Supporting Information

Synthesis of Substituted Cy5 Phosphoramidite Derivatives and Their Incorporation into Oligonucleotides Using Automated DNA Synthesis

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Section 1: Additional Structural Figures

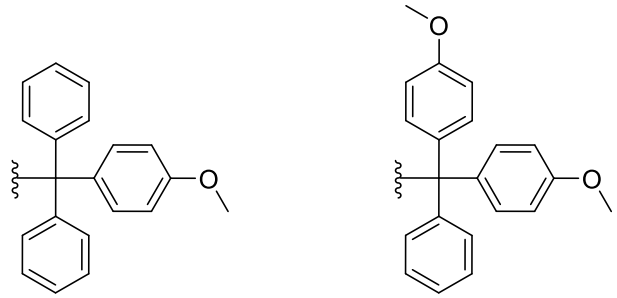


Figure S1: Mono-methoxy trityl (left) versus Di-methoxy trityl (DMTr, right). Under acidic hydrolysis conditions, DMTr cleavage proceeds approximately 10-fold faster than MMTr cleavage.[4]

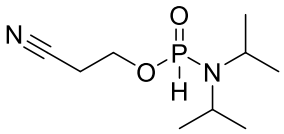


Figure S2: cyanoethyl-*N*,*N*-diisopropyl-H-phosphonamidate impurity generated during phosphoramidite conversion. NMR shown below in Figures S48-S50.

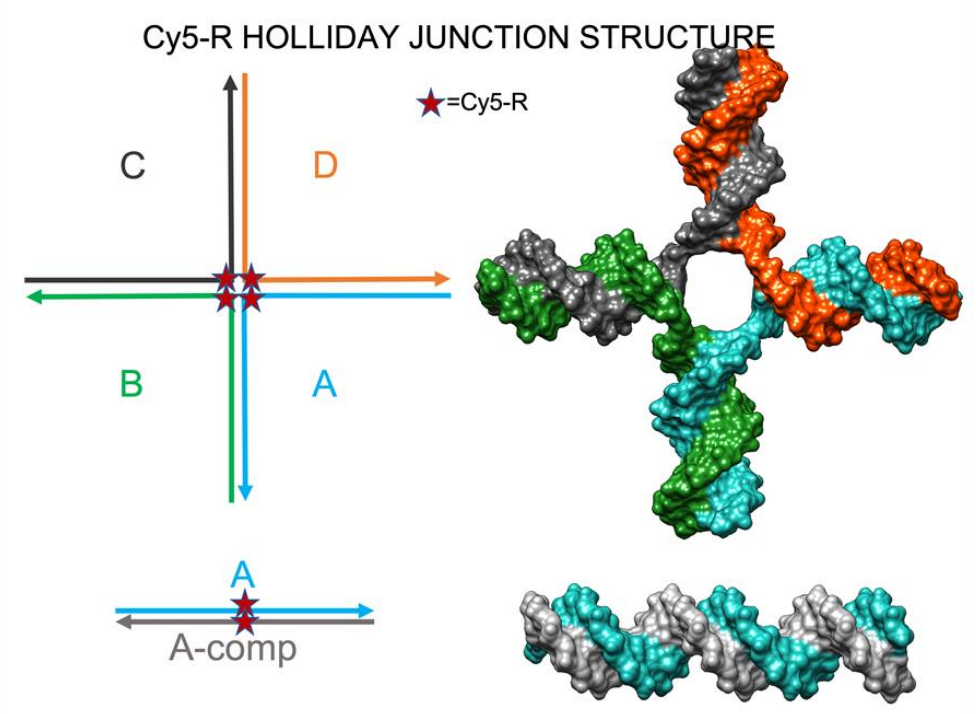
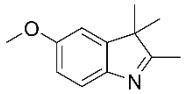


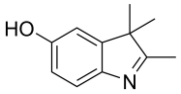
Figure S3: Structure of the 4-way Holliday junction. The four DNA sequences (A-D) synthesized with modified Cy5s self-assemble into a tetrameric Holliday junction structure. The fifth strand - A-comp – is complementary to A strand and allows the formation of a linear duplex to perform comparative studies with

the tetramer. Left: 2D line model of the Holliday junction and duplex. Right: 3D space-filled molecular model of the Holliday junction and duplex.

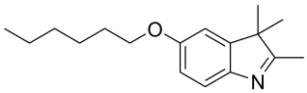
Section 2: Synthetic Procedures and Additional Compounds



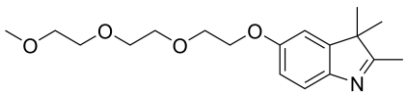
5-methoxy-2,3,3-trimethylindolenine (1-OMe). A mixture of *p*-methoxyphenylhydrazine hydrochloride (8.00 g, 45.8 mmol) and 3-methyl-2-butanone (5.00 mL, 46.4 mmol) in ethanol (160 mL) was refluxed for 19 h under N_2. The solution was allowed to cool to room temperature, and the solvent was evaporated. The residue was chromatographed on silica gel with $CHCl_3$ → $CHCl_3$:MeOH (25:1), yielding a pale, red-tinted solid (8.143 g, 94%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.43 (d, 1H, J = 8.0 Hz, Ph), 6.79-6.85 (m, 2H, Ph), 3.83 (s, 3H, CH_3O), 2.25 (s, 3H, CH_3), 1.29 (s, 6H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 185.8, 158.0, 147.5, 147.3, 120.1, 112.1, 108.2, 55.7, 53.8, 23.3, 15.3; LCMS (ESI): m/z $[M+H]^+$ Calcd for $C_{12}H_{16}NO$ 190.1237; Found 190.15.



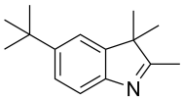
5-hydroxy-2,3,3-trimethylindolenine (**1-OH)**. A mixture of 5-methoxy-2,3,3-trimethylindolenine **1-OMe** (4.04 g, 21.3 mmol) and 48% HBr (70 mL) was refluxed for 2 h under N_2. After cooling, the reaction mixture was poured into 280 mL of deionized water. Solid $NaHCO_3$ was slowly added until the solution was basified. The product was extracted with $CHCl_3$ (3 × 100 mL). The combined $CHCl_3$ layers were washed with brine, and dried over Na_2SO_4. The solid was filtered off, and the solvent was evaporated to give a brown solid (3.29 g, 88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35 (d, 1H, J = 8.4 Hz, Ph), 6.82 (d, 1H, J = 1.6 Hz, Ph), 6.76 (dd, 1H, J = 8.2, 1.8 Hz, Ph), 2.26 (s, 3H, CH_3), 1.29 (s, 6H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 186.0, 155.8, 147.4, 145.2, 120.0, 114.3, 109.9, 53.8, 23.3, 15.1; LCMS (ESI): m/z $[M+H]^+$ Calcd for $C_{11}H_{14}NO$ 176.1081; Found 176.12.



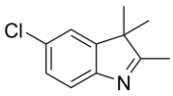
5-*n*-hexyloxy-2,3,3-trimethylindolenine (2-Hex). A mixture of 5-hydroxy-2,3,3-trimethylindolenine **1-OH** (6.69 g, 38.2 mmol), 1-bromo-hexane (7.56 g, 45.8 mmol) and Cs_2CO_3 (14.92 g, 45.8 mmol) in dry DMF (135 mL) was stirred at 60°C for 2 hours under N_2. After cooling, DMF was removed in vacuo. Resultant red solid was dissolved in CH_2Cl_2/water (1:1), then the aqueous layer was drained. The organic layer was then washed with water, brine, dried (Na_2SO_4) and concentrated to a pale brown oil. The oil solidified upon thorough drying, yielding a tan brown solid (9.69g, 98%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40 (d, 1H, J = 8.3 Hz, Ph), 6.83 (d, 1H, J = 2.4 Hz, Ph), 6.80 (dd, 1H, J_1 = 8.3, J_2 = 2.4 Hz, Ph), 3.96 (t, 2H, J = 6.6, CH_2), 2.23 (s, 3H, CH_3), 1.74-1.82 (m, 2H, CH_2), 1.42-1.52 (m, 2H, CH_2), 1.31-1.38 (m, 4H, CH_2), 1.27 (s, 6H, CH_3), 0.87-0.94 (m, 3H, CH_3). ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 185.7, 157.6, 147.5, 147.4, 120.2, 112.7, 108.9, 68.6, 53.9, 31.8, 29.5, 25.9, 23.4, 22.7, 15.4, 14.2. LCMS (ESI): m/z $[M+H]^+$ Calcd for $C_{17}H_{26}NO$ 260.2020; Found 260.34.



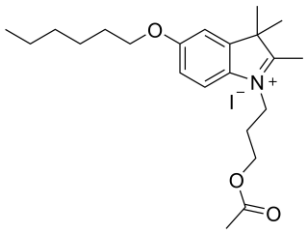
5-{2-[2-(2-methoxyethoxy)ethoxy]ethoxy}-2,3,3-trimethylindolenine (2-Peg). A mixture of 5-hydroxy-2,3,3-trimethylindolenine **1-OH** (4.00 g, 22.8 mmol), 1-bromo-2-[2-(2-methoxyethoxy)ethoxy]ethane **S1** (6.18 g, 27.2 mmol) and Cs_2CO_3 (9.71 g, 29.8 mmol) in dry DMF (70 mL) was stirred at 60°C for 6 days under N_2. After cooling, the inorganic salt was filtered off and further washed with acetone. The solvent was removed in vacuo. The residue was chromatographed on silica gel with $CHCl_3$:MeOH (50:1), yielding a viscous oil (6.90 g, 94%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.42 (d, 1H, J = 8.4 Hz, Ph), 6.87 (d, 1H, J = 2.4 Hz, Ph), 6.82 (dd, 1H, J = 8.4, 2.4 Hz, Ph), 4.15 (t, 2H, J = 5.0 Hz, CH_2), 3.87 (t, 2H, J = 4.8 Hz, CH_2), 3.73-3.77 (m, 2H, CH_2), 3.64-3.71 (m, 4H, CH_2), 3.55 (t, 2H, J = 4.6 Hz, CH_2), 3.38 (s, 3H, CH_3O), 2.25 (s, 3H, CH_3), 1.28 (s, 6H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 186.0, 157.2, 147.7, 147.4, 120.1, 112.9, 109.1, 72.1, 70.9, 70.8, 70.7, 70.0, 68.0, 59.1, 53.9, 23.3, 15.4; LCMS (ESI): m/z $[M+H]^+$ Calcd for $C_{18}H_{28}NO_4$ 322.2024; 322.35.



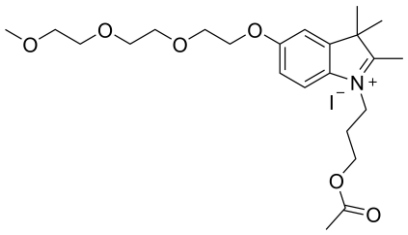
5-*tert*-butyl-2,3,3-trimethylindolenine (2-tBu). A mixture of (4-*tert*-butylphenyl)hydrazine hydrochloride (9.56 g, 47.6 mmol) and 3-methyl-2-butanone (5.10 mL, 47.6 mmol) was dissolved in ethanol (105 mL) then refluxed for 19 h in darkness, under N_2. The solution was allowed to cool to room temperature, and the solvent was evaporated. The resultant red-brown oil was dissolved in CH_2Cl_2 then washed with water, brine, dried (Na_2SO_4) and concentrated. Column chromatography [silica, hexanes/ethyl acetate (4:1)] yielded a pale brown oil that solidified after thorough drying and standing undisturbed overnight (7.97g, 78%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.44 (d, 1H, J = 7.6 Hz, Ph), 7.32 (dd, 1H, J_1 = 2.0 Hz, J_2 = 8.1 Hz, Ph), 7.29 (d, 1H, J = 2.0 Hz, Ph), 2.26 (s, 3H, CH_3), 1.35 (s, 9H, CH_3), 1.30 (s, 6H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 187.5, 151.7, 148.4, 145.5, 124.6, 119.2, 118.3, 53.7, 35.0, 31.9, 23.4, 15.5; LCMS (ESI): m/z $[M+H]^+$ Calcd for $C_{15}H_{22}N$ 216.1758; Found 216.26.



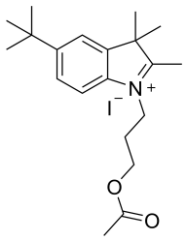
5-chloro-2,3,3-trimethylindolenine (2-Cl). A mixture of *p*-chlorophenylhydrazine hydrochloride (10.00 g, 55.9 mmol) and 3-methyl-2-butanone (5.68 g, 66.0 mmol) in ethanol (60 mL) and conc. H_2SO_4 (5.6 mL) was refluxed for 5 h under N_2. The solution was allowed to cool to room temperature, and the solvent was evaporated. H_2O (~150 mL) was added to the residue. The aqueous solution was washed with hexanes (2 times) and neutralized with Na_2CO_3. The product was extracted with $CHCl_3$ (3 times). The combined organic layers were dried over Na_2SO_4. The inorganic salt was filtered off, and the solvent was evaporated to give oil as the product (10.40 g, 96%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.44 (d, 1H, J = 8.0 Hz, Ph), 7.23-7.29 (m, 2H, Ph), 2.27 (s, 3H, CH_3), 1.30 (s, 6H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 188.4, 152.3, 147.4, 130.9, 127.7, 122.0, 120.8, 54.1, 22.9, 15.4; LCMS (ESI): m/z $[M+H]^+$ Calcd for $C_{11}H_{12}NCl$ 194.0737; Found 194.15.



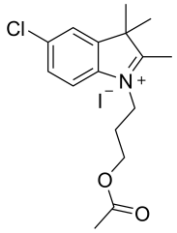
1-[3-(acetoxy)propyl]-5-*n*-hexyloxy-2,3,3-trimethylindolinium iodide (3-Hex). A mixture of 5-hexyloxy-2,3,3-trimethylindolenine **2-Hex** (9.69g, 37.4 mmol) and freshly prepared 3-iodopropyl acetate **S2** (9.48g, 41.6 mmol) was stirred at 100°C for 3.5 h under N_2. The reaction mixture was allowed to cool to room temperature. The resultant black, tar-like solid was dissolved in CH_2Cl_2:MeOH (1:1), transferred to a 250 mL round bottom flask and concentrated. The crude material was dissolved in CH_2Cl_2 (~15-20 mL) then forced to precipitate by the rapid addition of hexanes, while swirling the flask. The dissolution/precipitation was performed a second time, followed by extraction of the solid using diethyl ether (3 x 100 mL), discarding the supernatant each time. The sticky brown solid was then dried thoroughly, yielding a brown foamy solid (15.58g, 86%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.71 (d, 1H, J = 9.5 Hz, Ph), 7.01-7.07 (m, 2H, Ph), 4.83 (t, 2H, J = 7.3 Hz, CH_2), 4.23 (t, 2H, J = 5.9 Hz, CH_2), 4.02 (t, 2H, J = 6.5 Hz, CH_2), 3.10 (s, 3H, CH_3) 2.34-2.44 (m, 2H, CH_2), 2.02 (s, 3H, OAc) 1.76-1.86 (m, 2H, CH_2), 1.64 (s, 6H, CH_3), 1.43-1.52 (m, 2H, CH_2), 1.31-1.40 (m, 4H, CH_2), 0.89-0.96 (m, 3H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 193.1, 170.6, 161.2, 143.6, 134.1, 116.6, 115.1, 109.9, 69.2, 61.1, 54.5, 47.4, 31.6, 29.1, 27.4, 25.7, 23.5, 22.6, 21.1, 16.9, 14.1; LCMS (ESI): m/z $[M]^+$ Calcd for $C_{22}H_{34}NO_3$ 360.2544; Found 360.49.



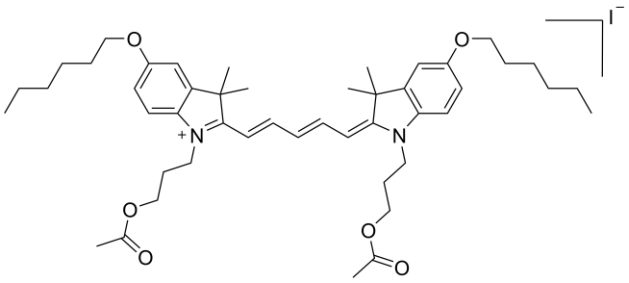
1-[3-(acetoxy)propyl]-5-{2-[2-(2-methoxyethoxy)ethoxy]ethoxy}-2,3,3-trimethylindolinium iodide (3-Peg). A mixture of 5-{2-[2-(2-methoxyethoxy)ethoxy]ethoxy}-2,3,3-trimethylindolenine **2-Peg** (1.28 g, 3.97 mmol) and freshly prepared 3-iodopropyl acetate **S2** (1.20 g, 5.26 mmol) was stirred at 105 °C for 7 h under N_2. The reaction mixture was allowed to cool to room temperature. $CHCl_3$ (1.0 mL) and MeOH (0.1 mL) were added to the reaction mixture to dissolve the tar-like oily residue. Then ether (~50 mL) was added, and the supernatant was discarded. This washing process was repeated twice. The viscous dark brown oil was further dried under vacuum for a yield of 2.15 g (98%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.62 (d, 1H, J = 8.8 Hz, Ph), 7.05-7.12 (m, 2H, Ph), 4.83 (t, 2H, J = 7.2 Hz, CH_2), 4.21 (m, 4H, CH_2), 3.89 (t, 2H, J = 4.4 Hz, CH_2), 3.72-3.77 (m, 2H, CH_2), 3.64-3.72 (m, 4H, CH_2), 3.54-3.59 (m, 2H, CH_2), 3.38 (s, 3H, CH_3O), 3.07 (s, 3H, CH_3), 2.36 (quint, 2H, J = 6.4 Hz, CH_2), 2.04 (s, 3H, CH_3CO), 1.63 (s, 6H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 193.4, 170.6, 160.8, 143.6, 134.2, 116.5, 115.3, 110.2, 71.9, 70.8, 70.6, 70.5, 69.4, 68.6, 61.0, 59.0, 54.5, 47.2, 27.3, 23.4, 21.0, 16.7; LCMS (ESI): m/z $[M]^+$ Calcd for $C_{23}H_{36}NO_6$ 422.2548; Found 422.46.



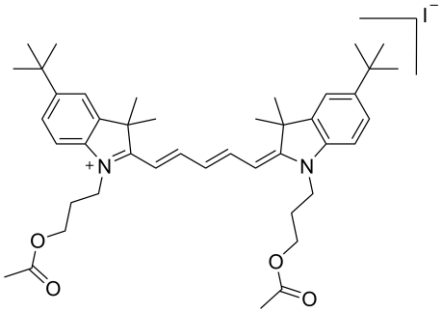
1-[3-(acetoxy)propyl]-5-*tert*-butyl-2,3,3-trimethylindolinium iodide (3-tBu). A mixture of 5-*tert*-butyl-2,3,3-trimethylindolenine **2-tBu** (2.15g, 10.0 mmol) and freshly prepared 3-iodopropyl acetate **S2** (2.28 g, 10.0 mmol) was stirred at 100 °C for 3 h under N_2. The reaction mixture was allowed to cool to room temperature. The resultant black, tar-like solid was dissolved in CH_2Cl_2:MeOH (1:1), transferred to a 100 mL round bottom flask and concentrated. The crude material was dissolved in CH_2Cl_2 (~5 mL) then forced to precipitate by the rapid addition of diethyl ether (30 mL), while swirling the flask. The dissolution/precipitation was repeated five times in total, until the supernatant no longer indicated extraction of highly colored impurities. The sticky light brown solid was then dried thoroughly, yielding a pale brown foam (3.17g, 72%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.64 (d, 1H, J = 8.6 Hz, Ph), 7.50 (dd, 1H, J_1 = 1.8 Hz, J_2 = 8.6 Hz, Ph) 7.51 (d, 1H, J = 1.8 Hz, Ph), 4.87 (t, 2H, J = 7.3 Hz, CH_2), 4.22 (t, 2H, J = 5.8 Hz, CH_2), 3.15 (s, 3H, CH_3), 2.33-2.41 (m, 2H, CH_2), 1.99 (s, 6H, OAc), 1.65 (s, 6H, CH_3), 1.36 (s, 9H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 195.7, 170.6, 154.6, 141.5, 138.9, 127.0, 120.1, 115.0, 61.1, 54.8, 47.4, 35.5, 31.5, 27.3, 23.5, 21.0, 17.2; LCMS (ESI): m/z $[M]^+$ Calcd for $C_{20}H_{30}NO_2$ 316.2282; Found 316.36.



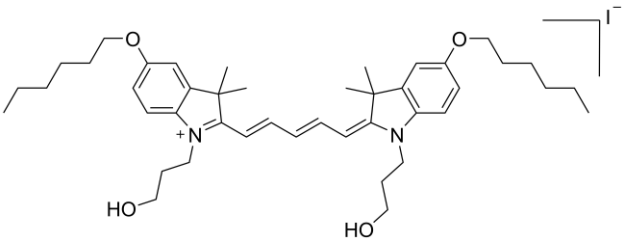
5-chloro-1-[3-(acetoxy)propyl]-2,3,3-trimethylindolinium iodide (3-Cl). A mixture of 5-chloro-2,3,3-trimethylindolenine **2-Cl** (1.00 g, 5.16 mmol) and freshly prepared 3-iodopropyl acetate **S2** (1.31 g, 5.74 mmol) was degassed under vacuum for 5 min, backfilled with N_2, then stirred at 105 °C for 21 h under N_2. The reaction mixture was allowed to cool to room temperature. $CHCl_3$ (1.0 mL) and MeOH (0.1 mL) were added to the reaction mixture to dissolve the oil residue. Then ether (~50 mL) was added, and the supernatant was discarded. This washing process was repeated 3 times. The viscous oil was further dried under vacuum for a final yield 2.05 g (94%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.68 (d, 1H, J = 8.4 Hz, Ph), 7.51-7.61 (m, 2H, Ph), 4.86 (t, 2H, J = 7.2 Hz, CH_2), 4.21 (t, 2H, J = 5.8 Hz, CH_2), 3.12 (s, 3H, CH_3), 2.35 (quint, 2H, J = 6.4 Hz, CH_2), 2.04 (s, 3H, CH_3CO), 1.69 (s, 6H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 196.9, 170.6, 143.3, 139.7, 136.7, 130.0, 124.0, 117.2, 61.1, 55.0, 47.6, 27.3, 23.3, 21.1, 17.3; LCMS (ESI): m/z $[M]^+$ Calcd for $C_{16}H_{21}NO_2Cl$ 294.126; Found 294.23.



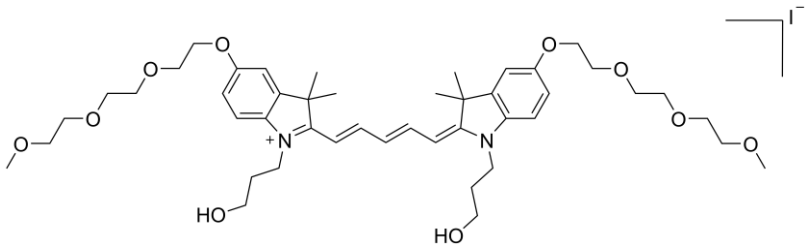
1,1'-bis(3-acetoxypropyl)-5,5'-bis(*n*-hexyloxy)-3,3,3',3'-tetramethyl-indodicarbocyanine iodide (4-Hex). A mixture of 1-[3-(acetoxy)propyl]-5-hexyloxy-2,3,3-trimethylindolinium iodide **3-Hex** (6.44g, 13.2 mmol), malonaldehyde dianilide hydrochloride (1.63g, 6.29 mmol) and sodium acetate (1.55g, 18.87 mmol), was suspended in acetic anhydride (52 mL) then stirred vigorously at 120°C for 2 h. After cooling to room temp, the solvent was evaporated by short-path distillation. Crude blue solid was dissolved in CH_2Cl_2 then washed with aqueous sodium iodide (2 molar equivalents, ~ 2 grams), dried (Na_2SO_4) and concentrated. Crude material was then split into two equal portions for column chromatography [silica, $CHCl_3$:MeCN (4:1)] yielding a blue solid (4.58g, 82%) when combined. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.91 (t, 2H, J = 13.0 Hz, =CH-), 6.98 (d, 2H, J = 8.7 Hz, Ph), 6.88 (d, 2H, J = 2.4 Hz, Ph), 6.80 (dd, 2H, J_1 = 2.4 Hz, J_2 = 8.7 Hz, Ph), 6.71 (t, 1H, J = 12.5 Hz, =CH-) 6.24 (d, 2H, J = 13.7 Hz, =CH-), 4.16 (t, 4H, J = 7.0 Hz, CH_2), 4.11 (t, 4H, J = 6.0 Hz, CH_2), 3.91(t, 4H, J = 6.5 Hz, CH_2), 2.09-2.17 (m, 4H, CH_2), 1.98 (s, 6H, OAc), 1.68-1.76 (m, 4H, CH_2) 1.65 (s, 12H, CH_3), 1.35-1.46 (m, 4H, CH_2), 1.24-1.32 (m, 8H, CH_2), 0.82-0.87 (m, 6H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 171.7, 170.7, 157.6, 152.1, 142.7, 135.3, 125.4, 113.8, 111.0, 109.6, 103.3, 68.8, 61.2, 49.4, 41.5, 31.5, 29.1, 28.2, 26.5, 25.6, 22.5, 21.0, 14.0; LCMS (ESI): m/z $[M]^+$ Calcd for $C_{47}H_{67}N_2O_6$ 755.5005; Found 755.50.



1,1'-bis(3-acetoxypropyl)-5,5'-bis(*tert*-butyl)-3,3,3',3'-tetramethyldicarboindocyanine iodide (4-tBu). A mixture of 1-[3-(acetoxy)propyl]-5-*tert*-butyl-2,3,3-trimethylindolinium iodide **3-tBu** (5.75 g, 13.0 mmol), malonaldehyde dianilide hydrochloride (1.67 g, 6.50 mmol), was dissolved in dichloromethane (130 mL) then treated with acetic anhydride (3.69 mL, 39.0 mmol) and triethylamine (9.06 mL, 65.0 mmol). The solution was stirred vigorously at room temp for 4.5 h, then aqueous sodium iodide (1 molar equivalent, ~ 2 g) was added, and stirring was continued for 12 hr. The two phases were then separated, dried (Na_2SO_4) and concentrated. Iterative column chromatography [silica, CH_2Cl_2:MeCN (8:1)→ (5:1)] yielded a blue solid (4.24g, 82%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02 (t, 2H, J = 13.1 Hz, =CH-), 7.34-7.38 (m, 4H, Ph), 7.00 (d, 2H, J = 7.7 Hz, Ph), 6.94 (t, 1H, J = 12.5 Hz, =CH-), 6.44 (d, 2H, J = 13.7 Hz, =CH-), 4.24 (t, 4H, J = 7.0 Hz, CH_2), 4.17 (t, 4H, J = 6.1 Hz, CH_2), 2.16-2.24 (m, 4H, CH_2), 2.02 (s, 6H, OAc), 1.75 (s, 12H, CH_3), 1.33 (s, 18H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 172.9, 170.9, 153.2, 149.1, 141.1, 140.0, 126.5, 125.7, 119.3, 109.9, 104.0, 61.5, 49.6, 41.7, 35.1, 31.7, 28.4, 26.8, 21.2; LCMS (ESI): m/z $[M]^+$ Calcd for $C_{43}H_{59}N_2O_4$ 667.4480; Found 667.72.

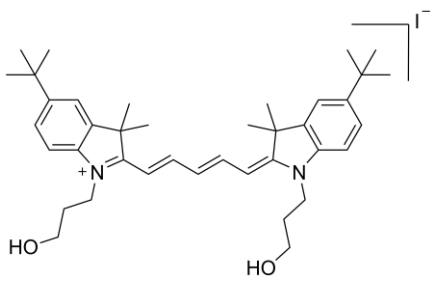


1,1'-bis(3-hydroxypropyl)-5,5'-bis(*n*-hexyloxy)-3,3,3',3'-tetramethyl-indodicarbocyanine iodide (5-Hex). A sample of 1,1'-bis(3-acetoxypropyl)-5,5'-bis(*n*-hexyloxy)-3,3,3',3'-tetramethyl-dicarboindocyanine iodide **4-Hex** (4.00g, 4.53 mmol) was dissolved in MeOH (400 mL) then treated with acetyl chloride (8.87g, 113 mmol) and allowed to stir at room temperature, in darkness, for 22 hours. The reaction mixture was carefully neutralized with $NaHCO_3$ (sat'd, aq.) while chilled in an ice bath. $CHCl_3$ was added to the mixture and the aqueous layer drained. Organic layer was then washed with aqueous sodium iodide (2 molar equivalents, ~ 1.5 g), dried (Na_2SO_4) and concentrated. Crude material was then split into two equal portions for column chromatography [silica, $CHCl_3$/MeCN (2:1)] yielding a blue solid (2.69g, 74%) when combined. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75 (t, 2H, J = 13.1 Hz, =CH-), 7.13 (d, 2H, J = 8.7 Hz, Ph), 6.77-6.90 (m, 5H, =CH- and Ph), 6.34 (d, 2H, J = 13.6 Hz, =CH-), 4.22 (t, 4H, J = 7.5 Hz, CH_2), 3.90 (t, 4H, J = 6.5 Hz, CH_2), 3.87-3.82 (m, 4H, CH_2), 3.74 (s, 2H, OH), 2.03-2.09 (m, 4H, CH_2), 1.69-1.78 (m, 4H, CH_2), 1.62 (s, 12H, CH_3), 1.38-1.47 (m, 4H, CH_2), 1.30-1.36 (m, 8H, CH_2), 0.87-0.93 (m, 6H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 171.4, 157.7, 151.4, 142.7, 135.8, 126.0, 113.7, 111.5, 109.8, 103.5, 68.9, 58.7, 49.3, 42.1, 31.7, 30.3, 29.4, 28.2, 25.8, 22.7, 14.2; LCMS (ESI): m/z $[M]^+$ Calcd for $C_{43}H_{63}N_2O_4$ 671.4793; Found 671.78.

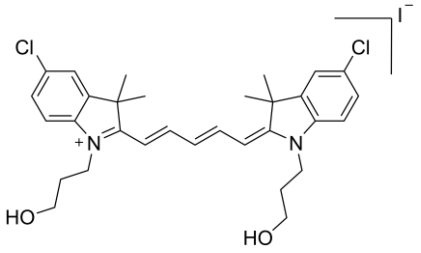


1,1'-bis(3-hydroxypropyl)-5,5'-bis{2-[2-(2-methoxyethoxy)ethoxy]ethoxy}-3,3,3',3'-tetramethyl-indodicarbocyanine iodide (5-Peg). 1-[3-(Acetoxy)propyl]-5-{2-[2-(2-methoxyethoxy)ethoxy]ethoxy}-2,3,3-trimethylindolinium iodide (6.194 g, 11.3 mmol), malonaldehyde dianilide hydrochloride (1.45 g, 5.60 mmol), acetic anhydride (3.8 mL, 40.0 mmol), triethylamine (1.9 mL, 14.0 mmol) and ethanol (60 mL) were added to a 250-mL round-bottom flask, and the mixture was refluxed for 2 h under N_2. After cooling, the solvent was evaporated. The residue was mixed with ether (~50 mL), and the supernatant was discarded (3 times). The crude intermediate **4-Peg** was dissolved in methanol (90 mL), then 4M HCl (90 mL) was added dropwise. The reaction mixture was stirred at room temperature in the dark for 38 h under N_2. Methanol was evaporated. The product was extracted with $CHCl_3$ (5 times). The combined organic layers were dried over Na_2SO_4. The inorganic salt was filtered off, and the solvent was evaporated. The residue was chromatographed on silica gel with $CHCl_3$:MeOH (8:1), yielding a blue solid (3.00 g, 65%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78 (t, 2H, J = 12.8 Hz, =CH-), 7.06 (d, 2H, J = 8.8 Hz, Ph), 6.86-6.98 (m, 5H, Ph and =CH-), 6.43 (d, 2H, J = 13.2 Hz, =CH-), 4.20 (t, 4H, J = 7.2 Hz, CH_2), 4.15 (t, 4H, J = 4.6 Hz, CH_2), 3.87 (t, 4H, J = 4.8 Hz, CH_2), 3.82 (t, 4H, J = 5.2 Hz, CH_2), 3.72-3.77 (m, 4H, CH_2), 3.68-3.72 (m, 4H, CH_2), 3.64-3.68 (m, 4H, CH_2), 3.39 (s, 6H, CH_3O), 2.07 (quint, 4H, J = 6.0 Hz, CH_2), 1.65 (s, 12H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 171.5, 157.2, 151.5, 142.6, 135.9, 125.7, 114.0, 111.5, 109.9,

103.3, 71.9, 70.8, 70.6, 70.5, 69.7, 68.3, 59.0, 58.5, 49.3, 41.9, 30.2, 28.1; LCMS (ESI): m/z $[M]^+$ Calcd for $C_{45}H_{67}N_2O_{10}$ 795.4790; Found 795.48.

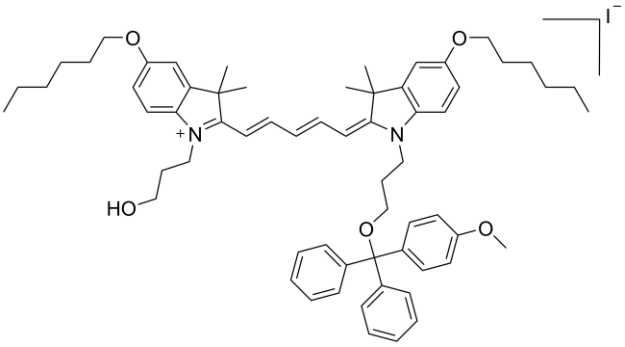


1,1'-bis(3-hydroxypropyl)-5,5'-bis(*tert*-butyl)-3,3,3',3'-tetramethyl-indodicarbocyanine iodide (5-tBu). A sample of 1,1'-bis(3-acetoxypropyl)-5,5'-bis(*tert*-butyl)-3,3,3',3'-tetramethyl-indodicarbocyanine iodide **4-tBu** (3.91 g, 5.00 mmol) was dissolved in MeOH (150 mL) then treated with acetyl chloride (5.89g, 75.0 mmol) and allowed to stir at room temperature, in darkness, for 16 hours. The reaction mixture was carefully neutralized with $NaHCO_3$ (sat'd, aq.) while chilled in an ice bath. $CHCl_3$ was added to the mixture and the aqueous layer drained. Organic layer was then washed with aqueous sodium iodide (2 molar equivalents, ~ 1.5 g), dried (Na_2SO_4) and concentrated. Column chromatography [silica, CH_2Cl_2/MeOH (40:1 → 10:1)] yielded a blue solid (2.54 g, 73%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.35 (t, 2H, J = 13.2 Hz, =CH-), 7.71 (d, 2H, J = 1.9 Hz, Ph), 7.44 (dd, 2H, J_1 = 1.9 Hz, J_2 = 8.4 Hz, Ph), 7.31 (d, 2H, J = 8.4 Hz, Ph), 6.54 (t, 1H, J = 12.4 Hz, =CH-), 6.32 (d, 2H, J = 13.9 Hz, =CH-), 4.78 (t, 2H, J = 5.0 Hz, CH_2), 4.15 (t, 4H, 7.1 Hz, CH_2), 3.36 (s, 3H, CH_3), 1.83-1.94 (m, 4H, CH_2), 1.72 (s, 12H, CH_3), 1.36 (s, 18H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ 173.6, 154.4, 148.8, 142.0, 140.9, 126.2, 126.0, 120.5, 111.3, 103.8, 58.6, 50.0, 41.7, 35.7, 32.4, 31.2, 28.2; LCMS (ESI): m/z $[M]^+$ Calcd for $C_{39}H_{55}N_2O_2$ 583.4269; Found 583.68.



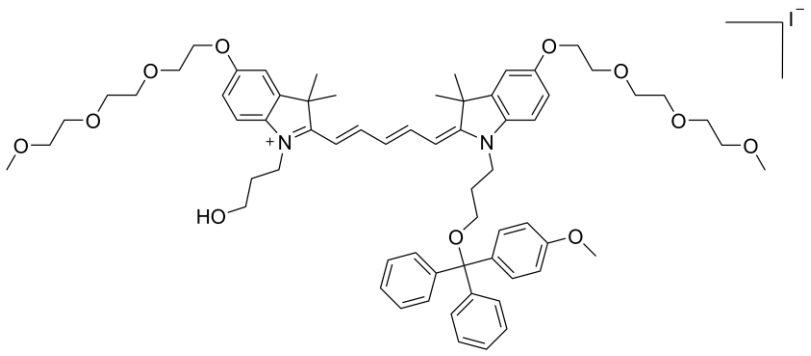
5,5'-dichloro-1,1'-bis(3-hydroxypropyl)-3,3,3',3'-tetramethyl-indodicarbocyanine iodide (5-Cl). Samples of 1-[3-(acetoxy)propyl]-5-chloro-2,3,3-trimethylindolinium iodide (2.52 g, 5.98 mmol), malonaldehyde dianilide hydrochloride (0.770 g, 2.98 mmol), acetic anhydride (2.00 mL, 21.2 mmol), triethylamine (1.00 mL, 7.17 mmol) were added to a 250-mL round-bottom flask, dissolved in ethanol (40 mL), and the contents refluxed for 2 h under N_2. After cooling, the solvent was evaporated. The residue was mixed with hexanes (~50 mL), and the supernatant was discarded. Then, the residue was further mixed with ether (~50 mL), and the supernatant was discarded (2 times). The crude product was dried under vacuum and used for the subsequent acetyl deprotection without further purification. The crude intermediate **4-Cl** was dissolved in dry methanol (15 mL) and CH_2Cl_2 (5.0 mL), then acetyl chloride (1.20 mL, 16.9 mmol) was added dropwise at room temperature under N_2. The reaction mixture was stirred in the dark for 15.5 h under N_2, then mixed with $CHCl_3$ (100 mL) and washed with brine (50 mL). The aqueous layer was washed with $CHCl_3$ (4~5 times). The combined organic layers were dried over Na_2SO_4. The inorganic salt was filtered off, and the solvent was evaporated. The residue was chromatographed on

silica gel with $CHCl_3$:MeOH (8:1 → 5:1), yielding a blue solid (1.40 g, 81%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85 (br s, 2H, =CH-), 7.34 (dd, 2H, J = 8.4, 2.0 Hz, Ph), 7.29 (d, 2H, J = 2.0 Hz, Ph), 7.12 (br s, 1H, =CH-), 7.09 (d, 2H, J = 8.4 Hz, Ph), 6.68 (br s, 2H, =CH-), 4.26 (t, 4H, J = 7.0 Hz, CH_2), 3.80 (t, 4H, J = 4.8 Hz, CH_2), 2.05 (m, 4H, CH_2), 1.66 (s, 12H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 172.2, 153.2, 142.6, 140.6, 130.4, 128.7, 126.9, 122.5, 112.1, 104.0, 58.3, 49.2, 41.9, 30.0, 27.8; LCMS (ESI): m/z $[M]^+$ Calcd for $C_{31}H_{37}Cl_2N_2O_2$ 539.2227; Found 539.45.

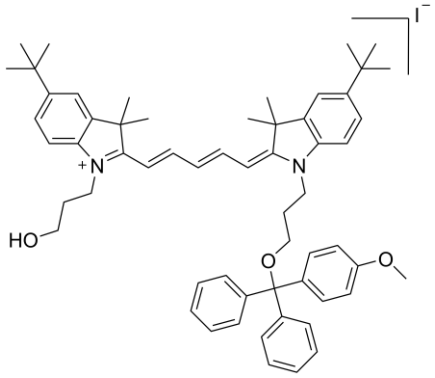


1-(3-hydroxypropyl)-1'-[3-(4-monomethoxytrityloxy)propyl]-5,5'-bis(*n*-hexyloxy)-3,3,3',3'-tetramethyl-indodicarbocyanine iodide (Cy5-Hex). A sample of 1,1'-bis(3-hydroxypropyl)-5,5'-bis(*n*-hexyloxy)-3,3,3',3'-tetramethyl-indodicarbocyanine iodide **5-Hex** (1.53g, 1.92 mmol) was dissolved in 25 mL of dry pyridine, then treated with 4-monomethoxytrityl chloride (1.18g, 3.82 mmol) and allowed to stir for 4 days at room temperature, in darkness. The pyridine was removed in vacuo then the resultant blue solid was dissolved in CH_2Cl_2, washed with aqueous sodium iodide (1 molar equivalent, ~ 0.9 g), dried (Na_2SO_4) and concentrated. Iterative column chromatography [silica, $CHCl_3$:MeCN (3:1)] yielded a blue solid (509.3 mg, 25%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.94 (t, 1H, J = 13.1 Hz, =CH-), 7.85 (t, 1H, J = 12.9 Hz, =CH-), 7.39-7.44 (m, 4H, Ph), 7.22-7.33 (m, 8H, Ph)*, 7.15 (d, 1H, J = 8.6 Hz, Ph), 6.95 (d, 1H, J = 8.7 Hz, Ph), 6.85-6.91 (m, 3H, Ph), 6.82 (d, 2H, J = 8.9 Hz, Ph), 6.73 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.7 Hz, Ph), 6.26 (t, 1H, J = 12.4 Hz, =CH-), 6.18 (d, 1H, J = 13.4 Hz, =CH-), 6.07 (d, 1H, J = 13.6 Hz, =CH-), 4.30 (t, 2H, J = 7.6 Hz, CH_2), 4.11 (t, 2H, J = 7.0 Hz, CH_2), 3.92-3.99 (m, 4H, CH_2), 3.82-3.89 (m, 2H, CH_2), 3.80 (s, 3H, OCH_3), 3.45 (s, 1H, OH), 3.10 (t, 2H, J = 5.6 Hz, CH_2), 2.05-2.13 (m, 4H, CH_2), 1.74-1.83 (m, 4H, CH_2), 1.66 (s, 6H, CH_3), 1.64 (s, 6H, CH_3), 1.43-1.51 (m, 4H, CH_2), 1.32- 1.38 (m, 8H, CH_2), 0.89-0.94 (m, 6H, CH_3);^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 171.4, 171.1, 158.1, 157.2, 156.9, 151.5, 151.0, 144.0, 142.29, 142.25, 135.0, 134.8, 134.7, 129.8, 127.7, 127.4, 126.5, 124.2, 113.5, 113.3, 112.6, 111.5, 111.0, 109.0, 108.9, 102.4, 102.3, 86.3, 68.3, 59.3, 57.8, 54.9, 49.1, 48.9, 41.7, 40.7, 31.0, 29.8, 28.7, 27.7, 27.5, 27.1, 25.1, 22.0, 13.6; LCMS (ESI): m/z $[M]^+$ Calcd for $C_{63}H_{79}N_2O_5$ 943.5994; Found 943.93.

