Supporting Information

# Modular Synthesis of Cell Permeating 2-Ketoglutarate Esters

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**Supporting Figures**

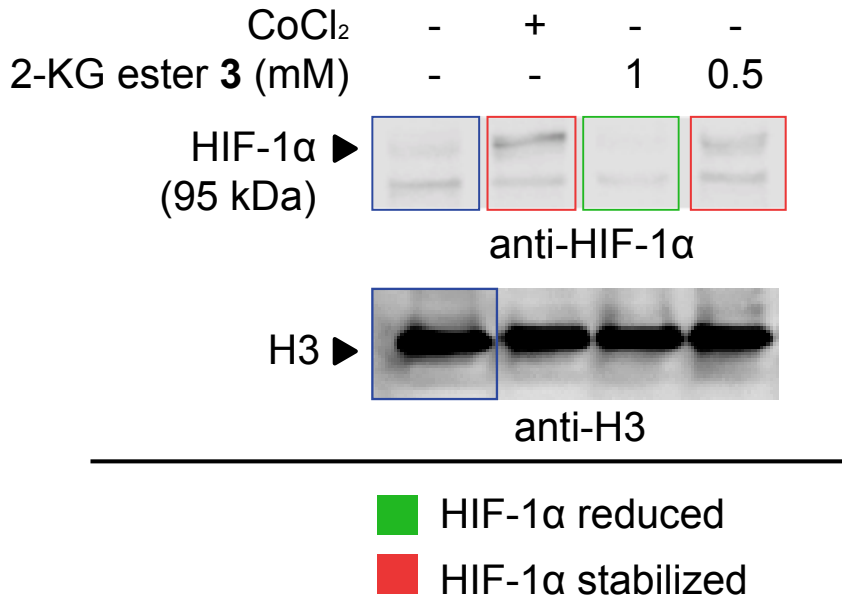


**Figure S1**. Immunoblot confirming effects of 2-KG diester **3** on cellular HIF-1α levels. HIF-1α-ODD-luciferase HeLa cells were treated with either vehicle, (lane 1), known HIF-1α stabilizer $CoCl_2$ (0.5 mM, lane 2) 2-KG diester **3** (1 mM, lane 3; 0.5 mM, lane 4). Lane 1 was aligned next to lane 2 for clarity; full blot data is available upon request. Green box indicates observation of HIF-1α degradation via immunoblot, red box indicates stabilization of HIF-1α, blue box indicates baseline (untreated) HIF-1α levels. Bottom panel shows anti-histone-H3 (H3) blot as loading control.

Concentration dosed

| Compound | 1000 μM | 500 μM | 250 μM | 125 μM |
|---|---|---|---|---|
| 2-KG | 105.7 | 115.7 | 111.3 | 105.9 |
| | 95.6 | 101.2 | 110.4 | 101.3 |
| | 111.9 | 109.4 | 104.6 | 102.6 |
| **1** | 4.9 | 222.7 | 328.3 | 267.2 |
| | 5.5 | 229.7 | 294.8 | 230.9 |
| | 7.4 | 259.9 | 319.6 | 252.4 |
| **2** | 119.1 | 119.3 | 101 | 101.5 |
| | 133.4 | 106.1 | 106 | 103.5 |
| | 121.5 | 95.8 | 103.3 | 102.9 |
| **3** | 41.4 | 581.4 | 420.2 | 127.3 |
| | 43.9 | 572.5 | 479.6 | 139.3 |
| | 41.4 | 580.3 | 451.8 | 106.9 |
| octyl-KG | 26.4 | 159.7 | 161.4 | 128.2 |
| | 19.1 | 166.8 | 163.4 | 139.1 |
| | 13.8 | 155.6 | 157.3 | 120.6 |
| dimethyl-KG | 118.3 | 120.3 | 119 | 108.2 |
| | 123.2 | 114.4 | 117.7 | 110.1 |
| | 120.3 | 106.5 | 115.8 | 114.6 |
| TFMB-OH | 144.9 | 102.3 | 93.2 | 88 |
| | 141.8 | 113.5 | 96.6 | 89.3 |
| | 139.3 | 107.1 | 86.6 | 87.1 |

Percent HIF-1α (Relative to untreated control)

Color Key
HIF degraded
HIF unaffected
HIF stabilized

**Figure S2**. Concentration-dependent effects of 2-KG esters on HIF-luciferase activity. Values represent the percent HIF-luciferase activity relative to an untreated control. Values below 100% indicate less HIF-luciferase activity (anti-hypoxic, blue) values higher than 100% indicate greater HIF-luciferase activity (pro-hypoxic, red).

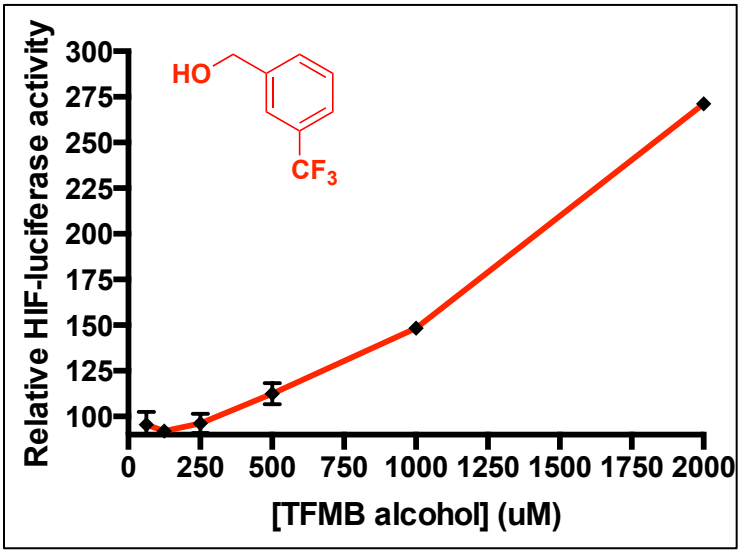


**Figure S3**. TFMB alcohol product produced by ester cleavage is a dose-dependent stabilizer of cellular HIF-luciferase activity. Note the y-axis *begins* at 100% activity.

**General Synthetic Procedures**

Chemicals were purchased from commercial sources (Sigma-Aldrich, Alfa Aesar, and TCI America) and used without further purification unless otherwise noted. *N*-bromosuccinimide was recrystallized in water and vacuum dried immediately before use. Concentration of *n*-BuLi in hexanes was determined by titrating the reagent against diphenylacetic acid in anhydrous THF. Anhydrous solvents were prepared by passage over activated alumina. Thin-layer chromatography (TLC) was conducted with E. Merck silica gel 60 F254 precoated plates (0.25 mm) and visualized by exposure to UV light (254 nm) or chemical staining. Flash column chromatography was performed using normal phase or reverse phase on a CombiFlash® Rf 200i (Teledyne Isco Inc). $^{1}$H NMR spectra were recorded at 400 or 500 MHz, and are reported relative to deuterated solvent signals. Data for $^{1}$H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity, coupling constant (Hz), and integration. $^{13}$C NMR spectra were recorded at 100 or 125 MHz. Data for $^{13}$C NMR spectra are reported in terms of chemical shift. All NMR spectra were standardized to the NMR solvent signal as reported by Gottlieb.[1] Analytical LC/MS was performed using a Shimadzu LCMS-2020 Single Quadrupole utilizing a Kinetex 2.6 µm C18 100 Å (2.1 x 50 mm) column obtained from Phenomenex Inc. Runs employed a gradient of 0→90% MeCN/0.1% aqueous formic acid over 4 minutes at a flow rate of 0.2 mL/min. High-resolution LC/MS analyses were conducted on a Thermo-Fisher LTQ-Orbitrap-XL hybrid mass spectrometer system with an Ion MAX API electrospray ion source in positive or negative ion mode. Separations were carried out on a narrow-bore (50 X 2.1 mm), Zorbax Rapid-Resolution, reversed-phase C18 (3.5 µm) column with a flow rate of 250 µL/min with a 10 min, 2-90% gradient of MeCN/$H_2O$ containing 0.1% HCOOH.

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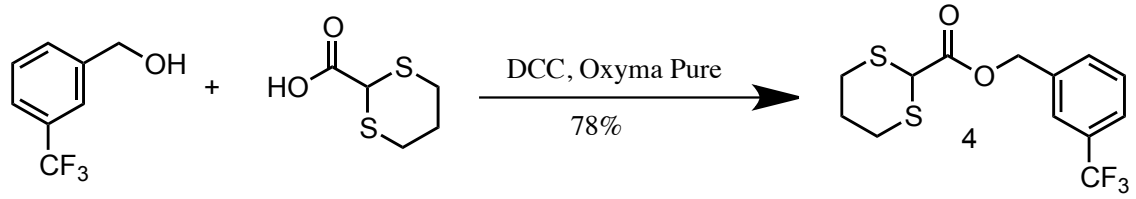
**Synthesis of 2-KG Esters**

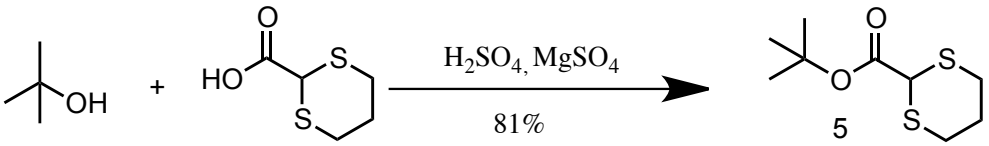
**(a) 3-(trifluoromethyl)benzyl 1,3-dithiane-2-carboxylate (4)**




1,3-dithiane-2-carboxylic acid (1.64 g. 10.0 mmol), 3-trifluoromethylbenzyl alcohol (1.60 g, 9.1 mmol), and Oxyma Pure (1.44 g, 10.1 mmol) were dissolved in DMF (50 mL) with stirring. This mixture was cooled to 0 °C and *N,N'*-dicyclohexylcarbodiimide (DCC) was added (2.30 g, 11.1 mmol) in a single portion. After 1 hour, the ice bath was removed and the reaction was allowed to warm to room temperature and stir overnight. The solvent was removed *in vacuo*, and the subsequent oil was re-dissolved in dichloromethane (50 mL) and washed with 5% $NaHCO_3$ (2 x 75 mL). The organic layer was dried over anhydrous $Na_2SO_4$, filtered, and concentrated under reduced pressure. The resultant oil was purified by flash chromatography (0→50% EtOAc:hexanes) to yield the product **4** as an off-white solid (2.28 g, 78%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.68 (s, 1H), 7.61 (dd, *J* = 12.4, 7.7 Hz, 2H), 7.52 (t, *J* = 7.6 Hz, 1H), 5.29 (s, 2H), 3.48-3.36 (m,

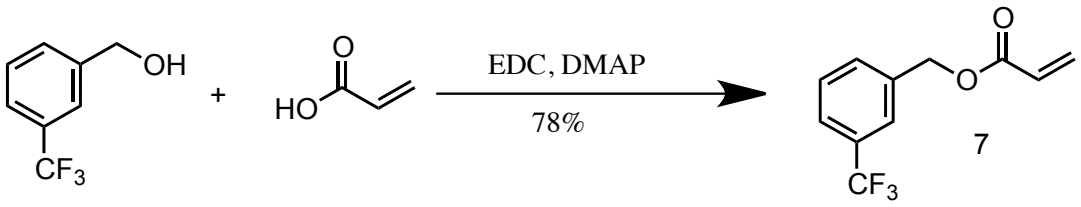
2H), 2.68-2.57 (m, 2H), 2.24-1.98 (m, 2H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 169.42, 136.46, 131.14, 129.15, 125.18, 125.15, 124.63, 124.60, 66.21, 39.57, 25.81, 24.92. HRMS (ESI+): Calcd for $C_{13}H_{14}F_3O_2S_2$: 322.0309. Measured: 323.0382

**(b) Tert-butyl 1,3-dithiane-2-carboxylate (5)**



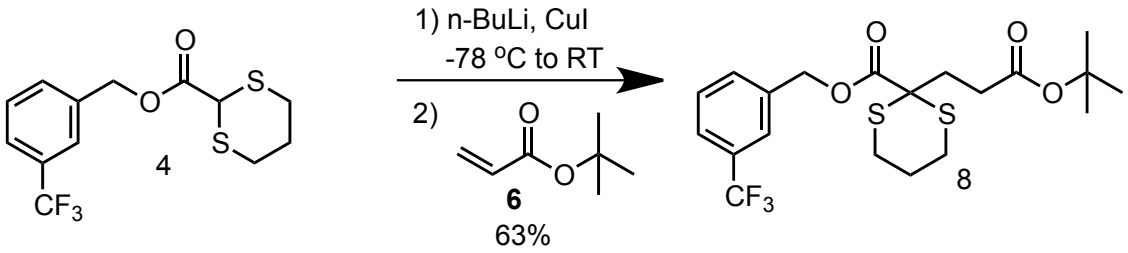

Tert-butyl ester **5** was prepared by a method adapted from Wright and coworkers.[2] Briefly, concentrated $H_2SO_4$ (0.055 mL, 1.0 mmol) was added to vigorously stirring anhydrous $MgSO_4$ (0.481 g, 4.0 mmol), in dichloromethane (4 mL). The reaction mixture was stirred for 15 minutes at room temperature, followed by sequential addition of 1,3-dithiane-2-carboxylic acid (164 mg. 1.0 mmol) and tert-butyl alcohol (0.48 mL, 5.0 mmol). The reaction mixture was stirred for 18 hours, quenched with 7.5 mL of 5% $NaHCO_3$, and stirred until homogenous. The organic phase was washed with brine, dried over anhydrous $Na_2SO_4$, filtered, and concentrated under reduced pressure. The product was purified by flash chromatography (0→50% EtOAc:hexanes) to yield the product **5** as an light yellow solid (1.78 g, 81%). $^1$H NMR (400 MHz, $CDCl_3$) δ 4.11 (s, 1H), 3.47-3.37 (m, 2H), 2.67-2.57 (m, 2H), 2.21-2.10 (m, 1H), 2.07-1.98 (m, 1H), 1.53 (s, 9H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 168.95, 82.31, 41.40, 27.94, 26.05, 25.16. HRMS (ESI+): Calcd for $C_9H_{16}O_2S_2$: 220.0592. Measured: 221.0664

**(c) 3-(trifluoromethyl)benzyl acrylate (7)**



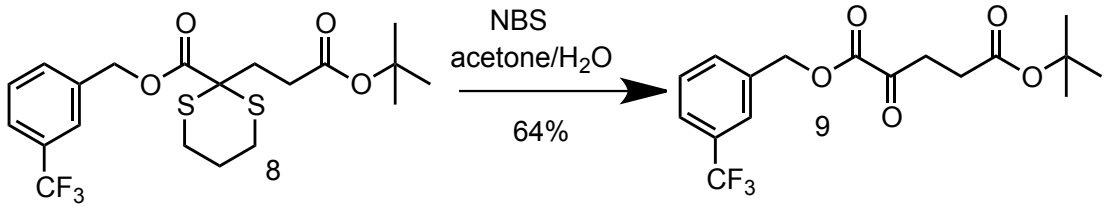

Acrylic acid (0.43 mL, 6.27 mmol), 3-trifluoromethylbenzyl alcohol (1.24 g, 7.0 mmol), dimethyl aminopyridine (DMAP) (0.153 g, 1.25 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.34 g, 7.0 mmol) were dissolved in DMF (7.5 mL), heated to 40 °C, and stirred overnight. The solvent was removed *in vacuo*, and the resultant oil was dissolved in dichloromethane (20 mL) and washed with 5% $NaHCO_3$ (2 x 10 mL The organic layer was dried over anhydrous $Na_2SO_4$, filtered, and concentrated under reduced pressure. The resultant oil was purified by flash chromatography (0→50% EtOAc:hexanes) to yield the product **7** as an oil (2.28 g, 78%). $^1$H NMR (400 MHz, $CDCl_3$) δ 7.69-7.48 (m, 4H), 6.50 (dd, $J$ = 17.4, 1.4 Hz, 1H), 6.21 (dd, $J$ = 17.3, 10.4 Hz, 1H), 5.92 (dd, $J$ = 10.5, 1.4 Hz, 1H), 5.27 (s, 2H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 165.79, 136.87, 131.59, 131.37, 129.08, 127.96, 125.09, 125.05, 124.82, 124.78, 65.40.

