Removal of Metabolic Liabilities Enables Development of Derivatives of Procaspase-Activating Compound 1 (PAC-1) with Improved Pharmacokinetics

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Supporting Information

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Biological Evaluation

Materials and Methods

Materials All reagents were obtained from Fisher unless otherwise indicated. All buffers were made with MilliQ purified water. Annexin V Binding Buffer contains 10 mM HEPES (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$. Bifunctional cell lysis/caspase activity buffer contains 200 mM HEPES (pH 7.4), 400 mM NaCl, 40 mM DTT, 0.4 mM EDTA disodium salt dihydrate, 1% TritonX, and 20 μM Ac-DEVD-AFC (Enzo Life Sciences).

Liver Microsome Stability Assay A mixture of 0.1 M potassium phosphate buffer pH 7.4, $NADP^+$ (final concentration 1.3 mM), $MgCl_2$ (final concentration 3.3 mM), glucose-6-phosphate (final concentration 3.3 mM), glucose-6-phosphate dehydrogenase (final concentration 0.4 U/mL), and a 10 mM solution of compound in DMSO (final concentration 10 μM; 0.1% DMSO) was incubated at 37°C in a shaking incubator for 5 min. The reactions were initiated by the addition of ice-cold liver microsomes (final protein concentration 1 mg/mL), to bring the total volume to 1 mL. A 450 μL aliquot was immediately removed, quenched with 450 μL of a 10 μM solution of **B-PAC-1**[3, 4] (internal standard for LC/MS; compound **3**{*18,7*} in ref.[3]) in MeCN, mixed by inversion, and centrifuged at 10,000 x *g* for 3 min. 750 μL of the supernatant was removed for LC/MS analysis. The reactions were incubated at 37°C in a shaking incubator for 3 h. A second 450 μL aliquot was removed, quenched with 450 μL of a 10 μM solution of **B-PAC-1** in MeCN, mixed by inversion, and centrifuged at 10,000 x *g* for 3 min. 750 μL of the supernatant was removed for LC/MS analysis. Samples from the liver microsome assay were analyzed by LC/MS using an Agilent 1200 HPLC with DAD (monitoring at 280 nm) and an Agilent 6230 TOF MS, with an Agilent C18 column, 3.0 x 50 mm. Mobile phase A was 0.1% HCO_2H in H_2O, B was 0.1% HCO_2H in MeCN. A gradient was run from 5-50% B over 18 min, then 50-95% B for 2 min, then constant 95% B for 4 min, then 95-5% B for 1 min, then constant 5% B for 2 min. The ratio of the areas of analyte:internal standard at 3 hours was compared to the ratio at 0 hours to determine the percent compound remaining after 3 hours.

Cell Culture U-937, Jurkat, and EL4 cells were obtained from the American Type Culture Collection. GL-1 cells were provided by Dr. Steve Suter (North Carolina State University, Raleigh, NC). All cultures were maintained at low passage number in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and grown at 37°C and 5% CO_2.

72hr IC_{50} Cell Death Assay To each well of a 96-well plate was added 49 μL of RPMI 1640 complete growth media. To each well was added 1 μL of compound stock solutions in DMSO at nine concentrations such that the cells were treated with half-log concentrations between 0.01 μM and 100 μM compound. 50 μL of a suspension of cells at 300,000 cells/mL (for U-937, EL4, GL-1, and OSW cells) or 500,000 cells/mL (for Jurkat

cells) were plated into the wells, for a final density of 15,000 or 25,000 cells per well, respectively. Each concentration was tested in triplicate per plate. In each plate 3 wells received 1 μL of a positive death control and 3 wells received 1 μL DMSO as a negative control. The plates were then incubated at 37°C with 5% CO_2 for 72 hours. After the 72 hour incubation period, the plates were analyzed using a Sulforhodamine B assay.[5] Specifically, to each well of the plate 25 μL of a 50% (w/v) solution of trichloroacetic acid in H_2O was added and the plates were incubated for 4 hours at 4°C. The plates were then washed gently with H_2O five times. The plates were allowed to air dry after which 100 μL of a 0.057% (w/v) Sulforhodamine B in a 1% (v/v) acetic acid solution was added to each well for 30 minutes at room temperature. The plates were gently washed 5 times with 1% (v/v) acetic acid and air dried. 200 μL of 10 mM Tris base (pH 10.4) was added to each well and the plates were placed on a shaker for thirty minutes. For U-937 cells, the level of SRB was quantified fluorometrically (ex. 488 nm, em. 585 nm) on a Gemini EM Microplate Reader (Molecular Devices) plate reader. For all other cell lines, the level of SRB was quantified by absorbance at 510 nm on a SpectraMax Plus 384 Microplate Reader (Molecular Devices). The percent cell death was calculated and normalized to the positive control (100% cell death) and the negative control (0% cell death). The percent cell death was averaged for each compound concentration and plotted as a function of compound concentration. The data were fit to a logistical dose response curve using TableCurve 2D and the IC_{50} value was calculated. The experiment was repeated three times and the average of the calculated IC_{50} values was reported. The standard error of the mean (s.e.m.) was determined and reported for the triplicate experiments.

Induction of Apoptosis by Hit Compounds To each well of a 24-well plate for compound treatment was added 490 μL of RPMI-1640 complete growth media. To each well was then added 10 μL of 5 mM DMSO solutions to achieve a final compound concentration of 50 μM. 10 μL of DMSO was added to one well as a live cell control. 500 μL of a suspension of cells at 1.2 x 10^6 cells/mL were plated into the wells, for a final density of 600,000 cells per well. Wells not containing compounds were filled with 1 mL RPMI-1640 complete growth media, and spaces between wells were filled with 1 mL sterile PBS. The cells were incubated at 37 ºC with 5% CO_2 for 12 hours. The cells were harvested via centrifugation (200 x *g* for 5 min), washed with PBS (2 mL), and resuspended in 450 μL Annexin V Binding Buffer containing 3.5 μL of FITC-conjugated Annexin V stain (Southern Biotech) and 2.25 μL of a 1 mg/mL solution of propidium iodide (Sigma) to a final concentration of 5 μg/mL. Samples were stored on ice until assessment. Cell populations were analyzed on a Becton Dickinson LSR II cell flow cytometer. 10,000 events per sample were recorded.

EGTA Fluorescence Titration Assay This titration assay is based on a published protocol.[6] Buffer (50 mM HEPES, 100 mM KNO_3, 8.1 mM EGTA, pH 7.2) and solutions of compounds (1 mM in DMSO) and $Zn(OTf)_2$ (100 μM-1 M in H_2O) were prepared. The compound solutions were diluted ten-fold with buffer

(final [compound] = 100 μM, final [EGTA] = 7.3 mM), and 198 μL of the resulting solution was added to each well of a 96-well plate. Each of 24 $Zn(OTf)_2$ solutions was added to four wells in each plate. The wells were allowed to equilibrate for 5 minutes, and the plates were analyzed via a Molecular Devices SpectraMax M3 fluorescent plate reader (ex. 410 nm, em. 475 nm). Fluorescence intensity at 475 nm of each of four technical replicates was plotted against free Zn^{2+} concentration ($[Zn^{2+}]_F$), calculated using the MaxChelator program (maxchelator.stanford.edu). The data were analyzed using OriginPro 9.1 and fitted to a formation curve based on Eq S1:[6]

$$I = (I_{min}K_d + I_{max}[Zn^{2+}]_F)/(K_d + [Zn^{2+}]_F) \qquad \text{Eq S1}$$

where I_{min} and I_{max} were defined as the fluorescence intensity of the free probe (**PAC-1** or derivative) and that of the Zn^{2+}-probe complex, respectively.

Caspase Activity in Cell Lysate To each inner well of two 96-well plates was added 100 μL of a suspension of U-937 cells in phenol red-free RPMI-1640 complete growth media at 500,000 cells/mL (50,000 cells/well). Inner wells without compound were filled with 200 μL of media, and outer wells were filled with 200 μL of sterile PBS. The plates were incubated at 37°C with 5% CO_2 for 15 hours. Solutions of compounds (**PAC-1**, **S-PAC-1**, **7**, **30**, **32**, **41**, **PAC-1a**, staurosporine, or DMSO alone) in media were prepared at 3x final concentrations (3% DMSO), and 50 μL of each solution was added to the appropriate wells. Each compound was tested in six wells per plate. In the first plate, 50 μL of bifunctional cell lysis/caspase activity buffer was added to each treatment well. Fluorescence (ex. 400 nm, em. 505 nm) was monitored on an Analyst AD 96-384 plate reader via a 60-minute kinetic read, and the slope was used to determine caspase activity. The second plate was incubated at 37°C with 5% CO_2 for 16 hours, at which time 50 μL of bifunctional cell lysis/caspase activity buffer was added to each treatment well, and fluorescence was monitored as above. The slopes at 16 hours were normalized to the slopes of each compound at 0 hours (0% activity) and the staurosporine-treated samples at 16 hours (100% activity) to give percent caspase activity.

Evaluation of Compound Tolerability in vivo. All experimental procedures were reviewed and approved by the University of Illinois Institutional Animal Care and Use Committee. 8-10 week old C57BL/6 mice were used in all experiments (Charles River). Mice (n = 3/cohort) were evaluated for their ability to tolerate a single 200 mg/kg intraperitoneal dosage of compounds, formulated at 5 mg/mL in 200 mg/mL hydroxypropyl-β-cyclodextrin (HPβCD) at pH 5.5. Mice were treated and observed for clinical signs over 24 hours; specifically, they were observed continuously for the first hour, then at hours 2, 4, 6, 8, 12 and 24 hours post-treatment. Mice were further allowed 1 week to demonstrate delayed effects of treatment. Toxicity was classified as inducing either an ‘excitatory’ or a ‘depressive’ phenotype. The extent of response was graded from mild to severe.

*Pharmacokinetics of **PAC-1** and Derivatives in Mice.* Compounds were formulated at 5 mg/mL in 200 mg/mL HPβCD at pH 5.5. C57/BL6 mice (n = 2 per cohort per time point) were treated with a 25 mg/kg dose of **PAC-1** or derivative via tail vein injection or oral gavage. At specified time points, mice were sacrificed and blood was collected, centrifuged, and the EDTA plasma was frozen at -80°C until analysis.

*Assessment of serum concentrations of **PAC-1** and derivatives*. 2 μL of a 10 μg/mL solution of internal standard in 60:40 methanol:water (**PAC-1** was used as an internal standard for analysis of **S-PAC-1**; **S-PAC-1** was used as an internal standard for analysis of all other compounds) was added to a 10 μL aliquot of serum. The proteins were precipitated by the addition of methanol (100 μL). The sample was mixed by vortex and centrifuged to remove the proteins. The resulting supernatant was evaporated to complete dryness with a SpeedVac. The dried solid was then reconstituted in 100 μL 60:40 methanol:water, followed by centrifugation. The supernatant was subject to instrument injection. Samples were analyzed with the 5500 QTRAP LC/MS/MS system (AB Sciex, Foster City, CA) in the Metabolomics Lab of the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. The 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) includes a degasser, an autosampler, and a binary pump. The LC separation was performed on a Phenomenex 4u Polar-RP 80A column (4.6 x 100mm, 4μm, Torrance, CA) with mobile phase A (0.1% formic acid in water) and mobile phase B (methanol). The flow rate was 0.8 mL/min. The linear gradient was as follows: 0-1 min, 0% B; 5 min, 70% B; 7.5-10.5 min, 100% B; 10.6-15 min, 0% B. The autosampler was set at 5°C. The injection volume was 2 μL. Mass spectra were acquired under negative electrospray ionization (ESI) with the ion spray voltage of -4500 V. The source temperature was 600°C. The curtain gas, ion source gas 1, and ion source gas 2 were 35, 50, and 65, respectively. Multiple reaction monitoring (MRM) was used for quantitation: **PAC-1**: m/z 391.1-->m/z 232.0; **S-PAC-1**: m/z 470.2-->m/z 311.1; compound **7**: m/z 423.1-->m/z 264.1; compound **30**: m/z 423.1-->m/z 246.1; compound **32**: m/z 448.1-->m/z 271.1; compound **41**: m/z 450.1-->m/z 271.1.

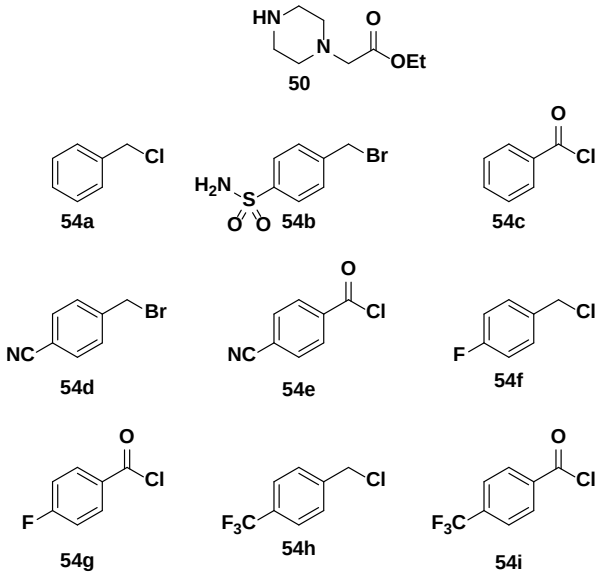
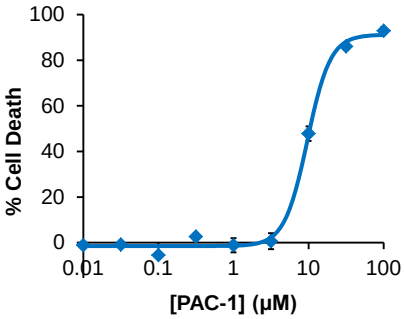
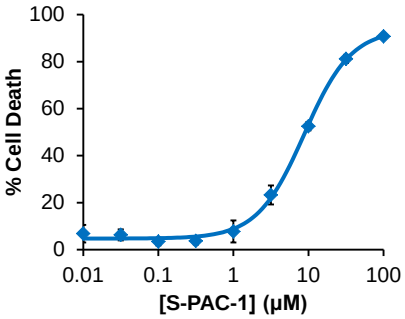


Figure S1. Starting materials used for synthesis of hydrazides.