*****multiplet overlaps with $CHCl_3$ residual, hydrogen count is assumed due to the accuracy of all remaining integrals. Resonance observed at 2.01 ppm attributed to MeCN.

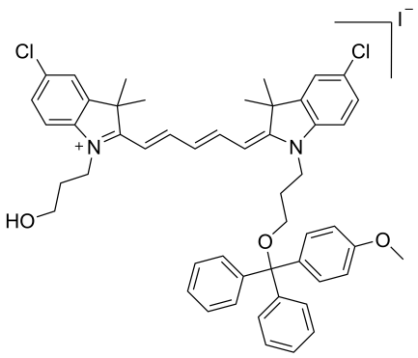


1-(3-hydroxypropyl)-1'-[3-(4-monomethoxytrityloxy)propyl]-5,5'-bis{2-[2-(2-methoxyethoxy)ethoxy]ethoxy}-3,3,3',3'-tetramethyl-indodicarbocyanine iodide (Cy5-Peg). A sample of **5-peg** (1.99 g, 2.39 mmol) was dissolved in 60 mL of dry CH_2Cl_2. Triethylamine (0.70 mL, 5.0 mmol) and 4-monomethoxytrityl chloride (0.89 g, 2.88 mmol) were added to the solution. The reaction mixture was stirred at room temperature in the dark for 3.5 h under N_2. The reaction was quenched by addition of methanol, and the solvent was evaporated. The residue was chromatographed on silica gel with $CHCl_3$:MeOH (8:1). The crude product was further purified by C18 reverse phase flash column chromatography (Biotage Selekt) with MeOH : 0.1 M triethylammonium acetate buffer (pH 7.0) (7:3 → 8:2). Methanol was evaporated from the product fractions. The product was extracted with $CHCl_3$ (3~4 times). The combined organic layers were washed with aqueous NaI solution and dried over Na_2SO_4. The inorganic salt was filtered off and the solvent was evaporated to give viscous dark blue oil as the product (0.92 g, 32%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.91 (t, 1H, J = 13.0 Hz, =CH-), 7.82 (t, 1H, J = 13.0 Hz, =CH-), 7.41 (d, 4H, J = 7.2 Hz, Ph), 7.20-7.34 (m, 8H, Ph), 7.15 (d, 1H, J = 8.8 Hz, Ph), 6.88-7.00 (m, 4H, Ph), 6.82 (d, 2H, J = 8.8 Hz, Ph), 6.73-6.79 (m, 1H, Ph), 6.27 (t, 1H, J = 12.6 Hz, =CH-), 6.18 (d, 1H, J = 14.0 Hz, =CH-), 6.09 (d, 1H, J= 13.2 Hz, =CH-), 4.29 (t, 2H, J = 7.2 Hz, CH_2), 4.08-4.20 (m, 6H, CH_2), 3.82-3.91 (m, 6H, CH_2), 3.80 (s, 3H, CH_3O), 3.72-3.78 (m, 4H, CH_2), 3.68-3.72 (m, 4H, CH_2), 3.63-3.68 (m, 4H, CH_2), 3.53-3.60 (m, 4H, CH_2), 3.39 (s, 6H, CH_3O), 3.11 (t, 2H, J = 5.4 Hz, CH_2), 2.03-2.14 (m, 4H, CH_2), 1.66 (s, 6H, CH_3), 1.64 (s, 6H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 171.9, 171.3, 158.4, 157.2, 156.9, 151.9, 151.3, 144.2, 142.5, 142.4, 135.6, 135.3, 135.2, 130.1, 128.0, 127.7, 126.8, 124.6, 114.0, 113.7, 113.0, 111.7, 111.0, 109.7, 109.6, 103.0, 102.5, 86.6, 71.7, 70.5, 70.3, 70.2, 69.4, 68.1, 68.0, 59.7, 58.8, 58.1, 55.2, 49.4, 49.1, 42.1, 41.1, 30.1, 28.0, 27.8, 27.4; LCMS (ESI): m/z $[M]^+$ Calcd for $C_{65}H_{83}N_2O_{11}$ 1067.5991; Found 1067.89.

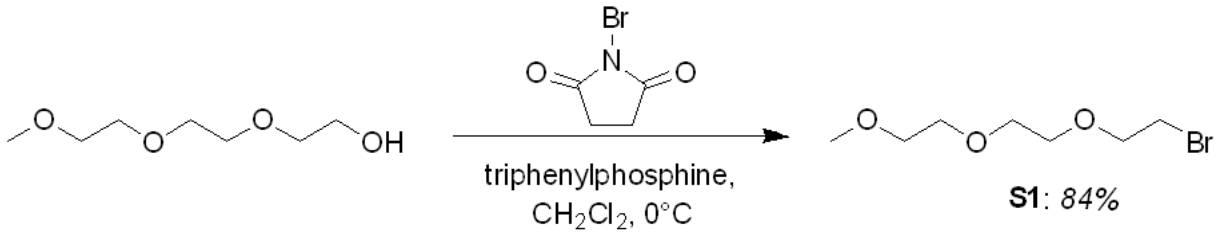


1-(3-hydroxypropyl)-1'-[3-(4-monomethoxytrityloxy)propyl]-5,5'-bis(*tert*-butyl)-3,3,3',3'-tetramethyl-indodicarbocyanine iodide (Cy5-tBu). A sample of 1,1'-bis(3-hydroxypropyl)-5,5'-bis(*tert*-

butyl)-3,3,3',3'-tetramethyl-indodicarbocyanine iodide **5-tBu** (2.50 g, 3.52 mmol) was dissolved in 50 mL of dry pyridine, then treated with 4-monomethoxytrityl chloride (816.0 mg, 2.64 mmol) and allowed to stir for 18 h at room temperature, in darkness. A second portion of 4-monomethoxytrityl chloride (544.0 mg, 1.76 mmol) was added, and the reaction was stirred an additional 2 h. The pyridine was removed in vacuo then the resultant blue solid was triturated with diethyl ether (100 mL). Crude was then dissolved in CH_2Cl_2, washed with aqueous sodium iodide (1 molar equivalent, ~ 0.9 g), dried (Na_2SO_4) and concentrated. Column chromatography [silica, $CHCl_3$/MeCN (8:1)→(3:1)→(2:1)] yielded a blue solid (1.10 g, 32%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.07 (t, 1H, J = 13.0 Hz, =CH-), 8.00 (t, 1H, J = 13.0 Hz, =CH-), 7.34-7.46 (m, 7H, Ph), 7.20-7.33 (m, 10H, Ph), 7.06 (d, 1H, J = 8.4 Hz, Ph), 6.81 (d, 2H, J = 9.0 Hz, Ph), 6.11-6.21 (m, 3H, =CH-), 4.27-4.34 (m, 2H, CH_2), 4.19-4.25 (m, 2H, CH_2), 3.85-3.92 (m, 2H, CH_2), 3.78 (s, 3H, OCH_2), 3.02-3.09 (m, 2H, CH_2), 2.07-2.23 (m, 4H, CH_2), 1.76 (s, 6H, CH_3), 1.69 (s, 6H, CH_3), 1.33 (s, 18H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 172.8, 172.2, 158.2, 152.7, 152.1, 148.7, 148.2, 144.2, 140.7, 140.6, 139.6, 139.3, 135.1, 130.0, 127.9, 127.6, 126.7, 125.4, 125.0, 124.9, 118.7, 112.8, 110.4, 110.1, 103.1, 102.7, 86.5, 59.5, 58.1, 55.1, 53.5, 49.4, 49.1, 41.9, 40.8, 34.6, 34.5, 31.4, 31.3, 30.1, 28.0, 27.8, 27.3; LCMS (ESI): m/z $[M]^+$ Calcd for $C_{59}H_{71}N_2O_3$ 855.5470; Found 855.85.



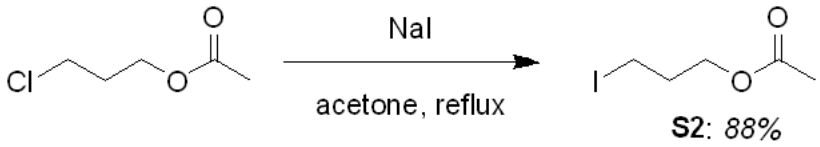
5,5'-dichloro-1-(3-hydroxypropyl)-1'-[3-(4-monomethoxytrityloxy)propyl]-3,3,3',3'-tetramethyl-indodicarbocyanine iodide (**Cy5-Cl)**. A sample of **5-Cl** (0.63 g, 1.10 mmol) was dissolved in 40 mL of dry CH_2Cl_2. Triethylamine (0.32 mL, 2.3 mmol) and 4-monomethoxytrityl chloride (0.340 g, 1.10 mmol) were added to the solution. The reaction mixture was stirred at room temperature in the dark for 2 h under N_2. The reaction was washed with aqueous NaI solution (1 molar equivalent, ~0.17g), and dried over Na_2SO_4. The inorganic salt was filtered off, and the solvent was evaporated. The residue was chromatographed [silica, $CHCl_3$:CH_3CN (2:1)] to obtain a dark blue solid (0.52 g, 50%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.12 (t, 1H, J = 13.0 Hz, =CH-), 8.02 (t, 1H, J = 13.0 Hz, =CH-), 7.39 (d, 4H, J = 7.2 Hz, Ph), 7.13-7.36 (m, 13H, Ph and =CH-), 7.00 (d, 1H, J = 8.4 Hz, Ph), 6.81 (d, 2H, J = 8.8 Hz, =CH-), 6.48 (t, 1H, J = 12.2 Hz, =CH-), 6.33 (d, 1H, J = 13.6 Hz, =CH-), 6.20 (d, 1H, J = 13.6 Hz, =CH-), 4.33 (t, 2H, J = 7.4 Hz, CH_2), 4.16 (t, 2H, J = 6.6 Hz, CH_2), 3.84 (t, 2H, J = 5.4 Hz, CH_2), 3.80 (s, 3H, CH_3O), 3.10 (t, 2H, J = 5.4 Hz, CH_2), 2.02-2.15 (m, 4H, CH_2), 1.69 (s, 6H, CH_3), 1.66 (s, 6H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 173.0, 172.2, 158.6, 153.9, 153.3, 144.3, 142.7, 140.7, 140.5, 135.3, 131.0, 130.4, 130.2, 128.9, 128.5, 128.2, 127.9, 127.0, 126.6, 122.7, 113.2, 112.4, 111.8, 104.2, 103.7, 86.9, 59.7, 58.2, 55.4, 49.6, 49.3, 42.4, 41.4, 30.1, 28.1, 27.9, 27.6; LCMS (ESI): m/z $[M]^+$ Calcd for $C_{51}H_{53}Cl_2N_2O_3$ 811.3428; Found 811.64.



1-Bromo-2-[2-(2-methoxyethoxy)ethoxy]ethane (S1). [1] A mixture of triethyleneglycol monomethyl ether (22.36 g, 136 mmol) and triphenylphosphine (42.86 g, 163 mmol) in CH_2Cl_2 was cooled to 0 °C in an ice bath under N_2. N-Bromosuccinimide (29.05 g, 163 mmol) was added portionwise to the reaction mixture, which was further stirred at 0 °C for 2 h. The solvent was evaporated, and ether (~200 mL) was added to the residue. The reaction mixture was sonicated for 1 h, and the solid was filtered off. The filtrate was cooled in an ice-bath, and the white precipitate was filtered off. This process was repeated several times until solid no longer precipitated. After the solvent was evaporated, the residue was chromatographed on silica gel with $CHCl_3$ → $CHCl_3$:MeOH (50:1). Upon concentration, compound **S1** was yielded as a viscous, colorless oil (26.08 g, 84%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.82 (t, 2H, J = 6.4 Hz, CH_2), 3.64-3.71 (m, 6H, CH_2), 3.54-3.58 (m, 2H, CH_2), 3.48 (t, 2H, J = 6.4 Hz, CH_2), 3.39 (s, 3H, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 72.0, 71.3, 70.7, 70.6, 59.1, 30.4. Unable to acquire LCMS data.

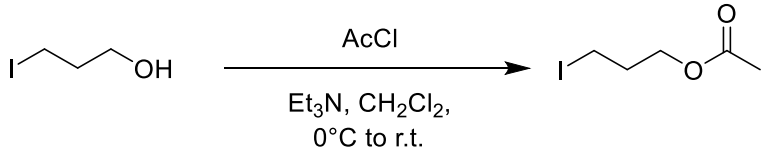
3-Iodopropyl acetate (S2).

Method 1 (modified from Brush) [2]



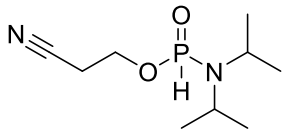
A mixture of 3-chloropropyl acetate (15.04 g, 110 mmol) and sodium iodide (33.00 g, 220 mmol) in acetone was refluxed for 25.5 h under N_2. After cooling, the solid was filtered off and further washed with acetone. The combined filtrate was evaporated. Ether was added to the residue. The solid was filtered off and further washed with ether. The filtrate was combined and evaporated to give **S2** as a clear, colorless oil (22.03g, 88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.14 (t, 2H, J = 6.0 Hz, CH_2), 3.23 (t, 2H, J = 6.8 Hz, CH_2), 2.15 (quint, 2H, J = 6.4 Hz, CH_2), 2.06 (s, 3H, CH_3CO); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 171.0, 64.2, 32.5, 21.0, 1.5. Unable to acquire LCMS data.

Method 2 (Modified from Baba)[3]



A mixture of 3-iodopropanol (9.30g, 50.0 mmol), Et_3N (7.35 ml, 52.5 mmol) was dissolved in dried dichloromethane (50 mL), covered in aluminum foil and cooled to 0°C, under N_2. Then, acetyl chloride (3.75 mL, 52.5 mmol) was added slowly over ~10 minutes. The solution was stirred at 0°C for 10 minutes then the ice bath was removed, stirring was continued for 1.5 h. Resultant cloudy solution was transferred to a separatory funnel and diluted with an addition 50 mL dichloromethane, the remaining solid was dissolved in water and added to the separatory funnel.

The organic phase was washed with water, brine, then dried (Na_2SO_4) and concentrated while covered with aluminum foil. The pale brown oil was dissolved in hexanes then filtered through a short column [silica, hexanes:dichloromethane (1:1)], yielding **S2** as a clear colorless oil (9.48 g, 83%) upon concentration. ^{1}H NMR resonances were consistent with those reported for Method 1.



cyanoethyl-N,N-diisopropyl-H-phosphonamidate This byproduct is formed during Cy5-phosphoramidite synthesis reactions. Note that the provided ^{31}P NMR (Figure S50) was not calibrated, nor decoupled, and was included only to demonstrate two highly (and identically) split but discrete resonances, corresponding to the single P(V) center. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.95 (d, J = 639.5 Hz, PH), 4.08-4.25 (m, 2H, CH_2), 3.43-3.59 (m, 2H, CH), 2.69-2.82 (m, 2H, CH_2), 1.28 (d, 6H, J = 5.6 Hz, CH_3), 1.26 (d, 6H, J = 5.6 Hz, CH_3); ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ 117.0 (s), 58.3 (d, J = 5.9 Hz), 45.5 (d, J = 6.6 Hz), 23.0-23.2 (m), 20.3 (d, J = 6.6 Hz); ^{31}P NMR (162 MHz, $CDCl_3$): 16.0-16.6 (m), 12.0-12.4 (m); LCMS (ESI): m/z $[M+Et_3NH]^+$ Calcd for $C_{15}H_{35}N_3O_2P$ 320.2466; Found 320.39.

Section 3: ^{1}H and ^{13}C NMR Spectra

NMR spectra are presented in order of appearance according to schema. Individual spectra are scaled to allow for easier visualization, in some cases residual solvent resonances corresponding to $CHCl_3$, TMS or DMSO exceed the viewing window. Commonly observed residual solvent resonances are $CHCl_3$, CH_2Cl_2, MeCN and water in $CDCl_3$ and DMSO in d_6-DMSO, see Fulmer *et al.* [5] for corresponding δppm values.

