Supplementary Information

Development of a Light-Activated STING Agonist

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Synthesis

All chemicals obtained from commercial sources Acros, Alfa Aesar, Fisher, Oakwood, Sigma Aldrich, and TCI Chemicals were used without purification. ^{1}H and ^{13}C NMR spectra were obtained from a Bruker Avance III 400 MHz or 500 MHz spectrometer with chemical shifts reported relative to residual $CDCl_3$ (7.26 ppm), CD_3OD (3.31 ppm), or DMSO-d_6 (2.50 ppm). HRMS was performed on a Q-Exactive (Thermo Scientific) mass spectrometer by University of Pittsburgh facilities.

6-(1H-Imidazol-1-yl) pyridazine-3-carboxylic acid (6). Methyl 6-chloropyridazine-3-carboxylate (**5**, 1.0 g, 5.79 mmol) was dissolved in dry DMF (10.0 mL) under argon, then imidazole (394.2 mg, 5.79 mmol, 1.0 eq) and K_2CO_3 (935.7 mg, 6.77 mmol, 1.17 eq) were added. The reaction mixture was stirred at 120 °C for 3 hours. The reaction mixture was cooled to room temperature and a solution of LiOH (2.8 mL, 6.95 mmol, 1.2 eq, 2.5 M) was added. The reaction mixture was then stirred at 60 °C for 1 hour. Once complete the mixture was cooled to room temperature and 1 *M* HCl was added to acidify the reaction to a pH 4-5. The resulting precipitate was filtered and washed with cold water to yield **6** (900.0 mg, 82%) as an off-white solid which was used without further purification. All analytical data matched the literature reported data.[1]

Methyl 2-((*tert*-butoxycarbonyl)amino)-4,5-difluorobenzoate (9). Methyl 2-amino-4,5-difluorobenzoate (**8**, 1.50 g, 8.02 mmol) was dissolved in dry DCM (6 mL) under argon, then $(Boc)_2O$ (2.10 g, 9.62 mmol, 1.2 eq) and DMAP (77.9 mg, 0.64 mmol, 0.08 eq) were added. The solution was stirred at room temperature overnight. The volatiles were evaporated *in vacuo* and the product was purified by flash column chromatography on SiO_2 (2% EtOAc in hexanes) to yield the desired product **9** (822.0 mg, 36%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.27 (s, 2H), 8.41 (dd, 1 H, *J* = 13.6, 7.5 Hz), 7.81 (dd, 1 H, *J* = 11.0, 8.9 Hz), 3.92 (s, 3 H), 1.52 (s, 9 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.1, 153.1, 152.6, 140.0, 119.2, 119.0, 108.1, 107.9, 81.2, 52.6, 28.3. HRMS $(M-H)^-$ calcd for $C_{13}H_{14}O_4NF_2$ $(M-H)^-$ 286.0885, found 286.0897.

2-((*tert*-Butoxycarbonyl)amino)-4,5-difluorobenzoic acid (10). A solution of **9** (500 mg, 1.74 mmol) in MeOH (14 mL) was added to a NaOH solution in water (1.9 mL, 10% w/v). The reaction mixtures was stirred at room temperature overnight and the volatiles were evaporated *in vacuo.* The resulting crude residue was dissolved in water (30 mL) and resulting mixture was acidified to pH 4-5 with 1 *M* HCl. The aqueous solution was extracted with EtOAc (3 x 30 mL) and the organic phases were combined, dried over anhydrous Na_2SO_4, and concentrated to yield the desired

product **10** (462.0 mg, 97%) as a white solid without any further purification. ^{1}H NMR (500 MHz, CD_3OD) δ 8.07 (dd, 1 H, *J* = 8.2, 2.4 Hz), 7.74 (dd, 1 H, *J* = 11.8, 9.4 Hz), 1.41 (s, 9 H). ^{13}C (125 MHz, CD_3OD) δ 171.1, 153.2, 143.0, 137.9, 119.2, 105.9, 106.1, 79.6, 27.2. HRMS $(M-H)^-$ calcd for $C_{12}H_{12}O_4NF_2$ $(M-H)^-$ 272.0729, found 272.0742.

Benzyl 2-((*tert*-butoxycarbonyl)amino)-4,5-difluorobenzoate (11). Compound **10** (200.0 mg, 0.73 mmol) was dissolved in dry acetonitrile (3.0 mL) under argon, then benzyl bromide (172.2 µL, 1.46 mmol, 2.0 eq) and DIPEA (127.1 µL, 0.73 mmol, 1.0 eq) were added. The solution was stirred at room temperature overnight. The volatiles were removed *in vacuo* and the resulting crude residue was purified flash column chromatography on SiO_2 (1% EtOAc in hexanes) to yield **11** (135.0 mg, 51%) as an off-white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.26 (s, 1 H), 8.41 (dd, 1 H, *J* = 13.6, 7.5 Hz), 7.84 (dd, 1 H, *J* = 11.0, 8.9 Hz), 7.38 (m, 5 H), 5.34 (s, 2 H), 1.52 (s, 9 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.4, 152.6, 135.1, 128.8, 128.6, 128.3, 119.2, 119.0, 110.2, 108.1, 107.9, 81.2, 67.3, 28.3. HRMS $(M-H)^-$ calcd for $C_{19}H_{18}O_4NF_2$ $(M-H)^-$ 362.1198, found 362.1206.

Benzyl 2-amino-4,5-difluorobenzoate (14). Compound **11** (130.0 mg, 0.36 mmol) was dissolved in a solution of TFA in DCM (3.0 mL, 10% v/v). The solution was stirred for 3 hours at room temperature before a 10% KOH solution was added dropwise to neutralize the acid. The resulting aqueous solution was extracted with DCM (3 x 15 mL) before the organic layers were combined, dried over anhydrous Na_2SO_4, and concentrated. The resulting residue was purified by flash column chromatography on SiO_2 (10% EtOAc in hexanes) to afford **14** (68.0 mg, 72%) as an off-white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (dd, 1 H, *J* = 11.3, 9.0 Hz), 7.38 (m, 5 H), 6.45 (dd, 1 H, *J* = 11.9, 6.6), 5.30 (s, 2 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.5, 155.6, 148.4, 140.9, 135.9, 128.8, 128.6, 128.3, 119.2, 119.0, 104.2, 104.0, 66.5. HRMS $(M+H)^+$ calcd for $C_{14}H_{12}O_2NF_2$ $(M+H)^+$ 264.0831, found 264.0841

Benzyl 2-(6-(1H-imidazol-1-yl)pyridazine-3-carboxamido)-4,5-difluorobenzoate (2). Compound **6** (40.0 mg, 0.21 mmol) was dissolved in thionyl chloride (1.0 mL) under argon. The mixture was heated at 80 °C and stirred for 3 hours. The thionyl chloride was concentrated *in vacuo*, resulting in the crude material **7**, and then a solution of **14** (60.5 mg, 0.23 mmol, 1.1 eq) and DIPEA (109.6 µL, 0.63 mmol, 3 eq) in dry acetonitrile (1.0 mL) was added slowly, under argon. The reaction mixture was stirred at room temperature for 1 hour. The volatiles were then removed *in vacuo* and the resulting crude residue was purified by flash column chromatography on SiO_2 (2% MeOH in DCM) to yield the final product **2** (14.0 mg, 15%) as an off-white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.40 (s, 1 H), 8.95 (dd, overlap, 1 H, *J* = 12.9, 7.5 Hz) 8.94 (s, overlap, 1 H), 8.71 (d, 1 H, *J* = 9.1 Hz), 8.37 (s, 1 H), 7.99 (dd, 1 H, *J* = 10.8, 8.6 Hz), 7.56 (s, 1 H), 7.43 (m, 6 H), 5.47 (s, 2 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.0, 160.68, 151.9, 134.6, 129.2, 128.8, 128.8, 128.5, 119.9, 117.6, 110.3, 110.1, 67.8. HRMS $(M+H)^+$ calcd for $C_{22}H_{16}O_3N_5F_2$ $(M+H)^+$ 436.1216, found 436.1198.

2-Phenylpropyl 2-((*tert*-butoxycarbonyl)amino)-4,5-difluorobenzoate (12). The carboxylic acid **10** (300.0 mg, 1.10 mmol) was dissolved in dry DMF under argon, then HATU (673.0 mg, 1.77 mmol, 1.6 eq), DMAP (268.8 mg, 2.20 mmol, 2 eq), and TEA (306.6 µL, 2.20 mmol, 2 eq) were added. The solution was stirred for 10 minutes before 2-phenylpropan-1-ol (272.4 mg, 2.00 mmol, 1.8 eq) was added. The reaction was heated to 65 °C and stirred overnight. After it was cooled to room temperature the volatiles were evaporated *in vacuo* and purified by flash column

chromatography on SiO_2 (5% EtOAc in hexanes) to yield **12** (185.0 mg, 43%) as an off-white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.14 (s, 1 H), 8.38 (dd, 1 H, *J* = 13.5, 7.5 Hz), 7.66 (dd, 1 H, *J* = 11.0, 8.9 Hz), 7.35 (m, 2 H), 7.27 (m, 3 H), 4.38 (dd, 2 H, *J* = 7.0, 2.3 Hz), 3.24 (dq, 1 H, *J* = 14.0, 7.0 Hz), 1.39 (d, 3 H, *J* = 7.2 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.4, 152.6, 142.6, 140.0, 128.7, 127.2, 127.0, 118.8, 108.0, 107.9, 81.2, 70.4, 39.0, 28.3, 18.0. HRMS $(M+Na)^+$ calcd for $C_{21}H_{23}O_4NF_2Na$ 414.1487, found 414.1492.

2-Phenylpropyl 2-amino-4,5-difluorobenzoate (15). Compound **12** (180.0 mg, 0.46 mmol) was dissolved in a solution of TFA in DCM (3.5 mL, 10% v/v). The solution was stirred for 2 hours at room temperature before a 10% KOH solution was added dropwise to neutralize the acid. The resulting aqueous solution was extracted with DCM (3 x 20 mL) before the organic layers were combined, dried over anhydrous Na_2SO_4, and concentrated. The resulting residue was purified by flash column chromatography on SiO_2 (20% EtOAc in hexanes) to afford **15** (112.7 mg, 84%) as an off-white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.56 (dd, 1 H, *J* = 11.3, 9.0 Hz), 7.34 (m, 2 H), 7.25 (m, 3 H), 6.48 (dd, 1 H, *J* = 11.7, 6.6 Hz), 4.35 (dd, 2 H, *J* = 7.0, 3.1 Hz), 3.22 (dq, 1 H, *J* = 14.0, 6.9 Hz), 1.39 (d, 3 H, *J* = 7.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.5, 155.5, 153.6, 153.4, 143.0, 128.6, 127.3, 126.9, 119.0, 118.9, 104.9, 69.8, 39.0, 18.0. HRMS $(M+H)^+$ calcd for $C_{16}H_{16}O_2NF_2$ 292.1144, found 292.1154.

2-Phenylpropyl 2-(6-(1H-imidazol-1-yl)pyridazine-3-carboxamido)-4,5-difluorobenzoate (3). Compound **6** (65.0 mg, 0.34 mmol) was dissolved in thionyl chloride (1.70 mL) under argon. The mixture was heated at 80 °C and stirred for 3 hours. The thionyl chloride was concentrated *in vacuo* and then a solution of **15** (107.7 mg, 0.37 mmol, 1.1 eq) and DIPEA (177.5 µL, 1.02 mmol, 3 eq) in dry acetonitrile (1.70 mL) was added slowly, under argon. The reaction mixture was stirred at room temperature for 1 hour. The volatiles were then removed *in vacuo* and the resulting crude residue was purified by flash column chromatography on SiO_2 (5% MeOH in DCM) to yield the final product **3** (7.0 mg, 4%) as an off-white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 13.33 (s, 1 H), 9.76 (s, 1 H), 9.1 (dd, 1 H, *J* = 12.9, 7.5 Hz), 8.61 (d, 1 H, *J* = 9.0 Hz), 8.43 (d, 1 H, *J* = 8.6 Hz), 8.15 (s, 1 H), 7.79 (dd, 1 H, *J* = 10.8, 8.6 Hz), 7.46 (s, 1 H), 7.35 (m, 2 H), 7.27 (m, 3 H), 4.52 (dd, 2 H, *J* = 6.7, 6.7 Hz), 3.31 (dq, 1 H, *J* = 14.3, 7.0 Hz), 1.41 (d, 3 H, *J* = 7.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.0, 160.7, 153.1, 151.9, 142.6, 135.3, 132.0, 129.1, 128.7, 127.2, 127.0, 119.6, 119.5, 117.6, 116.2, 110.2, 110.0, 70.9, 53.6, 38.9. HRMS $(M+H)^+$ calcd for $C_{24}H_{20}O_3N_5F_2$ 464.1529, found 464.1527.

2-(2-Nitrophenyl)propyl 2-((*tert*-butoxycarbonyl)amino)-4,5-difluorobenzoate (13). The carboxylic acid **10** (50.0 mg, 0.18 mmol) was dissolved in dry DMF under argon, then HATU (110.0 mg, 0.29 mmol, 1.6 eq), DMAP (44.0 mg, 0.36 mmol, 2 eq), and TEA (50.2 µL, 0.36, 2 eq) were added. The solution was stirred for 10 minutes before 2-(2-nitrophenyl)propan-1-ol (59.7 mg, 0.33 mmol, 1.8 eq) was added. The reaction was heated to 65 °C and stirred overnight. After it was cooled to room temperature the volatiles were evaporated *in vacuo* and purified by flash column chromatography on SiO_2 (5% EtOAc in hexanes) to yield **13** (21.2 mg, 27%) as an off-white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.17 (s, 1 H), 8.38 (dd, 1 H, *J* = 13.5, 7.5 Hz), 7.79 (dd, 1 H, *J* = 8.2, 1.3 Hz), 7.60 (m, 2 H), 7.50 (d, 1 H, *J* = 2.8 Hz), 7.41 (m, 1 H), 4.47 (m, 2 H), 3.83 (q, 1 H, *J* = 6.84 Hz), 1.51 (s, 9 H), 1.44, (d, 3 H, *J* = 7.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) 166.4, 152.6, 150.4, 143.4, 140.1, 136.8, 132.8, 128.1, 127.8, 124.4, 118.8, 108.2, 107.9, 81.2, 69.3, 33.2, 28.2, 17.9. HRMS $(M-H)^-$ calcd for $C_{21}H_{21}O_6N_2F_2$ 435.1362, found 435.1367.

2-(2-Nitrophenyl)propyl 2-amino-4,5-difluorobenzoate (16). Compound **13** (33.0 mg, 0.08 mmol) was dissolved in a solution of TFA in DCM (1.0 mL, 10% v/v). The solution was stirred for

4 hours at room temperature before a 10% KOH solution was added dropwise to neutralize the acid. The resulting aqueous solution was extracted with DCM (3 x 5 mL) before the organic layers were combined, dried over anhydrous Na_2SO_4, and concentrated. The resulting residue was purified by flash column chromatography on SiO_2 (20% EtOAc in hexanes) to afford **16** (12.8 mg, 48%) as an off-white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.78 (dd, 1 H, *J* = 8.1, 1.2 Hz), 7.59 (dd, 1 H, *J* = 8.4, 8.4 Hz), 7.50 (m, 2 H), 7. 39 (dd, 1 H, *J* = 8.4, 8.4 Hz), 6.39 (dd, 1 H, *J* = 12.0, 6.6 Hz), 4.42 (dd, 2 H, *J* = 6.6, 1.1 Hz) 3.82 (q, 1 H, *J* = 6.8 Hz) 1.44 (d, 3 H, *J* = 7.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) 166.3, 154.7, 150.4, 148.3, 140.9, 137.1, 132.7, 128.2, 127.6, 124.3, 118.8, 104.2, 104.1, 68.5, 33.2, 18.0. HRMS $(M+H)^+$ calcd for $C_{16}H_{15}O_4N_2F_2$ 337.0994, found 337.1011.

2-(2-Nitrophenyl)propyl-2-(6-(1H-imidazol-1-yl)pyridazine-3-carboxamido)-4,5-difluorobenzoate (4). Compound **6** (6.0 mg, 0.032 mmol) was dissolved in thionyl chloride (200 µL) under argon. The mixture was heated at 80 °C and stirred for 3 hours. The thionyl chloride was concentrated *in vacuo*, resulting in the crude material **7**, then a solution of **16** (11.7 mg, 0.035 mmol, 1.1 eq) and DIPEA (16.5 µL, 0.10 mmol, 3 eq) in dry acetonitrile (200 µL) was added slowly, under argon. The reaction mixture was stirred at room temperature for 1 hour. The volatiles were then removed *in vacuo* and the resulting crude residue was purified by flash column chromatography on SiO_2 (5% MeOH in DCM) to yield the final product **4** (4.4 mg, 26%) as an off-white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.90 (s, 1 H), 8.77 (m, 2 H), 8.50 (d, 1 H, *J* = 9.1 Hz), 8.44 (d, 1 H, *J* = 9.1 Hz), 8.18 (s, 1 H) 7.76 (m, 4 H), 7.48 (dd, 1 H, *J* = 7.5, 7.5 Hz), 7.25 (s, 1 H), 4.56 (m, 2 H), 3.65 (q, 1 H, *J* = 7.1 Hz), 1.40 (d, 3 H, *J* = 7.1 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.5, 161.2, 153.9, 151.4, 150.7, 136.7, 136.4, 133.5, 131.6, 129.8, 129.1, 128.5, 124.1, 119.9, 119.6, 117.3, 113.6, 109.8, 109.6, 70.0, 33.2, 18.1. HRMS $(M-H)^-$ calcd for $C_{24}H_{17}O_5N_6F_2$ $(M-H)^-$ 507.1234, found 507.1217.

Photochemical Analysis

HPLC analysis of stability. Solutions of SR-717 (**1**, Cayman Chemical 3175910) or **4** (200 µM) were made in phosphate buffered saline (PBS) at pH 7.4 with 2% DMSO with or without 10% fetal bovine serum (FBS, Cytiva SH30396.03). Samples were incubated at 37 °C for 6 hours, with an aliquot (100 µL) removed each hour for analysis. Prior to analysis, ice-cold acetonitrile (100 µL) was added to each aliquot and the precipitate was pelleted by centrifugation at 10,000 g for 5 minutes. The supernatant from each sample was transferred to a mass spectrometry tube and analyzed by HPLC (**Fig. S1**) using an Agilent InfinityLab Poroshell 120 EC-C18 column (4.6 x 150 mm, 4 µm particles, P/N 693970-902) with a Phenomenex SecurityGuard C18 kit (KJ0-4282) using a gradient of 5 – 95% acetonitrile (0.1% TFA) in water (0.1% TFA), a flow rate of 1.1 mL/min, and a detection wavelength of 280 nm. The area of the peak corresponding to **4** was plotted over time using Prism 8.

