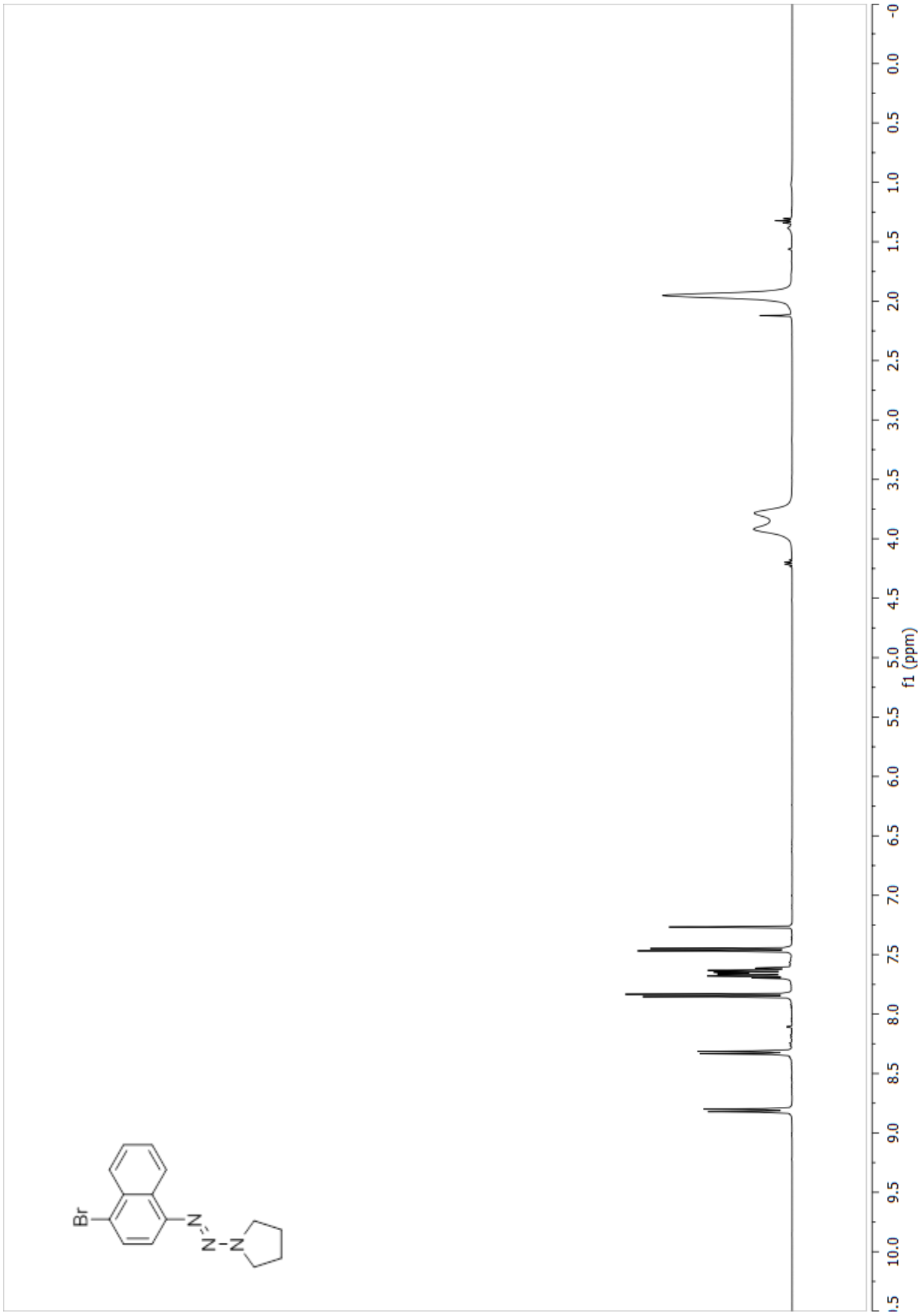


Figure S6: Cell permeability of **1** demonstrated during attempted live-cell cell-surface labeling by **1**. CHO K1 cells were incubated with 50 μM Ac_4ManNAl for 3 days. The cells were washed with 3 x 300 μL PBS and incubated with a solution of 200 μL PBS containing 300 μM BTTAA (from a 50 mM stock in H_2O), 50 μM $CuSO_4$ (from a 50 mM stock in H_2O), 2 mM sodium ascorbate (from a 100 mM stock in PBS), and 25 μM **1** (from a 5 mM stock in tBuOH) in the dark for either 5 or 15 minutes. After the indicated time, the reaction was quenched with the addition of 2 μL of 100 mM bathocuproine disulfonate in H_2O (1 mM final). The cells were washed with 2 x 300 μL PBS, then covered with 300 μL PBS and directly taken on for imaging. Insets show a four-fold magnification to highlight cell-surface labeling after 15 minutes. The green fluorescence inside cells indicates that compound **1** is cell permeable, but that this cell permeability may be a liability for cell-surface labeling. Exposure time = 150 ms. Scale bar = 50 μm. FITC filter, with fluorescence cut-off at 200 to 800.