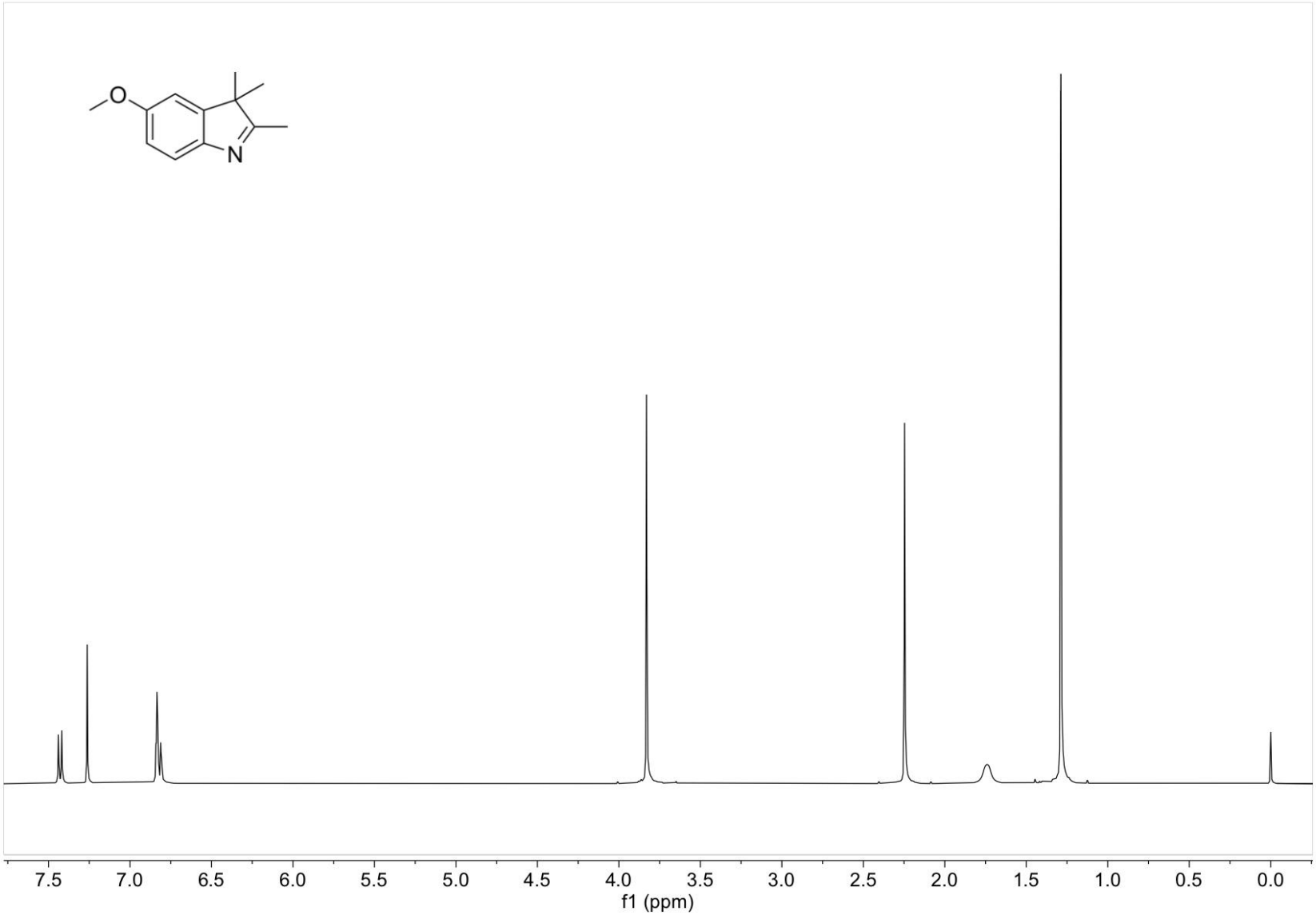


Figure S4: 1H NMR of 1-OMe

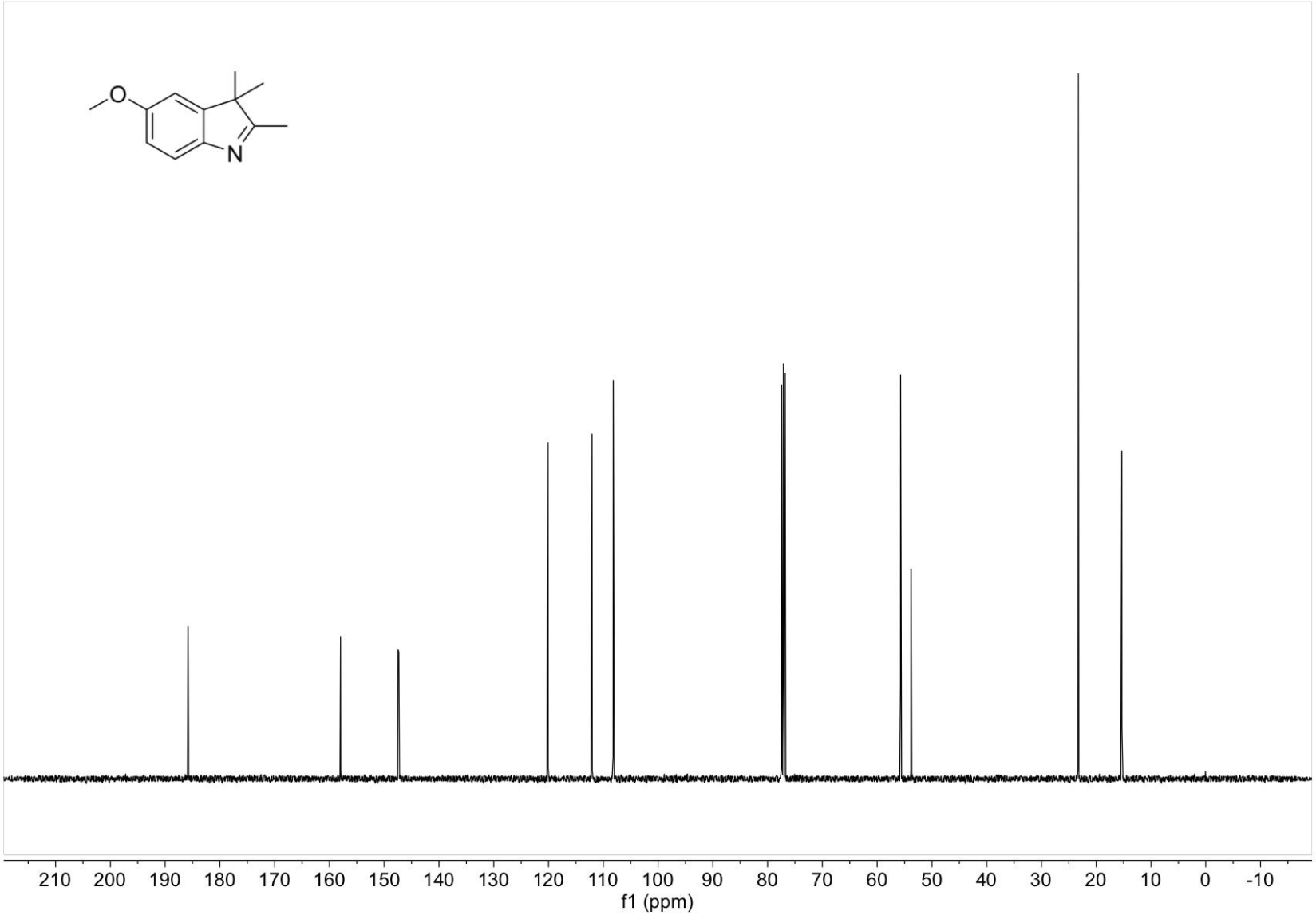


Figure S5: ^{13}C NMR of 1-OMe

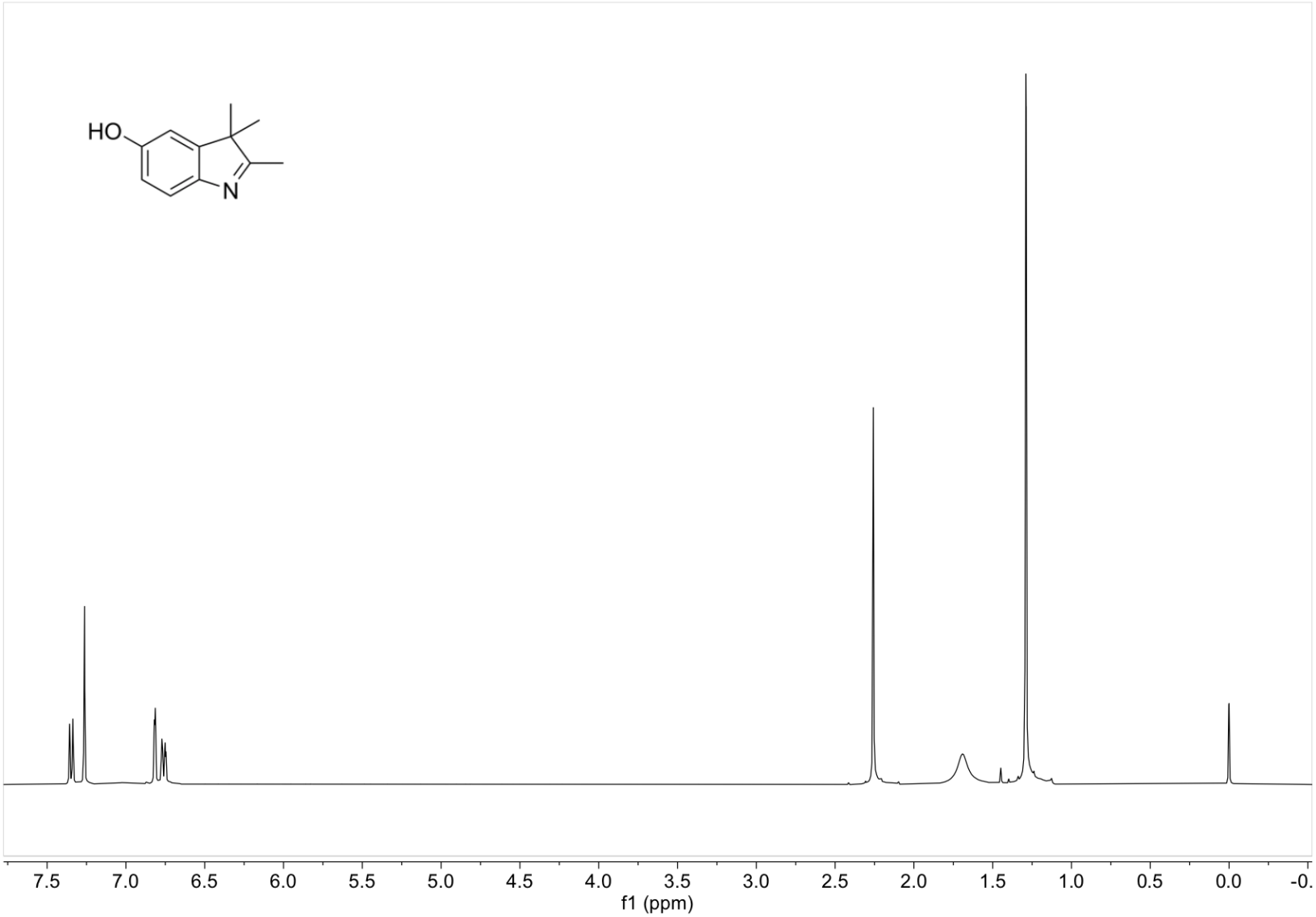


Figure S6: ^{1}H NMR of 1-OH

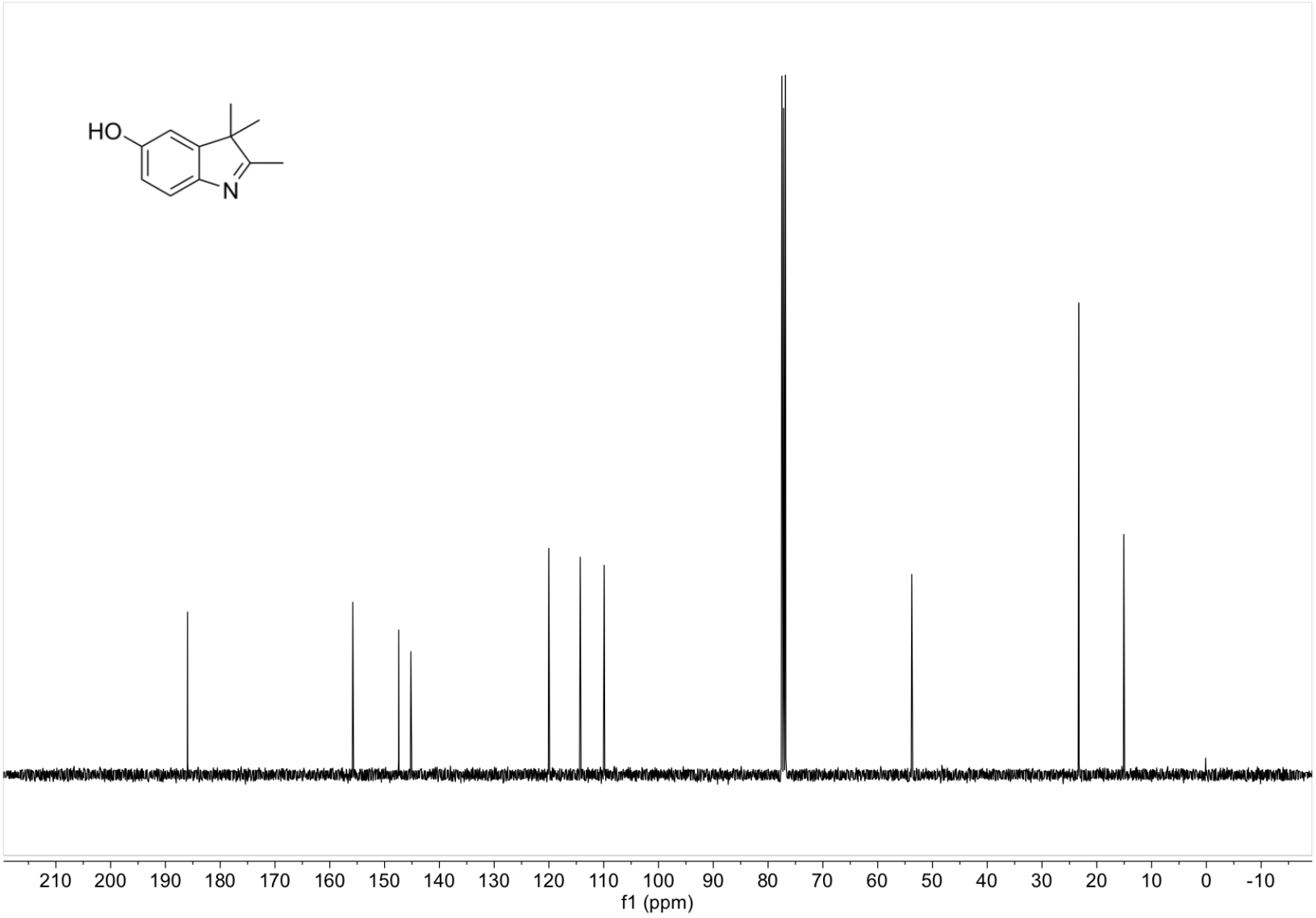


Figure S7: ^{13}C NMR of 1-OH

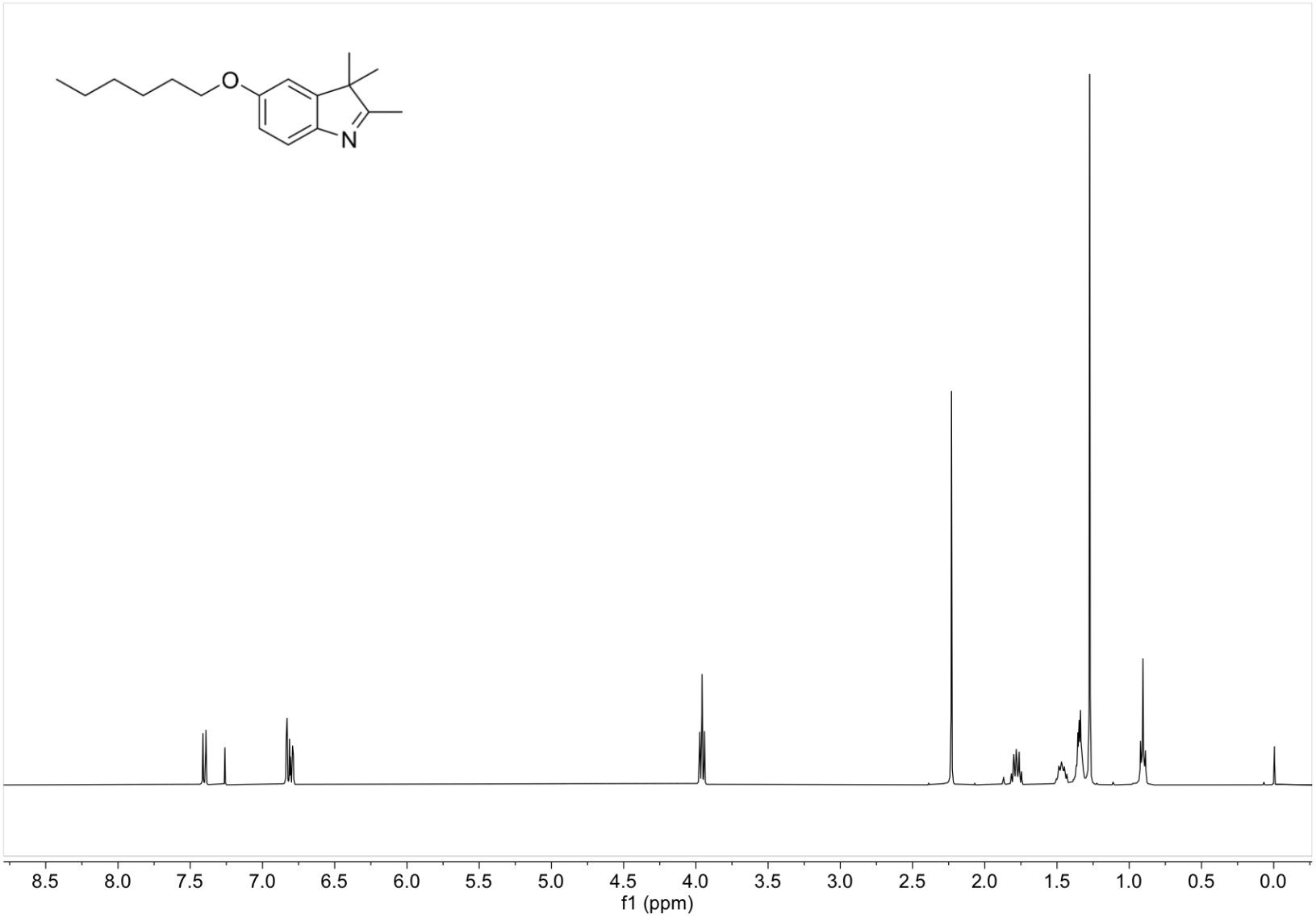


Figure S8: ^{1}H NMR of 2-hex

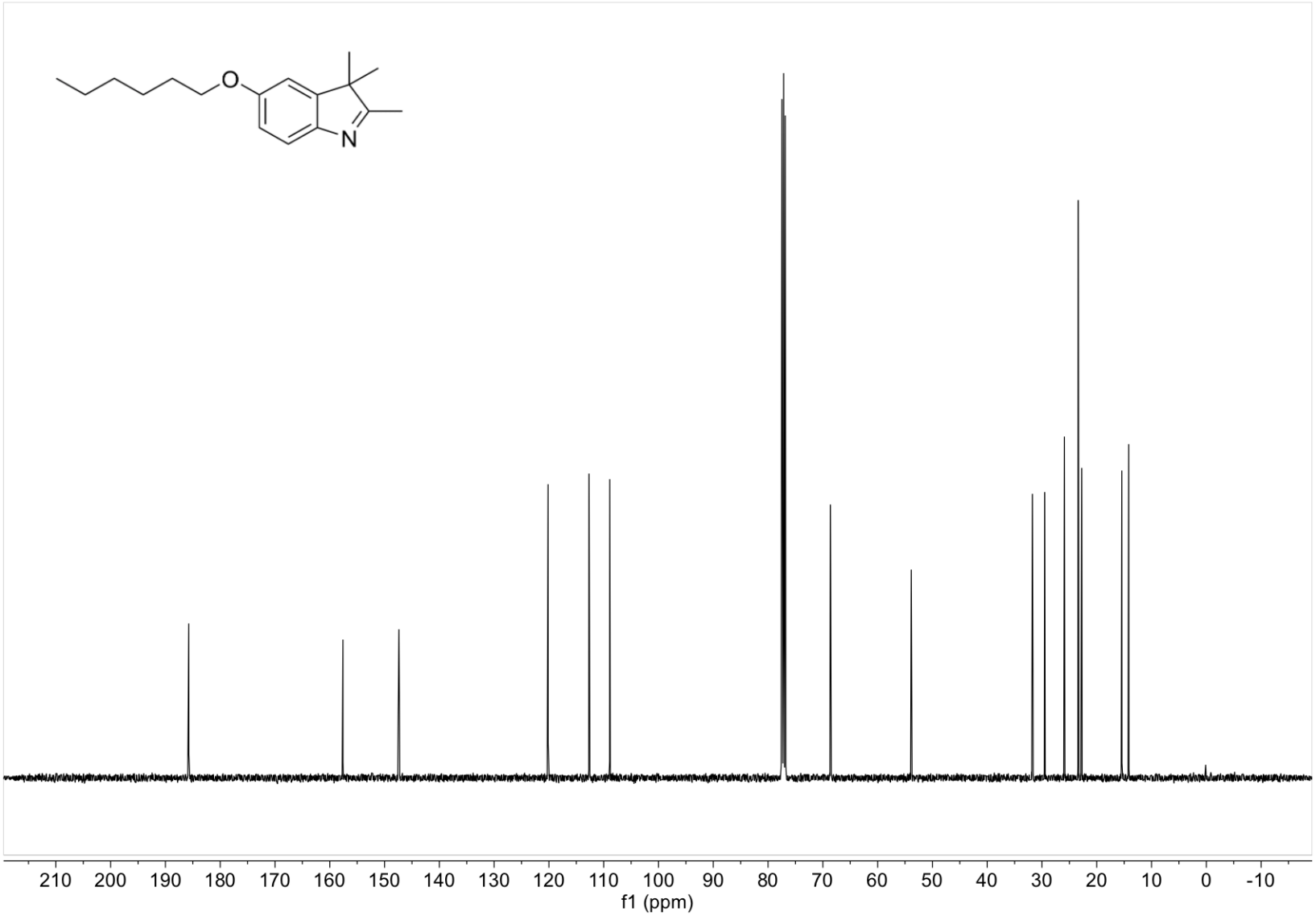


Figure S9: ^{13}C NMR of 2-hex

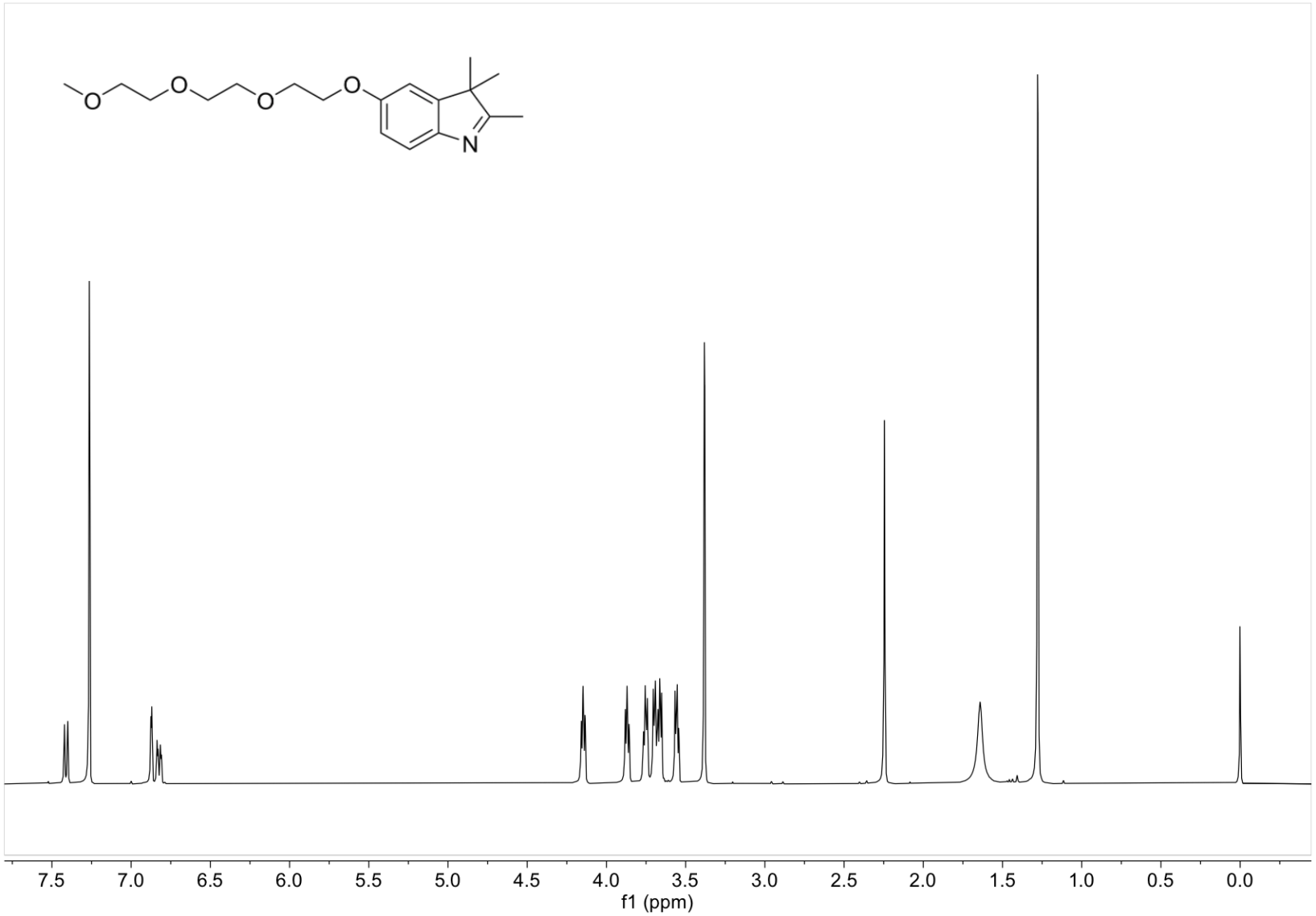


Figure S10: ^{1}H NMR of 2-Peg

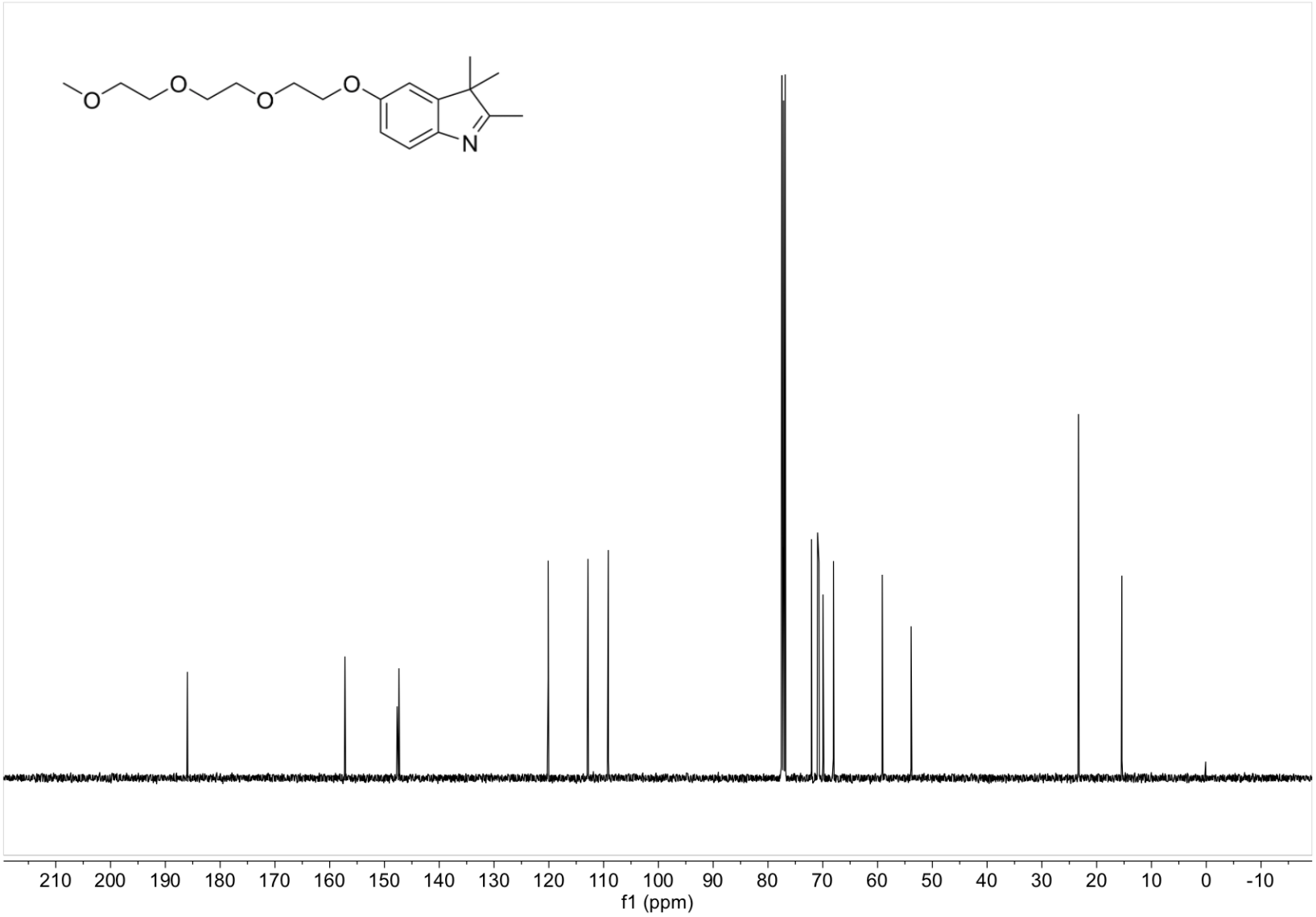


Figure S11: ^{13}C NMR of 2-Peg

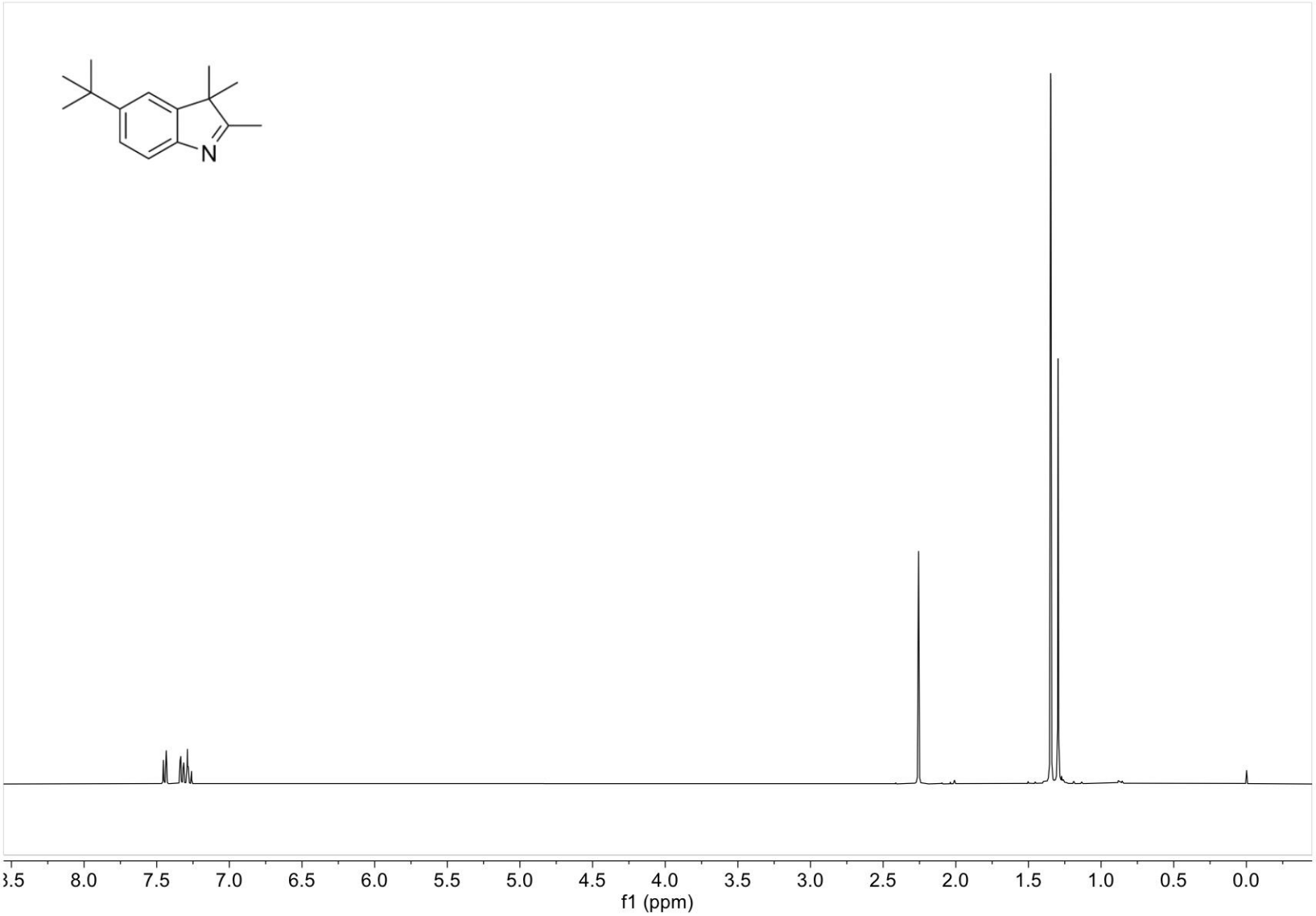


Figure S12: ^{1}H NMR of 2-tBu

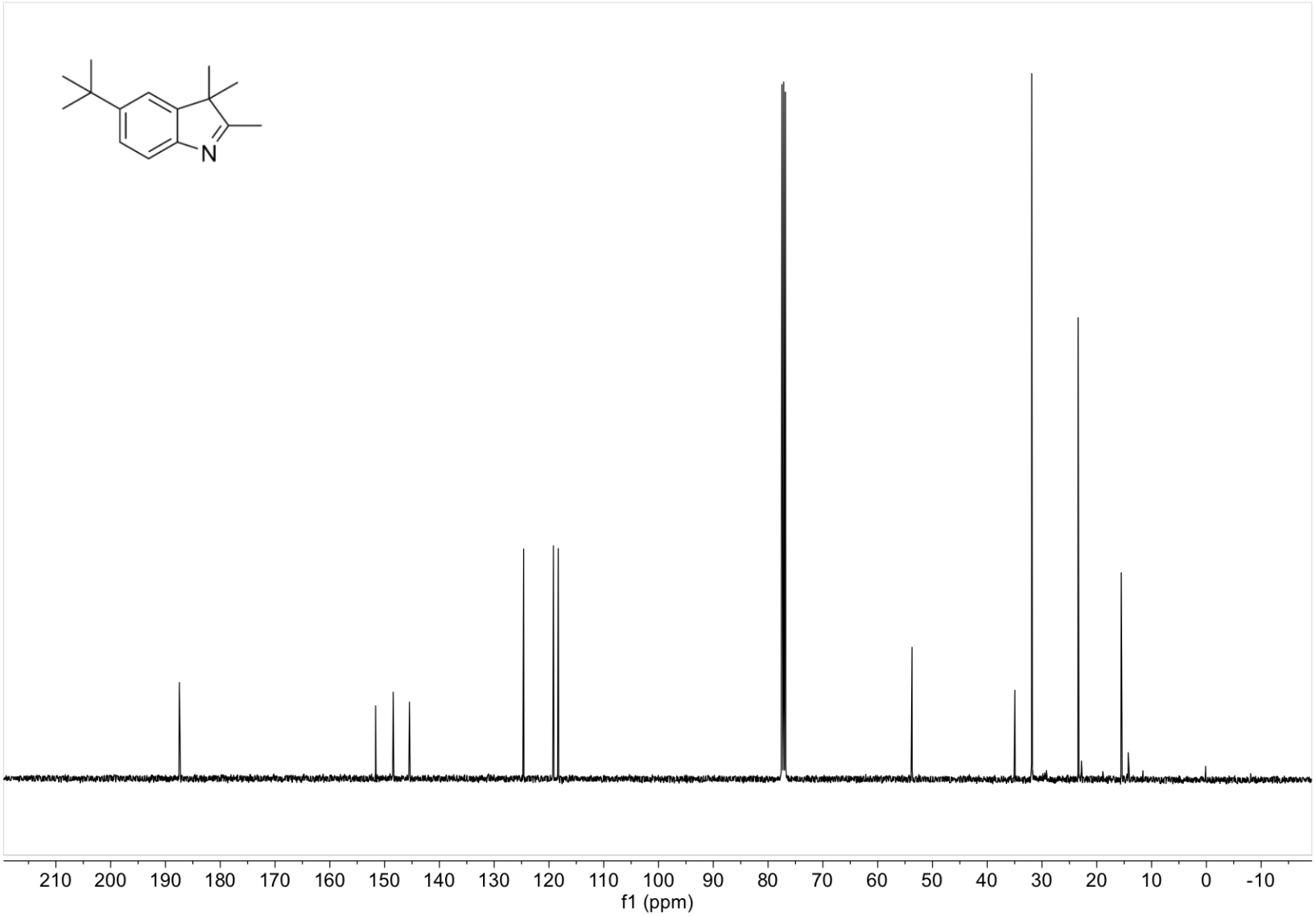


Figure S13: ^{13}C NMR of 2-tBu

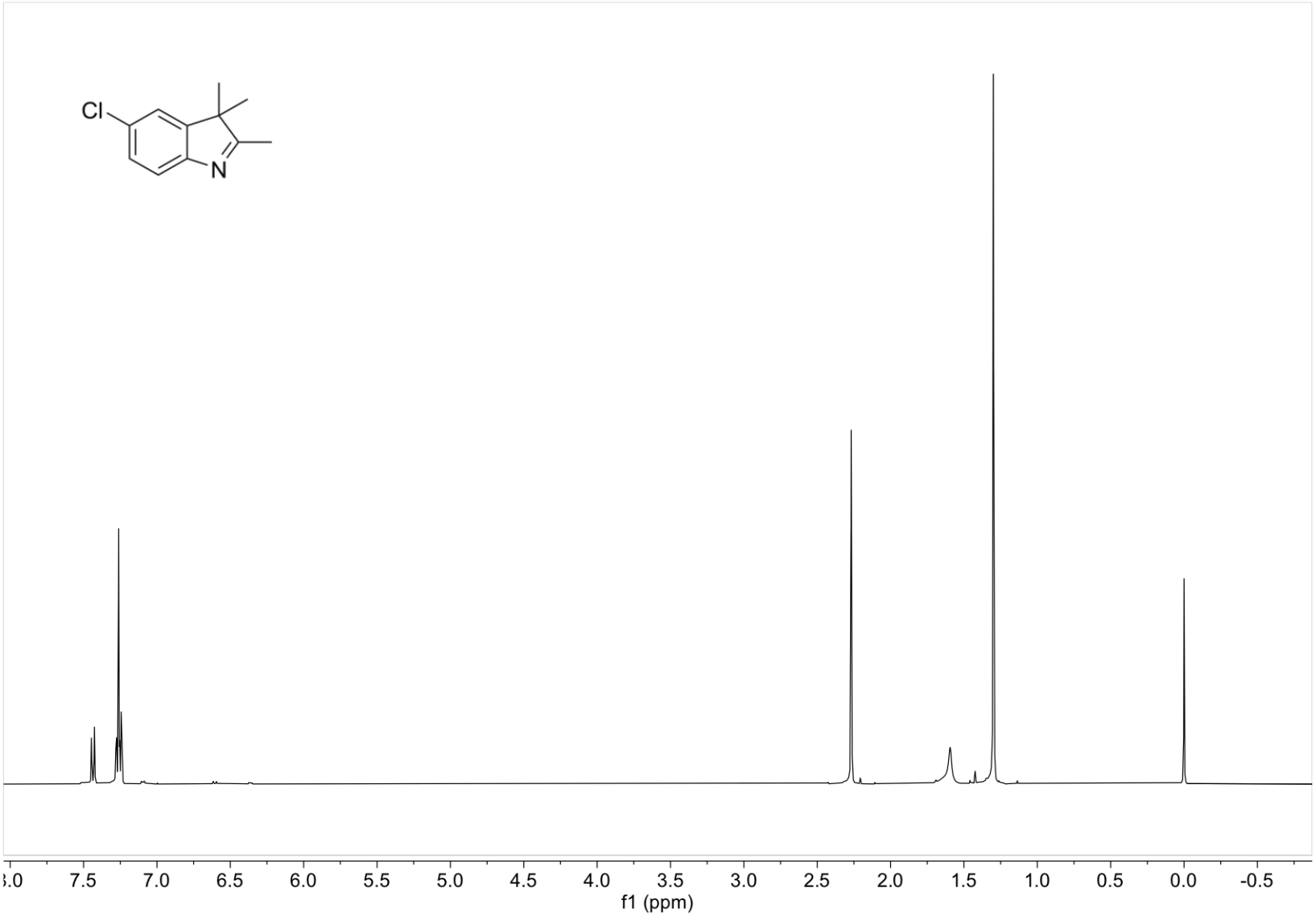


Figure S14: ^{1}H NMR of 2-Cl

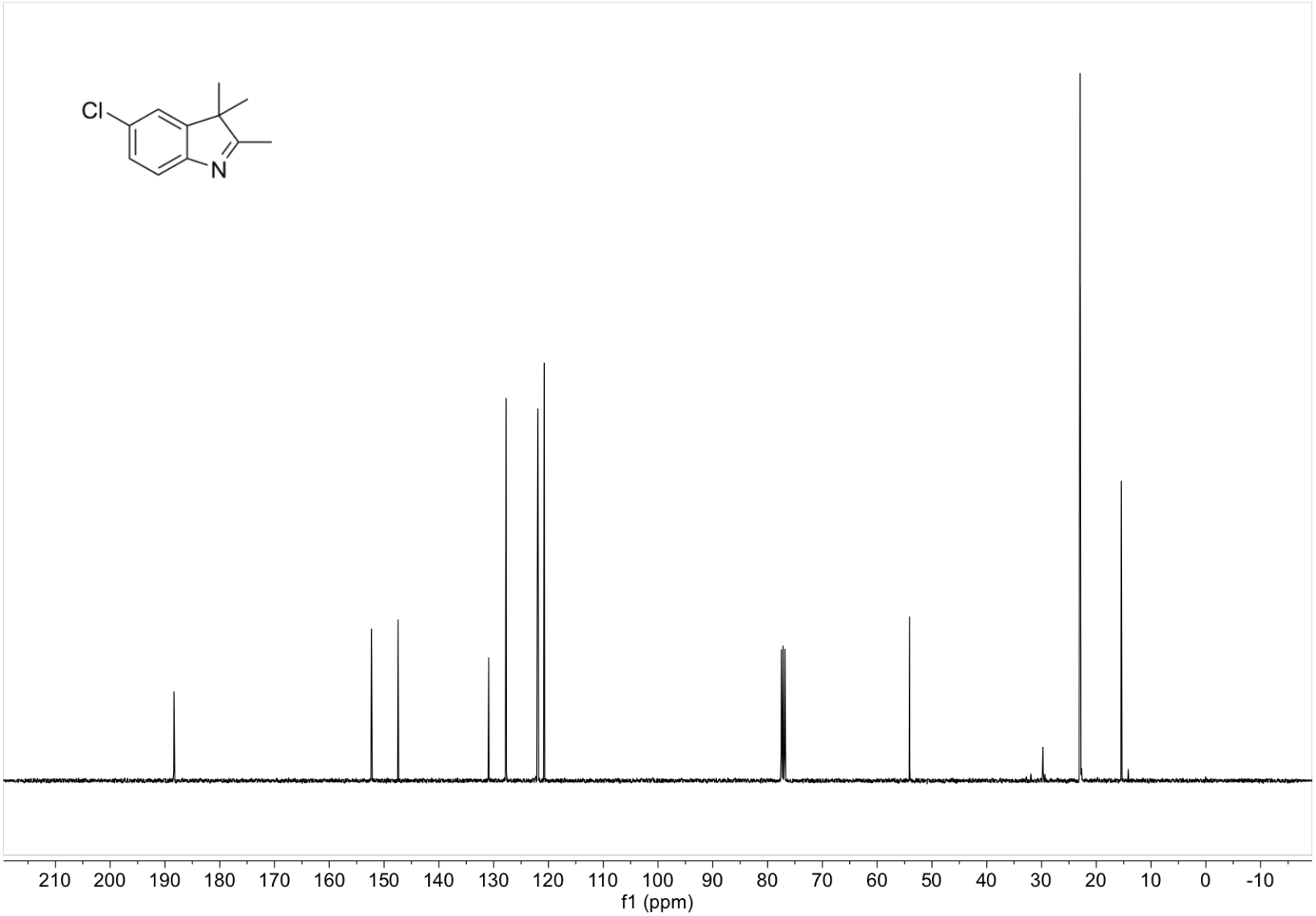


Figure S15: ^{13}C NMR of 2-Cl

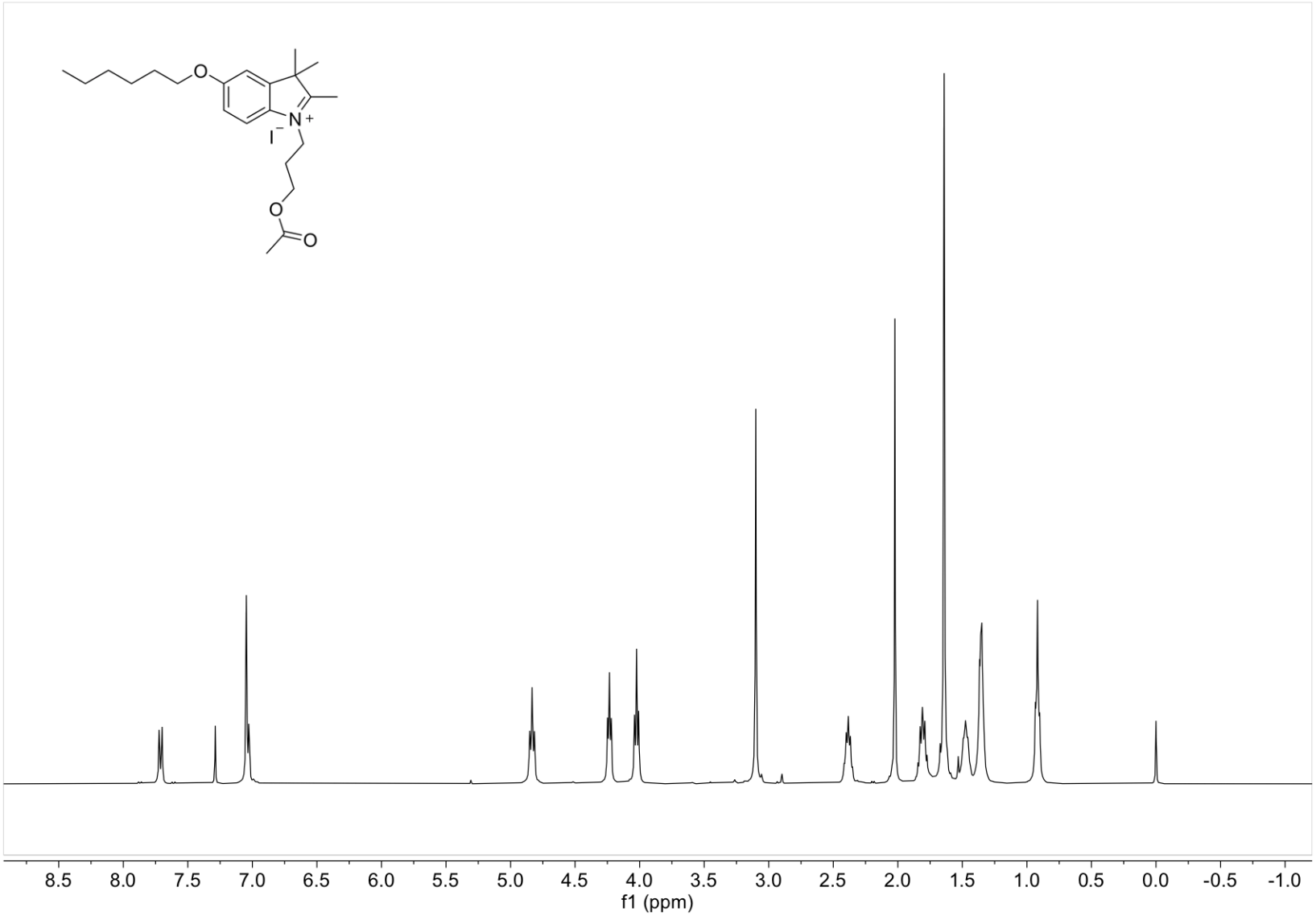


Figure S16: ^{1}H NMR of 3-hex

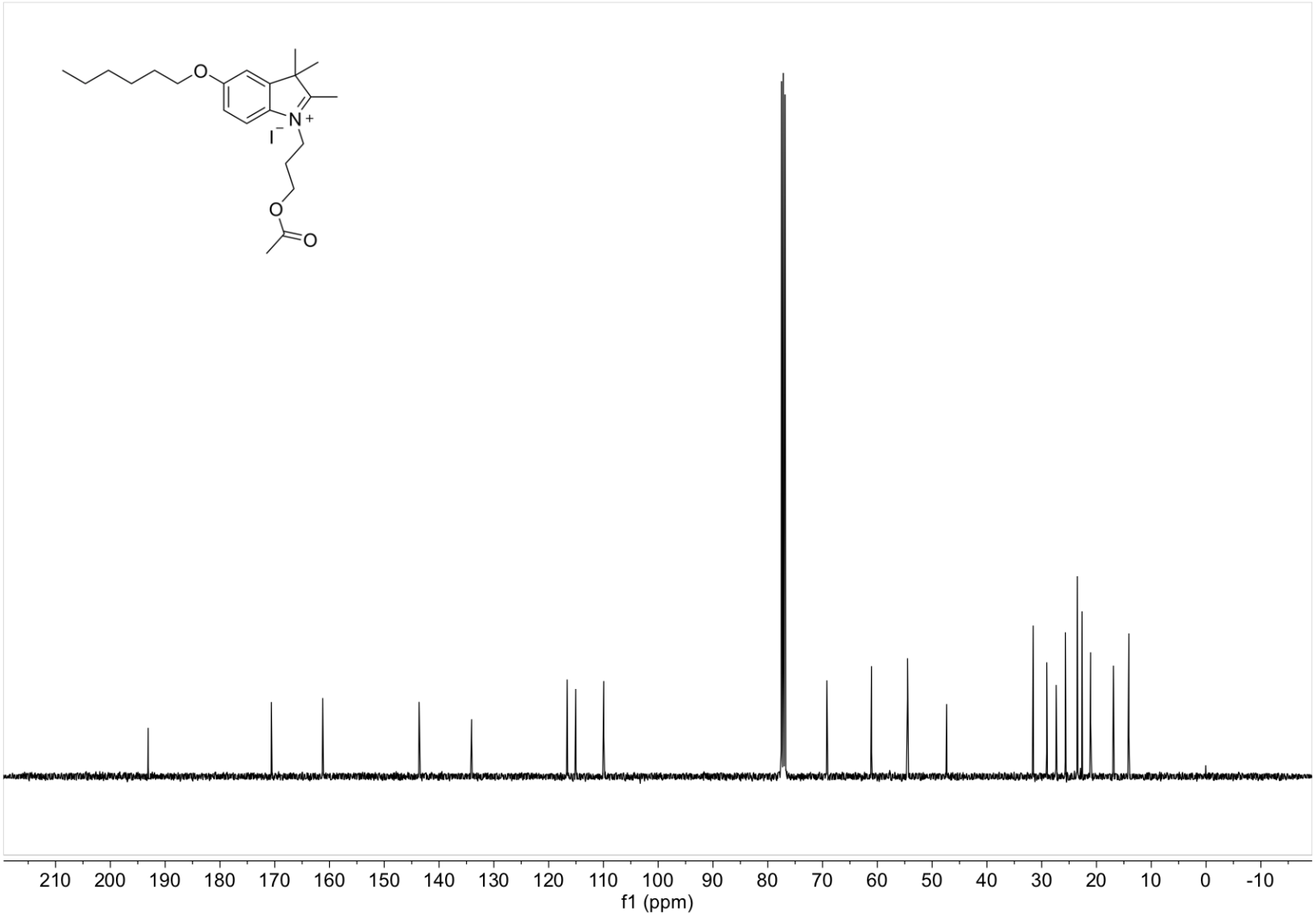


Figure S17: ^{13}C NMR of 3-hex

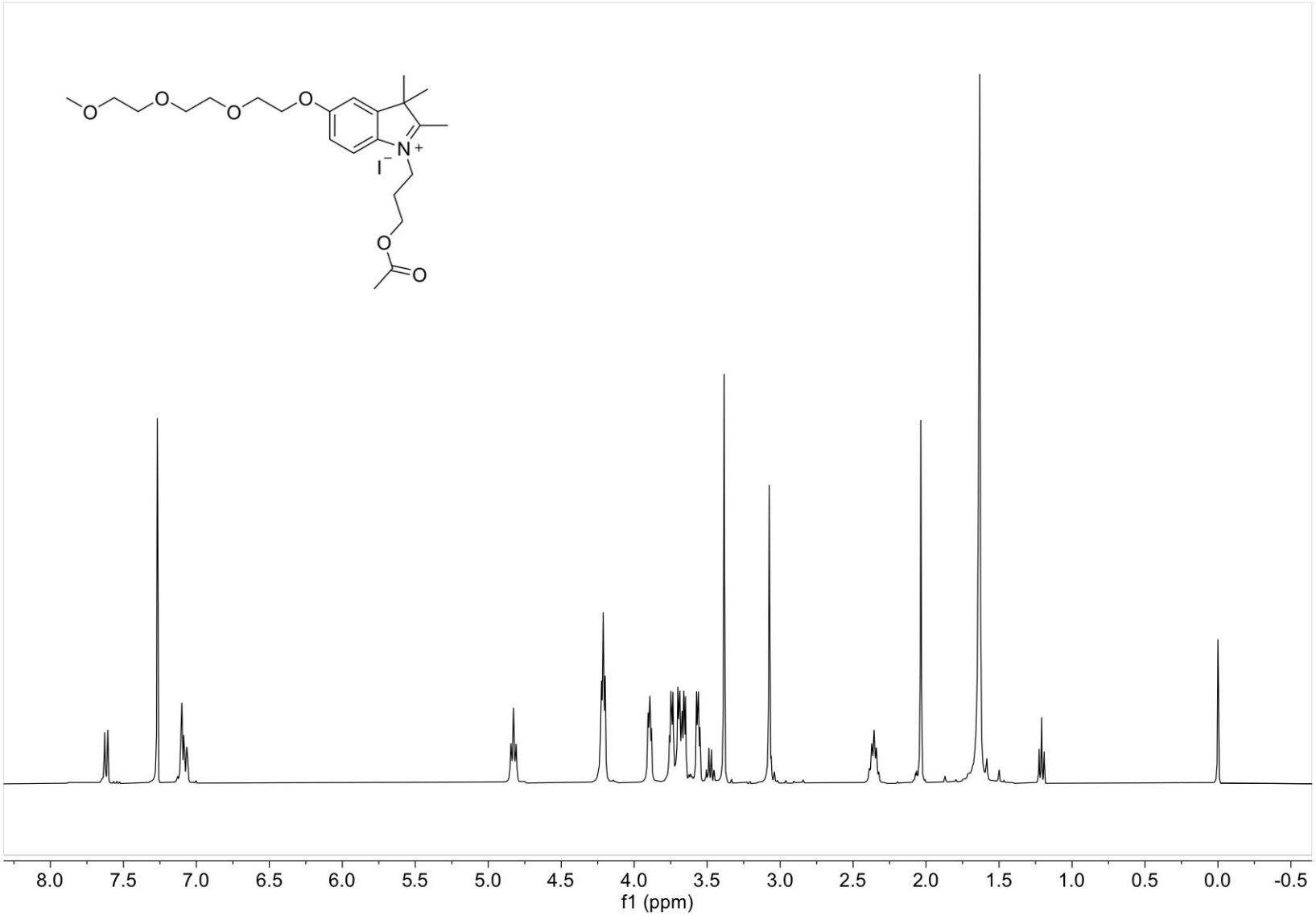


Figure S18: ^{1}H NMR of 3-Peg

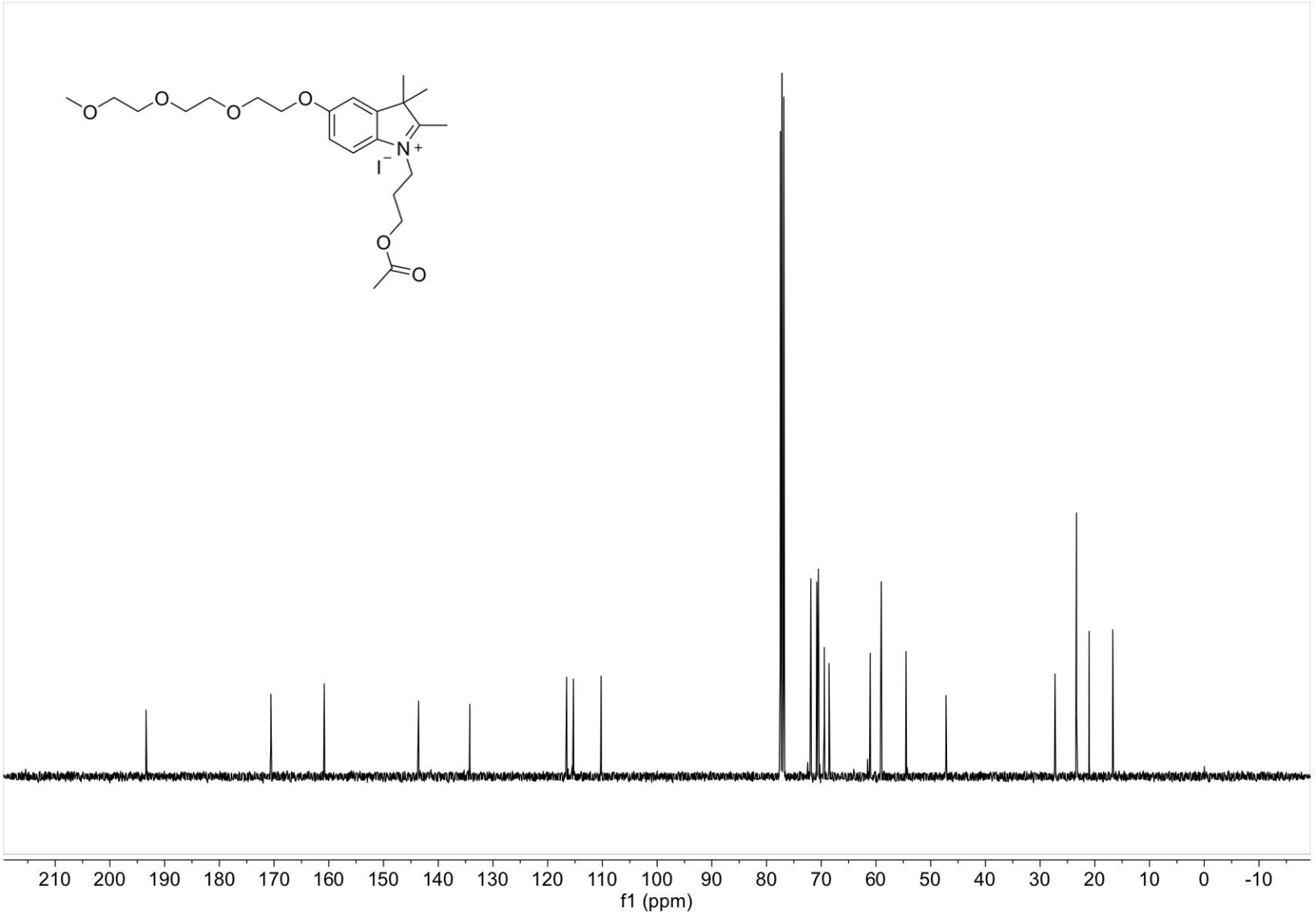


Figure S19: ^{13}C NMR of 3-Peg

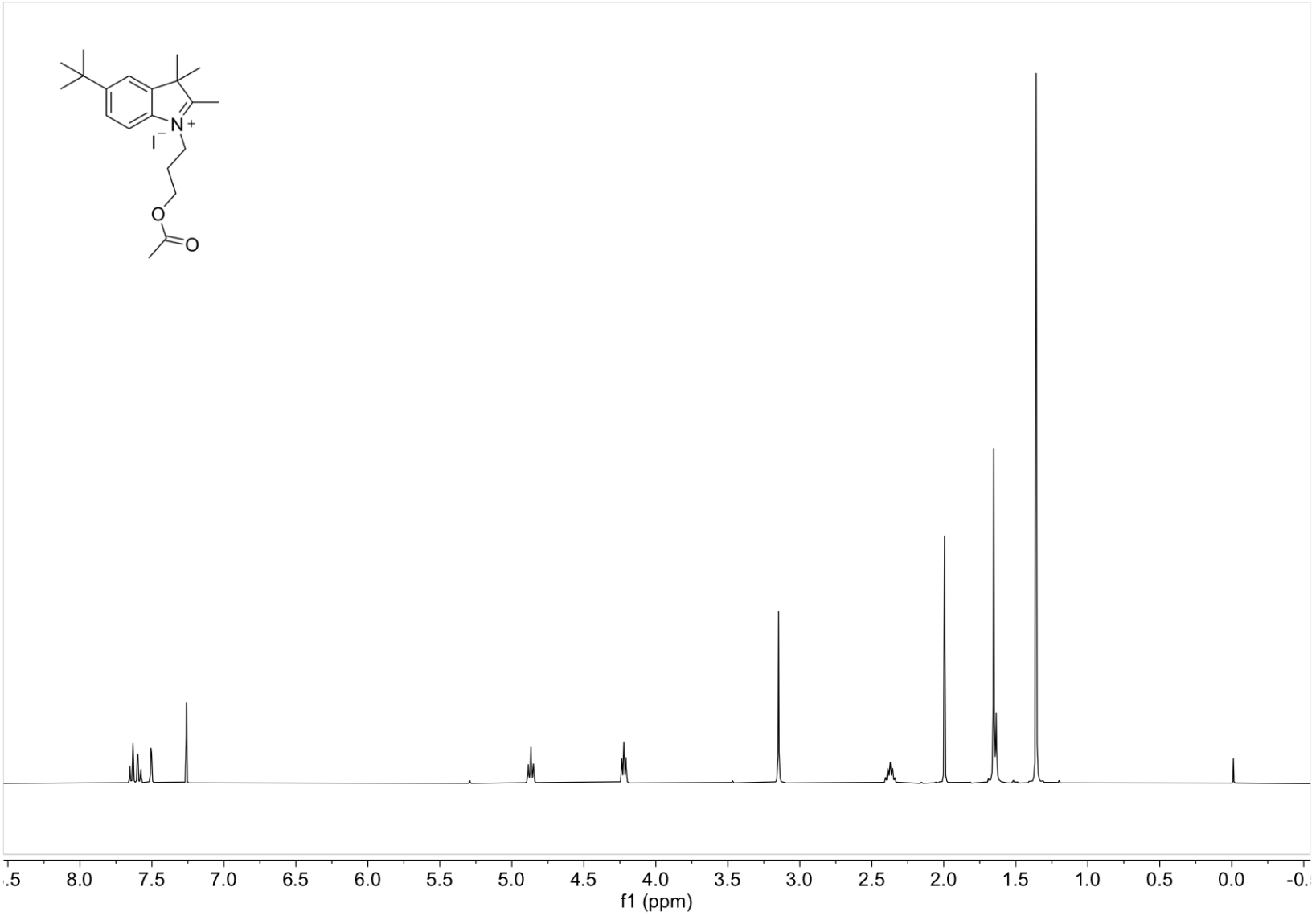


Figure S20: ^{1}H NMR of 3-tBu

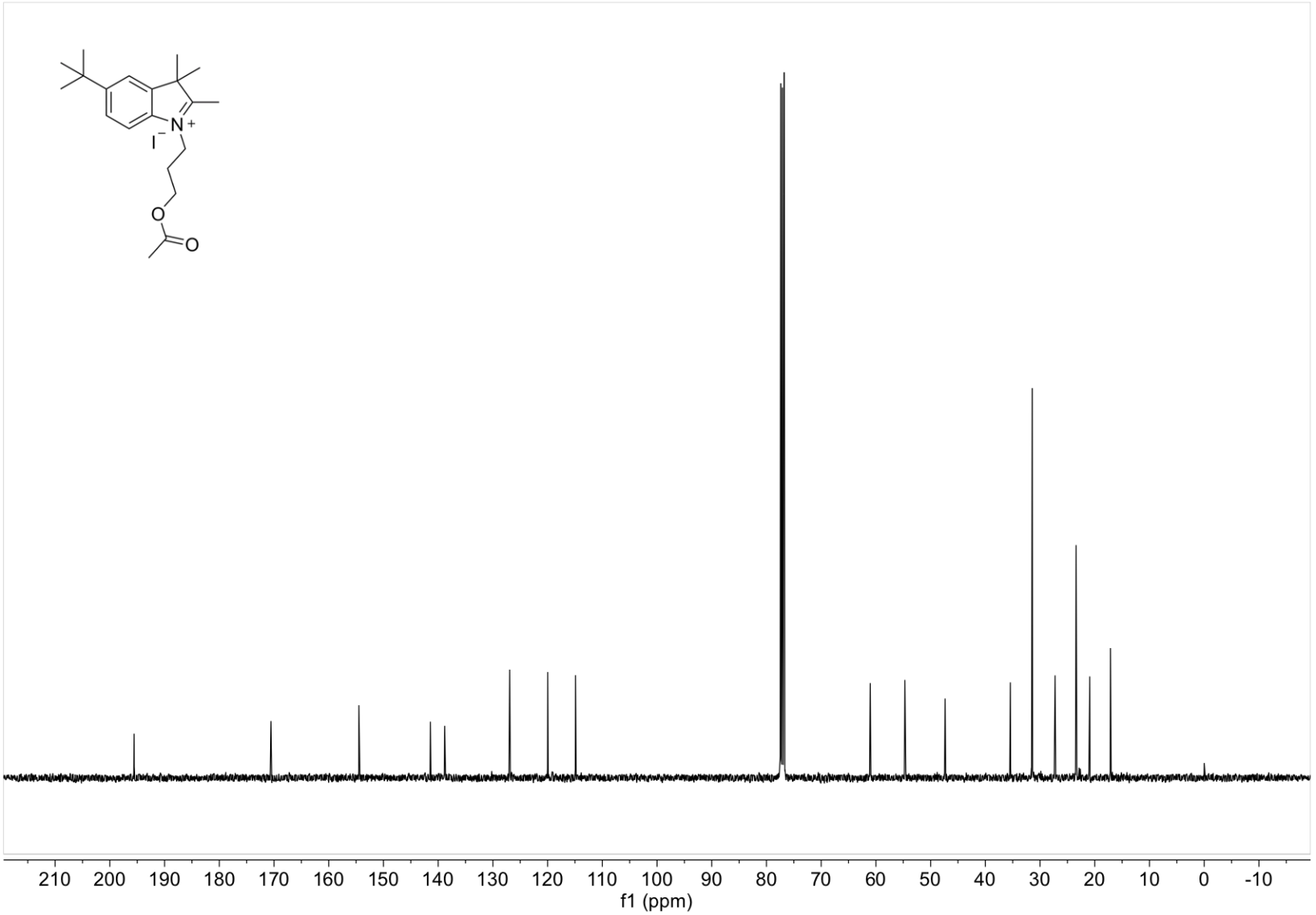


Figure S21: ^{13}C NMR of 3-tBu

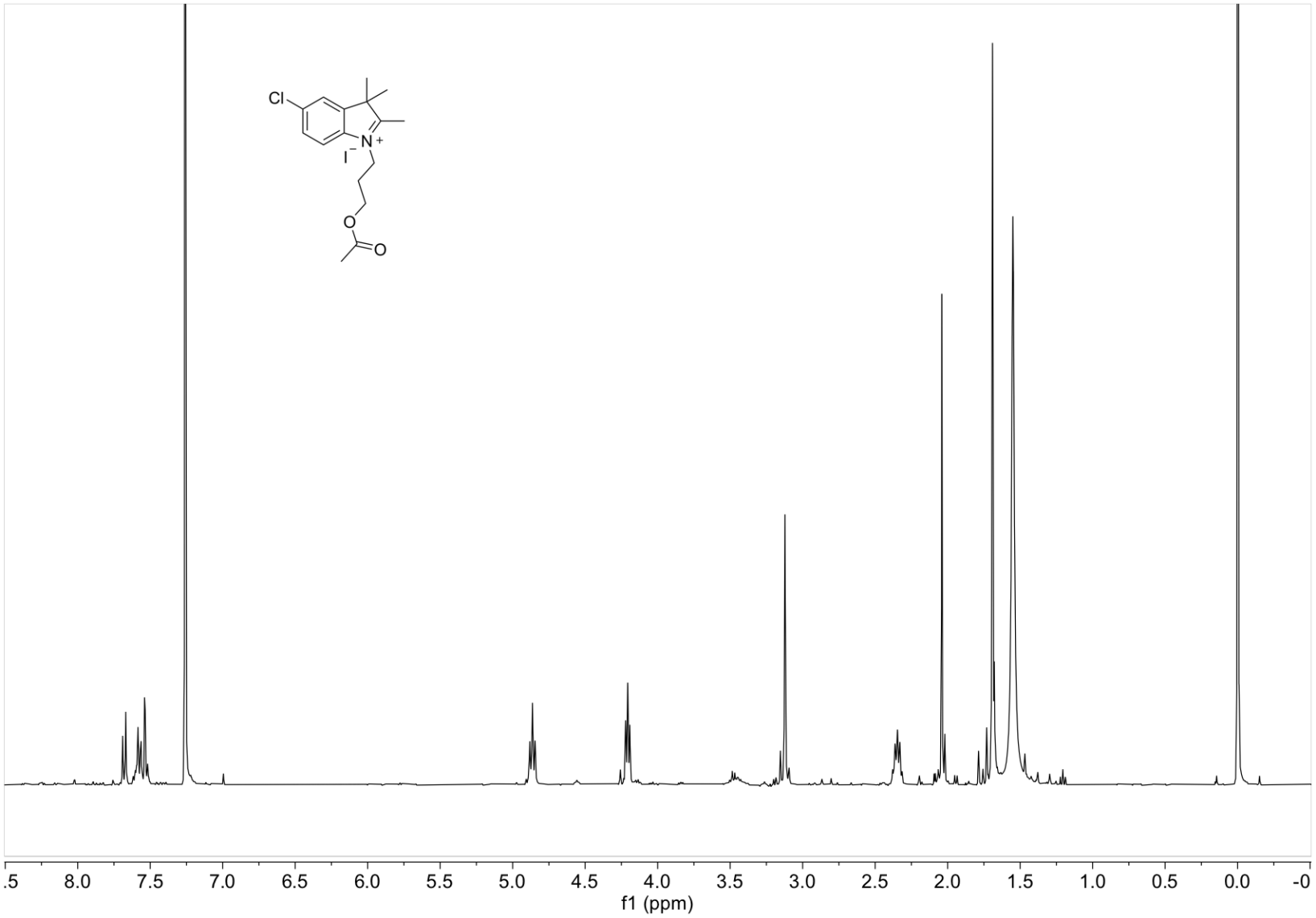


Figure S22: ^{1}H NMR of 3-Cl

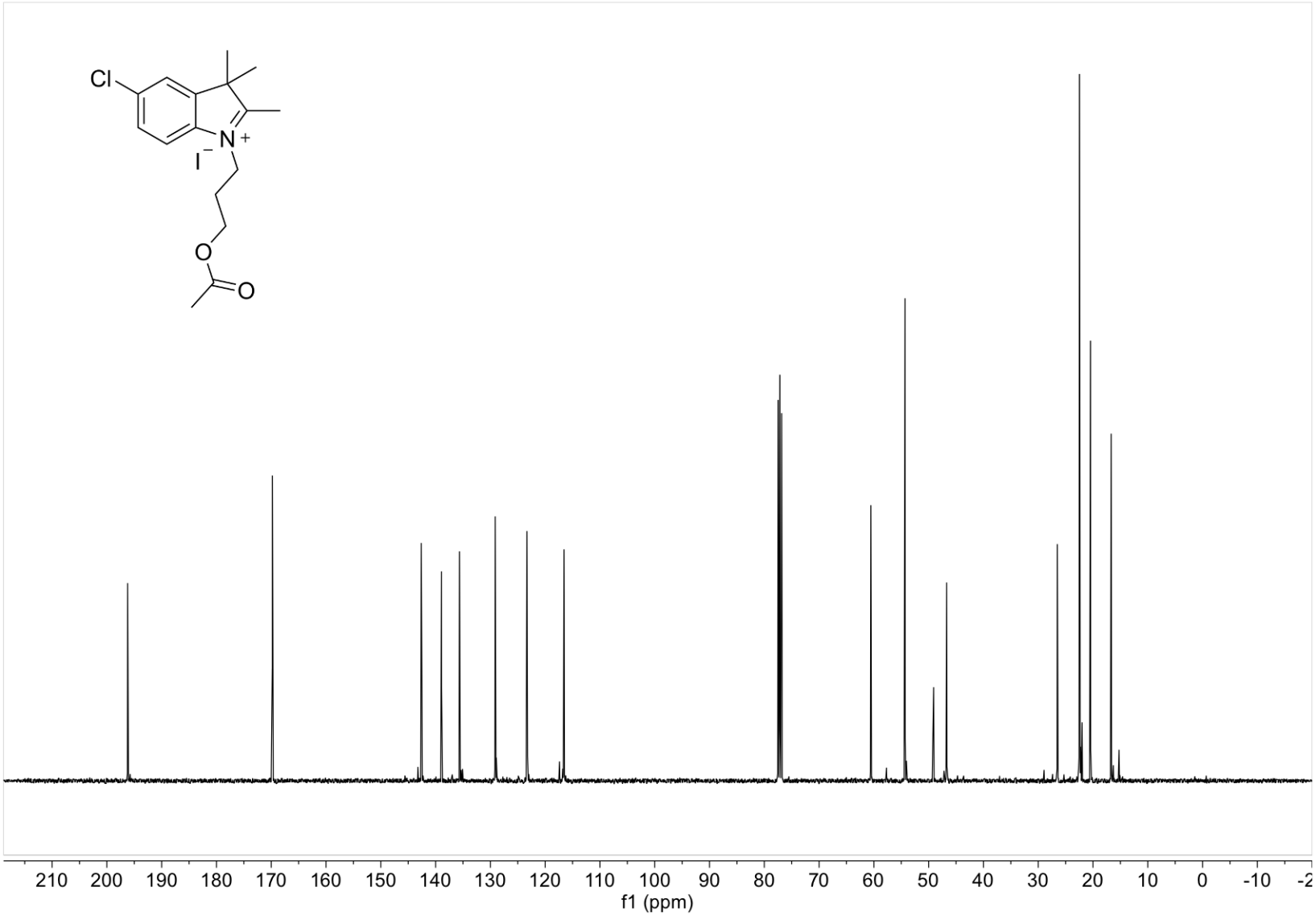


Figure S23: ^{13}C NMR of 3-Cl

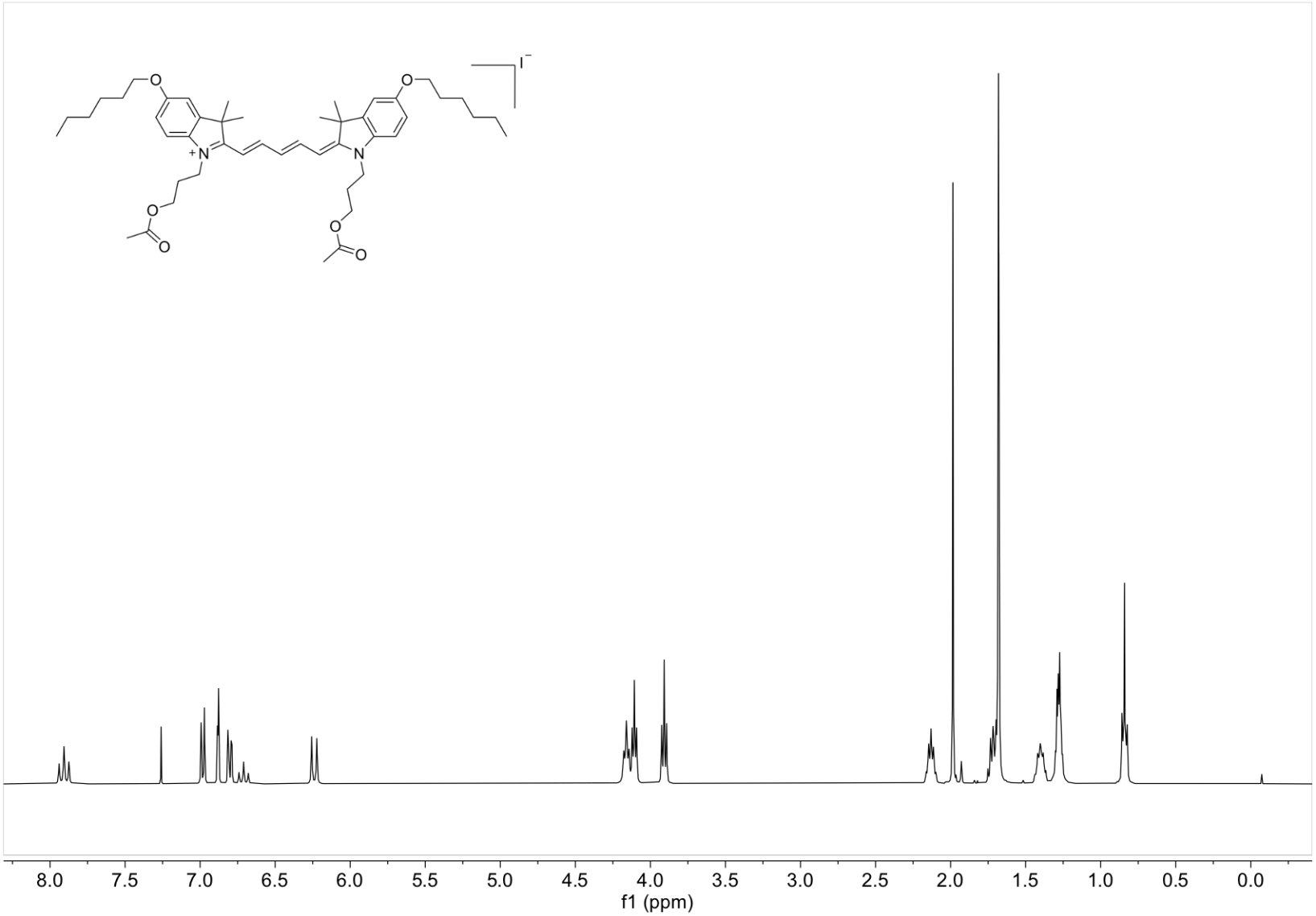


Figure S24: ^{1}H NMR of 4-hex

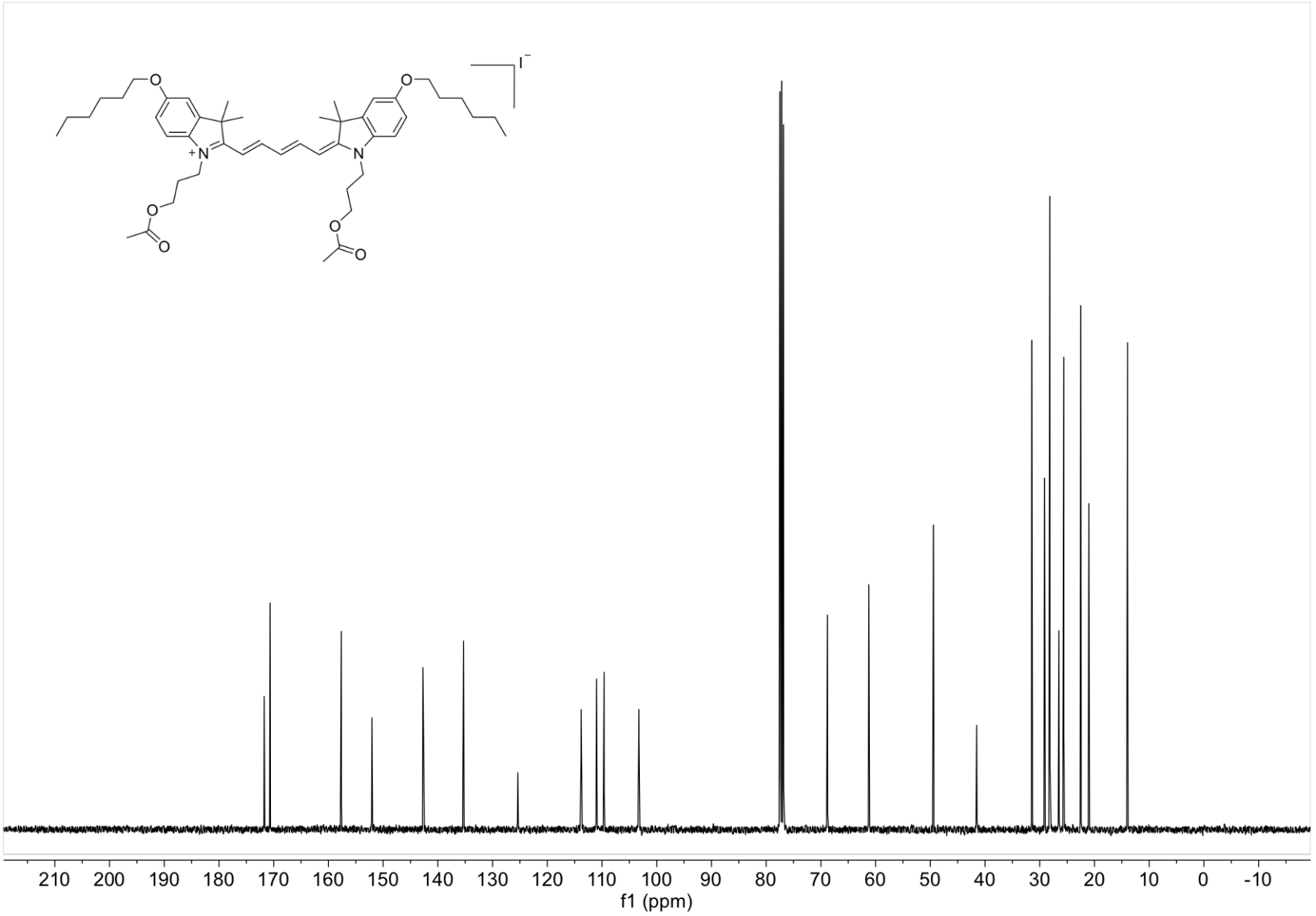


Figure S25: ^{13}C NMR of 4-hex

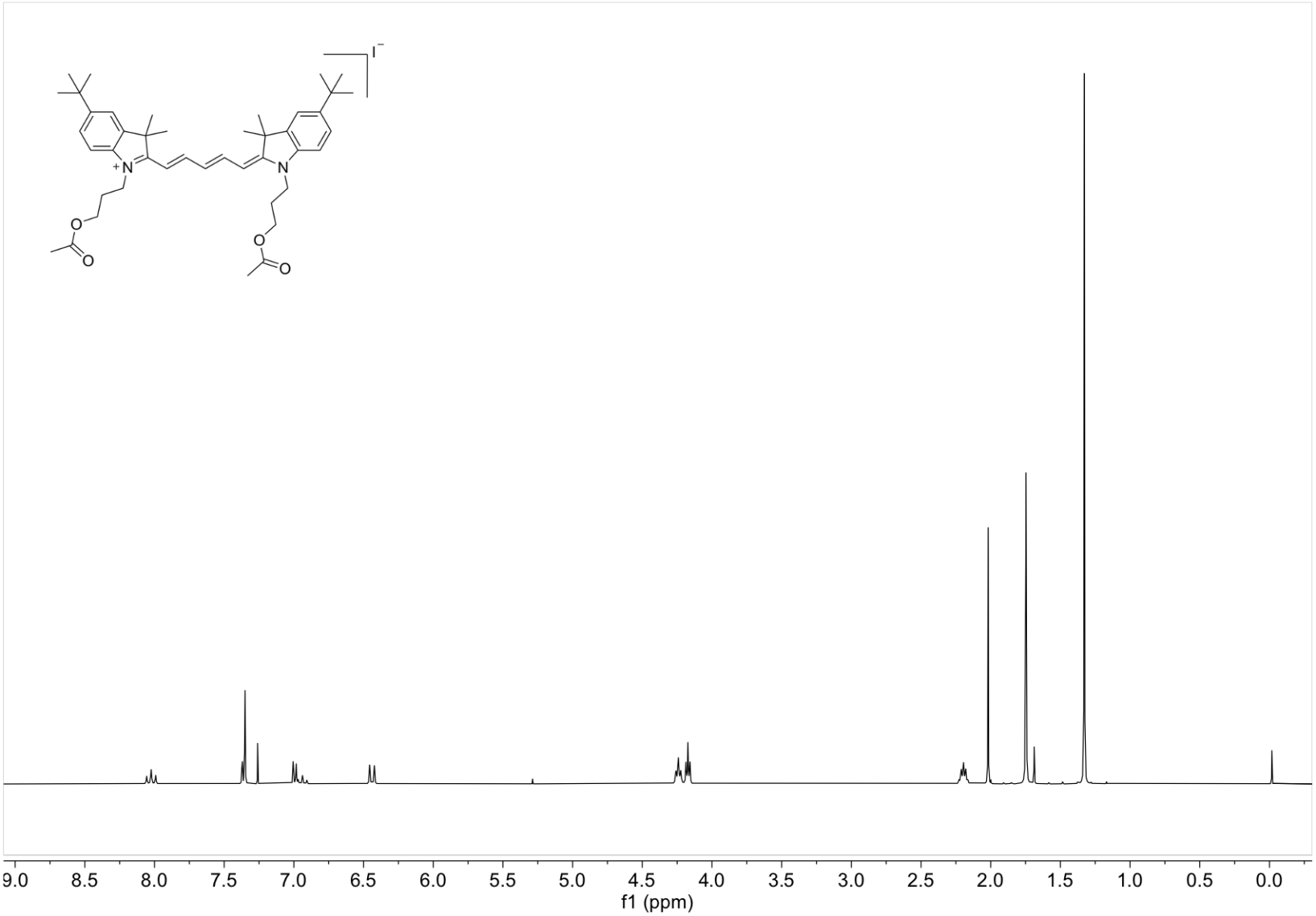


Figure S26: ^{1}H NMR of 4-tBu

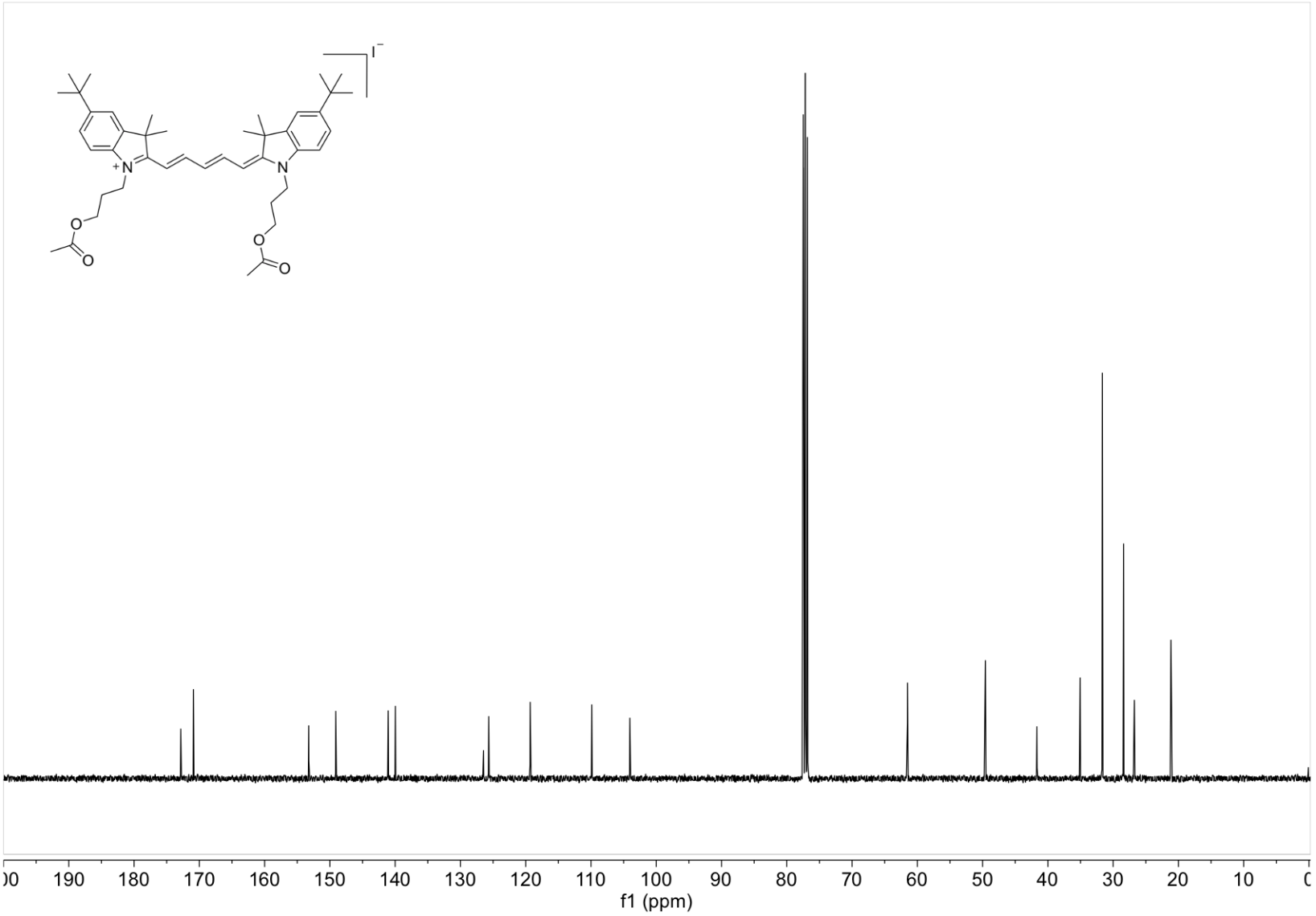


Figure S27: ^{13}C NMR of 4-tBu

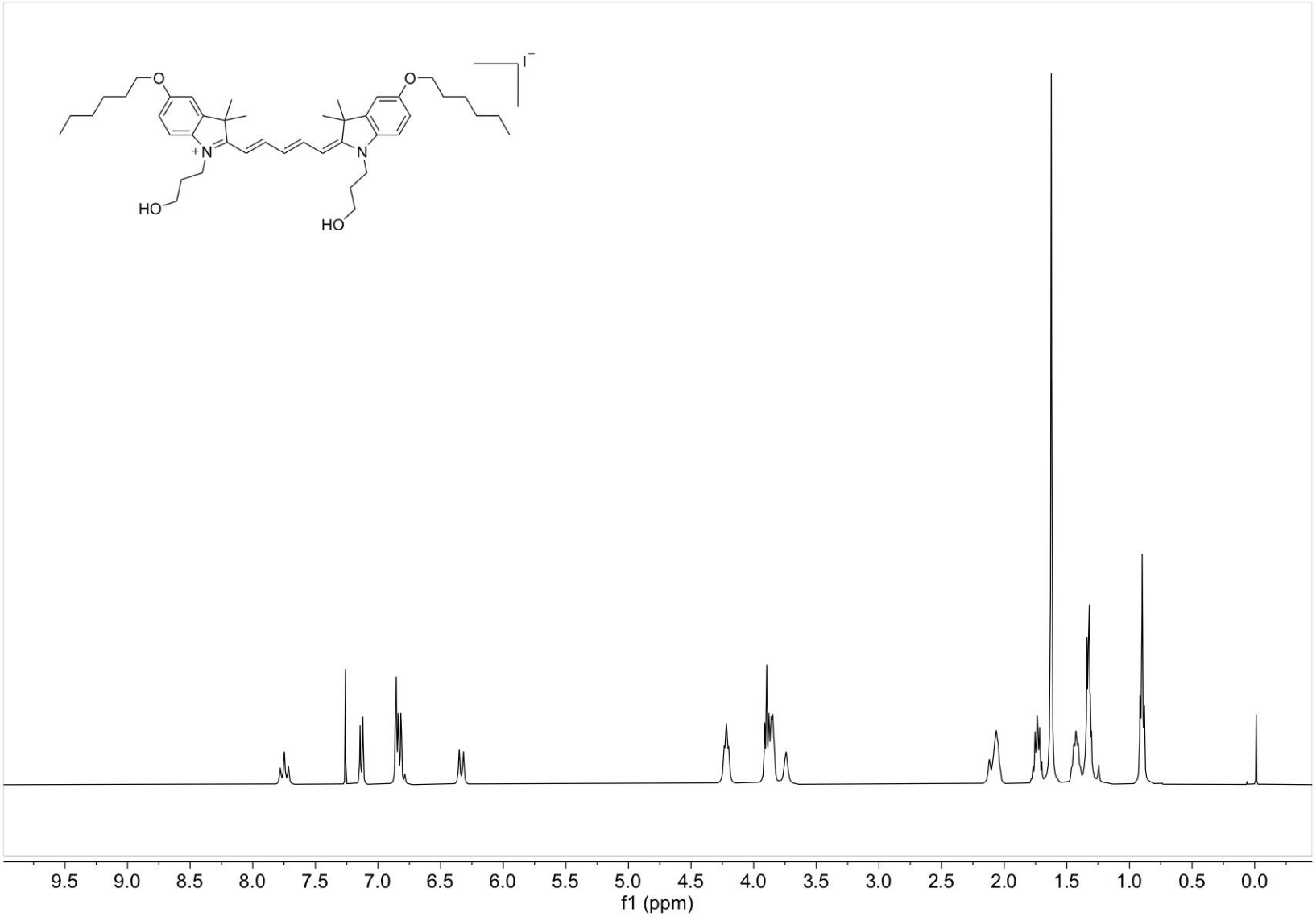


Figure S28: ^{13}C NMR of 5-hex

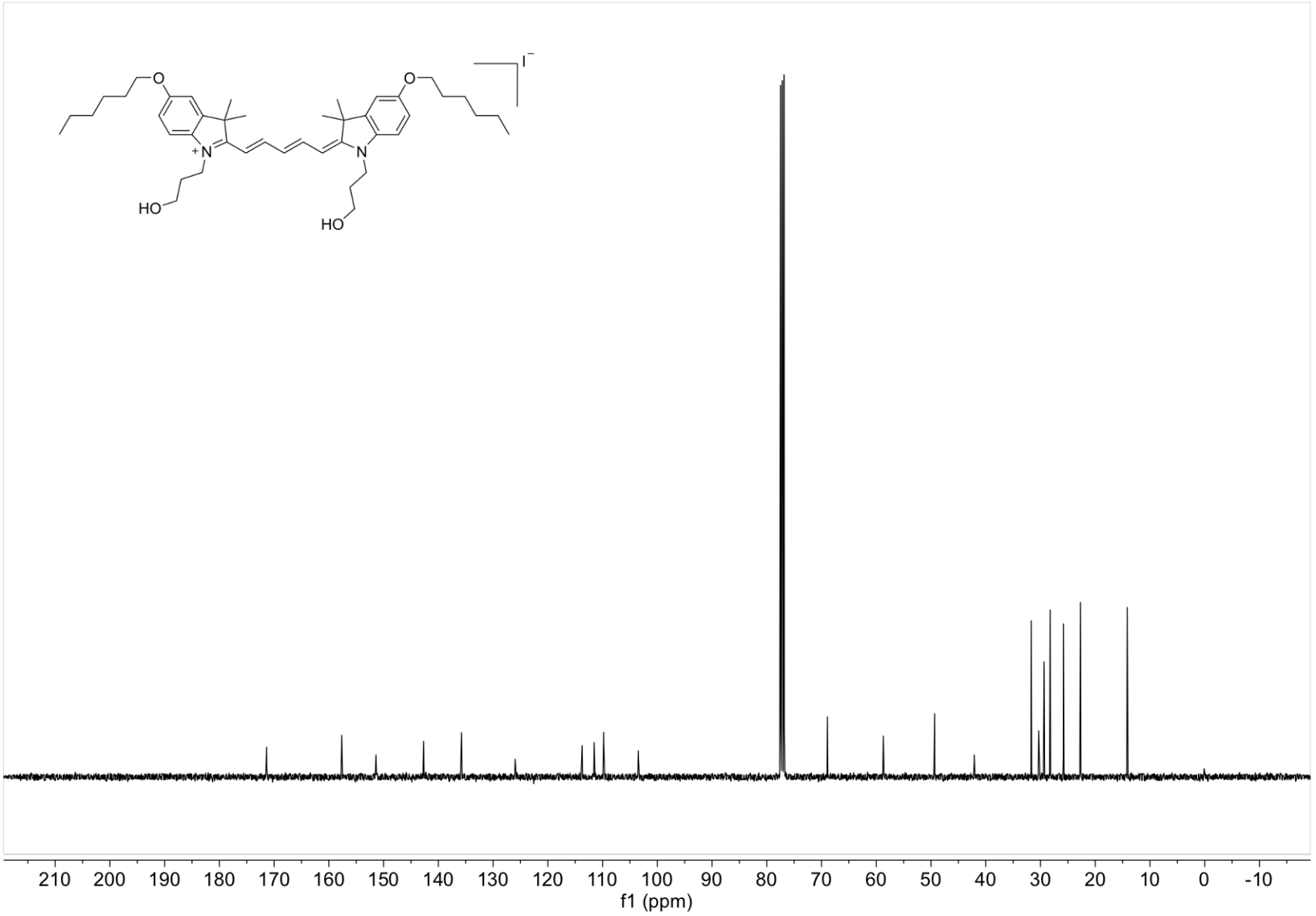


Figure S29: ^{13}C NMR of 5-hex

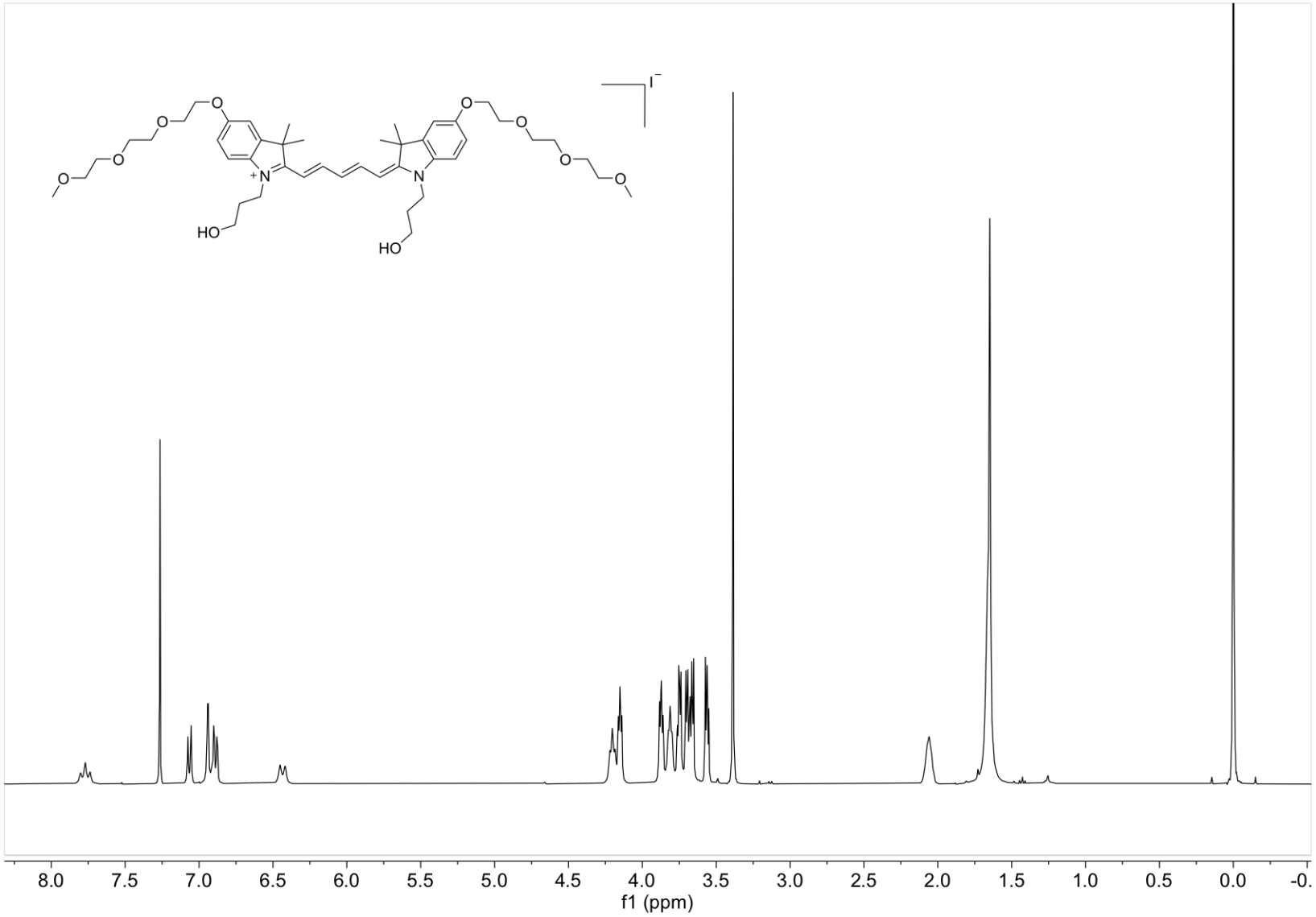


Figure S30: ^{1}H NMR of 5-Peg

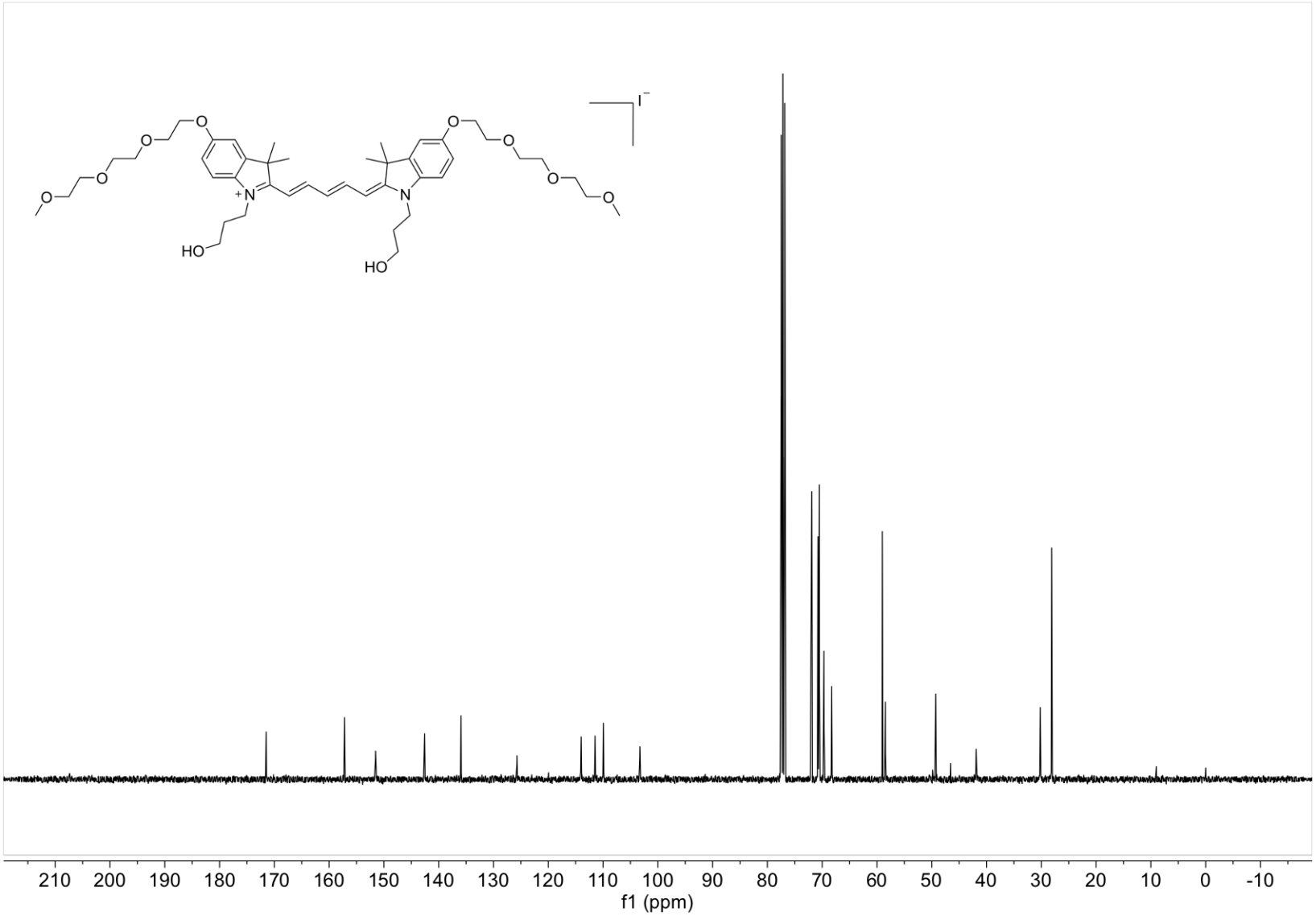


Figure S31: ^{13}C NMR of 5-Peg

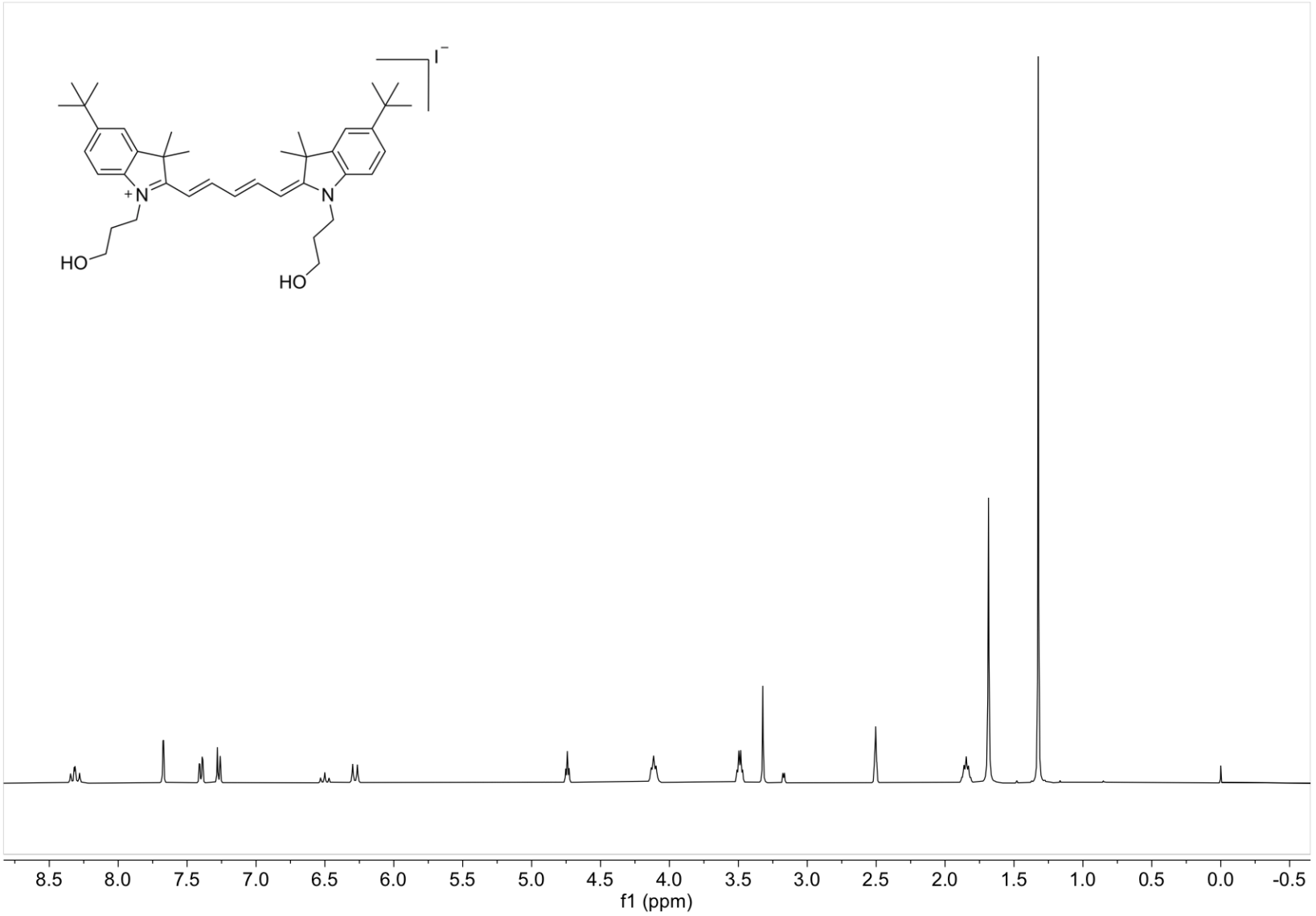


Figure S32: ^{1}H NMR of 5-tBu

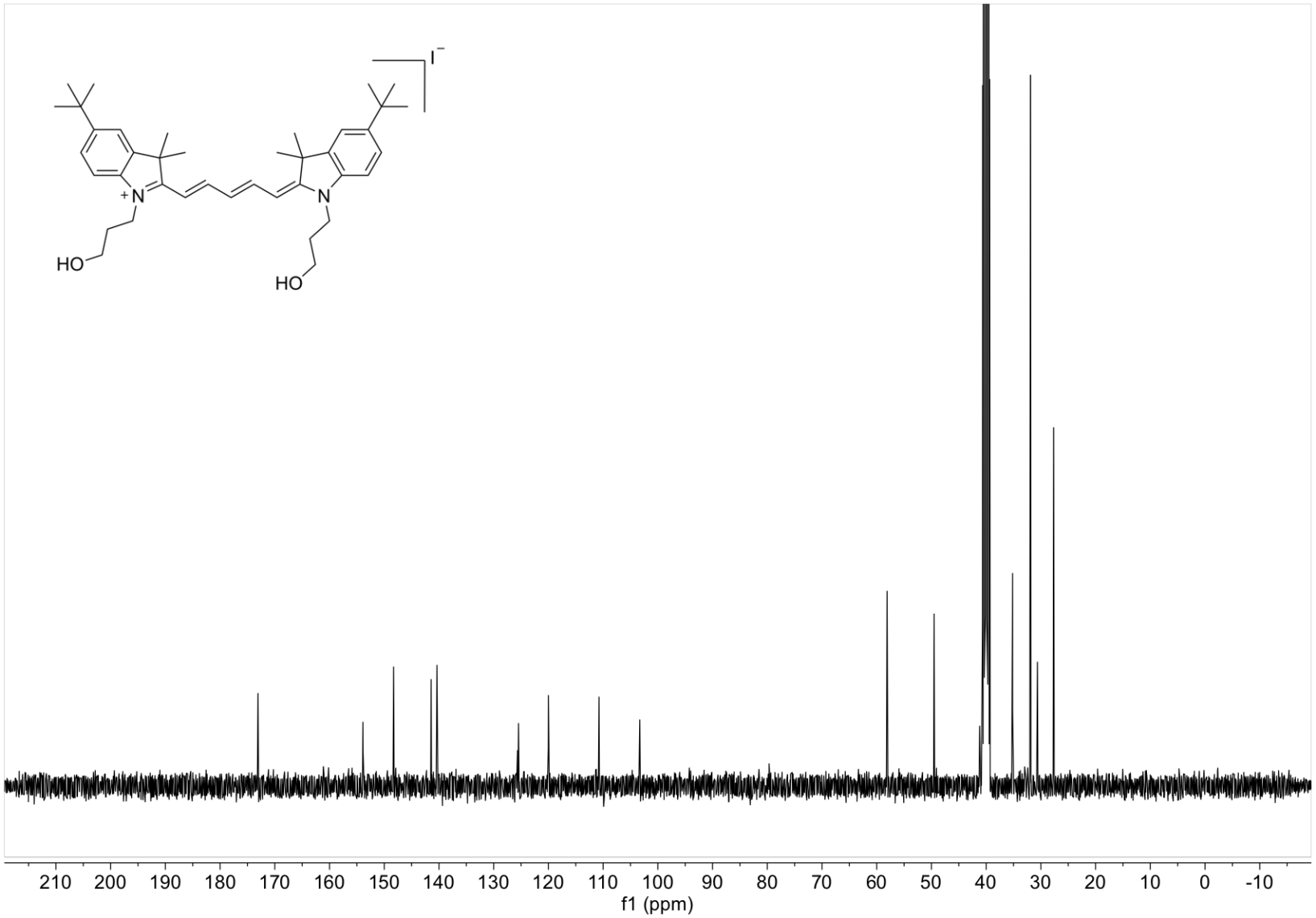


Figure S33: ^{13}C NMR of 5-tBu

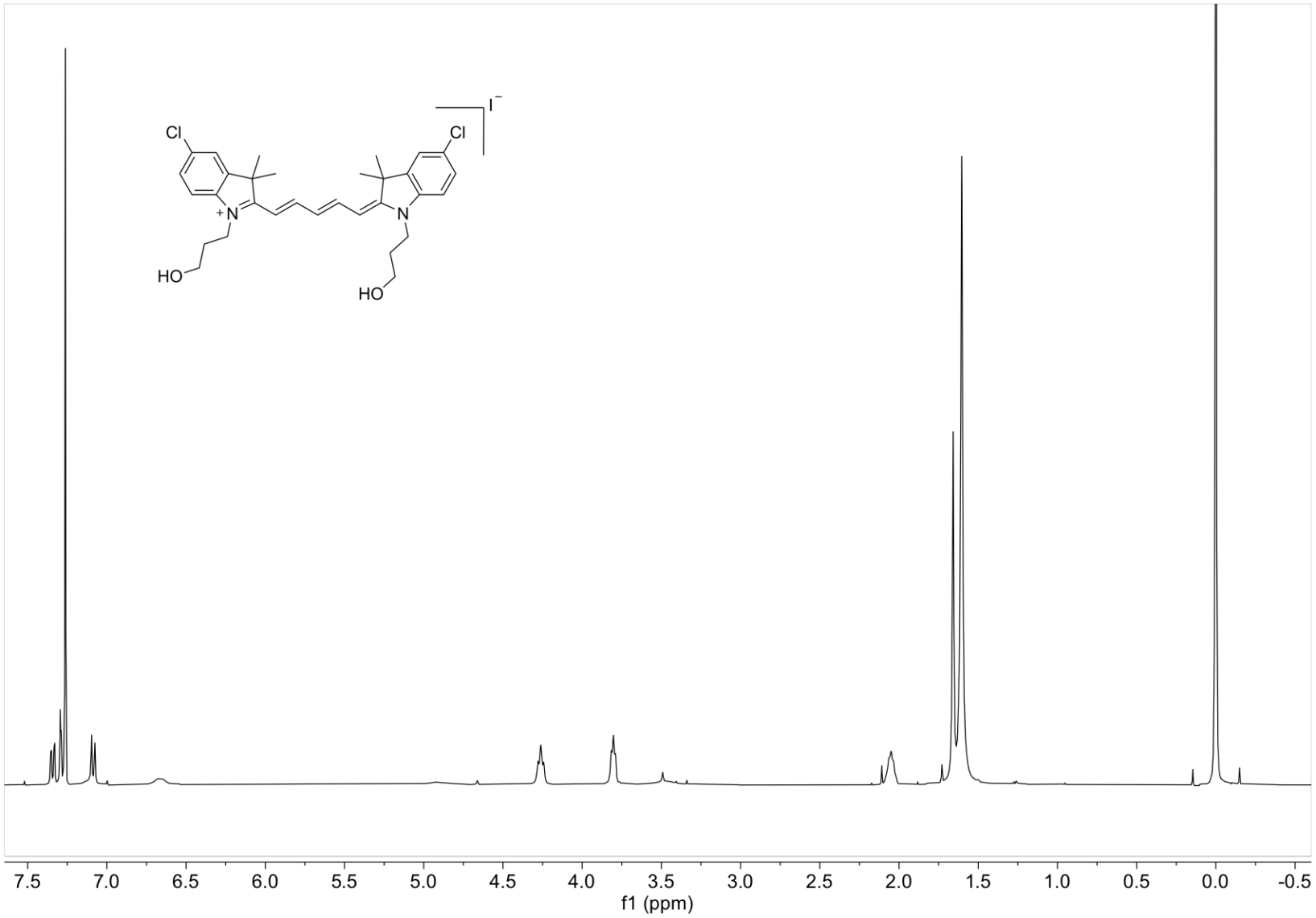


Figure S34: ^{13}C NMR of 5-Cl

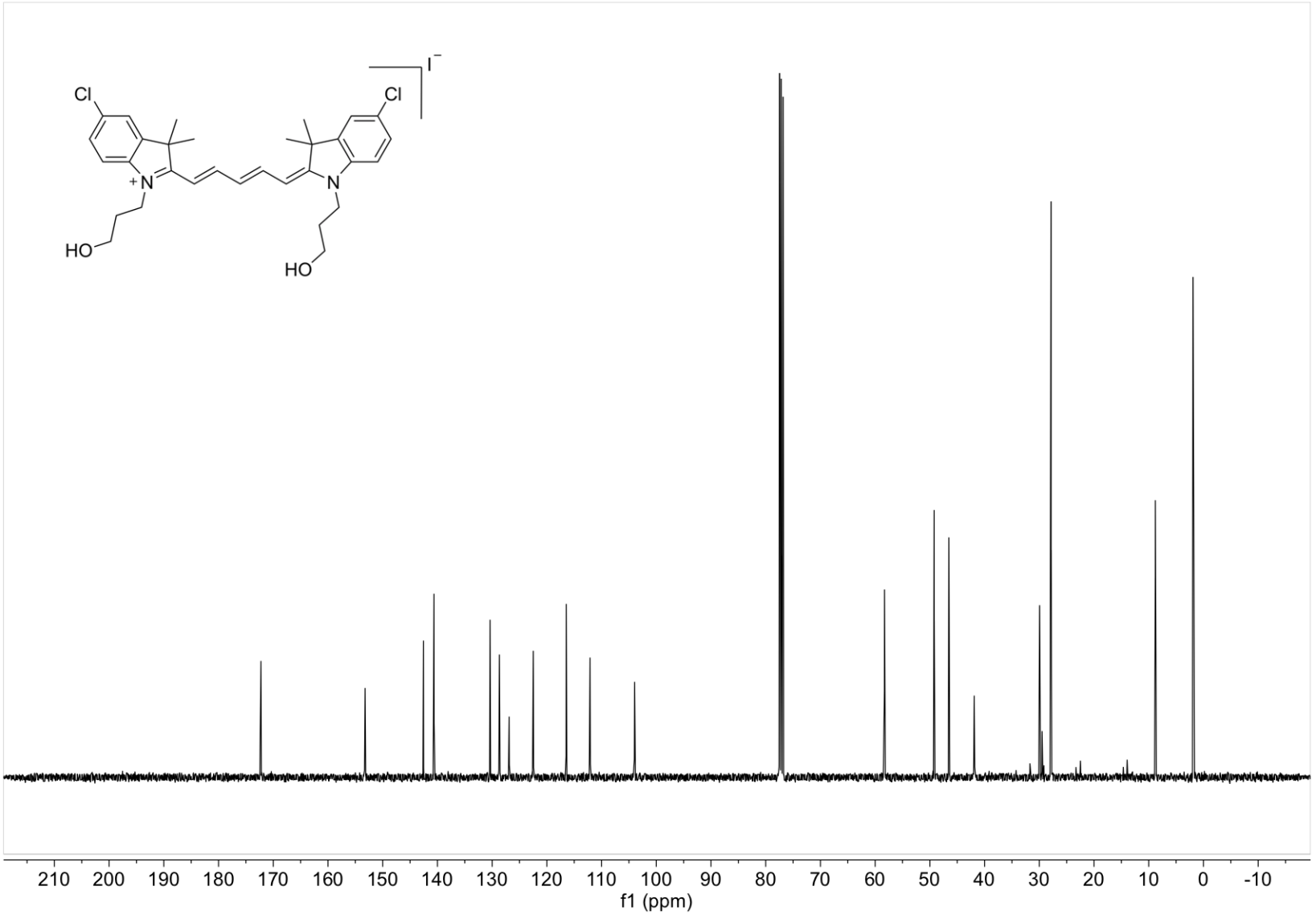


Figure S35: ^{13}C NMR of 5-Cl

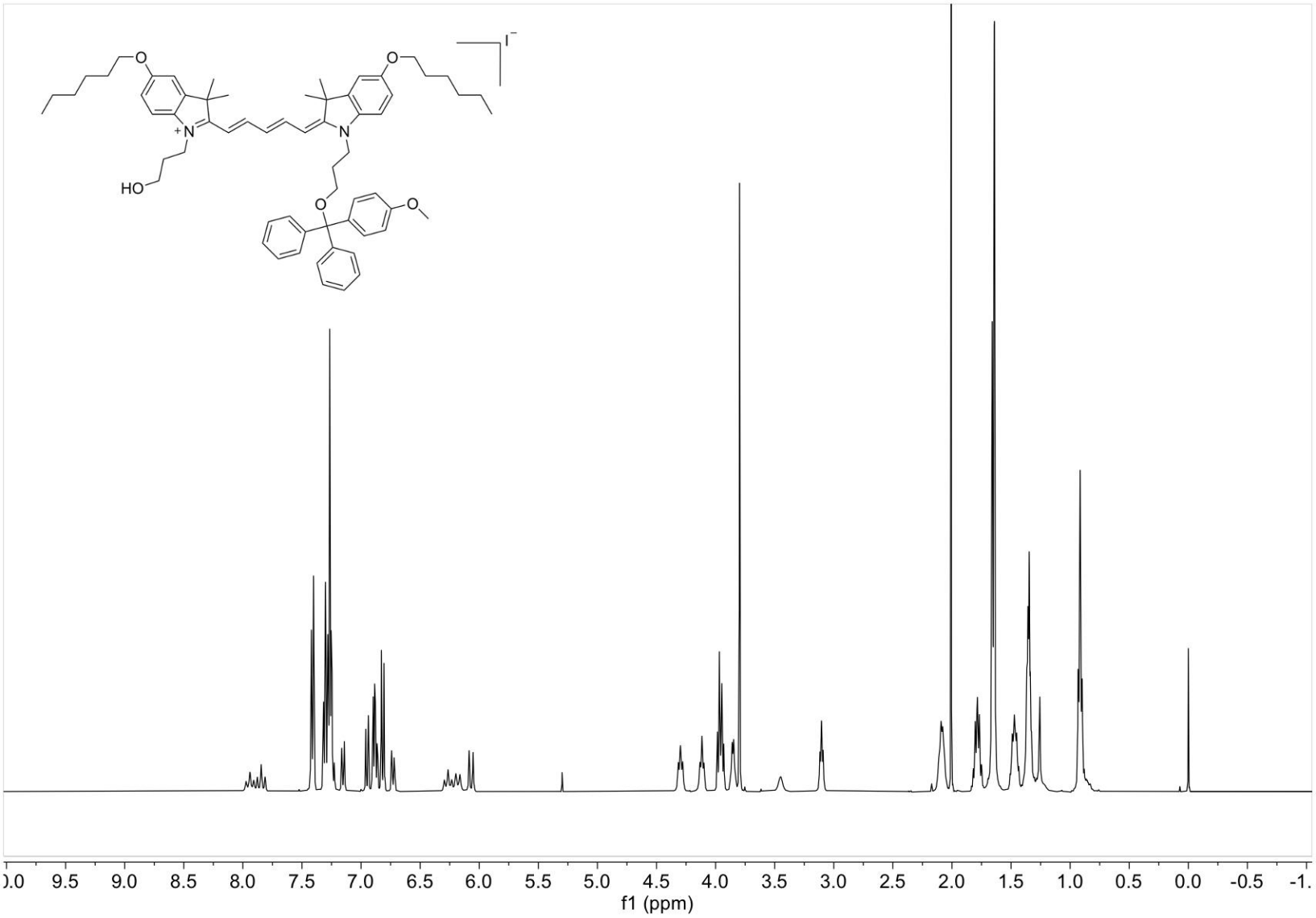


Figure S36: ^{1}H NMR of Cy5-hex

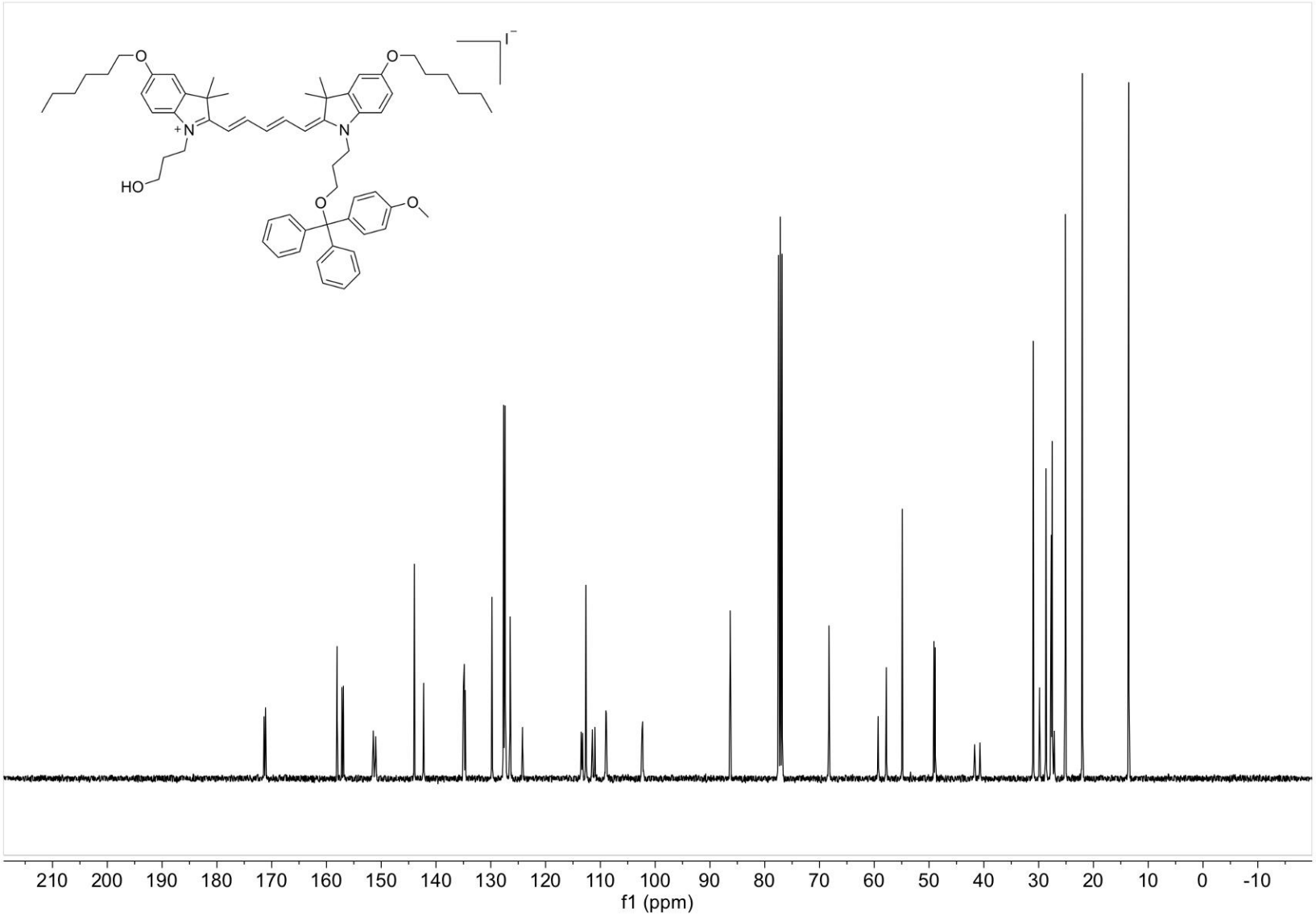


Figure S37: ^{13}C NMR of Cy5-hex

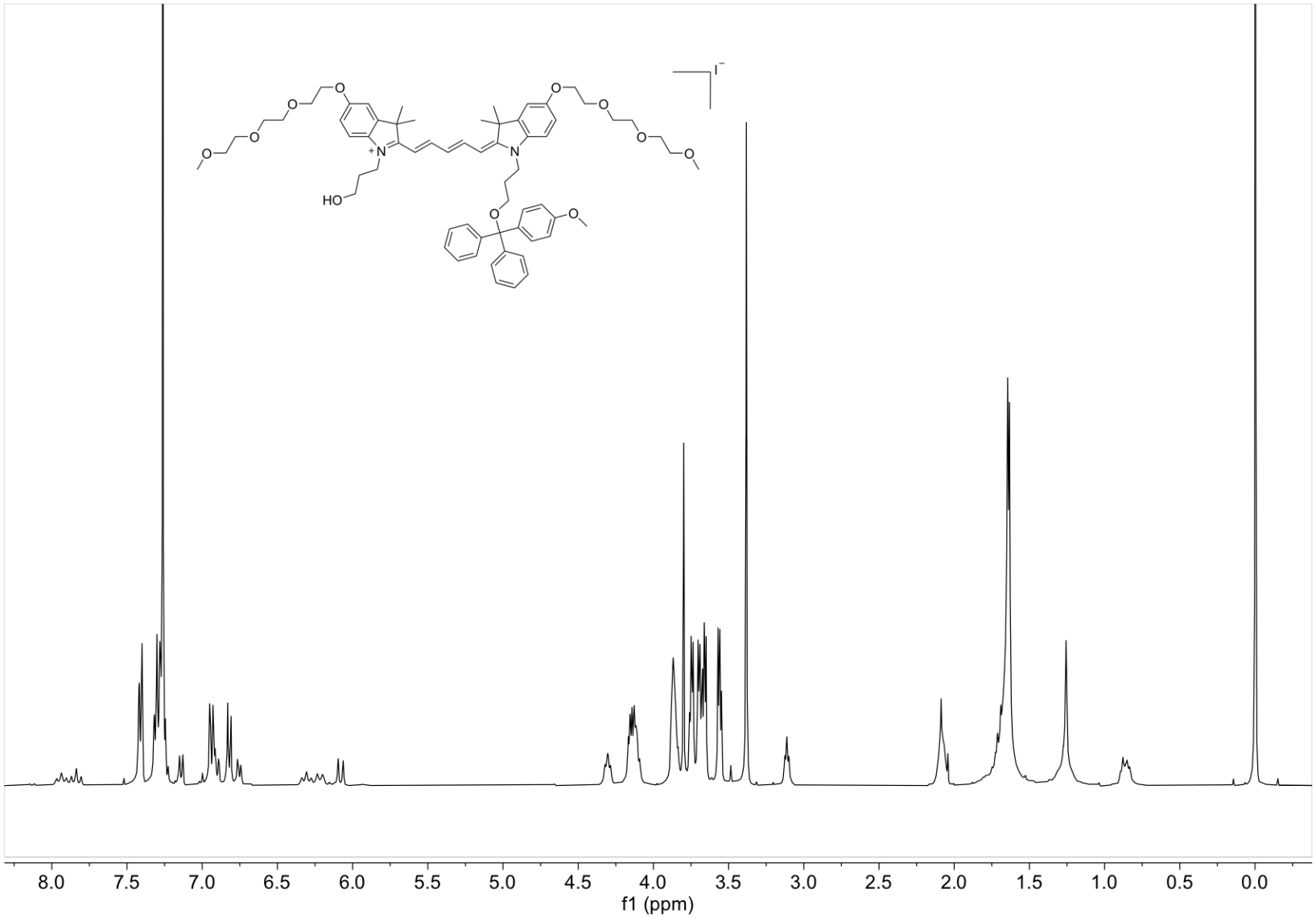


Figure S38: ^{1}H NMR of Cy5-Peg

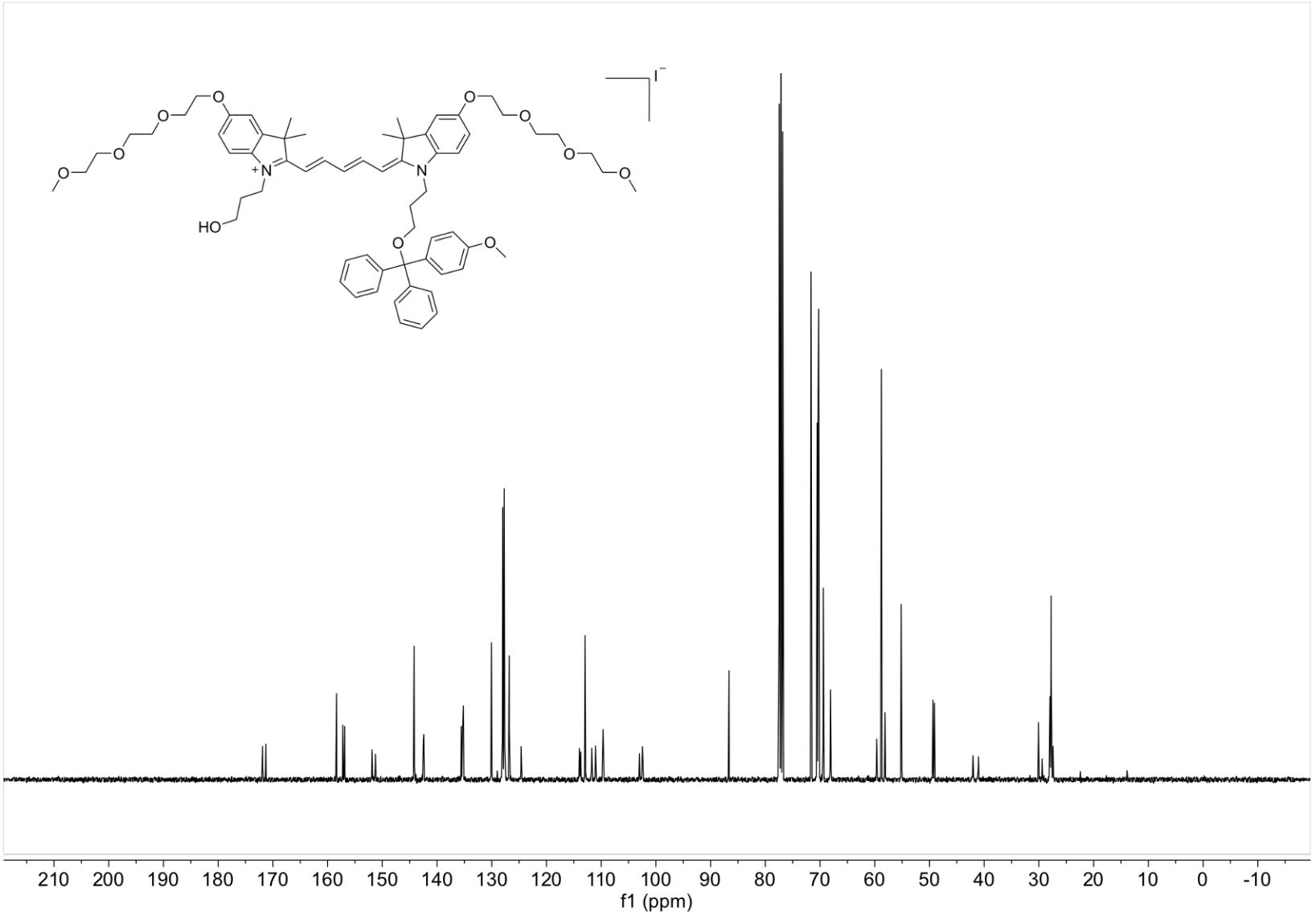


Figure S39: ^{13}C NMR of Cy5-Peg

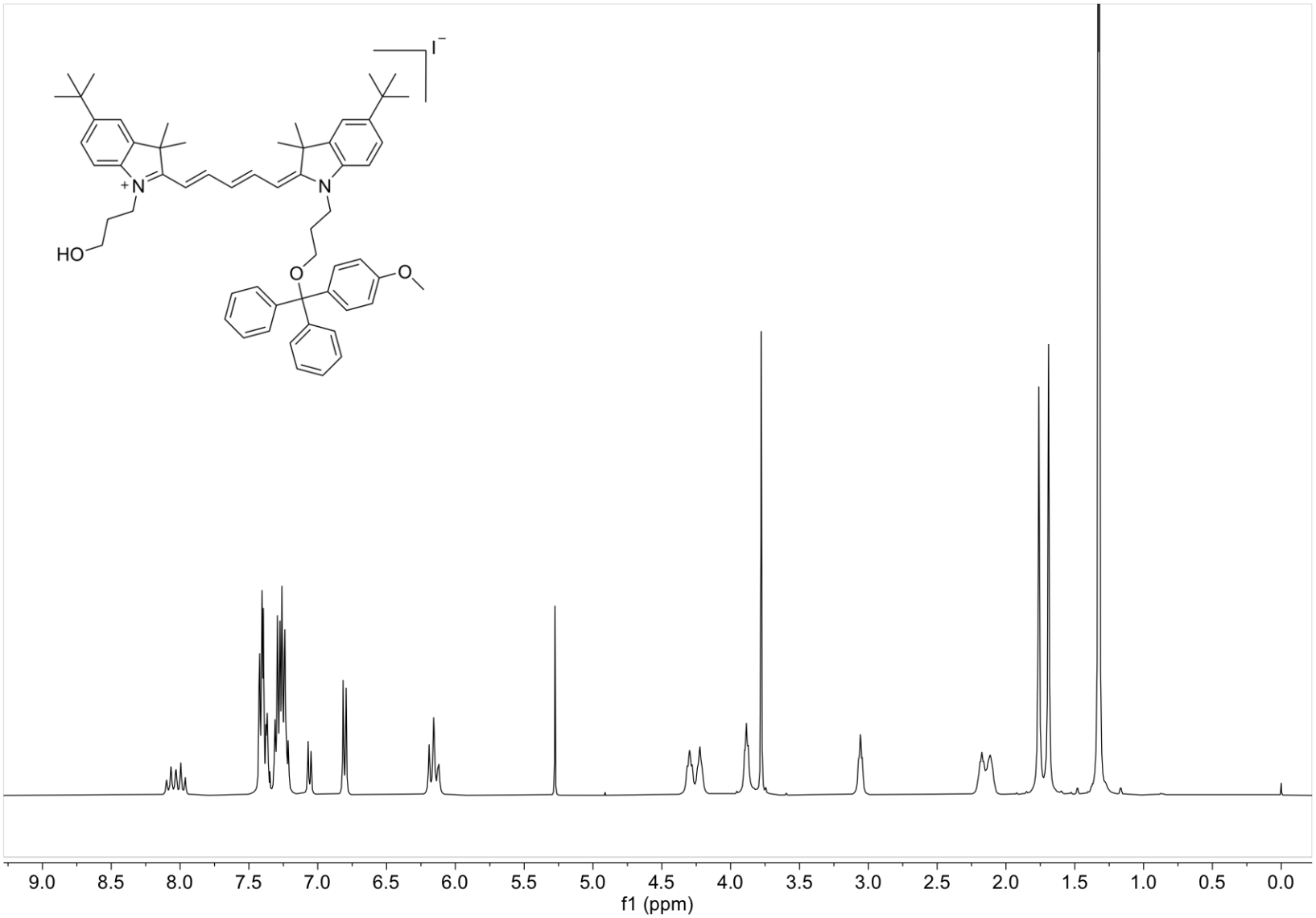