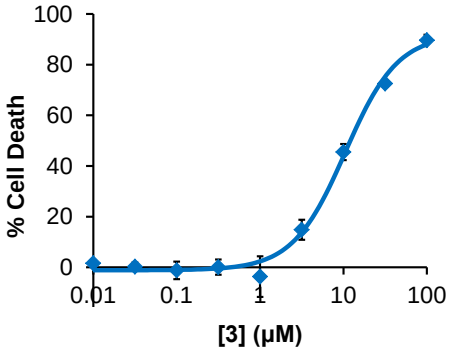
PAC-1: IC_{50} = 10.2 ± 0.3 μM



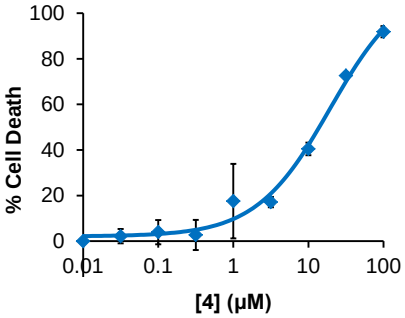
S-PAC-1: IC_{50} = 8.9 ± 0.6 μM



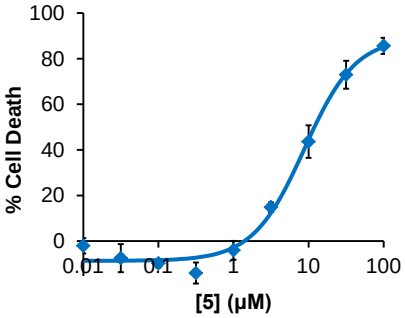
3: IC_{50} = 12.1 ± 1.3 μM



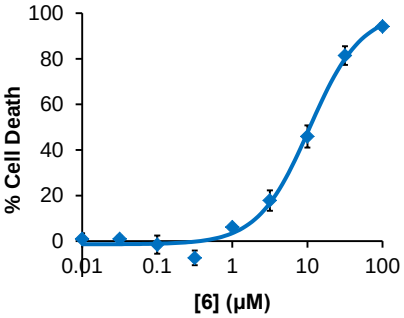
4: IC_{50} = 13.7 ± 0.9 μM



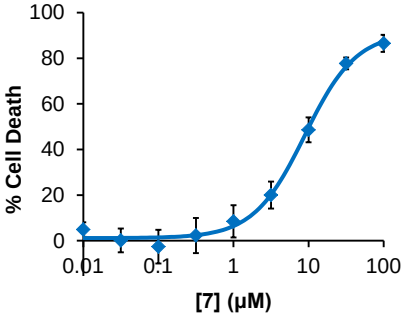
5: IC_{50} = 13.1 ± 3.7 μM



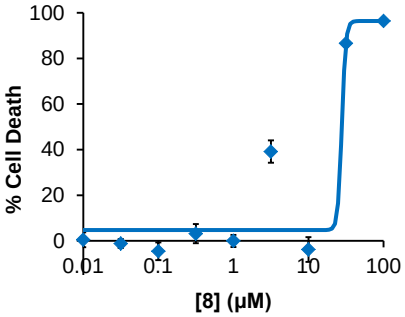
6: IC_{50} = 11.1 ± 2.1 μM



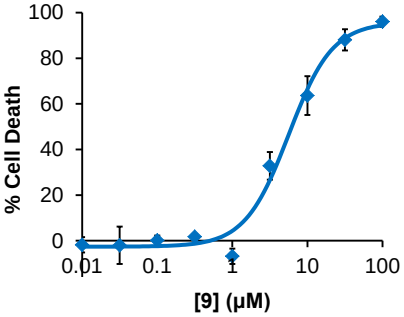
7: IC_{50} = 10.2 ± 1.7 μM



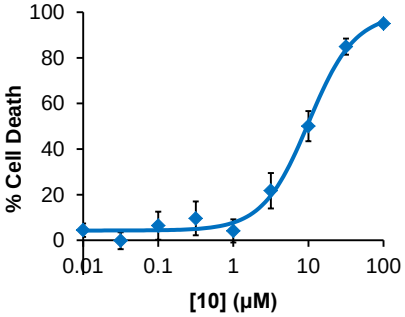
8: IC_{50} = 15.3 ± 6.7 μM



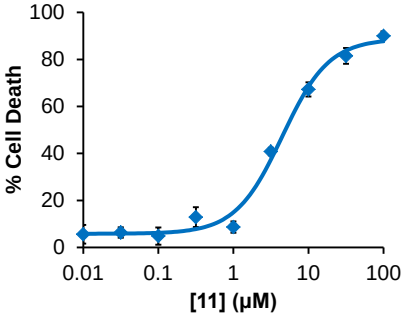
9: IC_{50} = 6.6 ± 1.9 μM



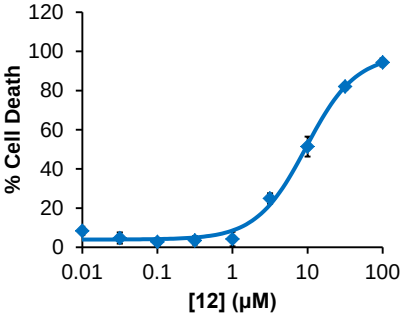
10: IC_{50} = 9.6 ± 2.1 μM



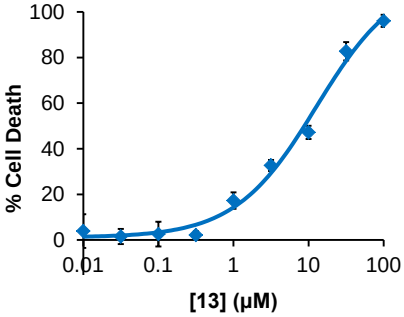
11: IC_{50} = 4.9 ± 0.4 μM



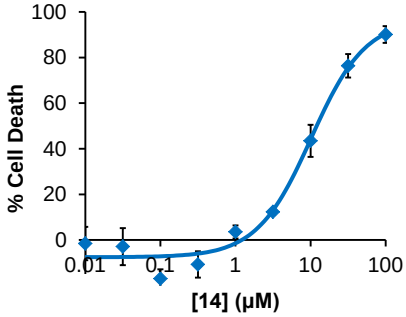
12: IC_{50} = 9.4 ± 1.3 μM



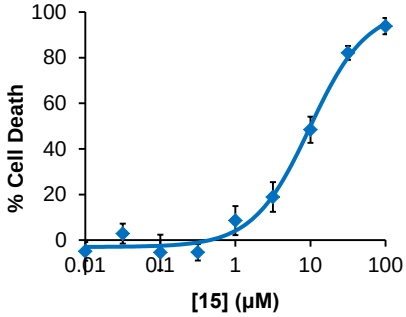
13: IC_{50} = 9.0 ± 1.2 μM



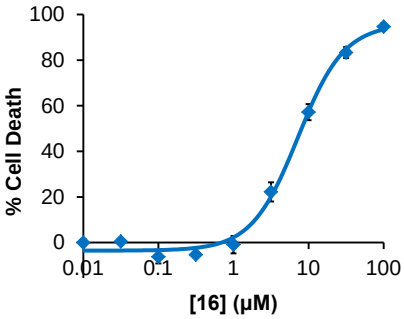
14: IC_{50} = 12.8 ± 2.7 μM



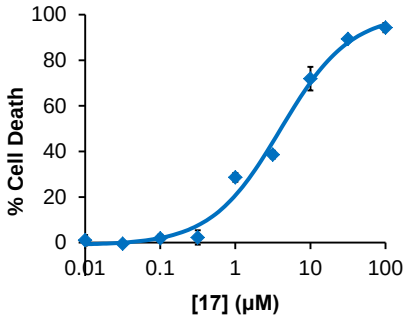
15: IC_{50} = 10.0 ± 1.7 μM



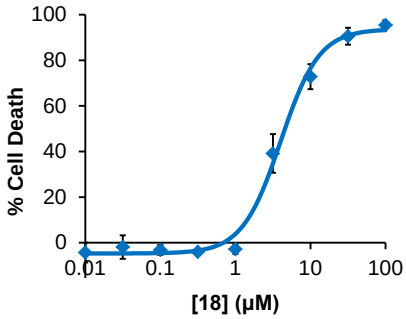
16: IC_{50} = 7.3 ± 0.9 μM



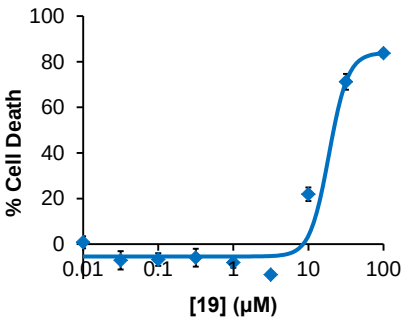
17: IC_{50} = 4.1 ± 0.4 μM



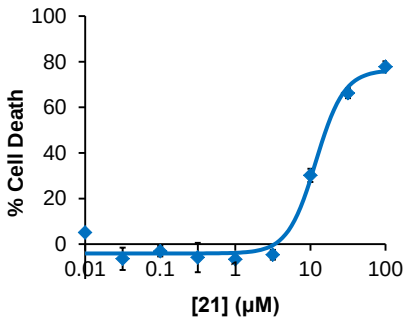
18: IC_{50} = 4.8 ± 1.2 μM



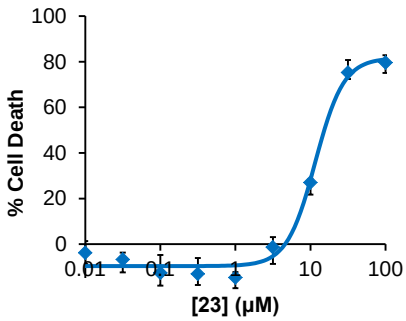
19: IC_{50} = 17.0 ± 1.4 μM



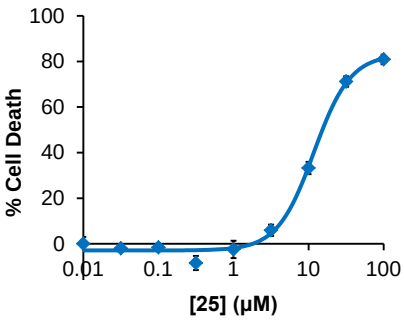
21: IC_{50} = 15.7 ± 2.6 μM



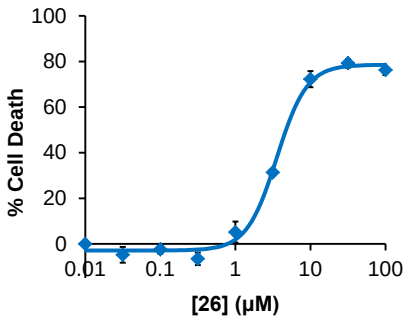
23: IC_{50} = 15.3 ± 1.3 μM



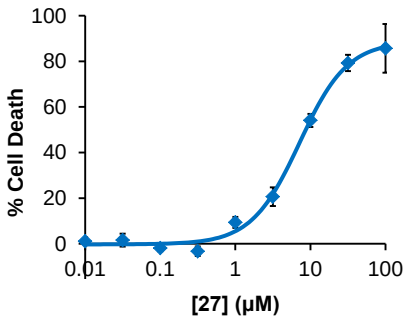
25: IC_{50} = 15.3 ± 0.8 μM



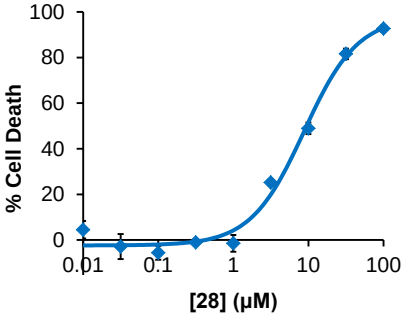
26: IC_{50} = 4.7 ± 0.3 μM



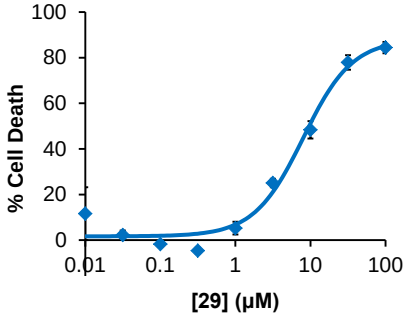
27: IC_{50} = 8.7 ± 0.5 μM



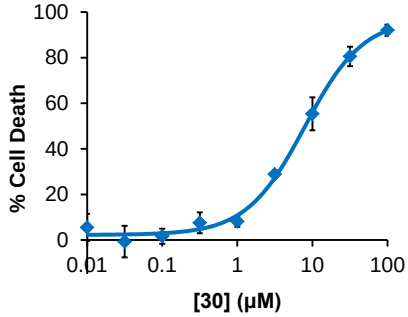
28: IC_{50} = 9.5 ± 0.9 µM



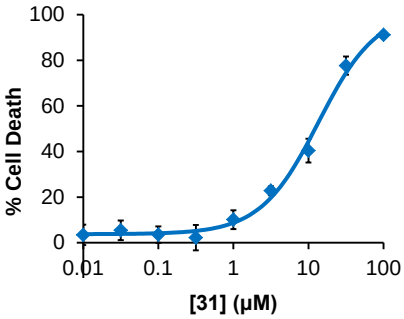
29: IC_{50} = 9.8 ± 1.3 µM



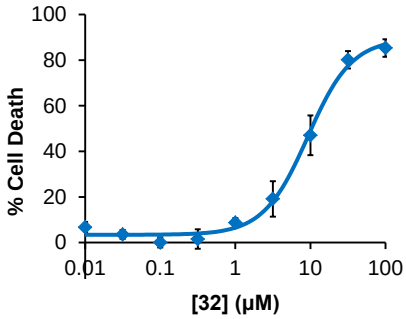
30: IC_{50} = 8.6 ± 2.0 µM



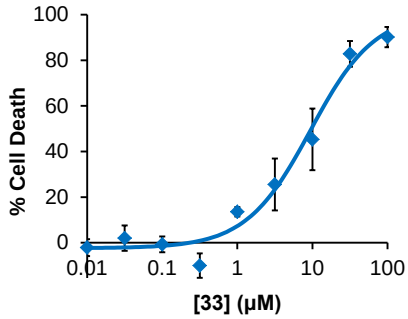
31: IC_{50} = 12.7 ± 2.0 µM



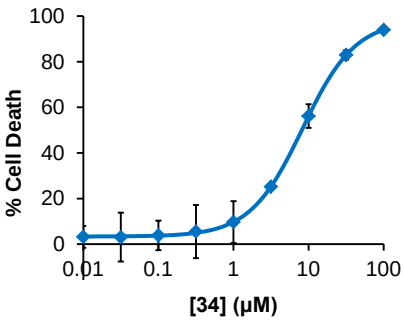
32: IC_{50} = 10.1 ± 2.0 µM



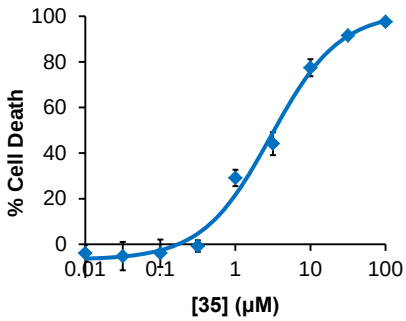
33: IC_{50} = 10.3 ± 4.1 µM



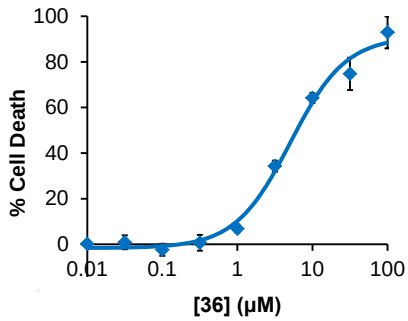
34: IC_{50} = 8.5 ± 1.4 µM



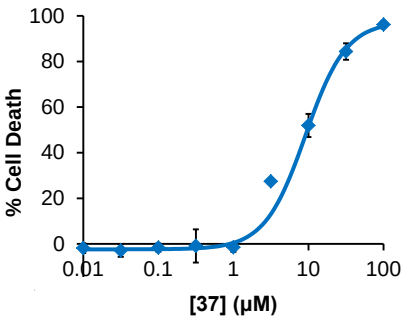
35: IC_{50} = 3.4 ± 0.6 µM



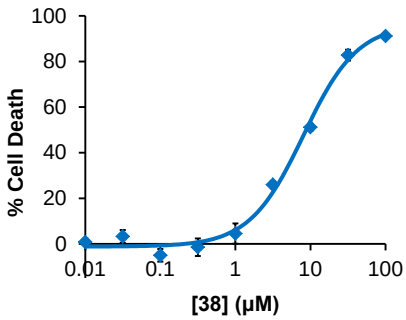
36: IC_{50} = 6.5 ± 0.6 µM



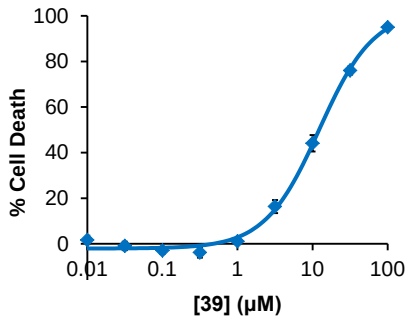
37: IC_{50} = 8.9 ± 1.2 µM



38: IC_{50} = 8.7 ± 0.4 µM



39: IC_{50} = 12.3 ± 1.0 µM



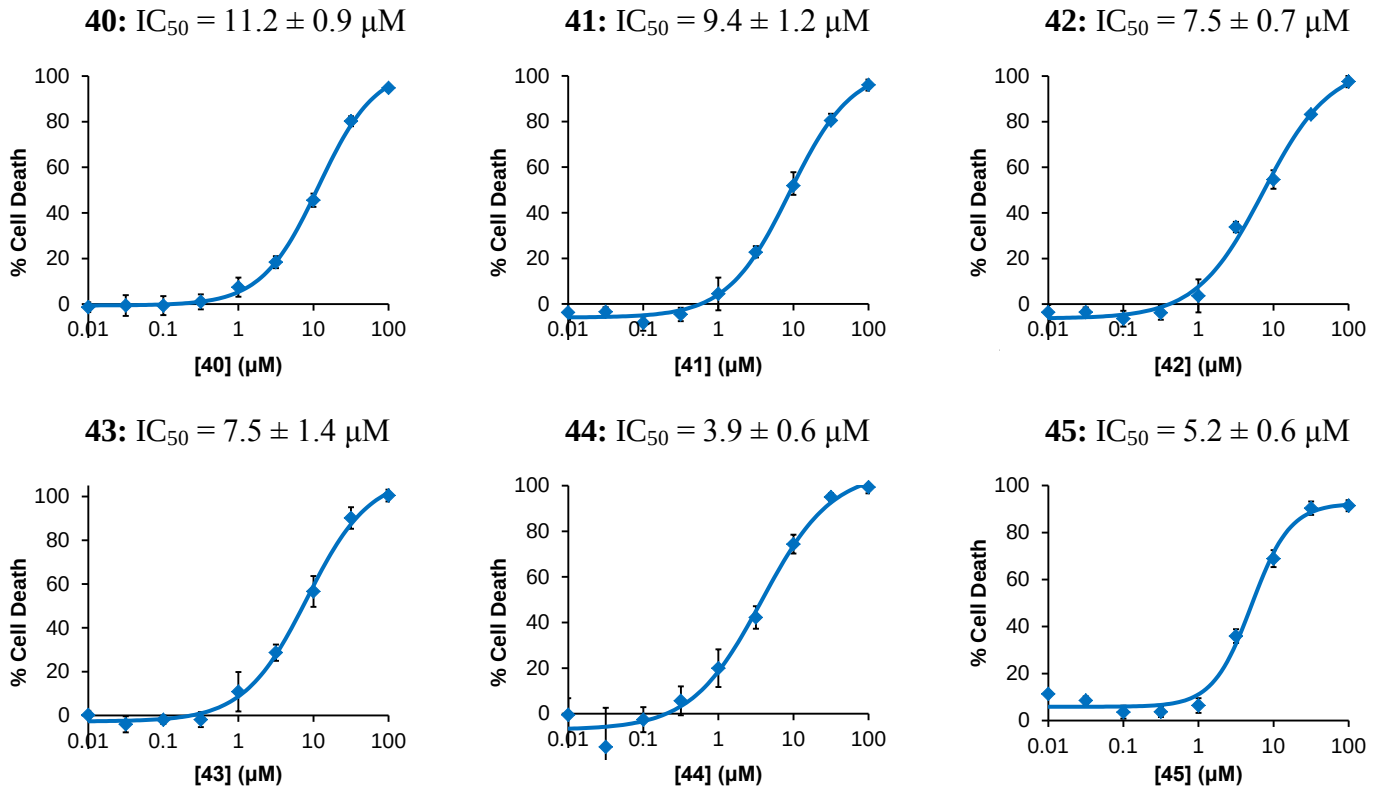
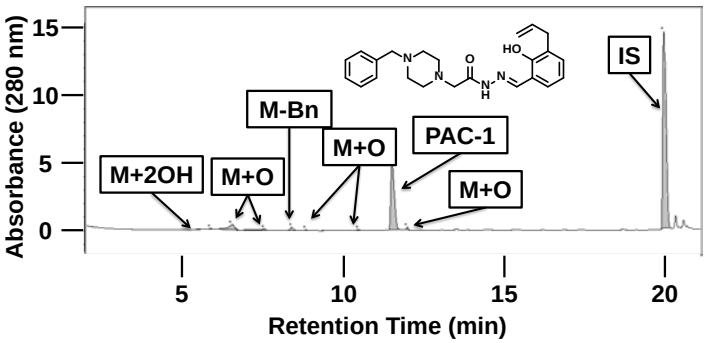


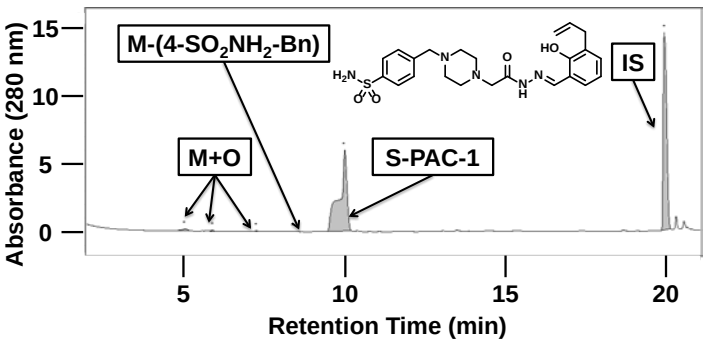
Figure S2. U-937 Cells treated with compounds for 72 hours. Biomass quantified by sulforhodamine B assay.

IC_{50} values shown are mean ± s.e.m. (n = 3).