Supplementary References:

1) Urano, Y., Kamiya, M., Kanda, K., Ueno, T., Hirose, K., Nagano, T.; *J. Am. Chem. Soc.* **2005**, *127*, 4888-4894.
2) Williams, A.T.R.; Winfield, S.A.; Miller, J.N.; *Analyst.* **1983,** *108,* 1067.
3) Hein, J.E.; Krasnova, L.B.; Iwasaki, M.; Fokin, V.V.; *Org. Synth.* **2011**, *88*, 238.
4) Codelli, J.A.; Baskin, J.M.; Agard, N.J.; Bertozzi, C.R.; *J. Am. Chem. Soc.* **2008**, *130*, 11486-11493.
5) Sletten, E.M.; Bertozzi, C.R.; *Org. Lett.* **2008,** *10*, 3097-3099.
6) Besanceney-Webler, C.; Jiang, H.; Zheng, T.; Feng, L.; Soriano del Amo, D.; Wang, W.; Klivansky, L.M.; Marlow, F.L.; Liu, Y.; Wu, P.; *Angew. Chem. Int. Ed.* **2011,** *50,* 8051-8056.
7) Chang, P.V.; Chen, X.; Smyrniotis, C.; Xenakis, A.; Hu, T.; Bertozzi, C.R.; Wu, P.; *Angew. Chem. Int. Ed.* **2009,** *48,* 4030-4033.