Figure S40: ^{1}H NMR of Cy5-tBu

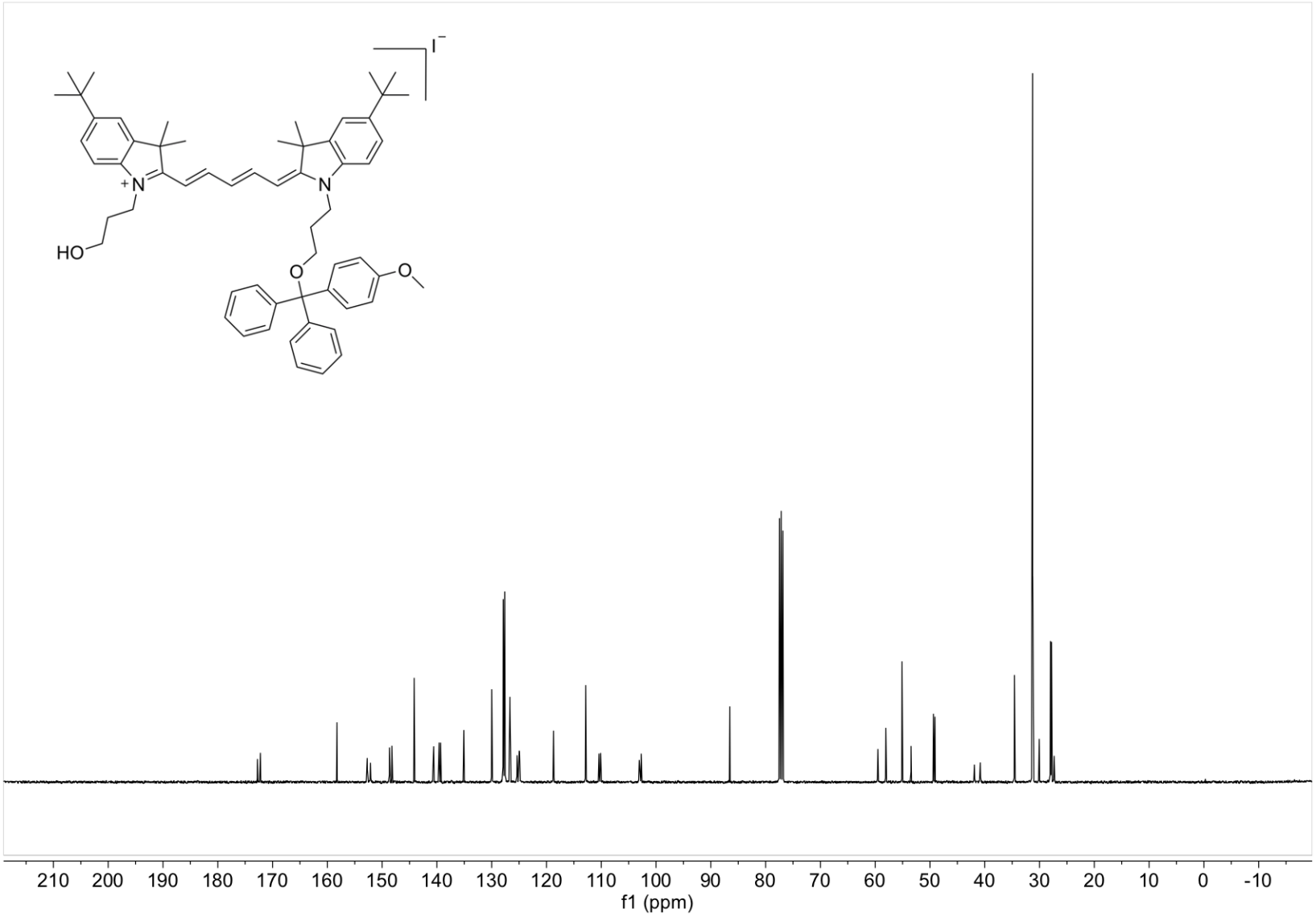


Figure S41: ^{13}C NMR of Cy5-tBu

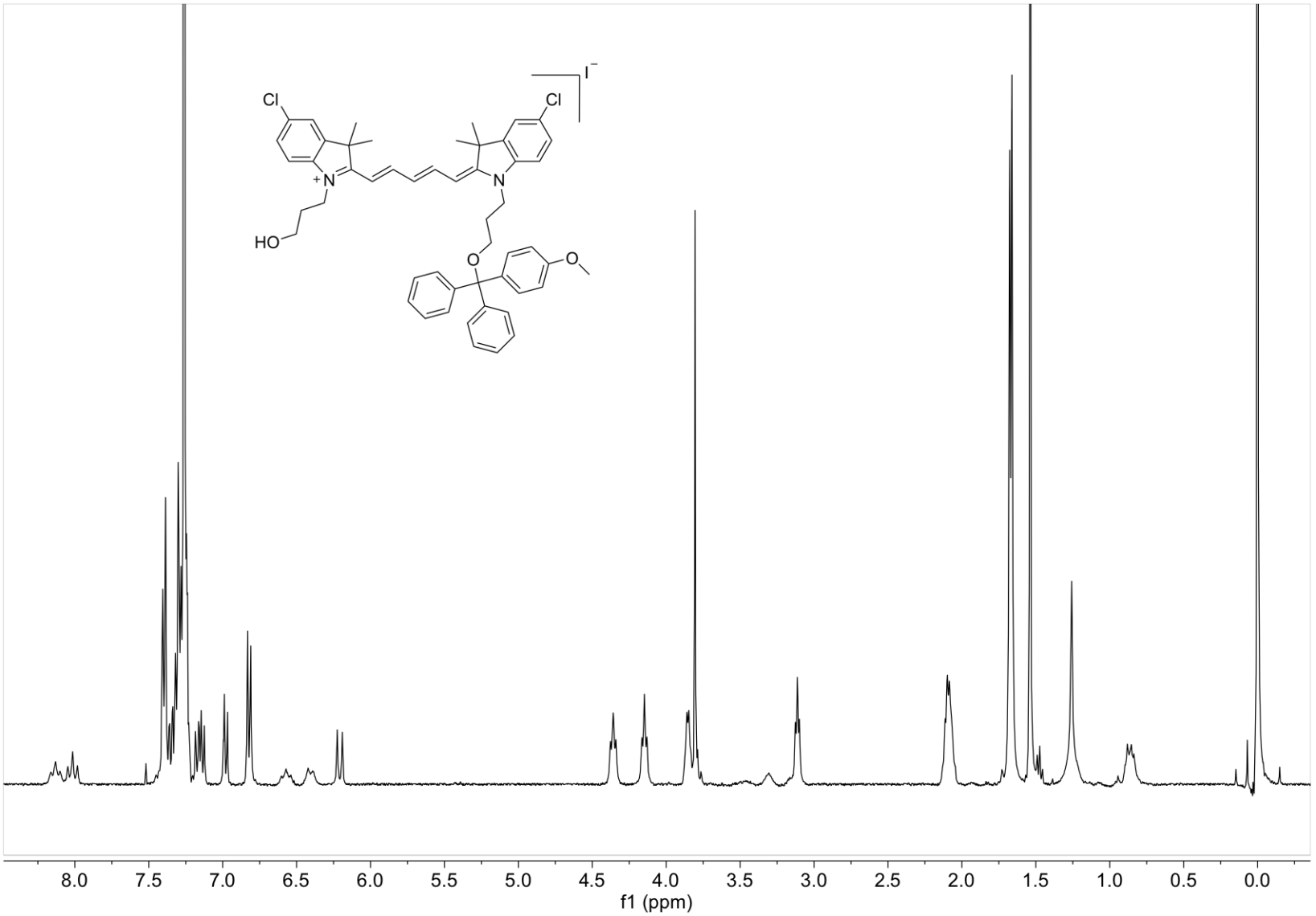


Figure S42: ^{1}H NMR of Cy5-Cl

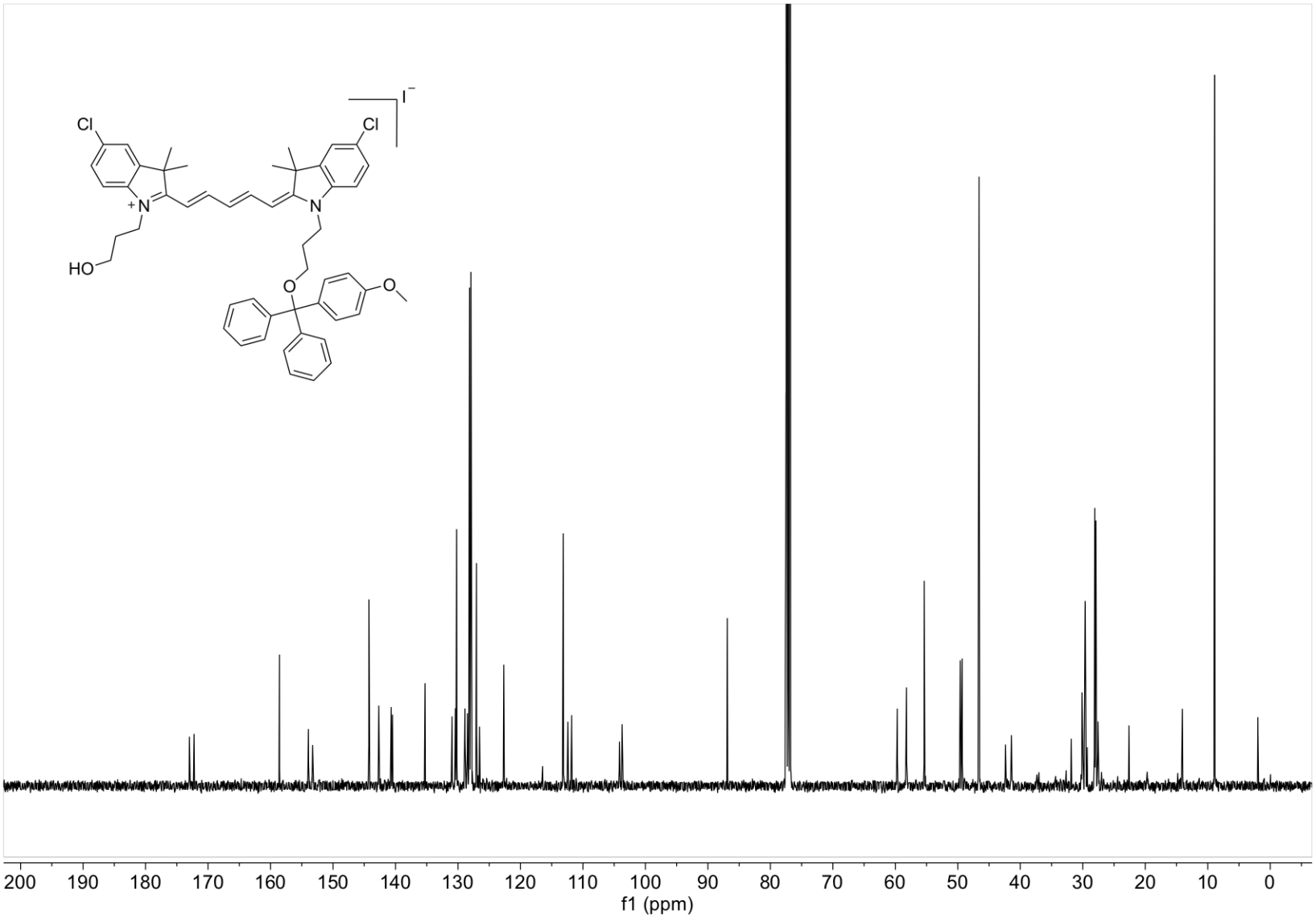


Figure S43: ^{13}C NMR of Cy5-Cl

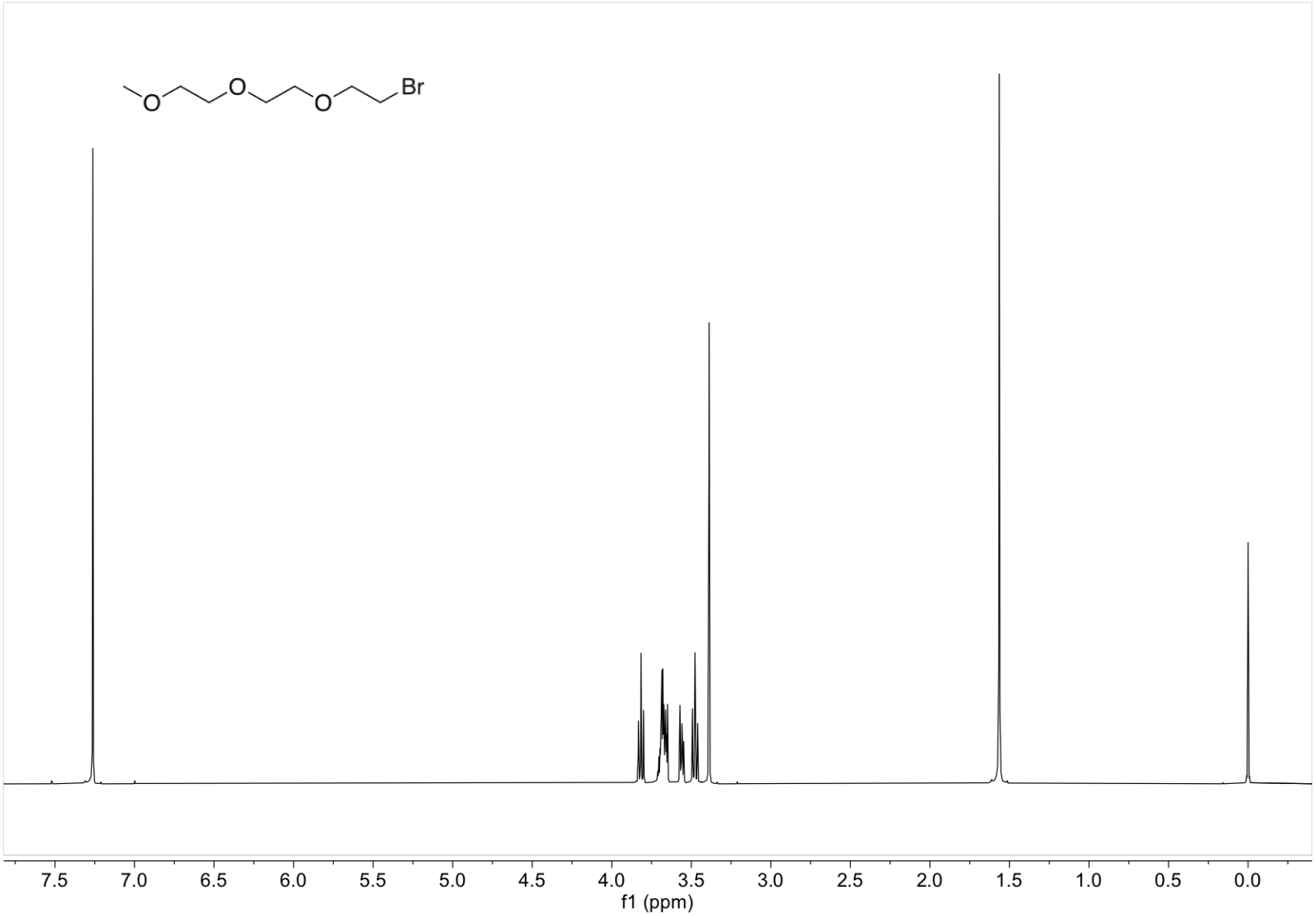


Figure S44: ^{1}H NMR of compound S1

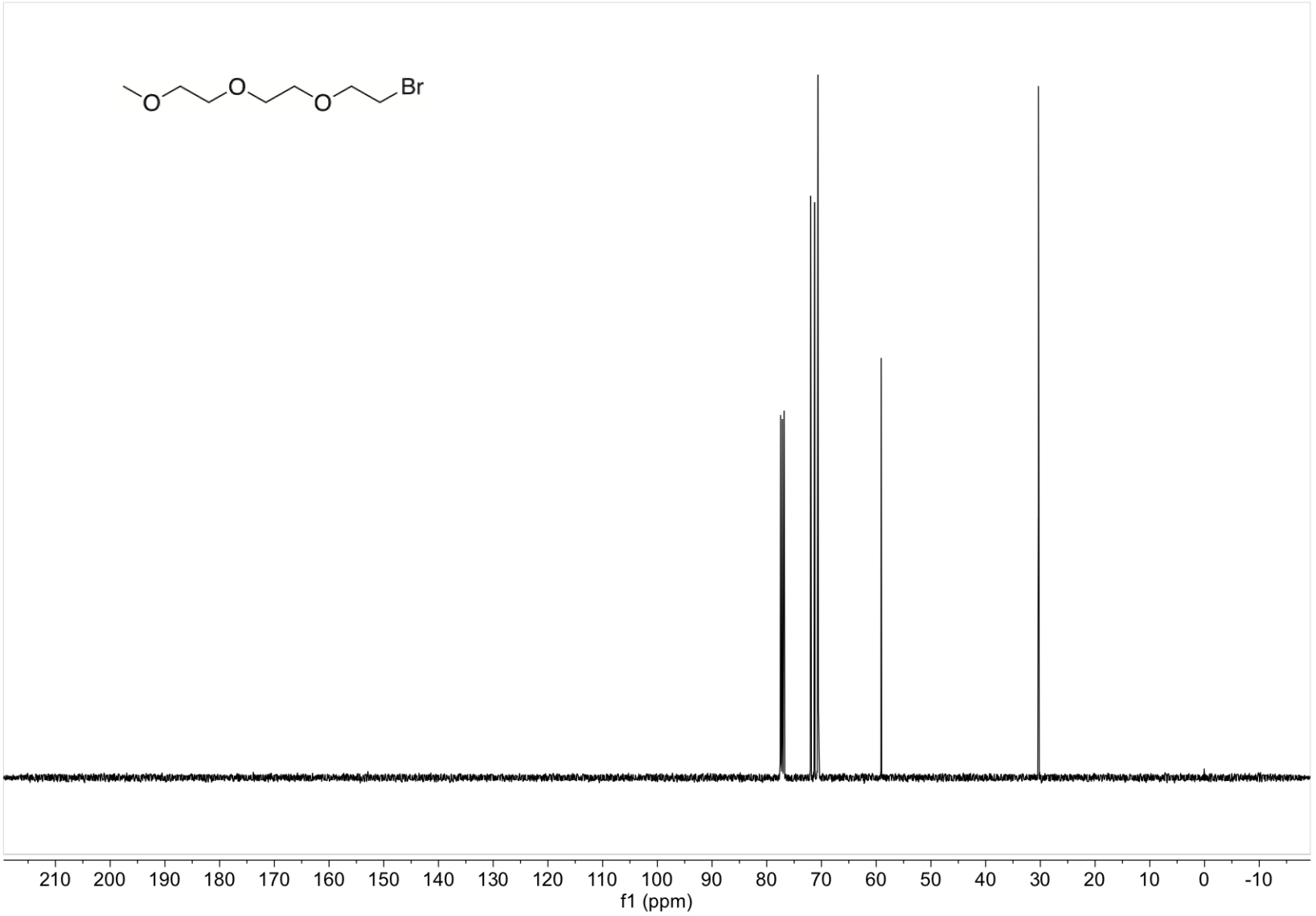


Figure S45: ^{13}C NMR of compound S1

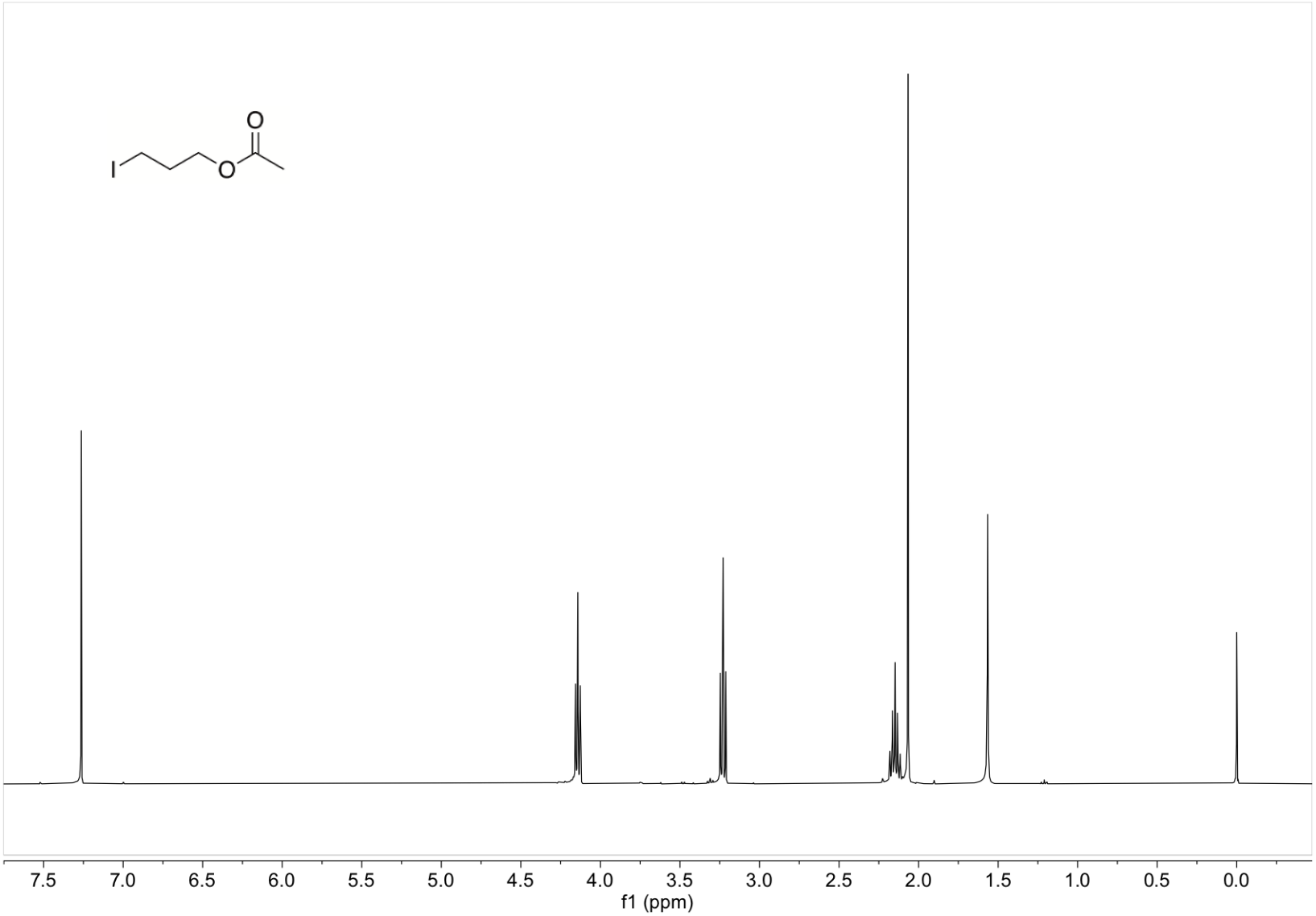


Figure S46: ^{1}H NMR of compound S2

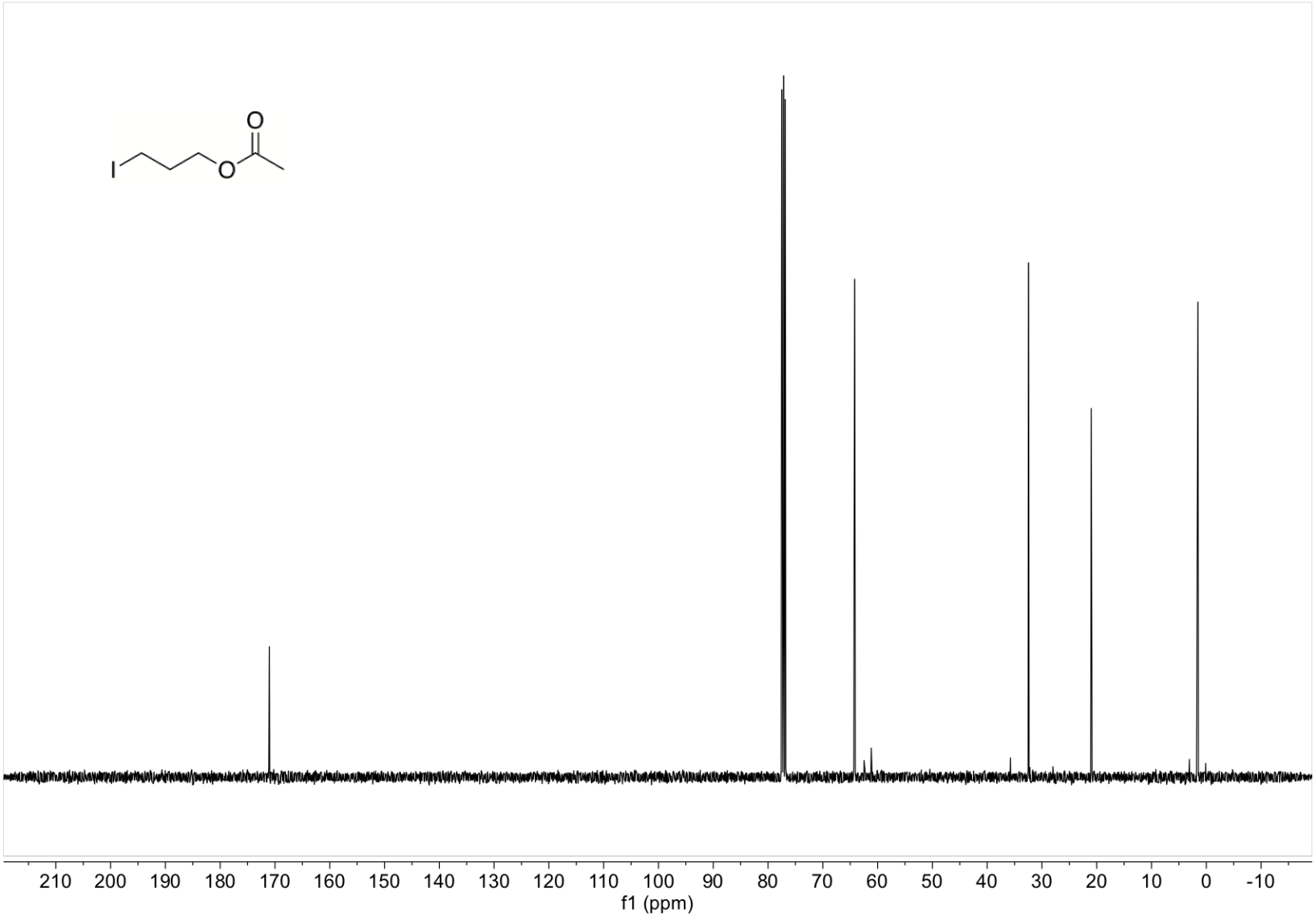


Figure S47: ^{13}C NMR of compound S2

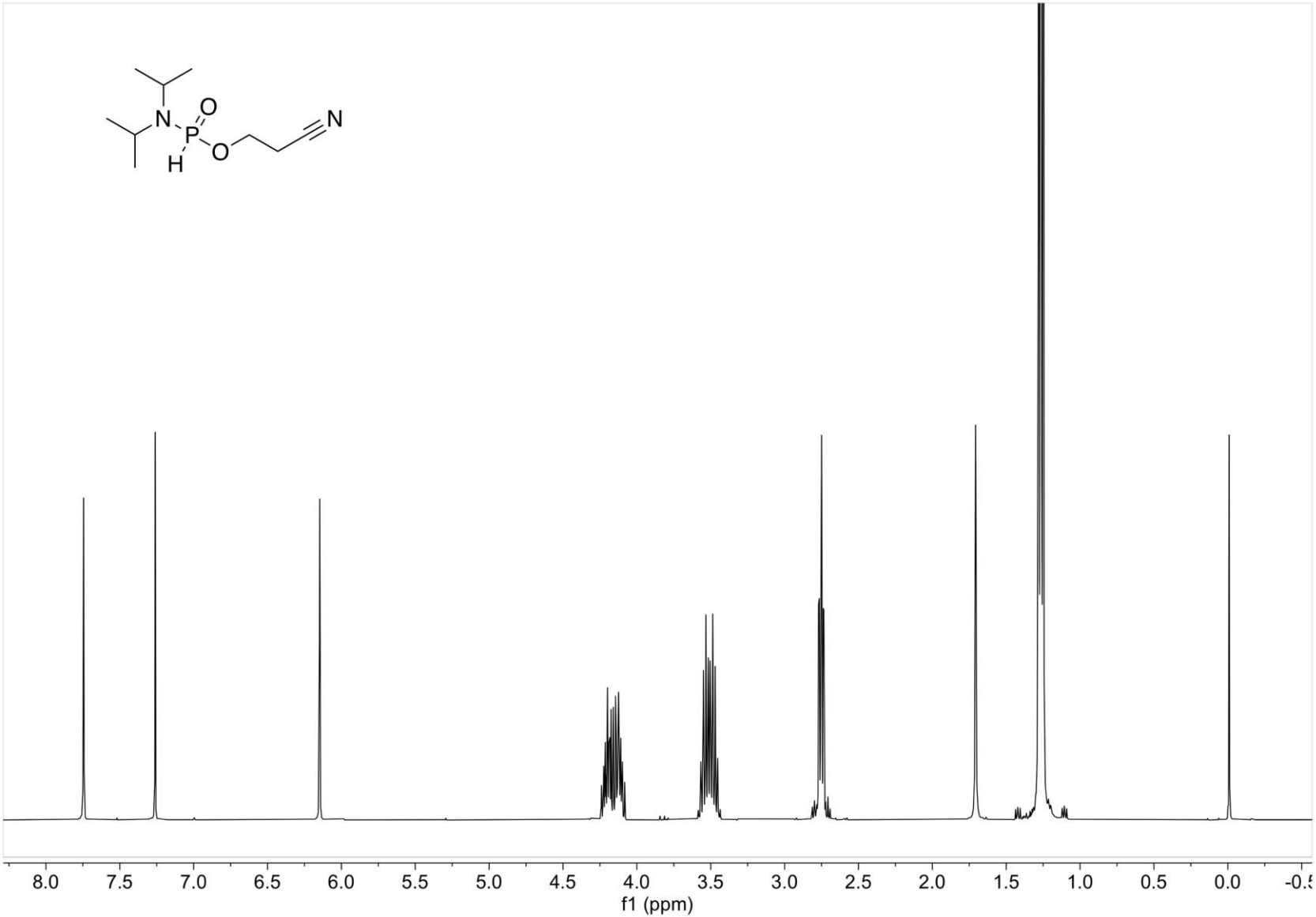


Figure S48: ^{1}H NMR of cyanoethyl-N,N-diisopropyl-H-phosphonamidate impurity

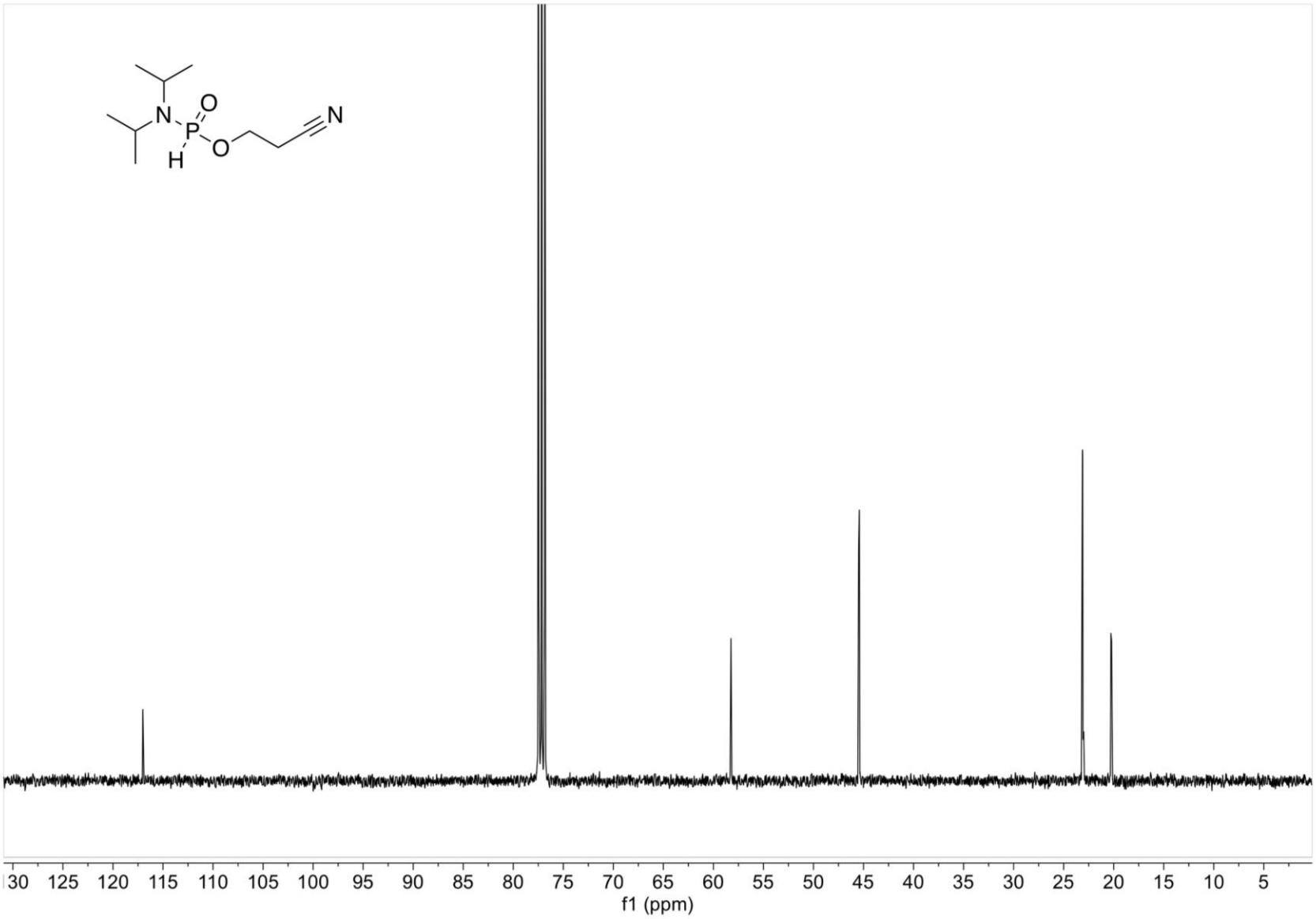


Figure S49: ^{13}C NMR of cyanoethyl-N,N-diisopropyl-H-phosphonamidate impurity

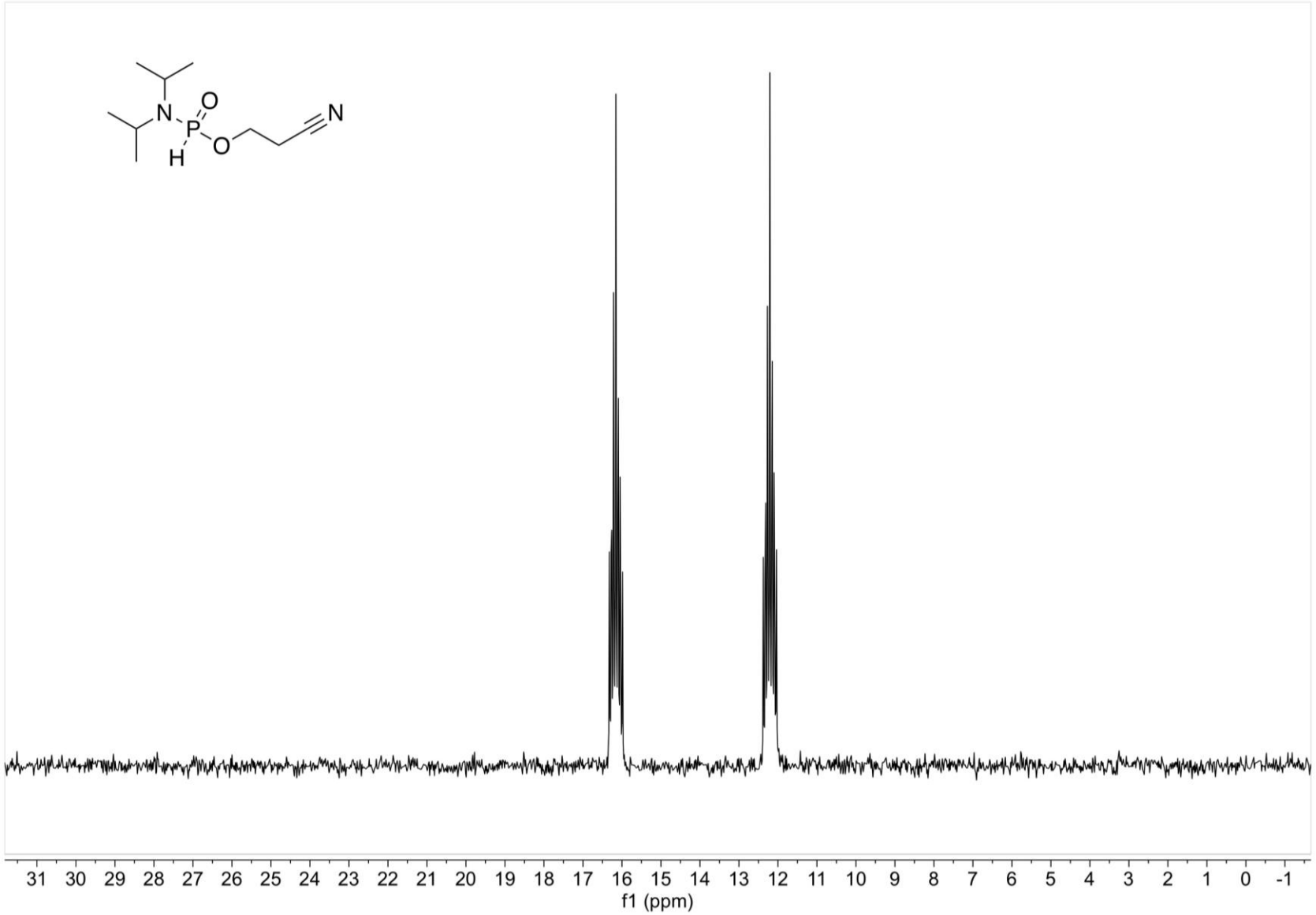
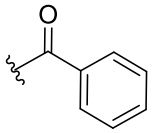
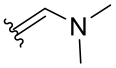


Figure S50: ^{31}P NMR of cyanoethyl-N,N-diisopropyl-H-phosphonamidate impurity

Section 4: Automated DNA Synthesis Phosphoramidites and Coupling Protocols

Coupling times for phosphoramidite monomer incorporation into oligomer are shown below. Protocols for ATCG are the commercially available phosphoramidites corresponding to the naturally occurring nucleobases, protocol for Cy5 corresponds to the unsubstituted, commercial phosphoramidite (included as a reference protocol), and Cy5-R are the novel phosphoramidites prepared in this work. These protocols are represented in the syntax of the Expedite 8909 synthesizer protocols. Commercial phosphoramidites ATCG and Cy5 purchased from Glen-Research (Sterling, VA) catalog numbers are in Table S1, below.

Table S1: Summary of commercial phosphoramidites utilized in oligonucleotide synthesis.

Phosphoramidite	Amine Protecting Group	Amine Protecting Group Structure	Catalog Number.
Adenosine	benzoyl		10-1000-1C
Thymidine	n/a	n/a	10-1030-1C
Cytidine	acetyl		10-1015-1C
Guanosine	dimethylformamidyl		10-1029-1C
Cyanine (unsubstituted)	n/a	n/a	10-5915-90

A

Syntax code	Line		Type	Amount	Time (s)	Comment
1	/*Wsh	*/	PULSE	5	0	"Flush system with Wsh"
2	/*Act	*/	PULSE	5	0	"Flush system with Act"
18	/*A +Act	*/	PULSE	5	0	"Monomer + Act to column"
18	/*A +Act	*/	PULSE	2	16	"Couple monomer"
2	/*Act	*/	PULSE	3	24	"Couple monomer"
1	/*Wsh	*/	PULSE	7	56	"Couple monomer"
1	/*Wsh	*/	PULSE	8	0	"Flush system with Wsh"

T

Syntax code	Line		Type	Amount	Time (s)	Comment
1	/*Wsh	*/	PULSE	5	0	"Flush system with Wsh"
2	/*Act	*/	PULSE	5	0	"Flush system with Act"
21	/*T +Act	*/	PULSE	6	0	"Monomer + Act to column"
21	/*T +Act	*/	PULSE	1	8	"Couple monomer"
2	/*Act	*/	PULSE	4	32	"Couple monomer"
1	/*Wsh	*/	PULSE	7	56	"Couple monomer"