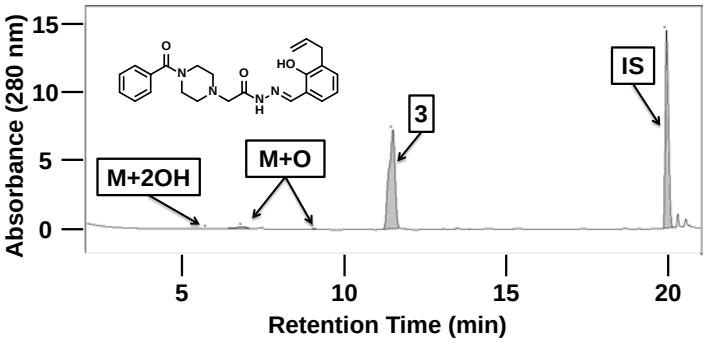
PAC-1: Stability = 38 ± 2%



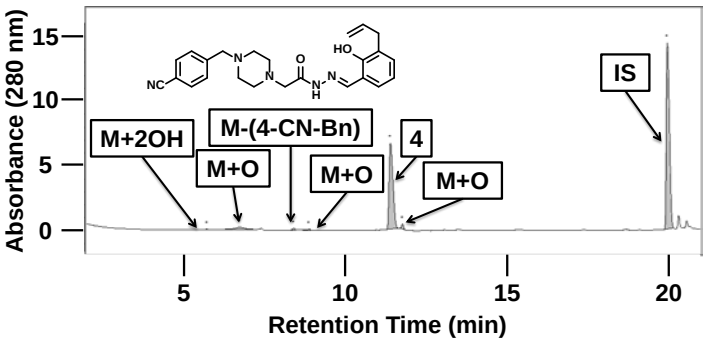
S-PAC-1: Stability = 84 ± 1%



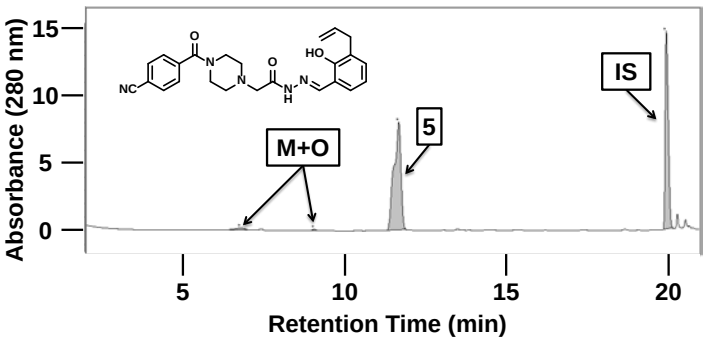
3: Stability = 89 ± 4%



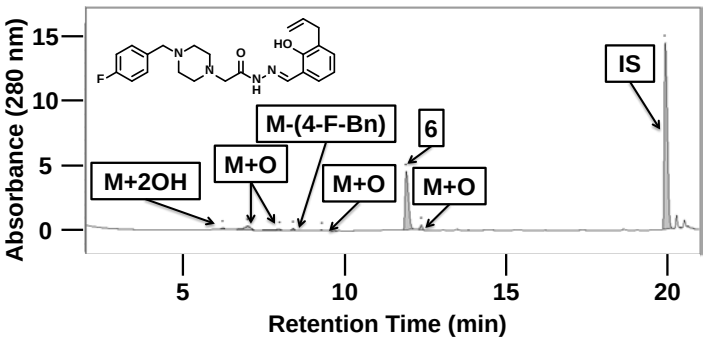
4: Stability = 48 ± 2%



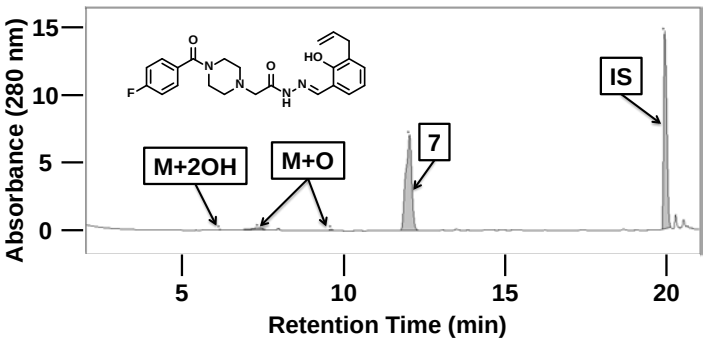
5: Stability = 90 ± 4%



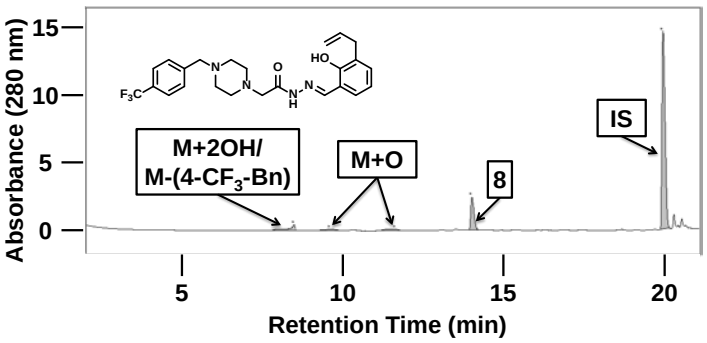
6: Stability = 31 ± 1%



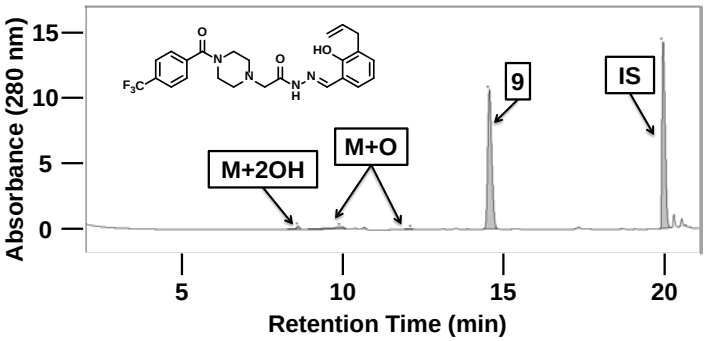
7: Stability = 86 ± 2%



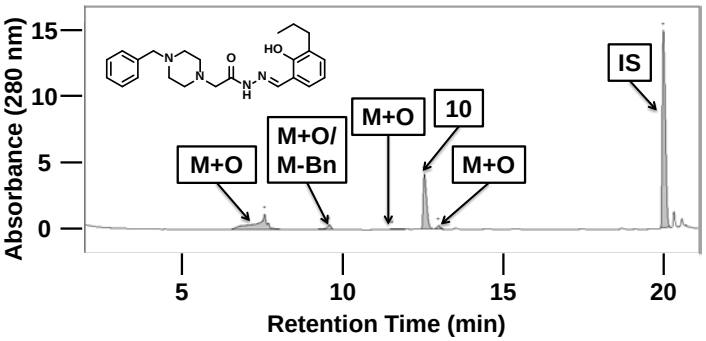
8: Stability = 16 ± 1%



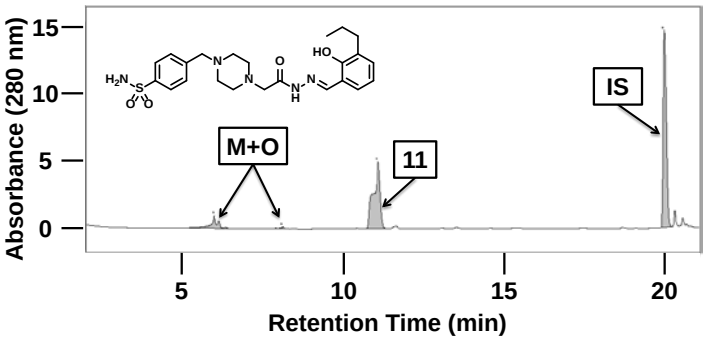
9: Stability = 85 ± 6%



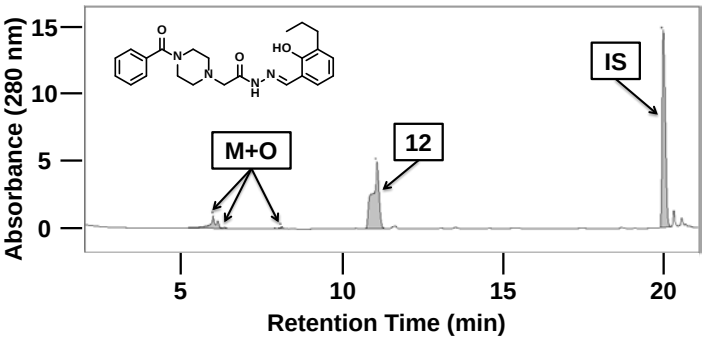
10: Stability = 30 ± 1%



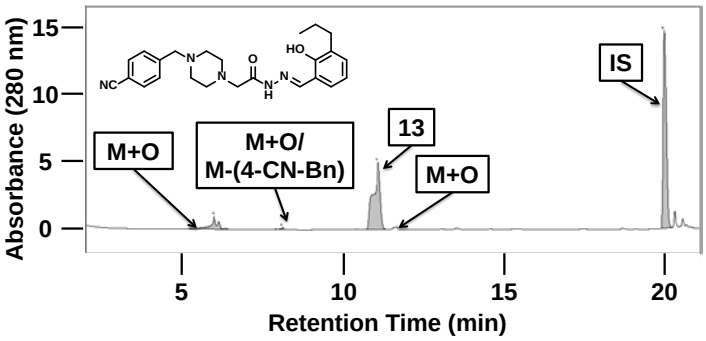
11: Stability = 61 ± 2%



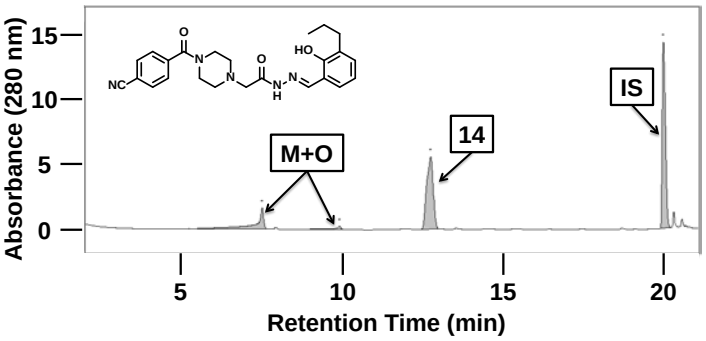
12: Stability = 71 ± 3%



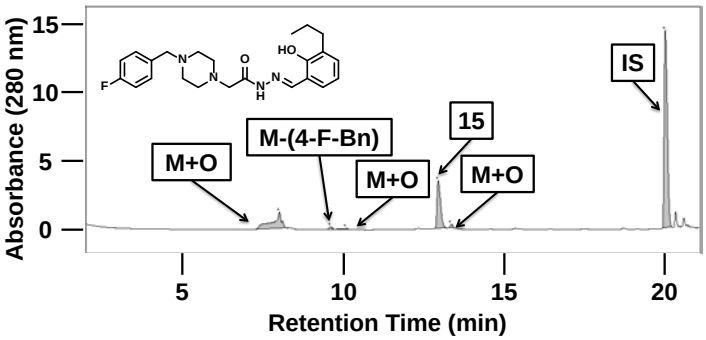
13: Stability = 30 ± 2%



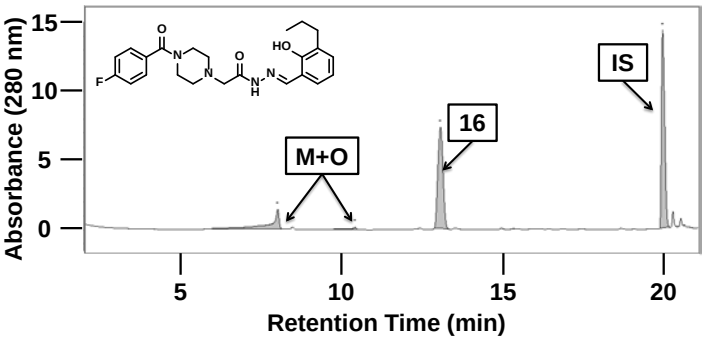
14: Stability = 61 ± 3%



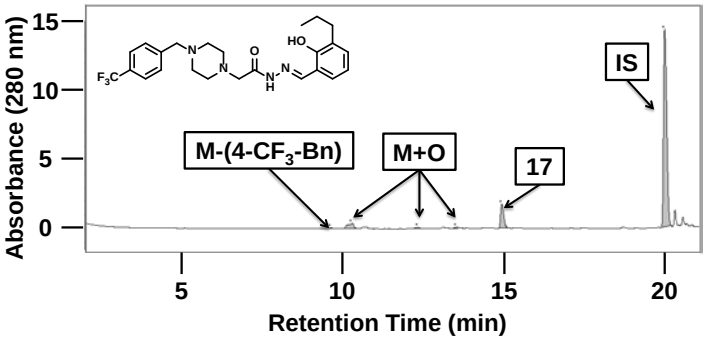
15: Stability = 24 ± 2%



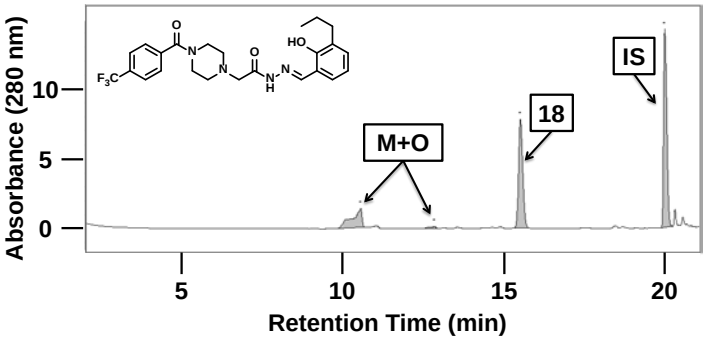
16: Stability = 69 ± 4%



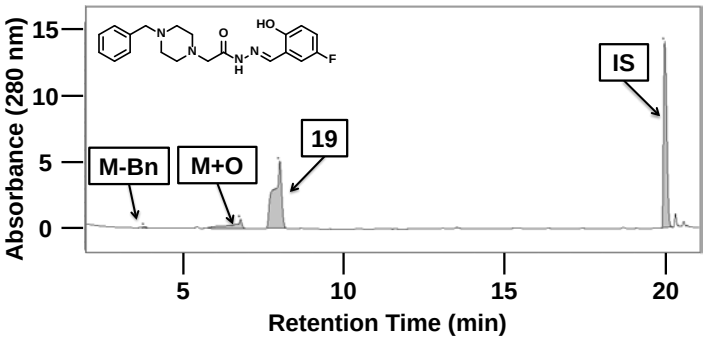
17: Stability = 15 ± 2%



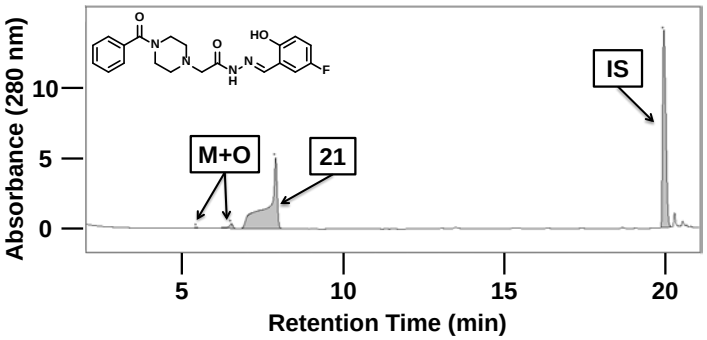
18: Stability = 64 ± 1%



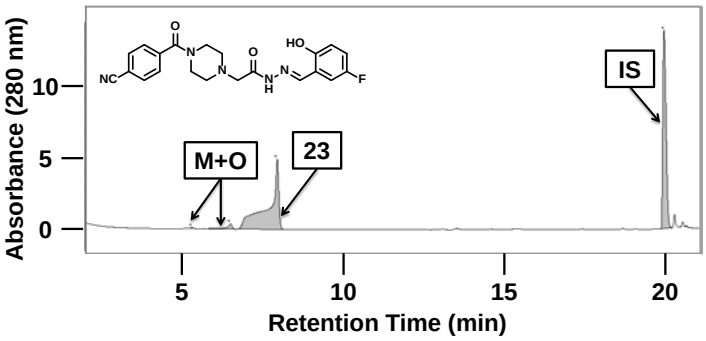
19: Stability = 64 ± 4%



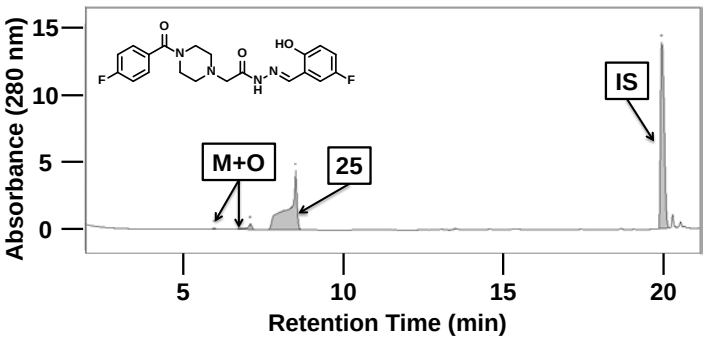
21: Stability = 88 ± 1%



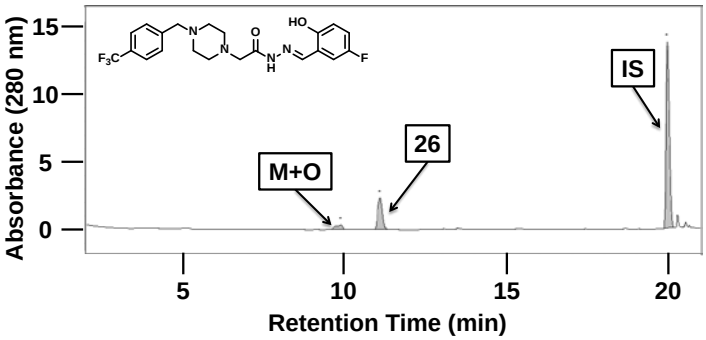
23: Stability = 88 ± 4%



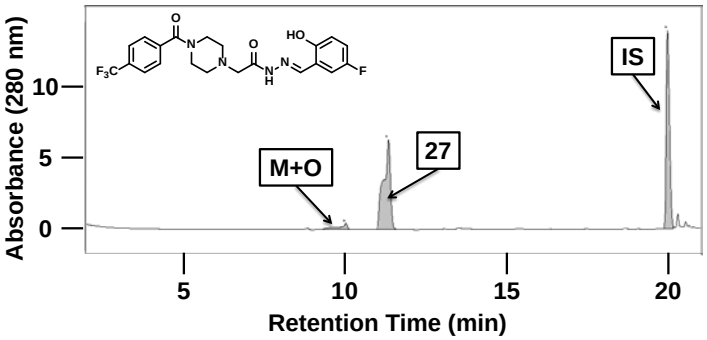
25: Stability = 86 ± 2%



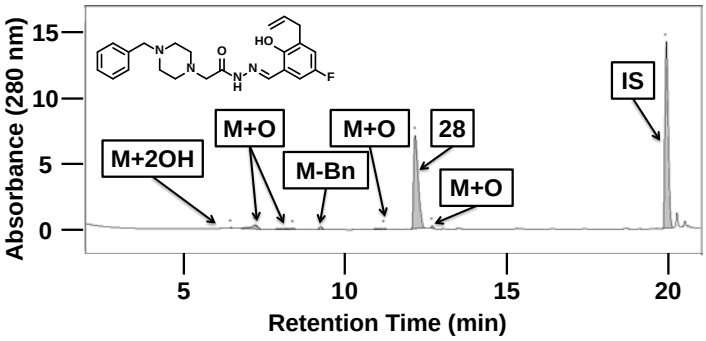
26: Stability = 30 ± 5%



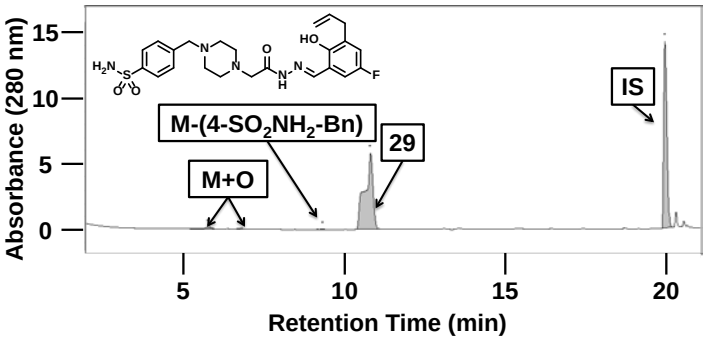
27: Stability = 87 ± 3%



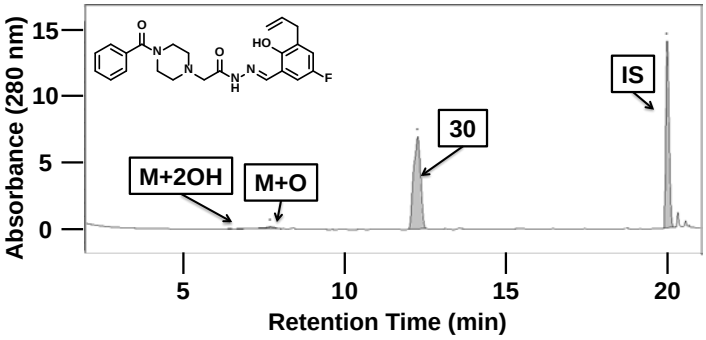
28: Stability = 56 ± 1%



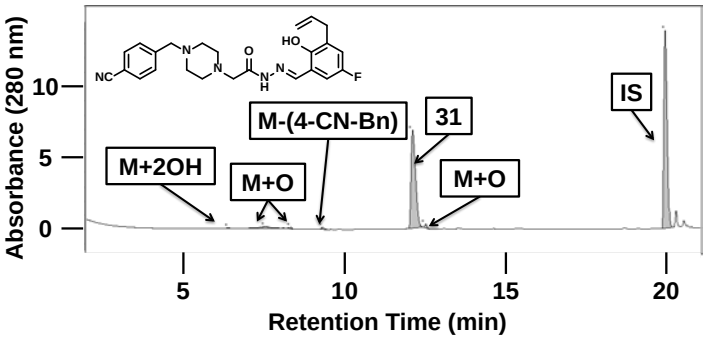
29: Stability = 89 ± 3%



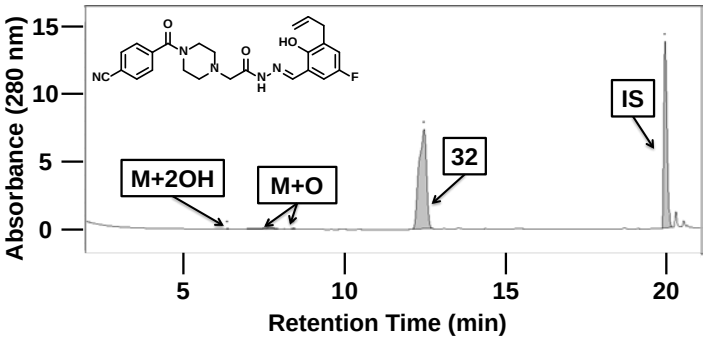
30: Stability = 93 ± 7%



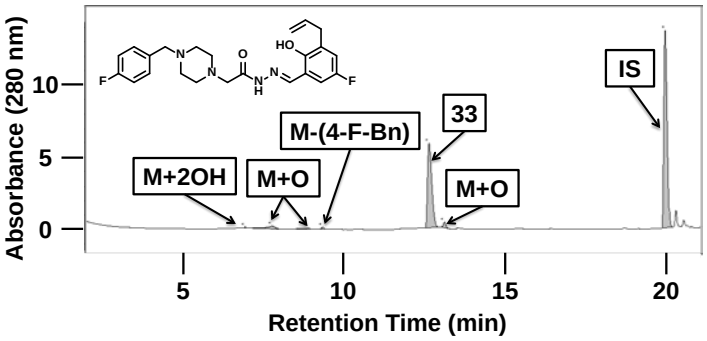
31: Stability = 65 ± 2%



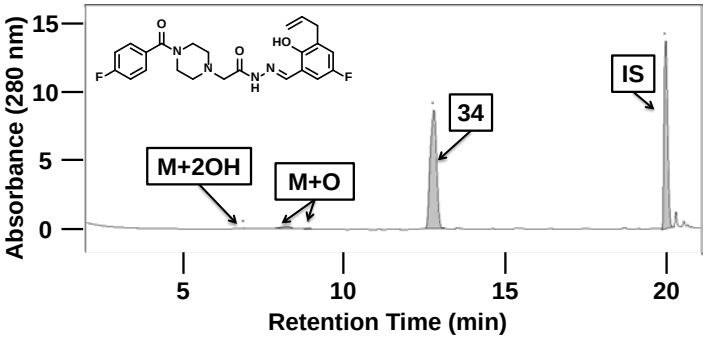
32: Stability = 95 ± 4%



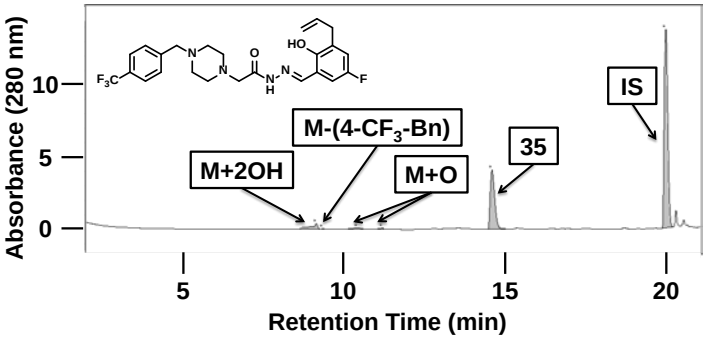
33: Stability = 57 ± 1%



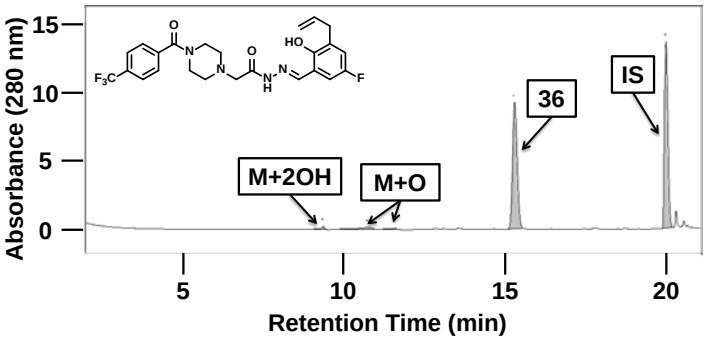
34: Stability = 92 ± 3%



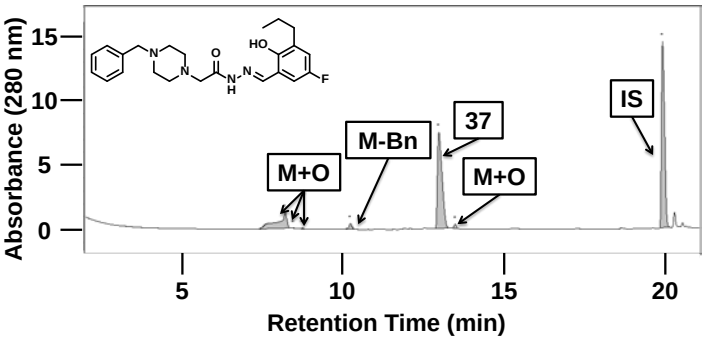
35: Stability = 49 ± 3%



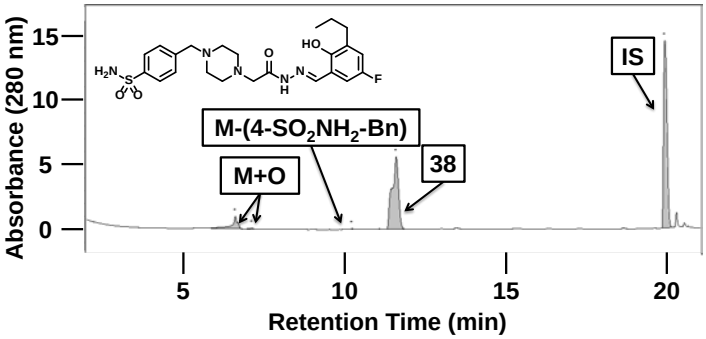
36: Stability = 90 ± 2%



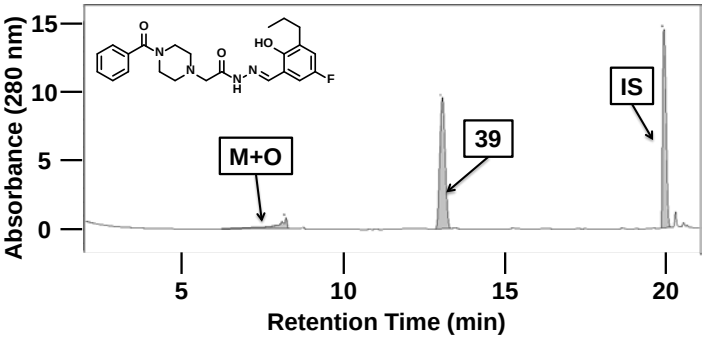
37: Stability = 49 ± 6%



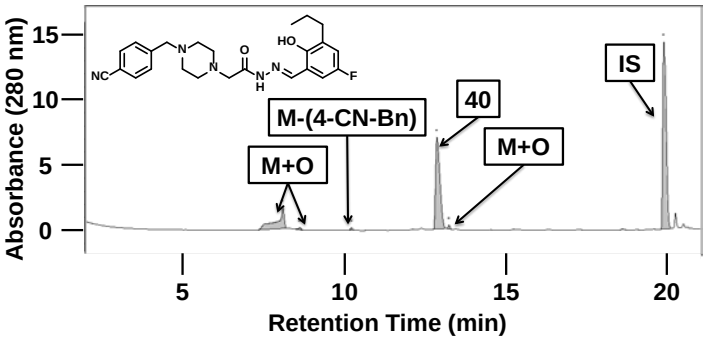
38: Stability = 62 ± 3%



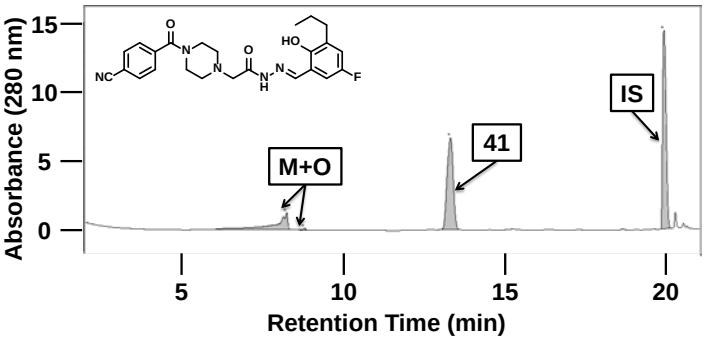
39: Stability = 86 ± 5%



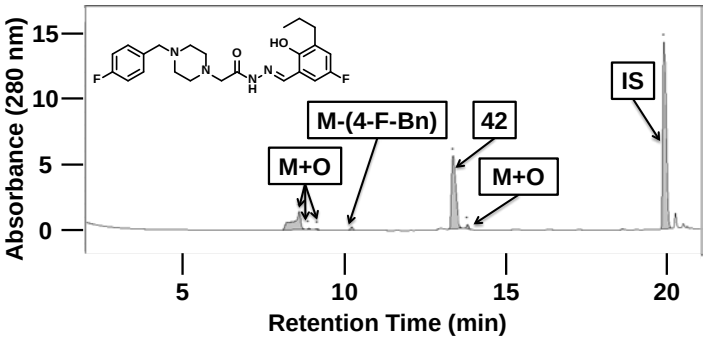
40: Stability = 49 ± 5%



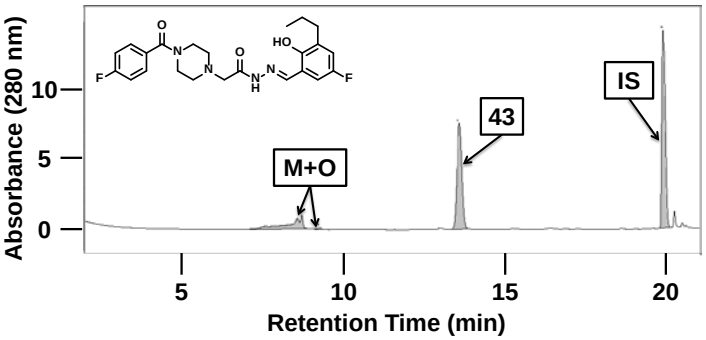
41: Stability = 66 ± 3%



42: Stability = 48 ± 1%



43: Stability = 67 ± 3%



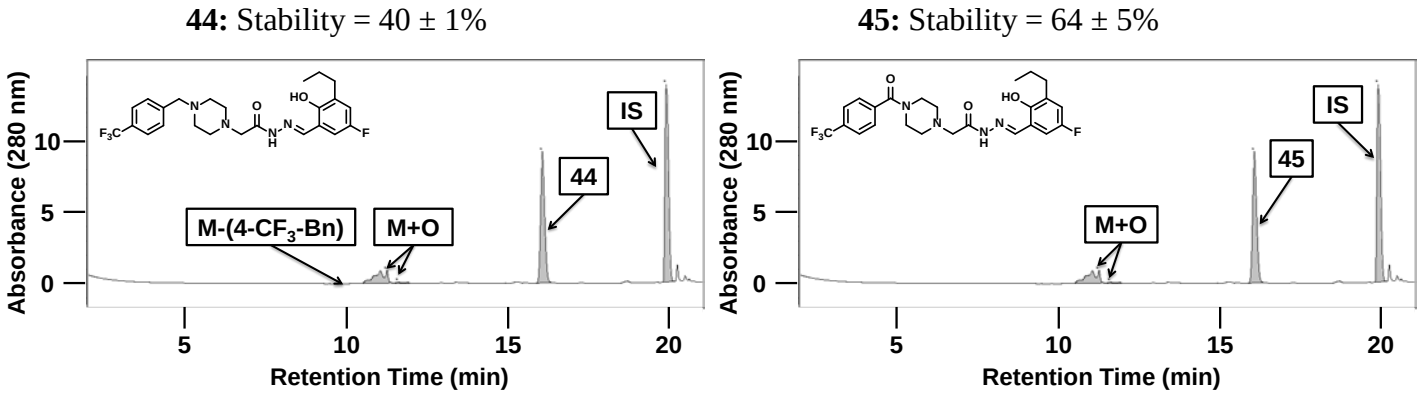
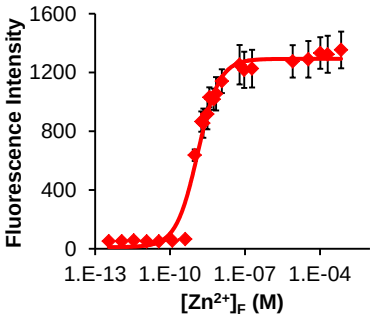
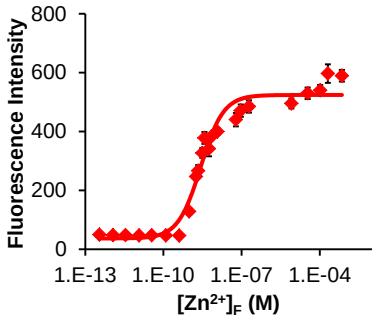


Figure S3. LC/MS results from liver microsome stability experiments of **PAC-1** analogues. IS = internal standard. Results are representative of three independent experiments. Values shown are mean ± s.e.m. (n = 3).

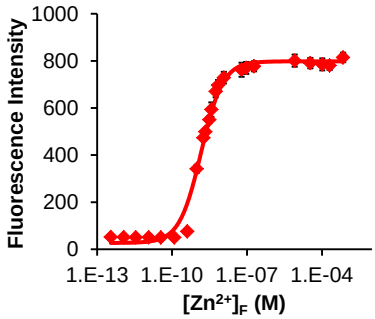
PAC-1: K_d = 1.28 ± 0.03 nM



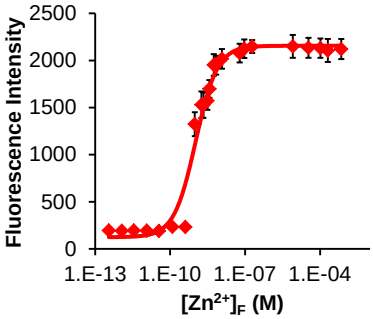
S-PAC-1: K_d = 2.72 ± 0.13 nM



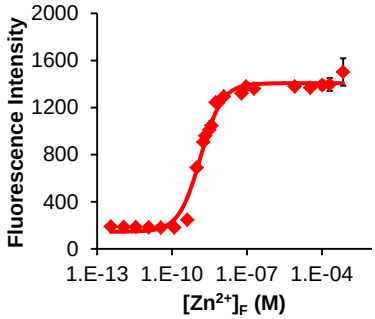
7: K_d = 1.46 ± 0.07 nM



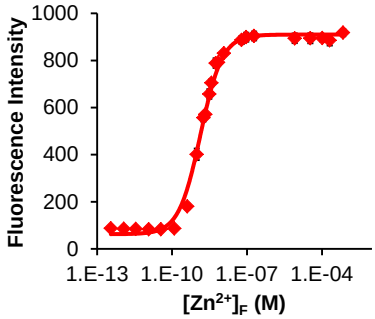
30: K_d = 1.07 ± 0.09 nM



32: K_d = 1.37 ± 0.10 nM



41: K_d = 1.37 ± 0.03 nM



PAC-1a: $K_d > 10^6$ nM

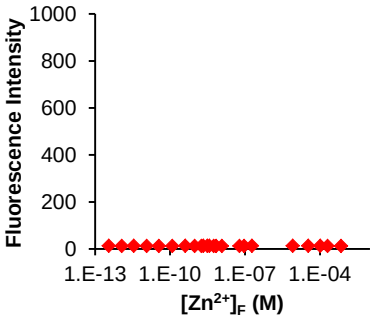


Figure S4. Increasing amounts of $Zn(OTf)_2$ added to a buffered solution of EGTA (7.3 mM) and **PAC-1** derivative (100 µM). K_d was determined by comparing fluorescence intensity (ex. 410 nm, em. 475 nm) and free zinc concentration. Values shown are mean ± s.e.m. (n = 3).

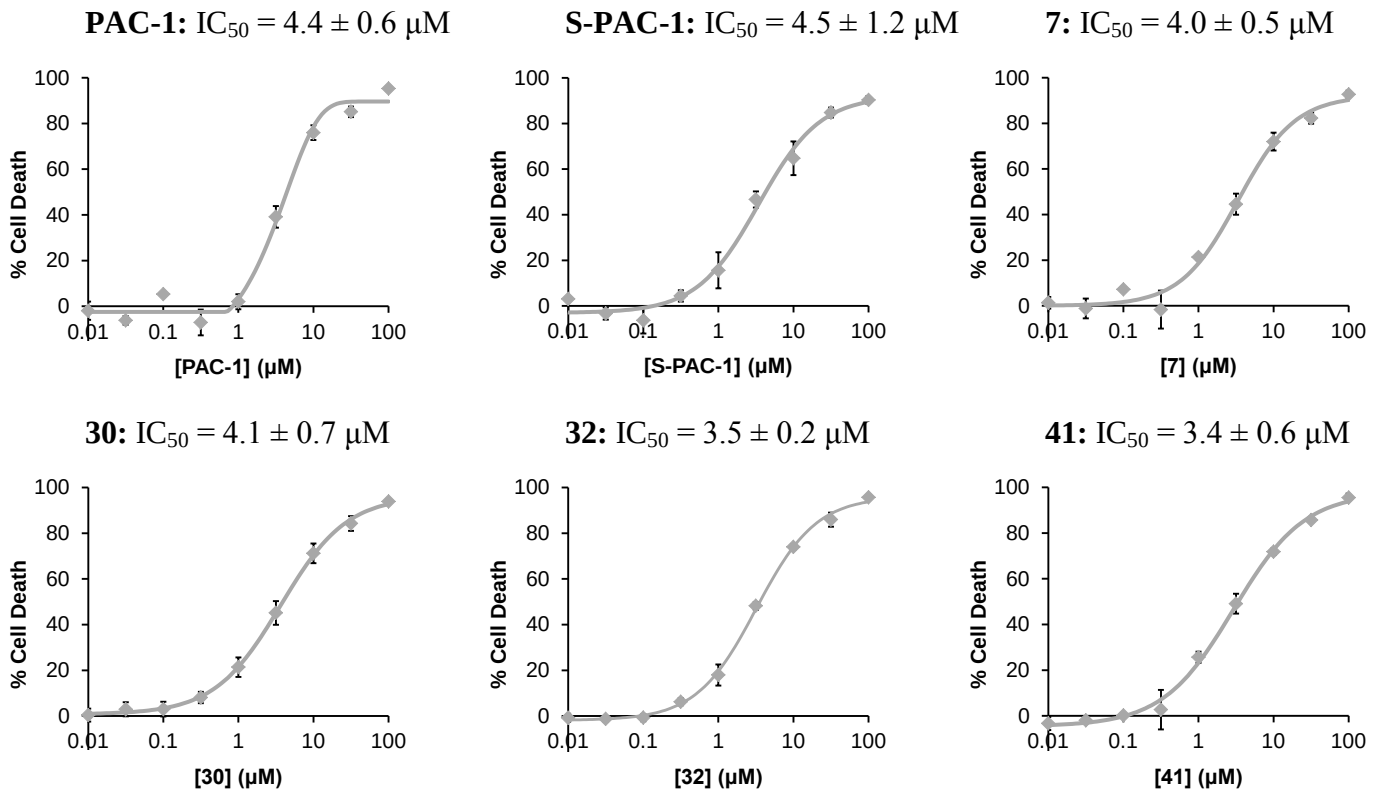


Figure S5. Jurkat cells treated with compounds for 72 hours. Biomass quantified by sulforhodamine B assay. IC_{50} values shown are mean ± s.e.m. (n = 3).

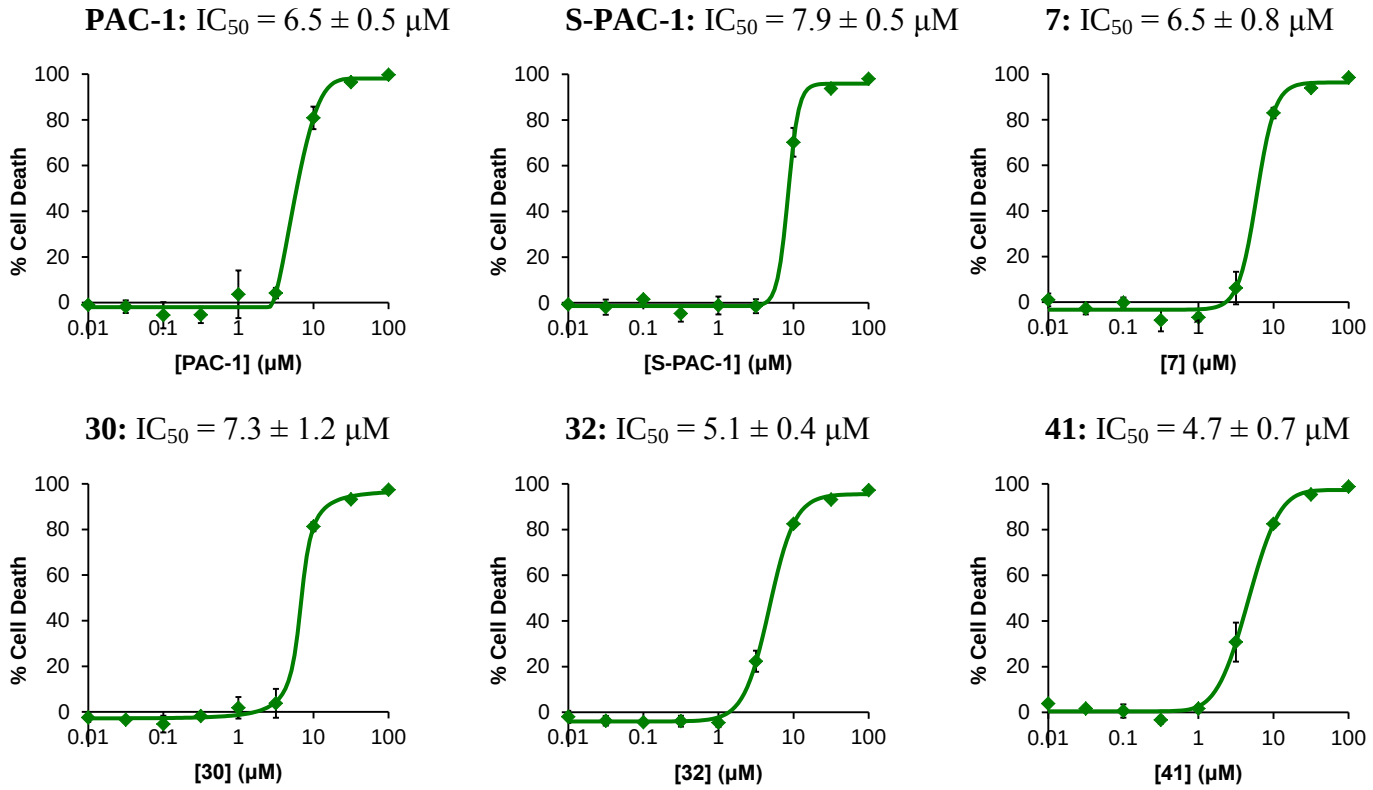
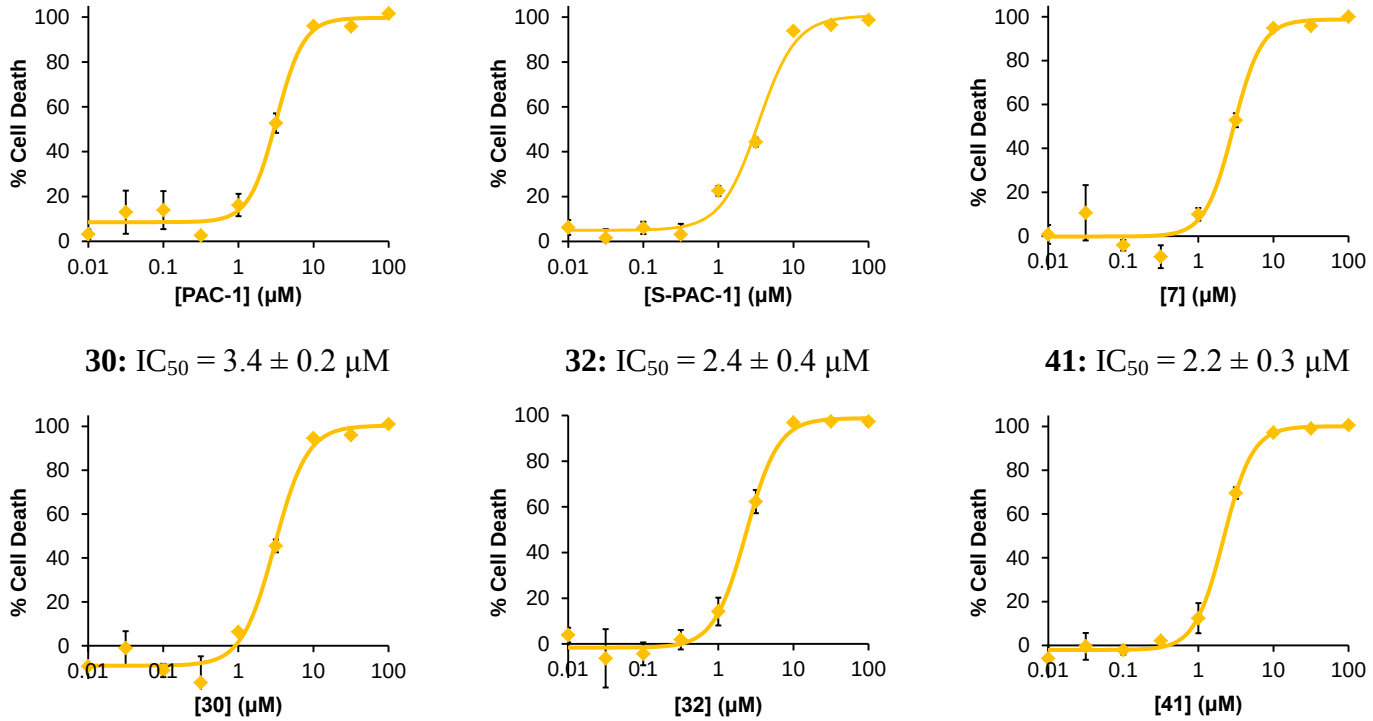


Figure S6. EL4 cells treated with compounds for 72 hours. Biomass quantified by sulforhodamine B assay. IC_{50} values shown are mean ± s.e.m. (n = 3).

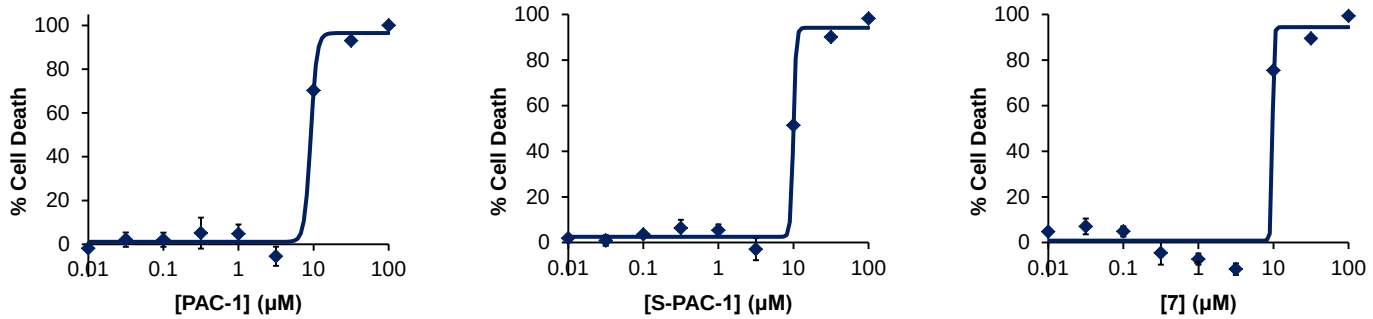
PAC-1: IC_{50} = 3.0 ± 0.1 μM **S-PAC-1:** IC_{50} = 3.2 ± 0.2 μM **7:** IC_{50} = 3.0 ± 0.1 μM



30: IC_{50} = 3.4 ± 0.2 μM **32:** IC_{50} = 2.4 ± 0.4 μM **41:** IC_{50} = 2.2 ± 0.3 μM

Figure S7. GL-1 cells treated with compounds for 72 hours. Biomass quantified by sulforhodamine B assay. IC_{50} values shown are mean ± s.e.m. (n = 3).