**(d) 3-(trifluoromethyl)benzyl 2-(3-(**tert**-butoxy)-3-oxopropyl)-1,3-dithiane-2-carboxylate (8)**




TFMB ester **4** (1.44 g, 4.5 mmol) was dissolved under argon in anhydrous diethyl ether (5 mL) and cooled to -78 °C. A solution of *n*-BuLi in hexanes (1.97 mL, 4.5 mmol) was quickly added and the reaction mixture was allowed to stir for 45 minutes at 0 °C. This solution was transferred via cannula to a cooled (-78 °C) suspension of CuI (0.456 g, 2.39 mmol) in ether,[2] which was allowed to stir for an additional hour. This mixture was warmed to -40 °C and a solution of tert-butyl acrylate **6** (0.22 mL, 1.50 mmol) in ether (8 mL) was added. The reaction was allowed to warm to room temperature over 2.5 hours, quenched with saturated $NH_4Cl$ (25 mL) for 15 minutes, and filtered through Celite. The mixture was extracted with ethyl acetate, dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The resultant oil was purified by flash chromatography (0→50% EtOAc:hexanes) to yield the product **8** as an oil (0.342 g, 63%). $^1$H NMR (400 MHz, $CDCl_3$) δ 7.68 (s, 1H), 7.61 (t, *J* = 7.3 Hz, 2H), 7.52 (t, *J* = 7.7 Hz, 1H), 5.30 (s, 2H), 3.28-3.16 (m, 2H), 2.73-2.61 (m, 2H), 2.54 – 2.46 (m, 2H), 2.42 – 2.33 (m, 2H), 2.18-2.07 (m, 1H), 1.93-1.79 (m, 1H), 1.45 (s, 9H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 171.66, 170.22, 136.67, 131.09, 129.17, 125.15, 125.12, 124.54, 124.51, 80.66, 66.40, 51.61, 33.30, 30.73, 28.06, 27.64, 24.31. HRMS (ESI+):Calcd for $C_{20}H_{25}F_3O_2S_2$: 450.1146. Measured: 451.1219

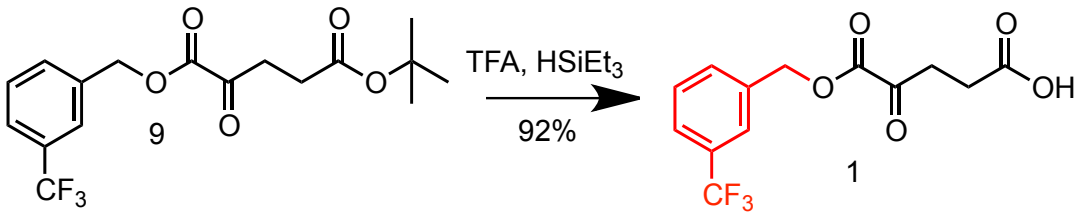
**(e) 5-tert-butyl 1-(3-(trifluoromethyl)benzyl) 2-oxopentanedioate (9)**




Diester **8** (71.9 mg, 0.16 mmol) was dissolved in acetone (0.8 mL) and added dropwise to a stirring, ice-cold solution of *N*-bromosuccinimide (228 mg, 1.3 mmol) in 95:5 acetone:water (4 mL).[3] Once the starting material had been consumed as judged by TLC analysis (typically ~5-15 minutes), the reaction was quenched with saturated sodium sulfite (5 mL) and 1:1 hexane:dichloromethane (5 mL). The solution was washed with 5% $NaHCO_3$, water, and brine, dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The resultant oil was purified by flash chromatography (0→50% EtOAc:hexanes) to yield the product **9** as an oil (36.7 mg, 64%). $^1$H NMR (400 MHz, $CDCl_3$) δ 7.69 (s, 1H), 7.62 (t, *J* = 7.3 Hz, 2H), 7.53 (t, *J* = 7.7 Hz, 1H), 5.35 (s, 2H), 3.13

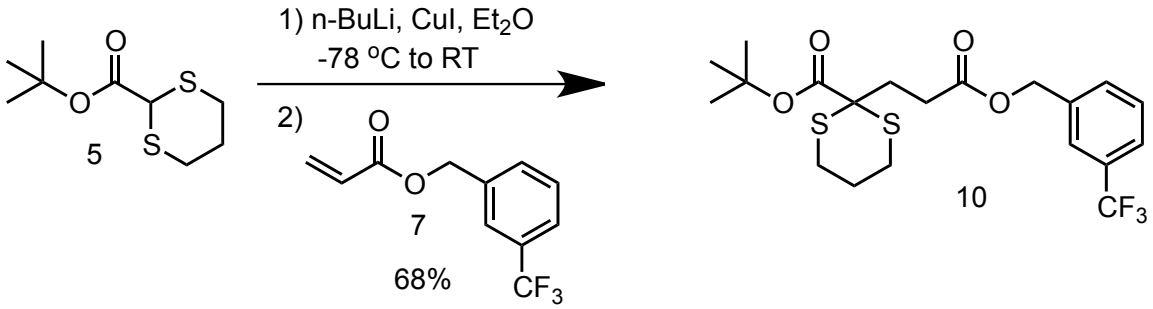
(t, *J* = 6.5 Hz, 2H), 2.63 (t, *J* = 6.5 Hz, 2H), 1.44 (s, 9H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 192.20, 171.12, 160.19, 135.39, 131.89, 129.30, 125.66, 125.63, 125.36, 81.19, 67.02, 34.32, 29.00, 28.00. HRMS (ESI+): Calcd for $C_{17}H_{19}F_3O_5$: 360.1185. Measured: 378.1525 (corresponds to hydrate), 383.1078 ($M+Na^+$)

**(f) 4,5-dioxo-5-((3-(trifluoromethyl)benzyl)oxy)pentatonic acid (1)**




Selective removal of the tert-butyl ester was accomplished following a method adapted from Mehta and coworkers.[4] Briefly, diester **9** (21.3 mg, 0.059 mmol) was dissolved in dichloromethane (0.5 mL), and a mixture of TFA (0.77 mmol) and triethylsilane (0.15 mmol) were directly added. The reaction was stirred for 15 minutes at room temperature, and directly loaded onto a disposable Combi*Flash* FR 200i column for flash chromatography purification using a 0→10% $CH_2Cl_2$:MeOH gradient. Concentration of eluted product under reduced pressure results in isolation of a mixture of the ketone and hydrate form of **1** (15.5 mg, 92%; estimated at ~60% ketone by $^1$H-NMR). Note: $^1$H NMR and $^{13}$C NMR values for both the ketone and hydrate are reported. $^1$H NMR (500 MHz, $CDCl_3$) δ 7.72-7.51 (m, 8H), 5.42-5.31 (m, 4H), 4.67 (s, 1H), 3.21 (t, *J* = 6.4 Hz, 2H), 2.90-2.70 (m, 6H), 2.40- 2.34 (m, 1H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 191.58, 175.24, 174.21, 168.22, 159.99, 135.25, 134.92, 131.89, 131.71, 131.49, 131.35, 131.23, 129.54, 129.34, 125.96 (q, *J* =3.8 Hz), 125.72 (q, *J* =3.8 Hz), 125.38 (q, *J* = 5Hz), 125.23 (q, *J* =3.8 Hz), 125.20, 125.17, 124.81, 101.17, 68.13, 67.16, 34.01, 30.66, 28.20, 26.91. HRMS (ESI-): Calcd for $C_{13}H_{11}F_3O_5$: 304.0559. Measured: 303.0485

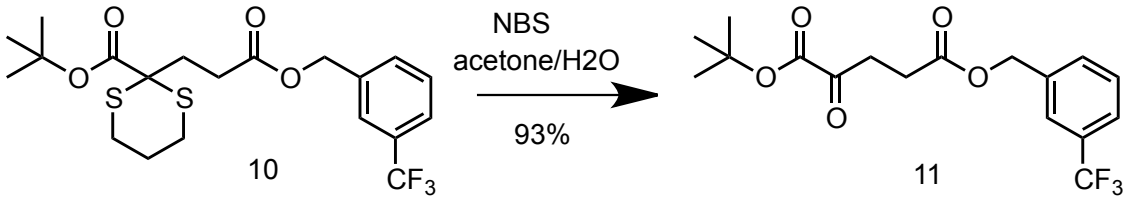
**(g) Tert-butyl 2-(3-oxo-3-((3-(trifluoromethyl)benzyl)oxy)propyl)-1,3-dithiane-2-carboxylate (10)**




Tert-butyl ester **5** (232 mg, 1.1 mmol) was dissolved under argon in anhydrous diethyl ether (1.3 mL) and cooled to -78 °C. A solution of *n*-BuLi in hexanes (0.45 mL, 1.1 mmol) was quickly added and the reaction mixture was allowed to stir for 45 minutes at 0 °C. This solution was transferred via cannula to a cooled (-78 °C) suspension of CuI (107 mg, 0.56 mmol) in ether (2.5 mL),[2] which was allowed to stir for an additional hour. This

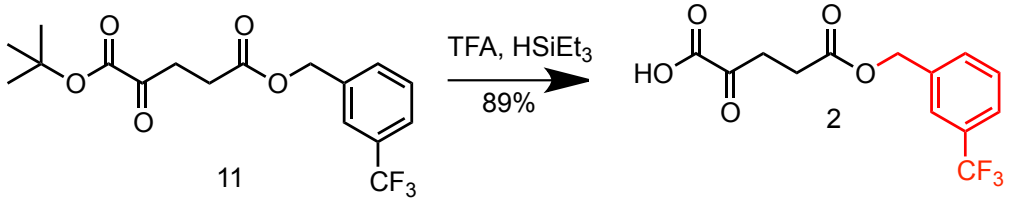
mixture was warmed to -40 °C and a solution of TFMB acrylate **7** (81.1 mg, 0.35 mmol) in ether was added. The reaction was allowed to warm to room temperature over 2.5 hours, quenched with saturated $NH_4Cl$ (5 mL) for 15 minutes, and filtered through Celite. The mixture was extracted with ethyl acetate, dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The resultant oil was purified by flash chromatography (0→50% EtOAc:hexanes) to yield the product **10** as an oil (0.107 g, 68%). $^1$H NMR (400 MHz, $CDCl_3$) δ 7.67-7.46 (m, 4H), 5.19 (s, 2H), 3.37-3.25 (m, 2H), 2.75-2.61 (m, 4H), 2.42-2.31 (m, 2H), 2.20-2.09 (m, 1H), 1.93-1.80 (m, 1H), 1.53 (s, 9H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 172.39, 169.46, 136.91, 131.37, 129.05, 125.02, 124.98, 124.82, 124.78, 82.64, 65.48, 51.95, 33.25, 29.66, 27.95, 27.55, 24.49. HRMS (ESI+): Calcd for $C_{20}H_{25}F_3O_4S_2$: 450.1146. Measured: 451.1221

**(h) 1-tert-butyl 5-(3-(trifluoromethyl)benzyl) 2-oxopentanedioate (11)**




Diester **10** (207 mg, 0.46 mmol) was dissolved in acetone (2.5 mL) and added dropwise to a stirring, ice-cold solution of *N*-bromosuccinimide (655 mg, 3.7 mmol) in 95:5 acetone:water (12 mL).[3] Once the starting material had been consumed as judged by TLC analysis (typically ~5-15 minutes), the reaction was quenched with saturated sodium sulfite (5 mL) and 1:1 hexane:dichloromethane (5 mL). The solution was washed with 5% $NaHCO_3$, water, and brine, dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The resultant oil was purified by flash chromatography (0→40% EtOAc:hexanes) to yield the product **9** as an oil (154 mg, 93%). $^1$H NMR (500 MHz, $CDCl_3$) δ 7.64-7.50 (m, 4H), 5.20 (s, 2H), 3.17 (t, $J$ = 6.5, 6.5 Hz, 2H), 2.74 (t, $J$ = 6.5, 6.5 Hz, 2H), 1.57 (s, 9H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 193.46, 171.83, 159.79, 136.69, 131.36, 129.11, 125.11, 125.08, 124.81, 124.78, 84.26, 65.74, 34.00, 27.78, 27.54. HRMS (ESI+): Calcd for $C_{17}H_{19}F_3O_5$: 360.1185. Measured: 378.1525 (corresponds to hydrate), 383.1078 ($M+Na^+$)

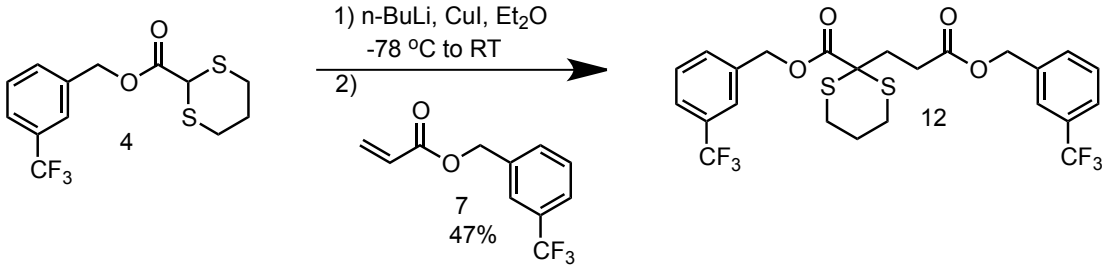
**(i) 2,5-dioxo-5-((3-(trifluoromethyl)benzyl)oxy)pentatonic acid (2)**




Diester **11** (68.6 mg, 0.19 mmol) was dissolved in dichloromethane (0.5 mL), and a mixture of TFA (0.19 mL, 2.49 mmol) and triethylsilane (0.076 mL, 0.48 mmol) were directly added.[4] The reaction was stirred for 30 minutes, concentrated under reduced pressure, and purified by flash chromatography (0→10% $CH_2Cl_2$:MeOH ) to yield **2** as an oil (51.4 mg, 89%). $^1$H NMR (400 MHz, $CDCl_3$) δ 7.54 (s, 2H), 7.48-7.38 (m, 2H), 5.11 (s, 2H), 3.21 (t, $J$ = 6.8, 5.8 Hz, 2H), 2.74 (t, $J$ = 6.8, 5.8 Hz, 2H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 194.16, 171.50, 158.48, 136.42, 131.41, 129.16, 125.25, 125.22, 124.87,

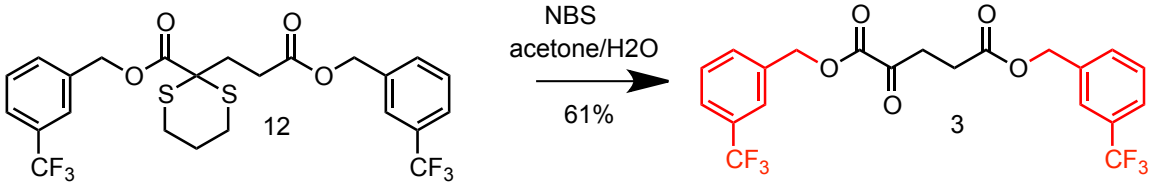
124.84, 66.01, 32.19, 27.82. HRMS (ESI+): Calcd for $C_{13}H_{11}F_3O_5$: 304.0559. Measured: 349.0270 ($M+2Na^+$)