1	/*Wsh	*/	PULSE	8	0	"Flush system with Wsh"

C

Syntax code	Line		Type	Amount	Time (s)	Comment
1	/*Wsh	*/	PULSE	5	0	"Flush system with Wsh"
2	/*Act	*/	PULSE	5	0	"Flush system with Act"
19	/*C +Act	*/	PULSE	5	0	"Monomer + Act to column"
19	/*C +Act	*/	PULSE	2	16	"Couple monomer"
2	/*Act	*/	PULSE	3	24	"Couple monomer"
1	/*Wsh	*/	PULSE	7	56	"Couple monomer"
1	/*Wsh	*/	PULSE	8	0	"Flush system with Wsh"

G

Syntax code	Line		Type	Amount	Time (s)	Comment
1	/*Wsh	*/	PULSE	5	0	"Flush system with Wsh"
2	/*Act	*/	PULSE	5	0	"Flush system with Act"
20	/*G +Act	*/	PULSE	6	0	"Monomer + Act to column"
20	/*G +Act	*/	PULSE	1	8	"Couple monomer"
2	/*Act	*/	PULSE	4	32	"Couple monomer"
1	/*Wsh	*/	PULSE	7	56	"Couple monomer"
1	/*Wsh	*/	PULSE	8	0	"Flush system with Wsh"

Cy5 (commercial)

Syntax code	Line		Type	Amount	Time (s)	Comment
1	/*Wsh	*/	PULSE	5	0	"Flush system with Wsh"
2	/*Act	*/	PULSE	5	0	"Flush system with Act"
22	/*5 +Act	*/	PULSE	6	0	"Monomer + Act to column"
22	/*5 +Act	*/	PULSE	1	32	"Couple monomer"
2	/*Act	*/	PULSE	4	128	"Couple monomer"
1	/*Wsh	*/	PULSE	7	200	"Couple monomer"
1	/*Wsh	*/	PULSE	8	0	"Flush system with Wsh"

Cy5-R

Syntax code	Line		Type	Amount	Time (s)	Comment
1	/*Wsh	*/	PULSE	5	0	"Flush system with Wsh"
2	/*Act	*/	PULSE	5	0	"Flush system with Act"
22	/*5 +Act	*/	PULSE	3	0	"Monomer + Act to column"
1	/*Wsh	*/	PULSE	10	0	"Push Monomer+Act"
1	/*Wsh	*/	PULSE	7	150	"Couple monomer"
1	/*Wsh	*/	PULSE	8	0	"Flush out Monomer+Act"
22	/*5 +Act	*/	PULSE	3	0	"Monomer + Act to column"
1	/*Wsh	*/	PULSE	9	0	"Push Monomer+Act"
1	/*Wsh	*/	PULSE	7	150	"Couple monomer"
1	/*Wsh	*/	PULSE	8	0	"Flush out Monomer+Act"
22	/*5 +Act	*/	PULSE	3	0	"Monomer + Act to column"
1	/*Wsh	*/	PULSE	9	0	"Push Monomer+Act"
1	/*Wsh	*/	PULSE	7	150	"Couple monomer"
1	/*Wsh	*/	PULSE	8	0	"Flush out Monomer+Act"
2	/*Act	*/	PULSE	4	300	"Couple monomer"
1	/*Wsh	*/	PULSE	7	150	"Couple monomer"
1	/*Wsh	*/	PULSE	8	0	"Flush system with Wsh"

Wsh (Anhydrous Acetonitrile); 5 (Cy5-R phosphoramidite); Act (Activator 0.25 M 5-Ethylthio-1H-Tetrazole (ETT) in Anhydrous Acetonitrile);

Section 5: Mass Spectral Summary and LC/MS Traces for DNA-Cy5 Sequences

All traces below were acquired on the continuous ACQUITY UPLC system, passing through detectors in the following order: PDA eλ, FLR, SQD2. ACUITY UPLC is operated by MassLynx 4.2 software. All samples were injected using the auto-sampler (ACQUITY FTN, 2.0 μL injection volume), with the elution program including isocratic phase (2 minutes of gradient initial solution), gradient phase (16 minutes), wash phase (8.5 minutes) and re-equilibration to starting conditions (5.5 minutes), with a constant flow rate of 0.2 mL/min. Each series of sequences utilized a different gradient of increasing methanol in 0.05M TEAA (aq.), the gradient is indicated below the corresponding figures in the following pages. Only Optima grade solvents were utilized. Sample purity determined by integration, using absorbance bands observed in the 260 nm channel. Spectra for each LC/MS trace are displayed as follows, Figure S47 is annotated as an example:

A) Fluorescence Emission, single channel emission focused on λ max emission for given Cy5, acquired on ACQUITY FLR
B) Absorbance Intensity, single channel absorption focused on λ max absorbance for given Cy5, acquired on ACQUITY PDA eλ Detector
C) Absorbance Intensity, single channel absorption focused at 260 nm, acquired on ACQUITY PDA eλ Detector
D) Total Absorbance Intensity, for all channels in range of 200-800 nm, acquired on ACQUITY PDA eλ Detector
E) Total Ion Current Chromatogram (TIC) for all channels in range of 200-3000 amu, acquired on ACQUITY SQD2
F) TIC for single channel focused on monoisotopic weight of target sequence, assuming charge of -4, acquired on ACQUITY SQD2
G) Combined mass spectrum over time period target band elutes (denoted by asterisk), obtained from full mass range TIC spectrum

All found and calculated m/z are summarized in Table S2. Sample isotope envelop (used to determine base peak) as generated by MassLynx 4.2 shown in Figure S52.

Table S2. Summary of target verification for Cy5 containing DNA sequences. Predicted masses are acquired from continuum spectrum of isotope envelope of given sequence (feature of MassLynx 4.2 software, see Figure S51 for example) where Z = -4. Observed masses correspond to the base peak where Z = -4, and are in agreement with the predicted masses ± 1 amu.

DNA Sequence	Predicted m/z (amu)	Observed m/z (amu)
Cy5-hex series:		
HJA	2168.15	2169.05
HJB	2204.40	2205.31
HJC	2121.36	2122.36
HJD	2203.89	2204.56
HJAcomp	2180.40	2181.20
Cy5-Peg series:		
HJA	2199.16	2199.36
HJB	2235.41	2236.19
HJC	2152.38	2153.18
HJD	2234.89	2235.35
HJAcomp	2211.41	2212.04
Cy5-tBu series:		
HJA	2146.11	2146.82
HJB	2182.39	2183.12
HJC	2099.34	2099.67
HJD	2181.87	2182.36
HJAcomp	2158.36	2158.86
Cy5-Cl series:		
HJA	2135.28	2135.37
HJB	2171.55	2171.94
HJC	2088.49	2088.87
HJD	2171.04	2171.43
HJAcomp	2147.55	2147.88

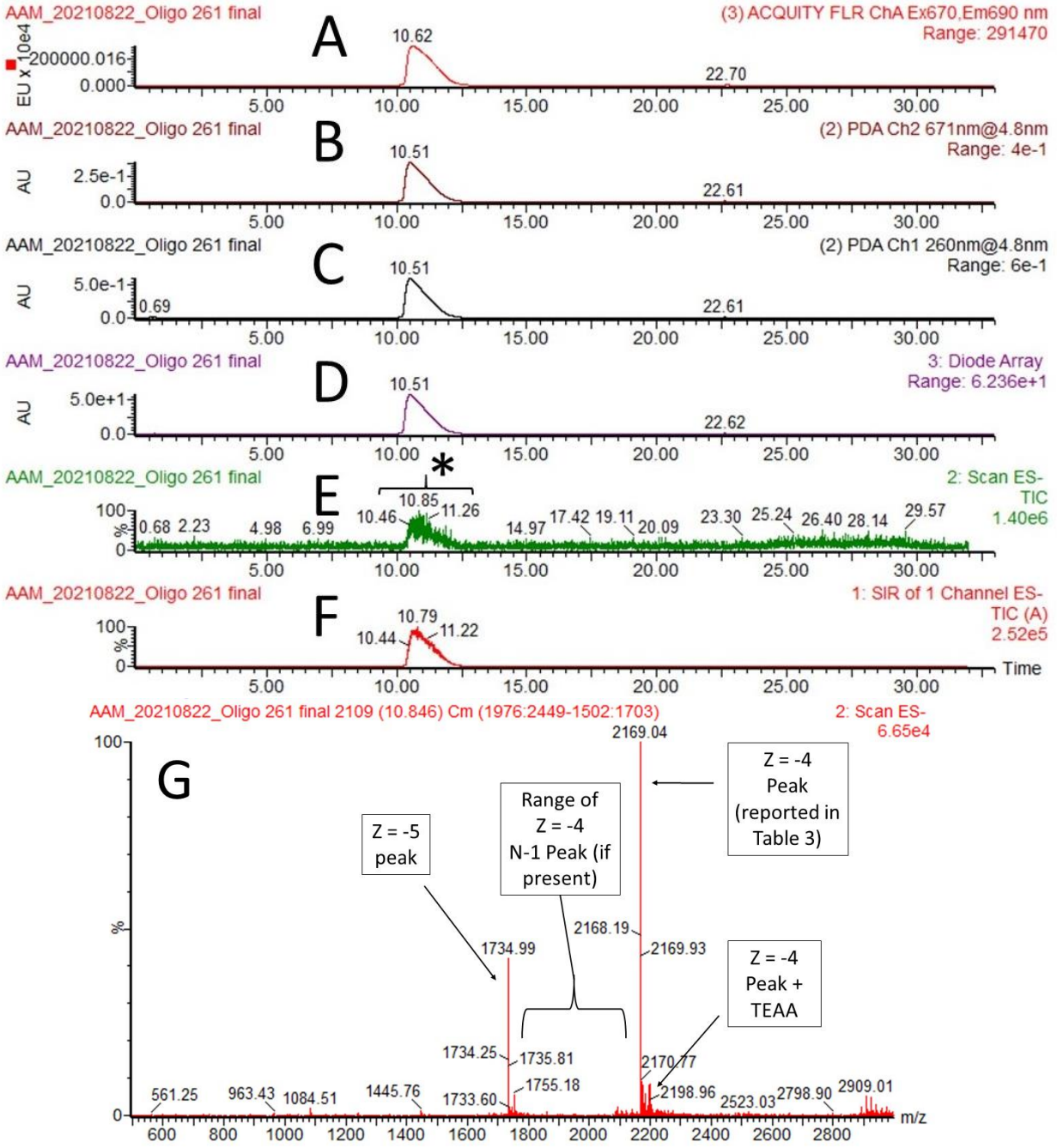


Figure S51: LC/MS Traces (top, A-F) and mass spectrum (bottom, G) of Purified HJA-Cy5hex, Gradient of 47-53% MeOH in 0.05 M TEAA (aq.)

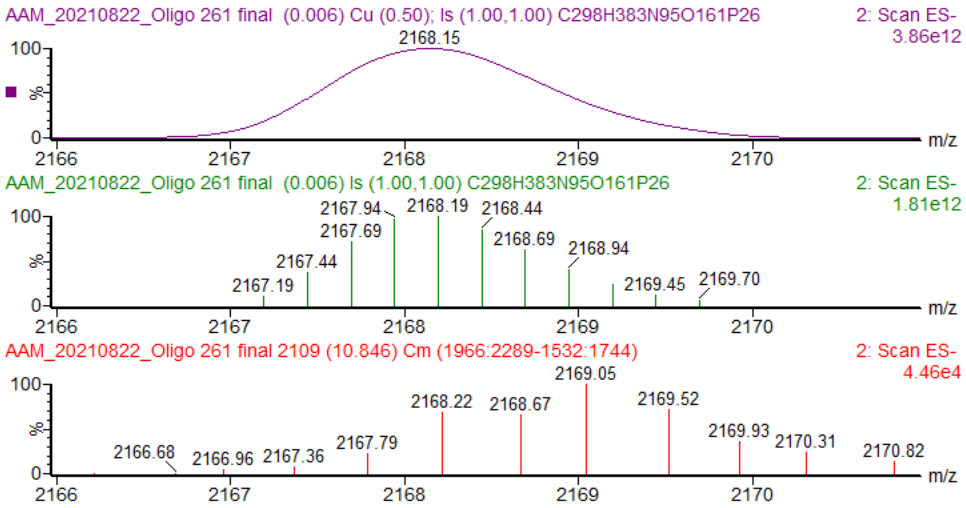


Figure S52: Predicted Isotope Envelope of HJA-Cy5hex (center, and top as continuum spectrum) in comparison to observed mass spectrum of target mass where z = -4 (bottom).

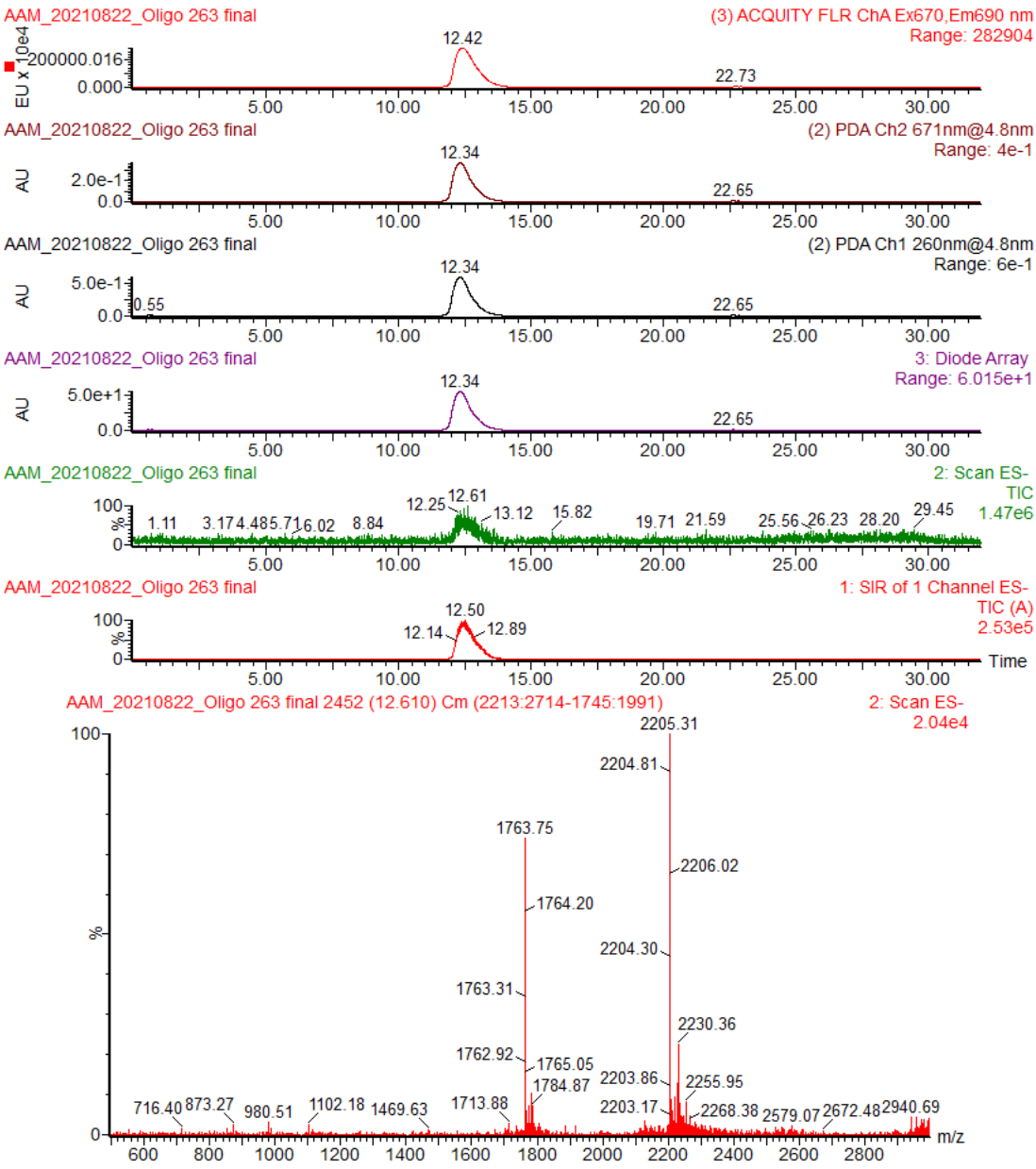


Figure S53: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJB-Cy5hex, Gradient of 47-53% MeOH in 0.05 M TEAA (aq.)