PAC-1: IC_{50} = 10.0 ± 0.8 μM **S-PAC-1:** IC_{50} = 9.8 ± 0.1 μM **7:** IC_{50} = 9.3 ± 0.2 μM



30: IC_{50} = 10.0 ± 0.6 μM **32:** IC_{50} = 9.5 ± 0.7 μM **41:** IC_{50} = 8.5 ± 0.7 μM

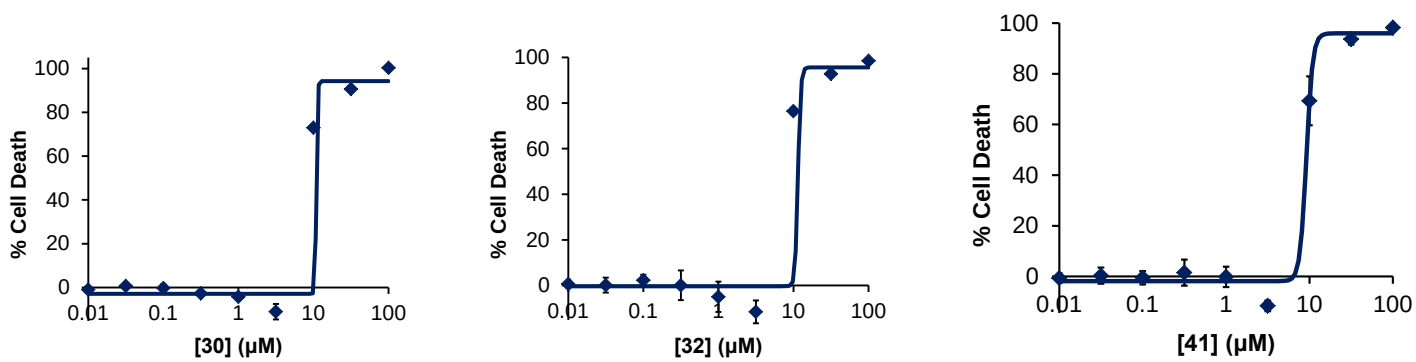


Figure S8. OSW cells treated with compounds for 72 hours. Biomass quantified by sulforhodamine B assay. IC_{50} values shown are mean ± s.e.m. (n = 3).

References

1. Putt, K. S.; Chen, G. W.; Pearson, J. M.; Sandhorst, J. S.; Hoagland, M. S.; Kwon, J. T.; Hwang, S. K.; Jin, H.; Churchwell, M. I.; Cho, M. H.; Doerge, D. R.; Helferich, W. G.; Hergenrother, P. J. Small-molecule activation of procaspase-3 to caspase-3 as a personalized anticancer strategy. *Nat. Chem. Biol.* **2006,** 2, 543-550.
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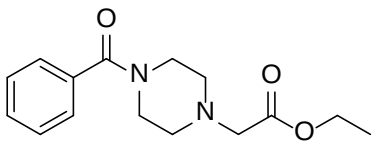
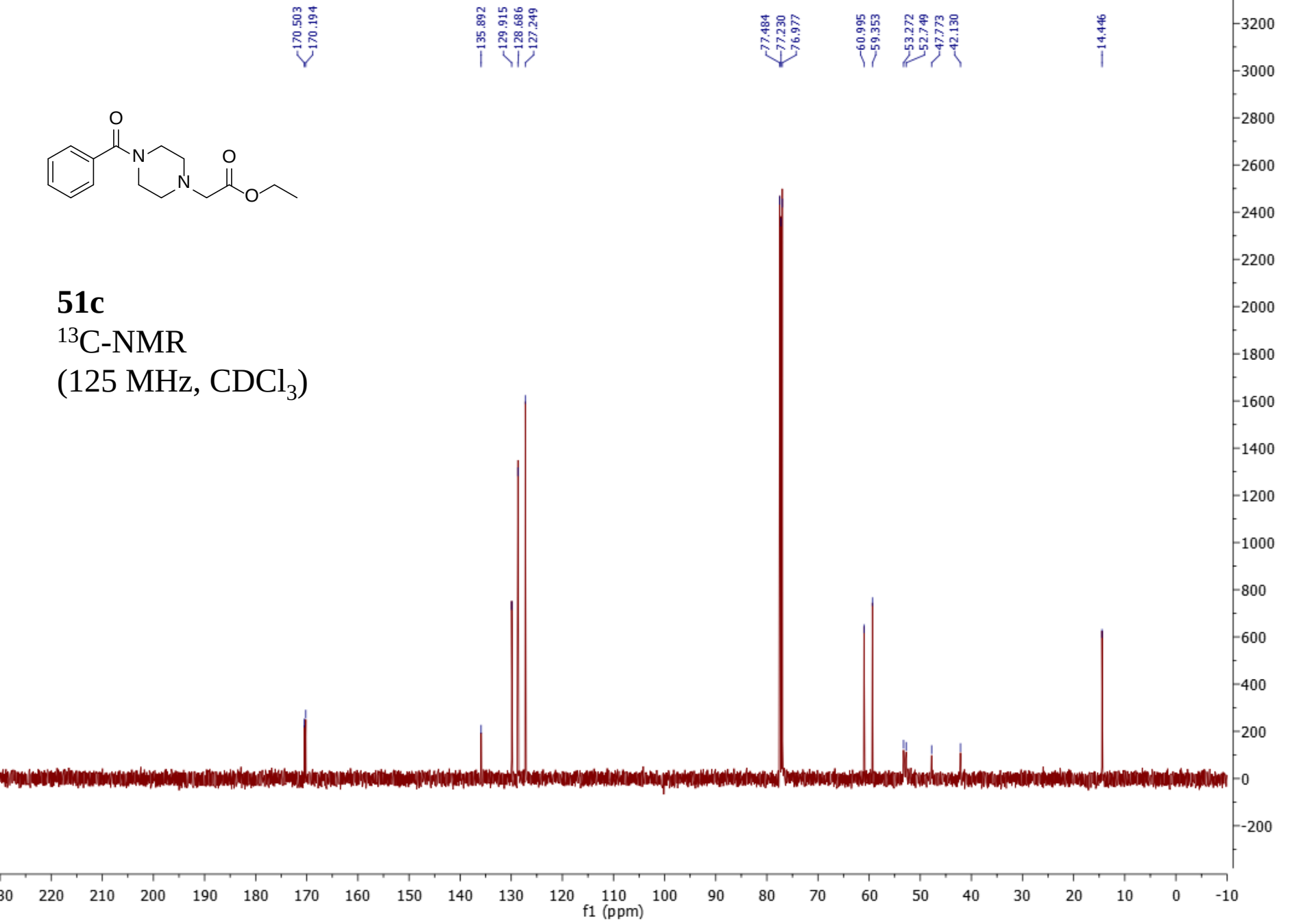
3. Hsu, D. C.; Roth, H. S.; West, D. C.; Botham, R. C.; Novotny, C. J.; Schmid, S. C.; Hergenrother, P. J. Parallel synthesis and biological evaluation of 837 analogues of Procaspase-Activating Compound 1 (PAC-1). *ACS Comb. Sci.* **2012,** 14, 44-50.
4. Patel, V.; Balakrishnan, K.; Keating, M. J.; Wierda, W. G.; Gandhi, V. Expression of executioner procaspases and their activation by a procaspase activating compound in chronic lymphocytic leukemia cells. *Blood* **2015,** 125, 1126-1136.
5. Vichai, V.; Kirtikara, K. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat. Protoc.* **2006,** 1, 1112-1116.
6. Huang, S.; Clark, R. J.; Zhu, L. Highly sensitive fluorescent probes for zinc ion based on triazolyl-containing tetradentate coordination motifs. *Org. Lett.* **2007,** 9, 4999-5002.

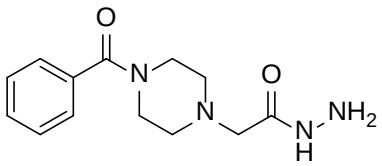


51c

^{1}H-NMR

(500 MHz, $CDCl_3$)

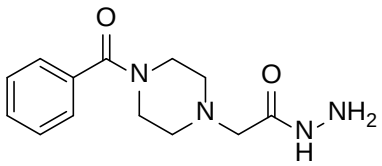

170.503
170.194
135.892
129.915
128.686
127.249
77.484
77.230
76.977
60.995
59.353
53.272
52.749
47.773
42.130
14.446
O
N
N
O
O
51c
13C-NMR
(125 MHz, CDCl3)
f1 (ppm)



46c

^{1}H-NMR

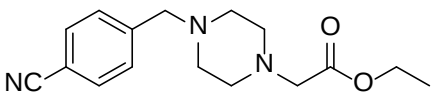
(500 MHz, $CDCl_3$)



46c

^{13}C-NMR

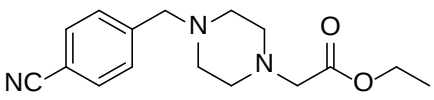
(125 MHz, $CDCl_3$)



51d

^{1}H-NMR

(500 MHz, $CDCl_3$)



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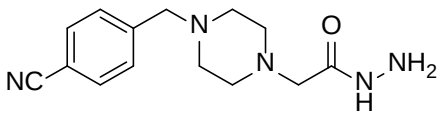
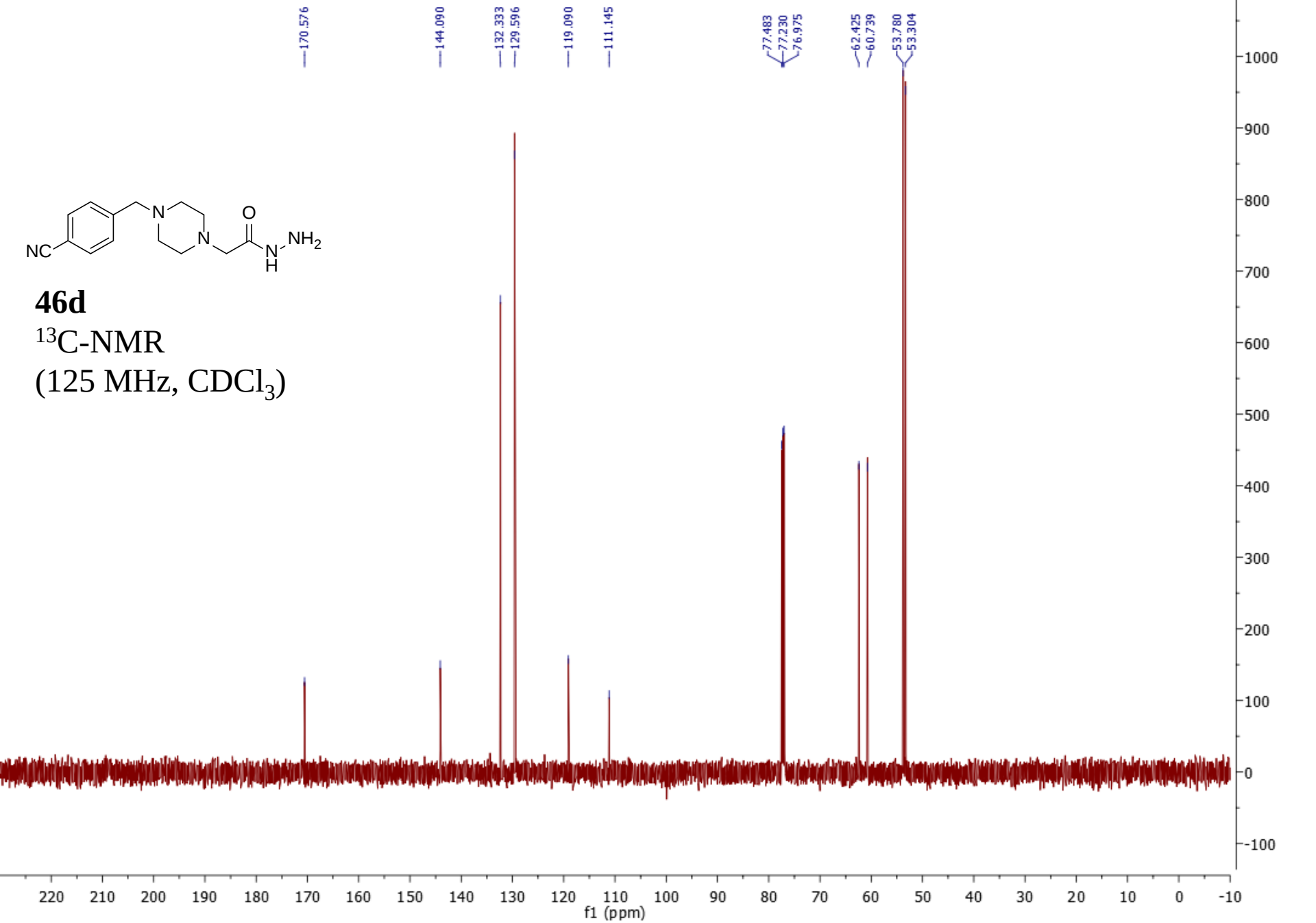
(125 MHz, $CDCl_3$)

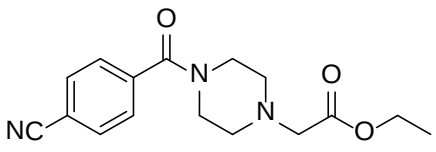


46d

^{1}H-NMR

(500 MHz, $CDCl_3$)

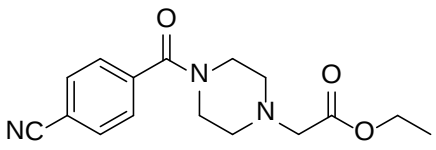

NC
N
N
O
N
H
NH2
46d
13C-NMR
(125 MHz, CDCl3)
170.576
144.090
132.333
129.596
119.090
111.145
77.483
77.230
76.975
62.425
60.739
53.780
53.304
f1 (ppm)



51e

^{1}H-NMR

(500 MHz, $CDCl_3$)



51e

^{13}C-NMR

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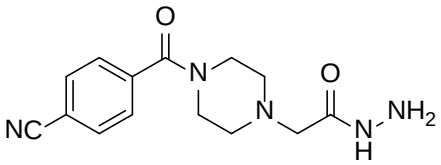

46e

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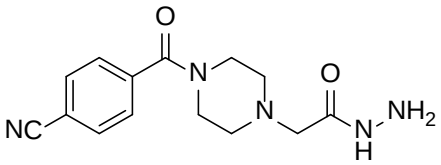
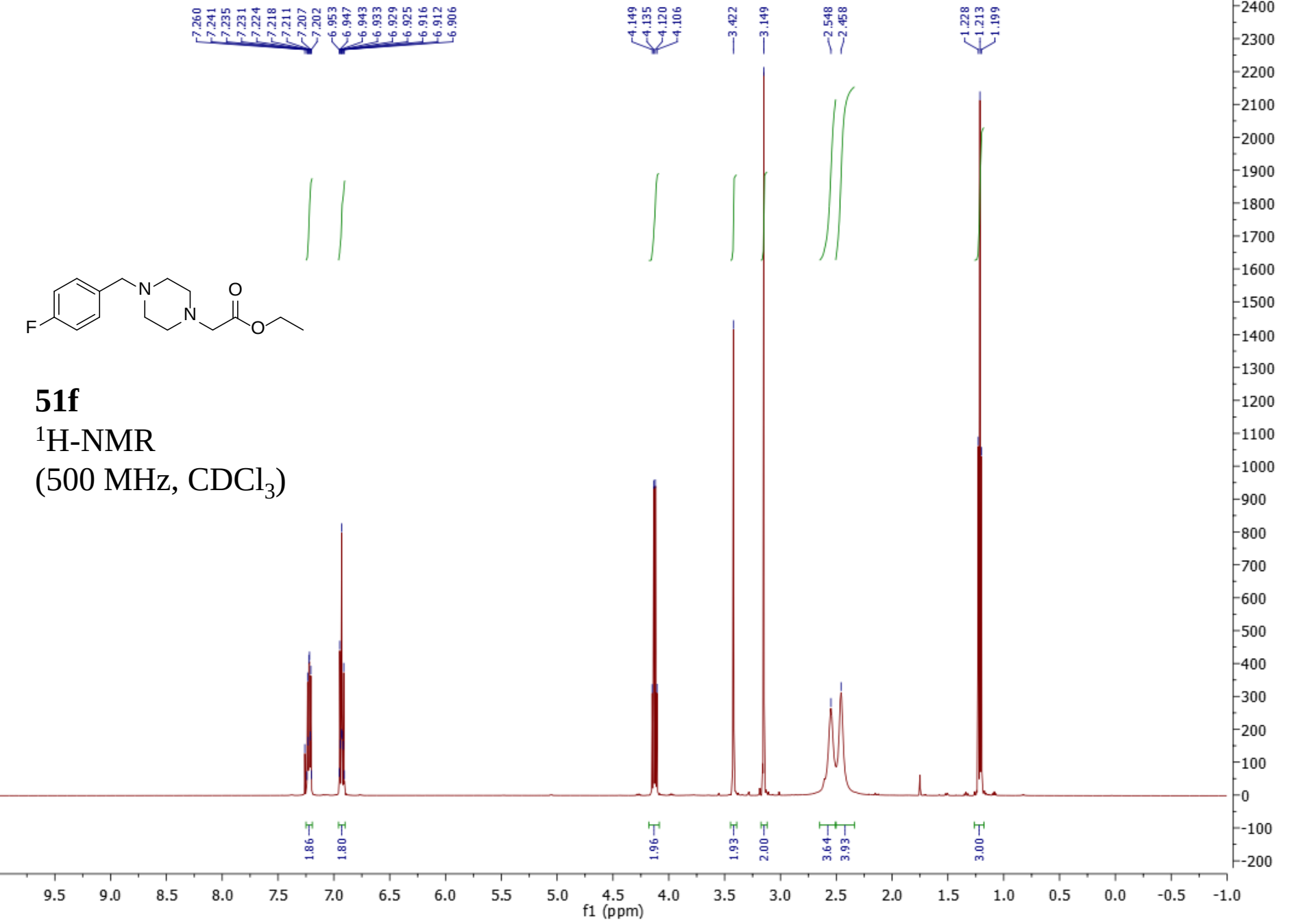


46e

^{13}C-NMR

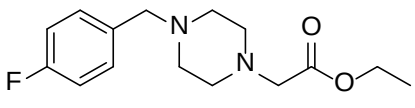
(125 MHz, $CDCl_3$)



51f

^{1}H-NMR

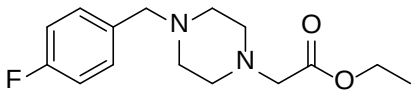
(500 MHz, $CDCl_3$)



51f

^{13}C-NMR

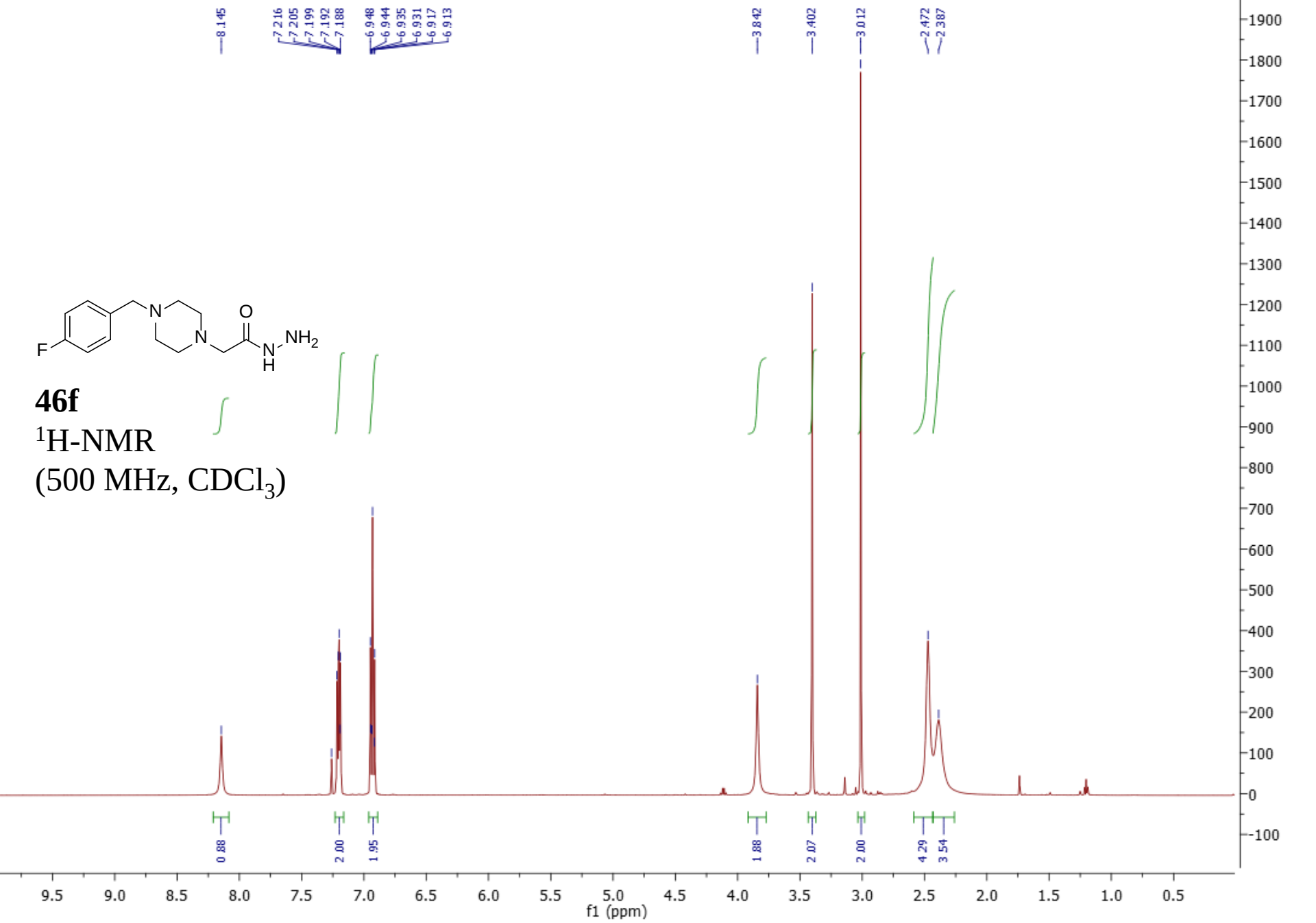
(125 MHz, $CDCl_3$)



51f

^{19}F-NMR

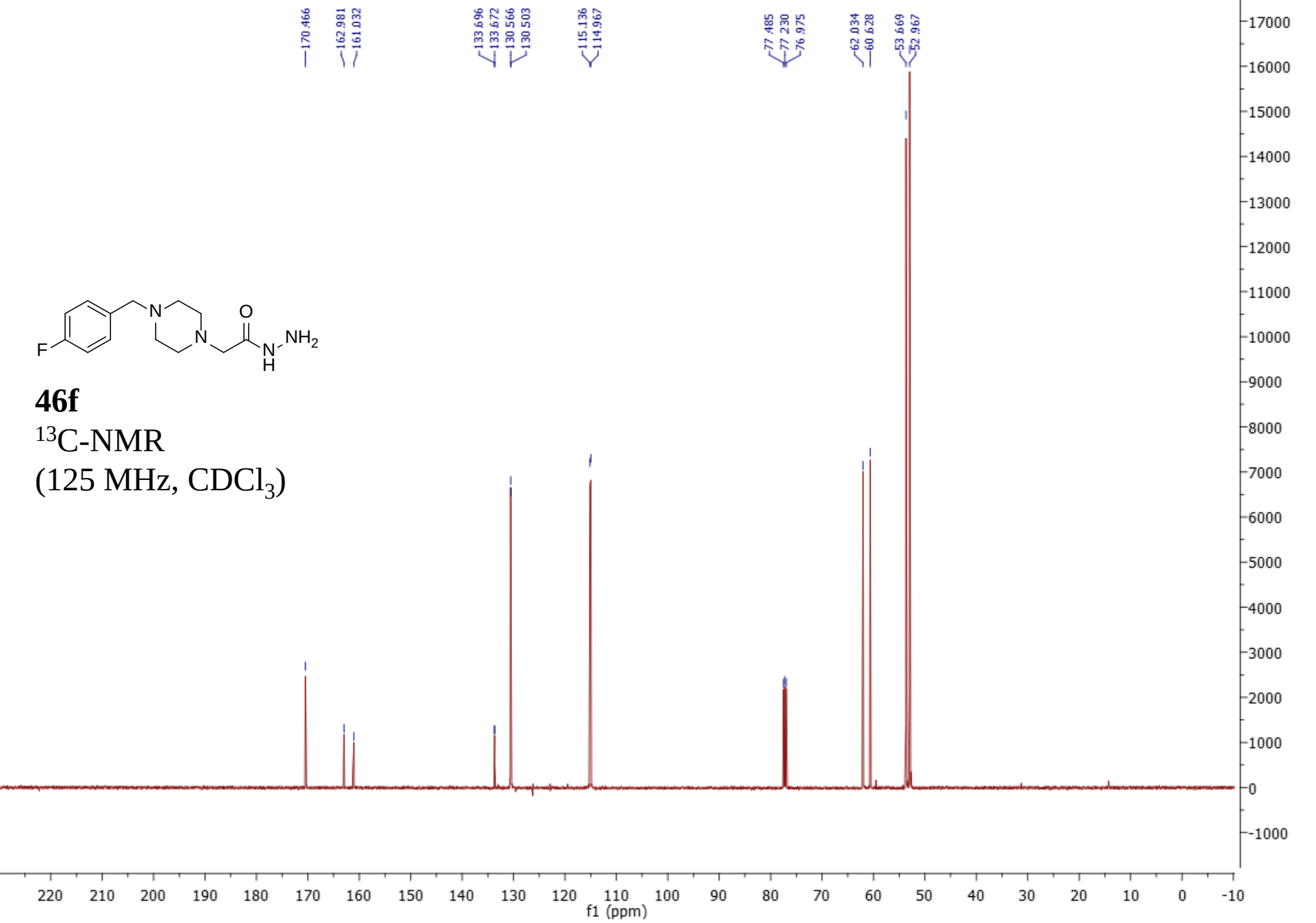
(470 MHz, $CDCl_3$)



46f

^{1}H-NMR

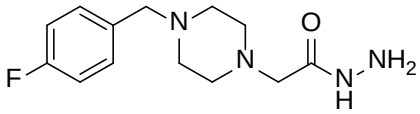
(500 MHz, $CDCl_3$)



46f

^{13}C-NMR

(125 MHz, $CDCl_3$)



46f

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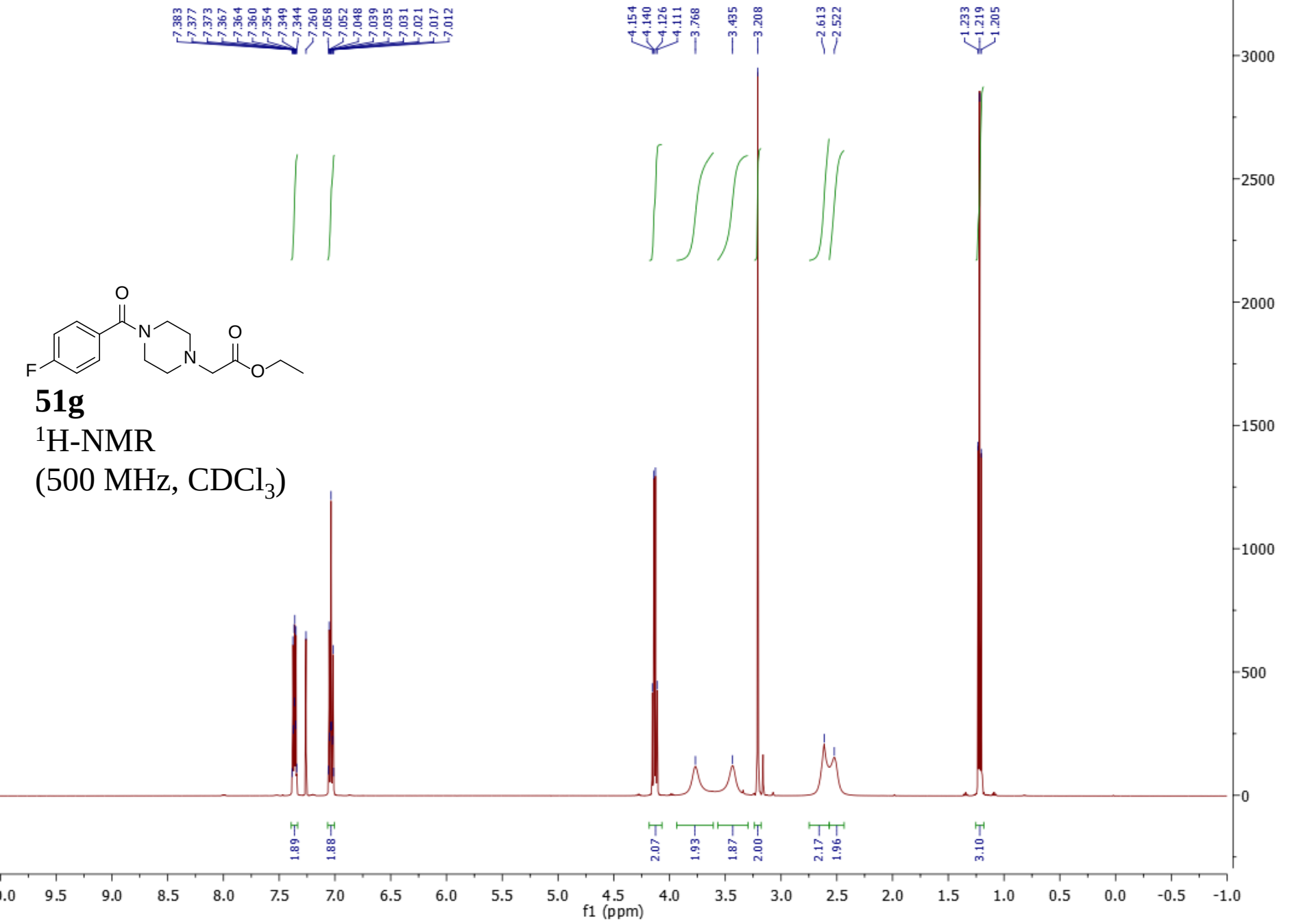
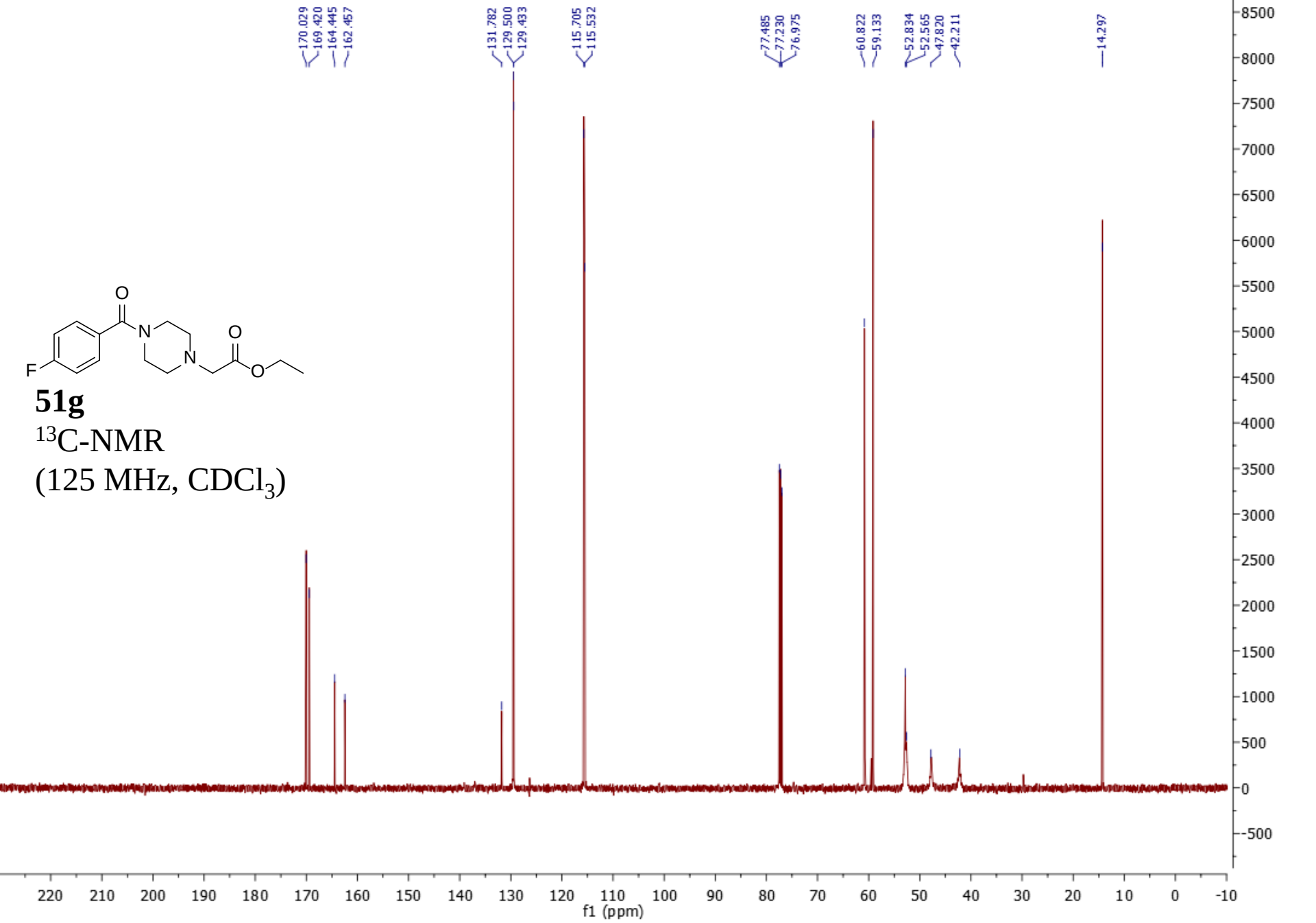