**(j) 3-(trifluoromethyl)benzyl 2-(3-oxo-3-((3-(trifluoromethyl)benzyl)oxy)propyl)-1,3-dithiane-2-carboxylate (12)**




TFMB ester **4** (670 g, 2.1 mmol) was dissolved under argon in anhydrous diethyl ether (1 mL) and cooled to -78 °C. A solution of *n*-BuLi in hexanes (0.83 mL, 2.1 mmol) was quickly added and the reaction mixture was allowed to stir for 45 minutes at 0 °C. This solution was transferred via cannula to a cooled (-78 °C) suspension of CuI (211 mg, 1.1 mmol) in ether (5 mL),[2] which was allowed to stir for an additional hour. This mixture was warmed to -40 °C and a solution of TFMB acrylate **7** (160 mg, 0.7 mmol) in ether was added. The reaction was allowed to warm to room temperature over 2.5 hours, quenched with saturated $NH_4Cl$ (10 mL) for 15 minutes, and filtered through Celite. The mixture was extracted with ethyl acetate, dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The resultant oil was purified by flash chromatography (0→50% EtOAc:hexanes) to yield the product **12** as an oil (0.182 g, 47%). $^1$H NMR (400 MHz, $CDCl_3$) δ 7.70-7.46 (m, 8H), 5.29 (s, 2H), 5.18 (s, 2H), 3.24 (t, *J* = 16.0 Hz, 2H), 2.76-2.58 (m, 4H), 2.45 (t, *J* = 8.0 Hz, 2H), 2.19-2.05 (m, 1H), 1.93-1.76 (m, 1H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 172.15, 170.12, 136.79, 136.56, 131.40, 131.10, 129.18, 129.06, 125.19, 125.16, 125.06, 125.03, 124.85, 124.82, 124.55, 124.52, 66.45, 65.56, 51.26, 32.99, 29.50, 27.58, 24.17. HRMS (ESI+): Calcd for $C_{24}H_{22}F_6O_4S_2$: 552.0864. Measured: 553.0930

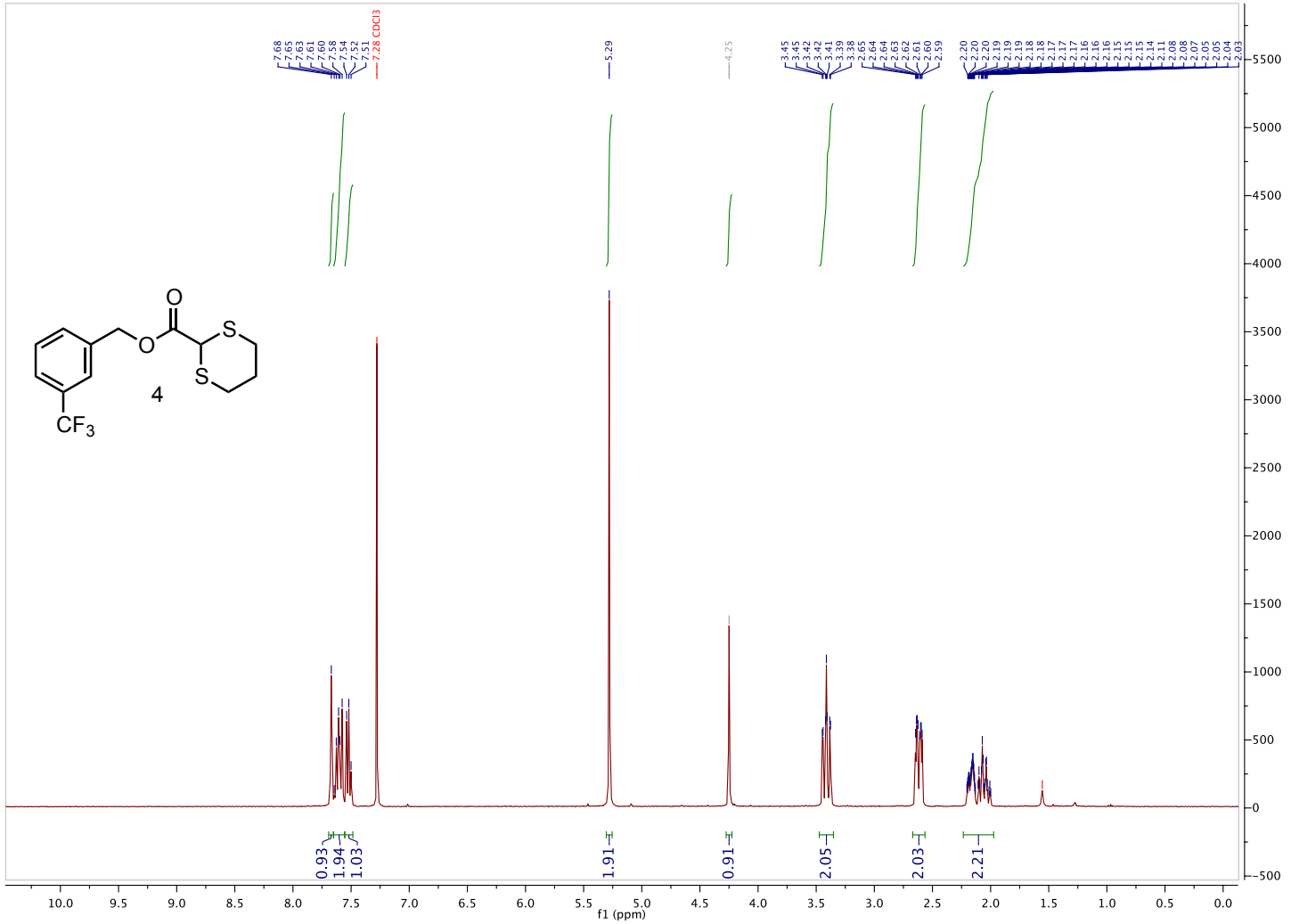
**(k) Bis(3-(trifluoromethyl)benzyl) 2-oxopentanedioate (3)**

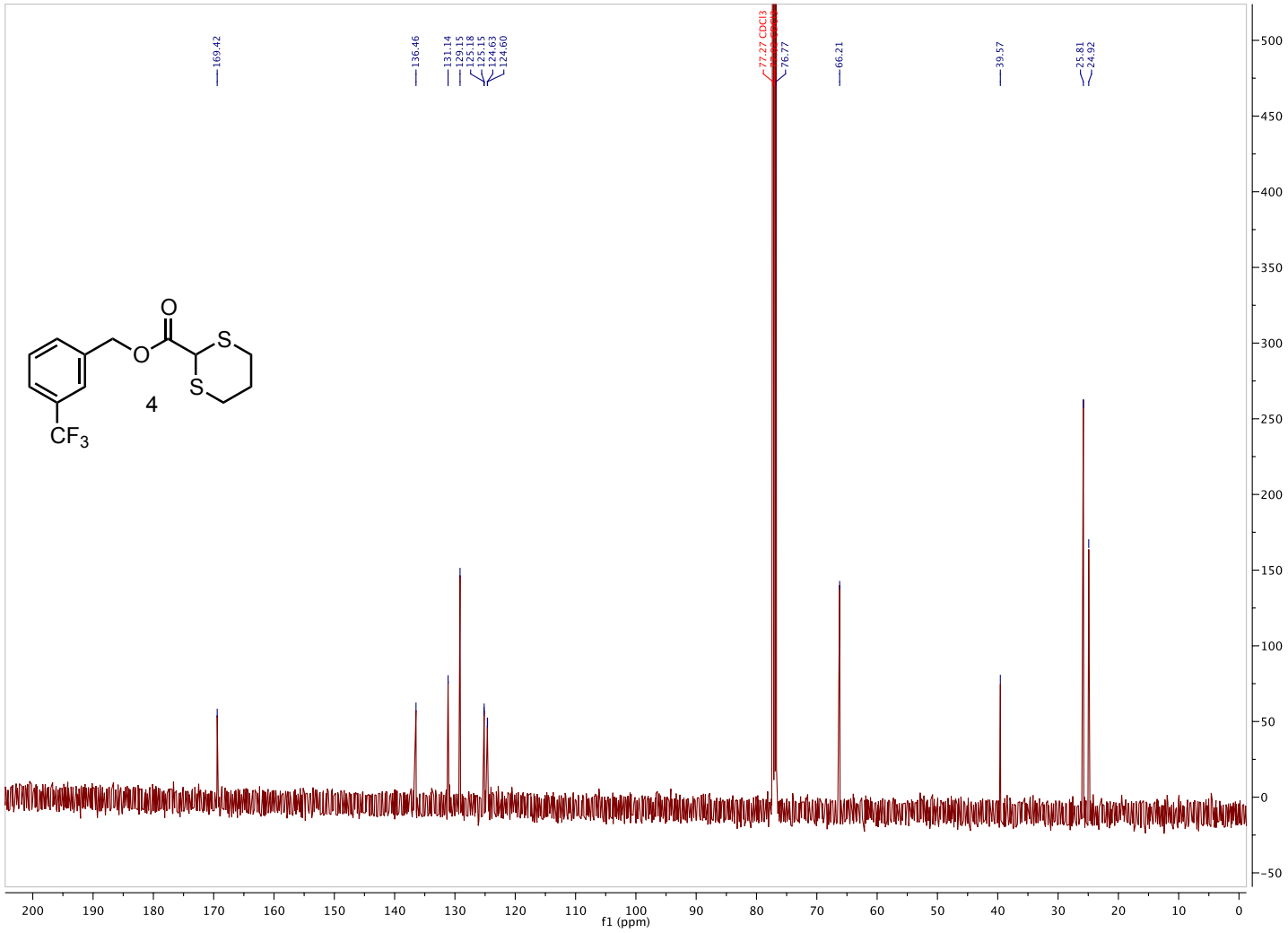



Diester **12** (55.5 mg, 0.10 mmol) was dissolved in acetone (0.5 mL) and added dropwise to a stirring, ice-cold solution of *N*-bromosuccinimide (142 mg, 0.8 mmol) in 95:5 acetone:water (2.5 mL).[3] Once the starting material had been consumed as judged by TLC analysis (typically ~5-10 minutes), the reaction was diluted with saturated sodium sulfite (5 mL) and 1:1 hexane:dichloromethane (5 mL). The solution was washed with 5% $NaHCO_3$, water, and brine, dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The resultant oil was purified by flash chromatography (0→50%

EtOAc:hexanes) to yield the product **3** as an oil (28.2 mg, 68%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.70-7.48 (m, 8H), 5.35 (s, 2H), 5.19 (s, 2H), 3.23 (t, *J* = 6.4 Hz, 2H), 2.78 (t, *J* = 6.4 Hz, 2H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 191.78, 171.64, 160.03, 136.57, 135.27, 131.87, 131.38, 129.32, 129.12, 125.70, 125.67, 125.36, 125.33, 125.16, 125.12, 124.83, 124.80, 122.72, 67.13, 65.85, 34.16, 27.60. HRMS (ESI+): Calcd for $C_{24}H_{22}F_6O_4S_2$: 462.0902. Measured: 463.0976