8) Sivakumar, K.; Xie, F.; Cash, B.M.; Barnhill, H.N.; Wang, Q.; *Org. Lett.* **2004**, *6,* 4604-4606.
9) Tokunaga, T.; Umezome, T.; Hume, W.E.; Nagata, R.; Okazaki, K.; Ueki, Y.; Kumagai, K.; *PCT Int. Appl.* **2000,** WO 2000010975 A1 20000302.
10) Grover, P.K.; Shah, G.D.; Shah, R.C.; *J. Chem. Soc. (Lond.)* **1955,** 3892-3895.
11) Mintaz, A. K., Kao, J. P. Y., Tsien, R. Y.;. *J. Biol. Chem.* **1989,** *265,* 8171-8178.
12) Melhuish, M.W.; Moodie, R.B.; *J. Chem. Soc. Perkin Trans. 2: Phys. Org. Chem. (1972-1999)*, **1989,** *6*, 667-673.
13) McCormick, K.D.; Boyce, C.W.; Shih, N.-Y.; Aslanian, R.G.; Chao, J.; Fevrier, S.; Mangiaracina, P.; Yu, Y.; Huang, C.-Y.; Liang, B.; Lu, h.; Liu, R.-Q.; *PCT Int. Appl.* **2008,** WO 2008100457 A2 20080821.
14) Rajesh, K.; Somasundaram, M.; Saiganesh, R.; Balasubramanian, K.K.; *J. Org. Chem.* **2007,** *72,* 5867-5869.
15) Salic, A.; Mitchison, T.J.; *Proc. Natl. Acad. Sci.* **2008,** *7,* 2415-2420.
16) Meng, Z.; Hendrickson, G.R.; Lyon, L.A.; *Macromolecules.* **2009,** *42,* 7664-7669.