HPLC analysis of decaging. Solutions of **1** or **4** (200 µM) were made in PBS at pH 7.4 with 2% DMSO. The photocaged sample, **4**, (200 µL) was irradiated with a 365 nm LED (Mouser Electronics single color luxigen LZ1 MCPCB, 66 mW/cm^2) for increasing durations (0 – 20 s) or with a 405 nm LED (Mouser Electronics, LZ1-10UB00-01U7, 392 mW/cm^2) for increasing durations (0 – 120 s). Light intensity measurements were performed using a ThorLabs power and energy meter console (PM200) with sensor (S170C). Following irradiation, each sample was analyzed by HPLC (**Fig. 5B, Fig. S2**) using an Agilent InfinityLab Poroshell 120 EC-C18 column (4.6 x 150 mm, 4 µm particles, P/N 693970-902) with a Phenomenex SecurityGuard C18 kit (KJ0-

4282) using a gradient of 5 – 95% acetonitrile (0.1% TFA) in water (0.1% TFA), a flow rate of 1.1 mL/min, and a detection wavelength of 280 nm. The peak corresponding to **1** was detected at 15.8 min and the peak corresponding to **4** was detected at 19.7 min. The new peak produced upon irradiation (18.3 min) was analyzed via HRMS to confirm the identity as the decaging byproduct. HRMS $(M+H)^-$ calcd for $C_9H_8NO_2$ 162.05496, found 164.05529.

Biological Studies

Cloning of STING expression plasmids. Site-directed mutagenesis was performed according to the Agilent Technologies QuikChange site-directed mutagenesis kit (#200518) protocol. Commercially available pMSCV-STING^{HAQ} (Addgene 102600) was used as a template to generate pMSCV-STING^{AQ} using primers P1/P2 and pMSCV-STING^{WT} (Addgene 102598) was used as a template to generate pMSCV-STING^{Q} using primers P3/P4 and pMSCV-STING^{REF} using primers P5/P6. For each reaction, 1 µL of Phusion polymerase (Thermo Scientific F530L) was added to a 49 µL reaction mixture containing template DNA (50 ng), 1 µL of each forward and reverse primer (10 µM, Sigma Aldrich), 10 µL of Phusion HF buffer (Thermo Scientific F530L), and 1 µL of dNTP mix (1 mM each, Thermo Scientific R0191). Each reaction was placed in a thermocycler (Bio-Rad T100) with the following cycling parameters: initial denaturing (95 °C, 30 sec), 16 cycles of denaturing (95 °C, 30 sec), annealing (Tm – 5 °C, 1 min), and elongation (72 °C, 8.1 min), followed by cooling (12 °C, 5 min). Template DNA was digested by adding 5 µL of 10X CutSmart buffer (NEB #B6004) and 0.5 µL DpnI (NEB R0176L) to 44.5 µL of each PCR reaction mixture with a 5 h incubation at 37 °C, followed by transformation into Top10 competent cells (Invitrogen). Cells were plated on 10 mL of LB agar containing 10 µL of ampicillin (100 mg/mL) and incubated overnight at 37 °C. Three colonies from each transformation were used to inoculate 5 mL of LB broth containing 5 µL ampicillin (100 mg/mL, Chem-Impex 0051625G) and grown overnight at 37 °C with shaking (250 rpm), followed by miniprep (Thermo Scientific K0503). All mutations were validated by Sanger sequencing at Genewiz using P7/P8. All primer sequences are listed in **Table S2**.

Dual luciferase assay. HEK293T cells (ATCC CRL-11268) were plated at 200,000 cells per well in a 6-well clear bottom plate and grown at 37 °C, 5% CO_2 in 2 mL per well of Dulbecco's modified Eagle media (DMEM, Cytiva SH30003.03) supplemented with 10% FBS and 1% streptomycin/penicillin (Corning 30-002-CI). At 80% confluency, the media was replaced with 2 mL of antibiotic-free DMEM (10% FBS) in preparation for transfection. The appropriate pMSCV-STING construct, pGL3-IFNb-Fluc (Addgene 102597), and pGL4.74-Rluc (Promega 9PIE692) were added in a 5:4:1 ratio (total plasmid DNA of 1000 ng/well) into OptiMEM (50 µL/well, Gibco 22600050). Lipofectamine 2000 (1 µL/well, Invitrogen 11668019) was added to OptiMEM (50 µL/well) in a separate tube. These two solutions were incubated at room temperature for 5 minutes, the Lipo/OptiMEM solution was pipetted into the DNA/OptiMEM solution and mixed by inverting. Following a 20-minute room temperature incubation, the transfection mix (100 µL) was added to each well. Approximately 18 hours after transfection, the media was carefully removed, and the cells were lifted with 500 µL of TrypLe (Gibco, 12604-021) and added to a 15 mL conical tube. The addition of 9.5 mL of DMEM inactivated TrypLe. Cells were pelleted at 1,000 g for 10 minutes at room temperature. DMEM was removed and cells were resuspended in 1 mL of Live Cell Imaging Solution (LCIS, Invitrogen A14291DJ). Cells were counted on a hemocytometer using Trypan Blue (to avoid counting dead cells). Cells were re-plated at 10,000 cells per well in a white, clear bottom 96-well plate in 90 µL of DMEM (without phenol red, Cytiva SH30284.01).

Dual luciferase assay – STING mutant comparison. HEK293T cells were transfected with either pMSCV-STING^{WT}, pMSCV-STING^{HAQ}, pMSCV-STING^{AQ}, pMSCV-STING^{Q}, or pMSCV-STING^{REF} along with firefly and *Renilla* luciferase plasmids (1 well each), and re-plated as

described above. Compound solutions in 100 µL of DMEM (– phenol red) each were prepared for DMSO or **1** (200 µM), then 10 µL of each 10X solution was added to experimental wells in triplicate to a final concentration of 20 µM with 2% DMSO. Compound treatment was followed by a 2-hour incubation period, and two washes with fresh LCIS to remove any additional compound from the well, and another 6 hour incubation. After the incubation period, the media was removed and replaced with 20 µL passive lysis buffer (Promega E1910). The plate was gently shaken at room temperature for 15 minutes, followed by analysis via Dual Luciferase assay (Promega E1980). As per the manufacturer's protocol, 100 µL of Luciferase assay reagent II was added to each well, firefly Luciferase activity was measured using a Tecan Infinite M1000 plate reader, 100 µL Stop & Glo reagent was added, and *Renilla* luciferase activity was measured again. Raw firefly luminescence values were normalized to *Renilla* luminescence values, then to the average value of the DMSO treated wells. Average fold-change with error bars representing standard deviation of triplicates was calculated (**Fig. 3**).

Western blot analysis. HEK293T cells were plated as described above, then transfected with either pMSCV-STING^{WT}, pMSCV-STING^{HAQ}, pMSCV-STING^{AQ}, pMSCV-STING^{Q}, or pMSCV-STING^{REF} (500 ng/well). Approximately 18 hours after transfection, the cells were gently resuspended and placed into Eppendorf tubes on ice, then cells were pelleted by centrifugation at 15000 g for 10 minutes. Cells were lysed with 200 µL of ice-cold RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS) supplemented with 2 µL of 100x protease inhibitor cocktail (Thermo Scientific, 78429) with 250 RPM shaking for 15 minutes. Cell debris was pelleted by centrifugation at 21000 g for 10 minutes at 4 °C and supernatant (150 µL) was combined with 50 µL of 4x SDS-PAGE sample loading buffer (200 mM Tris-HCl, pH 6.5, 400 mM DTT, 8% (w/v) SDS, 6 mM bromophenol blue, 4 M glycerol) and heated at 95 °C for 10 minutes.

Samples were separated by 10% (v/v) SDS-PAGE gel electrophoresis (60 V for 20 minutes, 175 V for 1 hour) with an ice pack placed inside the tank next to the gel cassette, followed by protein transfer to a 0.45 µm PDVF membrane (Millipore, IPVH00010) at 80 V for 90 minutes using ice-cold Transfer Buffer (25 mM Tris-HCl, 192 mM glycine, pH = 8.3). The membrane was blocked for 1 hour at room temperature with 10 mL of blocking buffer (5% milk in TBS with 0.1% (v/v) Tween 20, TBST) while rocking. After blocking, the blot was cut horizontally at the 70 kDa MW marker. Blots were probed with rabbit pAb anti-nucleolin (top piece, 1:2000 dilution, CST 14574S) and rabbit pAb anti-STING (bottom piece, 1:2000 dilution, ProteinTech 19851-1-AP) primary antibodies in 5 mL of fresh blocking buffer overnight with rocking at 4 °C. After washing three times by incubating the blots with 10 mL of ice-cold TBST at room temperature with rocking, membranes were incubated for 1 hour at room temperature with goat anti-rabbit IgG HRP-linked secondary antibody (1:5000 dilution, Cell Signaling 7074S) in 5 mL of TBST. After washing another three times with 10 mL of ice-cold TBST at room temperature with rocking, blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, 34580) by mixing 4 mL of the luminol/enhancer solution with 4 mL of the peroxide solution and incubating the membranes in the resulting solution for 5 minutes at room temperature with rocking. The blots were imaged on a BioRad ChemiDoc system with automated exposure times.

Dual luciferase assay – dose response against STING^{HAQ}. HEK293T cells were transfected with pMSCV-STING^{HAQ} along with firefly and *Renilla* luciferase plasmids (2 wells), and re-plated as described above. Compound solutions in 100 µL of DMEM (– phenol red) each were prepared for DMSO and varying concentrations of **1** or **4**, then 10 µL of each 10X solution was added to experimental wells in triplicate to various final concentrations of each compound with 2% DMSO. Compound treatment, incubation, and analysis were performed as described above (**Fig. 3C, Fig. S3**).

Dual luciferase assay – light-induced activation. HEK293T cells were transfected with pMSCV-STING^{HAQ} along with firefly and *Renilla* luciferase plasmids (2 wells), and re-plated as described above. Compound solutions in 100 µL of DMEM (– phenol red) each were prepared for DMSO, **1** (200 µM), or **4** (200 µM), then 10 µL of each 10X solution was added to experimental wells in triplicate to a final concentration of 20 µM with 2% DMSO. Compound treatment was followed by irradiation with a 365 nm LED (Mouser Electronics single color luxigen LZ1 MCPCB, 66 mW/cm^2) placed the top of each well (11 mm from sample) for various durations (0, 60, or 120 sec). Incubation and analysis were performed as described above (**Fig. 5C**).

Firefly luciferase transfection protocol. HEK293T cells were plated at 200,000 cells per well in a 6-well clear bottom plate and grown at 37 °C, 5% CO_2 in 2 mL per well of Dulbecco's modified Eagle media supplemented with 10% FBS and 1% streptomycin/penicillin. At 80% confluency, the media was replaced with 2 mL of antibiotic-free DMEM (10% FBS) in preparation for transfection. The appropriate pMSCV-STING^{HAQ} and pGL3-IFNb-Fluc were added in a 1:2 ratio (total plasmid DNA of 1000 ng/well) into OptiMEM (50 µL/well). Lipofectamine 2000 (1 µL/well) was added to OptiMEM (50 µL/well) in a separate tube. These two solutions were incubated at room temperature for 5 minutes, the Lipo/OptiMEM solution was pipetted into the DNA/OptiMEM solution and mixed by inverting. Following a 20-minute room temperature incubation, the transfection mix (100 µL) was added to each well. Approximately 18 hours after transfection, the media was carefully removed, and the cells were lifted with 500 µL of TrypLe and added to a 15 mL conical tube. The addition of 9.5 mL of DMEM inactivated TrypLe. Cells were pelleted at 1,000 g for 10 minutes at room temperature. DMEM was removed and cells were resuspended in 1 mL of LCIS. Cells were counted on a hemocytometer using Trypan Blue (to avoid counting dead cells). Cells were re-plated at 10,000 cells per well in a white, clear bottom 96-well plate in 90 µL of DMEM (without phenol red).

Firefly luciferase assay – dose response against STING^{HAQ}. HEK293T cells were transfected with pMSCV-STING^{HAQ} along with firefly and *Renilla* luciferase plasmids (2 wells), and re-plated as described above. Compound solutions in 100 µL of DMEM (– phenol red) each were prepared for DMSO and varying concentrations of **2** or **3**, then 10 µL of each 10X solution was added to experimental wells in triplicate to various final concentrations of each compound with 2% DMSO. Compound treatment, incubation, and analysis were performed as described above (**Fig. 4C and Fig. 4D**).

Cell viability assay. HEK293T cells were plated at 4,000 cells per well in a clear 384-well plate (Greiner, 781182) with varying concentrations of doxorubicin (positive control), **1**, or **4** with 2% DMSO in 40 µL of media (DMEM with 10% fetal bovine serum) in triplicate. After a 72-hour incubation, 16 µL of activated XTT reagent (8 µL of 1.7 mg/mL menadione diluted into 1 mL of XTT reagent solution) to each well. Absorbance was measured at 450 nm and 630 nm (Tecan M1000 plate reader) immediately following addition and again after 2 hours. Cell viability was determined using the following equation: $(Abs_{450} - Abs_{630})_{final} - (Abs_{450} - Abs_{630})_{initial}$, then each well was normalized to the DMSO-only control (**Fig. S4**).

Supplementary Figures and Tables

Table S1. Human STING mutants.

Mutation	71	230	232	293
$STING^{WT}$	R	G	R	R
$STING^{HAQ}$	H	A	R	Q
$STING^{REF}$	R	G	H	R
$STING^{AQ}$	R	A	R	Q
$STING^{Q}$	R	G	R	Q

Table S2. List of primers used to for cloning/sequencing. Mutations introduced by site-directed mutagenesis are highlighted in grey.

Primer	Sequence (5' to 3')
P1	gctgaggagctgcgccacatccactcca
P2	tggagtggatgtggcgcagctcctcagc
P3	ggccaaactcttctgccagacacttgaggacatcc
P4	ggatgtcctcaagtgtctggcagaagagtttggcc
P5	cagcagaccggtgaccacgctggcatcaaggatc
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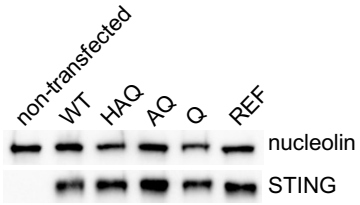


Fig. S1. Western blot analysis of relative STING expression levels following transfection in HEK293T cells.

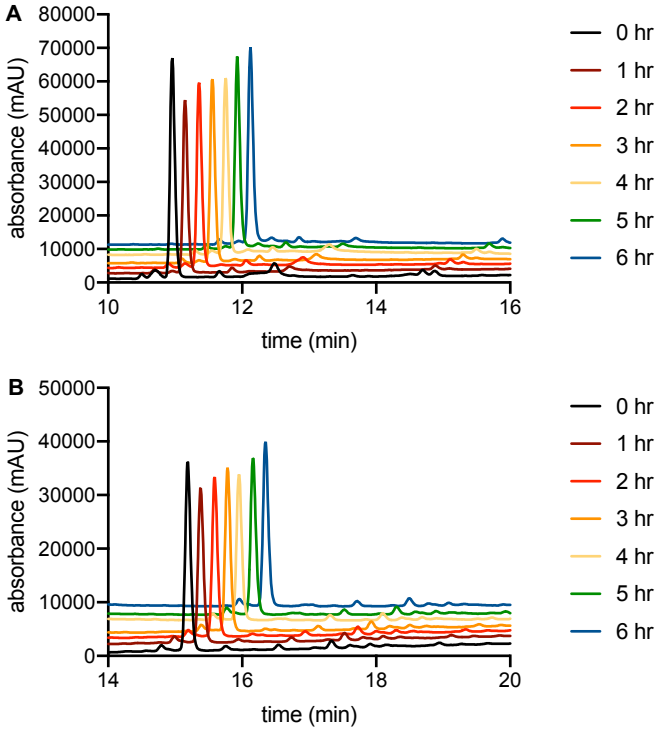


Fig. S2. Samples (100 μM) were prepared in PBS with 2% DMSO and 10% FBS of A) **1** or B) **4**. Samples were analyzed over the course of 6 h while incubating at 37 °C. No instability was observed.

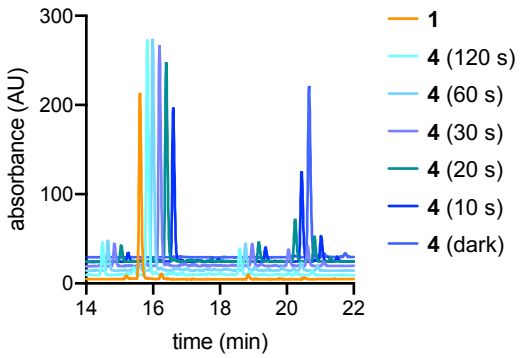


Fig. S3. HPLC decaging assay. Samples were prepared in PBS and irradiated for various amounts of time using a 405 nm LED. This analysis revealed complete decaging only with a 2 minute irradiation time, suggesting 365 nm provides for more efficient decaging.

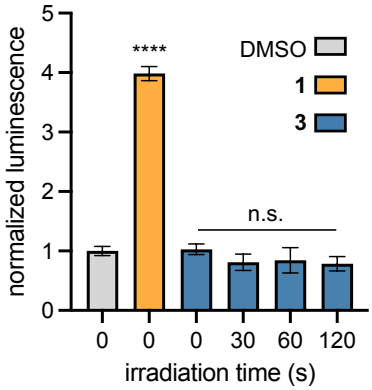


Fig. S4. No background activation in response to irradiation. Cells were treated with DMSO, **1** (20 μM), or with various concentrations of **3**.