**NMR Spectra for Final Products and Intermediates**

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124.60
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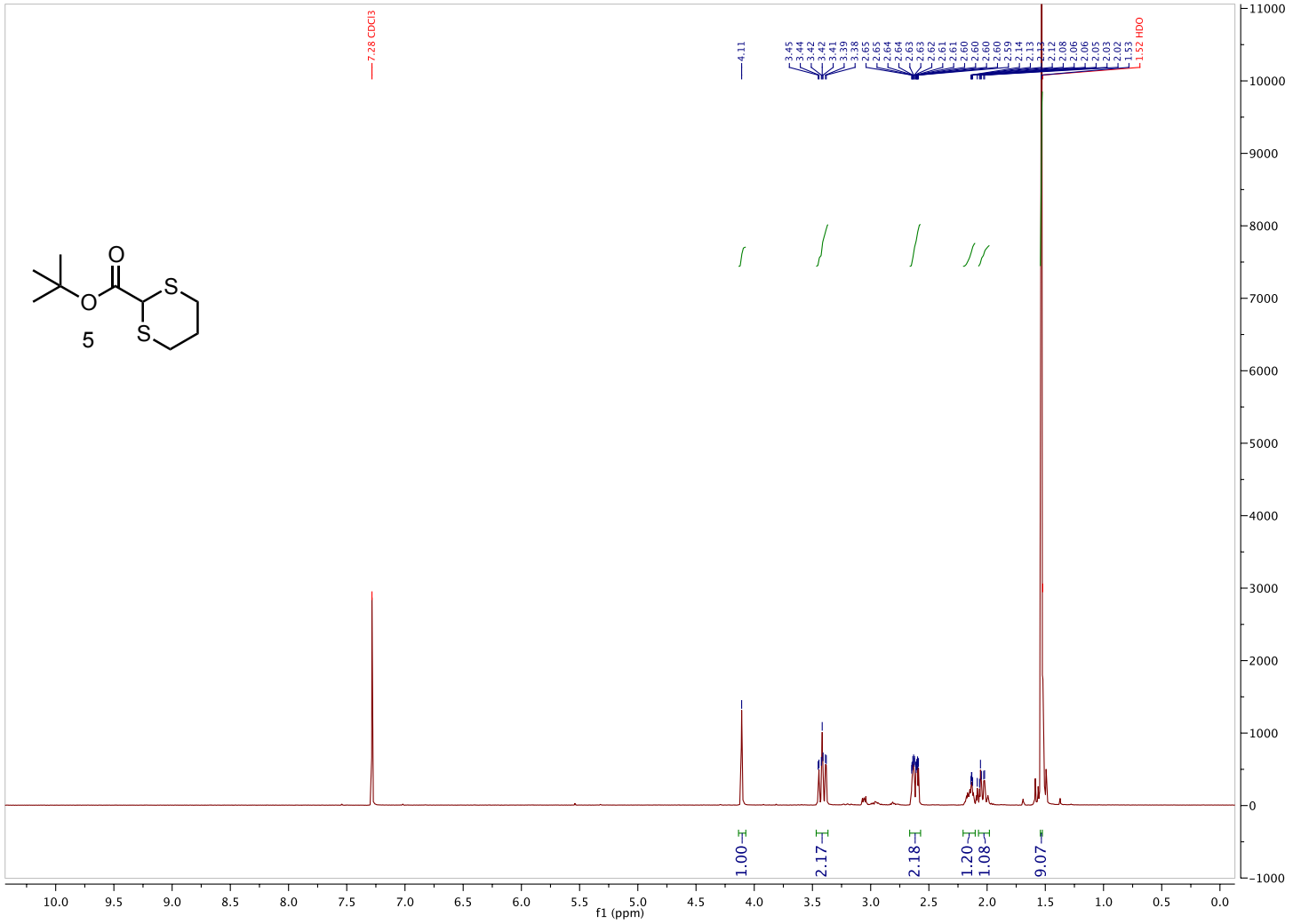
O
O
S
S
4
CF3
f1 (ppm)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4.11
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3.39
3.38
2.65
2.64
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2.63
2.63
2.62
2.61
2.61
2.60
2.60
2.60
2.60
2.59
2.14
2.13
2.13
2.12
2.08
2.06
2.06
2.05
2.03
2.02
1.53
1.52 HDO
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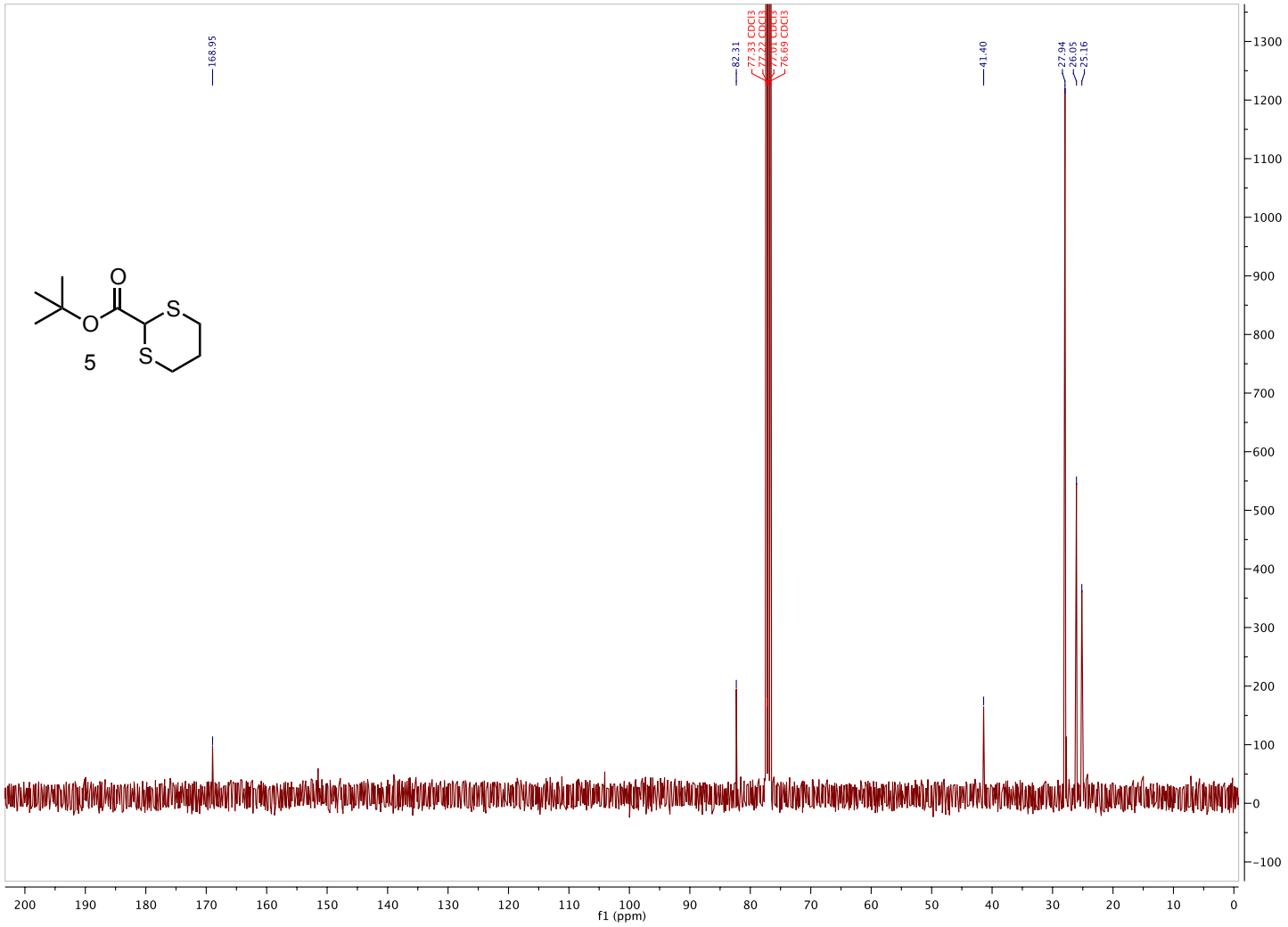
O
S
S
5
1.00
2.17
2.18
1.20
1.08
9.07
f1 (ppm)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82.31
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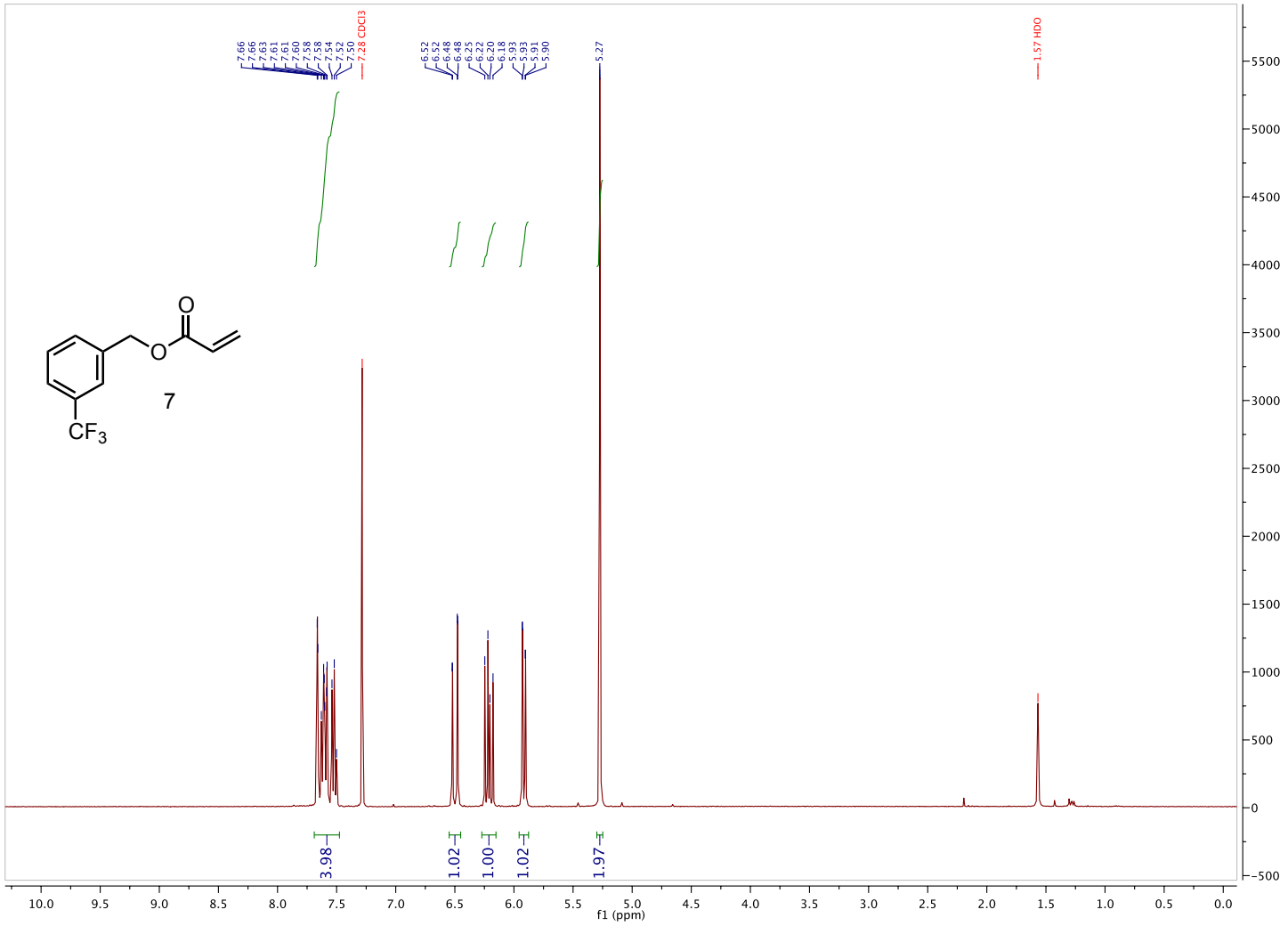
41.40
27.94
26.05
25.16
O
O
S
S
5
f1 (ppm)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7.66
7.63
7.61
7.61
7.60
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7.58
7.54
7.52
7.50
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6.52
6.52
6.48
6.48
6.25
6.22
6.20
6.18
5.93
5.93
5.91
5.90
5.27
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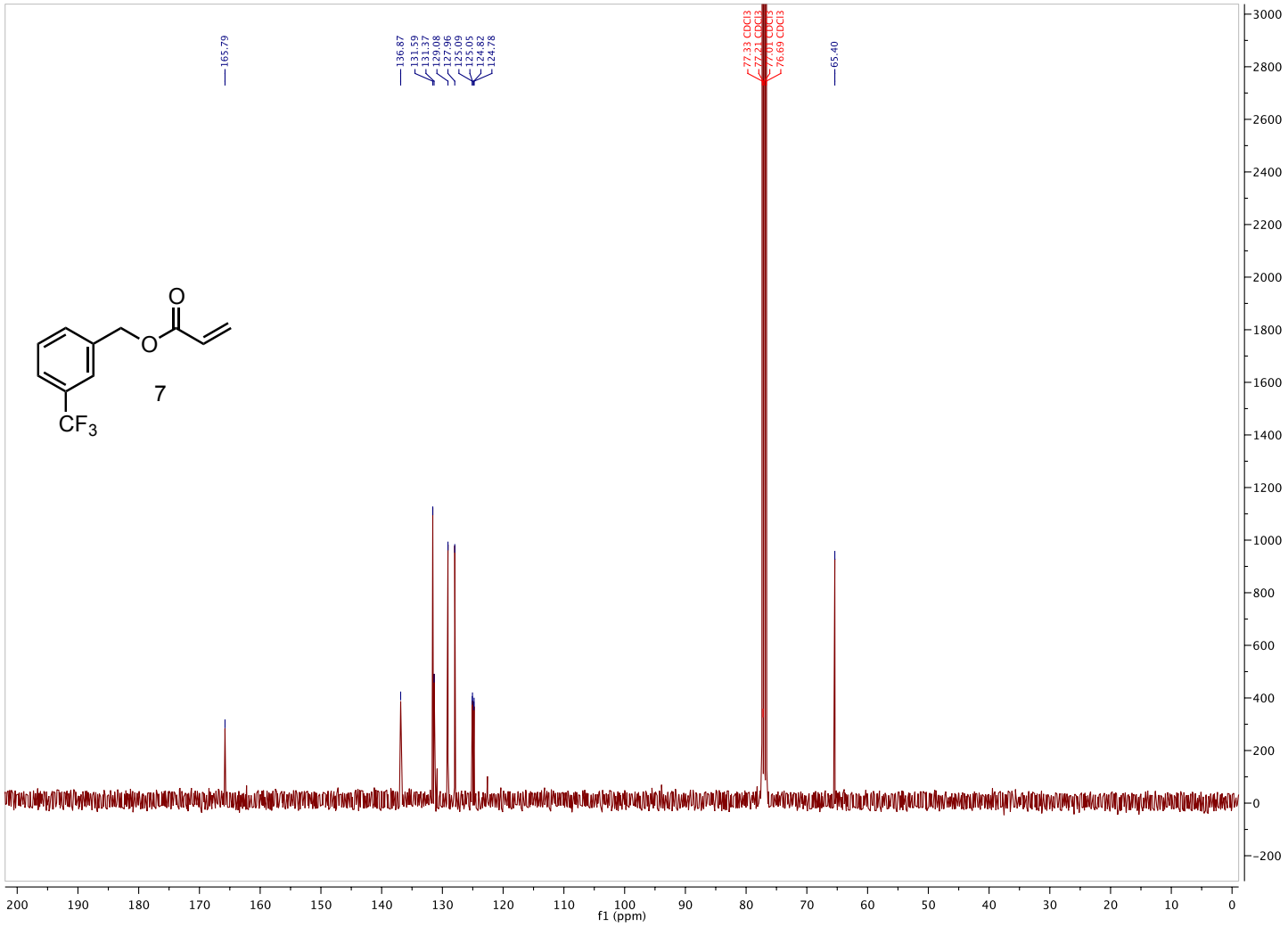
O
O
CF3
7
3.98
1.02
1.00
1.02
1.97
f1 (ppm)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125.05
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124.78
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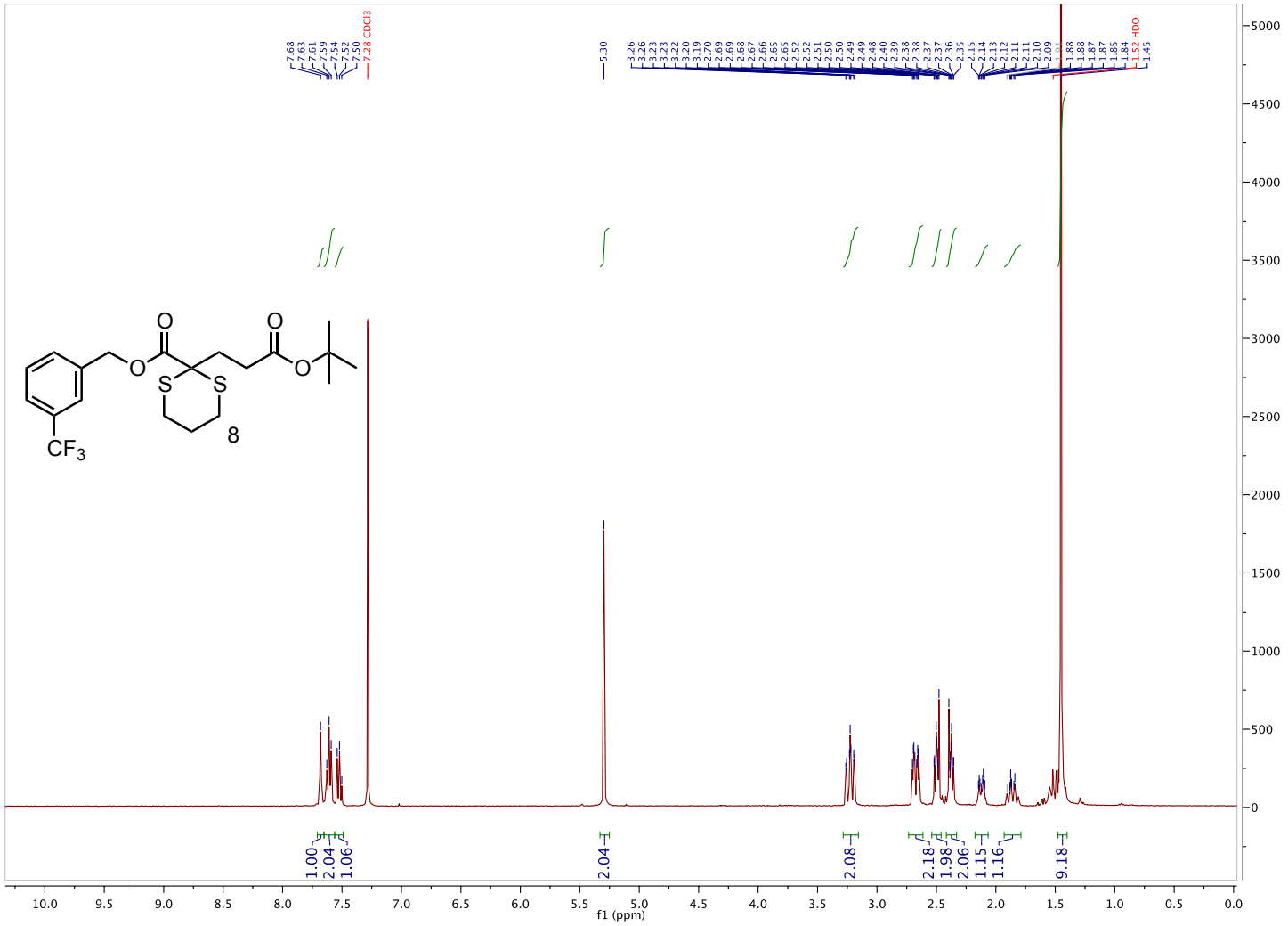
77.01 CDCl3
76.69 CDCl3
65.40
O
O