17) Hong, V.; Steinmetz, N. F.; Manchester, M.; Finn, M. G.; *Bioconj. Chem.* **2010,** *21,* 1912-1916.
18) Hsu, T.-L.; Hanson, S. R.; Kishikawa, K.; Wang, S.-K.; Sawa, M.; Wong, C.-H.; *Proc. Natl. Acad. Sci.* **2007,** *104,* 2614-2619.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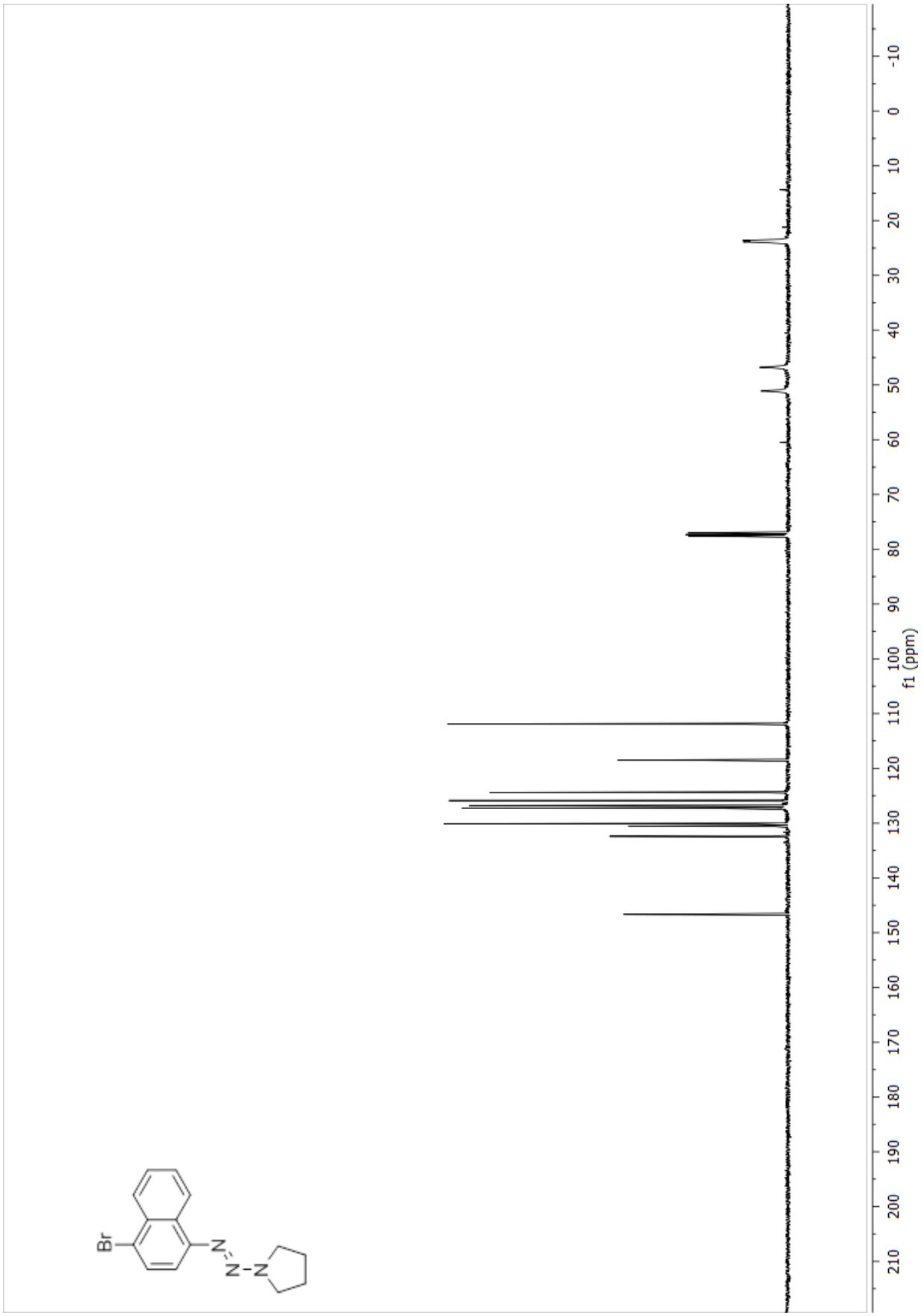
f1 (ppm)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N
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-10
0
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20
30
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160
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180
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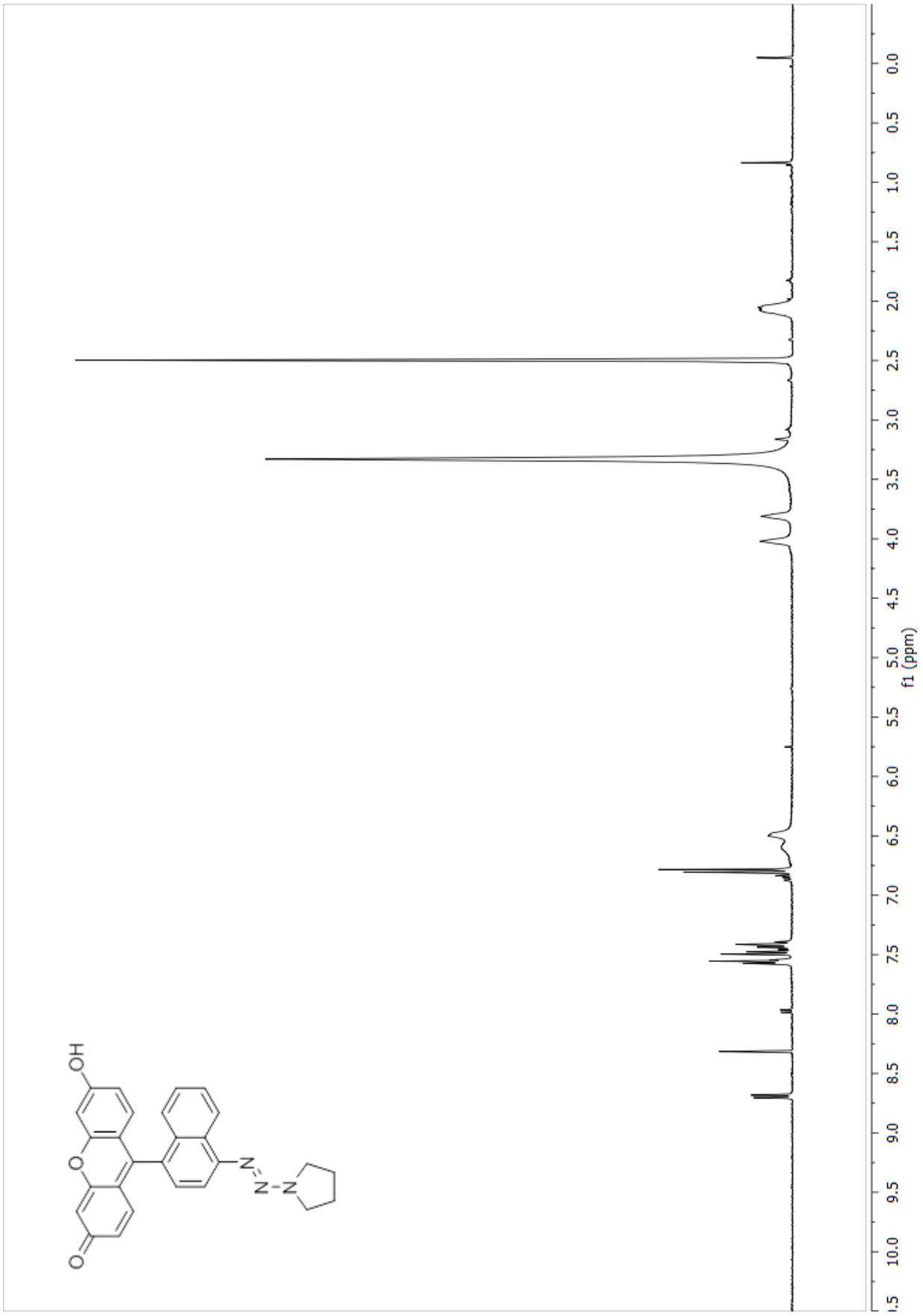
200
210
f1 (ppm)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O
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N
N
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9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