51g

^{1}H-NMR

(500 MHz, $CDCl_3$)



51g

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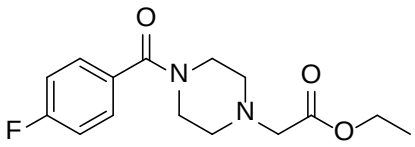
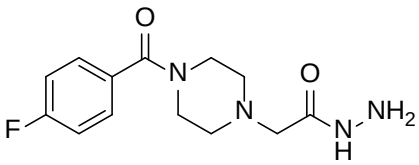


51g

^{19}F-NMR

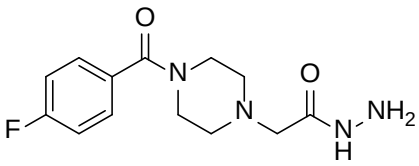
(470 MHz, $CDCl_3$)



46g

^{1}H-NMR

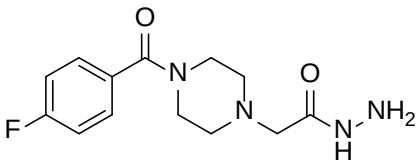
(500 MHz, $CDCl_3$)



46g

^{13}C-NMR

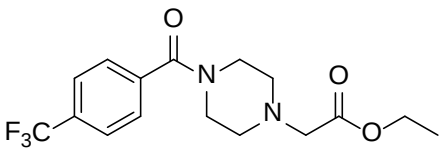
(125 MHz, $CDCl_3$)



46g

^{19}F-NMR

(470 MHz, $CDCl_3$)



51i

^{1}H-NMR

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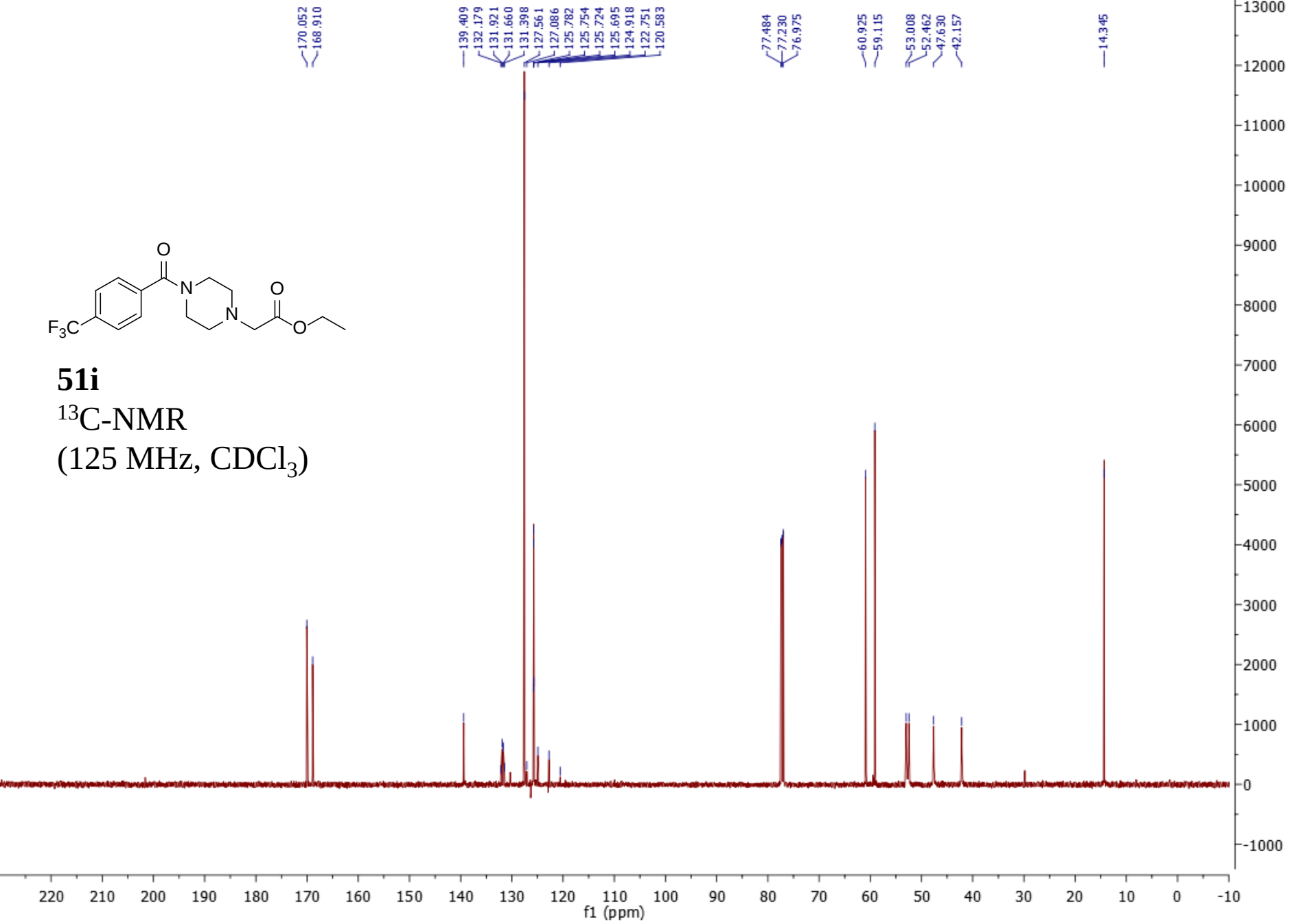

51i

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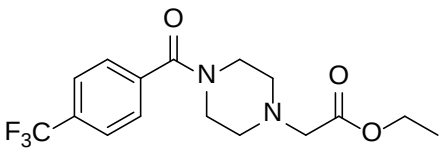

51i

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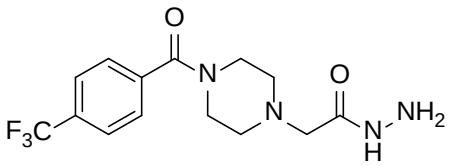
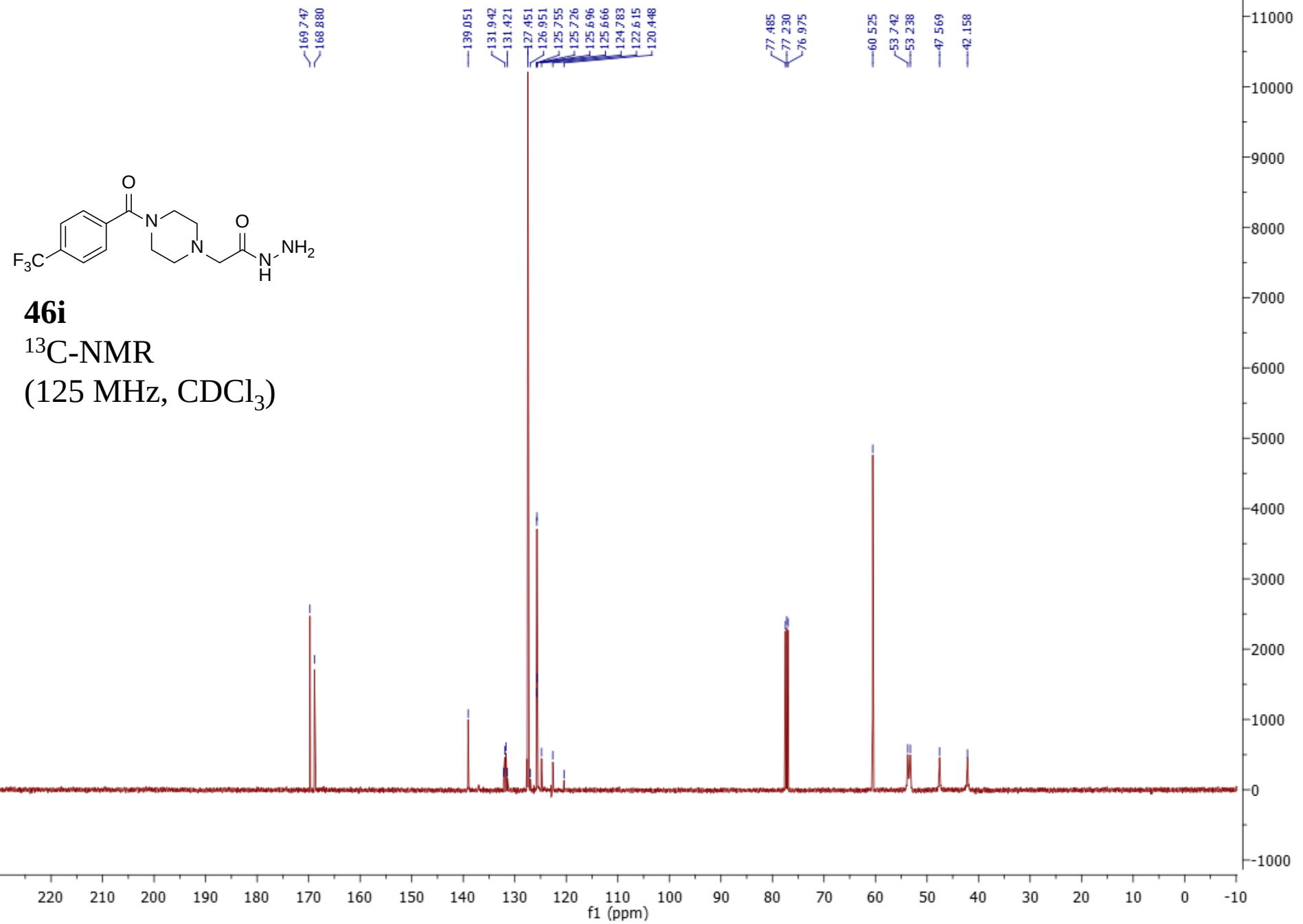
(470 MHz, $CDCl_3$)

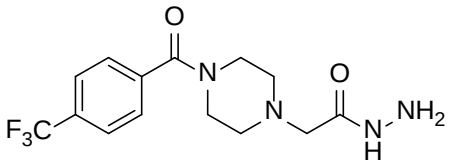


46i

^{1}H-NMR

(500 MHz, $CDCl_3$)

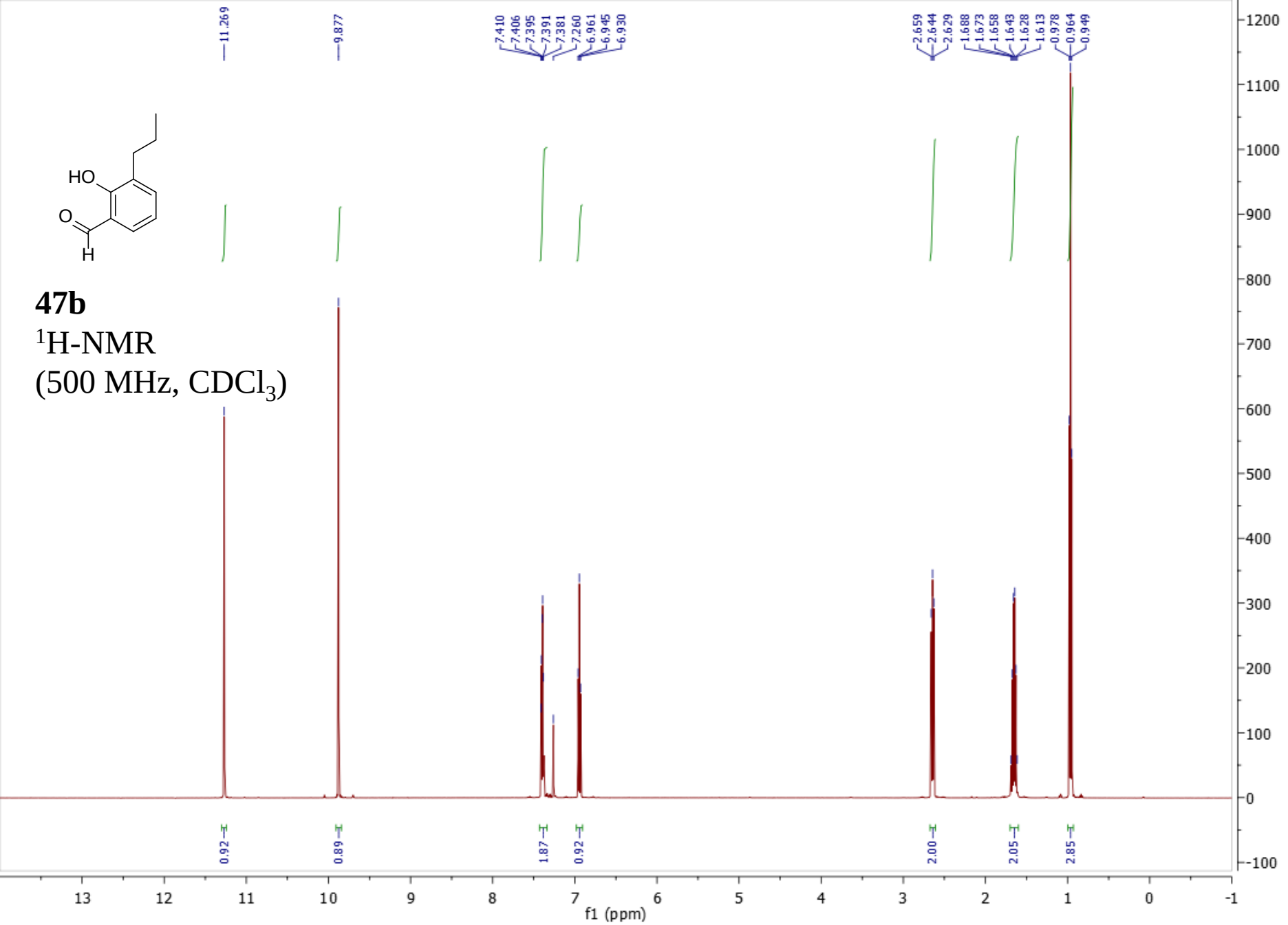

O
N
N
O
N
H
NH2
F3C
46i
13C-NMR
(125 MHz, CDCl3)
169.747
168.880
139.051
131.942
131.421
127.451
126.951
125.755
125.726
125.696
125.666
124.783
122.615
120.448
77.485
77.230
76.975
60.525
53.742
53.238
47.569
42.158
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)



46i

^{19}F-NMR

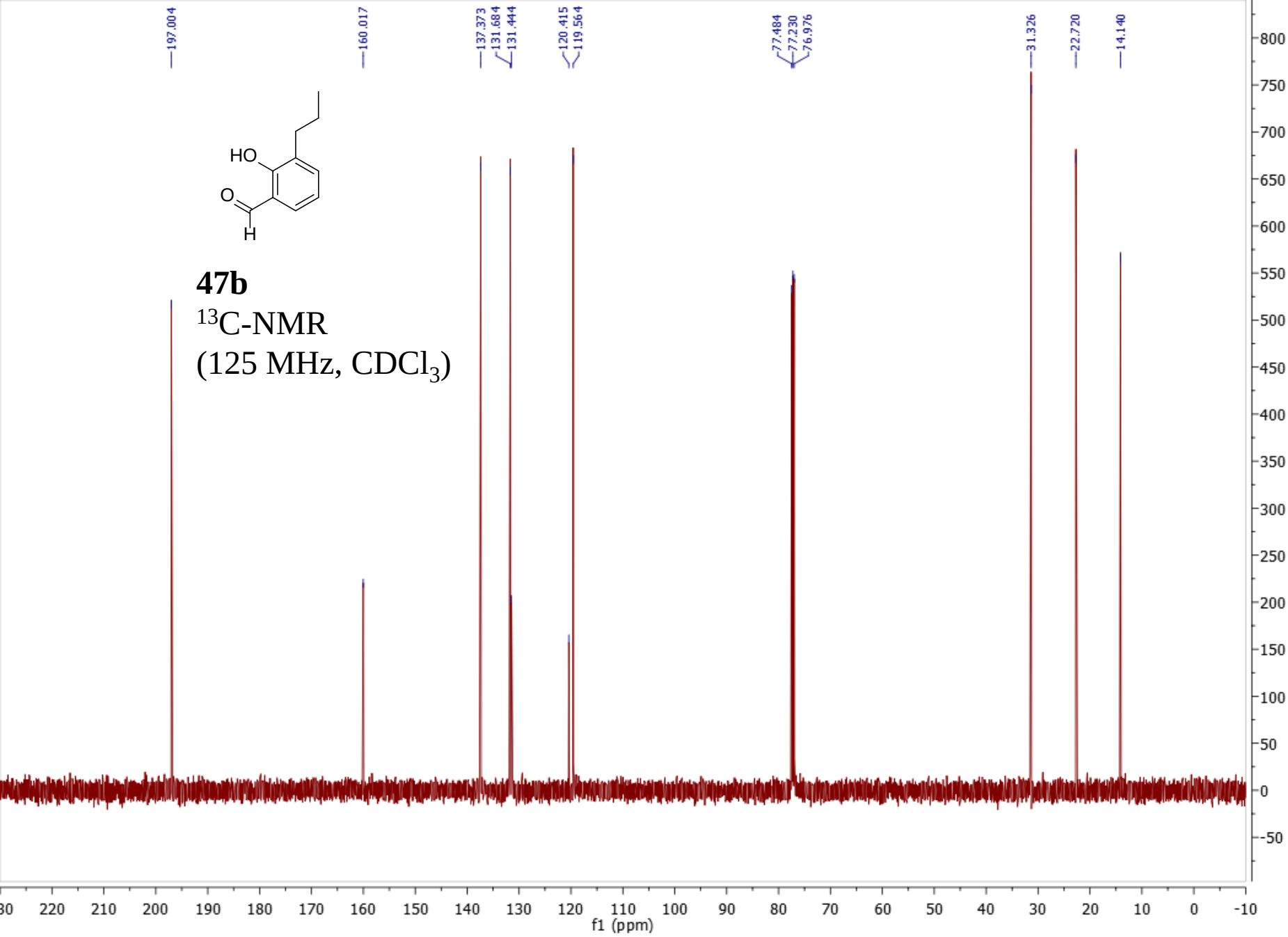
(470 MHz, $CDCl_3$)



47b

^{1}H-NMR

(500 MHz, $CDCl_3$)


197.004
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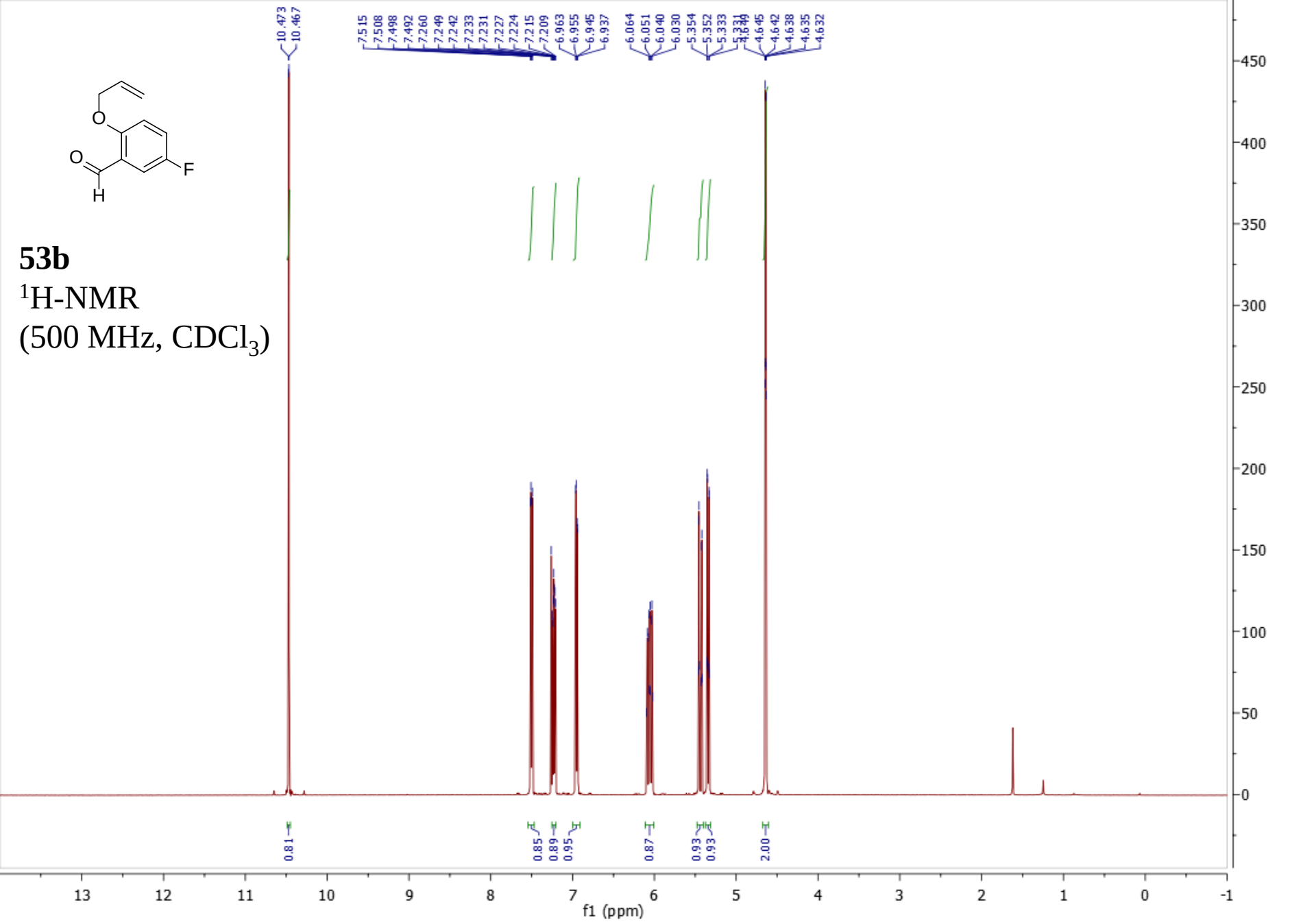
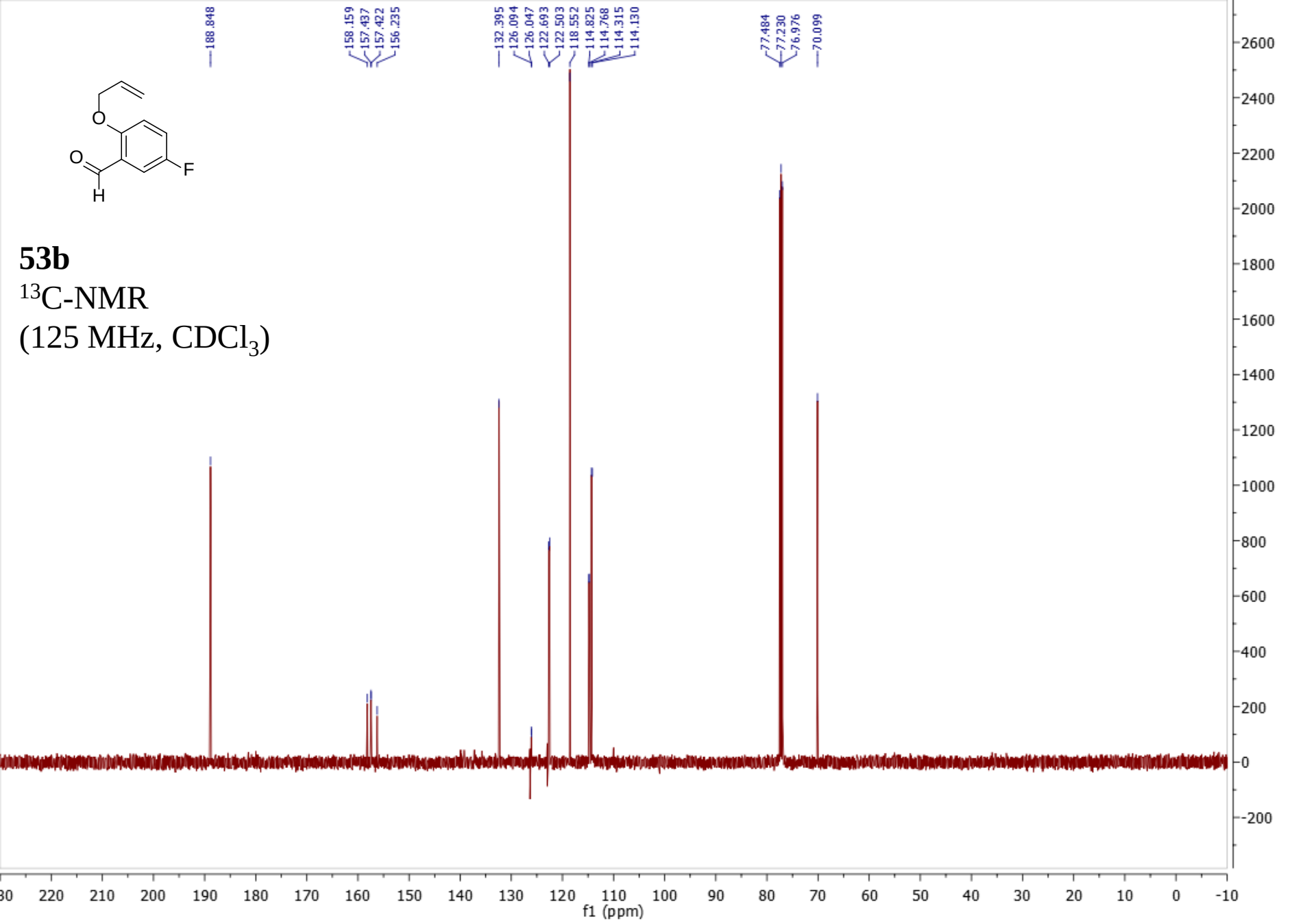
137.373
131.684
131.444
120.415
119.564
77.484
77.230
76.976
31.326
22.720
14.140
HO
O
H
47b
13C-NMR
(125 MHz, CDCl3)
f1 (ppm)