CF3
7
f1 (ppm)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3.26
3.23
3.22
3.20
3.19
2.70
2.69
2.68
2.67
2.66
2.65
2.52
2.51
2.50
2.49
2.48
2.40
2.39
2.38
2.37
2.36
2.35
2.15
2.14
2.13
2.12
2.11
2.10
2.09
1.88
1.87
1.85
1.84
1.52 HDO
1.45
O
O
O
O
S
S
8
CF3
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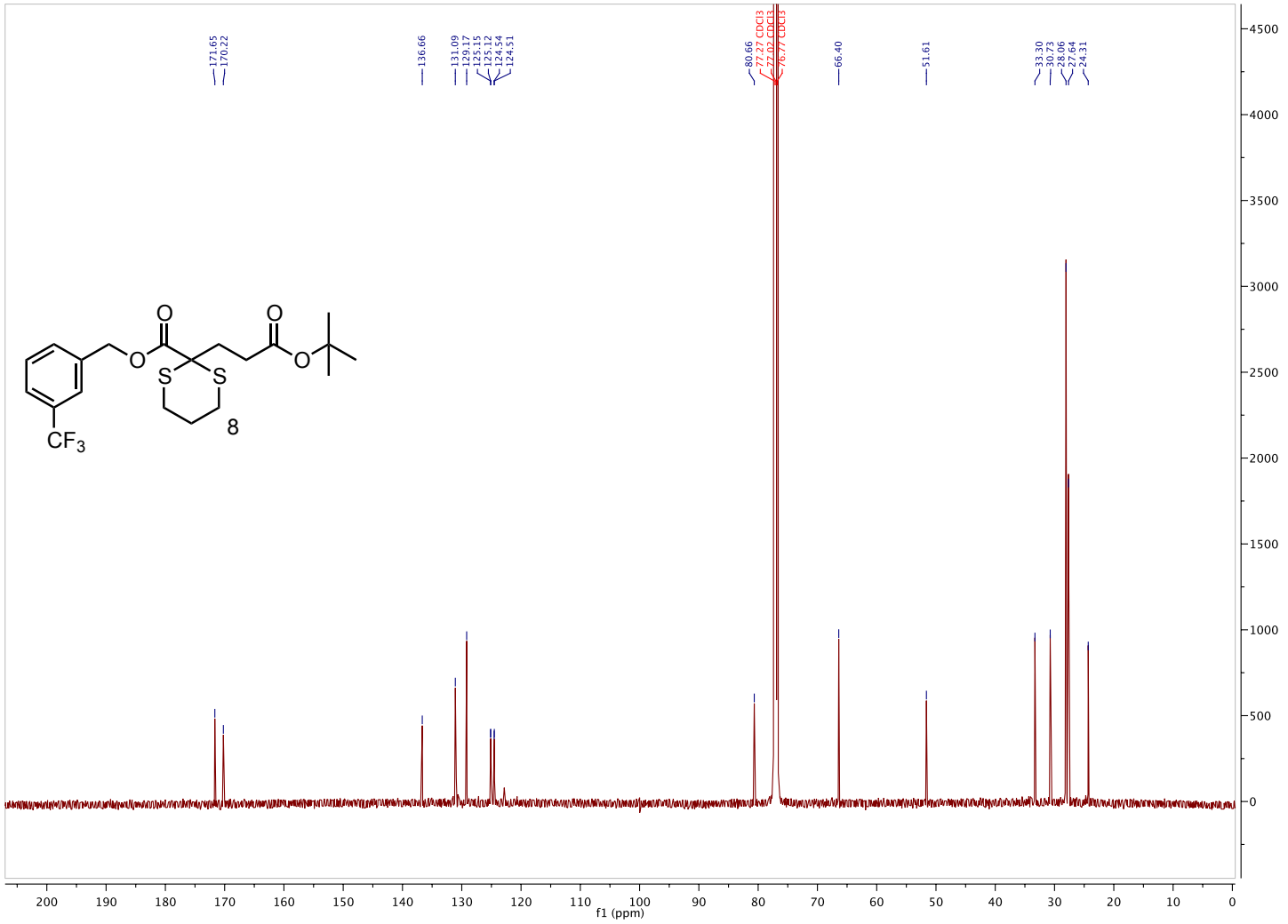
9.18
f1 (ppm)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80.66
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76.77 CDCl3
66.40
51.61
33.30
30.73
28.06
27.64
24.31
O
O
O
O
S
S
8
CF3
4500
4000
3500
3000
2500
2000
1500
1000
500
0
200
190
180
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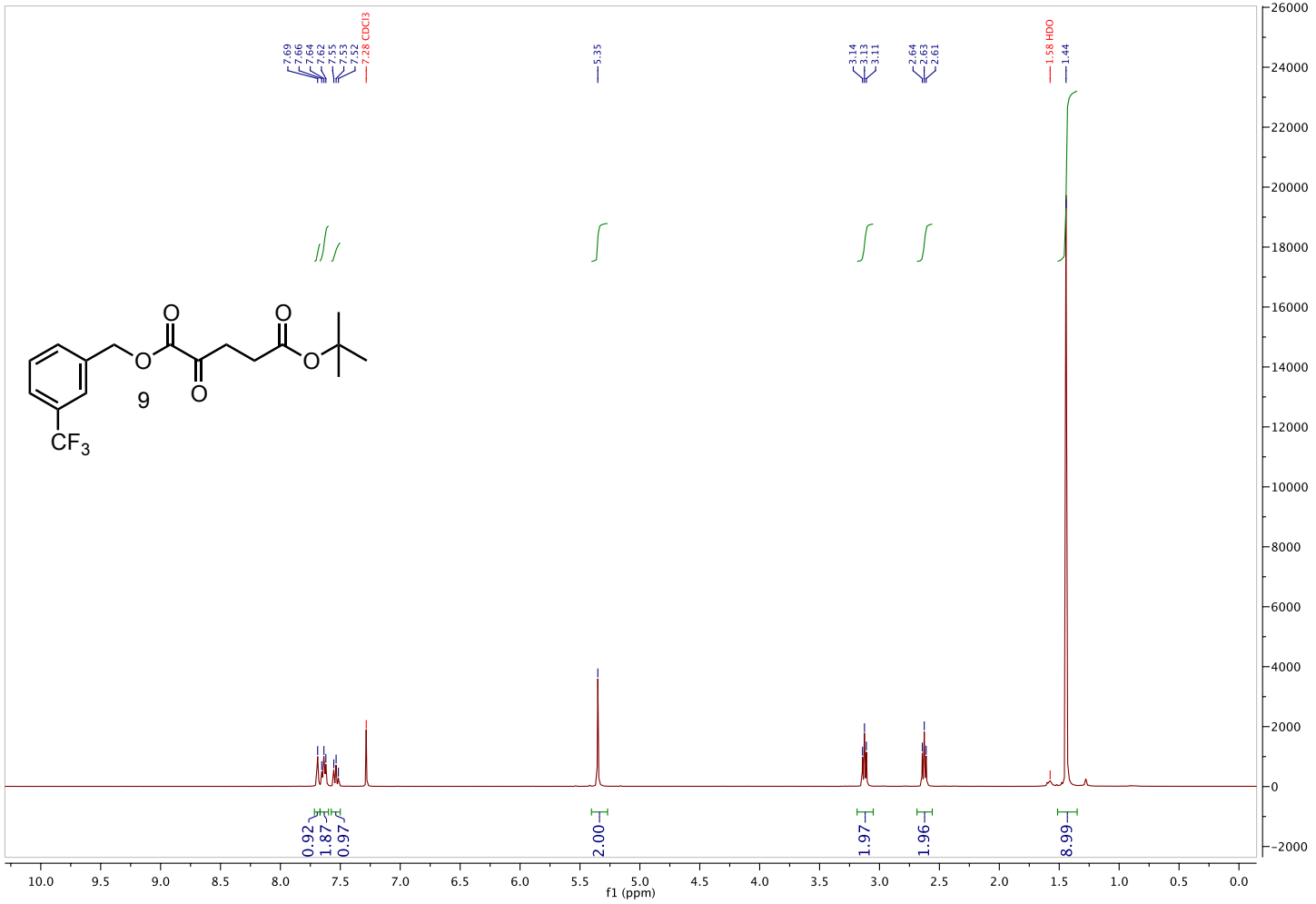
50
40
30
20
10
0
f1 (ppm)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7.52
7.28 CDCl3
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3.13
3.11
2.64
2.63
2.61
1.58 HDO
1.44
O
O
O
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O
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CF3
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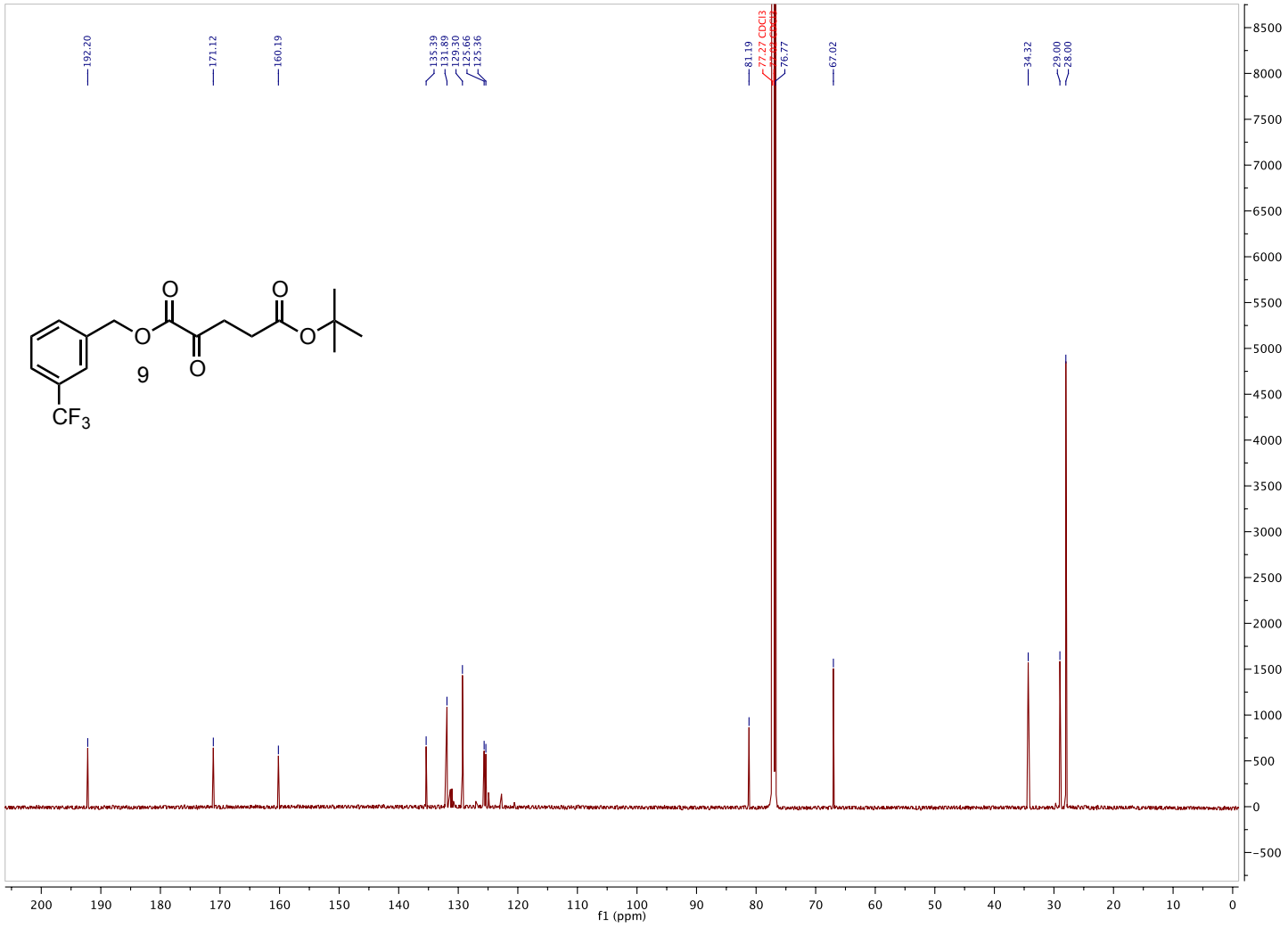
8.99
f1 (ppm)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125.36
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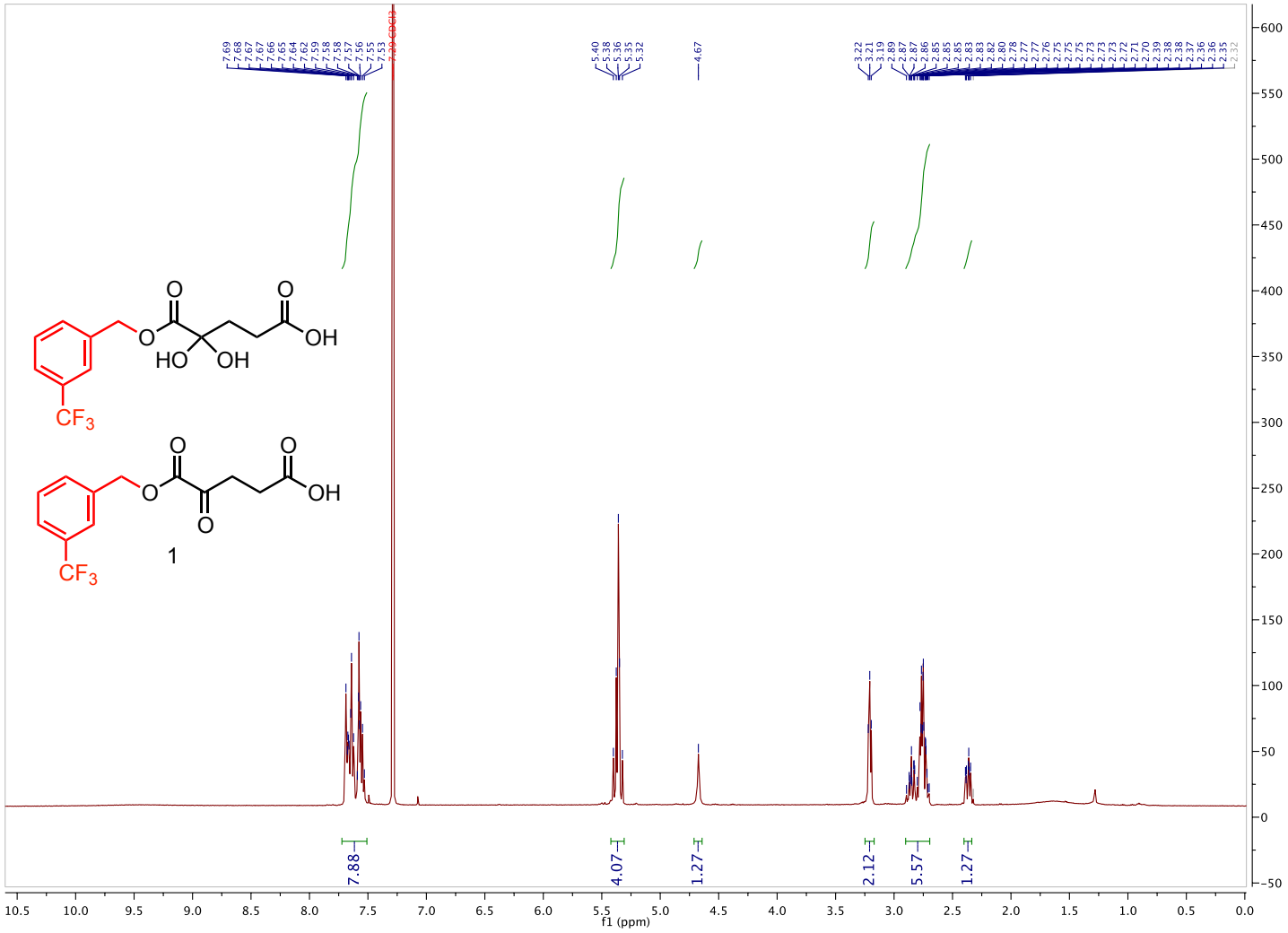
O
O
O
O
9
CF3
f1 (ppm)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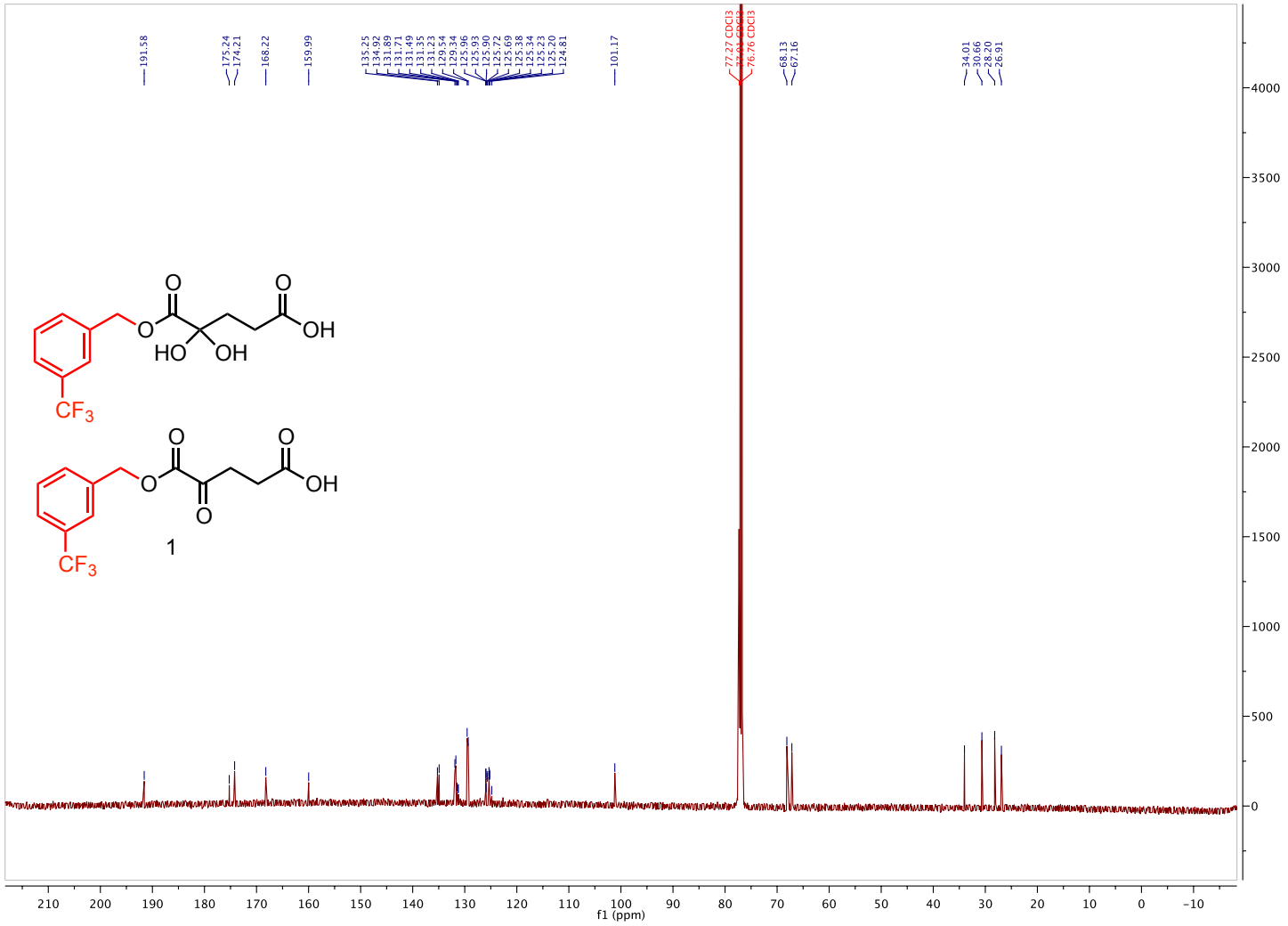
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131.35
131.23
129.54
129.34
125.96
125.93
125.90
125.72
125.69
125.38
125.34