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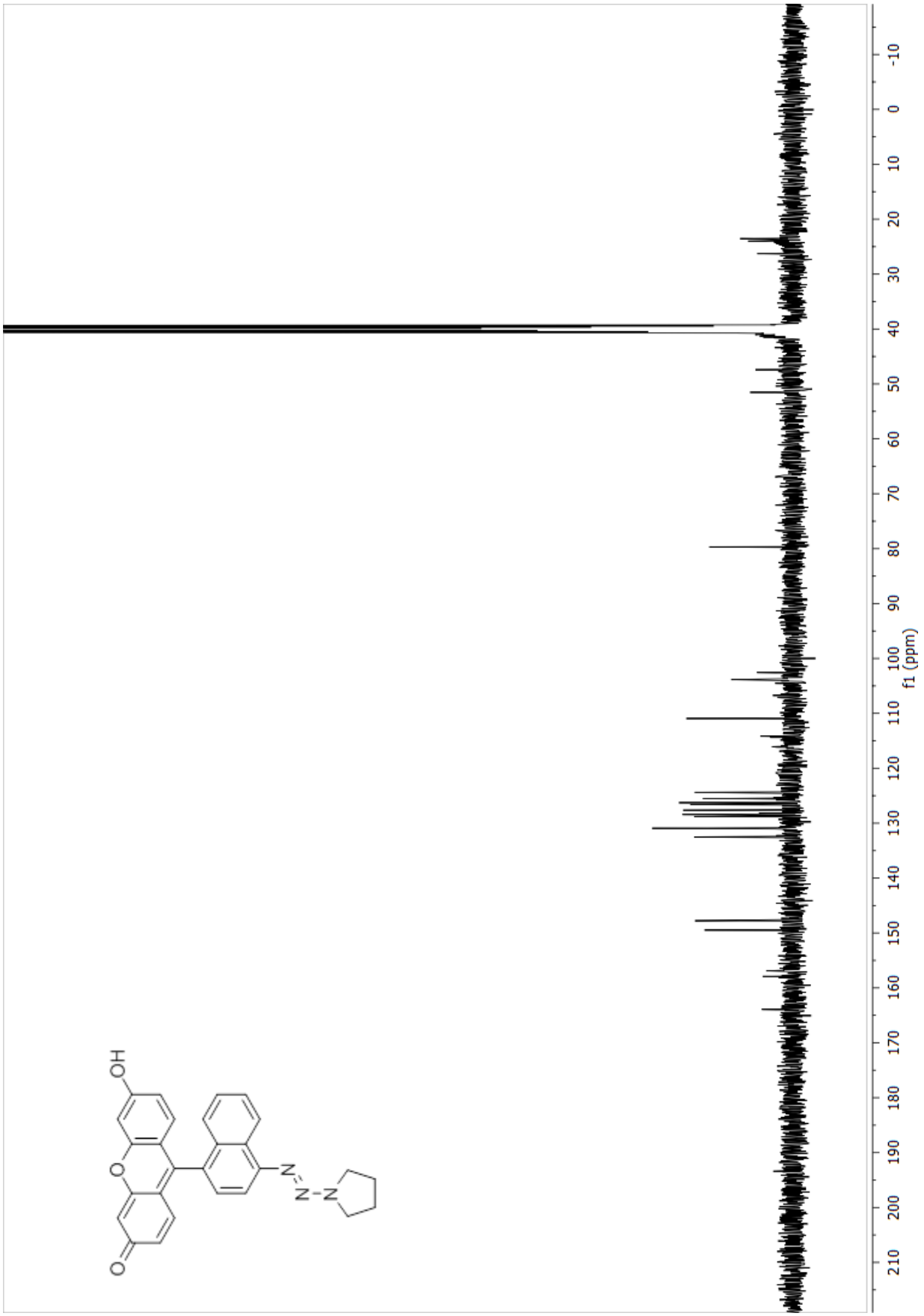
0.0
f1 (ppm)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200
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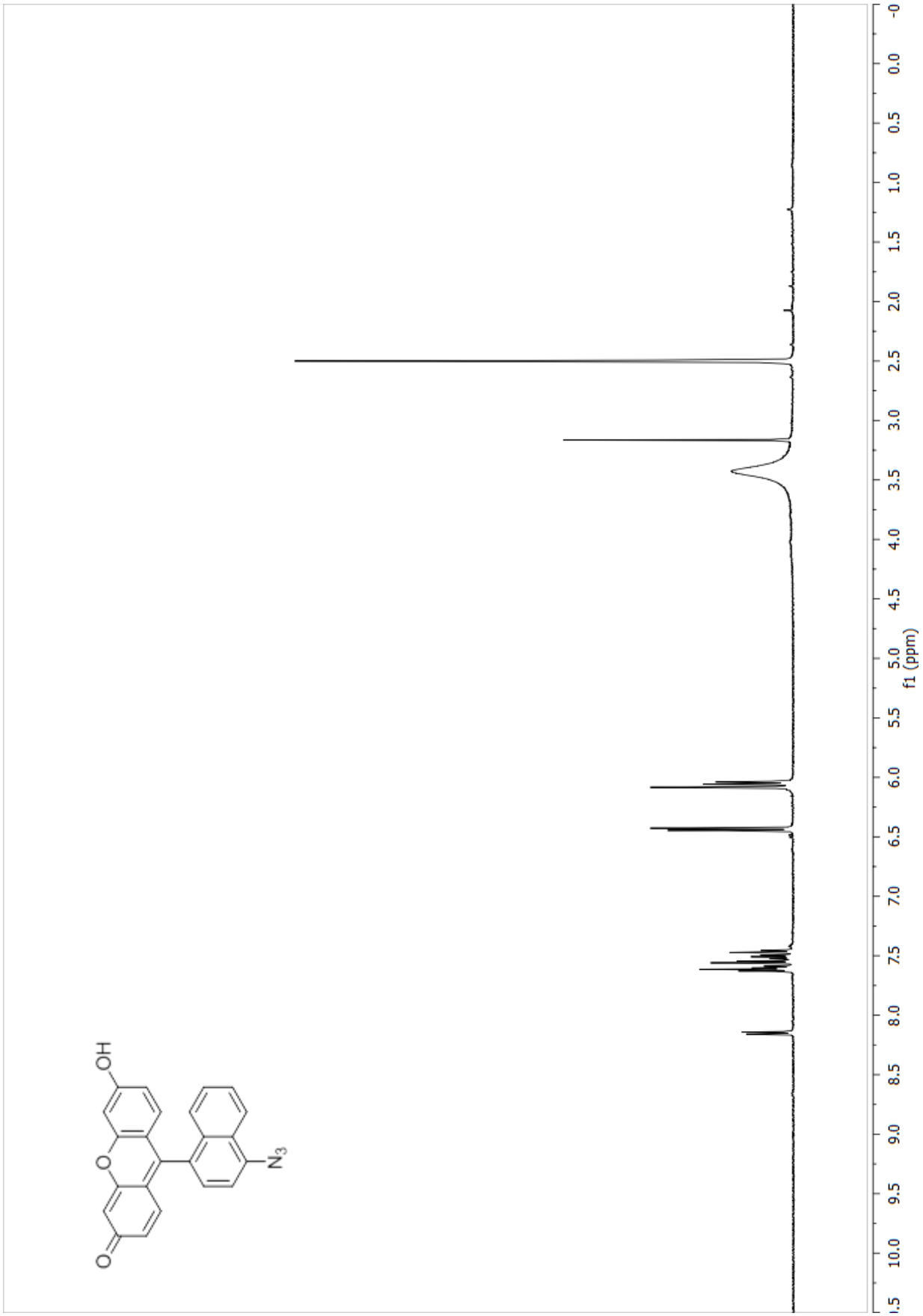
f1 (ppm)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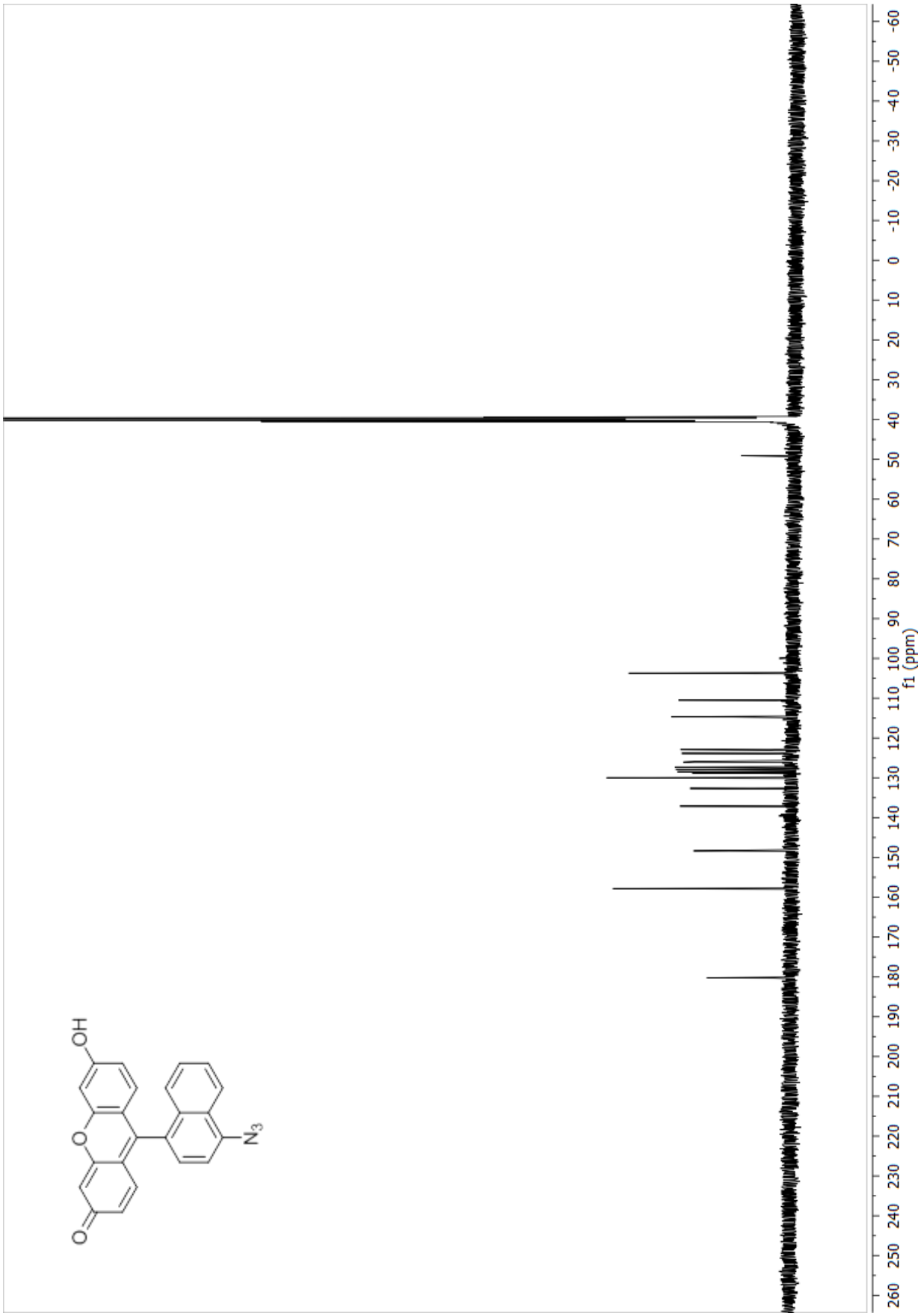
O
N3
f1 (ppm)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O
N3
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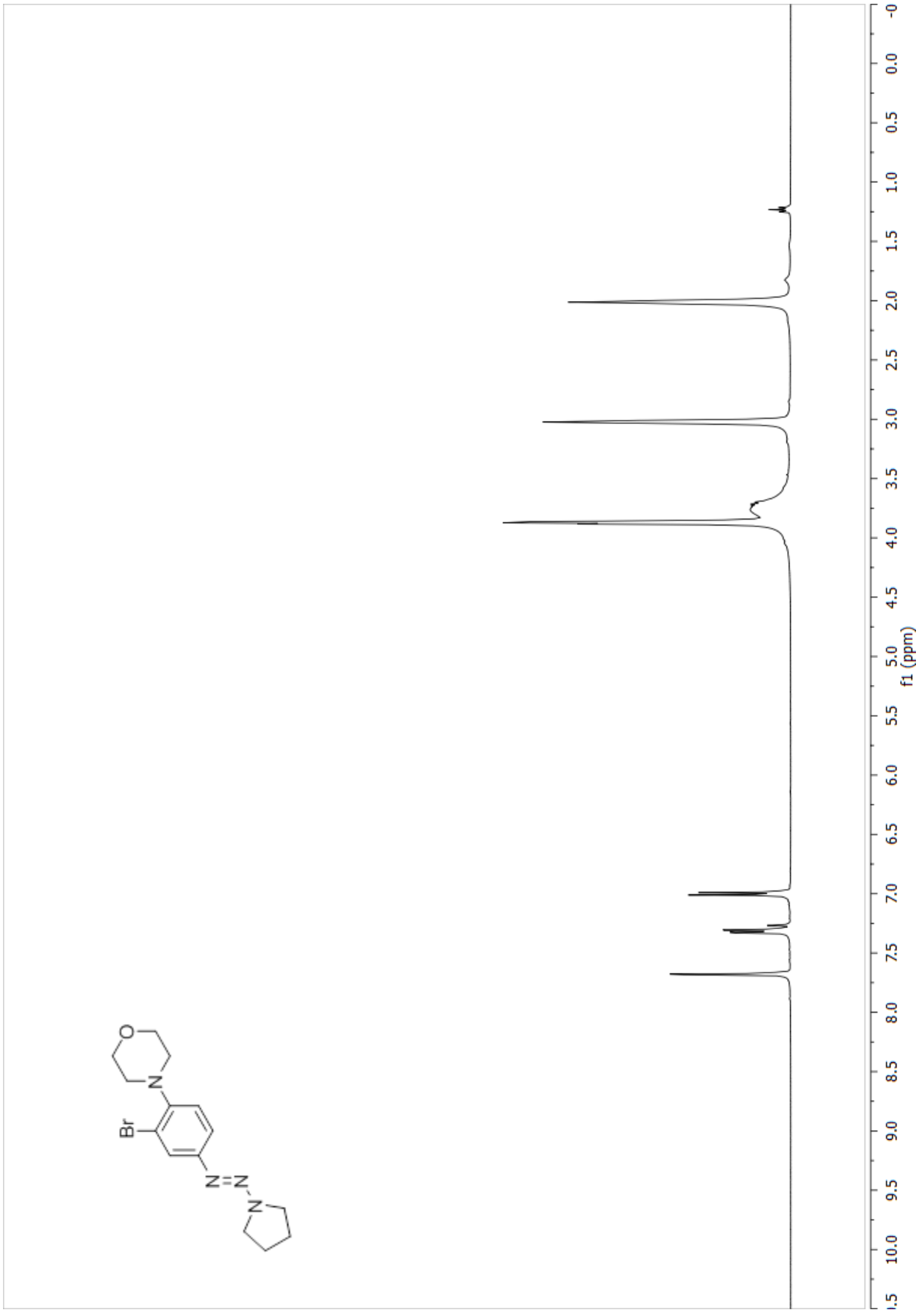
f1 (ppm)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Br
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N
-0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
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4.0
4.5
5.0
f1 (ppm)
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6.0
6.5
7.0
7.5
8.0
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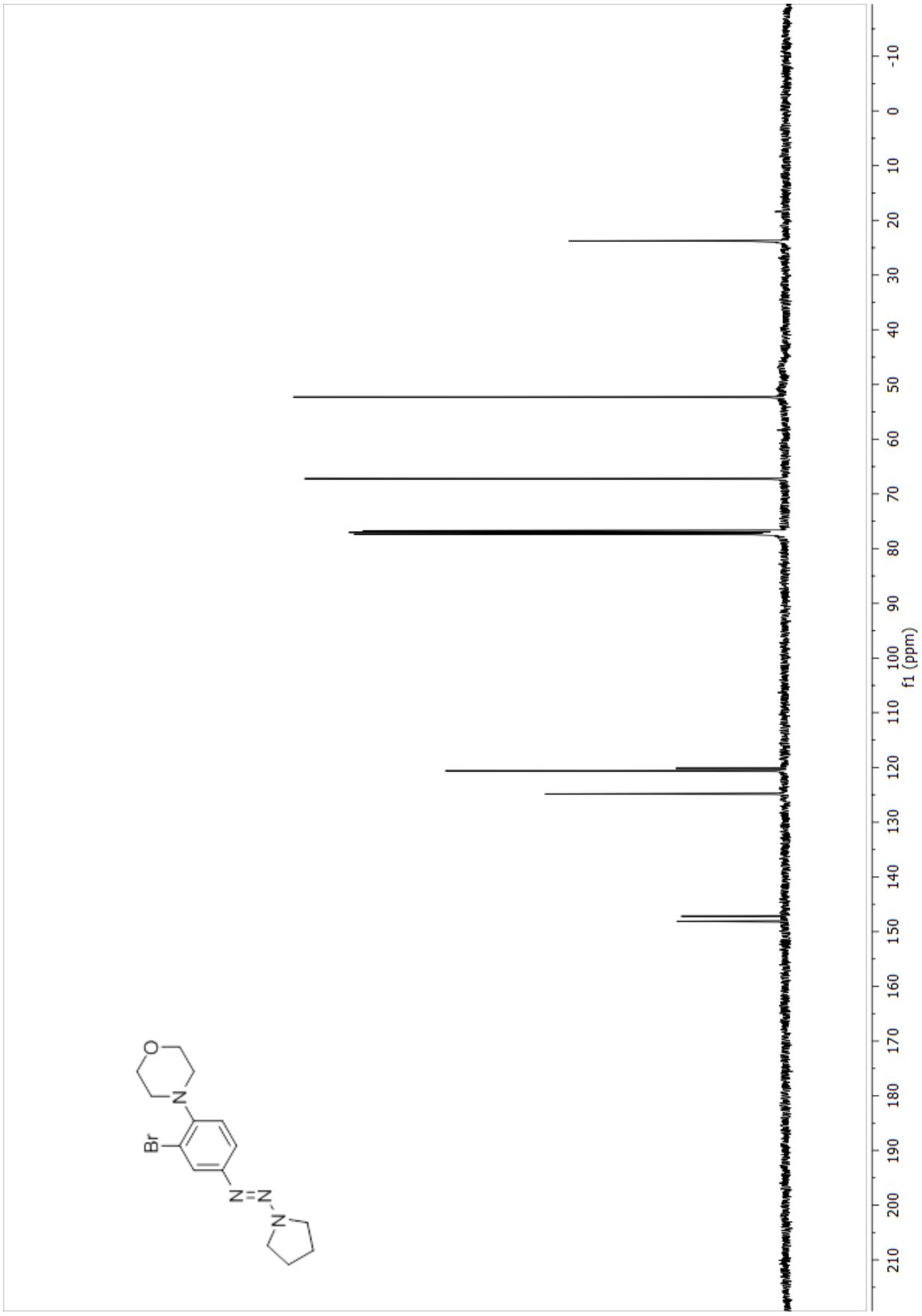
9.0
9.5
10.0


O
N
Br
N=N
N
-10
0
10
20
30
40