53b

^{1}H-NMR

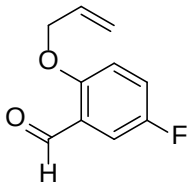
(500 MHz, $CDCl_3$)



53b

^{13}C-NMR

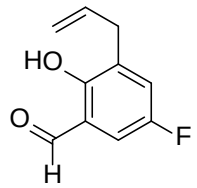
(125 MHz, $CDCl_3$)



53b

^{19}F-NMR

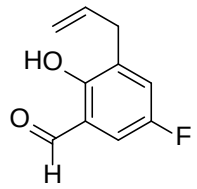
(470 MHz, $CDCl_3$)



47d

^{1}H-NMR

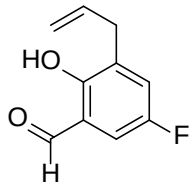
(500 MHz, $CDCl_3$)



47d

^{13}C-NMR

(125 MHz, $CDCl_3$)



47d

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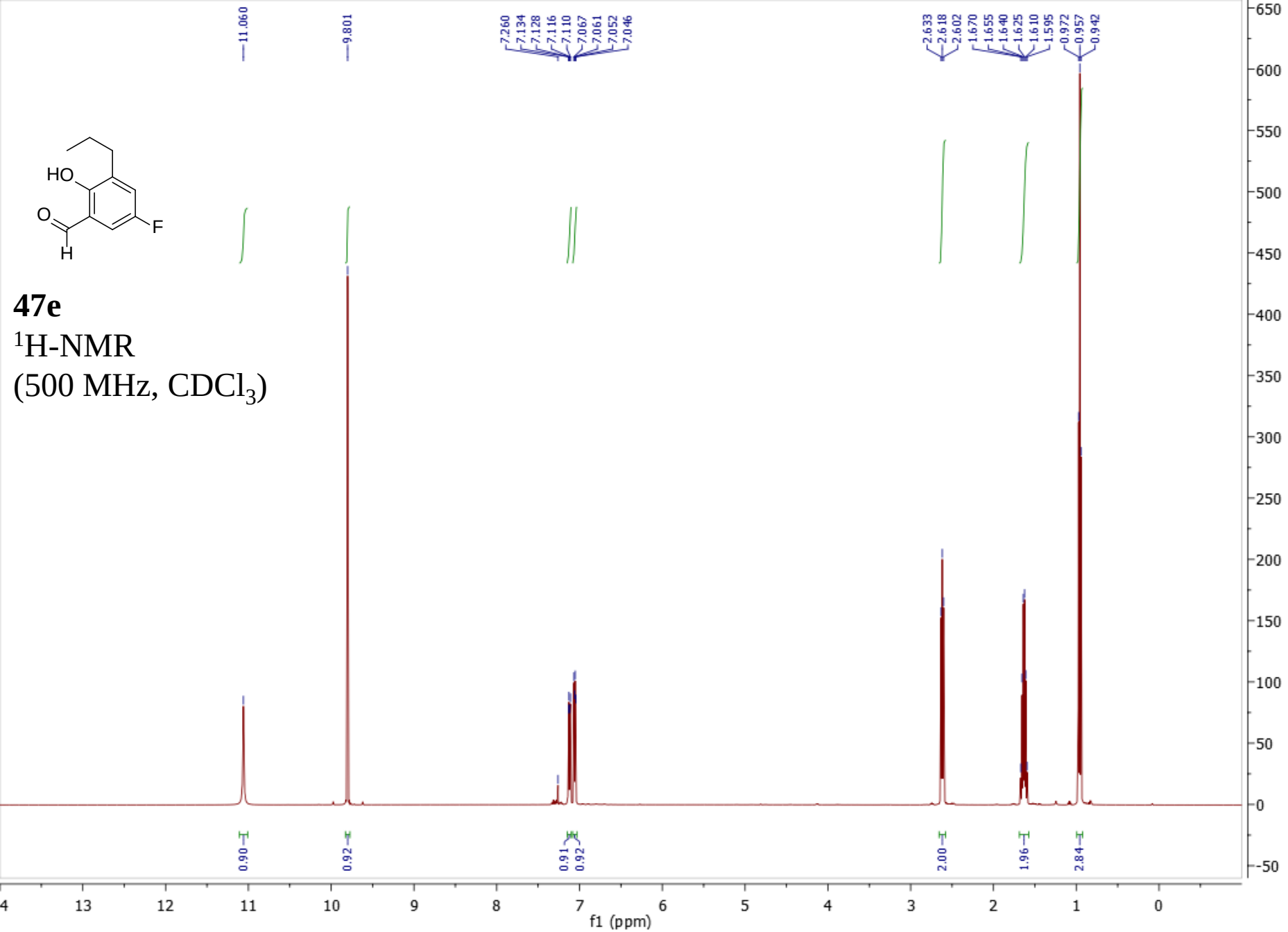
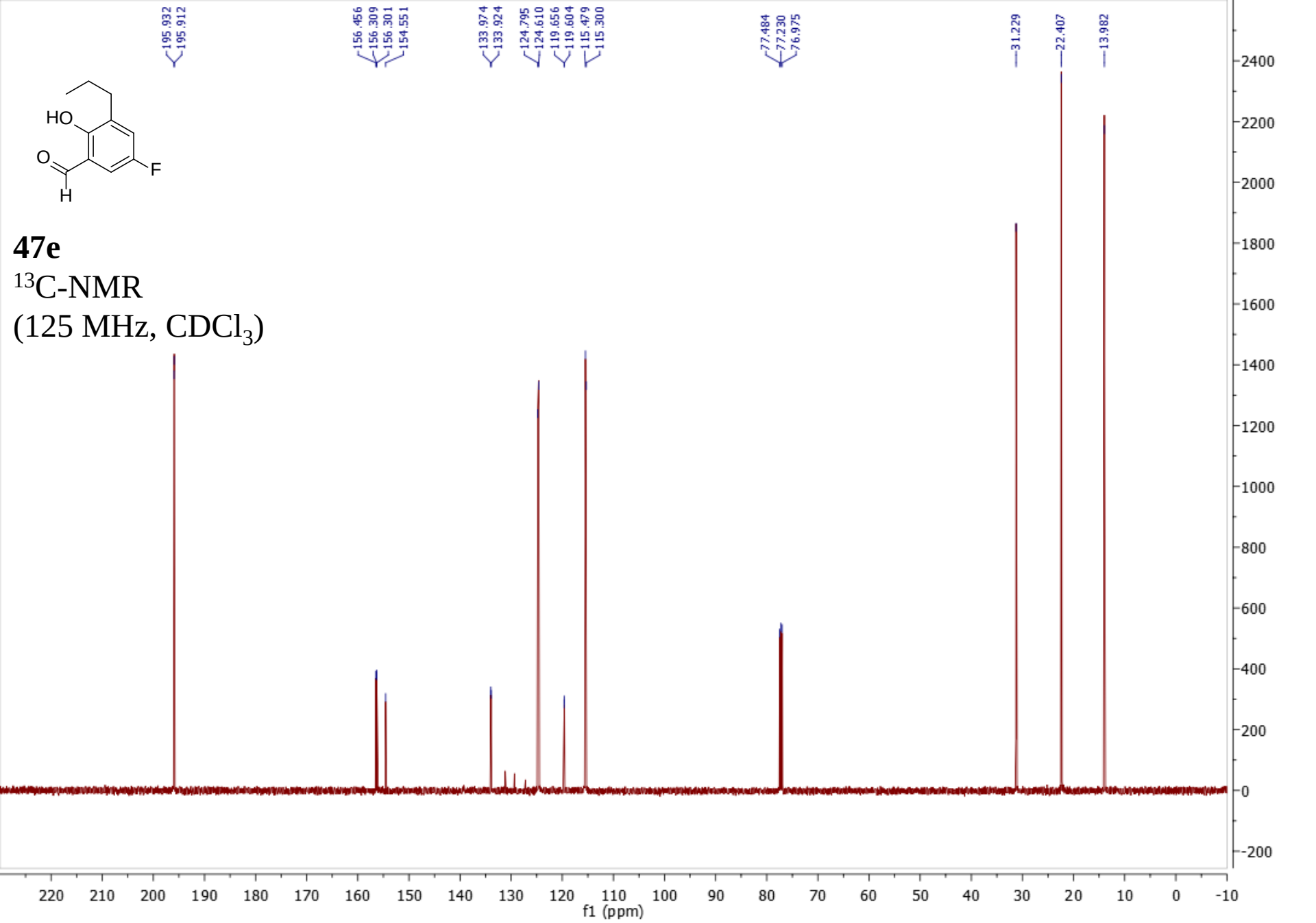
(470 MHz, $CDCl_3$)



47e

^{1}H-NMR

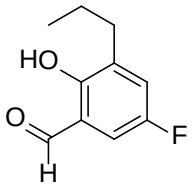
(500 MHz, $CDCl_3$)



47e

^{13}C-NMR

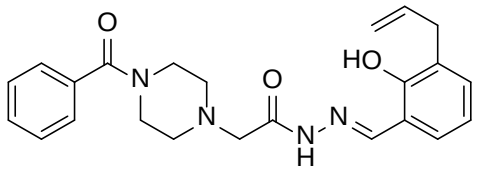
(125 MHz, $CDCl_3$)



47e

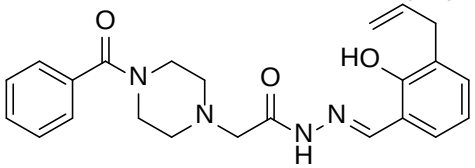
^{19}F-NMR

(470 MHz, $CDCl_3$)



3

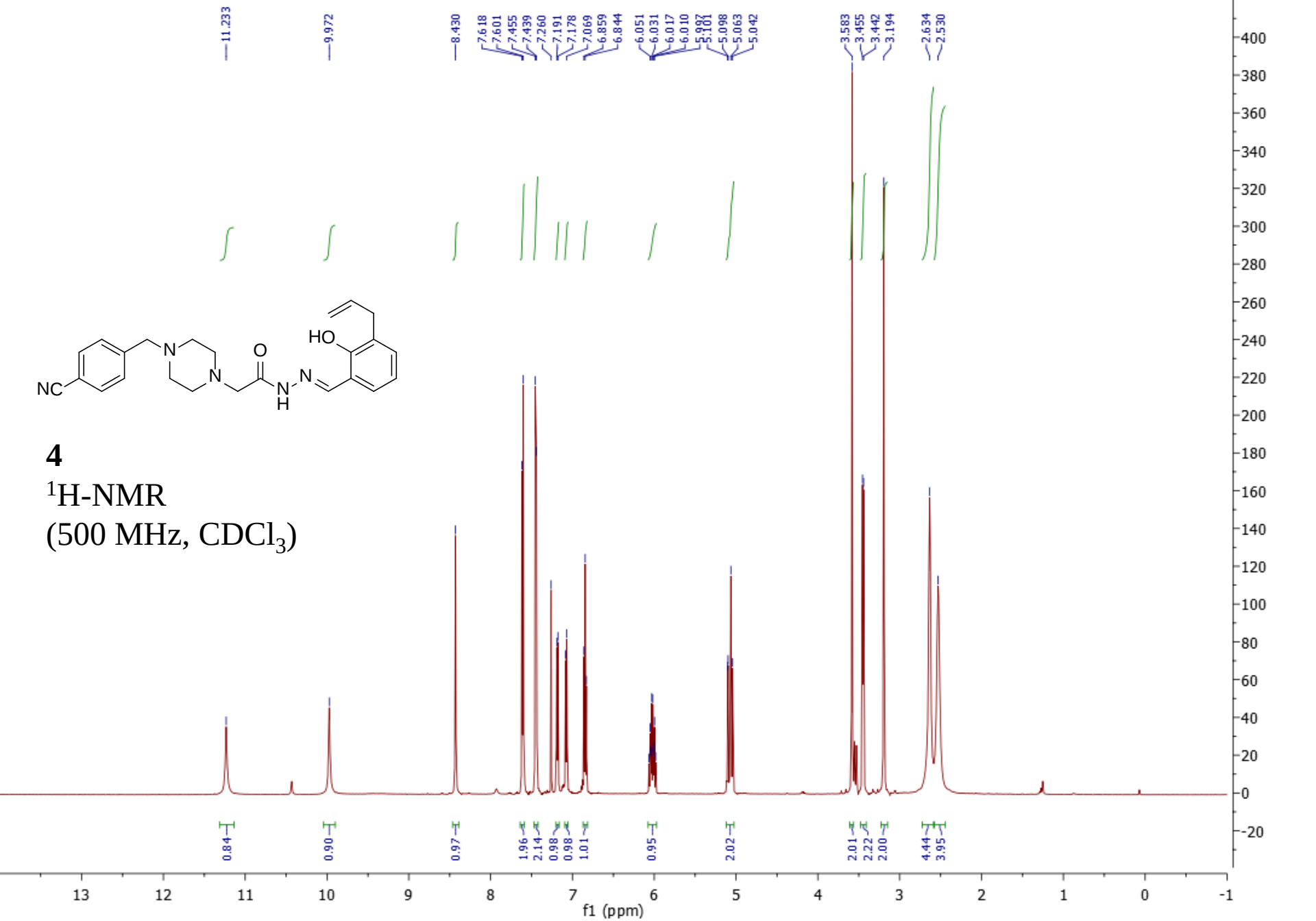
^{1}H-NMR
(500 MHz, $CDCl_3$)



3

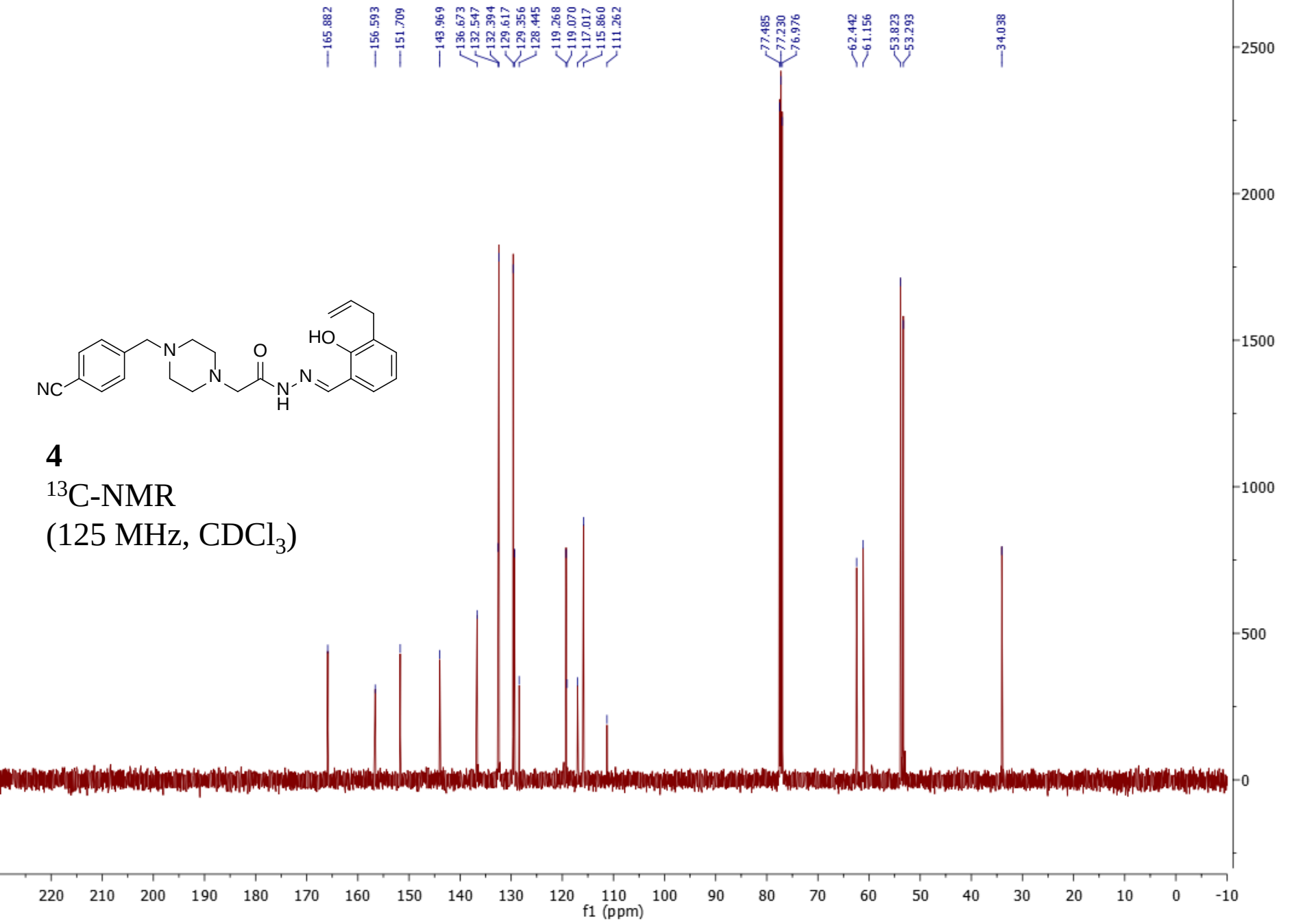
^{13}C-NMR

(125 MHz, $CDCl_3$)



4

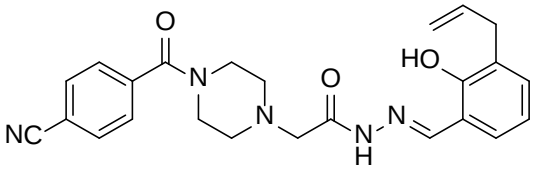
^{1}H-NMR
(500 MHz, $CDCl_3$)



4

^{13}C-NMR

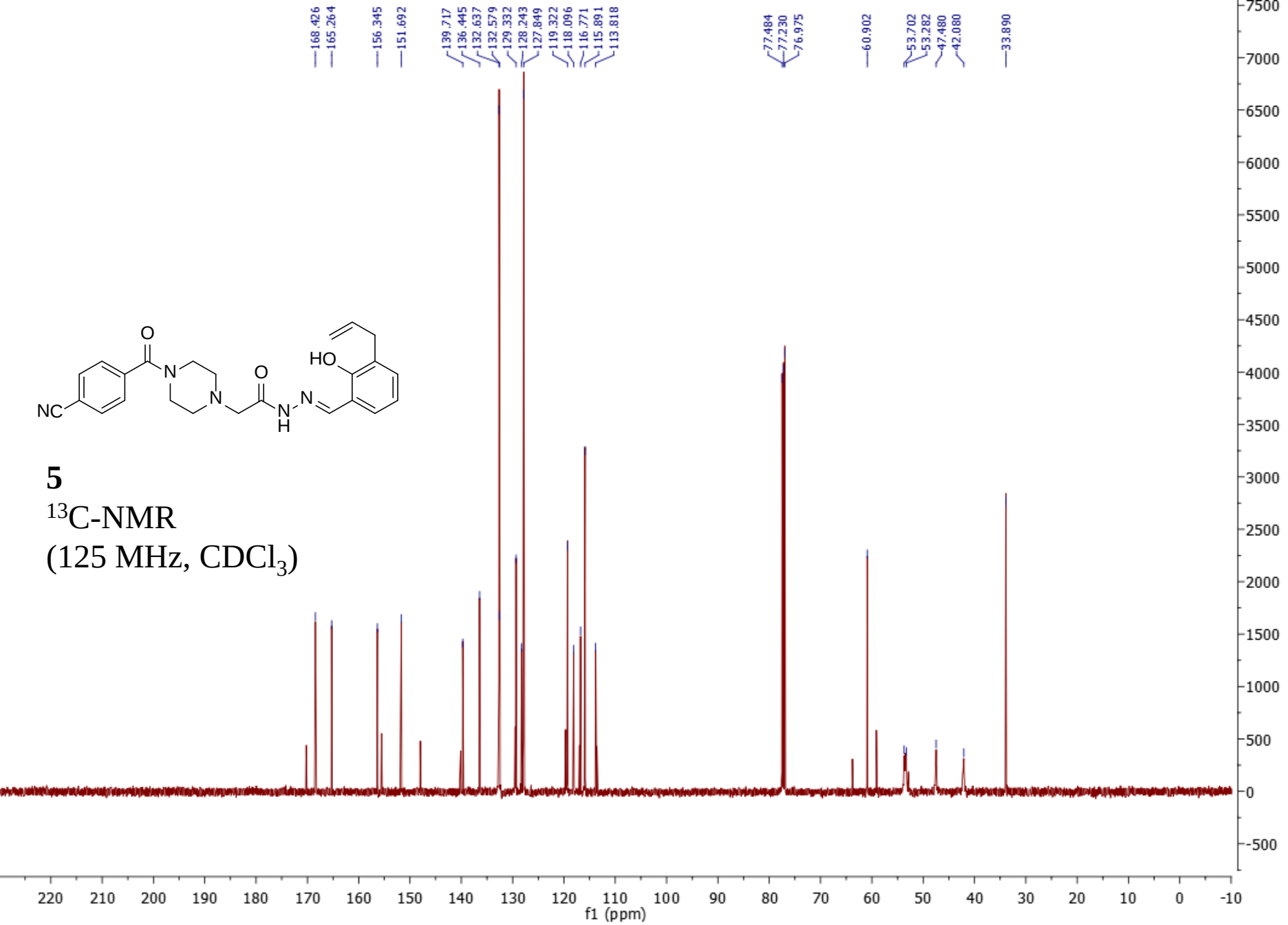
(125 MHz, $CDCl_3$)



5

^{1}H-NMR

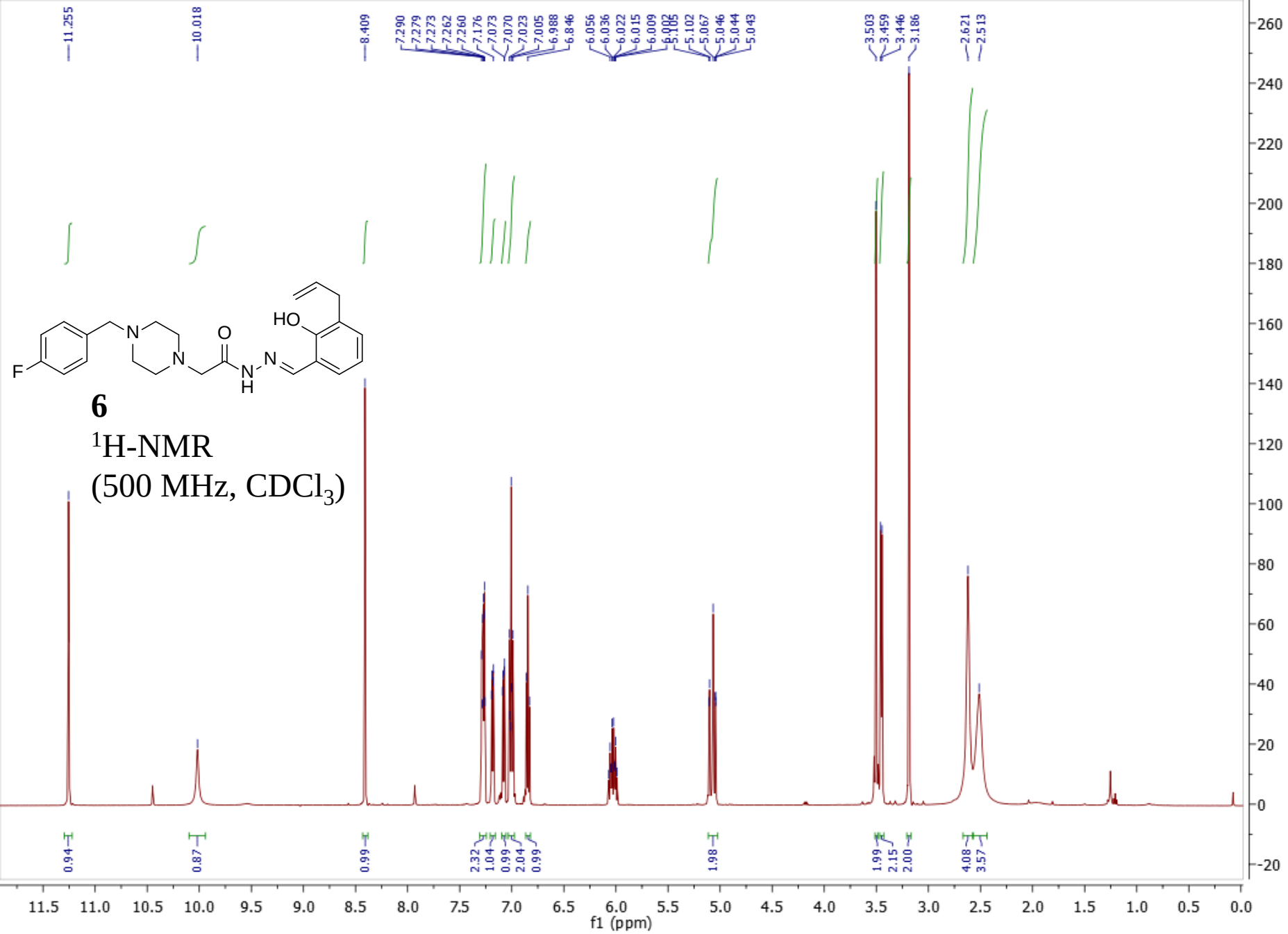
(500 MHz, $CDCl_3$)

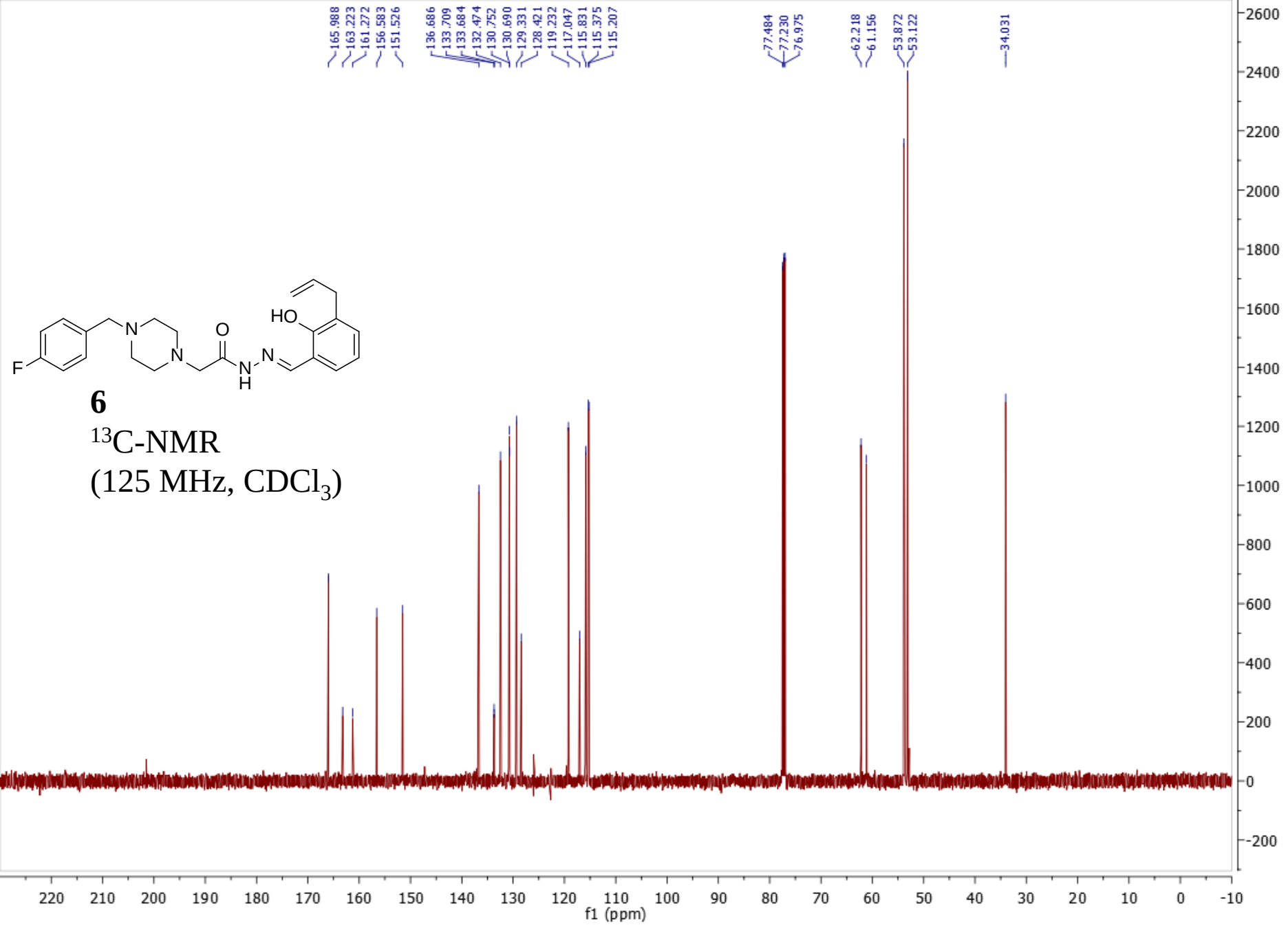


5

^{13}C-NMR

(125 MHz, $CDCl_3$)

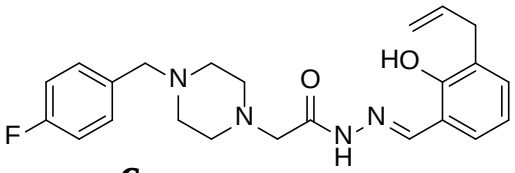

11.255
10.018
8.409
7.290
7.279
7.273
7.262
7.260
7.176
7.073
7.070
7.023
7.005
6.988
6.846
6.056
6.036
6.022
6.015
6.009
5.102
5.067
5.046
5.044
5.043
3.503
3.459
3.446
3.186
2.621
2.513
HO
O
N
N
N
H
F
6
1H-NMR
(500 MHz, CDCl3)
0.94
0.87
0.99
2.32
1.04
0.99
2.04
0.99
1.98
1.99
2.15
2.00
4.08
3.57
f1 (ppm)