125.23
125.20
124.81
101.17
77.27 CDCl3
77.01 CDCl3
76.76 CDCl3
68.13
67.16
34.01
30.66
28.20
26.91
4000
3500
3000
2500
2000
1500
1000
500
0
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
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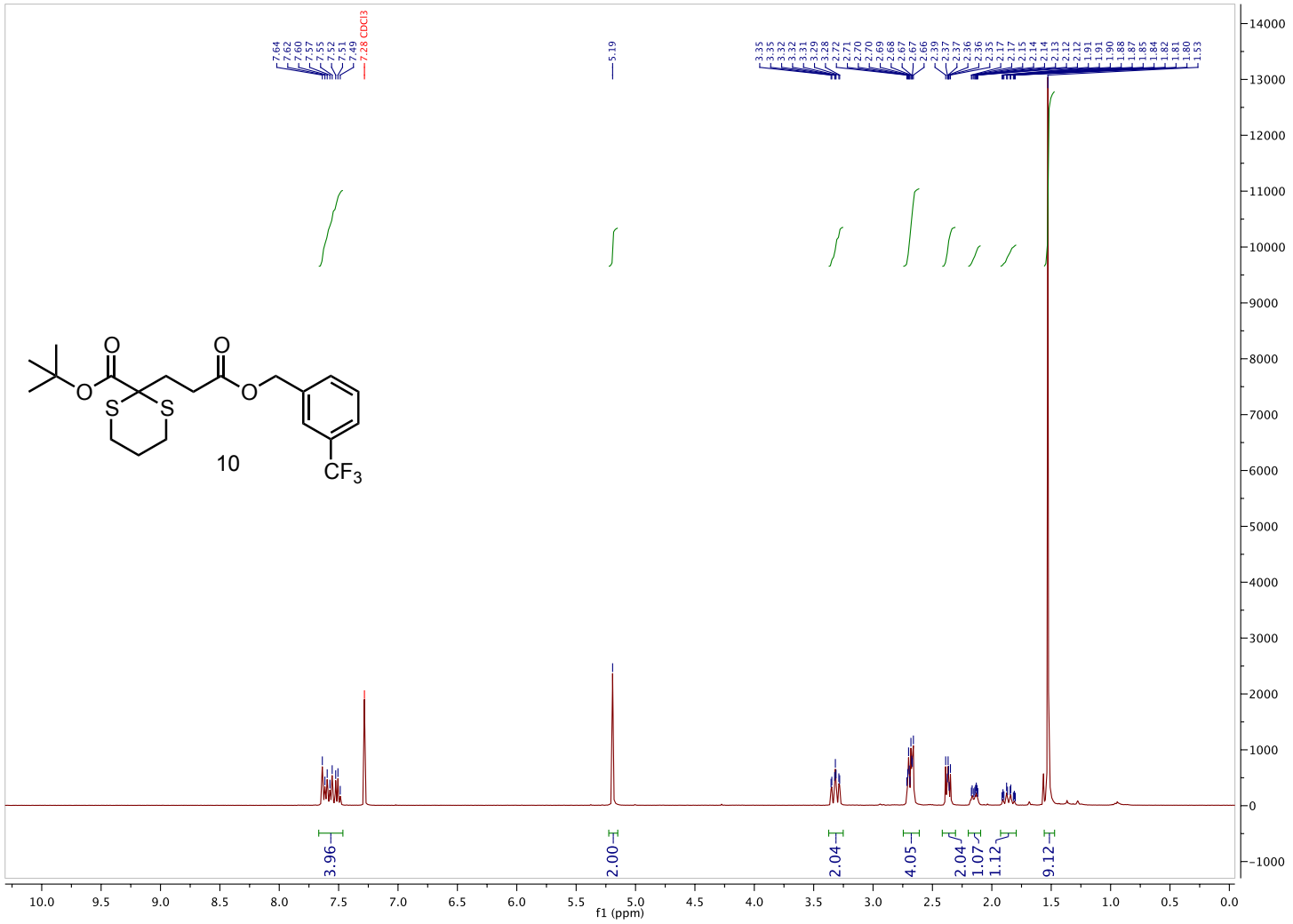
O
O
OH
HO
OH
CF3
O
O
O
OH
O
CF3
1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7.49
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3.32
3.32
3.31
3.29
3.28
2.72
2.71
2.70
2.70
2.68
2.68
2.67
2.67
2.66
2.39
2.37
2.37
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2.14
2.13
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1.91
1.91
1.90
1.88
1.87
1.85
1.84
1.82
1.81
1.80
1.53
O
O
O
O
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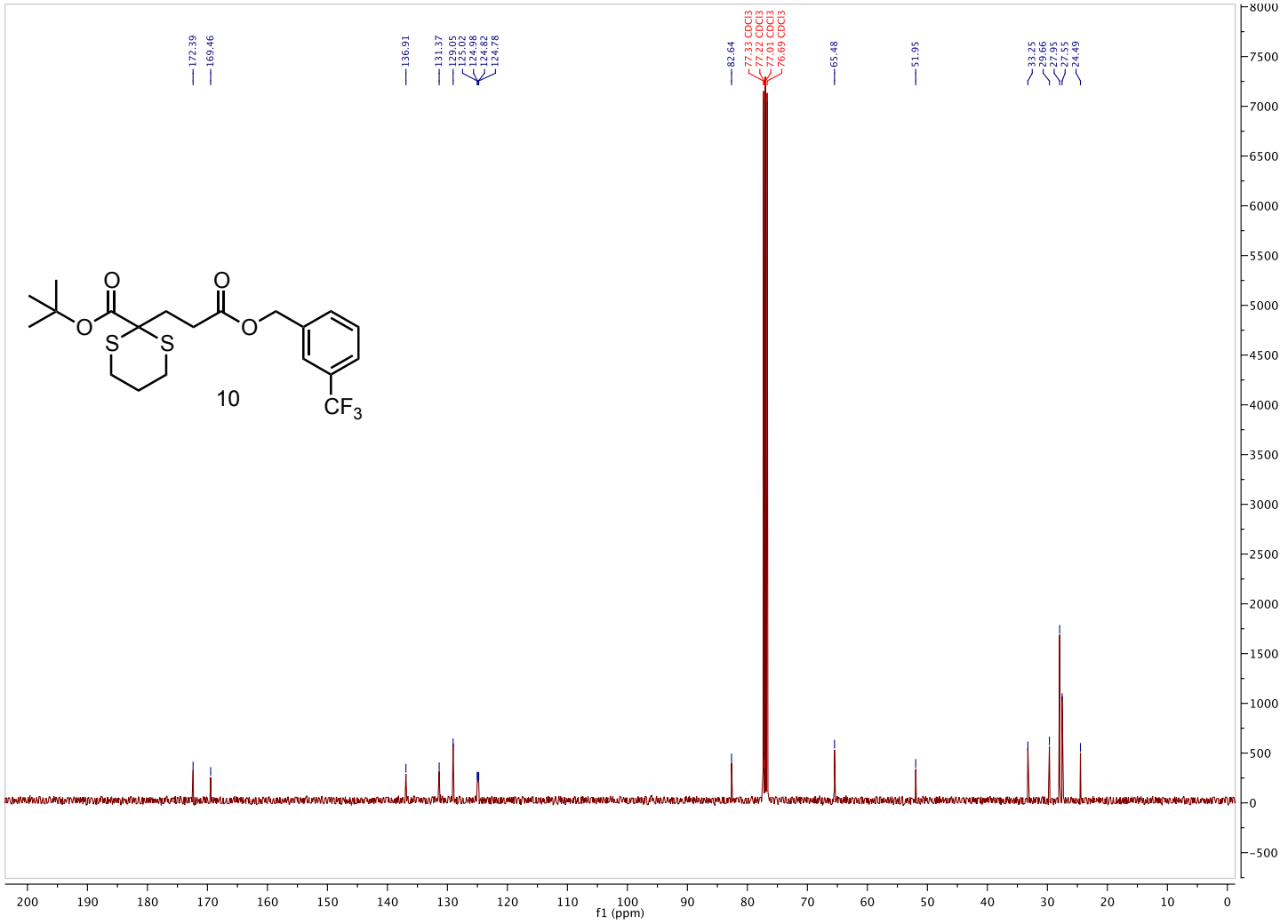
4.05
2.04
1.07
1.12
9.12
f1 (ppm)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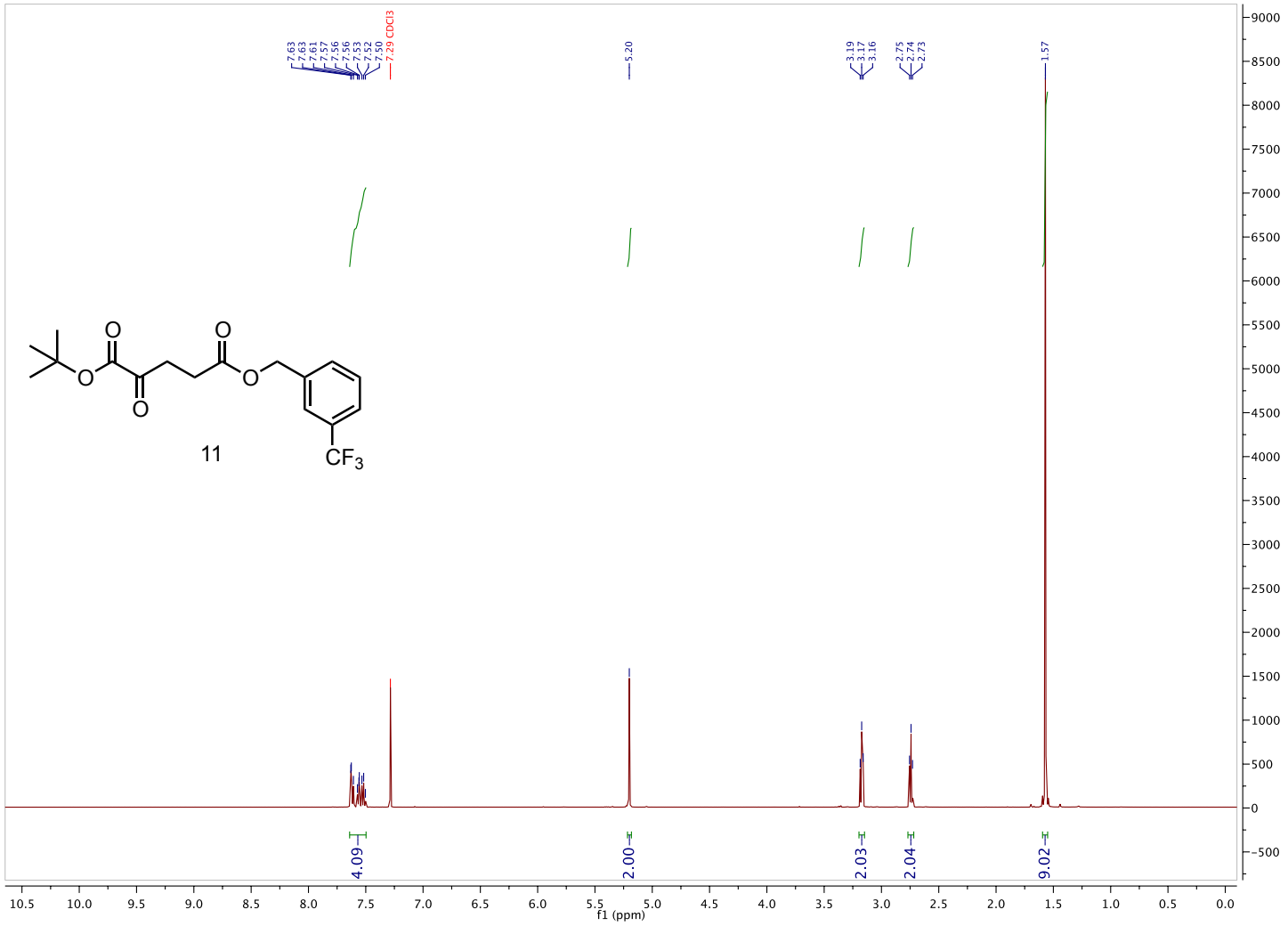
124.82
124.78
82.64
77.33 CDCl3
77.22 CDCl3
77.01 CDCl3
76.69 CDCl3
65.48
51.95
33.25
29.66
29.05
27.55
24.49
O
O
O
O
S
S
10
CF3
f1 (ppm)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7.56
7.56
7.53
7.52
7.50
7.29 CDCl3
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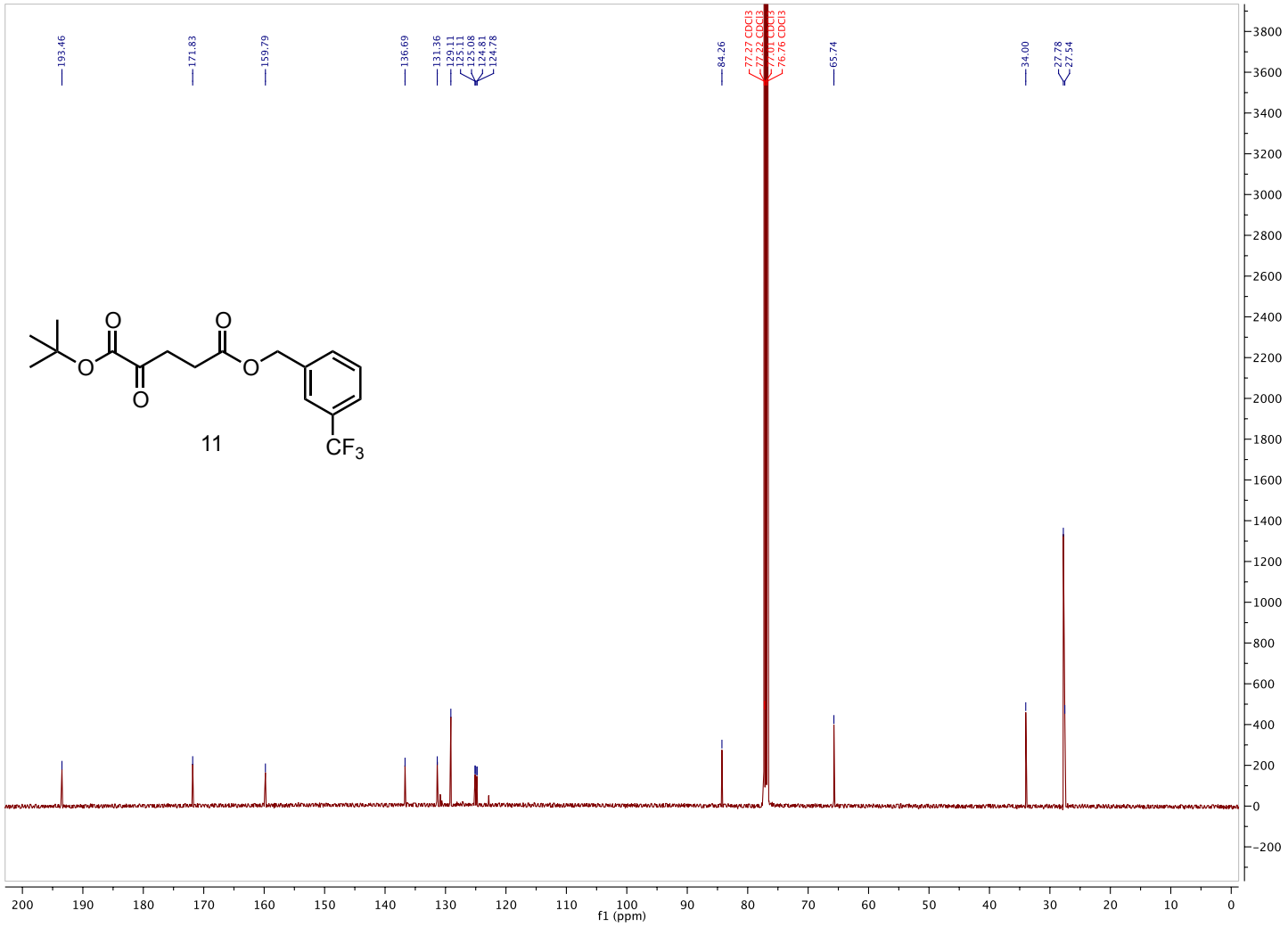
3.19
3.17
3.16
2.75
2.74
2.73
1.57
O
O
O
O
O
11
CF3
4.09
2.00
2.03
2.04
9.02
f1 (ppm)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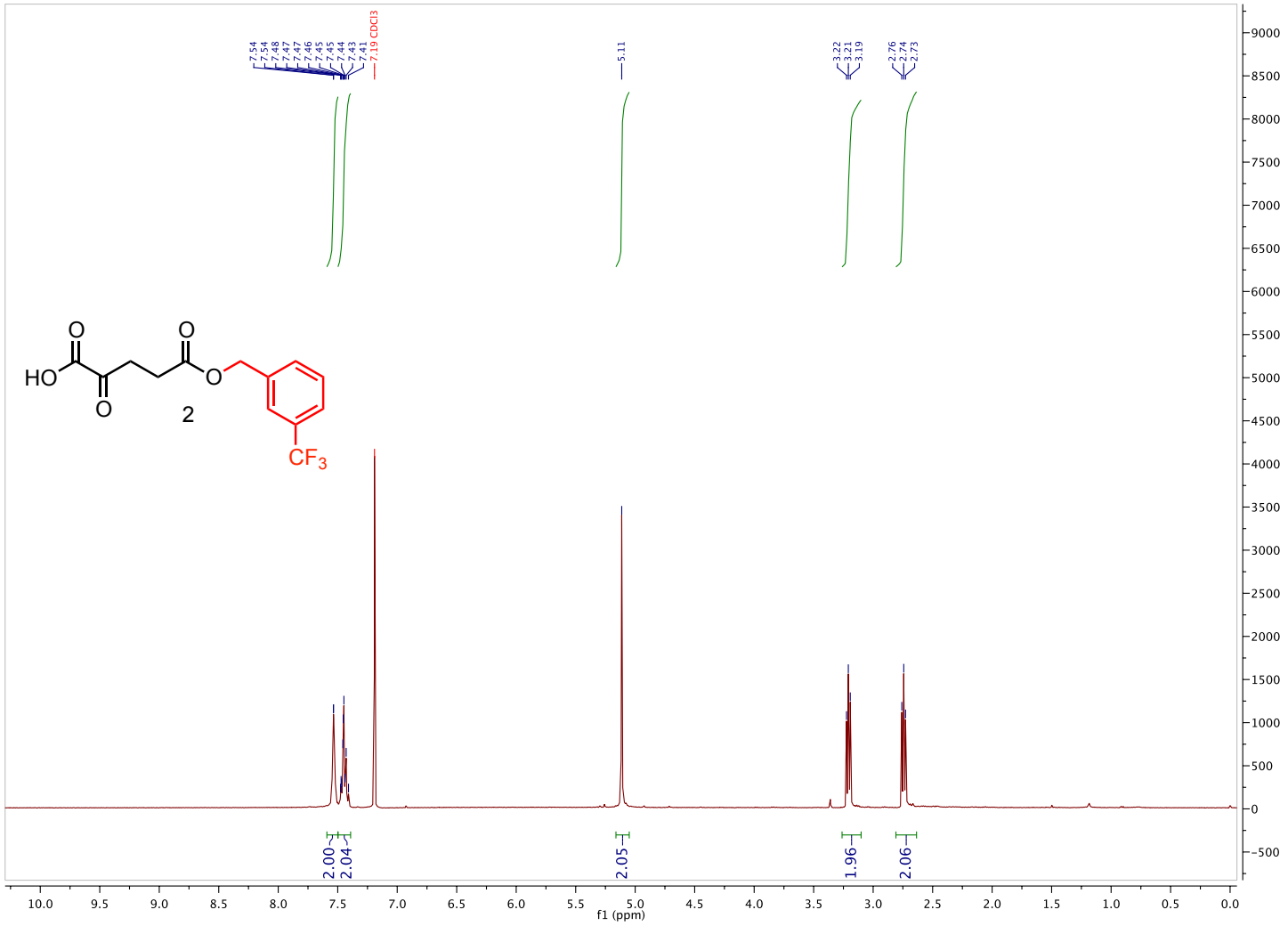
34.00
27.78
27.54
11
CF3
f1 (ppm)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3.22
3.21
3.19