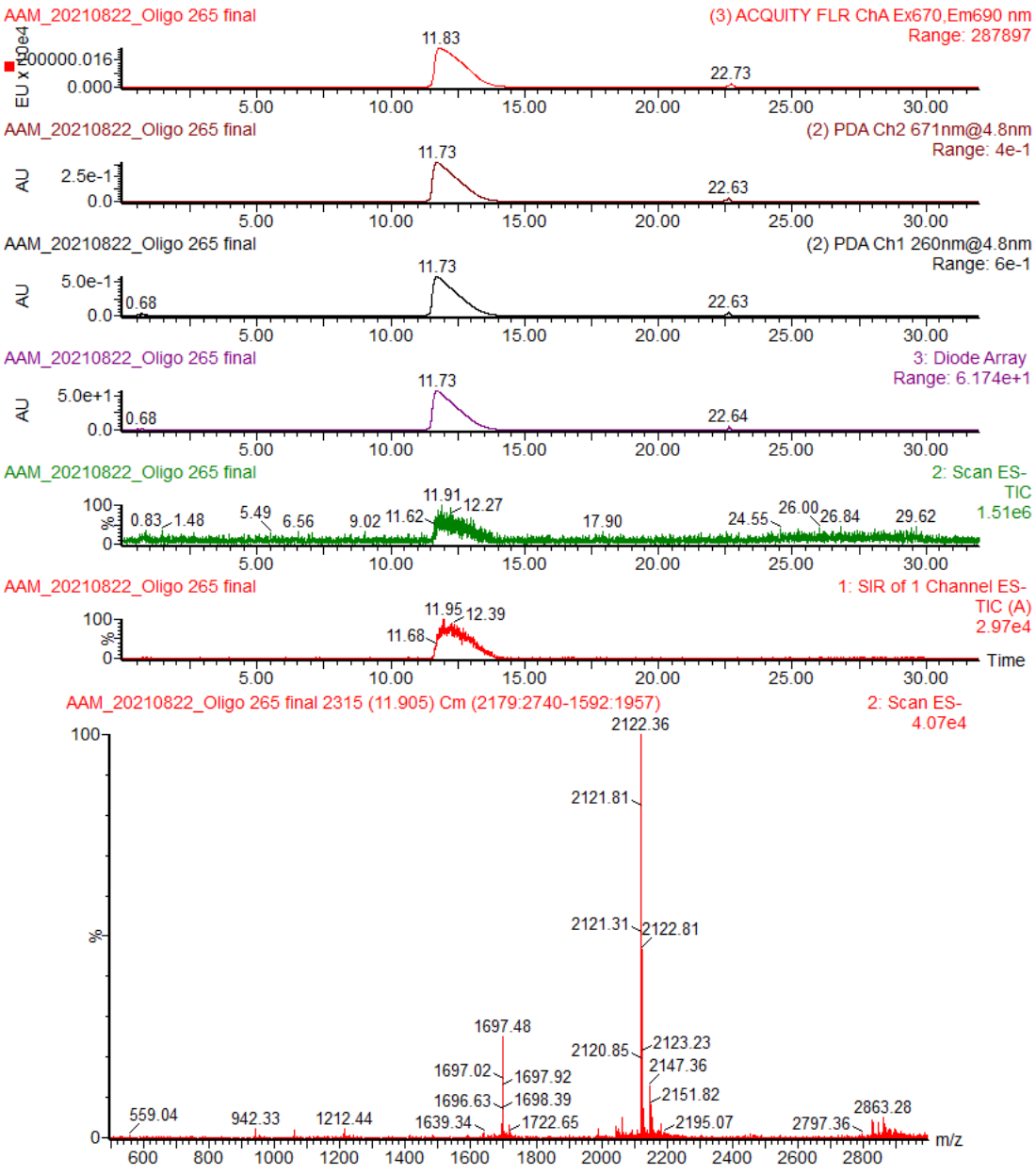


Figure S54: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJC-Cy5hex, Gradient of 47-53% MeOH in 0.05 M TEAA (aq.)

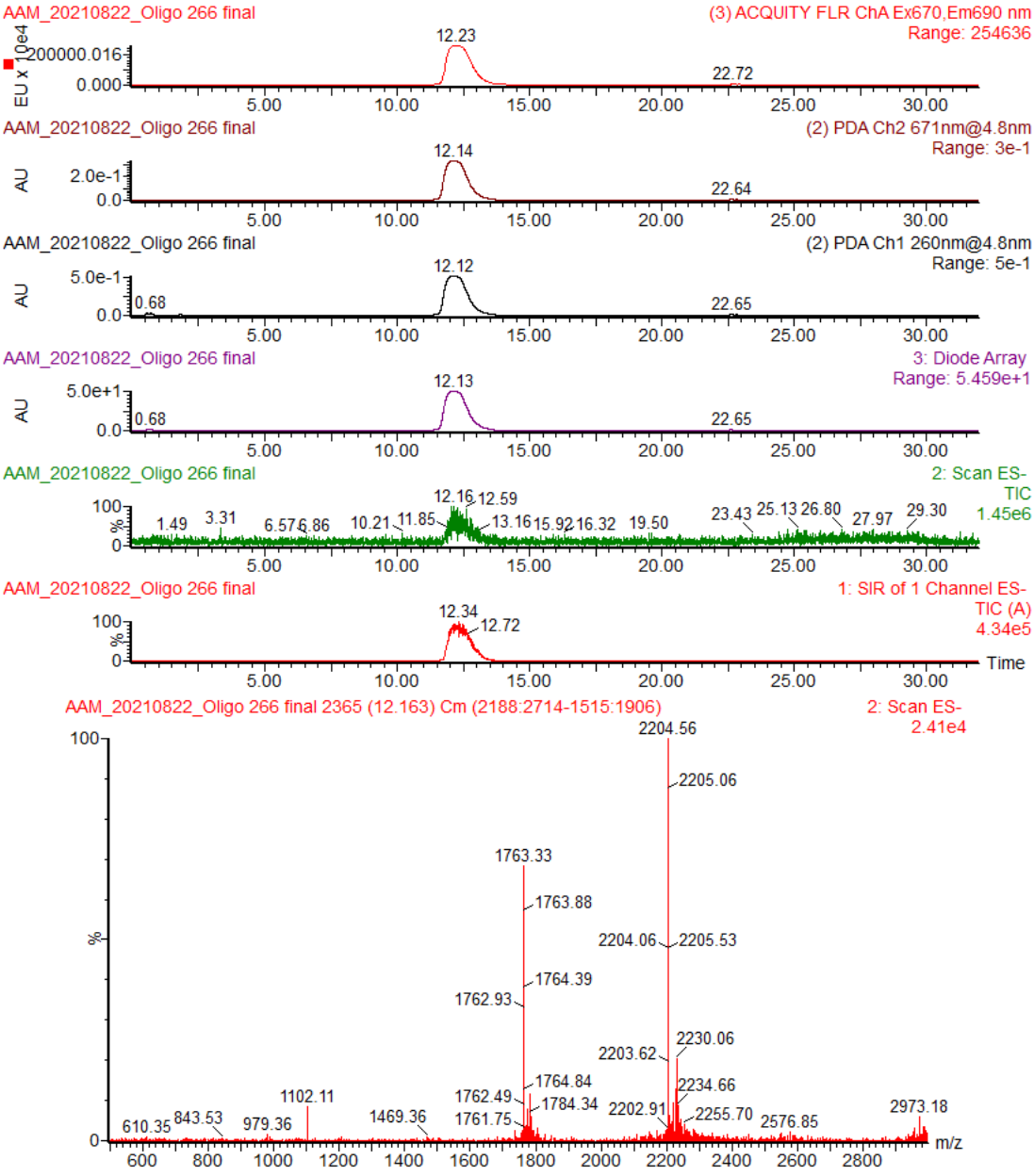


Figure S55: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJD-Cy5hex, Gradient of 47-53% MeOH in 0.05 M TEAA (aq.)

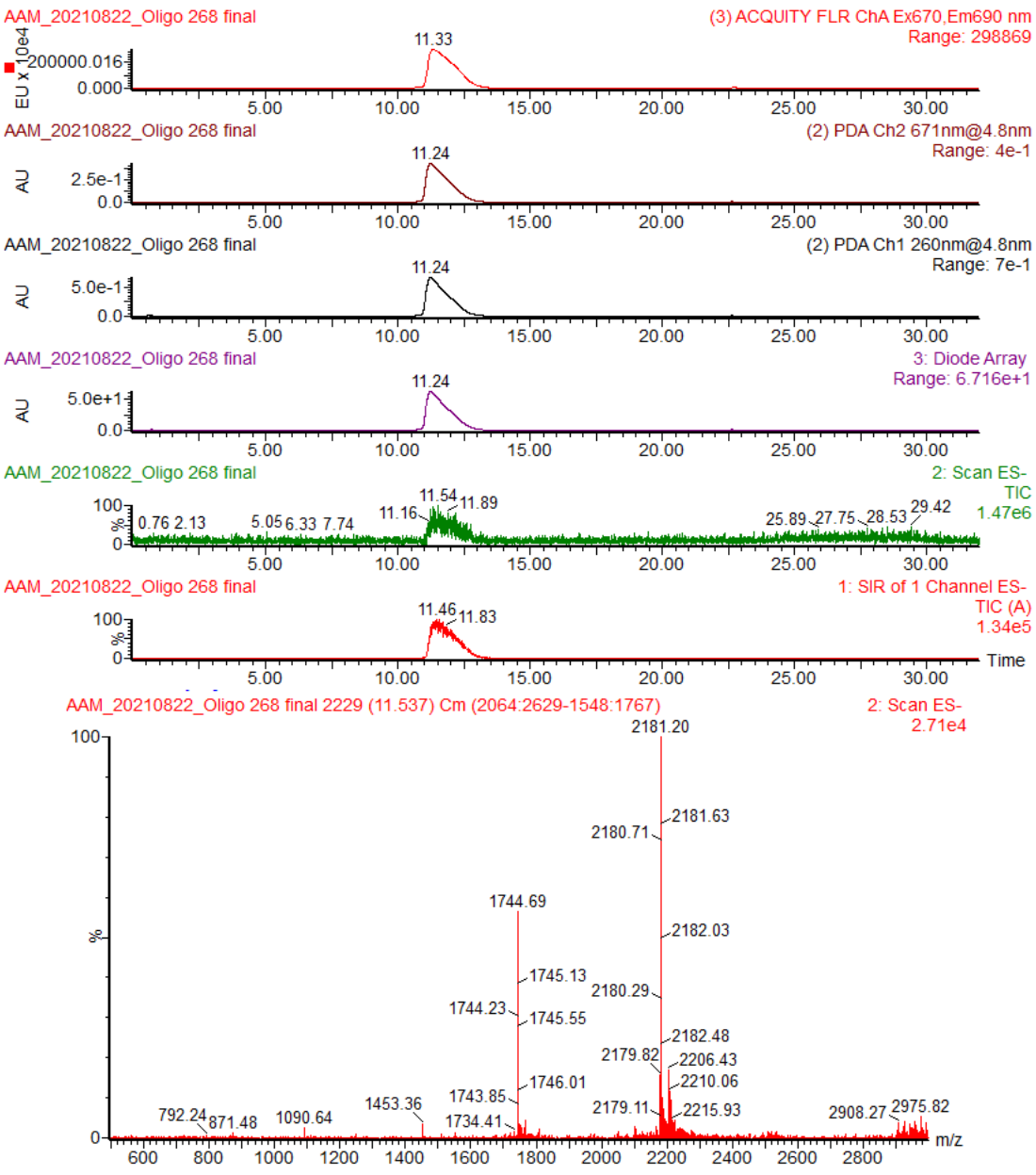


Figure S56: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJAcomp-Cy5hex, Gradient of 47-53% MeOH in 0.05 M TEAA (aq.)

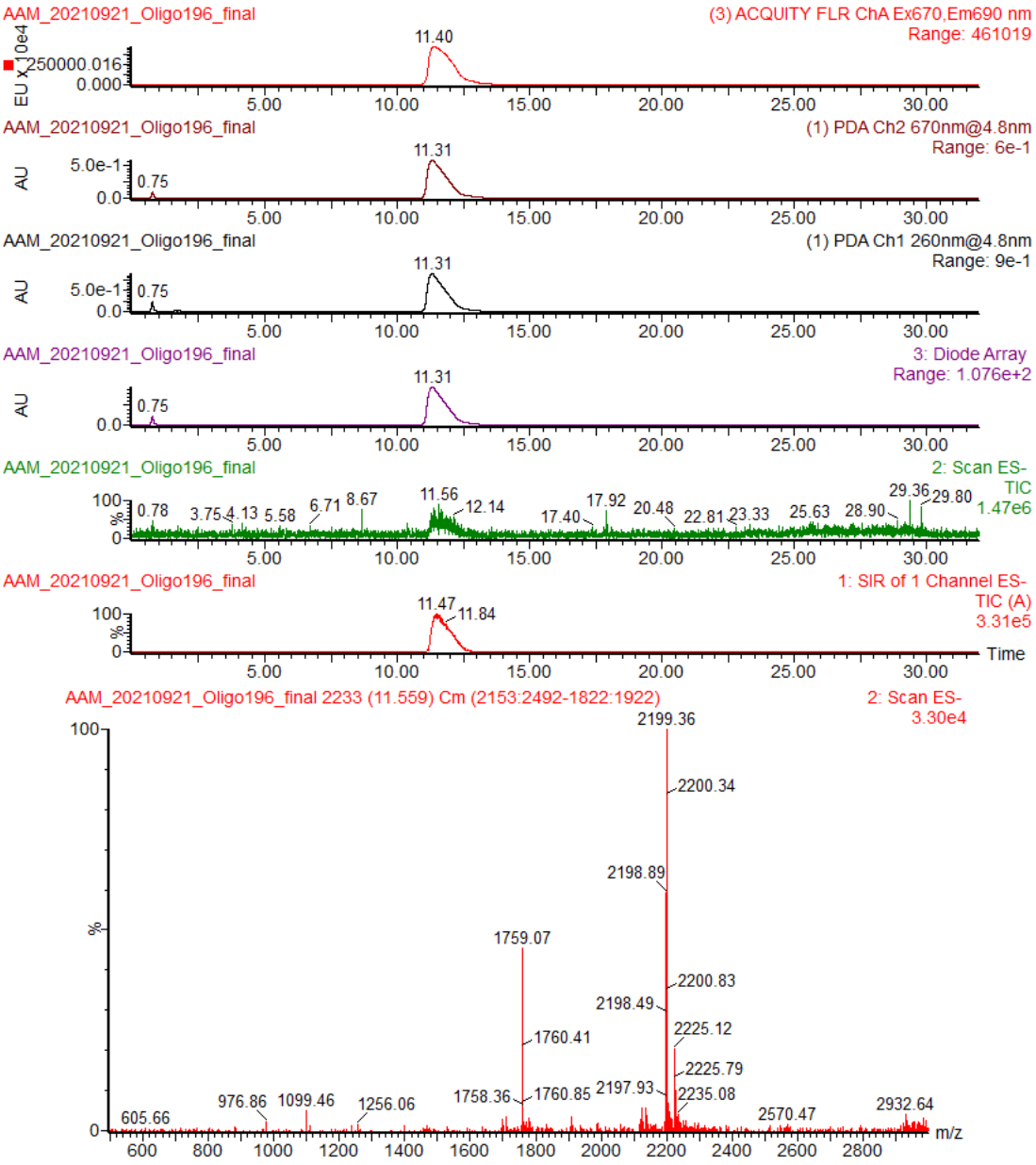


Figure S57: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJA-Cy5Peg, Gradient of 27.5-33.5% MeOH in 0.05 M TEAA (aq.)

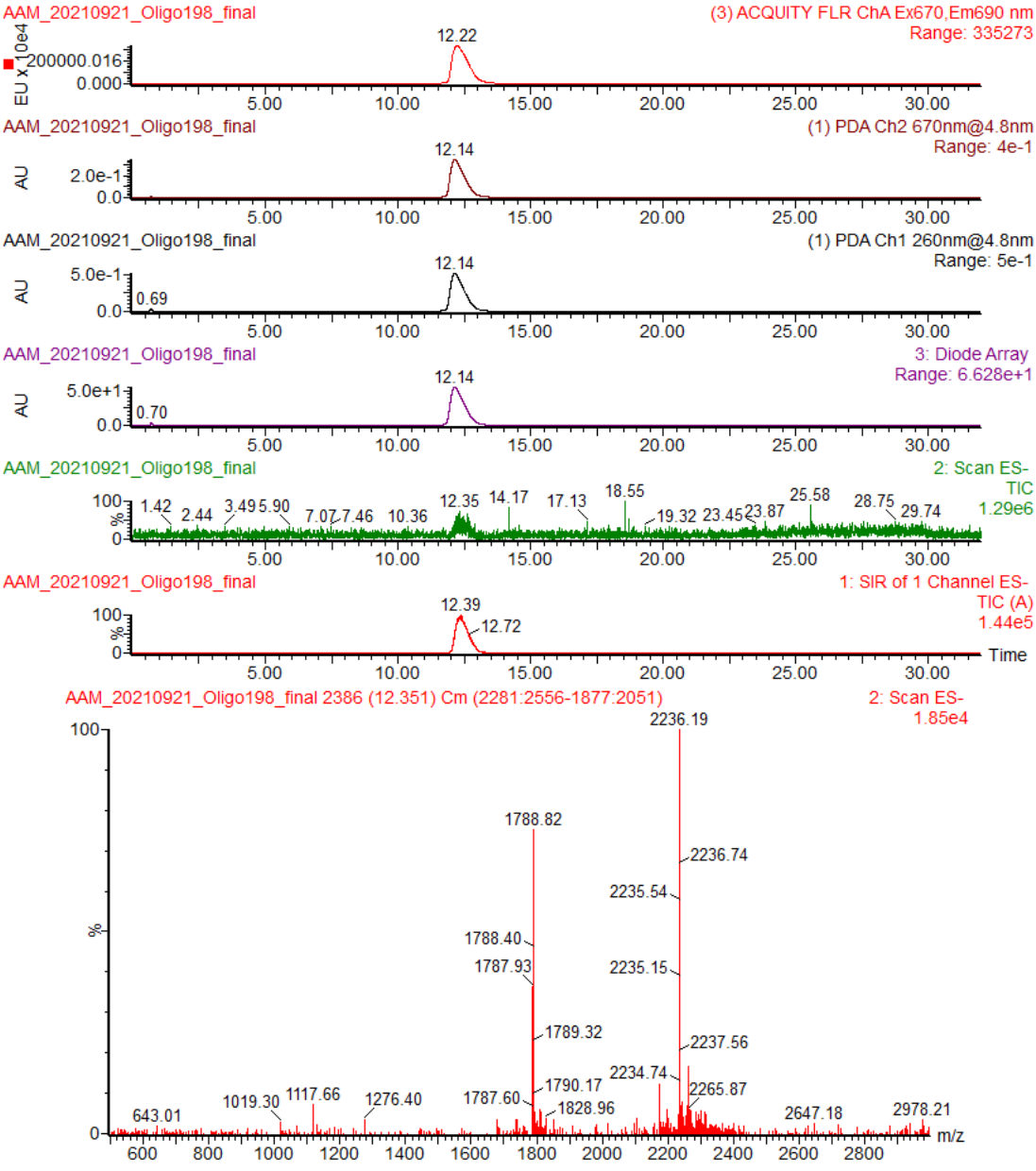


Figure S58: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJB-Cy5Peg, Gradient of 27.5-33.5% MeOH in 0.05 M TEAA (aq.)

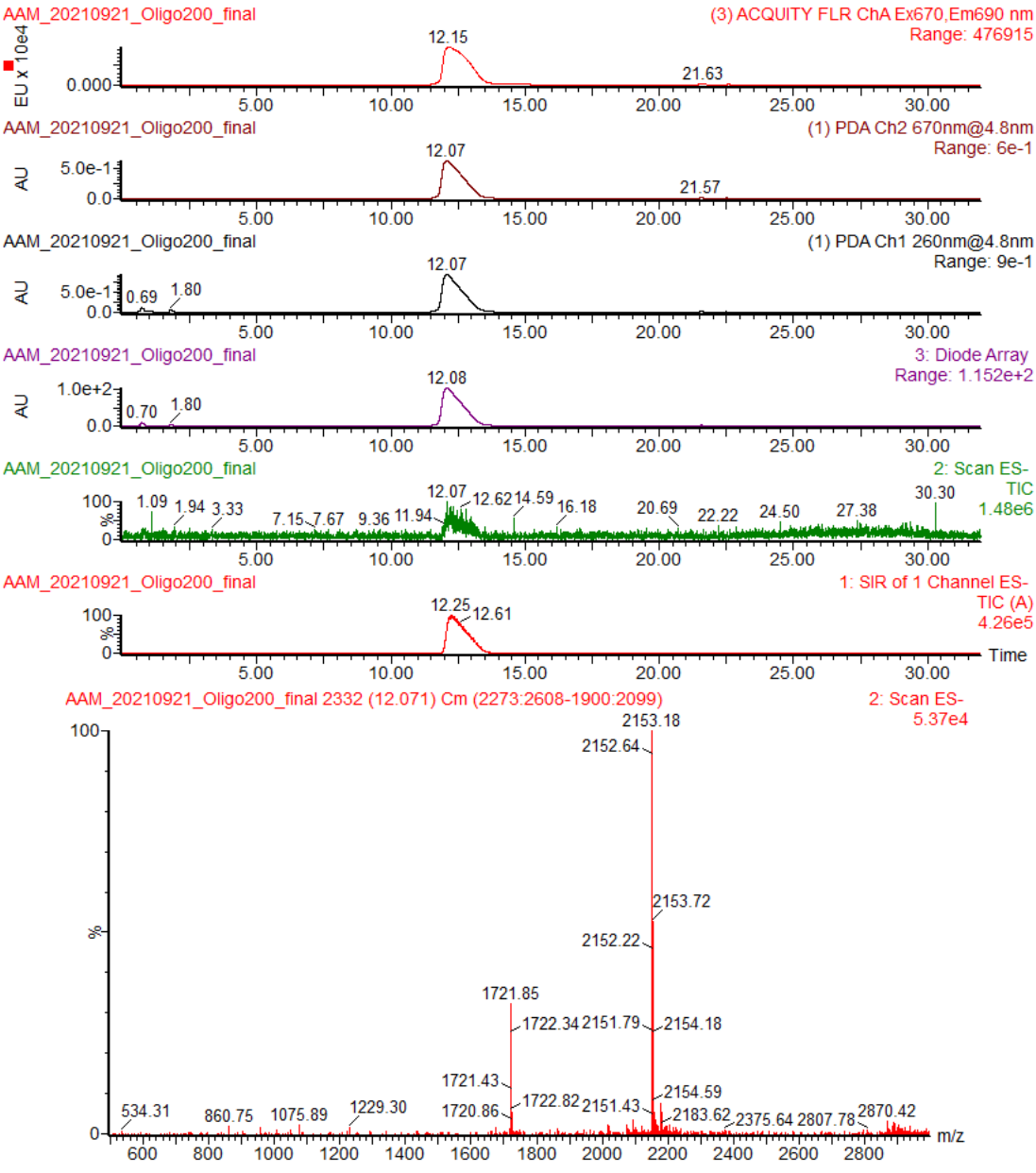


Figure S59: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJC-Cy5Peg, Gradient of 27.5-33.5% MeOH in 0.05 M TEAA (aq.)

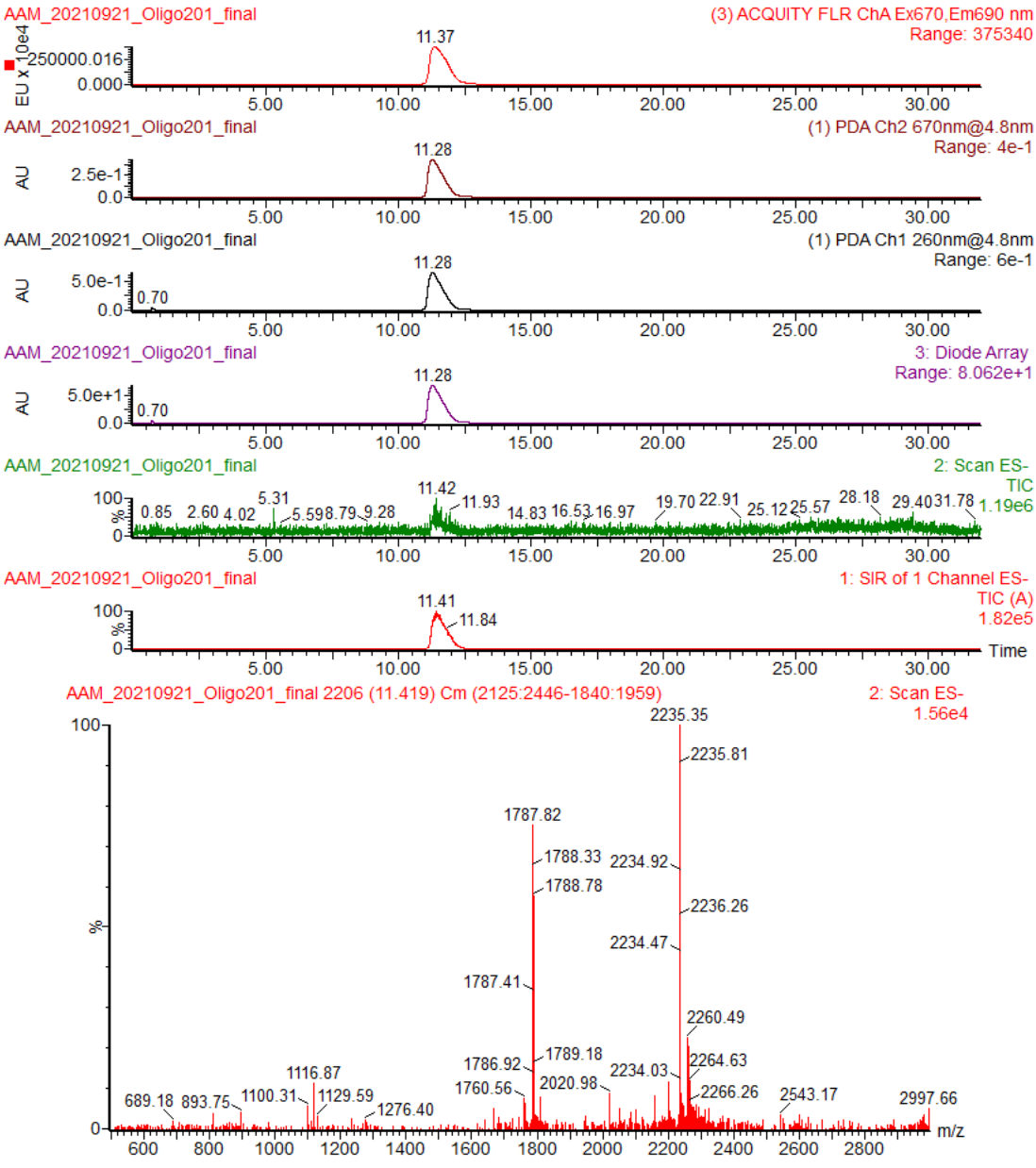


Figure S60: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJD-Cy5Peg, Gradient of 27.5-33.5% MeOH in 0.05 M TEAA (aq.)

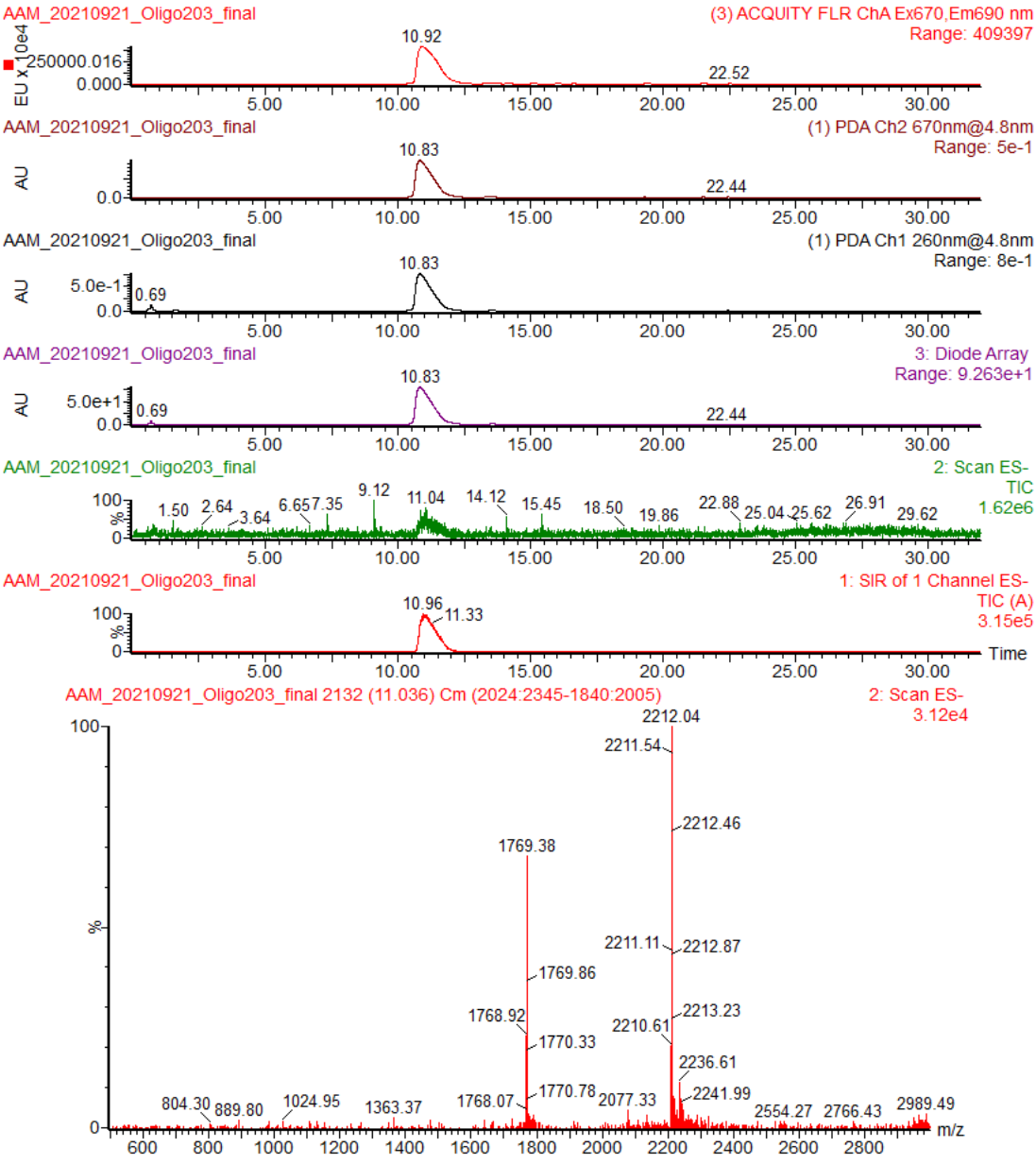


Figure S61: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJAcomp-Cy5Peg, Gradient of 27.5-33.5% MeOH in 0.05 M TEAA (aq.)

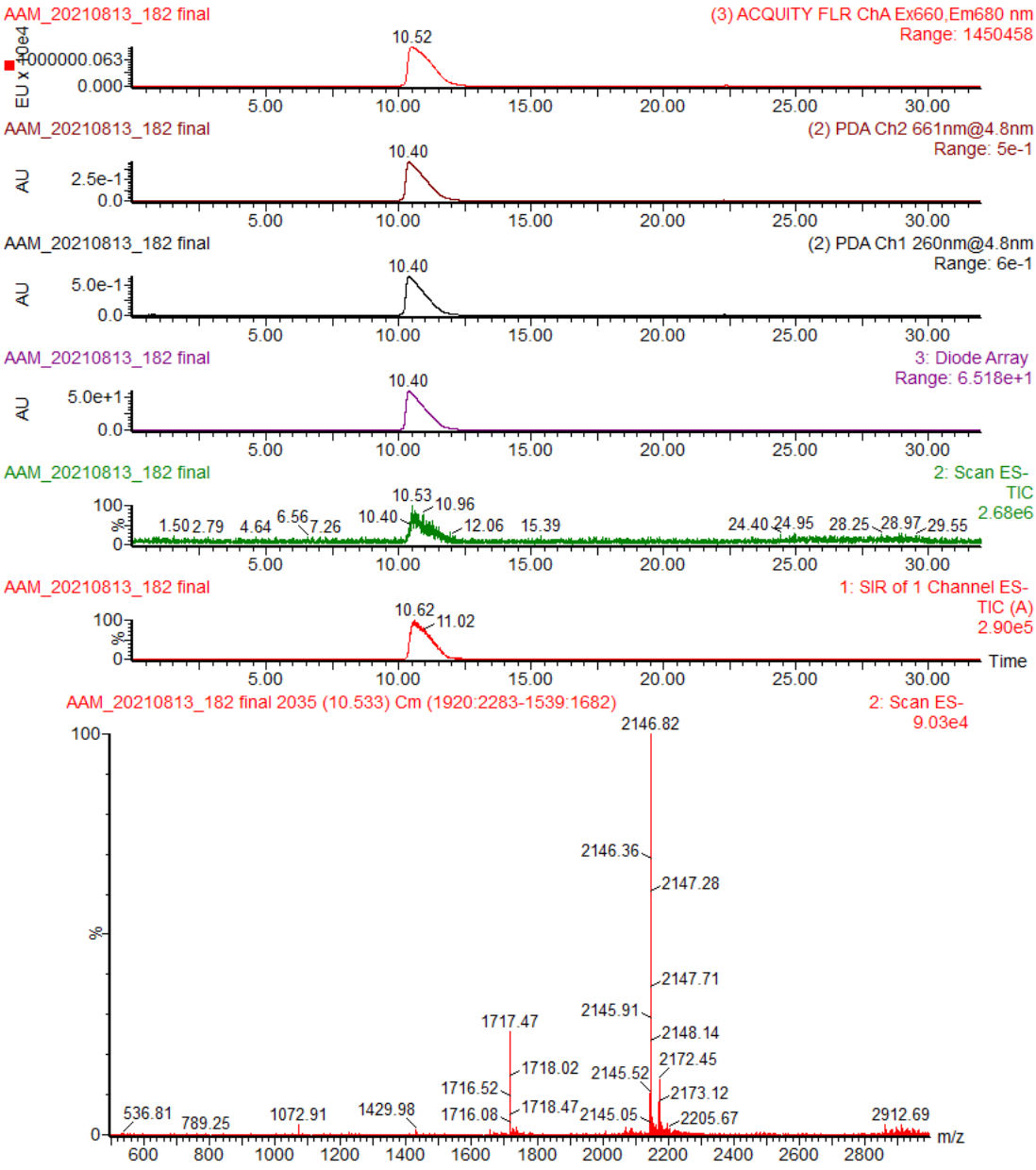


Figure S62: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJA-Cy5tBu, Gradient of 37-43% MeOH in 0.05 M TEAA (aq.)

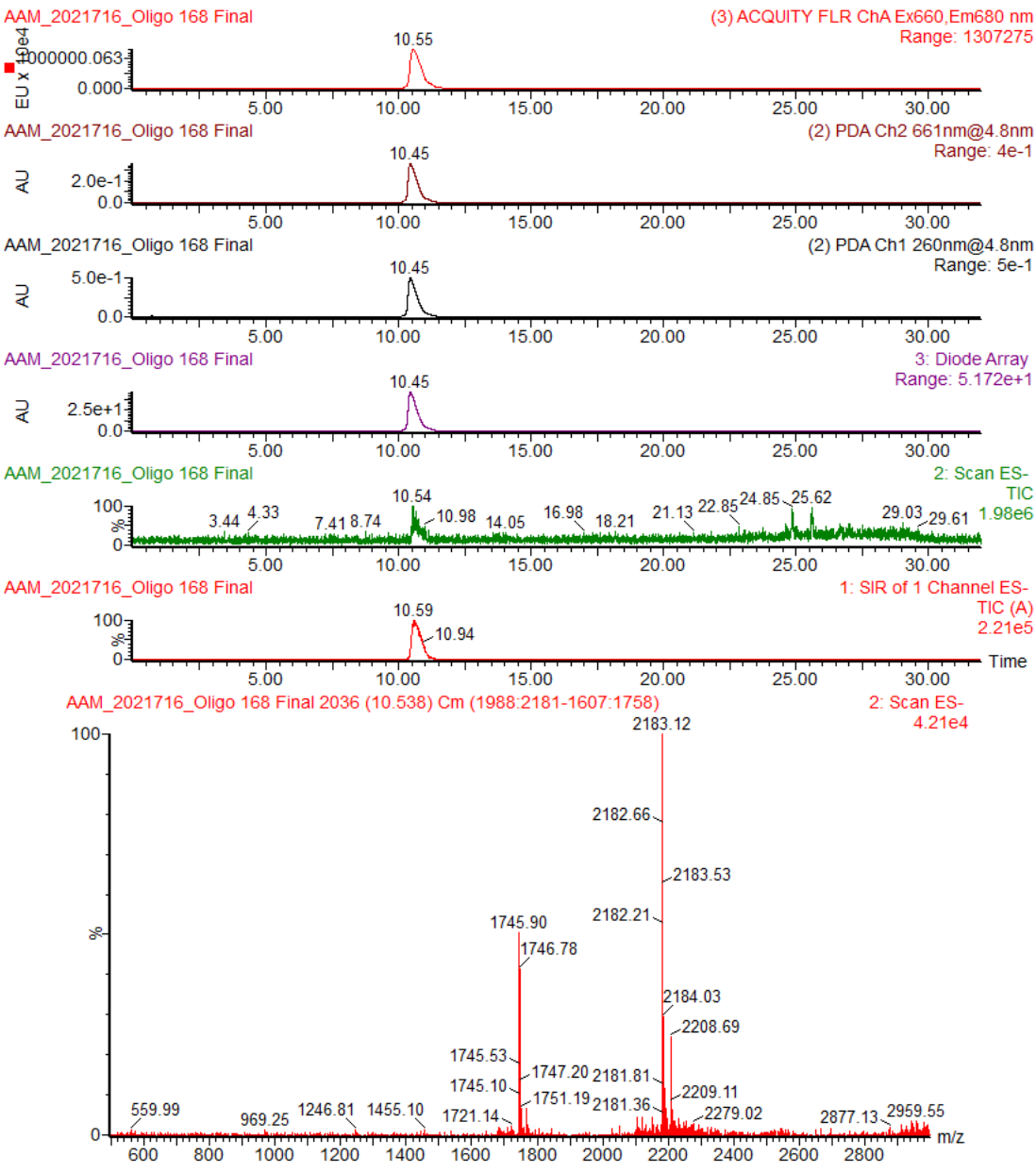


Figure S63: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJB-Cy5tBu, Gradient of 37-43% MeOH in 0.05 M TEAA (aq.)

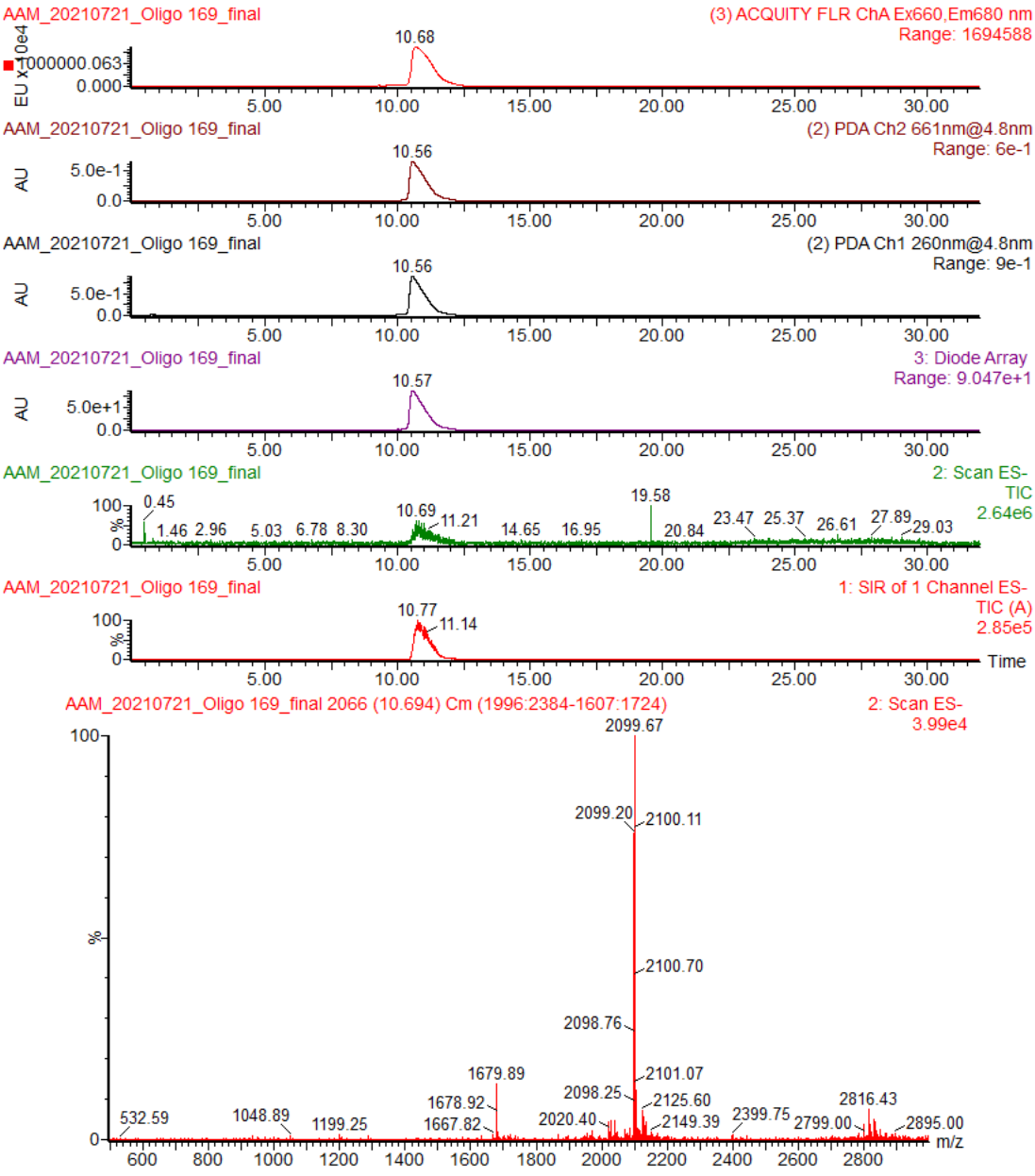


Figure S64: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJC-Cy5tBu, Gradient of 37-43% MeOH in 0.05 M TEAA (aq.)