6

^{13}C-NMR

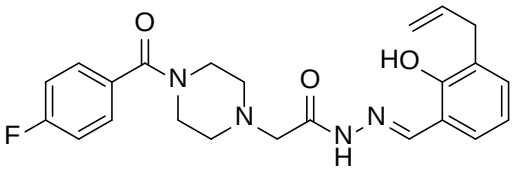
(125 MHz, $CDCl_3$)



6

^{19}F-NMR

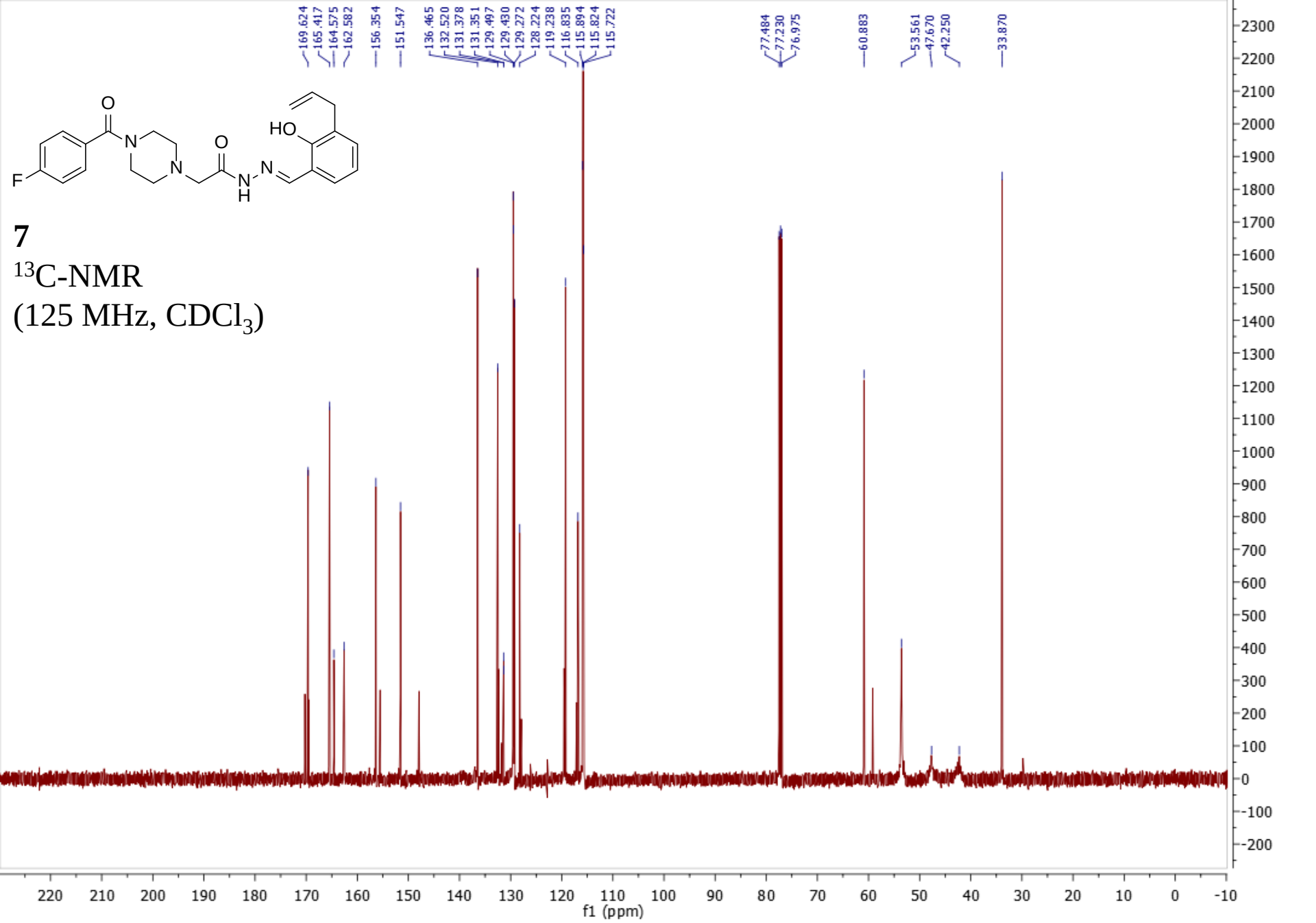
(470 MHz, $CDCl_3$)

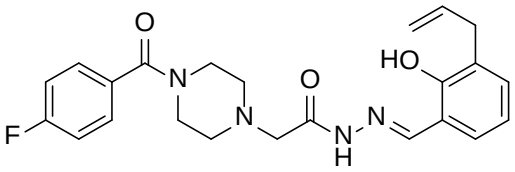


7

^{1}H-NMR

(500 MHz, $CDCl_3$)

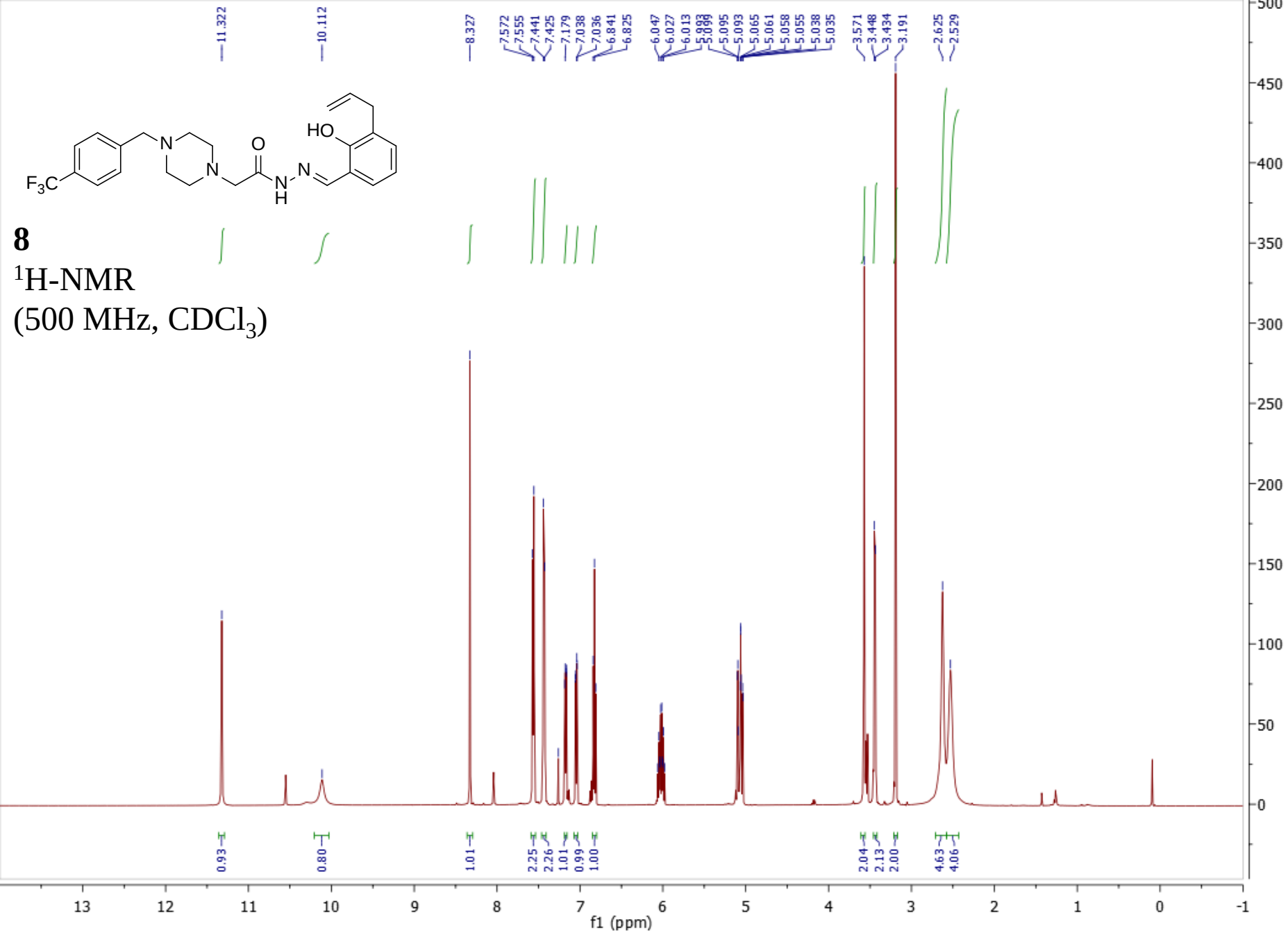

O
N
N
O
N
N
H
HO
F
7
13C-NMR
(125 MHz, CDCl3)
169.624
165.417
164.575
162.582
156.354
151.547
136.465
132.520
131.378
131.351
129.497
129.430
129.272
128.224
119.238
116.835
115.894
115.824
115.722
77.484
77.230
76.975
60.883
53.561
47.670
42.250
33.870
f1 (ppm)



7

^{19}F-NMR

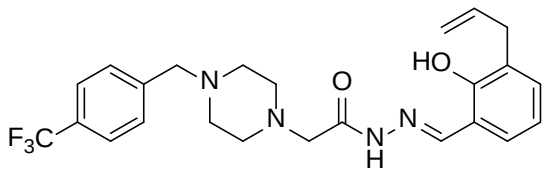
(470 MHz, $CDCl_3$)



8

^{1}H-NMR

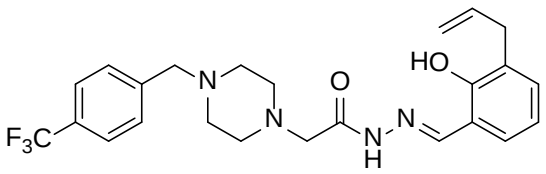
(500 MHz, $CDCl_3$)



8

^{13}C-NMR

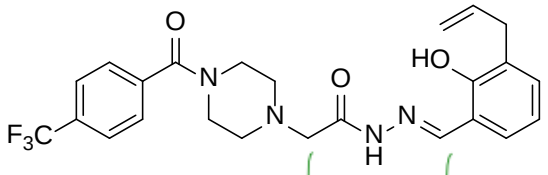
(125 MHz, $CDCl_3$)



8

^{19}F-NMR

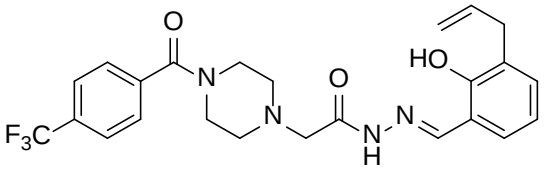
(470 MHz, $CDCl_3$)



9

^{1}H-NMR

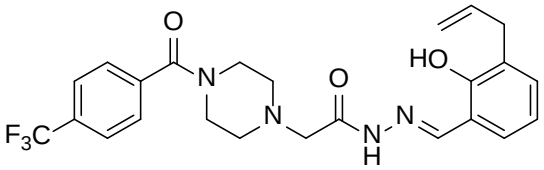
(500 MHz, $CDCl_3$)



9

^{13}C-NMR

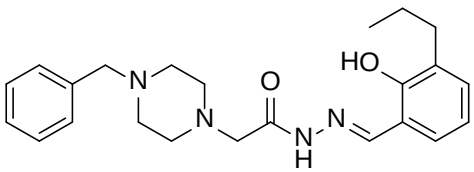
(125 MHz, $CDCl_3$)



9

^{19}F-NMR

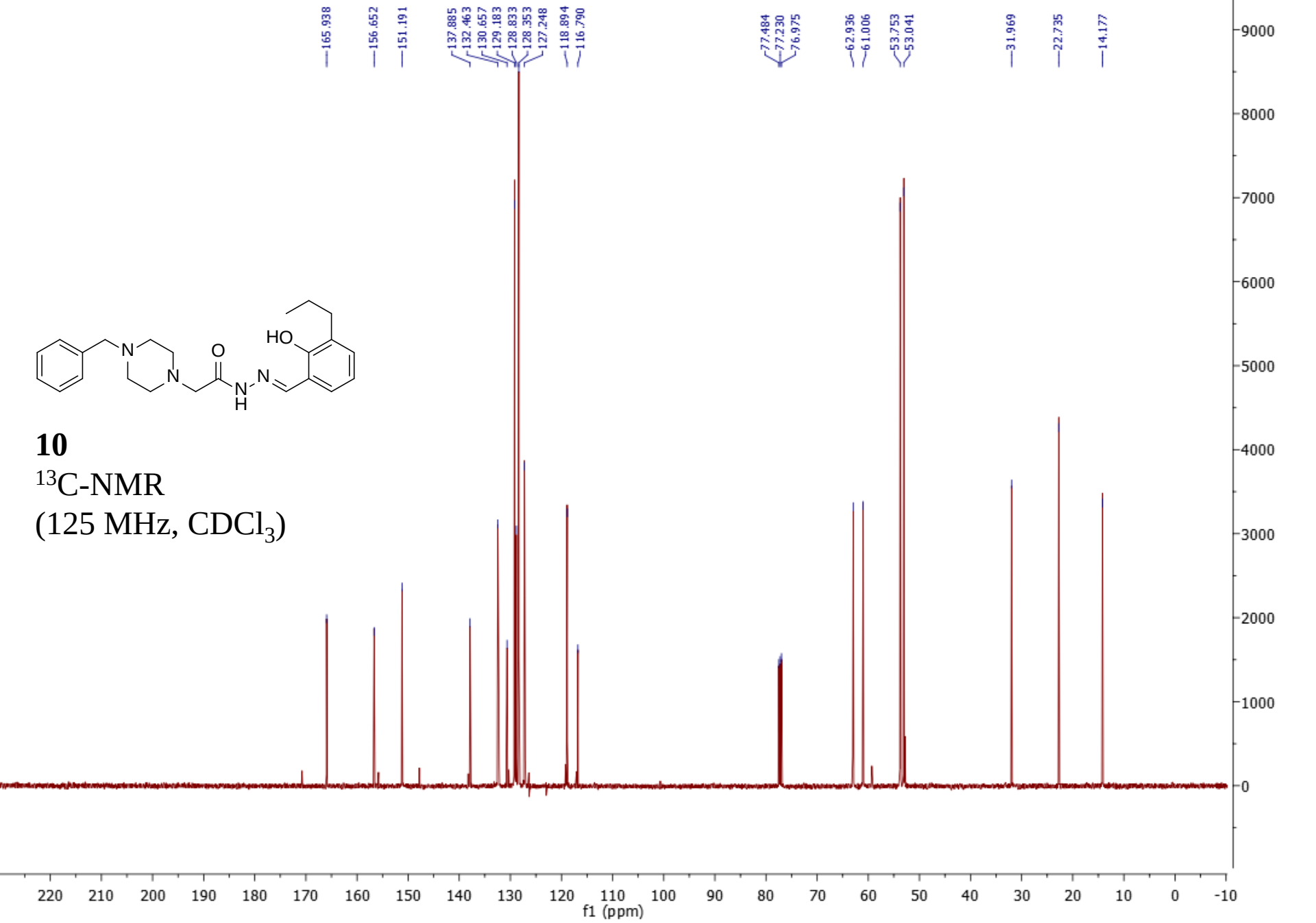
(470 MHz, $CDCl_3$)



10

^{1}H-NMR

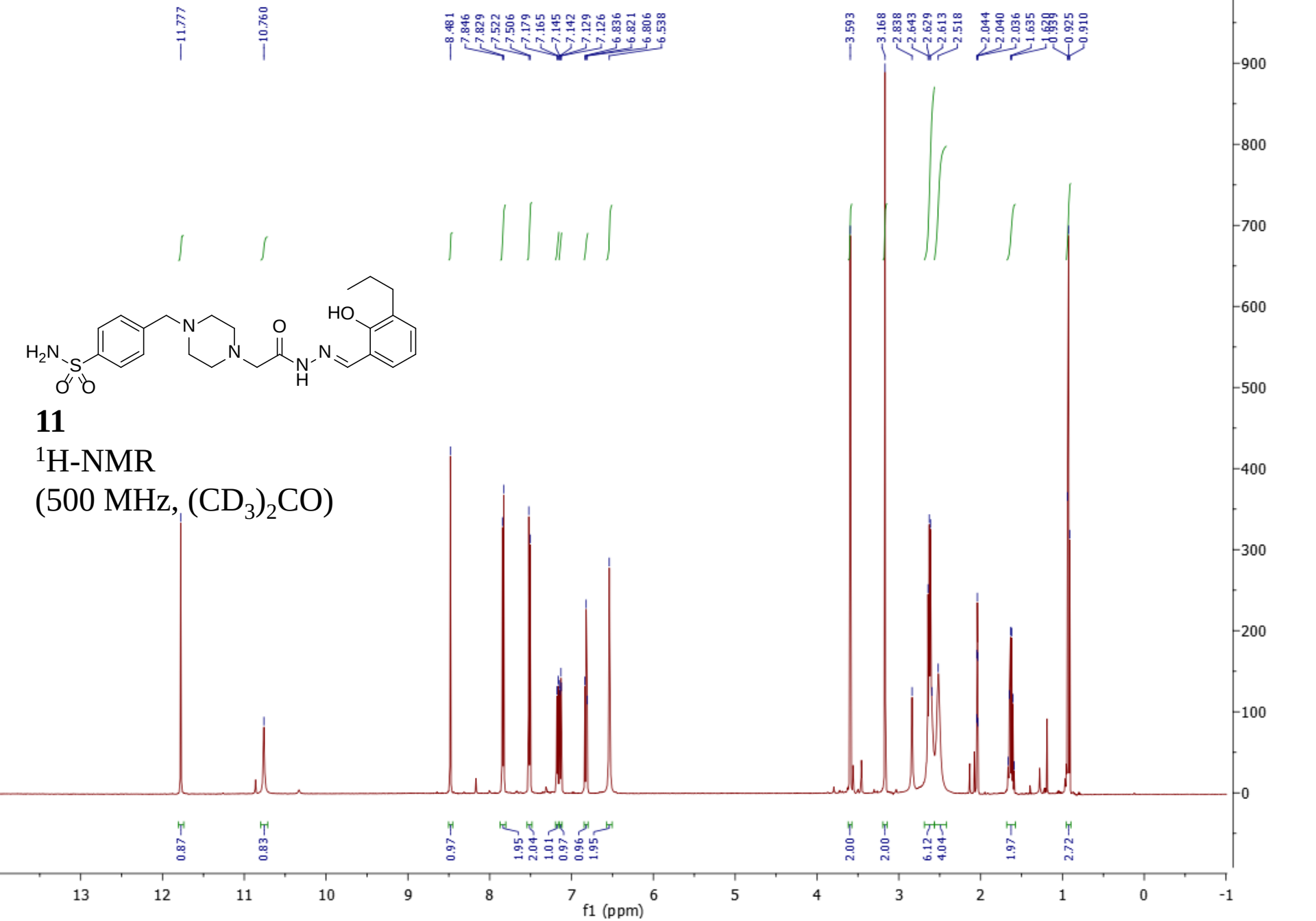
(500 MHz, $CDCl_3$)



10

^{13}C-NMR

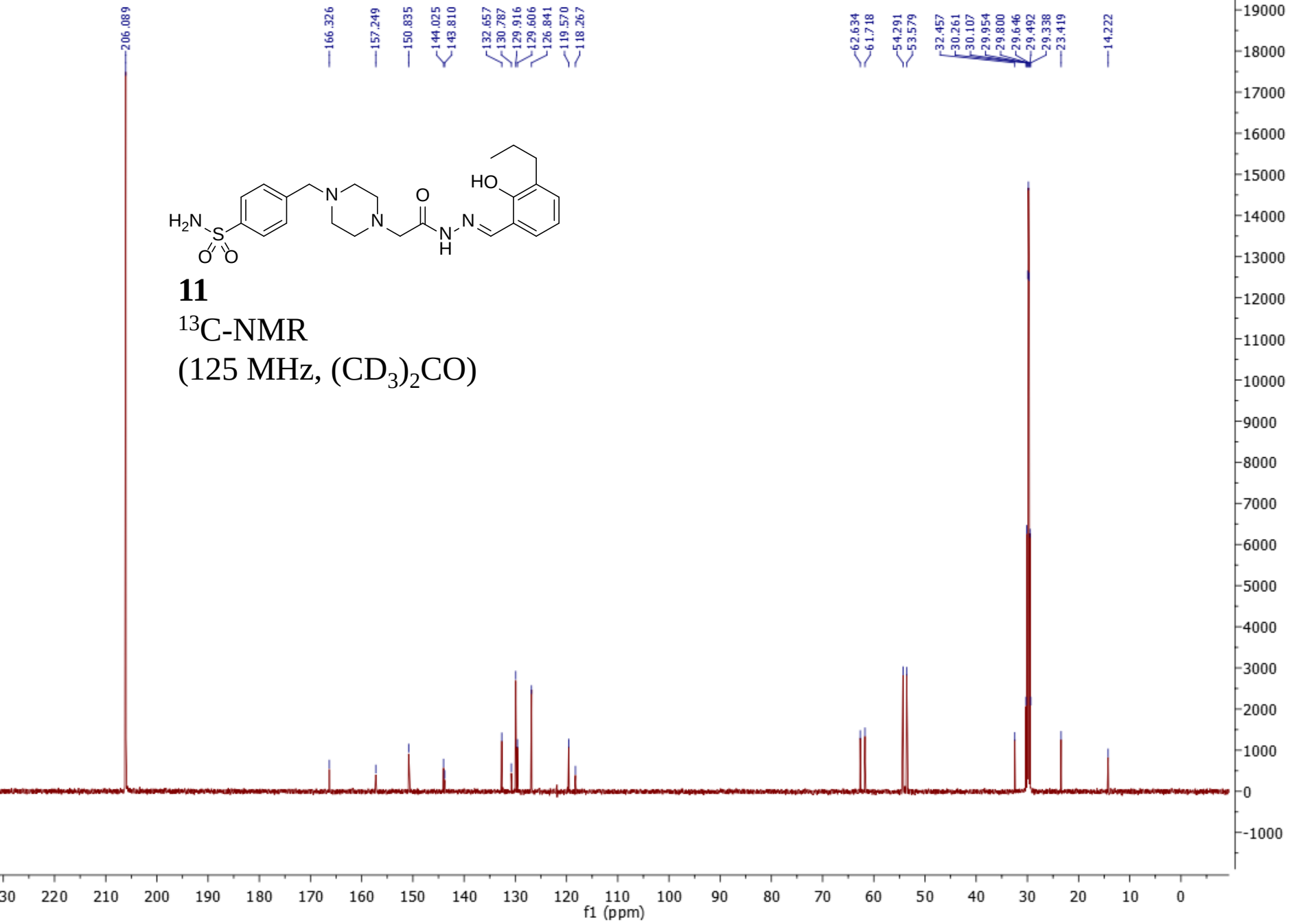
(125 MHz, $CDCl_3$)



11

^{1}H-NMR

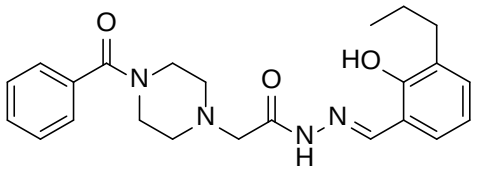
(500 MHz, $(CD_3)_2CO$)



11

^{13}C-NMR

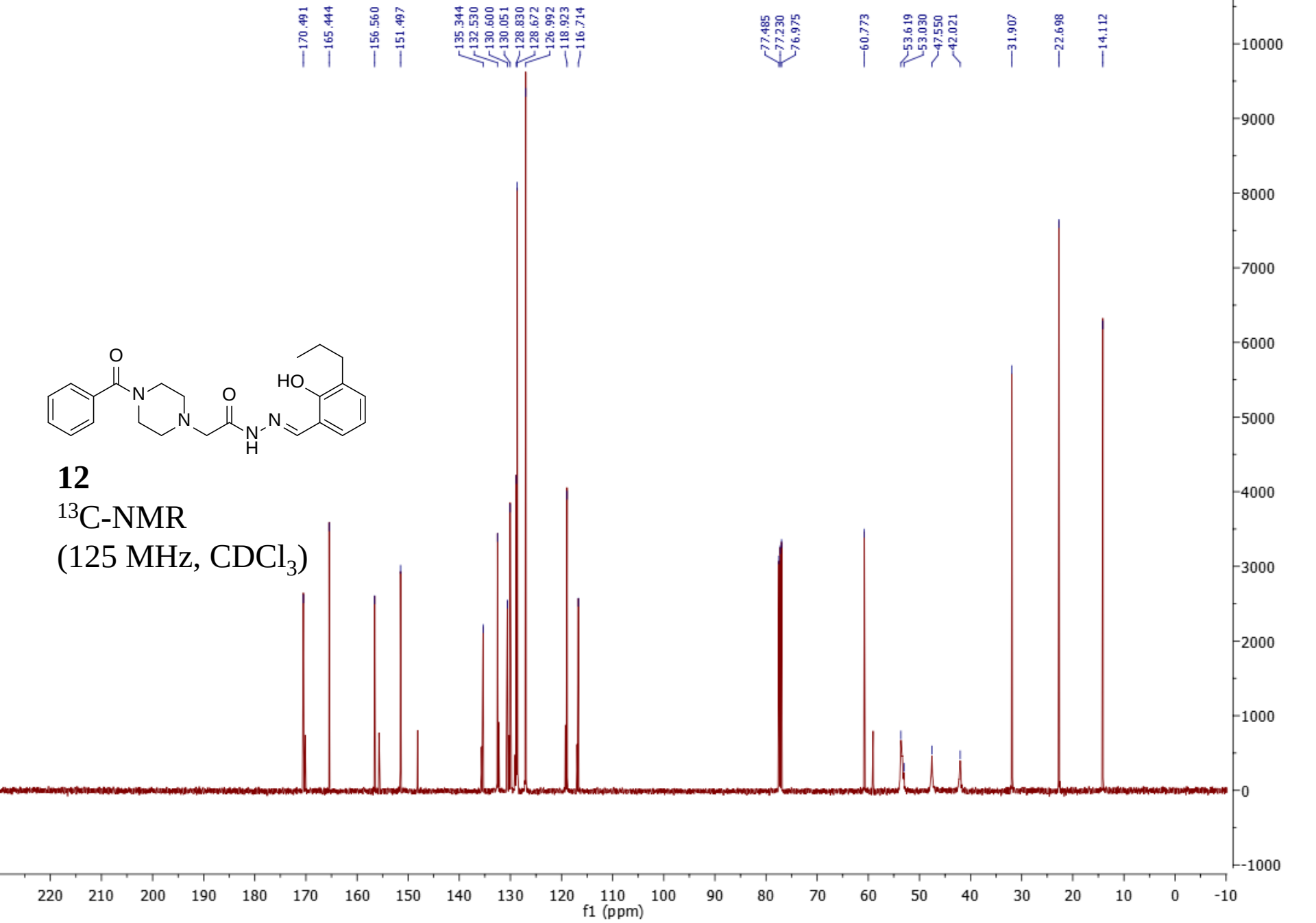
(125 MHz, $(CD_3)_2CO$)

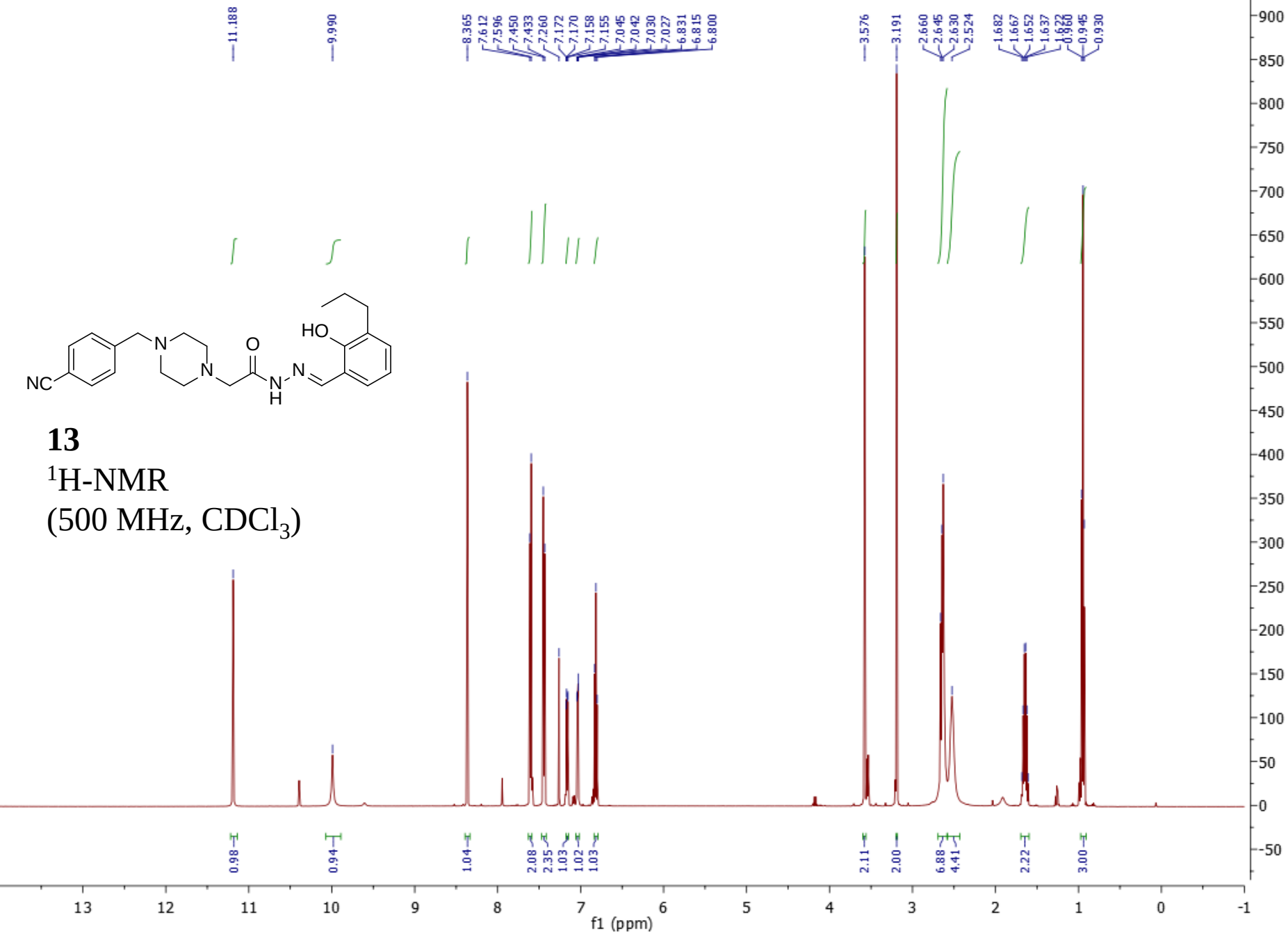


12

^{1}H-NMR

(500 MHz, $CDCl_3$)