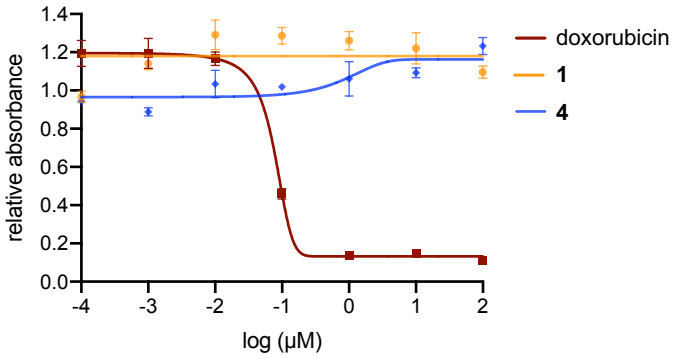
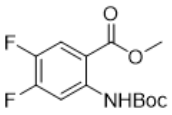
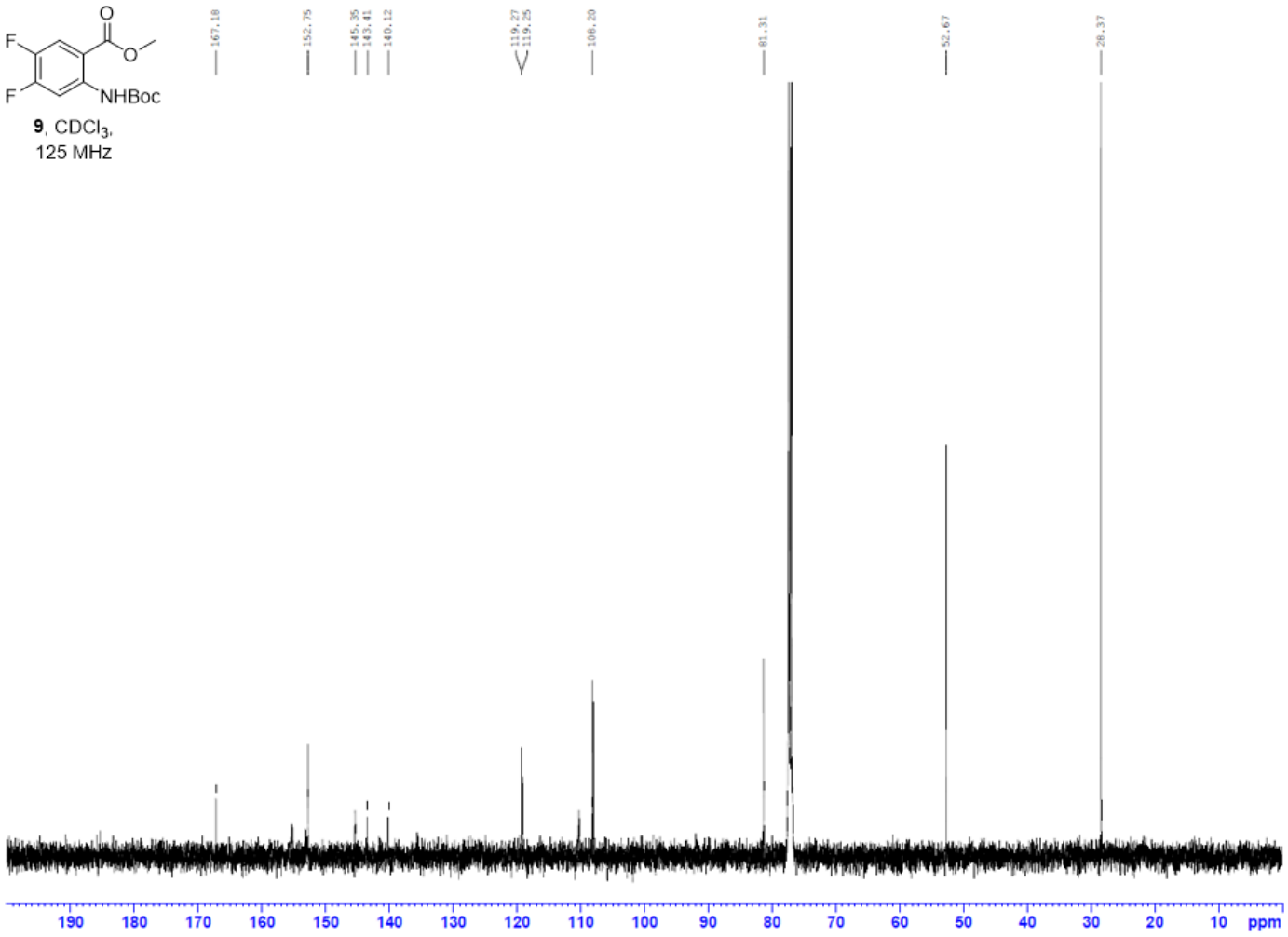


Fig. S5. Cell viability assay. Cells were treated with various concentrations of either doxorubicin, as a positive control for toxicity, **1**, or **4**. Both **1** and **4** were found to elicit no toxicity up to 100 μM. Error bars represent standard deviation from three biological replicates.