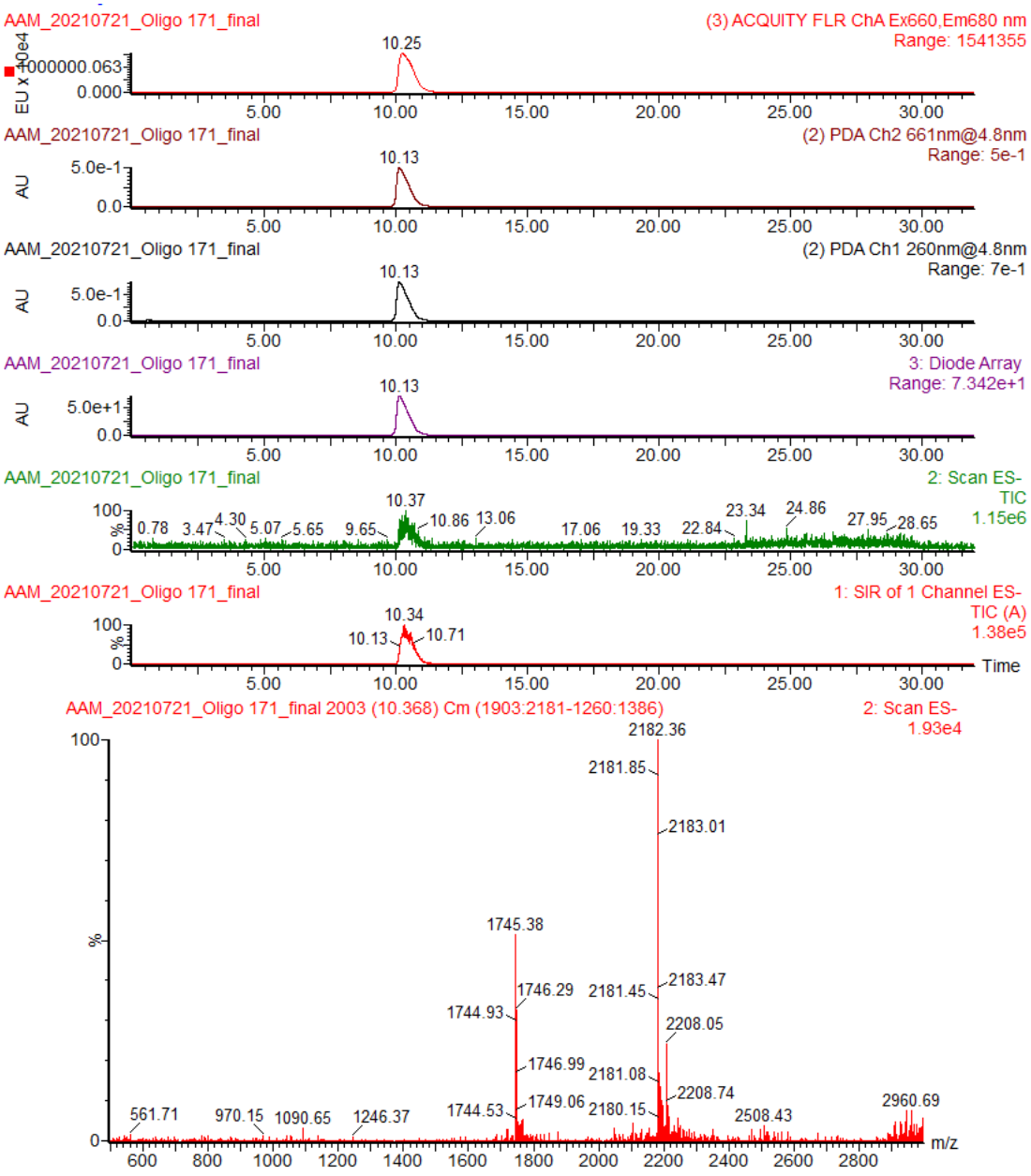


Figure S65: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJD-Cy5tBu, Gradient of 37-43% MeOH in 0.05 M TEAA (aq.)

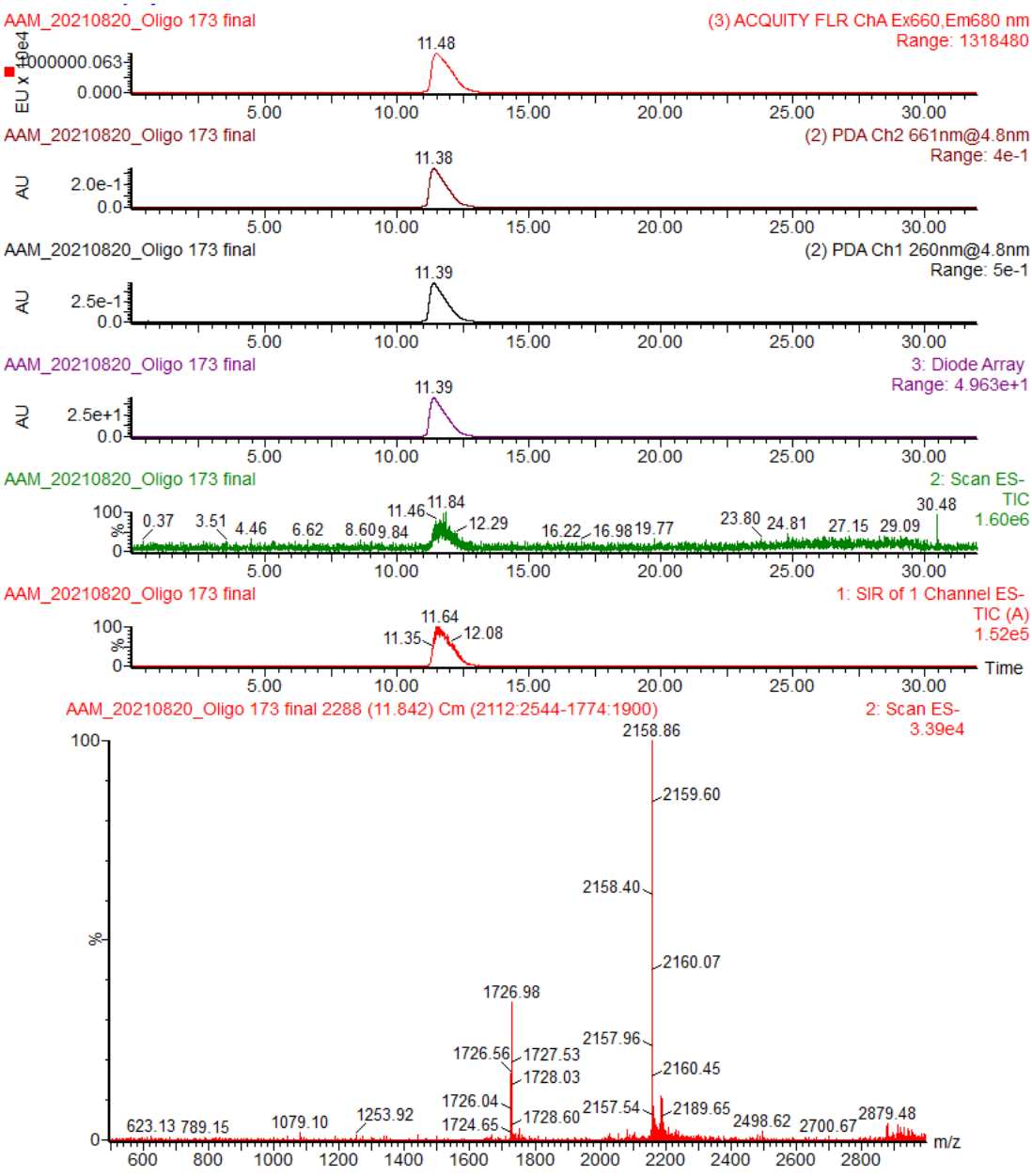


Figure S66: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJAcomp-Cy5tBu, Gradient of 37-43% MeOH in 0.05 M TEAA (aq.)

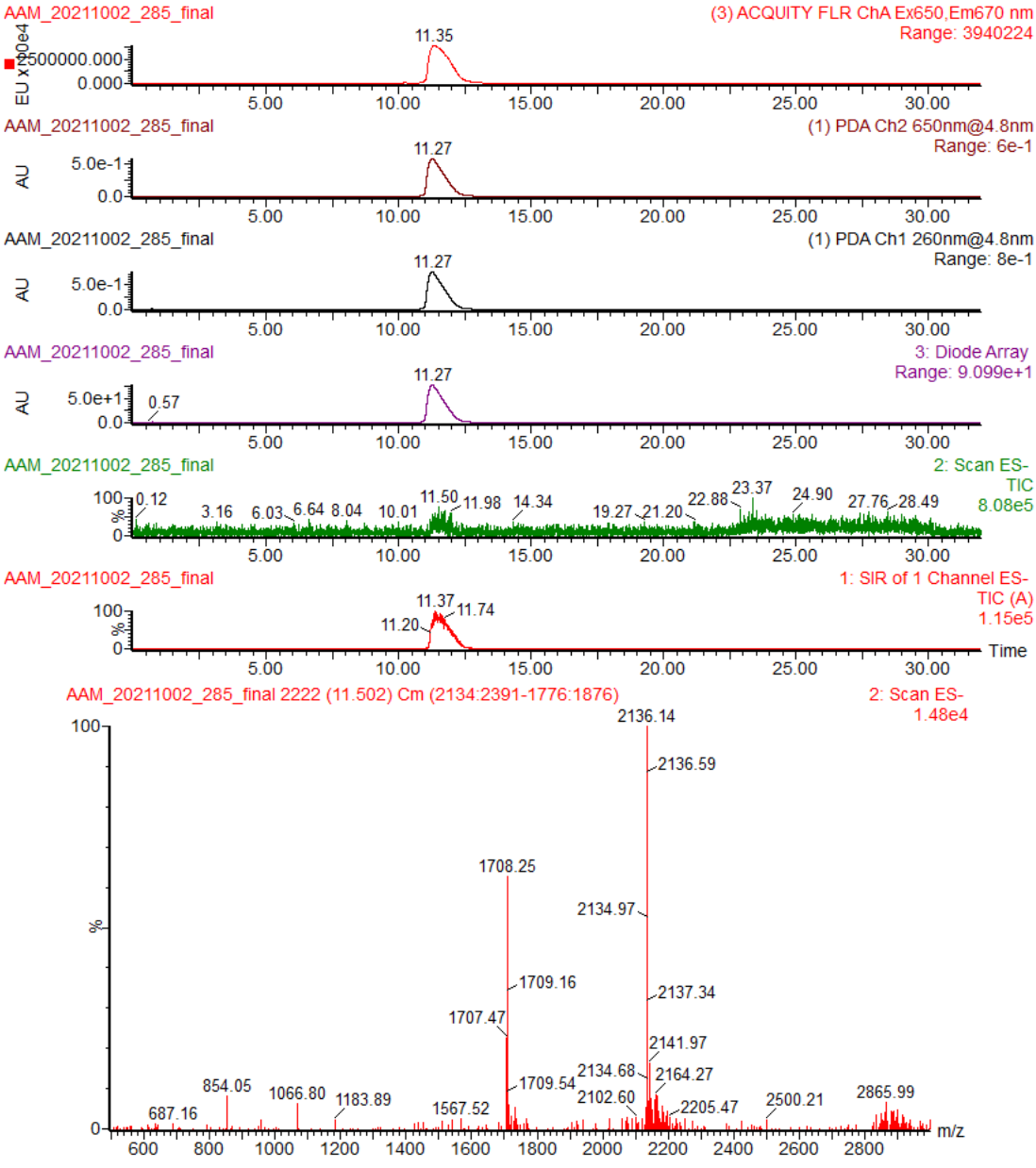


Figure S67: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJA-Cy5Cl, Gradient of 26.5-32.5% MeOH in 0.05 M TEAA (aq.)

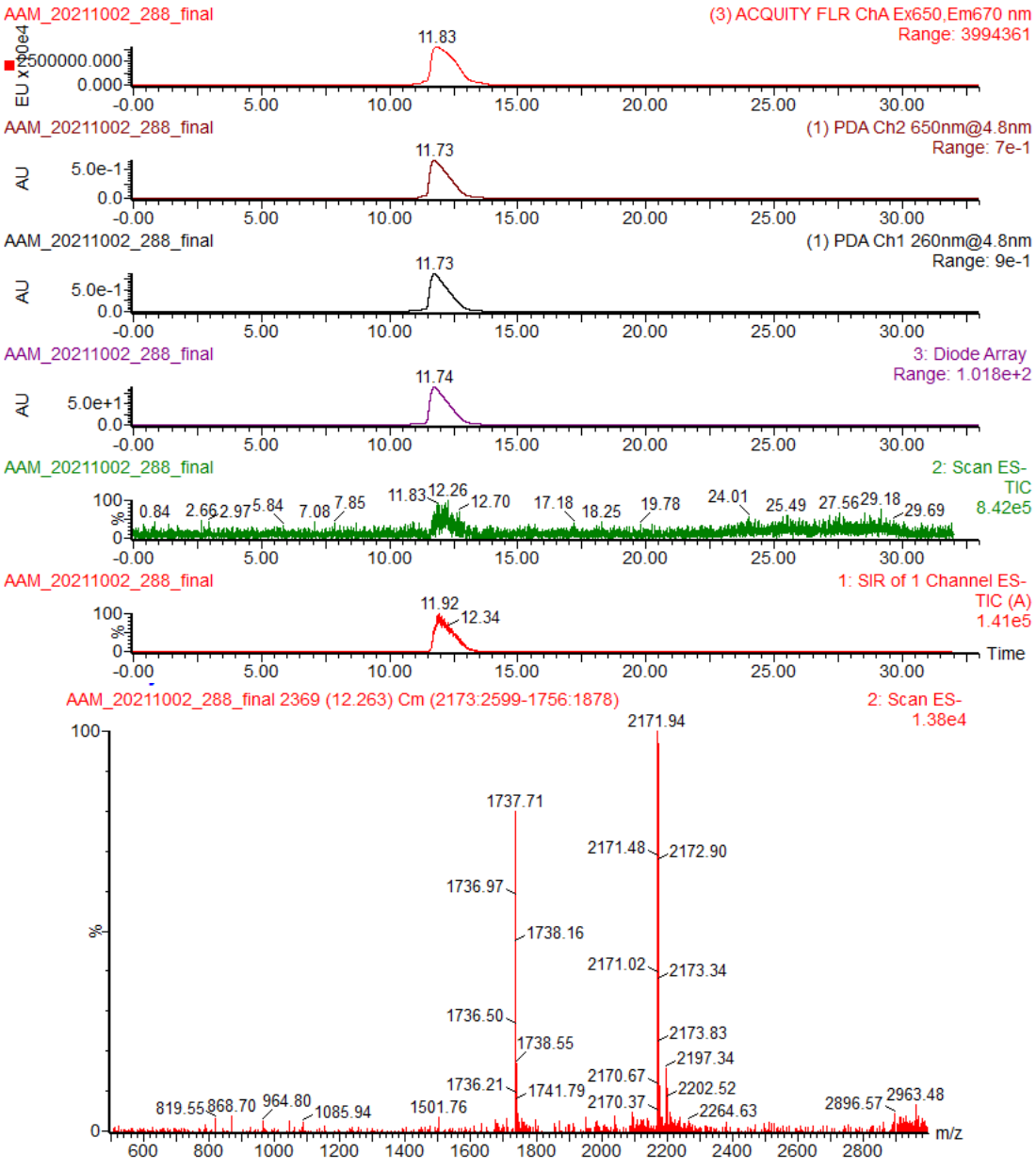


Figure S68: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJB-Cy5Cl, Gradient of 26.5-32.5% MeOH in 0.05 M TEAA (aq.)

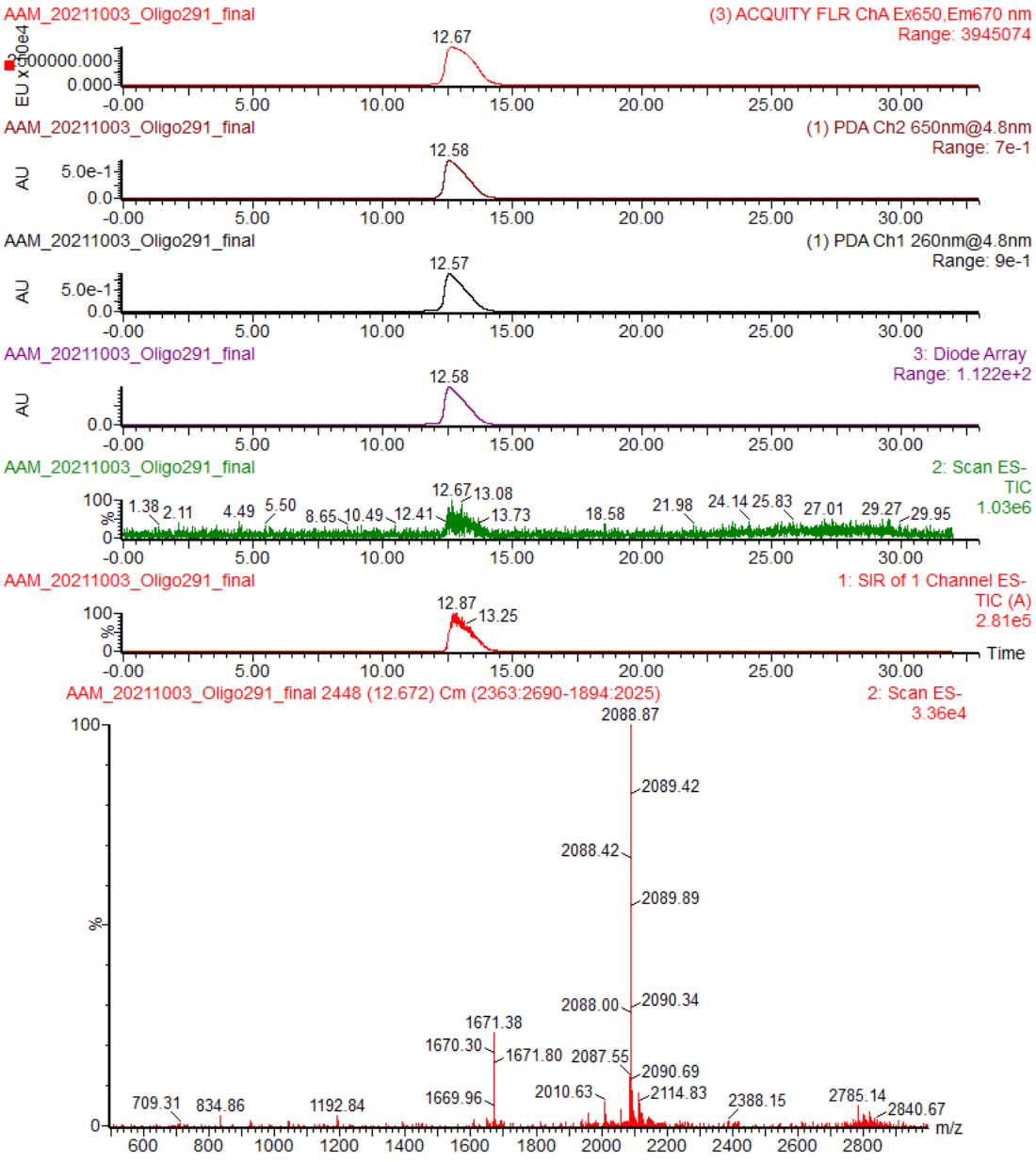


Figure S69: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJC-Cy5Cl, Gradient of 26.5-32.5% MeOH in 0.05 M TEAA (aq.)

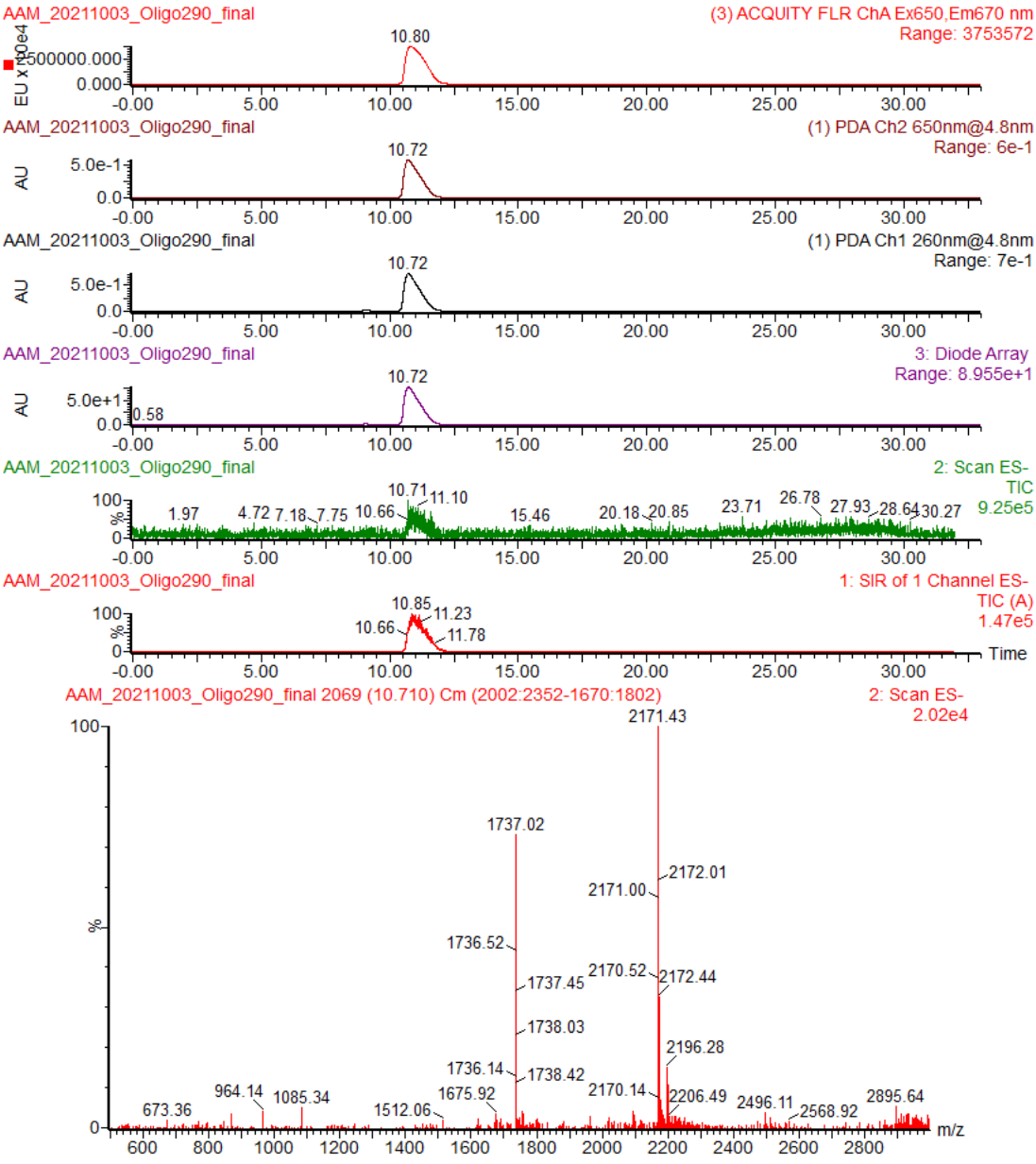


Figure S70: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJD-Cy5Cl, Gradient of 26.5-32.5% MeOH in 0.05 M TEAA (aq.)

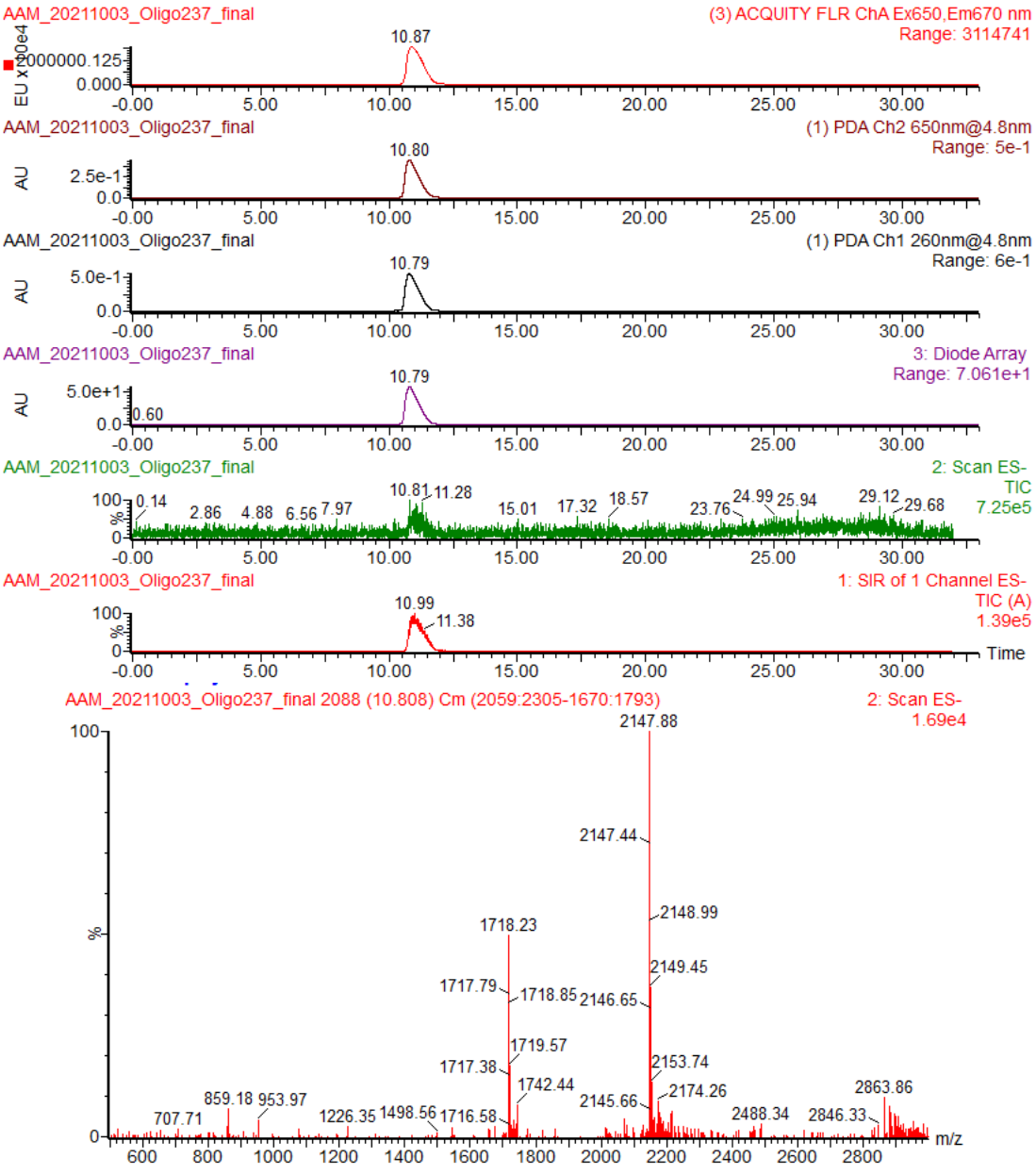


Figure S71: LC/MS Traces (top) and mass spectrum (bottom) of Purified HJAcomp-Cy5Cl, Gradient of 26.5-32.5% MeOH in 0.05 M TEAA (aq.)

Section 6: Additional Absorption Spectra and Unsubstituted Cy5-oligonucleotide Properties

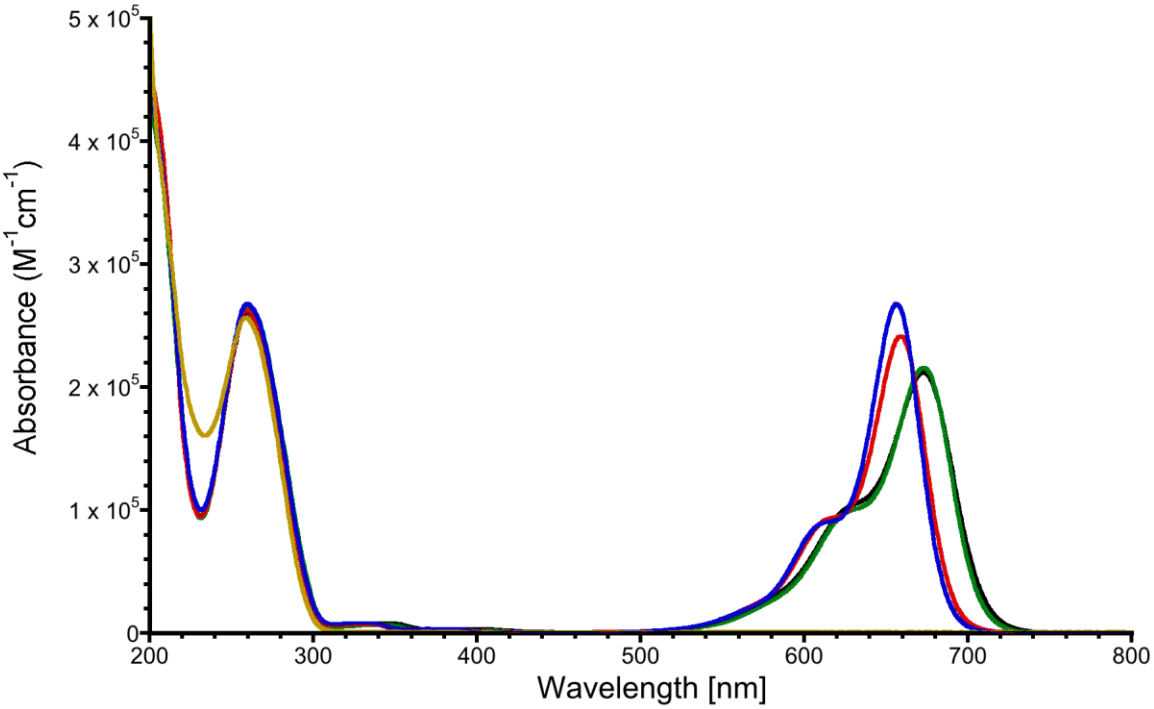


Figure S72: Absorption (extinction coefficient) spectra of HJA-Cy5hex (black), HJA-Cy5Peg (green), HJA-Cy5tBu (red), HJA-Cy5Cl (blue), HJA-unlabeled (gold). All spectra acquired in neat water where concentration in the range of 4-5 µM (A ~ 1.0).

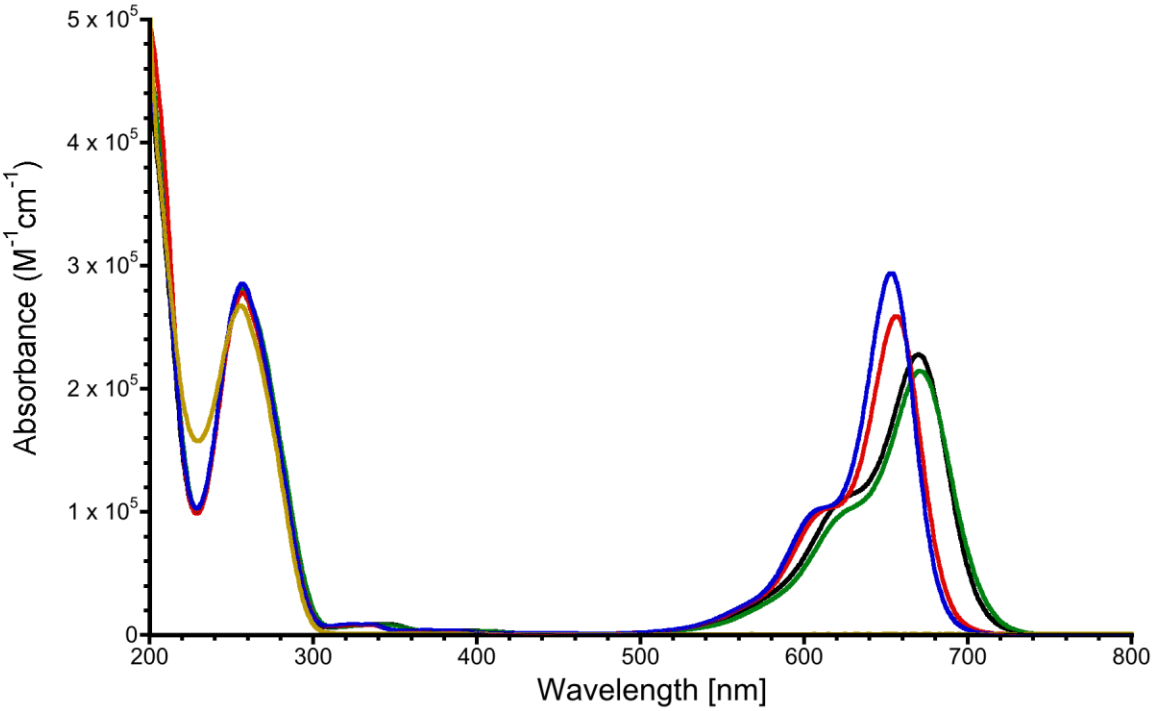


Figure S73: Absorption (extinction coefficient) spectra of HJB-Cy5hex (black), HJB-Cy5Peg (green), HJB-Cy5tBu (red), HJB-Cy5Cl (blue), HJB-unlabeled (gold). All spectra acquired in neat water where concentration in the range of 4-5 µM (A ~ 1.0).

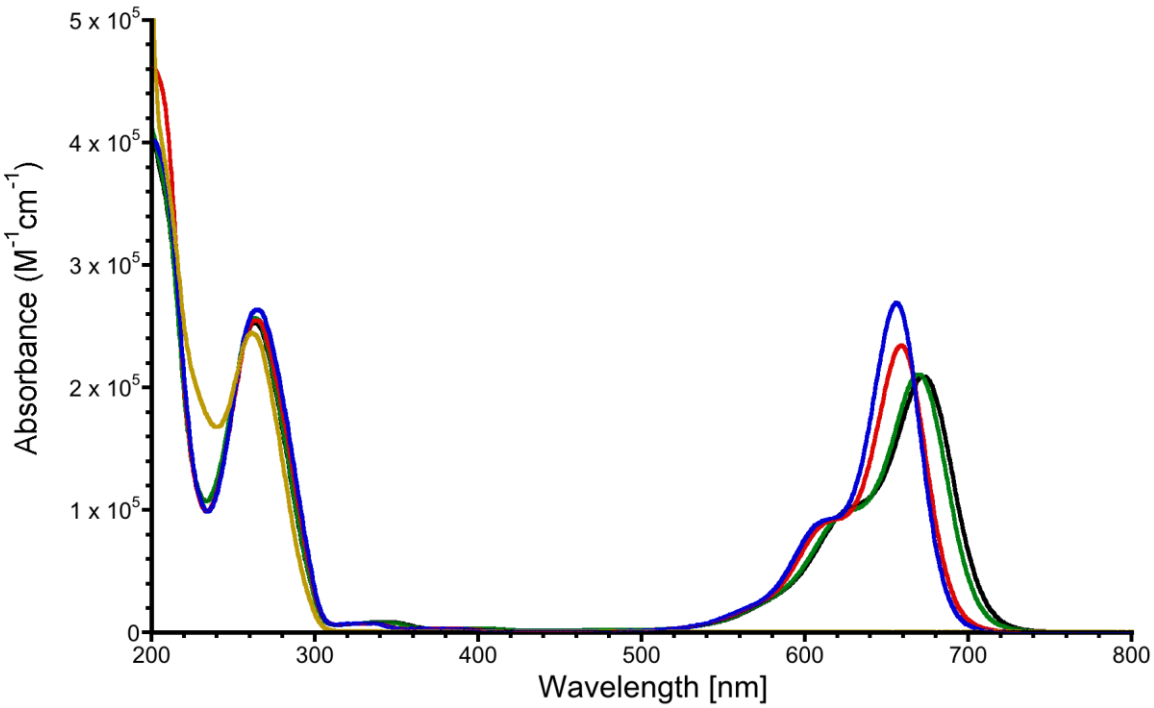


Figure S74: Absorption (extinction coefficient) spectra of HJC-Cy5hex (black), HJC-Cy5Peg (green), HJC-Cy5tBu (red), HJC-Cy5Cl (blue), HJC-unlabeled (gold). All spectra acquired in neat water where concentration in the range of 4-5 µM (A ~ 1.0).

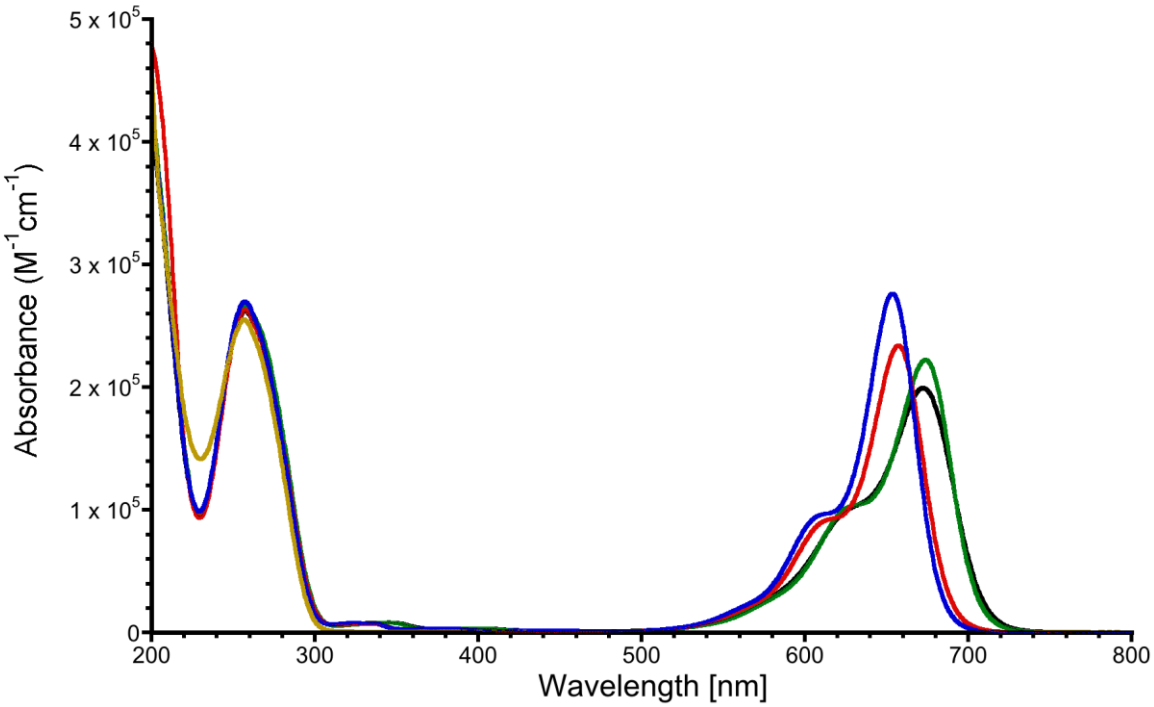


Figure S75: Absorption (extinction coefficient) spectra of HJD-Cy5hex (black), HJD-Cy5Peg (green), HJD-Cy5tBu (red), HJD-Cy5Cl (blue), HJD-unlabeled (gold). All spectra acquired in neat water where concentration in the range of 4-5 µM (A ~ 1.0).

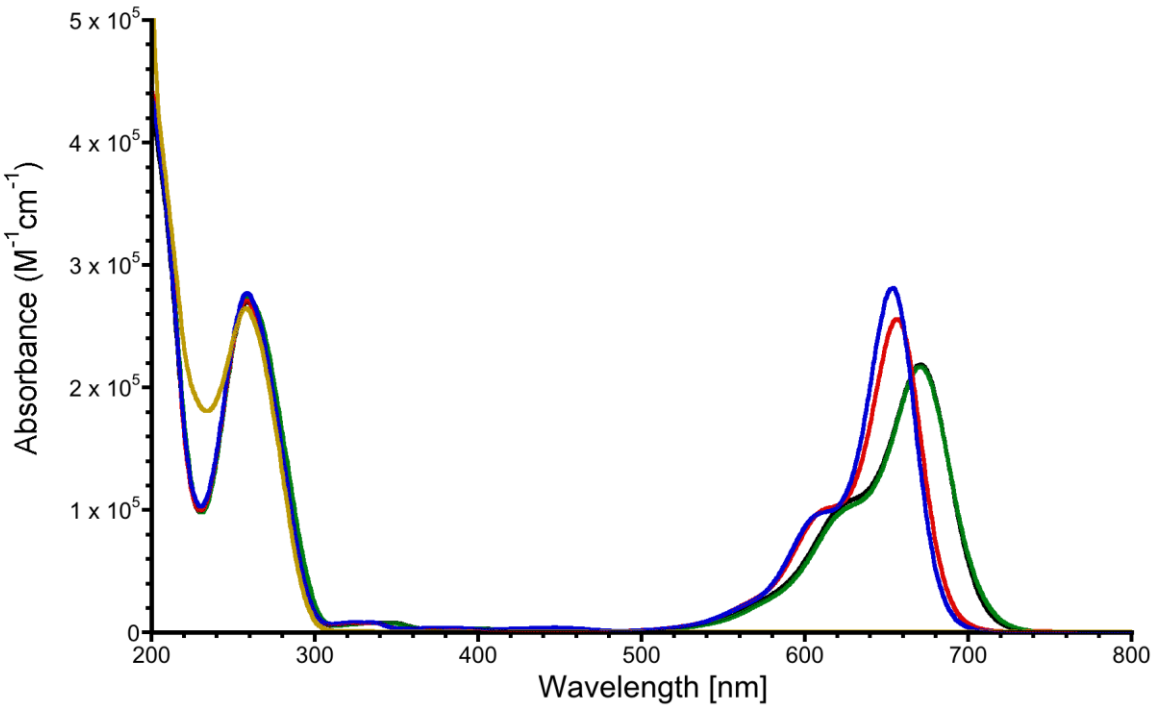


Figure S76: Absorption (extinction coefficient) spectra of HJAcomp-Cy5hex (black), HJAcomp-Cy5Peg (green), HJAcomp-Cy5tBu (red), HJAcomp-Cy5Cl (blue), HJAcomp-unlabeled (gold). All spectra acquired in neat water where concentration in the range of 4-5 μM (A ~ 1.0).

Table S3. Summary of properties for unsubstituted Cy5 sequences. All values determined in neat water. Extinction coefficients determined utilizing nearest-neighbor approximation for DNA absorbance at 260 nm,[6-8] while also accounting for Cy5 contribution. Cy5 percent contribution (0.02-0.04) was based on ratio of absorbance at 260 nm and at Cy5 λ_{max}, for each of the parent dyes, obtained in neat methanol. It was assumed that Cy5 percent contribution at 260 nm is constant between MeOH and H_2O. Fluorescence QY determined against 5,10,15,20-tetraphenylporphyrin standard (Φ_F = 0.07 in toluene) [9].

DNA sequence	λ_{max} abs (nm)	ε_{260} ($M^{-1}cm^{-1}$)	ε_{Cy5} ($M^{-1}cm^{-1}$)	λ_{max} em (nm)	Stokes' Shift (ν)	Φ_F	τ_{avg} (ns)	τ_1 (ns) / amplitude	τ_2 (ns) / amplitude
Cy5-unsubstitued series:									
HJA	647	266,500	247,600	666	441	0.37	1.45	0.66 / 0.26	1.73 / 0.74
HJB	649	277,000	231,900	666	393	0.40	1.82	0.73 / 0.18	2.06 / 0.82
HJC	649	254,100	240,000	665	371	0.41	1.89	1.04 / 0.15	2.04 / 0.85
HJD	649	265,100	249,700	665	371	0.33	1.64	0.59 / 0.15	1.83 / 0.85
HJAcomp	647	274,400	245,900	665	418	0.32	1.25	0.74 / 0.43	1.63 / 0.57

Section 7: Supporting References

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