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60
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80
90
100
110
120
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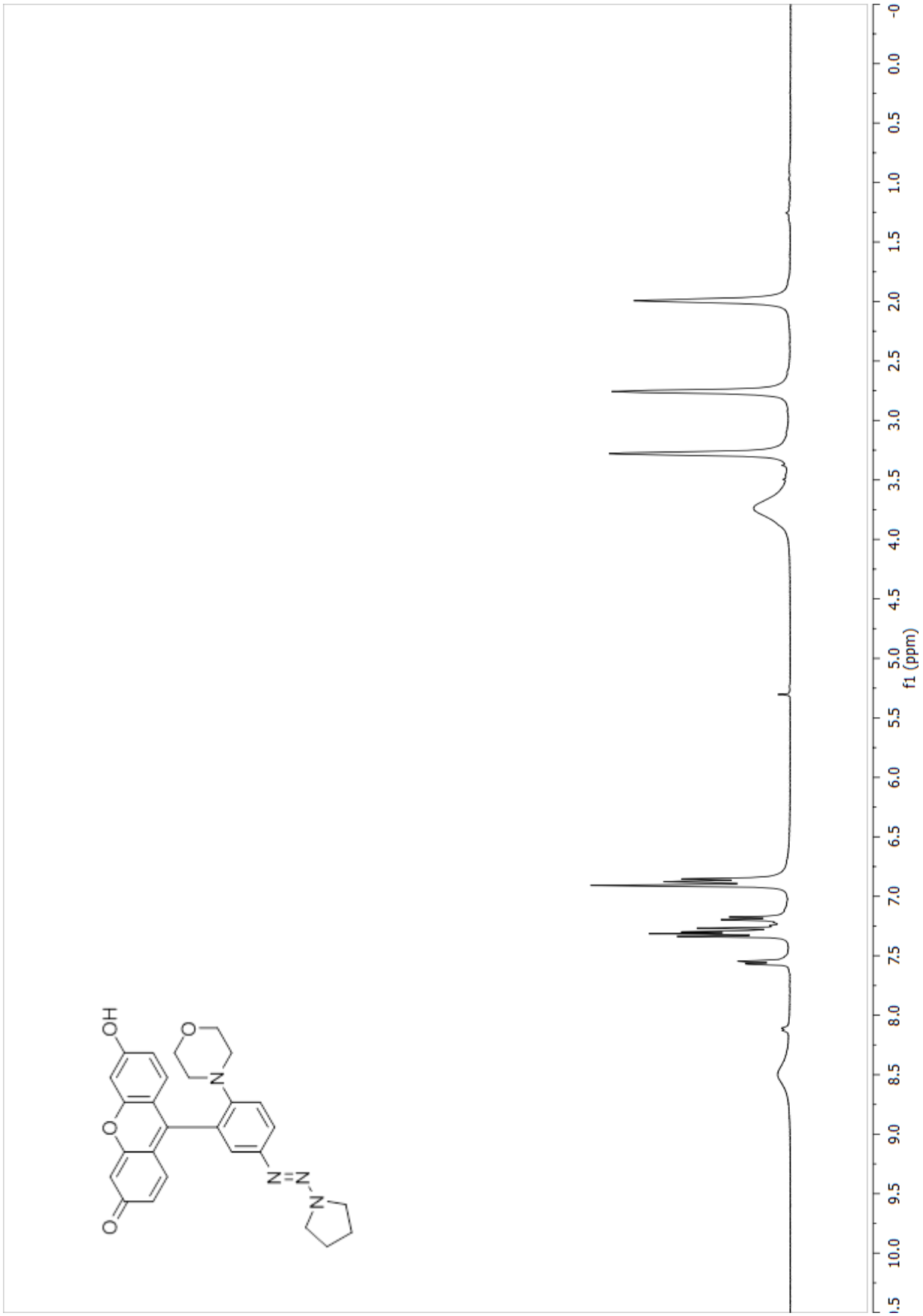
140
150
160
170
180
190
200
210
f1 (ppm)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O
N
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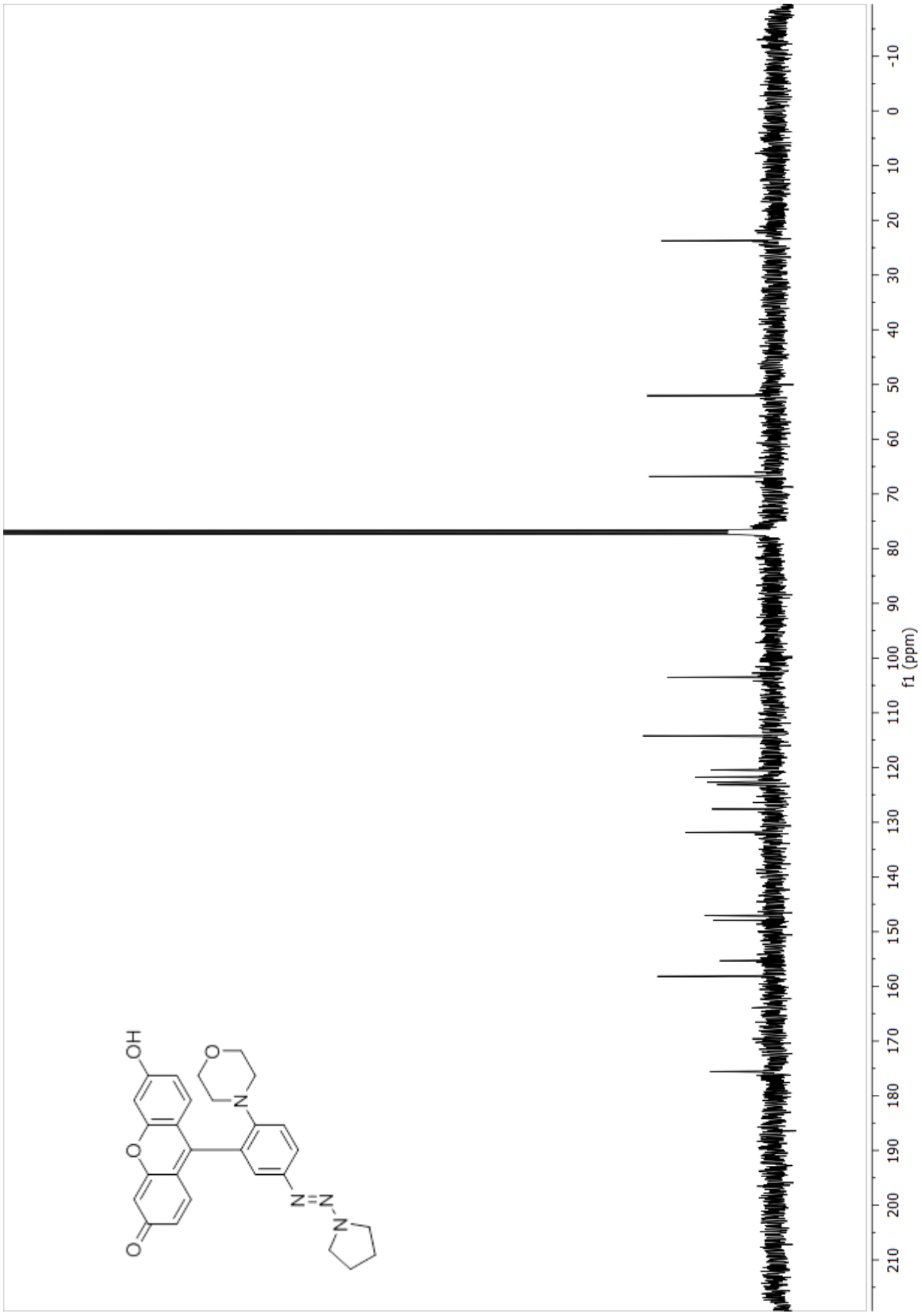
N
O
f1 (ppm)


OH
O
O
N
N=N
N
f1 (ppm)
-10
0
10
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30
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60
70
80
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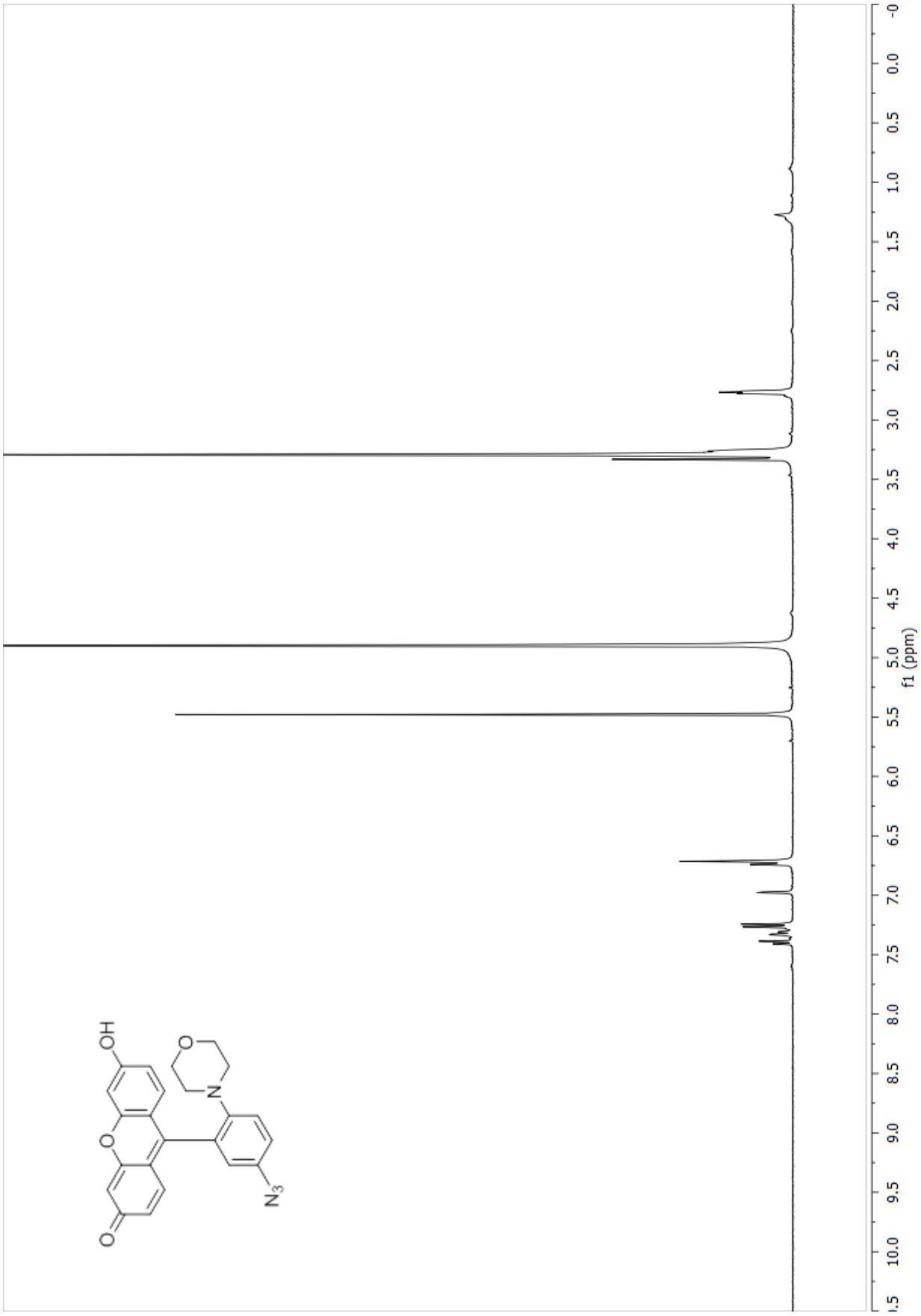
140
150
160
170
180
190
200
210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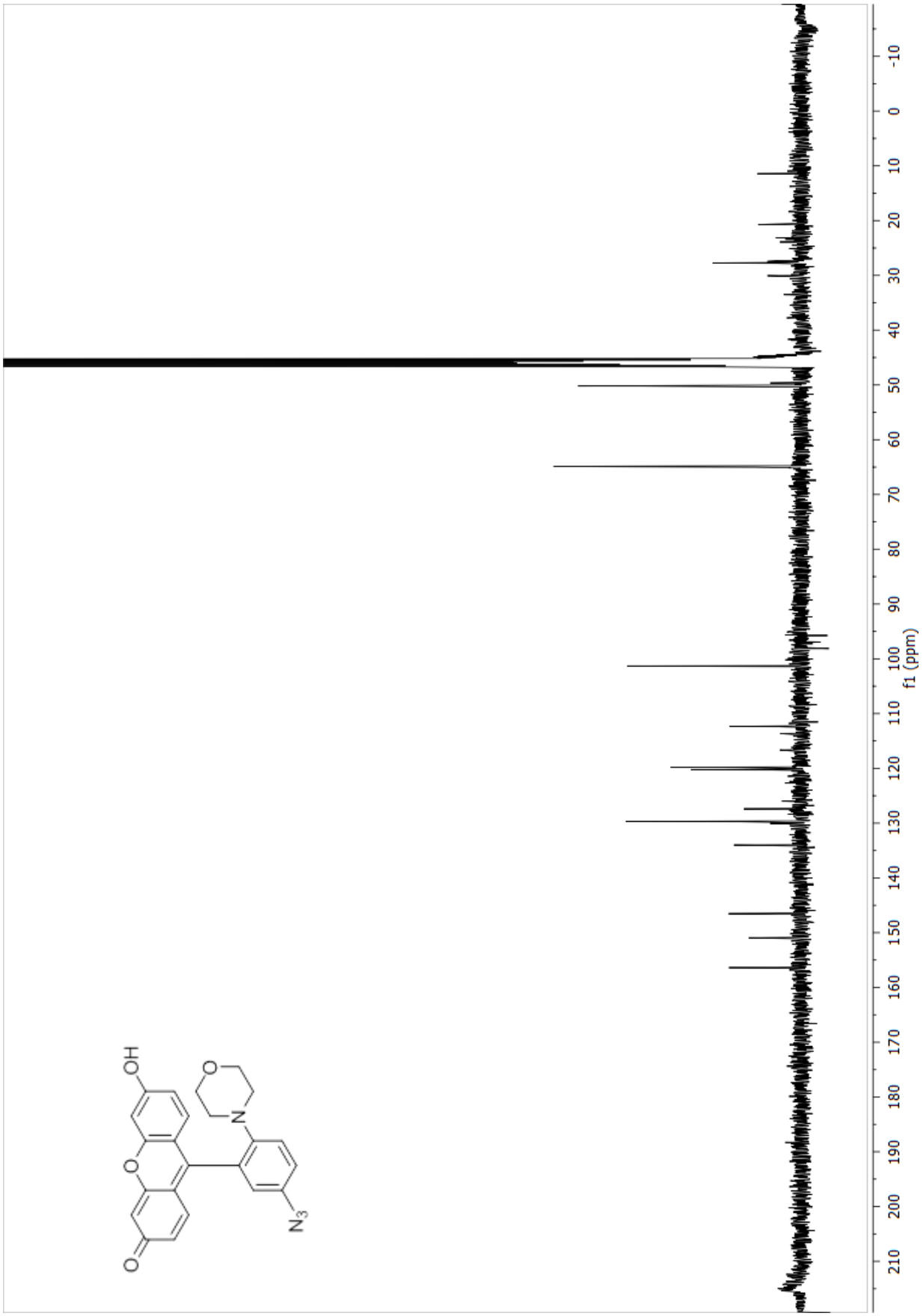
N
O
O
N_3
f1 (ppm)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O
O
N
N3
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0
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20
30