2.76
2.74
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O
O
2
CF3
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2.05
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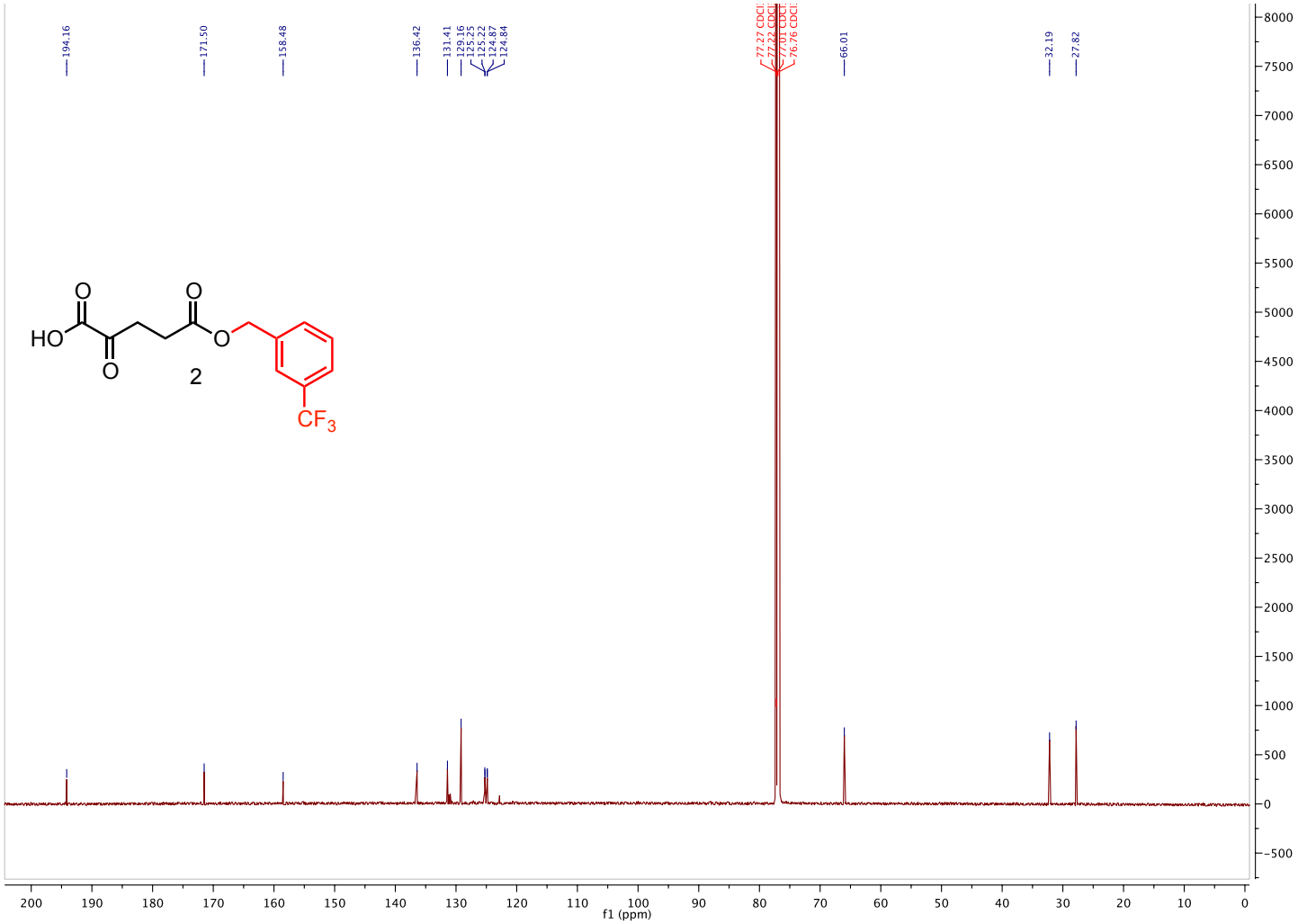
2.06
f1 (ppm)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O
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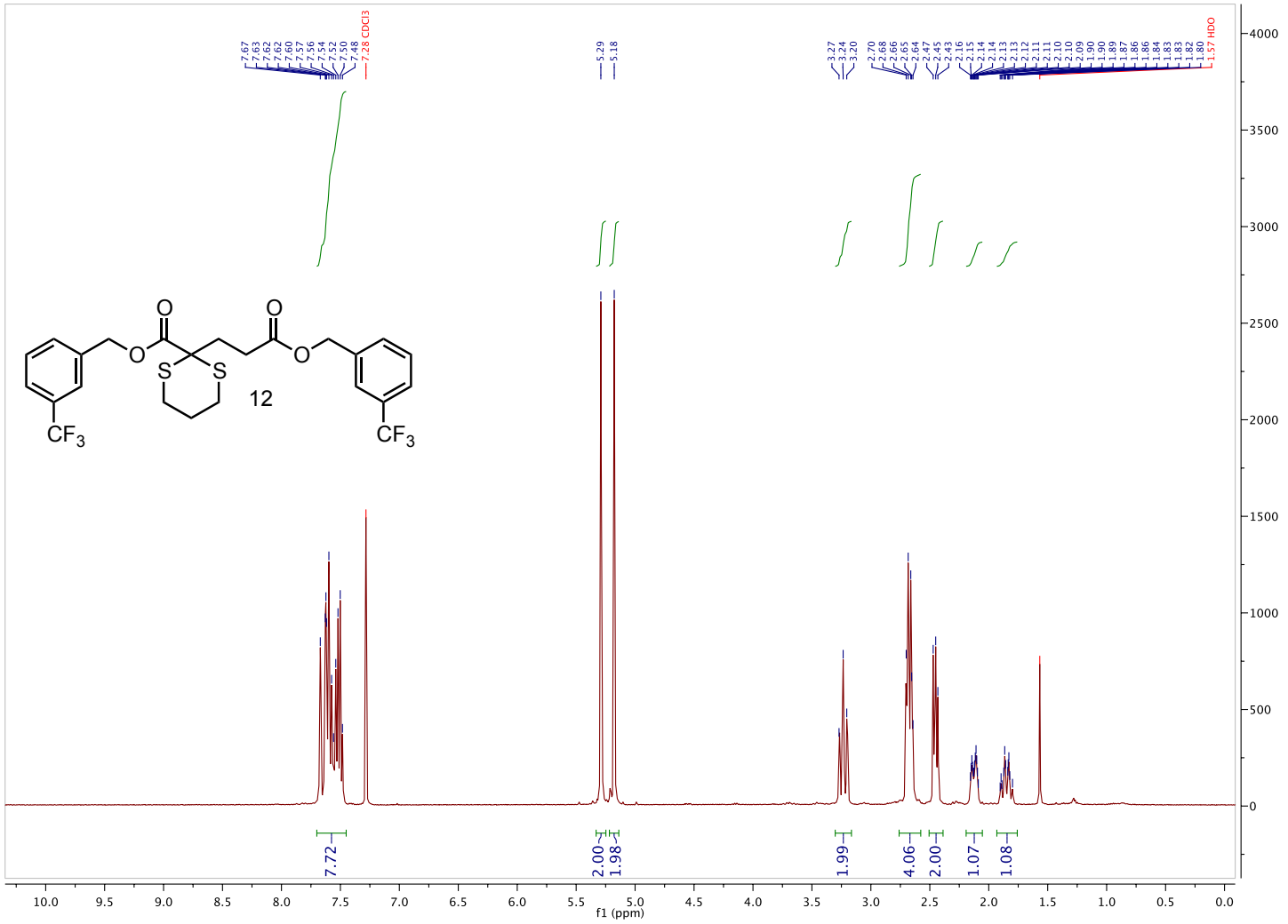
O
2
CF3
f1 (ppm)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CF3
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7.50
7.48
7.28 CDCl3
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5.18
3.27
3.24
3.20
2.70
2.68
2.66
2.65
2.64
2.47
2.45
2.43
2.16
2.15
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2.13
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1.87
1.86
1.86
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1.83
1.82
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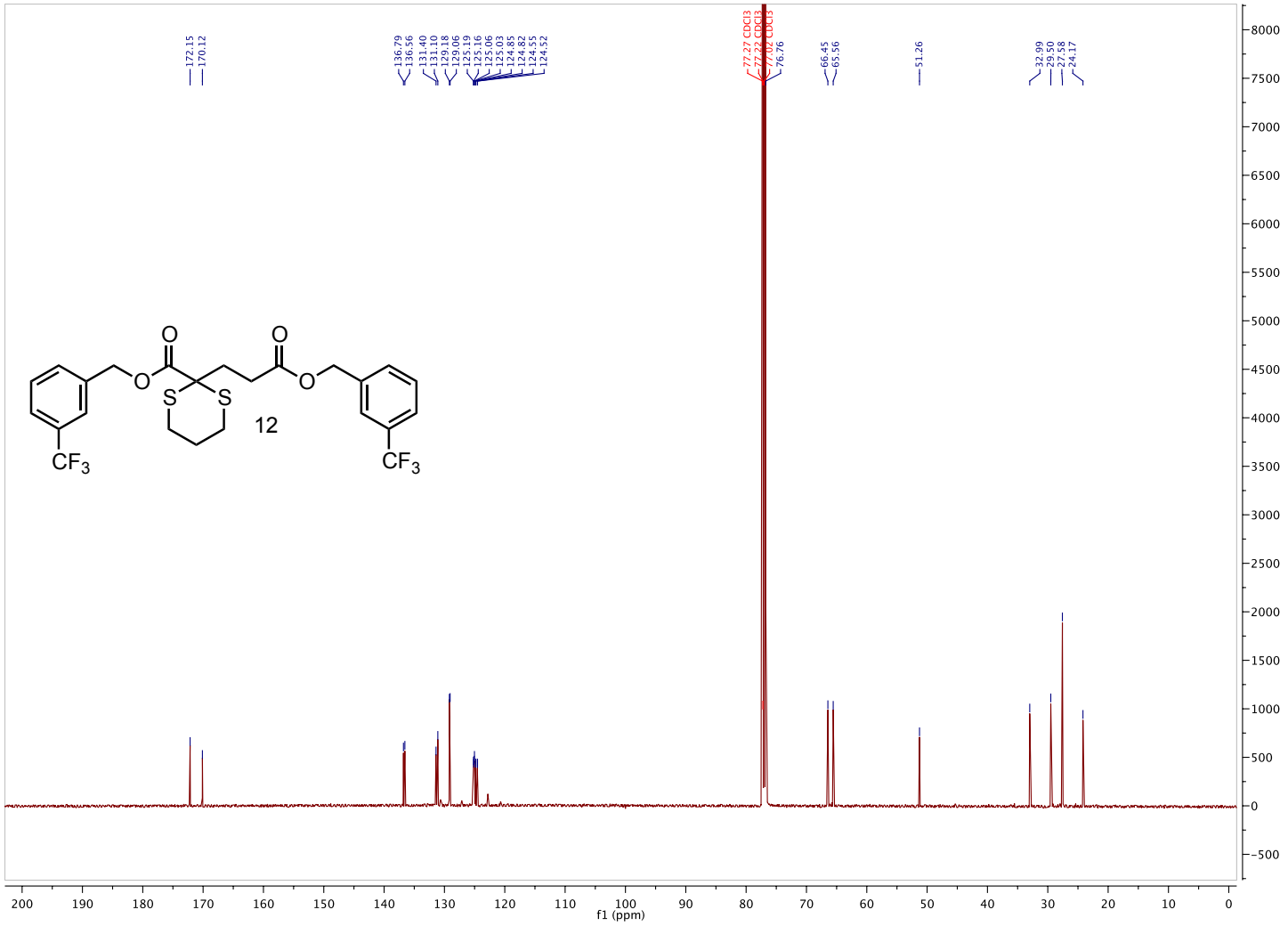
f1 (ppm)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124.55
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S
S
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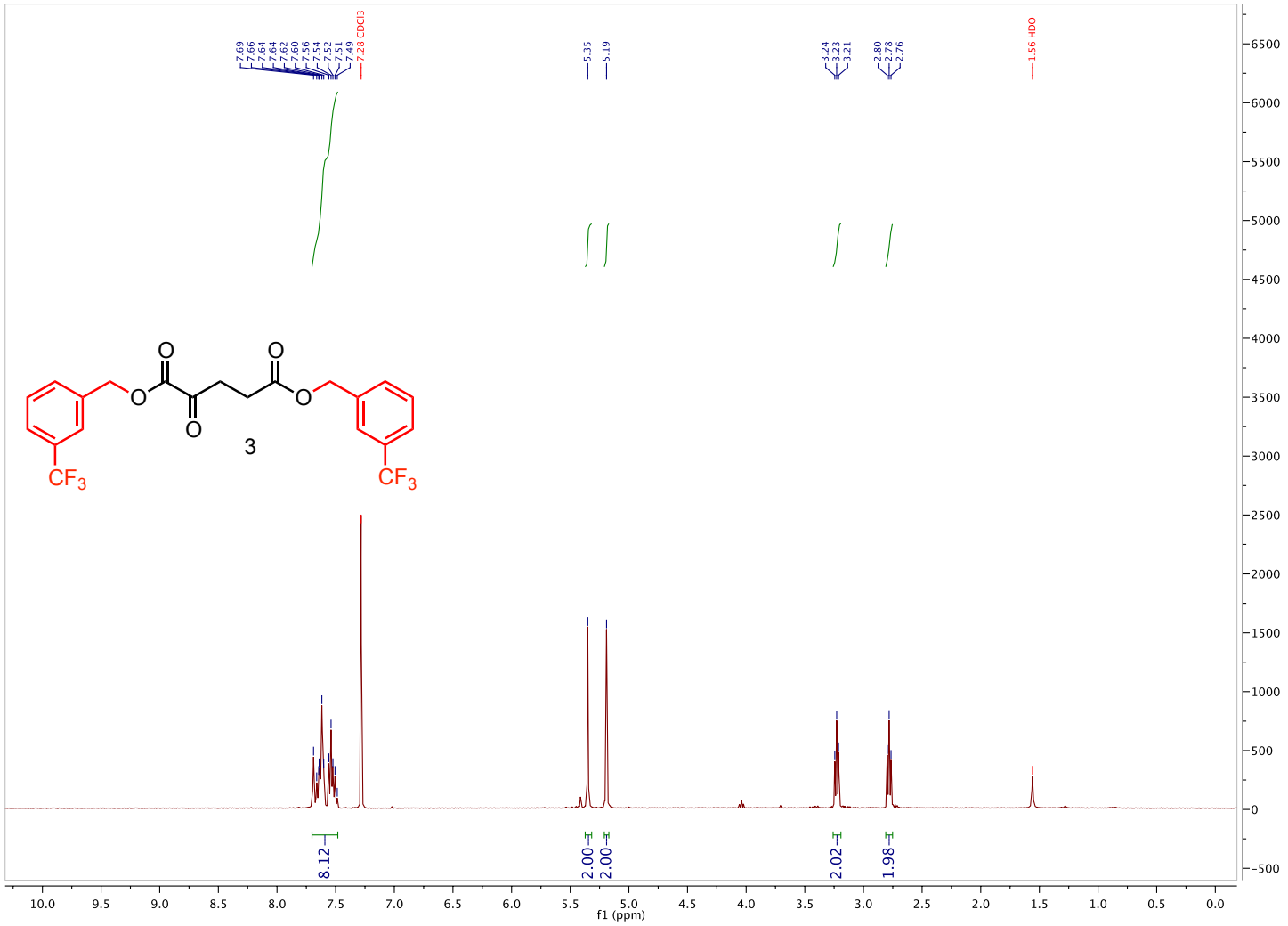
CF3
CF3
f1 (ppm)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2.00
2.00
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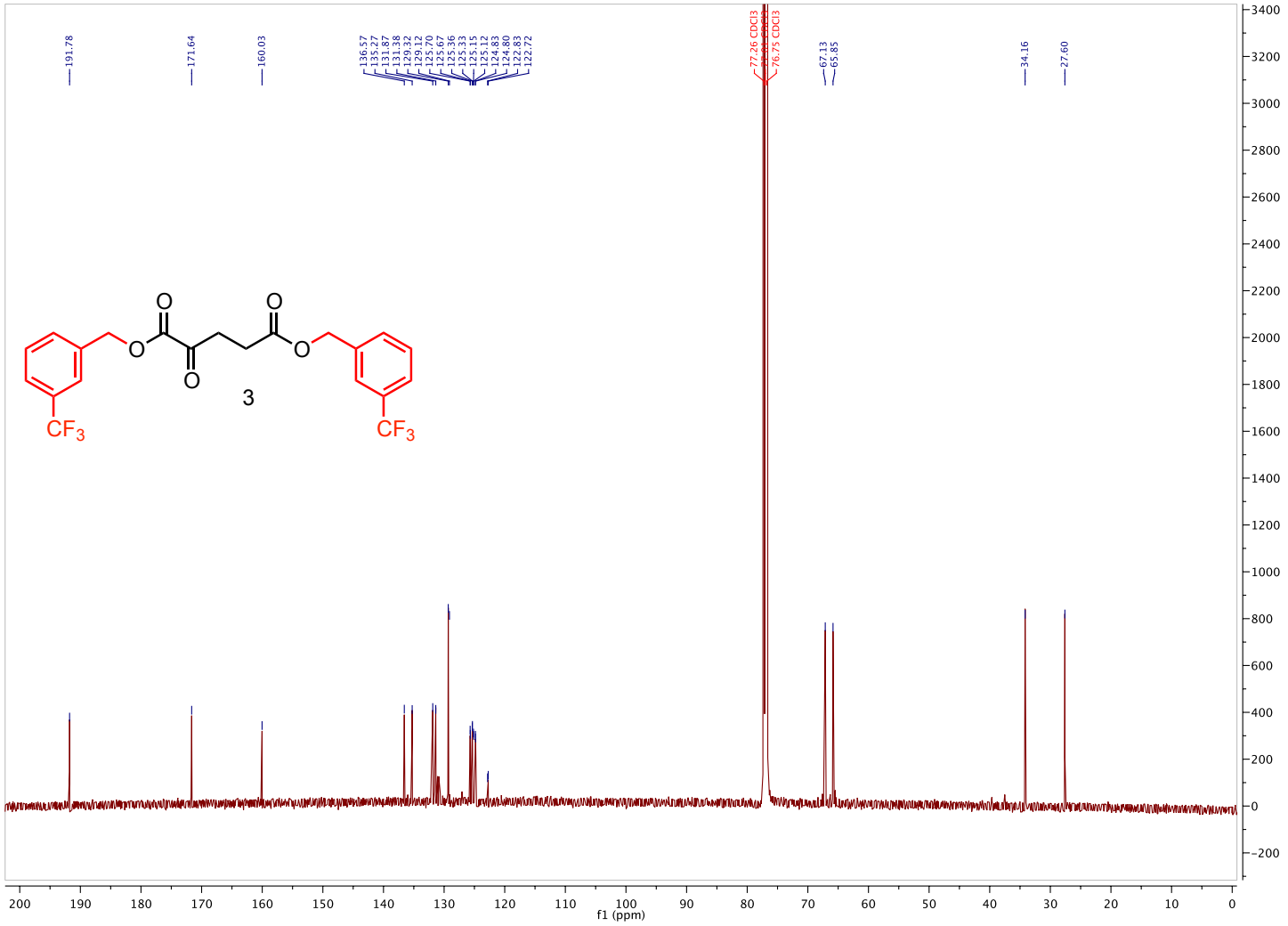
1.98
f1 (ppm)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125.15
125.12
124.83
124.80
122.83
122.72
77.26 CDCl3
76.75 CDCl3
67.13
65.85
34.16
27.60
O
O
O
O
O
3
CF3
CF3
f1 (ppm)