NMR Spectra



9, $CDCl_3$,
500 MHz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119.25
108.20
81.31
52.67
28.37
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140
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70
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40
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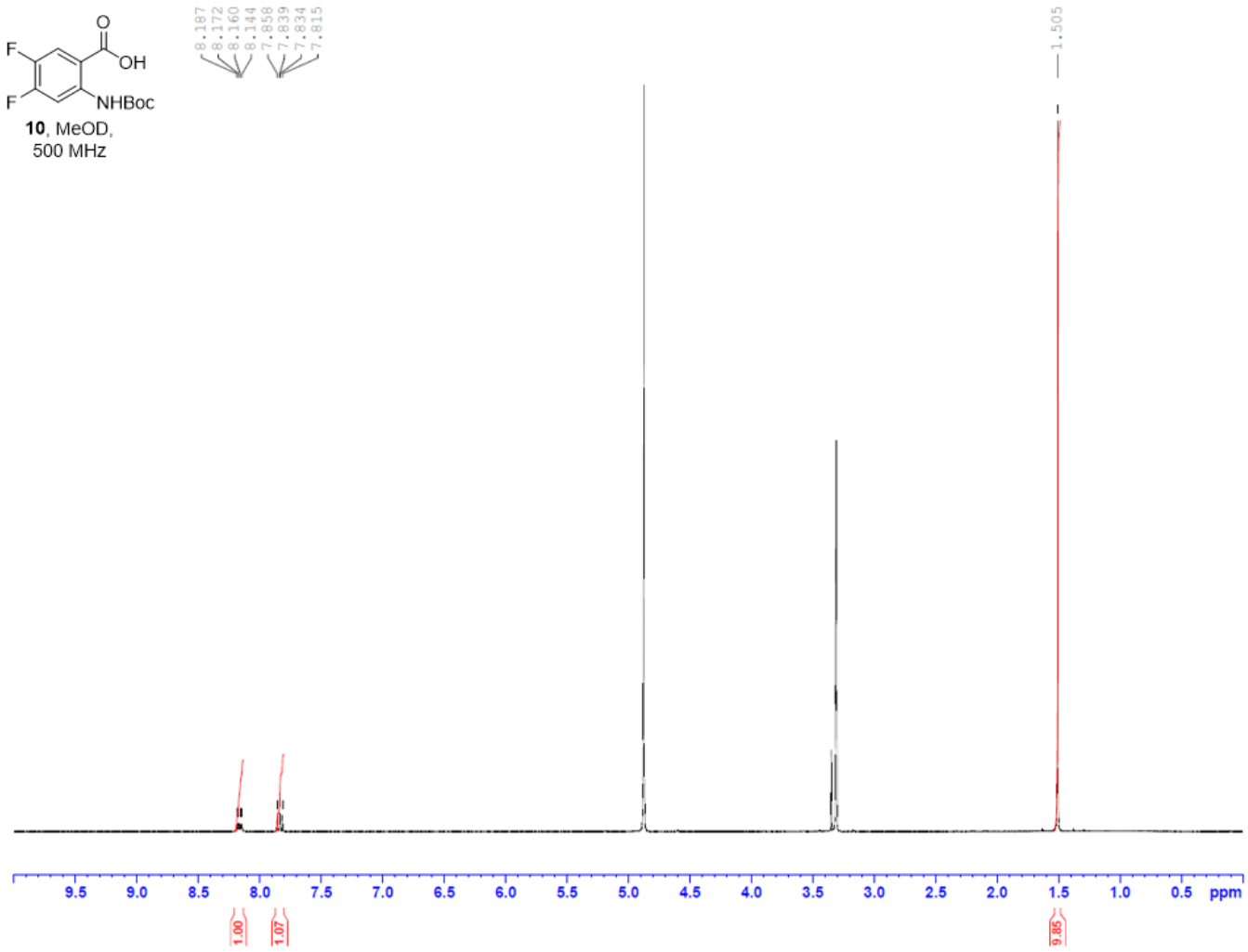
20
10
ppm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7.815
1.505
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9.0
8.5
8.0
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7.0
6.5
6.0
5.5
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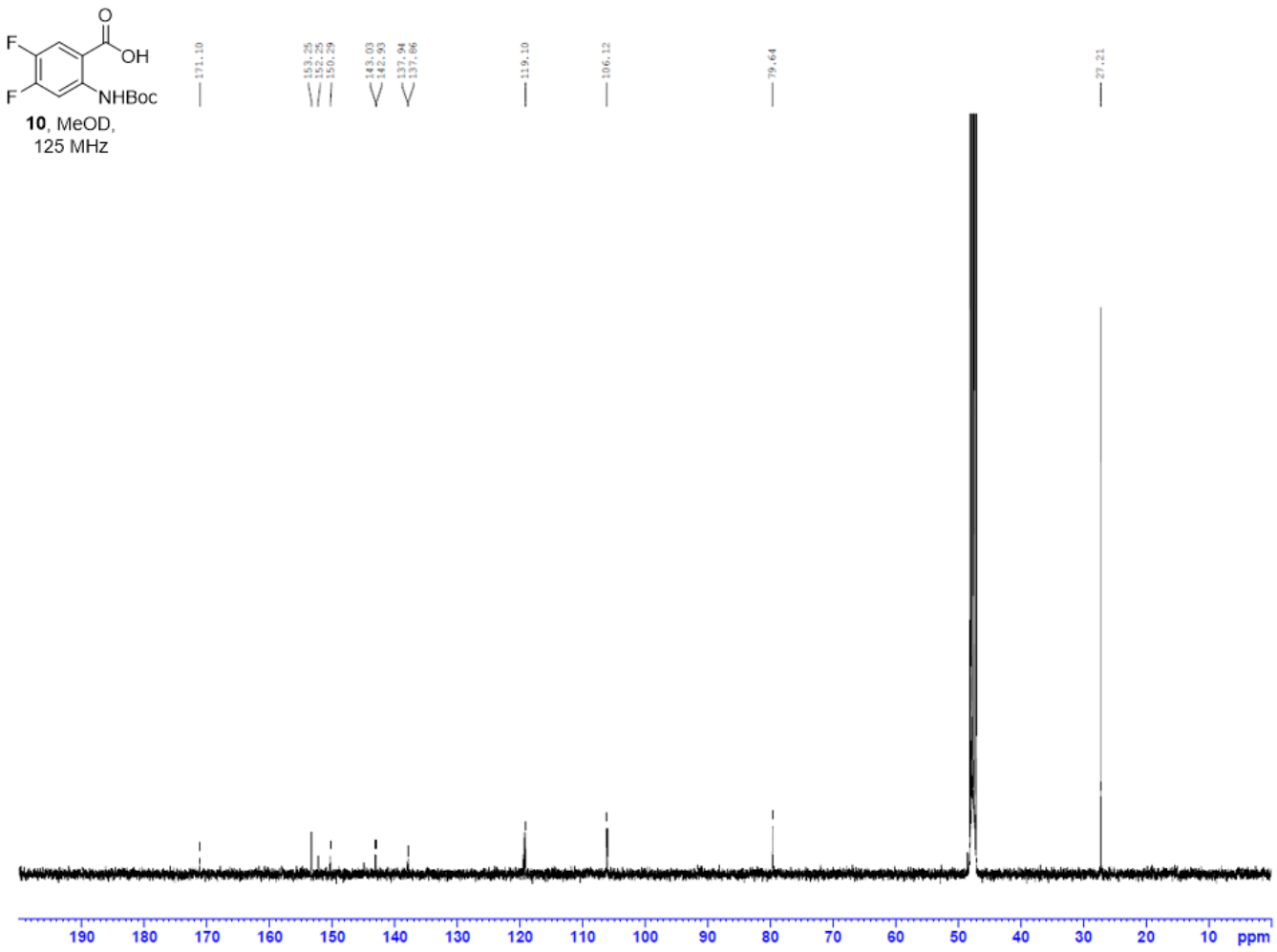
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F
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137.86
119.10
106.12
79.64
27.21
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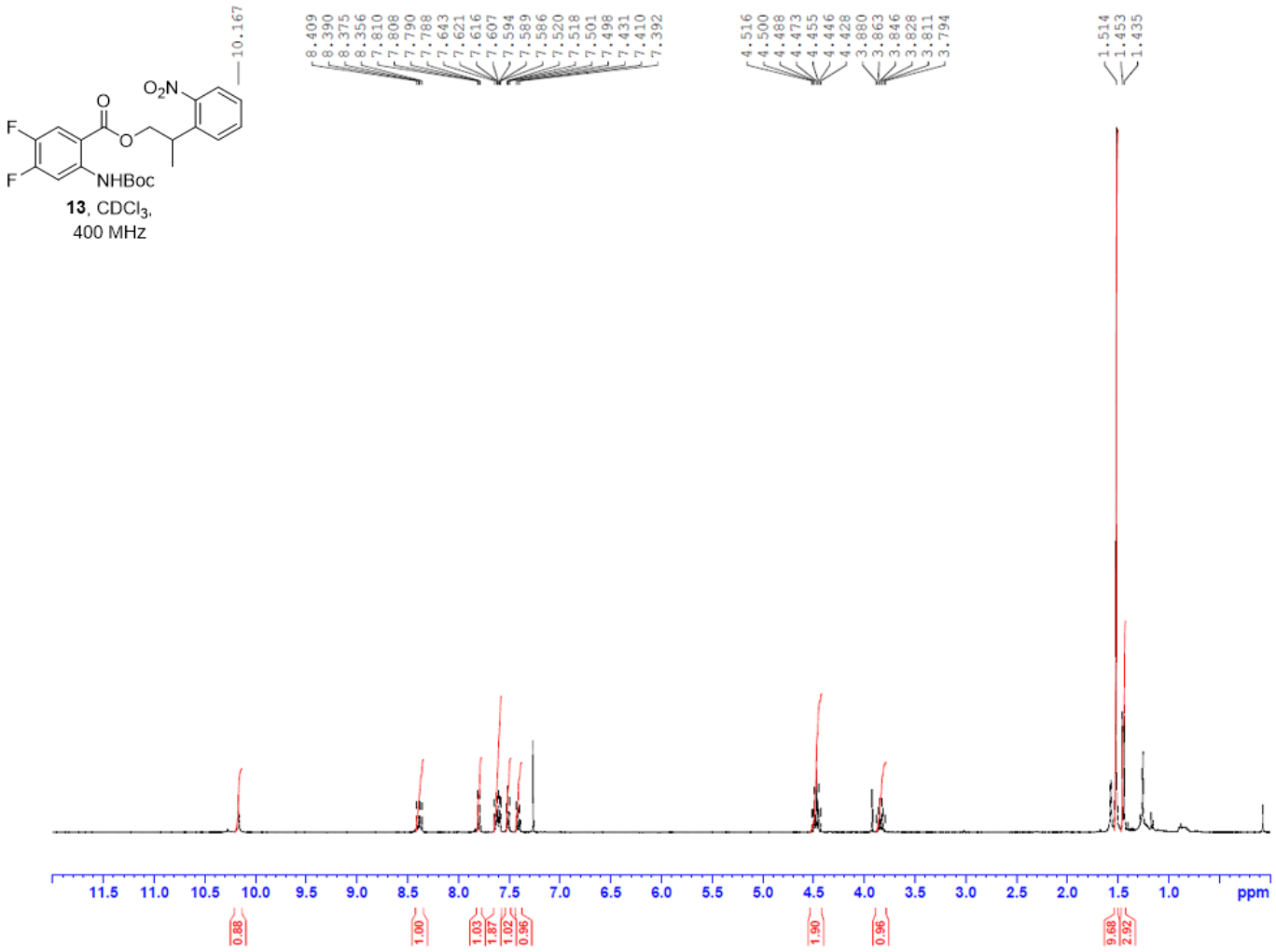
ppm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7.810
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7.790
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7.594
7.589
7.586
7.520
7.518
7.501
7.498
7.431
7.410
7.392
4.516
4.500
4.488
4.473
4.455
4.446
4.428
3.880
3.863
3.846
3.828
3.811
3.794
1.514
1.453
1.435
O2N
O
O
F
F
NHBoc
13, CDCl3,
400 MHz
11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
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0.96
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0.96
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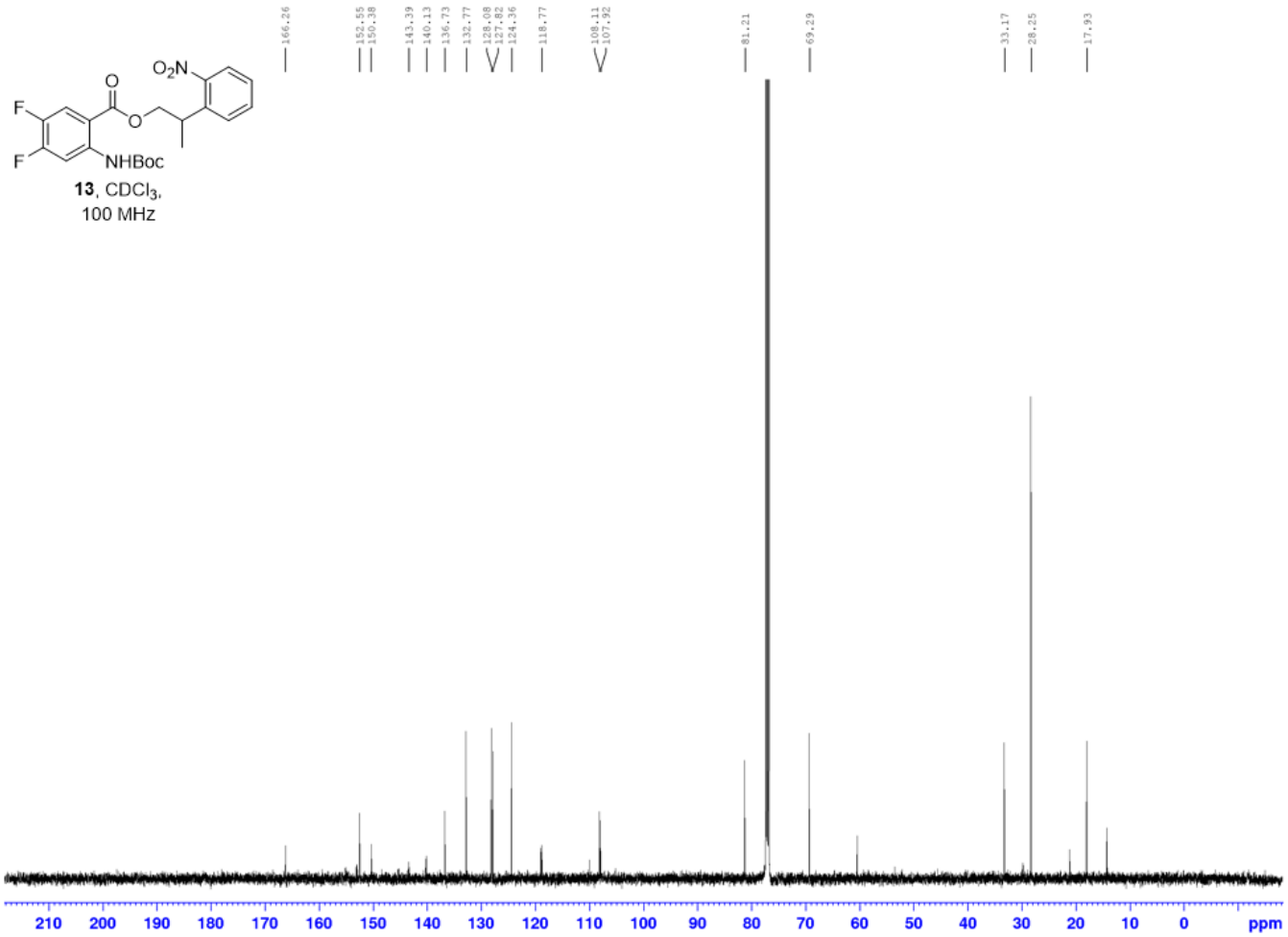
2.92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81.21
69.29
33.17
28.25
17.93
O2N
O
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F
F
NHBoc
13, CDCl3,
100 MHz