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80
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120
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160
170
180
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200
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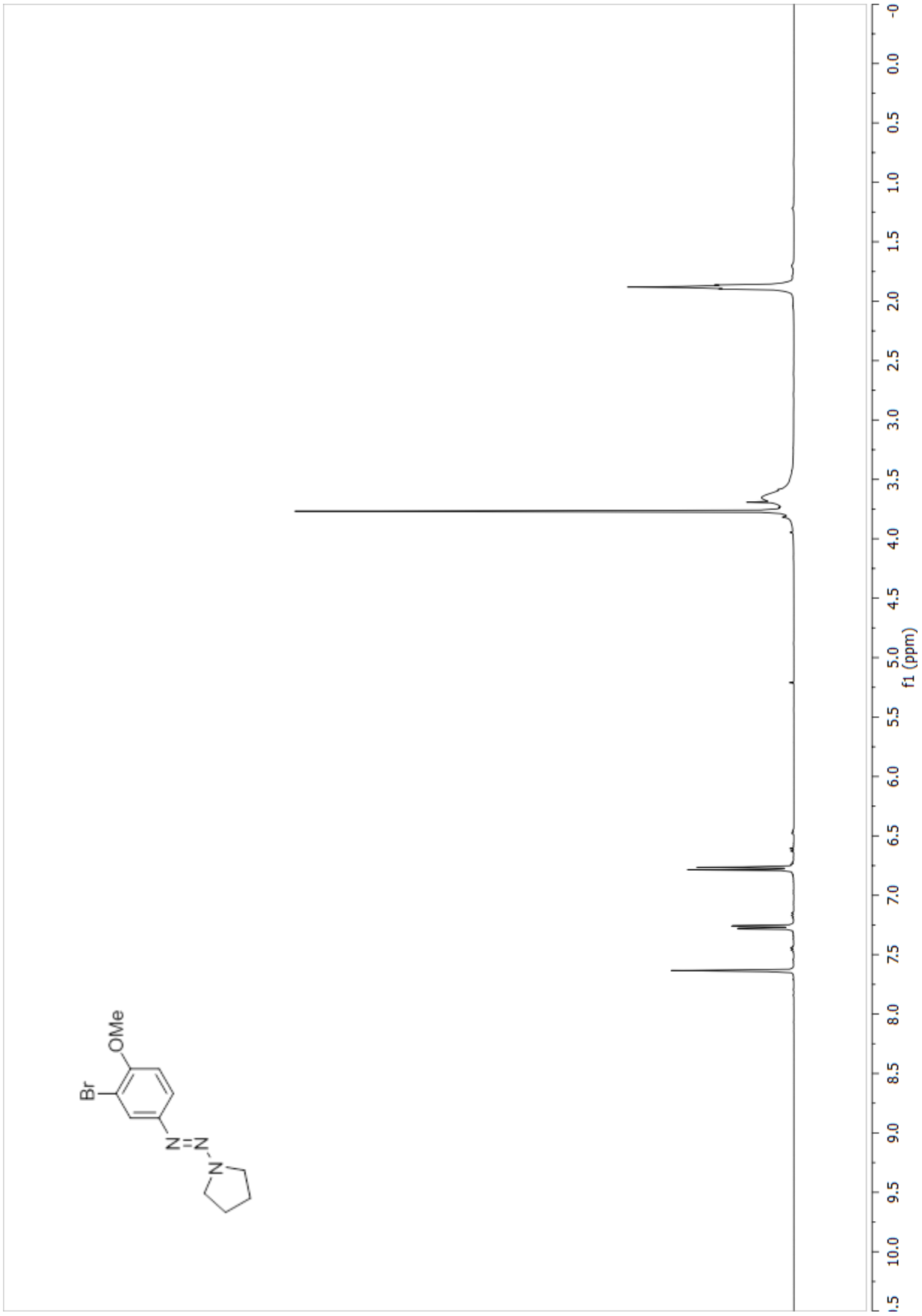
f1 (ppm)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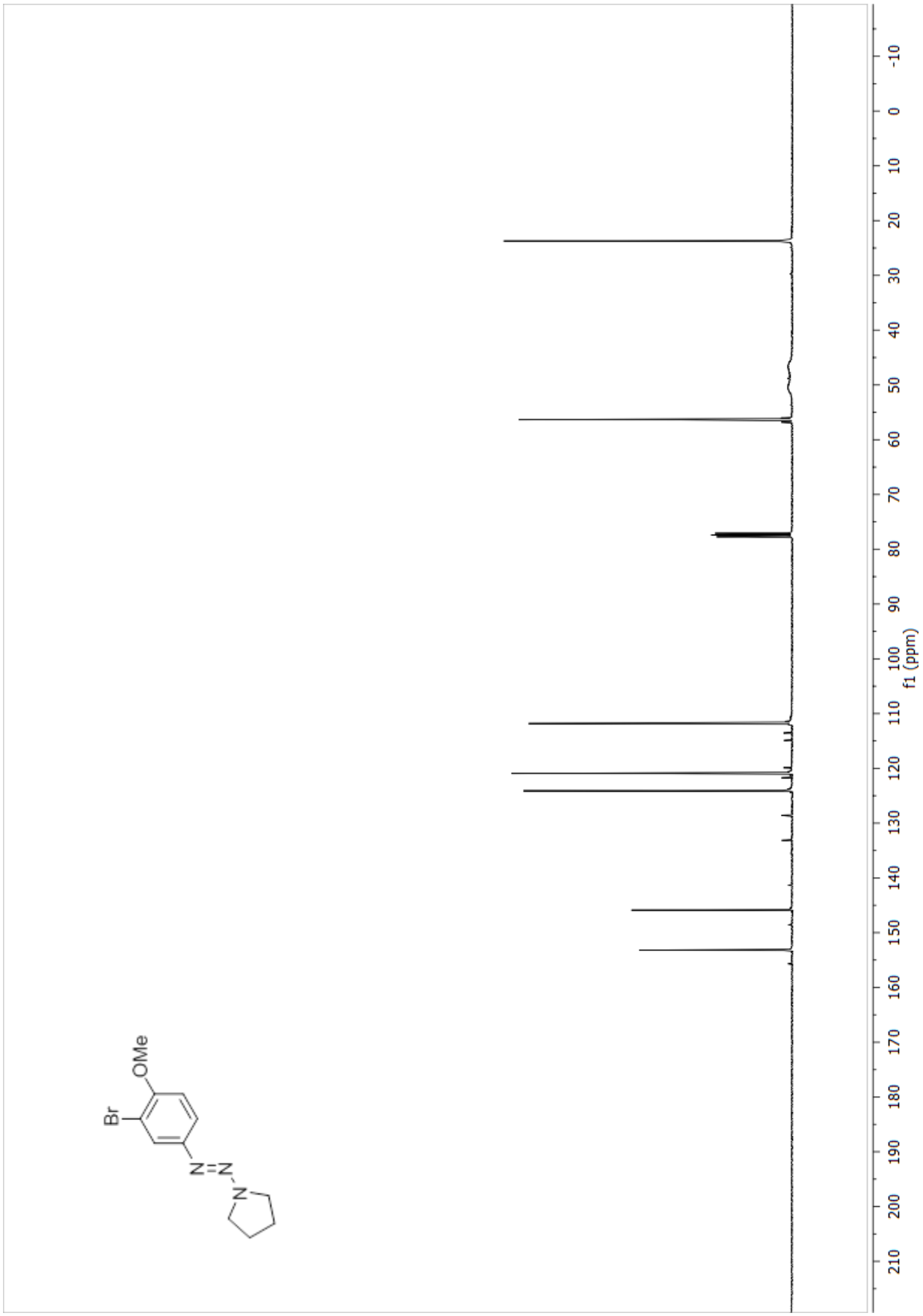
f1 (ppm)


OMe
Br
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200
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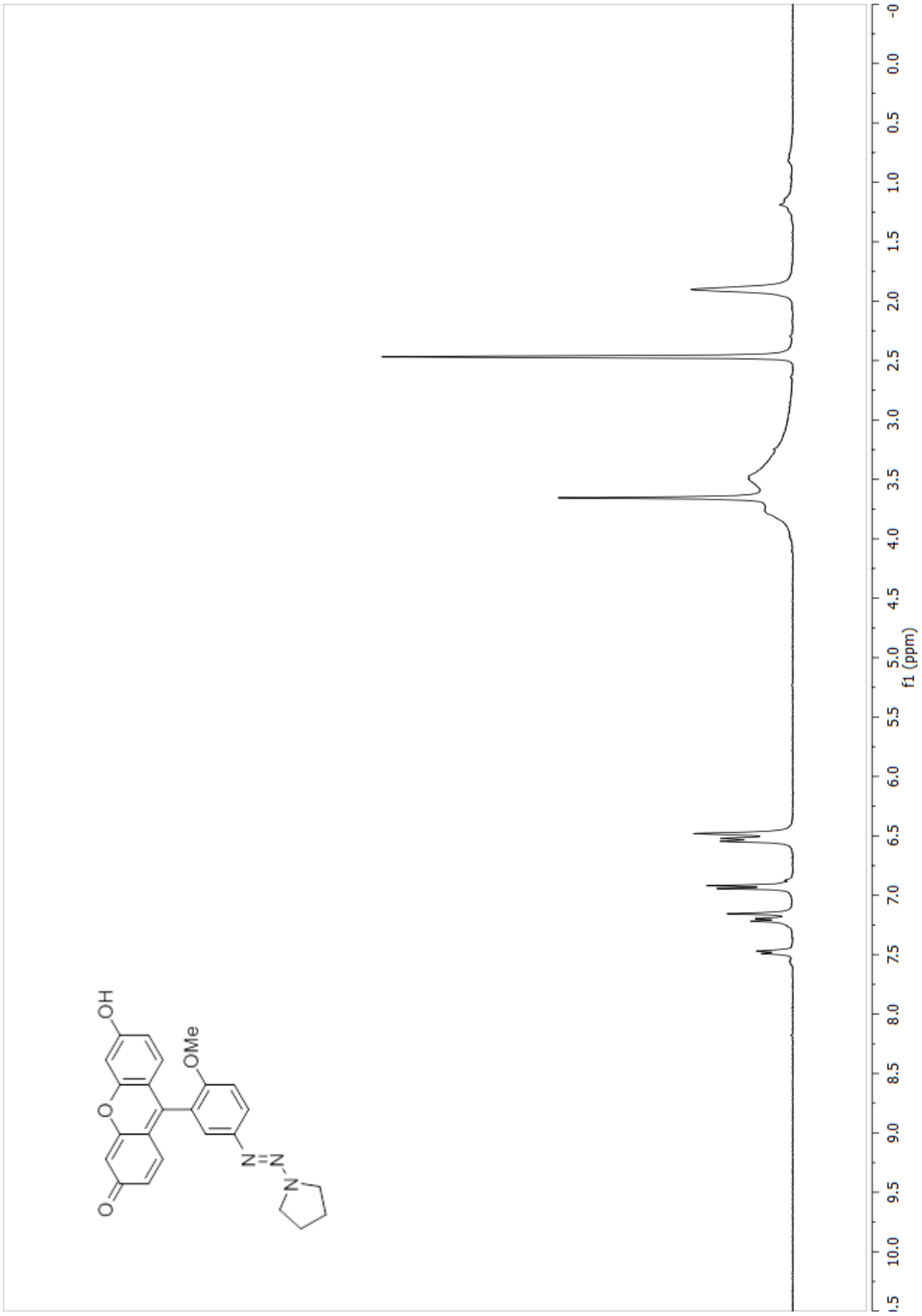
f1 (ppm)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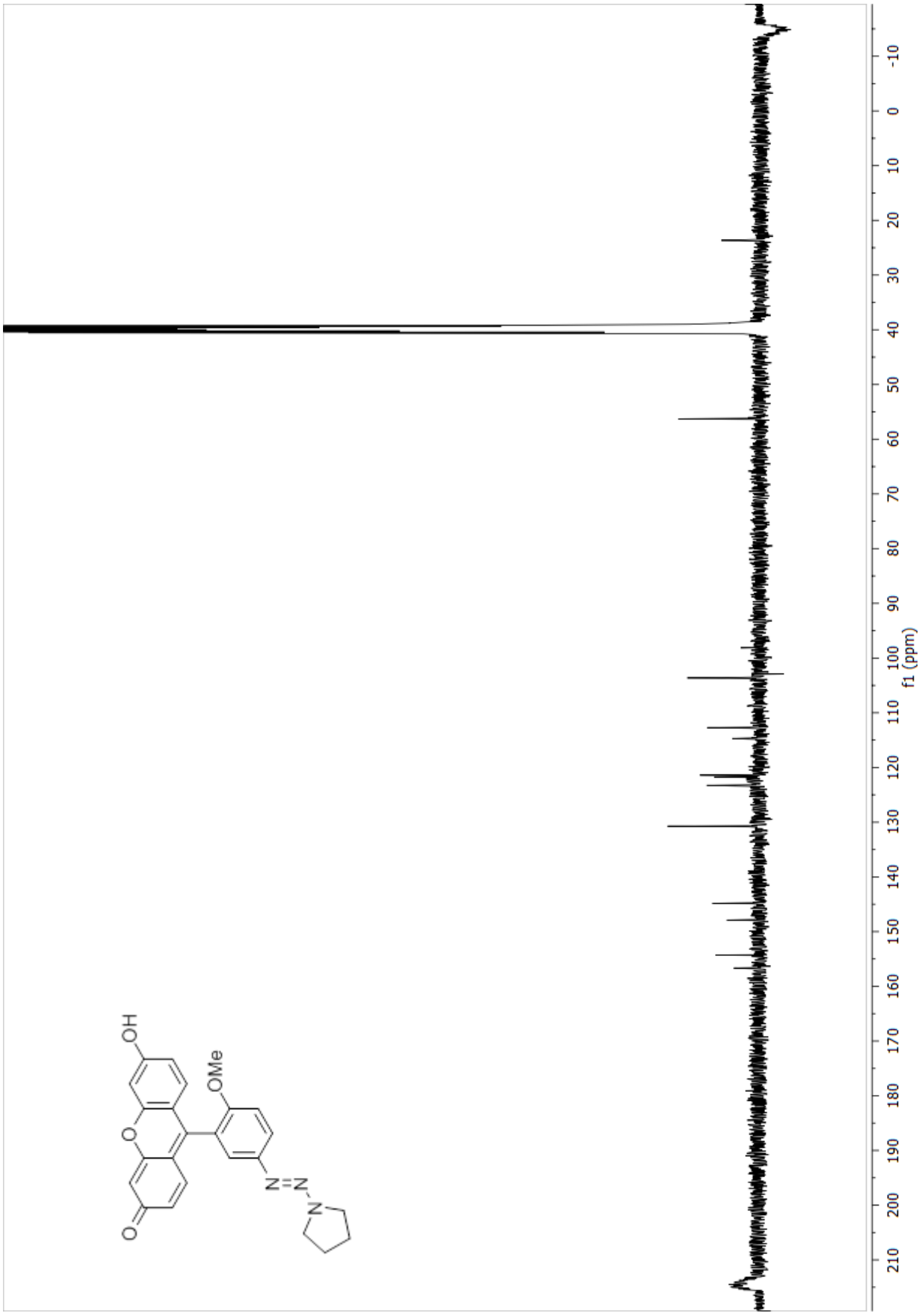
OMe
O
O
N=N
N
f1 (ppm)


OH
OMe
O
O
N=N
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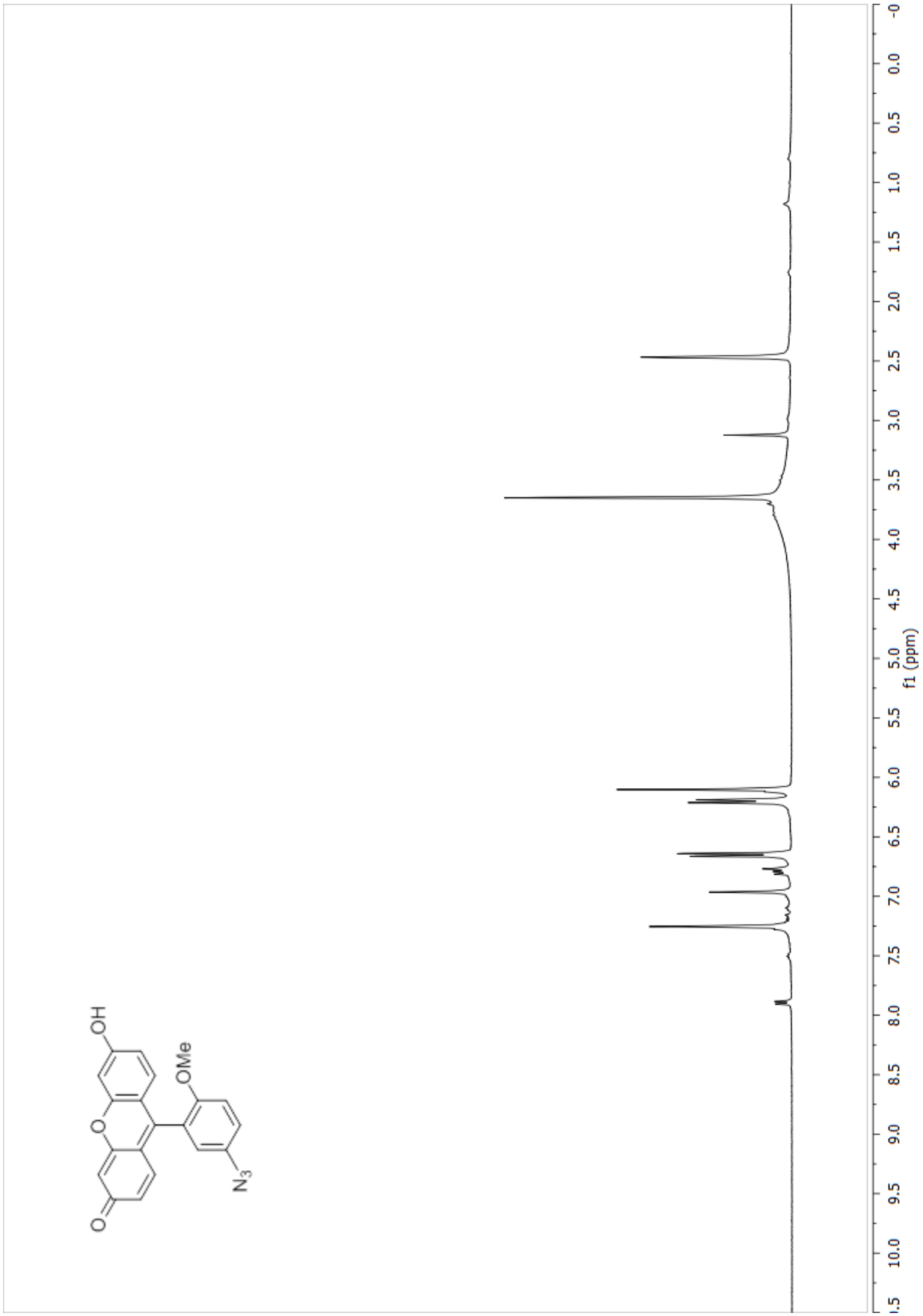
f1 (ppm)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OMe
N_3
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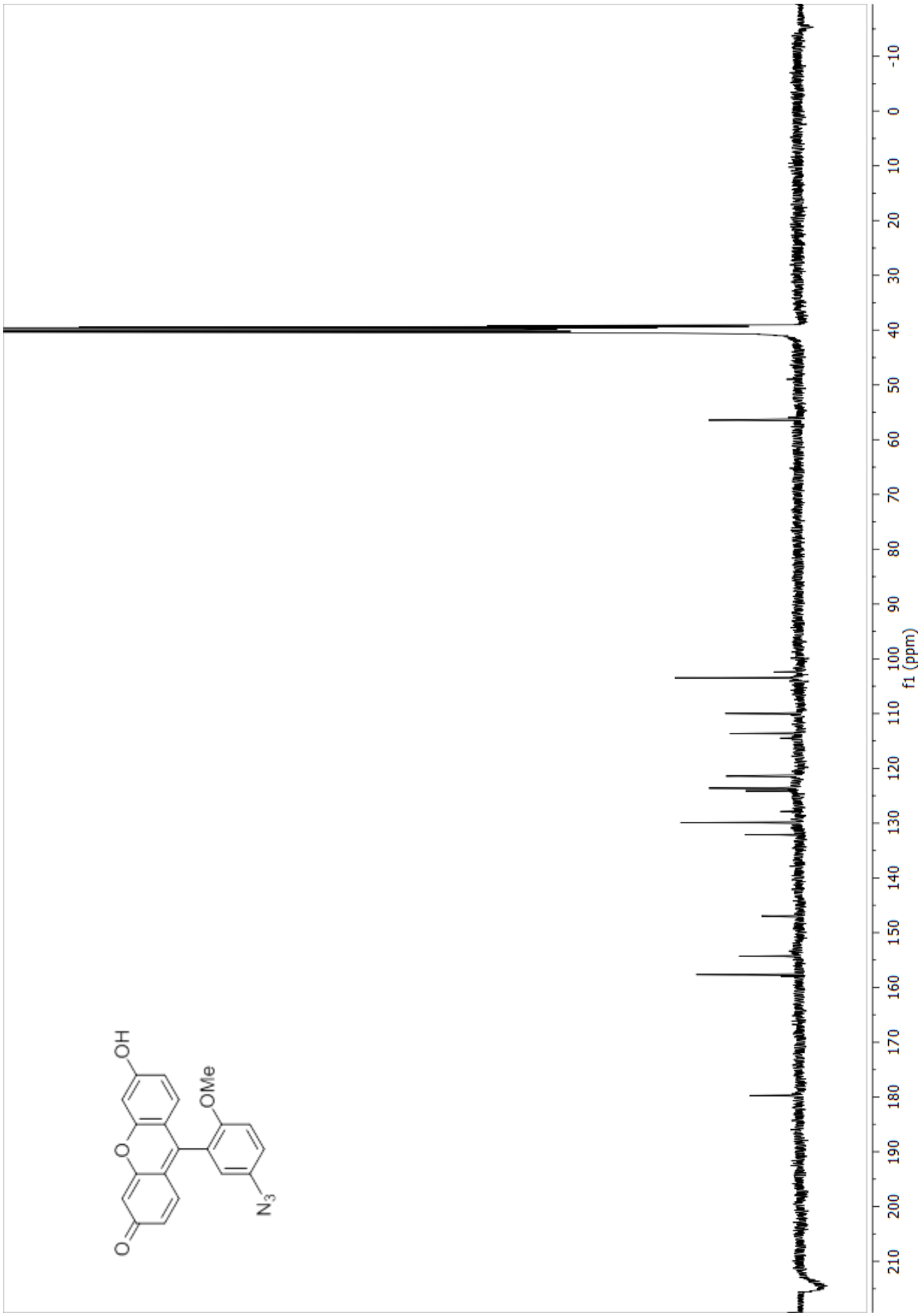