**General cell culture materials and methods**

HeLa cells containing a stable transfection of HIF-1α oxygen dependent-degradation domain (HIF-1α ODD) fused to luciferase (Luc) were obtained from Tsui-Fen Chou (UCLA).[6,7] Cells were grown in DMEM supplemented with 2.5% FBS, 2 mM glutamine at 37 °C and 5% $CO_2$. 2.5 μg/mL puromycin was added to media to maintain HIF-Luc selection expression. For all luciferase experiments, cells were plated on sterile, white-walled 96-well clear bottom plates (Corning #3610) luciferase activity detected using D-luciferin (Pierce # 88291). SDS-PAGE and Western blotting was performed using Bis-Tris NuPAGE gels (4-12%), MES running buffer in Xcell SureLock MiniCells (Invitrogen), and anti-HIF1α antibody (Cell Signaling # 3716) according to the manufacturer's instructions.

**Cell-based assay for HIF-1α-degradation**

To assay 2-KG dependent changes in HIF-1α levels, cells were seeded on 96-well plates (100 μL, 10,000-20,000 cells/well) and allowed to adhere for 16 hours. Cells were dosed with vehicle or test compounds (50 μl of a 2-4 mM stock in DMEM) and returned to the incubator. After 2 hours, cells were removed from the incubator, inspected for any signs of toxicity by microscopy, and D-luciferin (50 μl of 1 mg/ml in PBS) was added using a multichannel pipette. After five minutes, luminescense intensity was quantitated on a Biotek Synergy 2 plate reader (Biotek) using a 0.1-ms integration time.

**Ketoglutarate Ester Stablility Assay**

The relative stabilities of 1-TFMB-KG (**1**) and 5-TFMB-KG (**2**) were determined in phosphate buffered saline (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$ [pH 7.4] 137 mM NaCl, 2.7 mM KCl). Esters (2 mM) were incubated in PBS for 1 hour prior to analysis using a Biovision α-KG Assay (#K677-100) according to the manufacturer's instructions. The assay is based on enzymatic transamination of 2-KG to glutamate, which yields an equimolar amount of pyruvate, which is then consumed pyruvate oxidase to produce an equivalent of $H_2O_2$ and activate an oxidation-sensitive fluorophore ($\lambda_{ex}$ = 535 nm, $\lambda_{em}$ = 587 nm). A 2 mM solution of 2-ketoglutaric acid in PBS was used as a positive control representing 100% hydrolysis. Reported values represent the average of three measurements.

**Treatment and Harvesting of Nuclear Extracts for HIF-1α Western Blot**

Western blotting was used to verify the effects of 2-KG esters on HIF-1α levels in HeLa cells. Briefly, HeLa cells were seeded on 6-well plates (2.5 mL, $10^6$ cells/well), allowed to adhere overnight (16 hrs) and treated with either vehicle, $CoCl_2$ (0.5 mM; positive control for HIF-1α stabilization) or 2-KG diester **3** (0.5 mM, 1 mM). After 2 hours, cells were washed with ice cold PBS, harvested, and centrifuged (1400g, 5 min, 4°C). Supernatant (PBS) was removed and nuclear protein fractions were isolated using Thermo Scientific NE-PER kit (#PI-78835) according to the manufacturer's instructions. Protein concentrations were measured on a Qubit 2.0 fluorometer, and 15 μg of nuclear extract per well was found to give sufficient signal for detection of HIF1α stabilization or degradation. Western blotting materials and antibodies are given in "General cell culture materials and methods" (above).

**References**


(1) Gottlieb, H.E.; Kotlyar V.; Nudelman, A. *Journal of Organic Chemistry* **1997**, *62*, 7512-7515.

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