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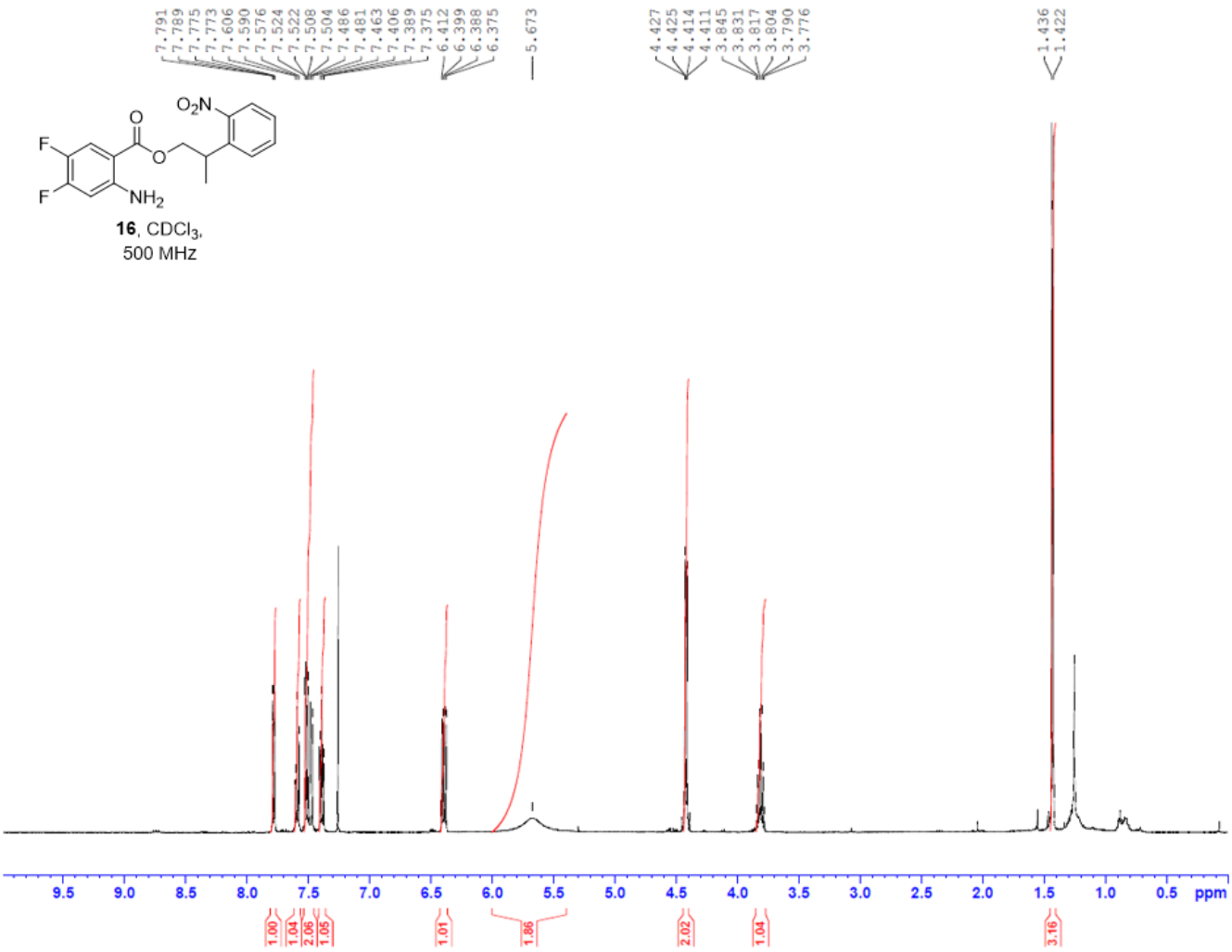
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6.388
6.375
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4.425
4.414
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3.831
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O
F
F
O
NH2
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500 MHz
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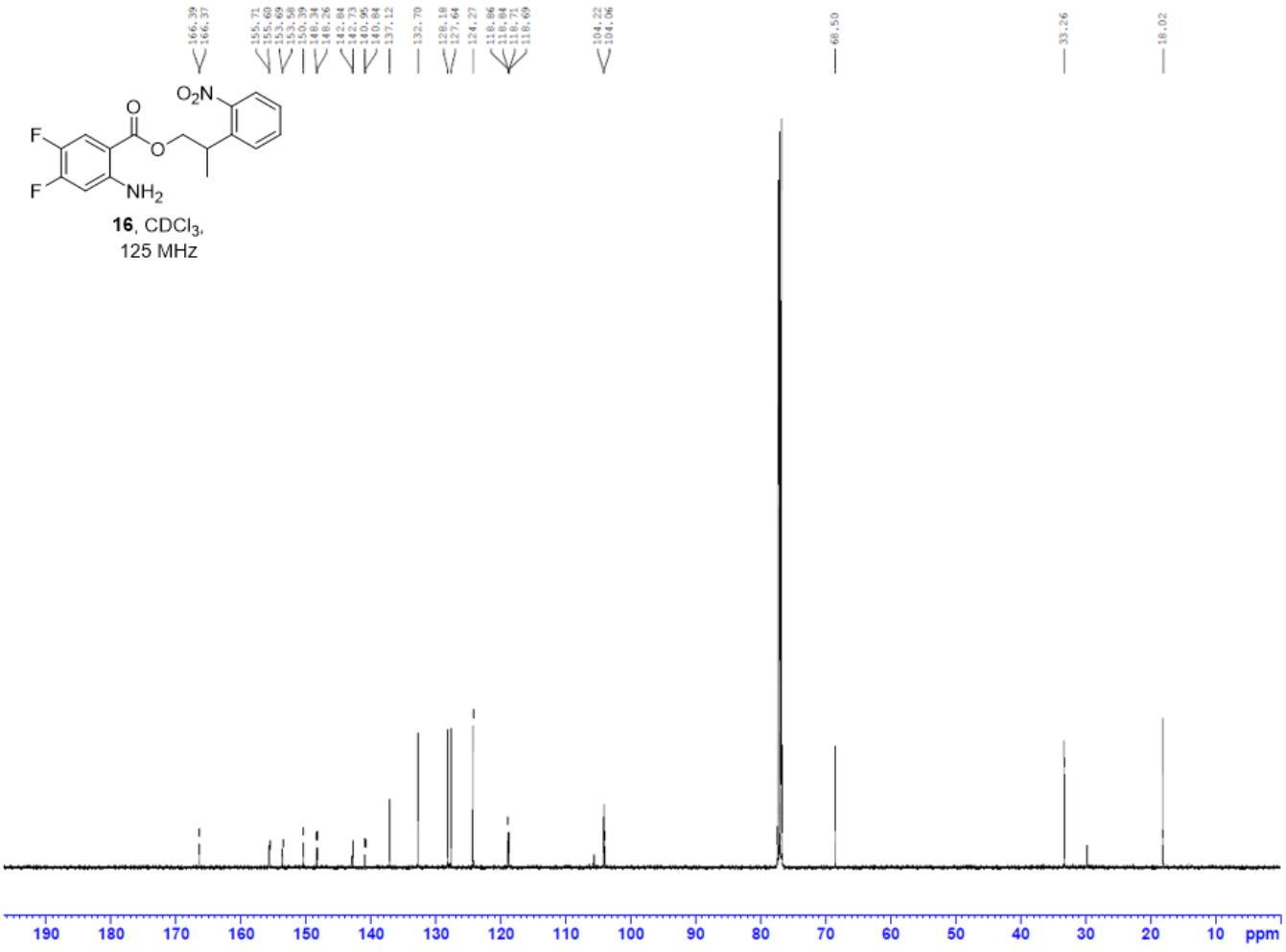
3.16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O
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NH2
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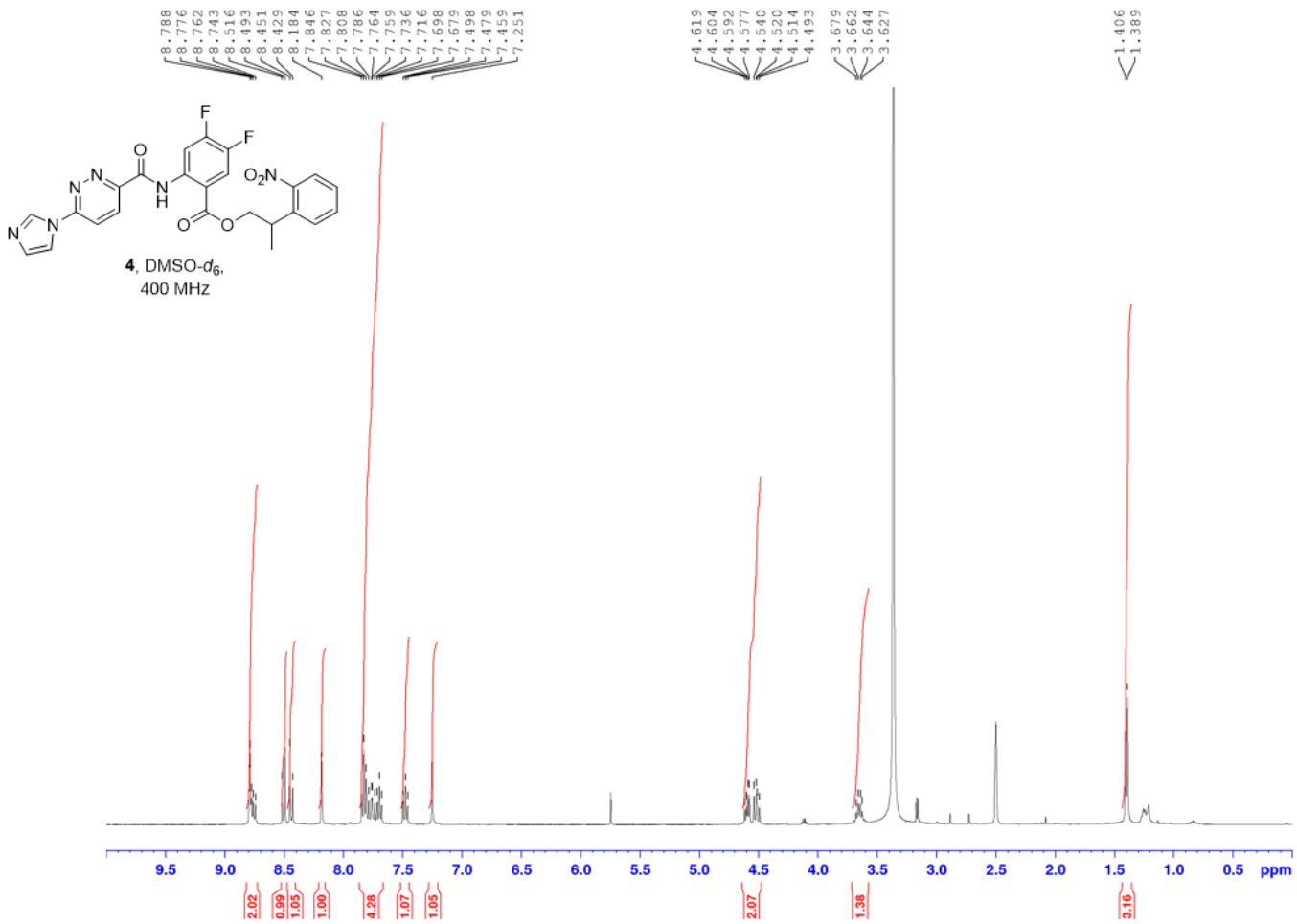
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H
O
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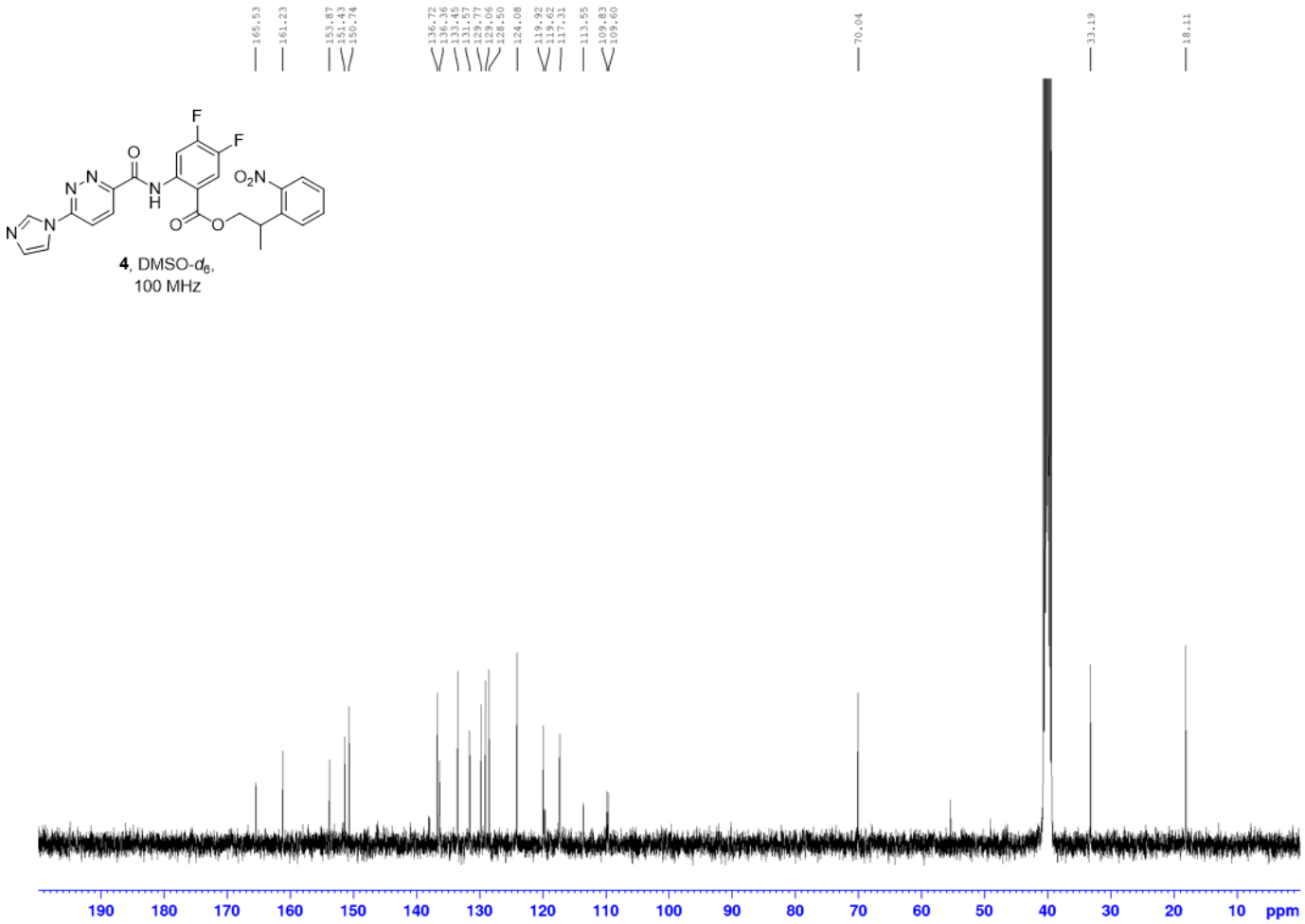
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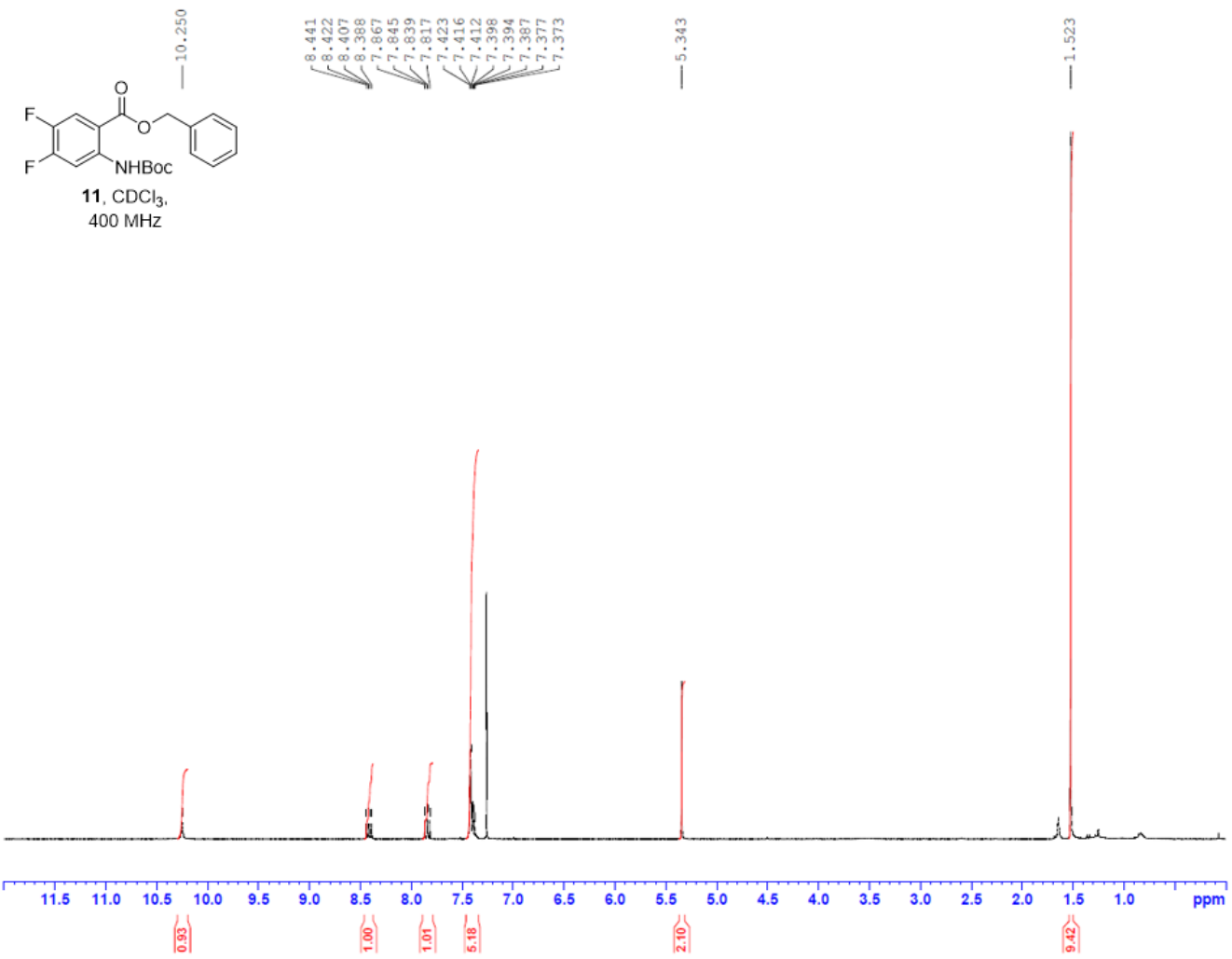
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O
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11, CDCl3,
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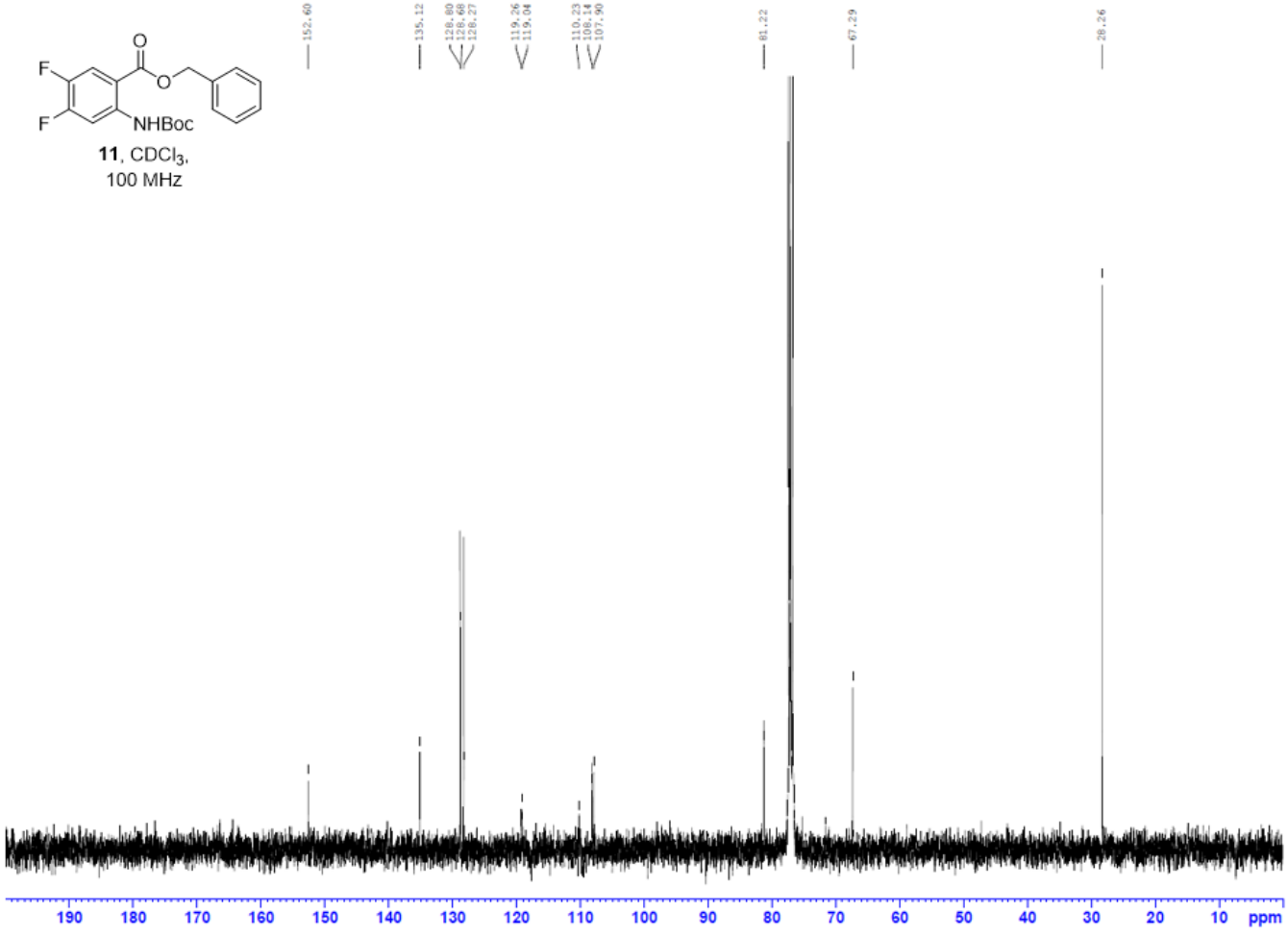
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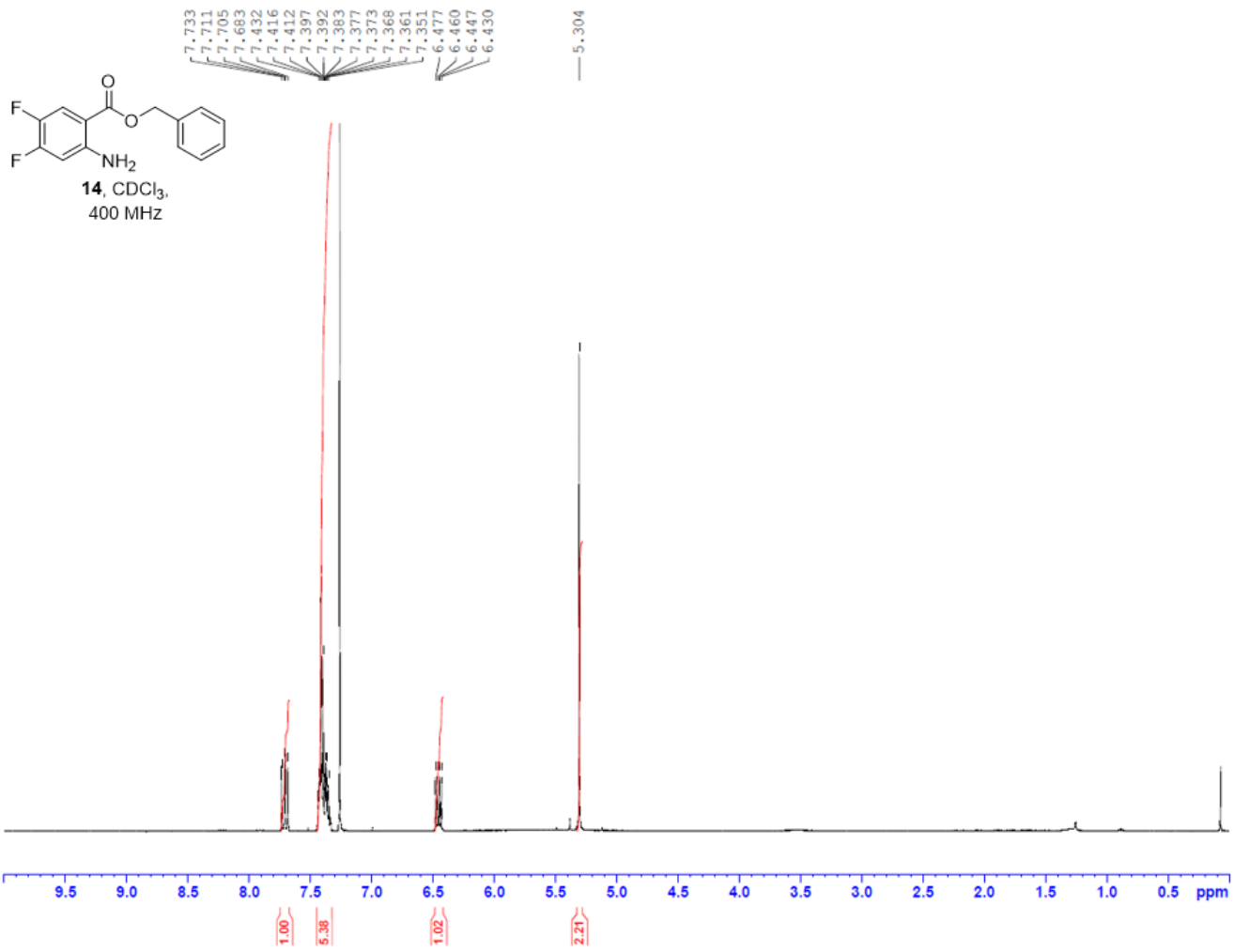
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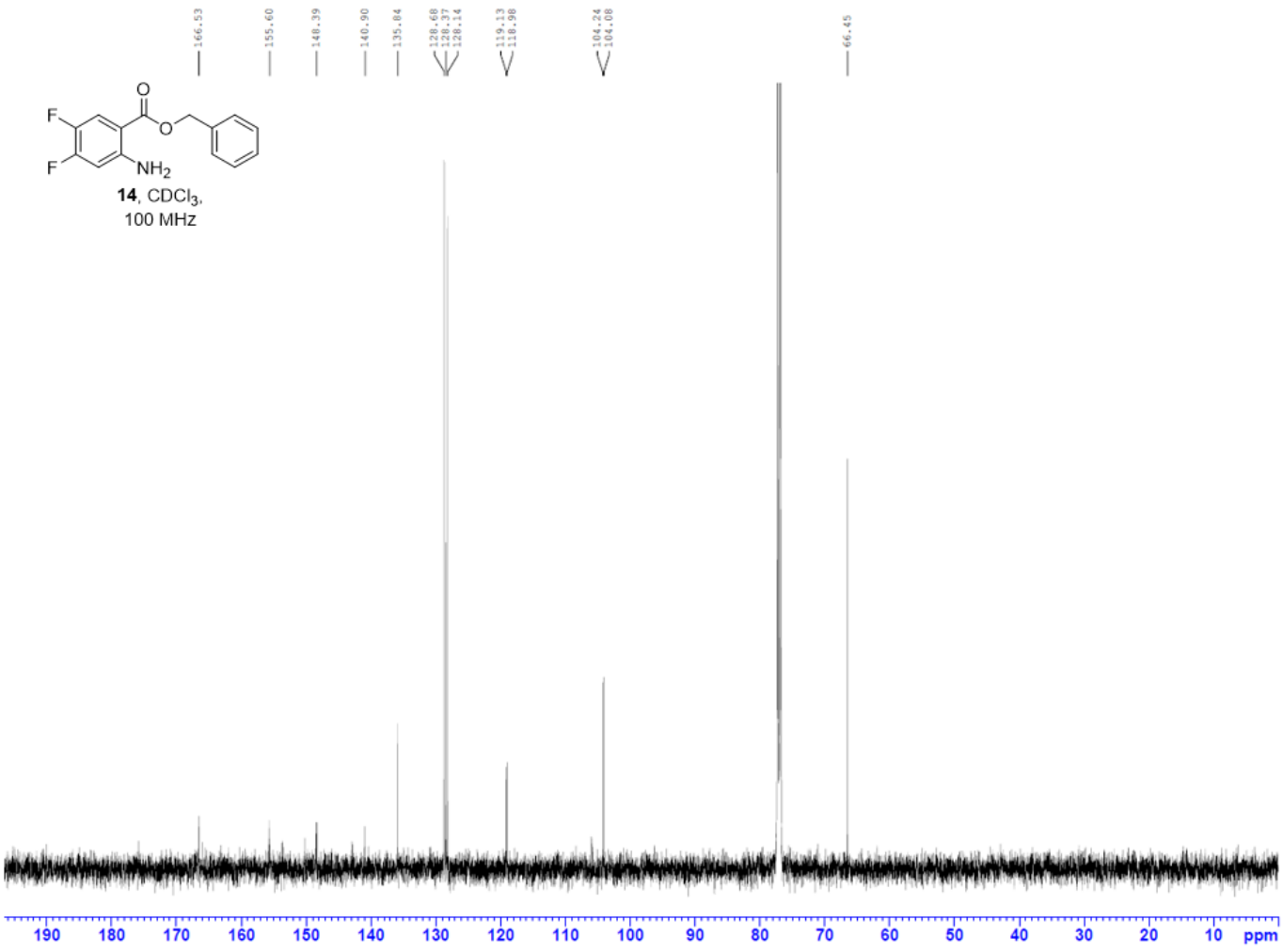
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100 MHz
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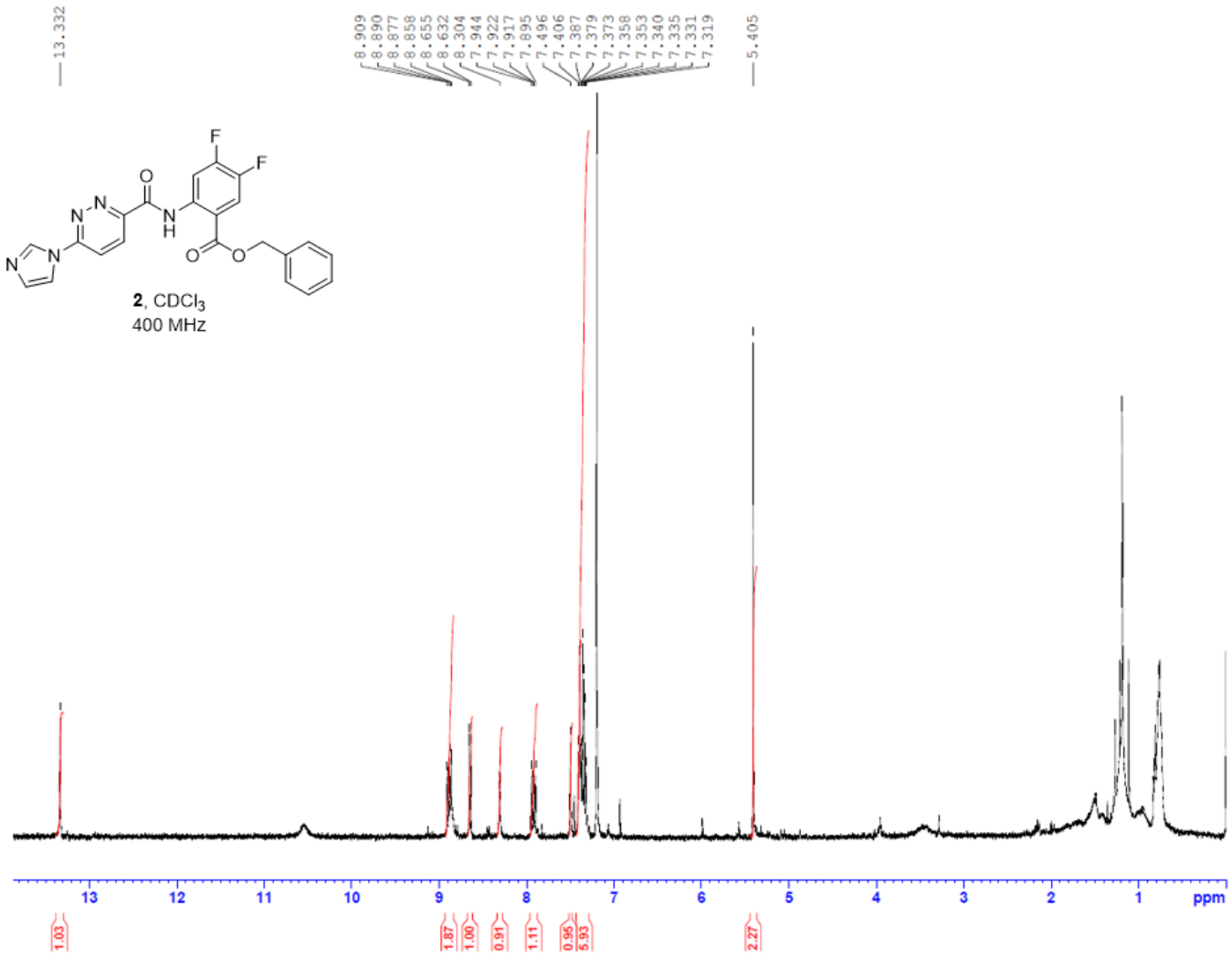
10
ppm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ppm
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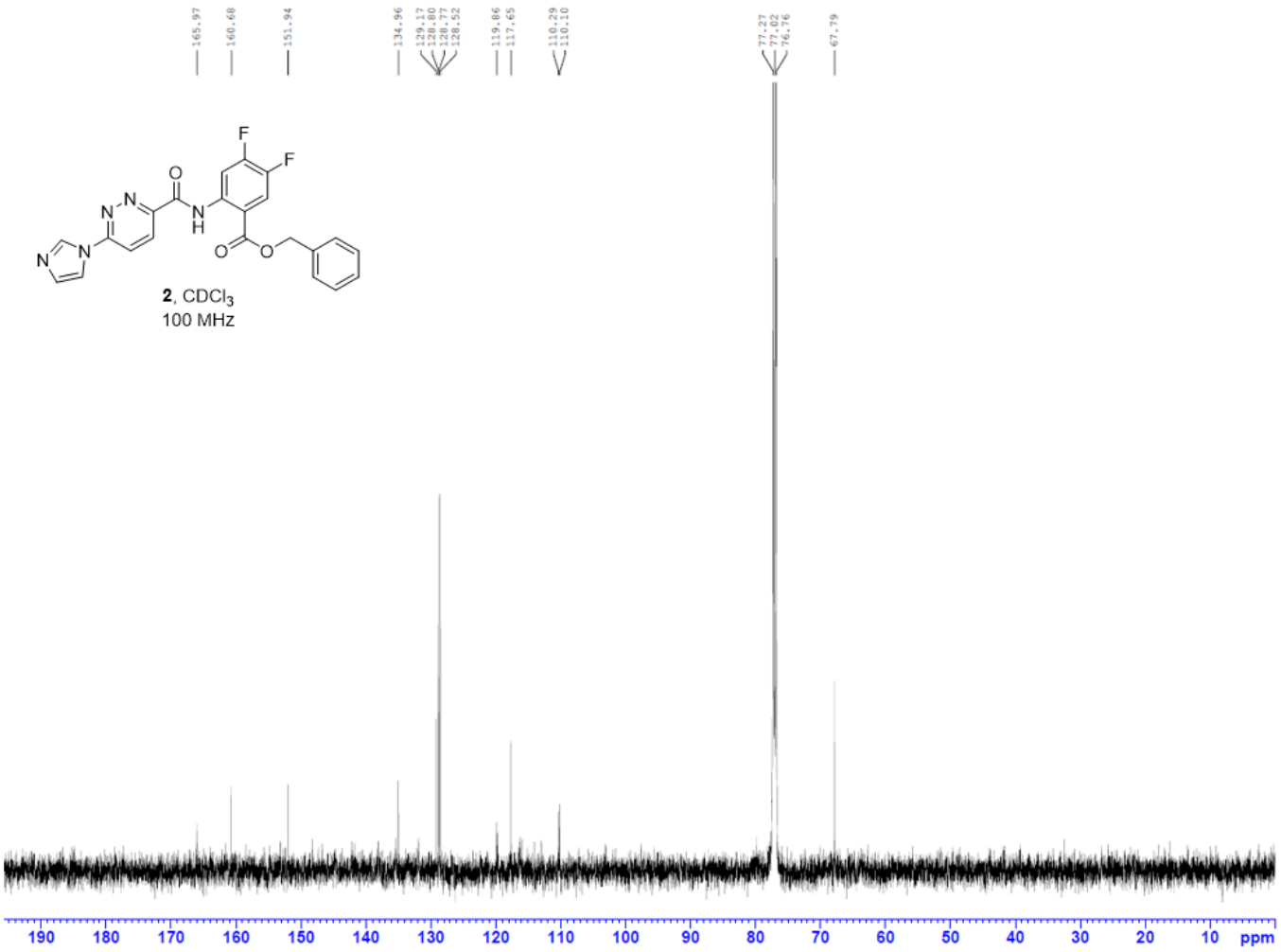
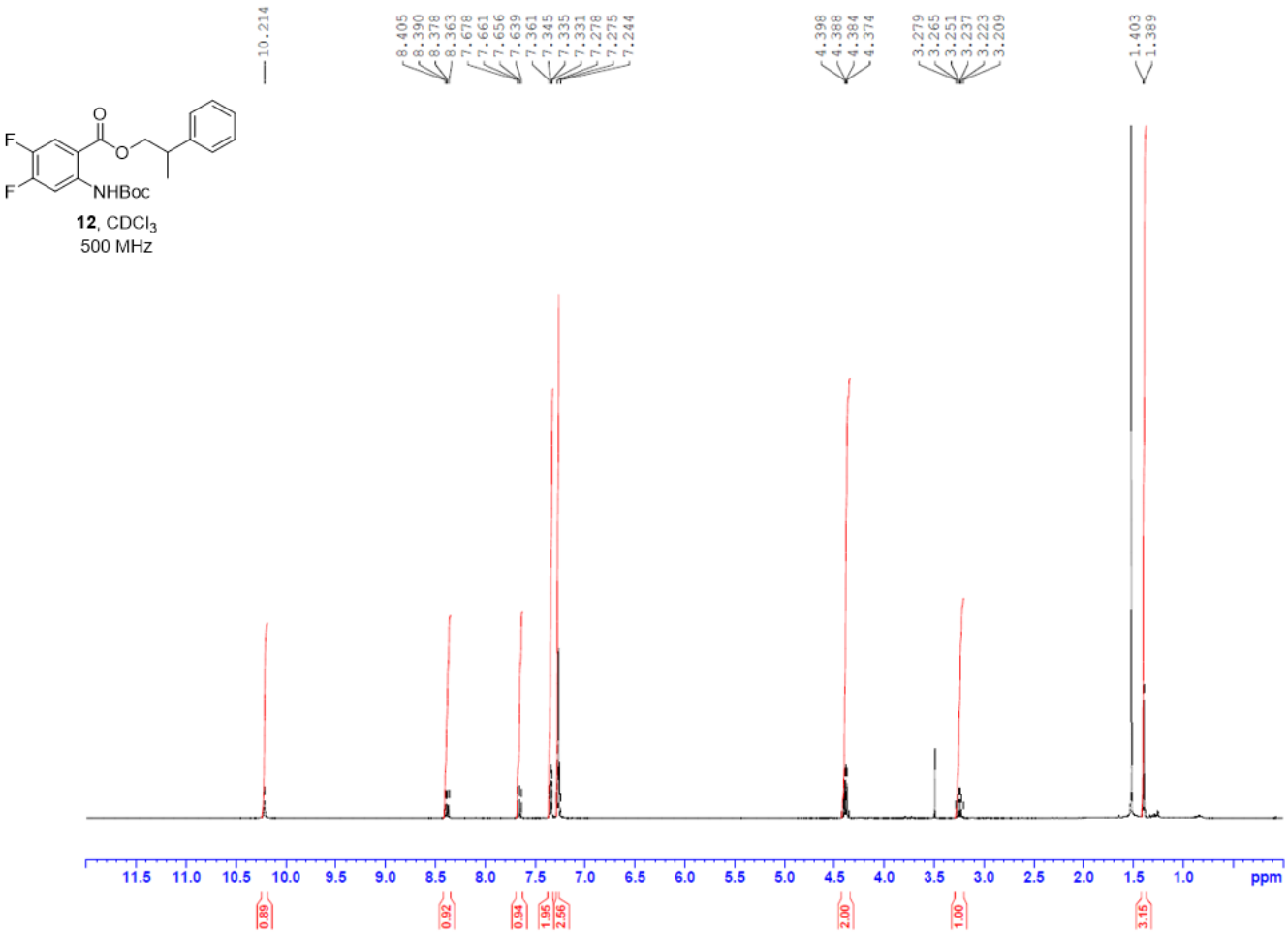
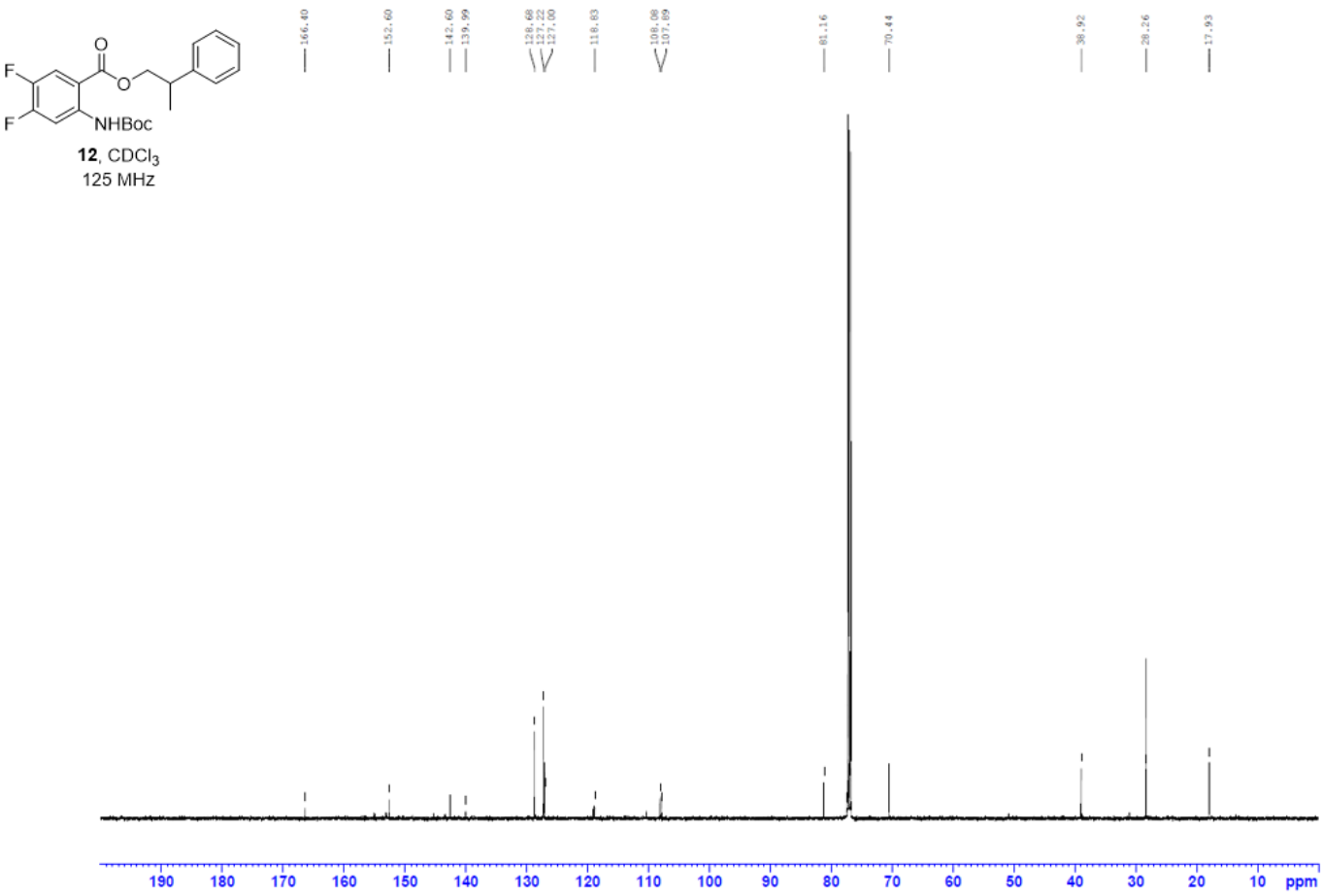
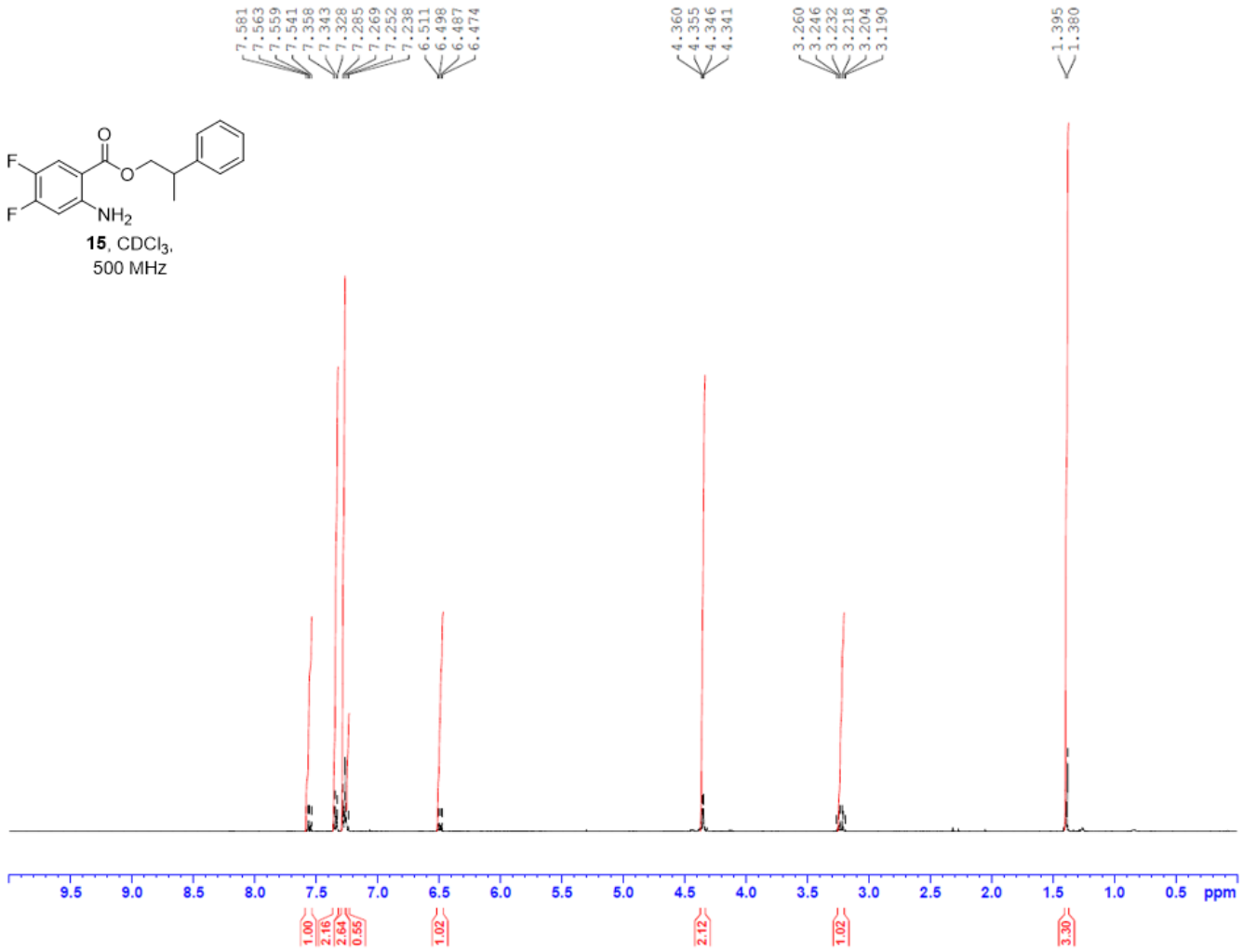
0.95
5.93
2.27