f1 (ppm)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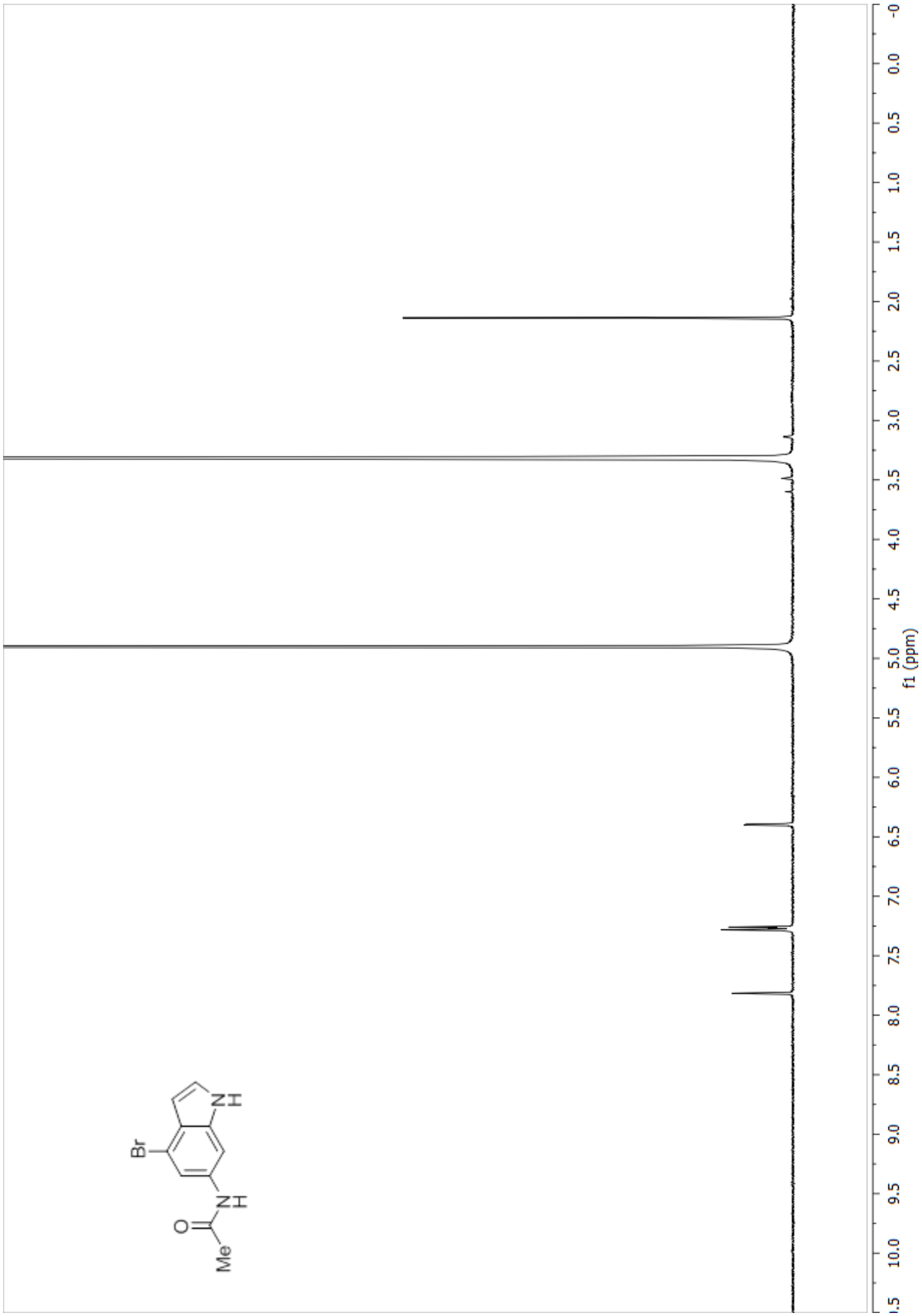
OMe
O
O
N3
f1 (ppm)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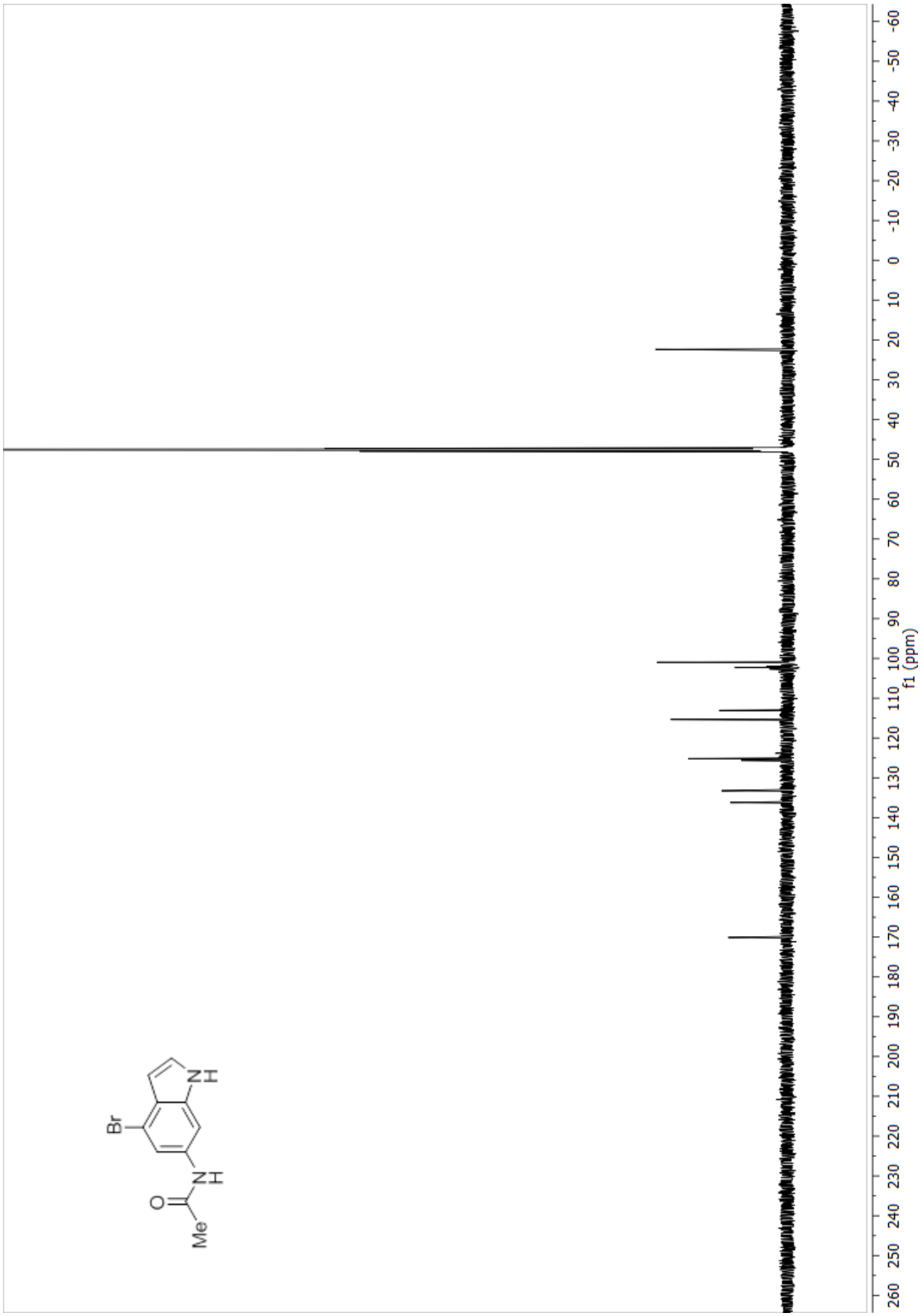
NH
O
Me
f1 (ppm)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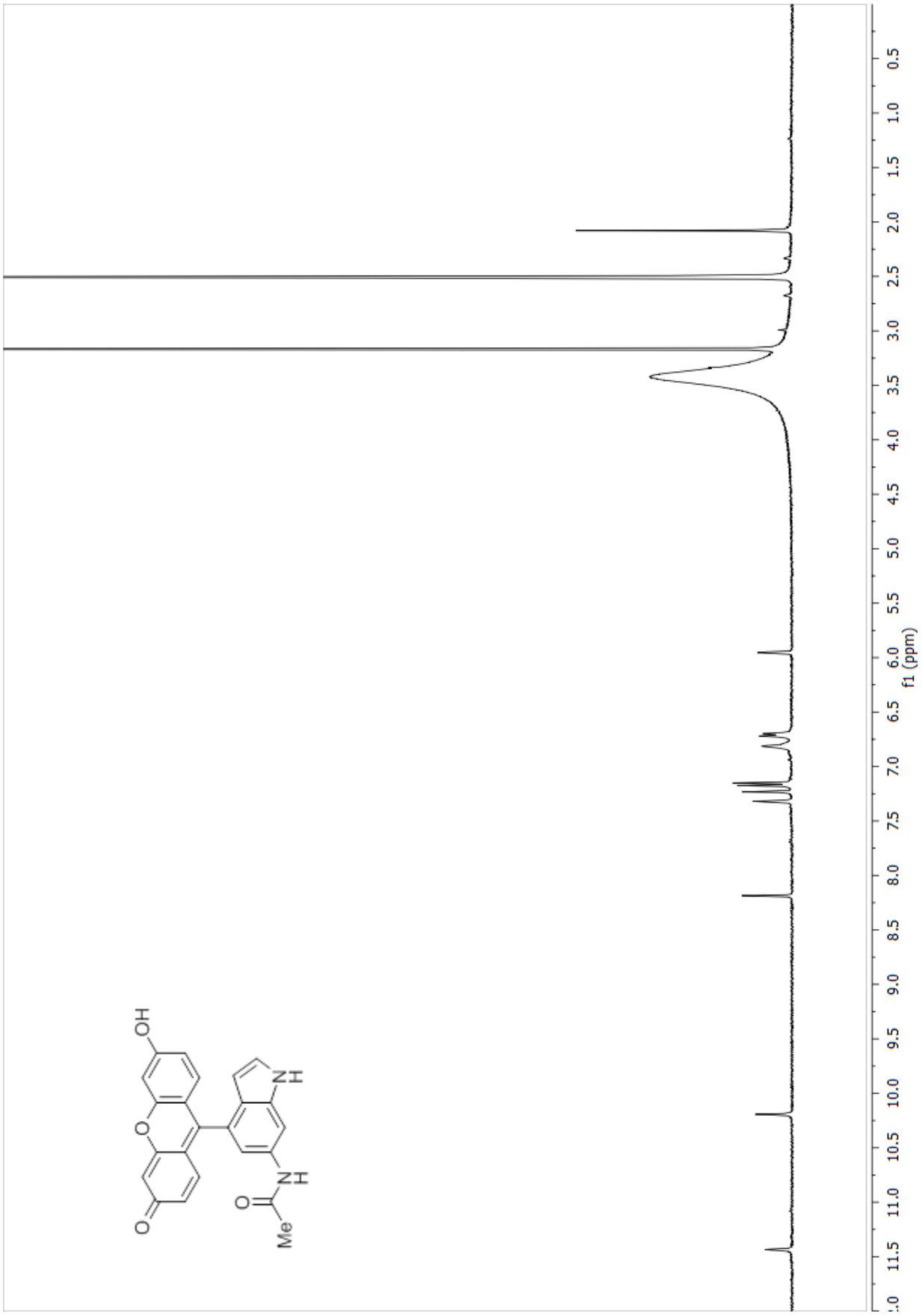
O
Me
f1 (ppm)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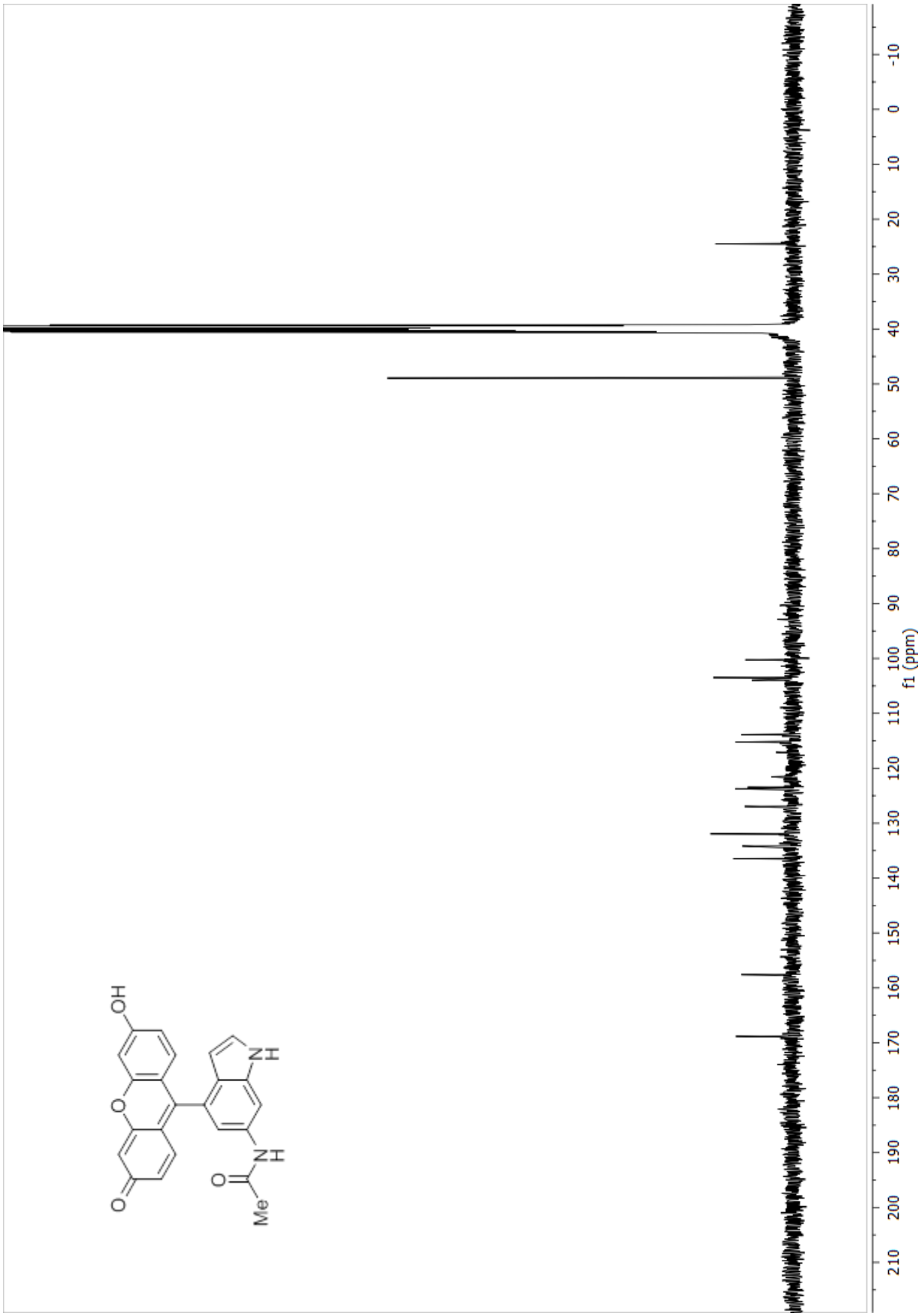
Me
f1 (ppm)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NH
NH
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Me
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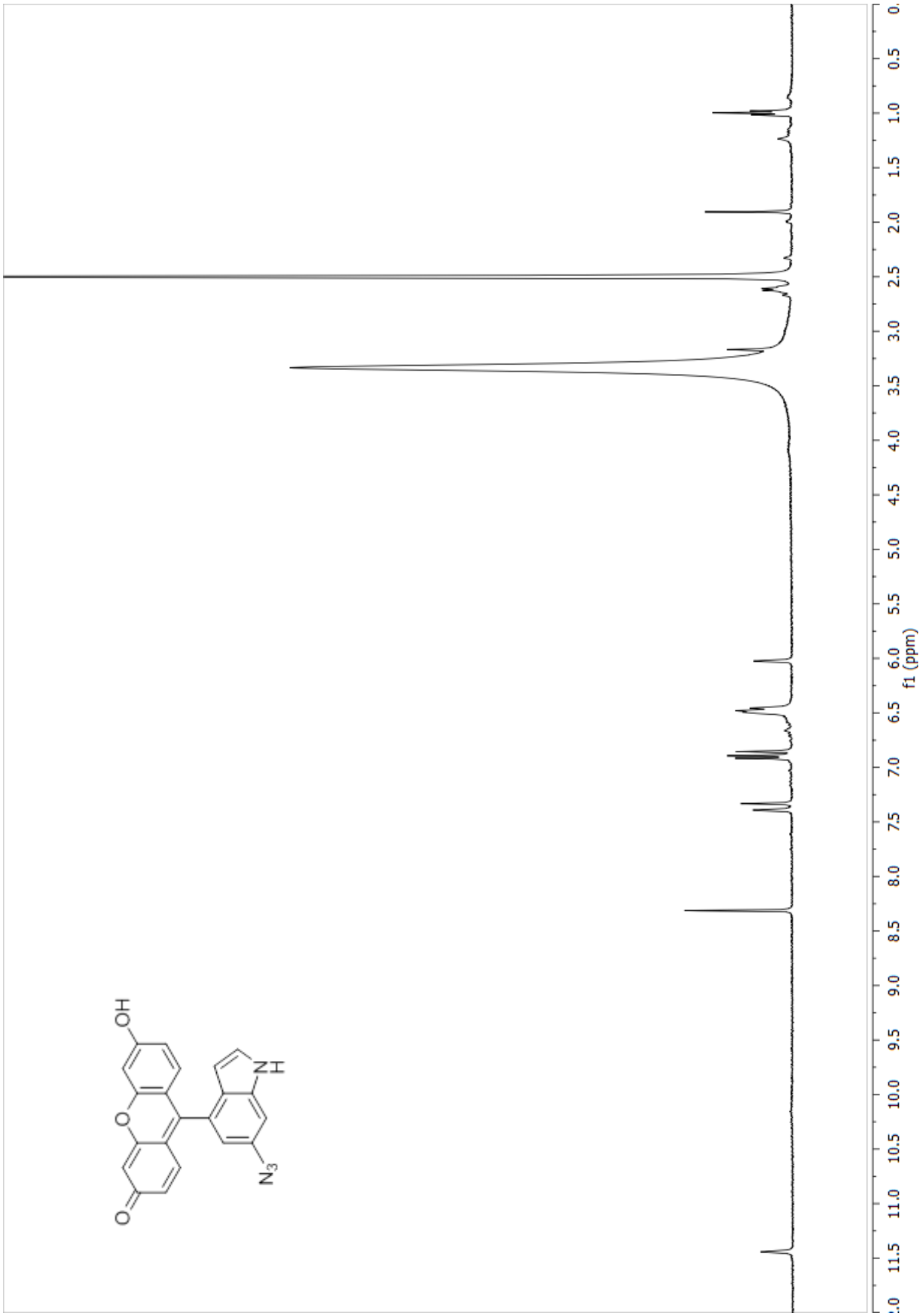
f1 (ppm)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O
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N3
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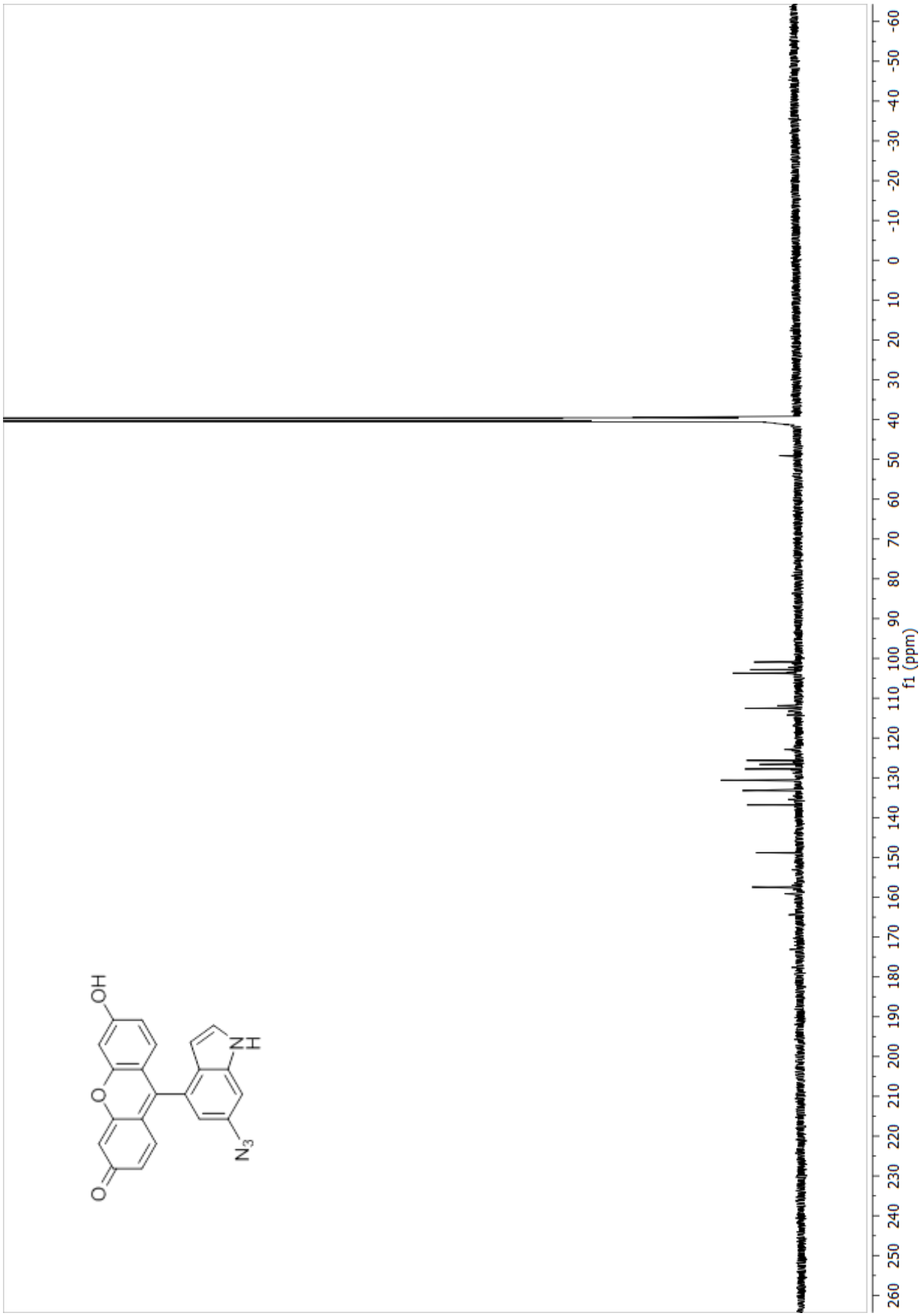
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O
NH
N3
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f1 (ppm)