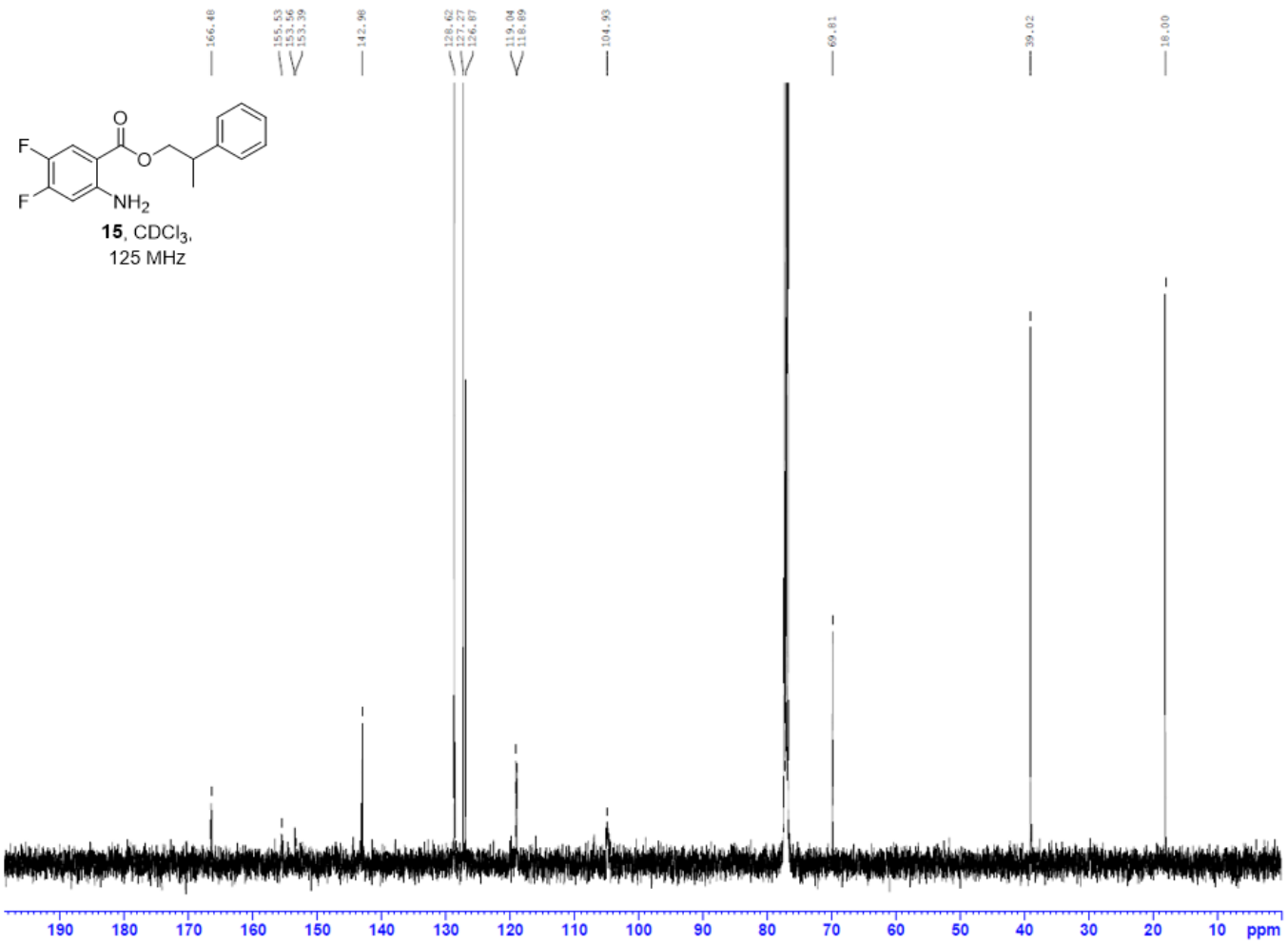

165.97
160.68
151.94
134.96
129.17
128.80
128.77
128.52
119.86
117.65
110.29
110.10
77.27
77.02
76.76
67.79
F
F
O
N
N
N
H
O
O
N
2, CDCl3
100 MHz
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm

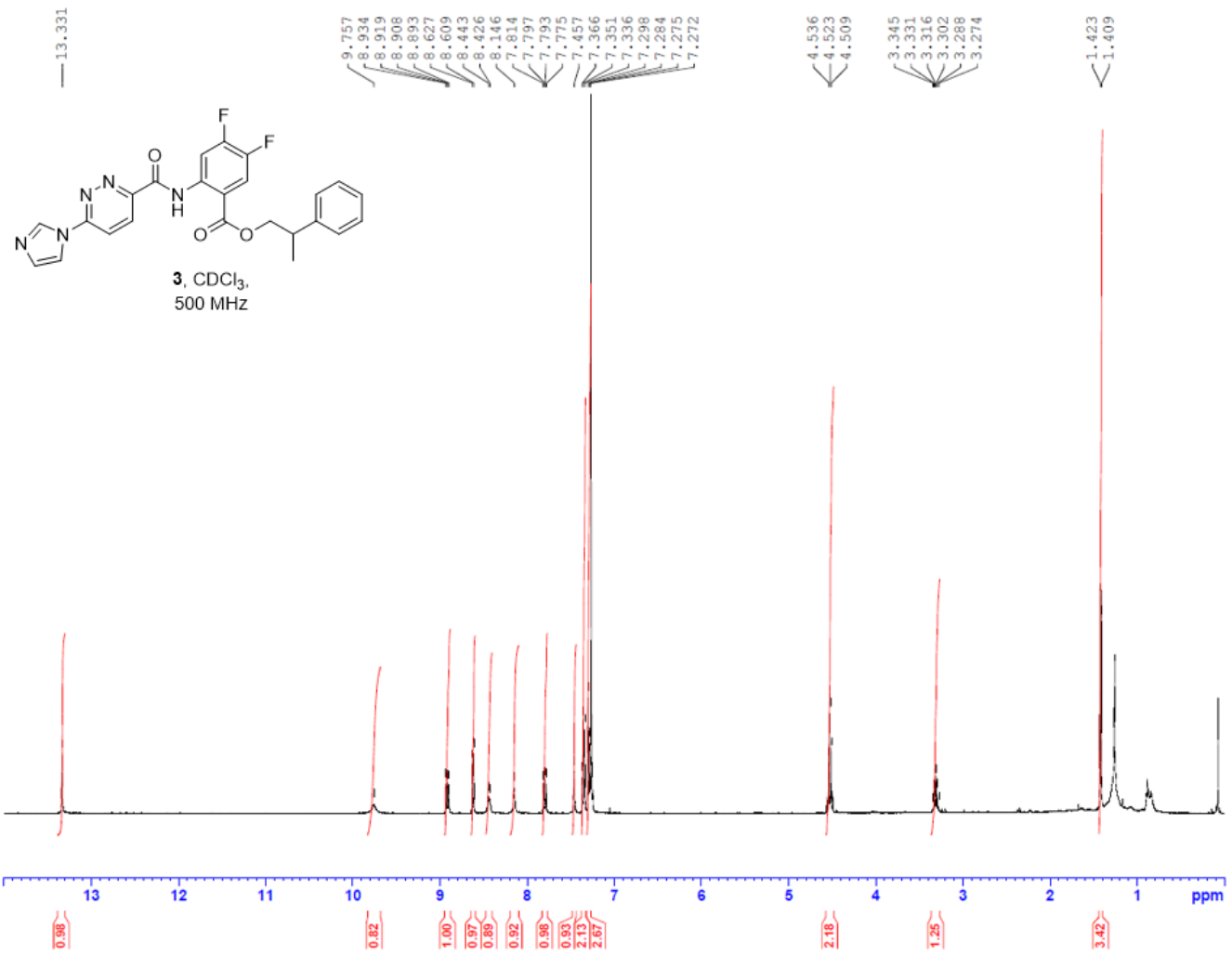

10.214
8.405
8.390
8.378
8.363
7.678
7.661
7.656
7.639
7.361
7.345
7.335
7.331
7.278
7.275
7.244
4.398
4.388
4.384
4.374
3.279
3.265
3.251
3.237
3.223
3.209
1.403
1.389
F
F
O
O
NHBoc
12, CDCl3
500 MHz
11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
0.89
0.92
0.94
1.95
2.56
2.00
1.00
3.15

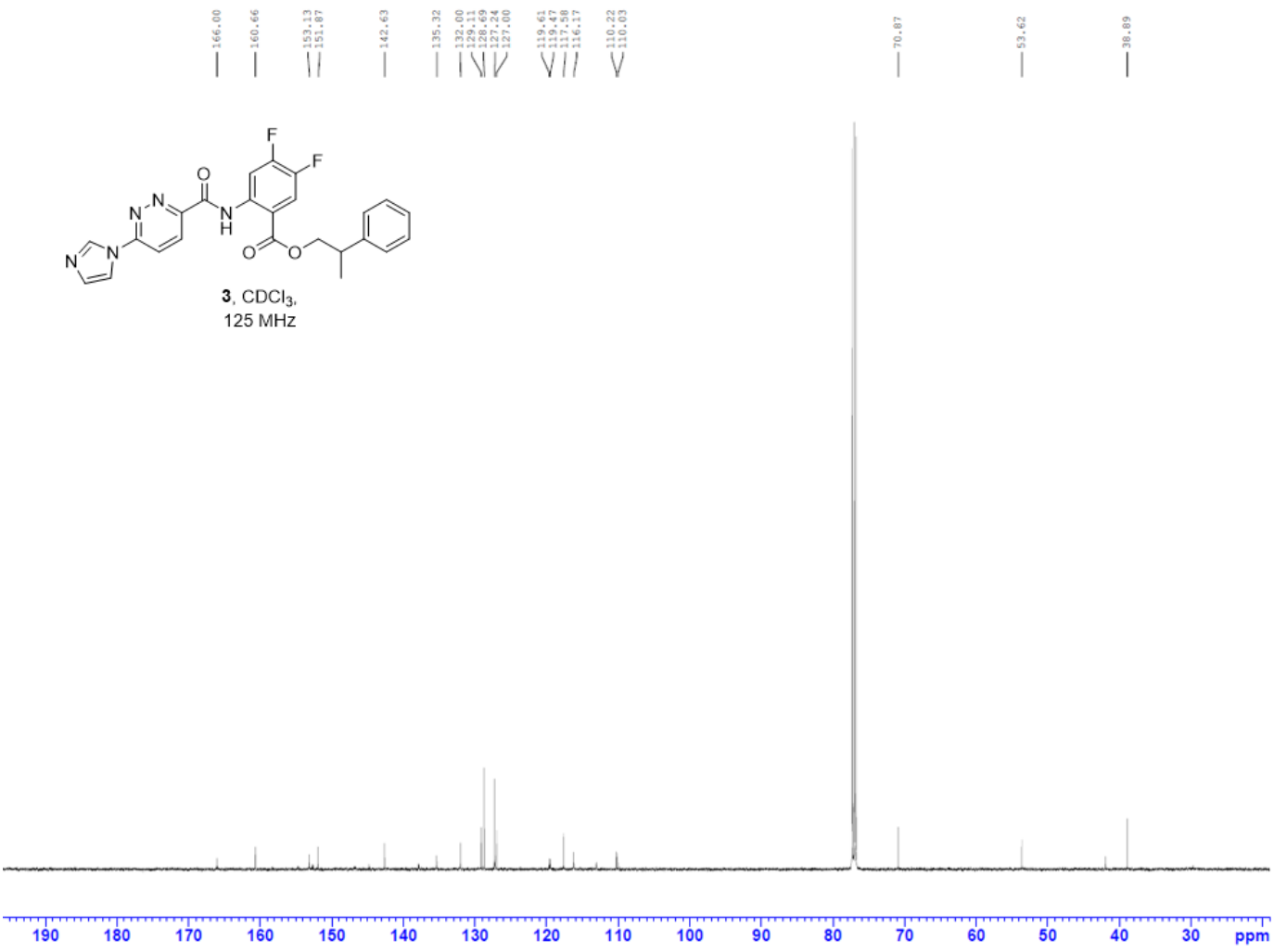


12, $CDCl_3$
125 MHz


7.581
7.563
7.559
7.541
7.358
7.343
7.328
7.285
7.269
7.252
7.238
6.511
6.498
6.487
6.474
4.360
4.355
4.346
4.341
3.260
3.246
3.232
3.218
3.204
3.190
1.395
1.380
F
F
O
O
NH2
15, CDCl3,
500 MHz
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
1.00
2.16
2.64
0.55
1.02
2.12
1.02
3.30

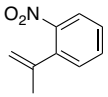

166.48
155.53
153.56
153.39
142.98
128.62
127.27
126.87
119.04
118.89
104.93
69.81
39.02
18.00
F
F
O
O
NH2
15, CDCl3,
125 MHz
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm

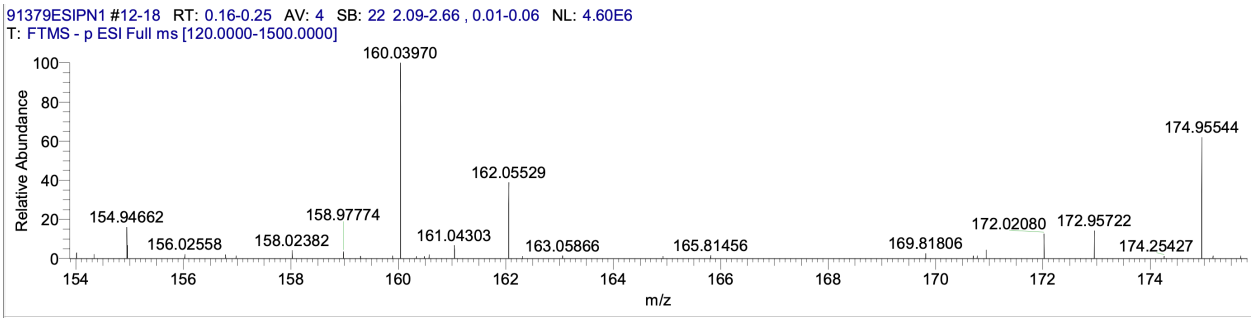

13.331
9.757
8.934
8.919
8.908
8.893
8.627
8.609
8.443
8.426
8.146
7.814
7.797
7.793
7.775
7.457
7.366
7.351
7.336
7.298
7.284
7.275
7.272
4.536
4.523
4.509
3.345
3.331
3.316
3.302
3.288
3.274
1.423
1.409
F
F
O
N
N
N
H
N
O
O
3, CDCl3,
500 MHz
13
12
11
10
9
8
7
6
5
4
3
2
1
ppm
0.98
0.82
1.00
0.97
0.89
0.92
0.98
0.93
2.13
2.67
2.18
1.25
3.42


166.00
160.66
153.13
151.87
142.63
135.32
132.00
129.11
128.69
127.24
127.00
119.61
119.47
117.58
116.17
110.22
110.03
70.87
53.62
38.89
F
F
O
N
N
N
H
N
O
O
3, CDCl3,
125 MHz
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm

HRMS

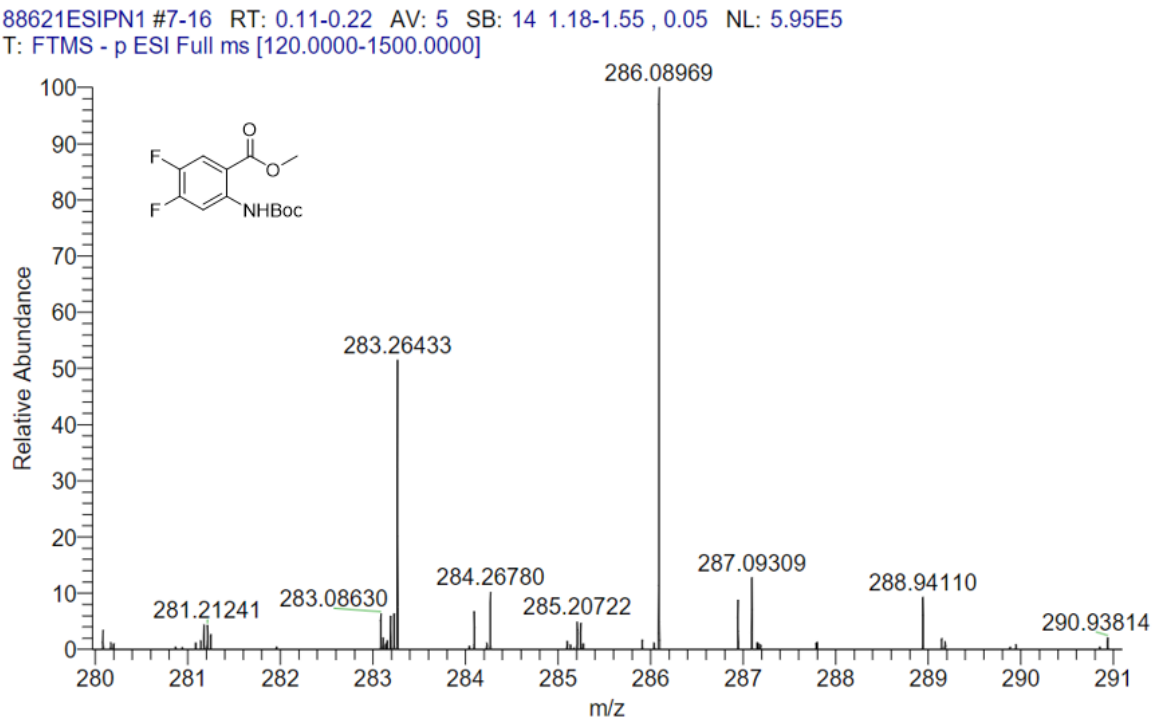
Decaging byproduct:





91379ESIPN1#12-18 RT: 0.16-0.25 AV: 4
SB: 22 2.09-2.66 , 0.01-0.06
T: FTMS - p ESI Full ms [120.0000-1500.0000]
m/z= 161.67462-162.60375

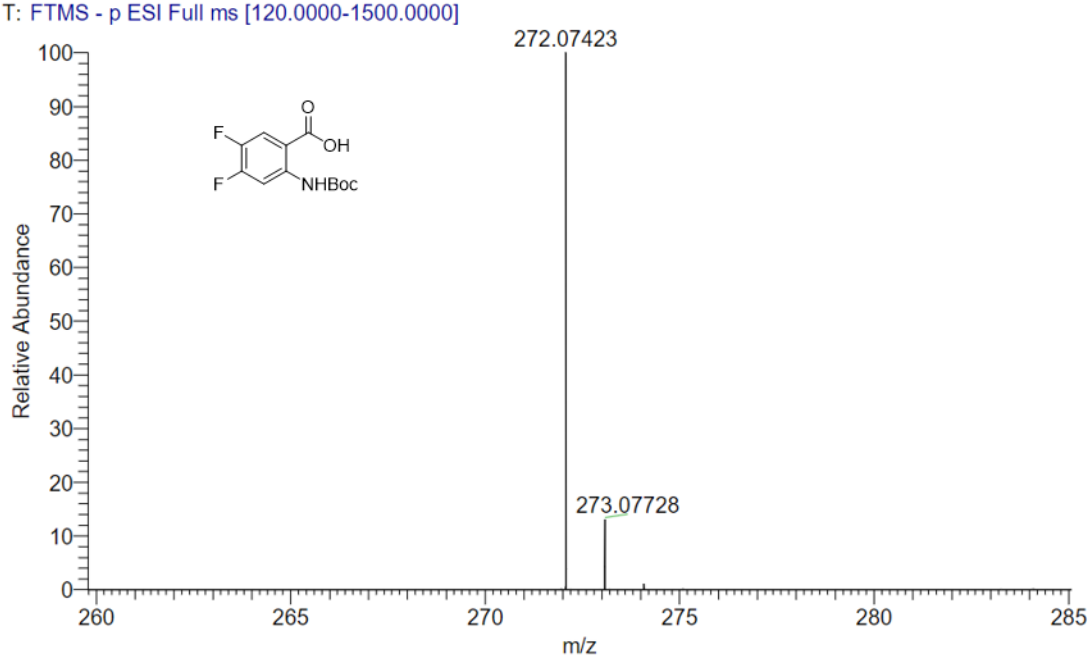
m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
162.05529	1844892.1	100.00	162.05496	2.07	$C_9H_8O_2N$



88621ESIPN1#8-16 RT: 0.11-0.22 AV: 5
SB: 14 1.18-1.55 , 0.05
T: FTMS - p ESI Full ms [120.0000-1500.0000]
m/z= 279.96781-291.08945

m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
286.08969	609072.2	100.00	286.08854	4.03	$C_{13}H_{14}O_4NF_2$

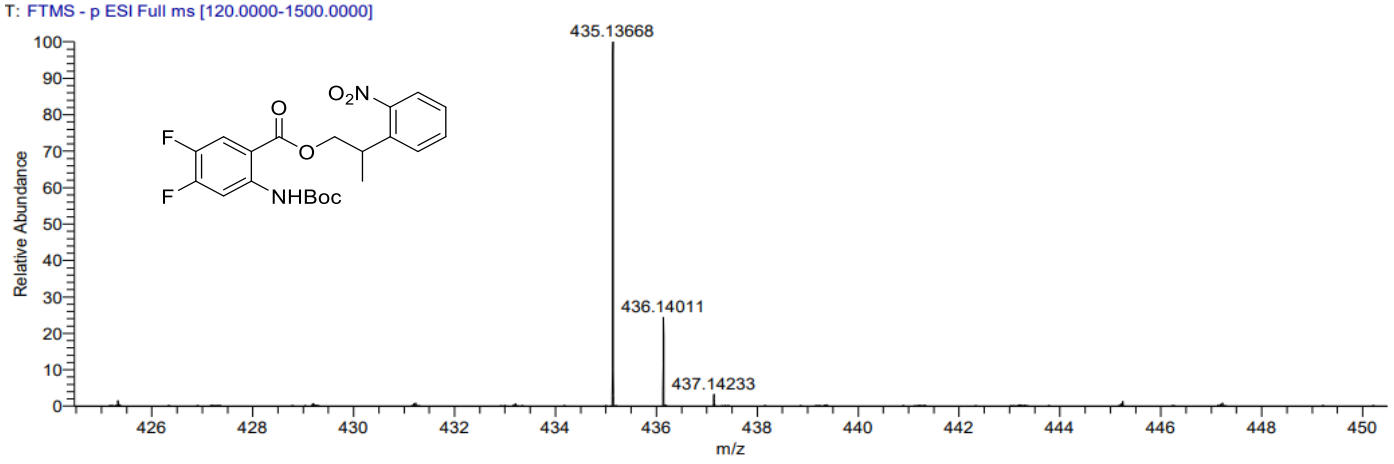
86473ESIPN1 #61-67 RT: 0.98-1.04 AV: 3 NL: 2.58E9
T: FTMS - p ESI Full ms [120.0000-1500.0000]



86473ESIPN1#62-66 RT: 0.98-1.04 AV: 3
T: FTMS - p ESI Full ms [120.0000-1500.0000]
m/z= 259.78689-285.06400

m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
272.07423	2664045568.0	100.00	272.07289	1.34	$C_{12}H_{12}O_4NF_2$

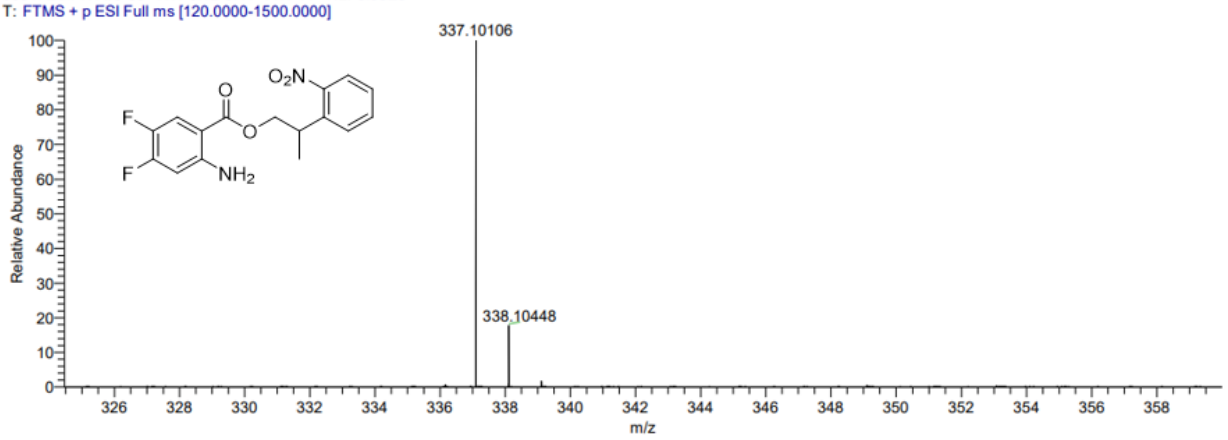
90940ESIPN1 #7-28 RT: 0.11-0.39 AV: 11 NL: 1.57E7
T: FTMS - p ESI Full ms [120.0000-1500.0000]



90940ESIPN1#8-30 RT: 0.11-0.42 AV: 12
T: FTMS - p ESI Full ms [120.0000-1500.0000]
m/z= 424.48039-450.48947

m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
435.13668	15050197.0	100.00	435.13622	1.06	$C_{21}H_{21}O_6N_2F_2$

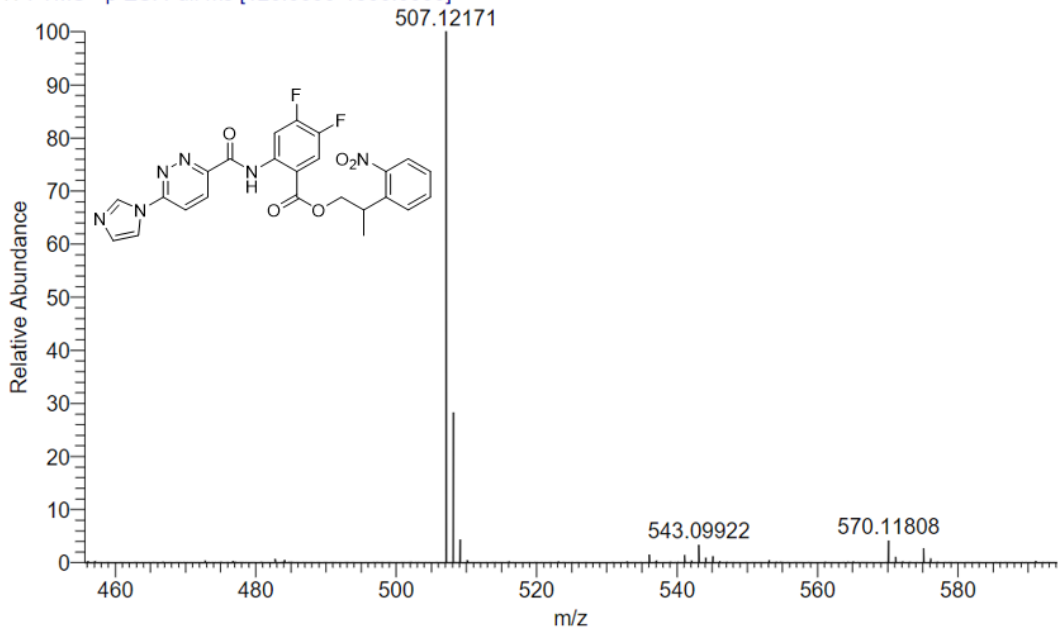
90947ESIPN1 #8-30 RT: 0.12-0.41 AV: 11 NL: 3.98E8
T: FTMS + p ESI Full ms [120.0000-1500.0000]



90947ESIPN1#9-29 RT: 0.12-0.41 AV: 11
T: FTMS + p ESI Full ms [120.0000-1500.0000]
m/z= 324.46297-359.99630

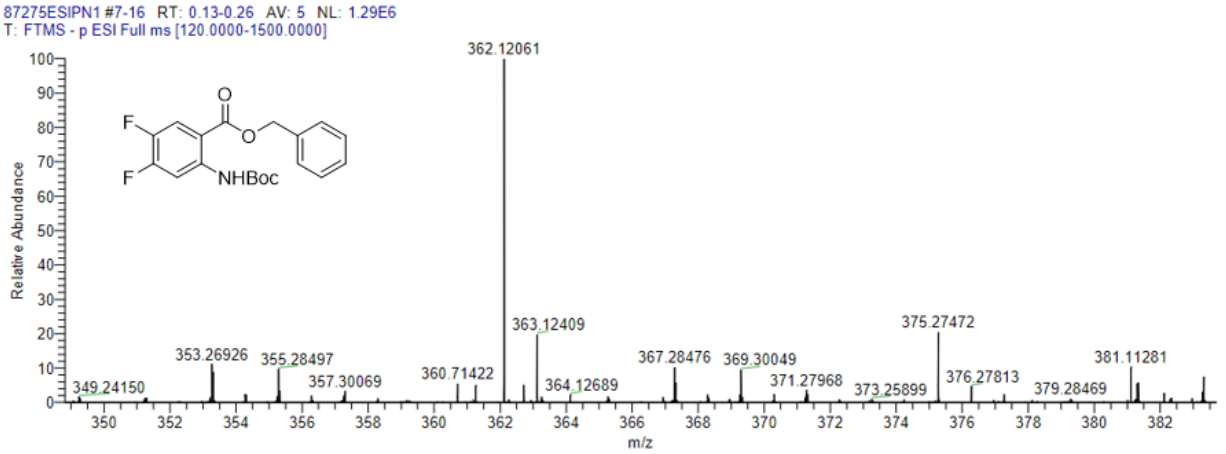
m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
337.10106	404110912.0	100.00	337.09944	4.81	$C_{16}H_{15}O_4N_2F_2$

86669ESIPN1 #6-16 RT: 0.10-0.26 AV: 6 NL: 6.45E7
T: FTMS - p ESI Full ms [120.0000-1500.0000]



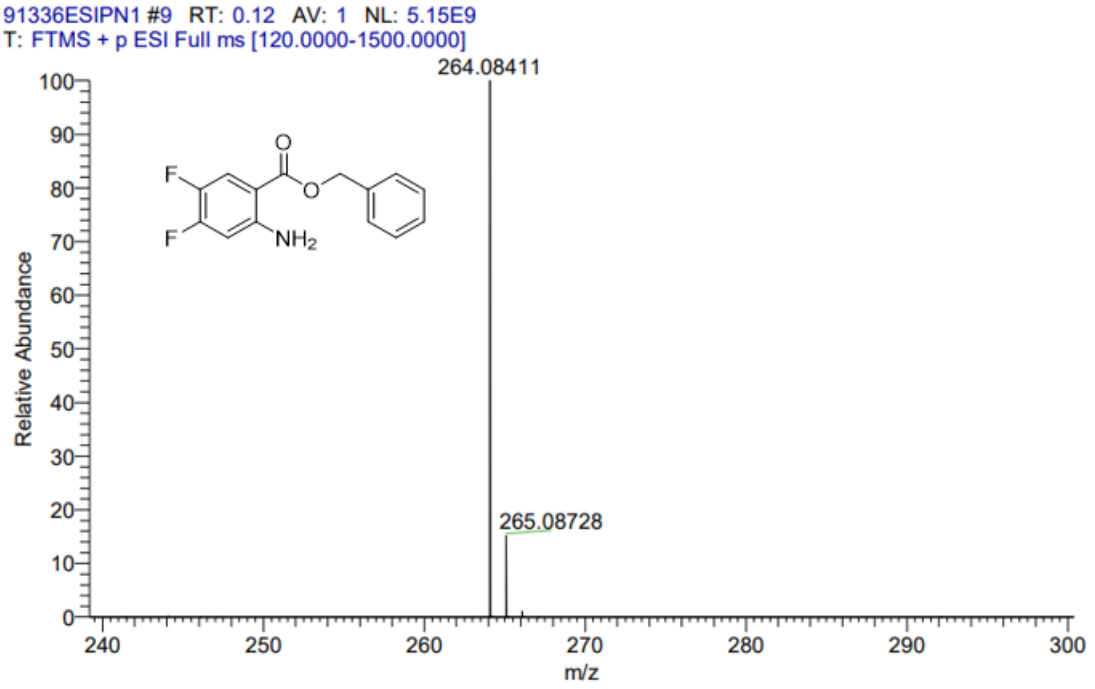
86669ESIPN1#6-16 RT: 0.10-0.26 AV: 6
T: FTMS - p ESI Full ms [120.0000-1500.0000]
m/z= 455.63298-594.06872

m/z	Intensity	Relative	Delta (ppm)	Composition
507.12171	65419900.0	100.00	-0.59	$C_{24}H_{17}O_5N_6F_2$



87275ESIPN1#8-16 RT: 0.13-0.26 AV: 5
T: FTMS - p ESI Full ms [120.0000-1500.0000]
m/z= 348.80942-383.69093

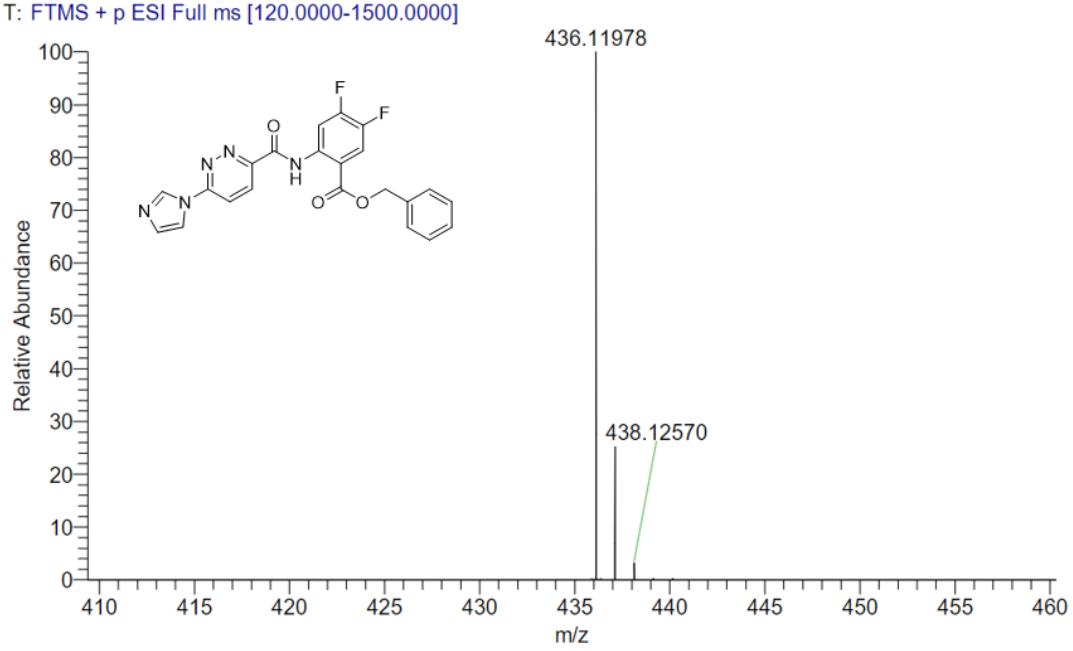
m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
362.12061	1287250.3	100.00	362.11984	2.11	$C_{19}H_{18}O_4NF_2$



91336ESIPN1#9 RT: 0.12
T: FTMS + p ESI Full ms [120.0000-1500.0000]
m/z= 239.18567-300.37681

m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
264.08411	5196764672.0	100.00	264.08306	3.96	$C_{14}H_{12}O_2NF_2$

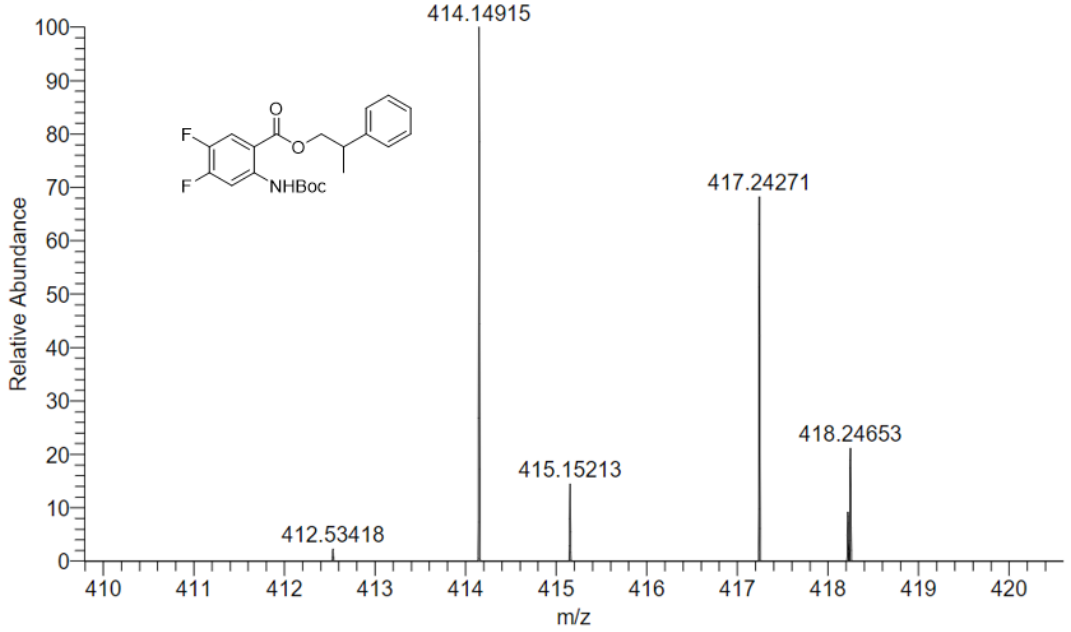
87300ESIPN1 #8-20 RT: 0.14-0.29 AV: 6 NL: 4.06E9
T: FTMS + p ESI Full ms [120.0000-1500.0000]



87300ESIPN1#9-19 RT: 0.14-0.29 AV: 6
T: FTMS + p ESI Full ms [120.0000-1500.0000]
m/z= 409.38423-460.29459

m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
436.11978	4115808256.0	100.00	436.12157	-1.80	$C_{22}H_{16}O_3N_5F_2$

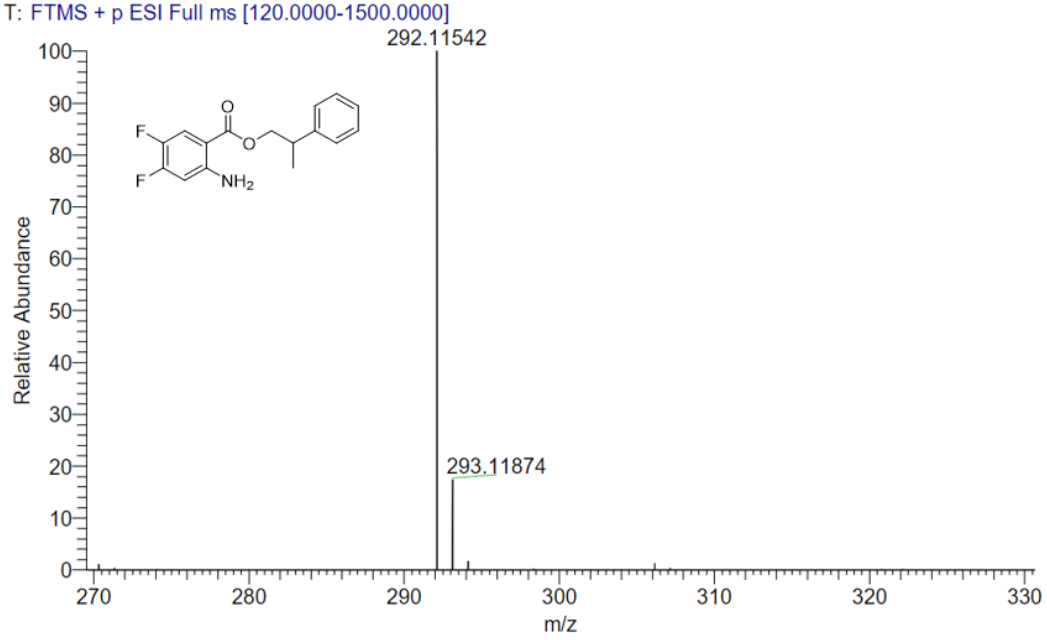
88591ESIPN2 #6-13 RT: 0.09-0.18 AV: 4 SB: 37 1.87-2.84 , 0.01-0.05 NL: 1.21E6
T: FTMS + p ESI Full ms [120.0000-1500.0000]



88591ESIPN2#7-13 RT: 0.09-0.18 AV: 4
SB: 37 1.87-2.84 , 0.01-0.05
T: FTMS + p ESI Full ms [120.0000-1500.0000]
m/z= 409.79242-420.59341

m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
414.14915	1214381.9	100.00	414.14874	1.00	$C_{21}H_{23}O_4NF_2Na$

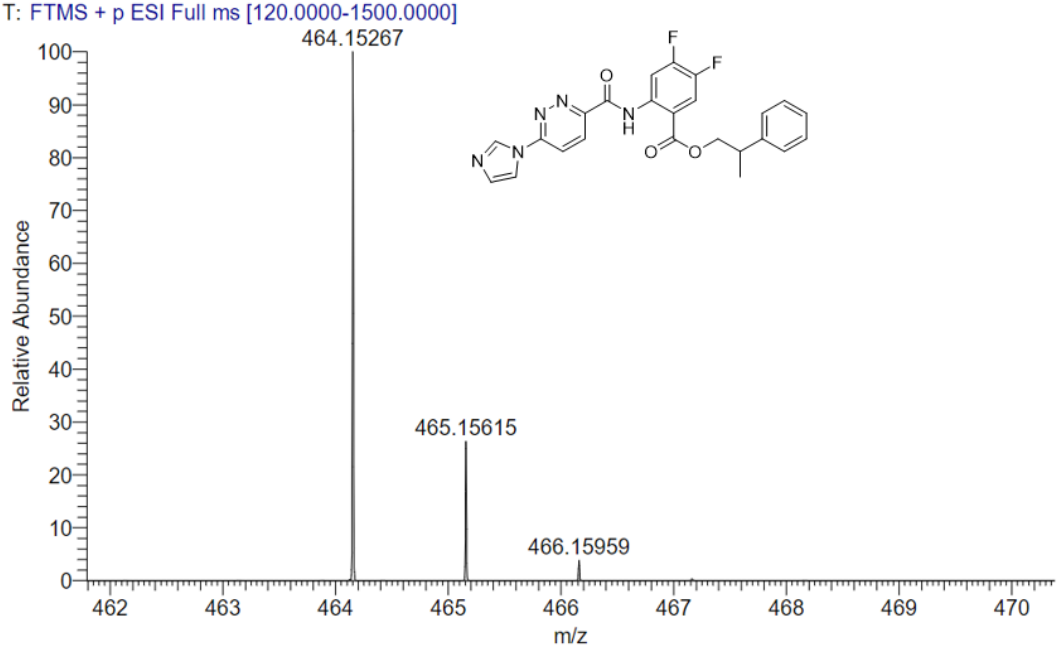
88617ESIPN1 #47 RT: 0.66 AV: 1 NL: 9.98E8
T: FTMS + p ESI Full ms [120.0000-1500.0000]



88617ESIPN1#47 RT: 0.66
T: FTMS + p ESI Full ms [120.0000-1500.0000]
m/z= 269.52032-330.62284

m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
292.11542	997874688.0	100.00	292.11436	3.61	$C_{16}H_{16}O_2NF_2$

88629ESIPN1 #8-13 RT: 0.12-0.18 AV: 3 NL: 9.34E9
T: FTMS + p ESI Full ms [120.0000-1500.0000]



88629ESIPN1#9-13 RT: 0.12-0.18 AV: 3
T: FTMS + p ESI Full ms [120.0000-1500.0000]
m/z= 460.82443-475.62619

m/z	Intensity	Relative	Theo. Mass	Delta (ppm)	Composition
464.15267	9360018432.0	100.00	464.15287	-0.43	$C_{24}H_{20}O_3N_5F_2$

References

1 Chin, E. N. *et al.* Antitumor activity of a systemic STING-activating non-nucleotide cGAMP mimetic. *Science* **369**, 993-999 (2020).