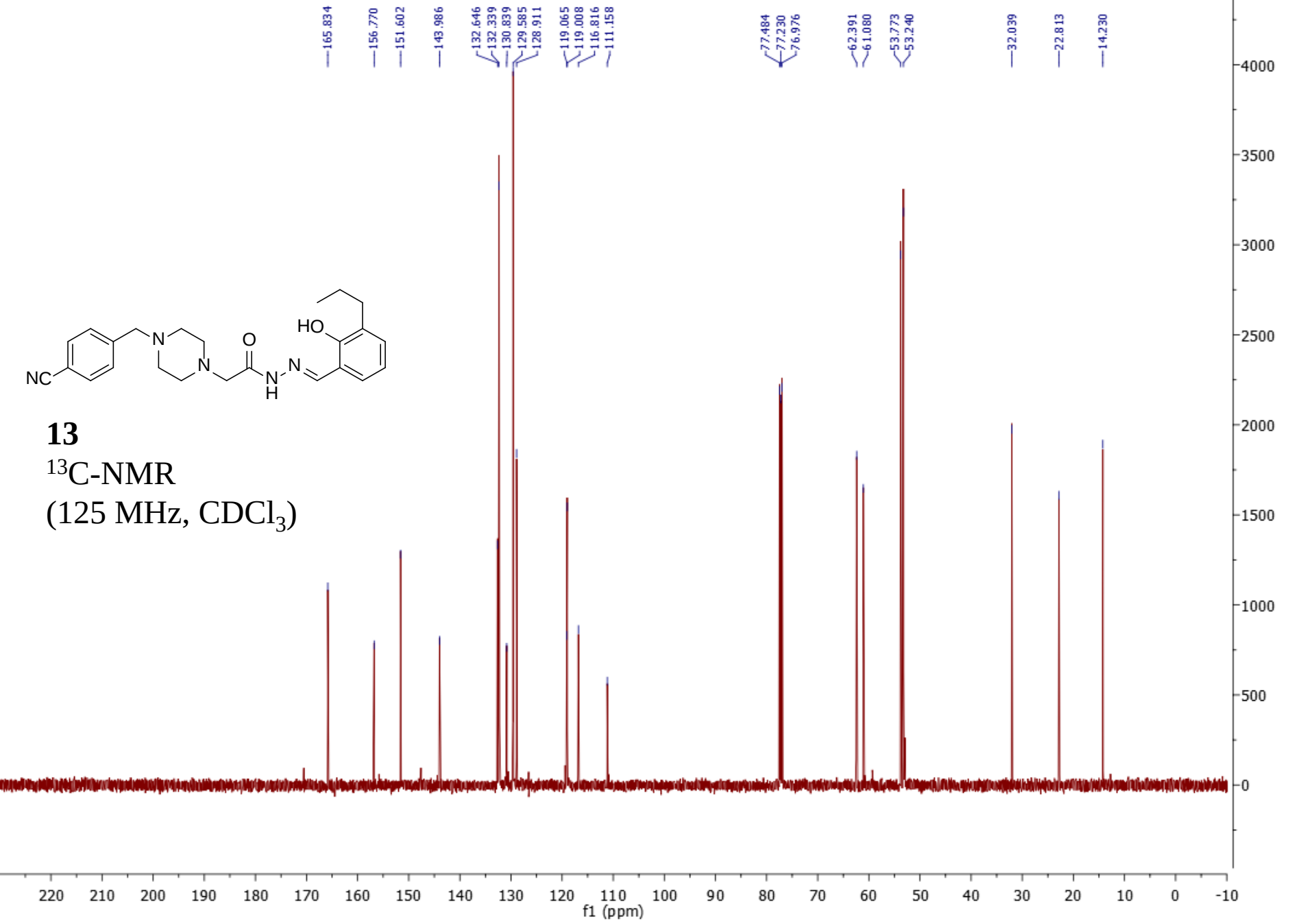

170.491
165.444
156.560
151.497
135.344
132.530
130.600
130.051
128.830
128.672
126.992
118.923
116.714
77.485
77.230
76.975
60.773
53.619
53.030
47.550
42.021
31.907
22.698
14.112
HO
O
N
O
N
N
H
12
13C-NMR
(125 MHz, CDCl3)
f1 (ppm)



13

^{1}H-NMR

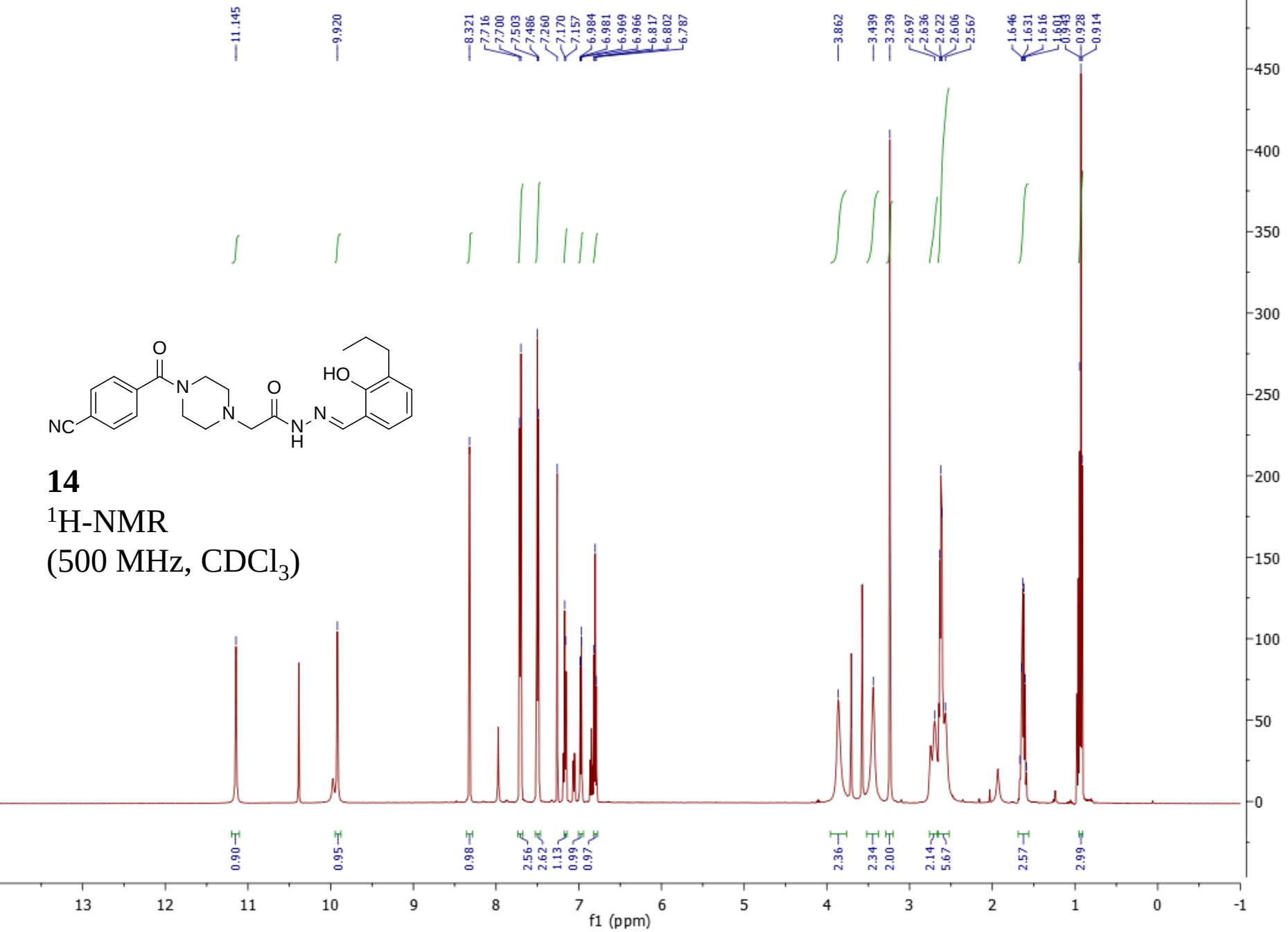
(500 MHz, $CDCl_3$)



13

^{13}C-NMR

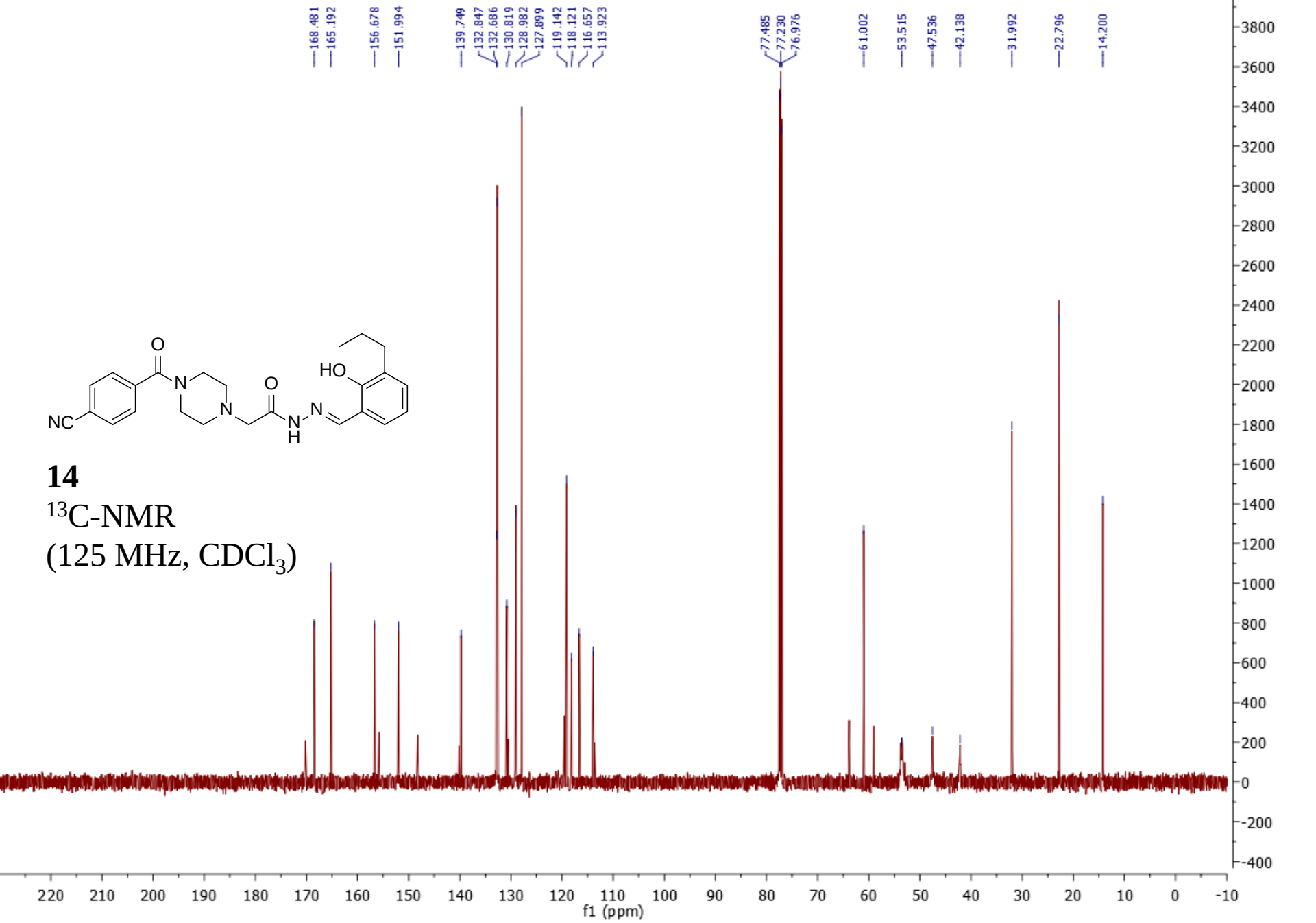
(125 MHz, $CDCl_3$)

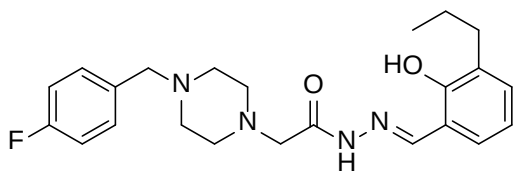


14

^{1}H-NMR

(500 MHz, $CDCl_3$)

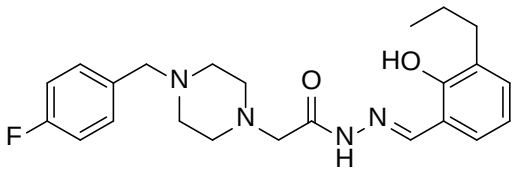

168.481
165.192
156.678
151.994
139.749
132.847
132.686
130.819
128.982
127.899
119.142
118.121
116.657
113.923
77.485
77.230
76.976
61.002
53.515
47.536
42.138
31.992
22.796
14.200
O
N
N
O
N
H
N
HO
NC
14
13C-NMR
(125 MHz, CDCl3)
f1 (ppm)



15

^{1}H-NMR

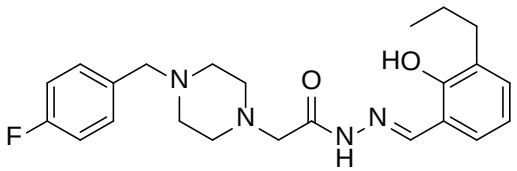
(500 MHz, $CDCl_3$)



15

^{13}C-NMR

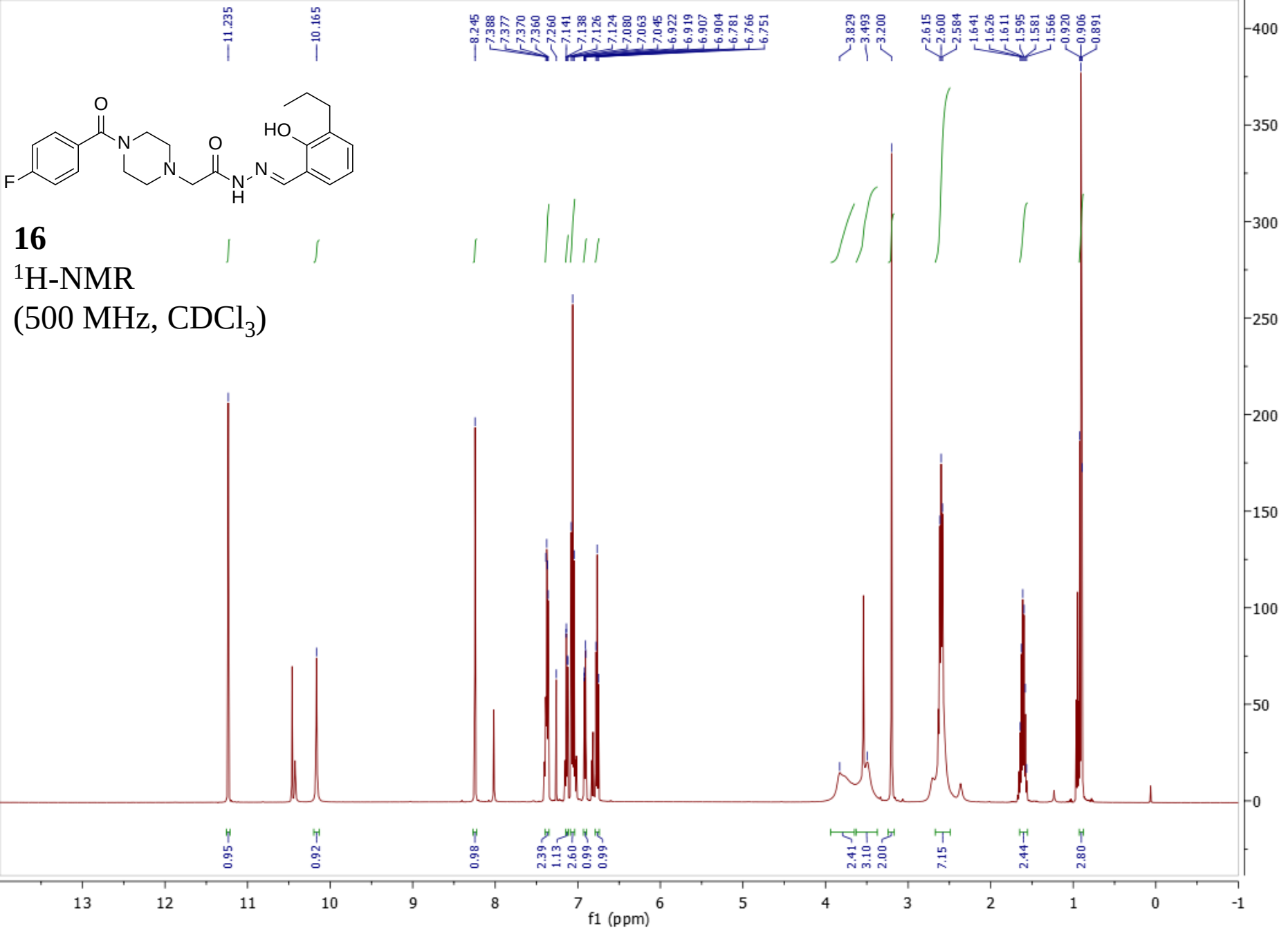
(125 MHz, $CDCl_3$)



15

^{19}F-NMR

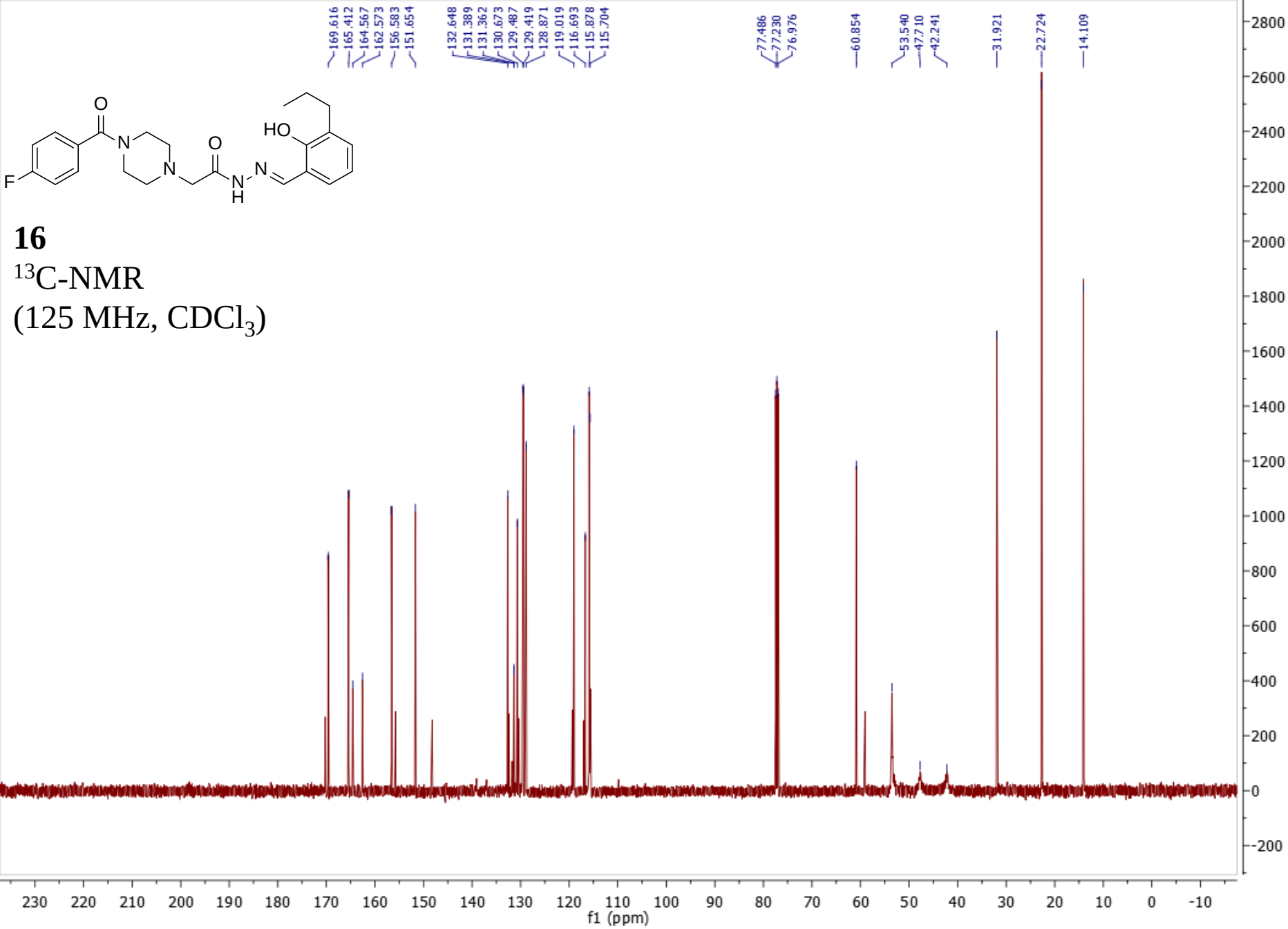
(470 MHz, $CDCl_3$)



16

^{1}H-NMR

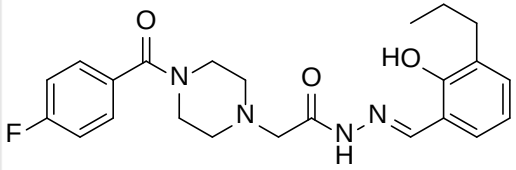
(500 MHz, $CDCl_3$)



16

^{13}C-NMR

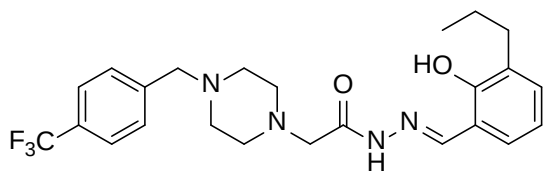
(125 MHz, $CDCl_3$)



16

^{19}F-NMR

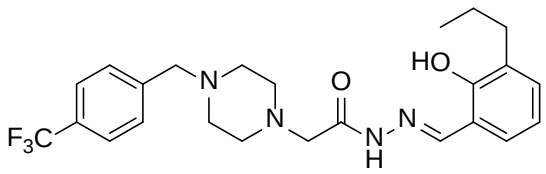
(470 MHz, $CDCl_3$)



17

^{1}H-NMR

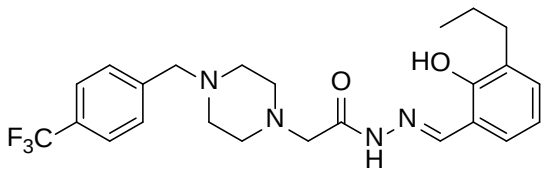
(500 MHz, $CDCl_3$)



17

^{13}C-NMR

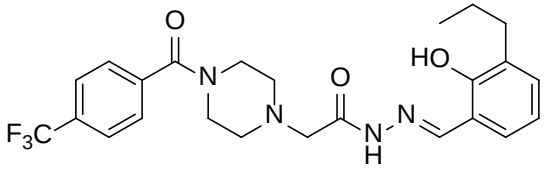
(125 MHz, $CDCl_3$)



17

^{19}F-NMR

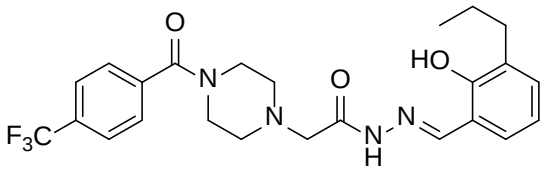
(470 MHz, $CDCl_3$)



18

^{1}H-NMR

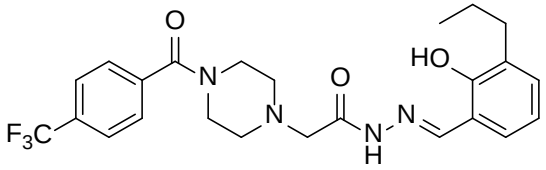
(500 MHz, $CDCl_3$)



18

^{13}C-NMR

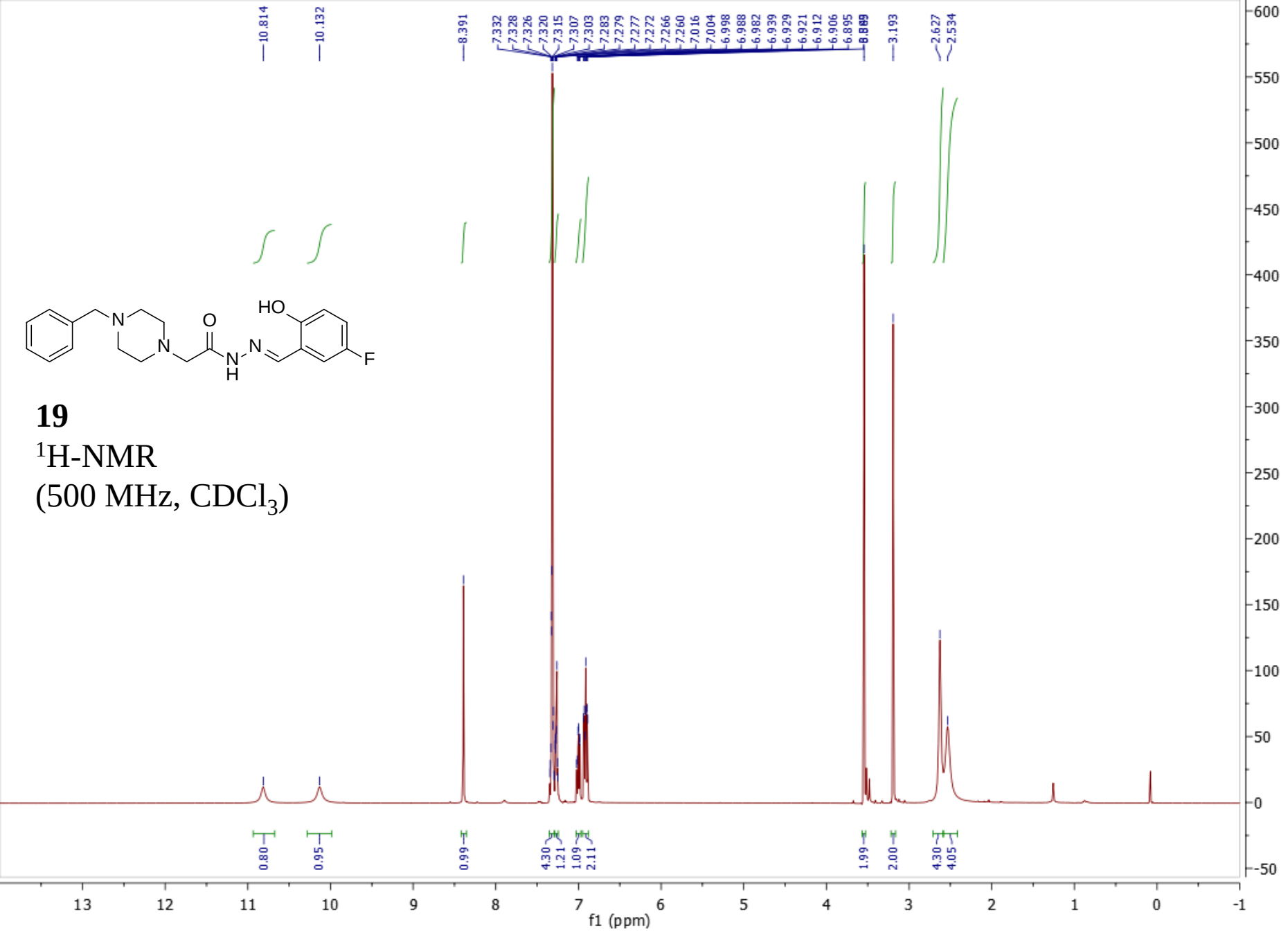
(125 MHz, $CDCl_3$)



18

^{19}F-NMR

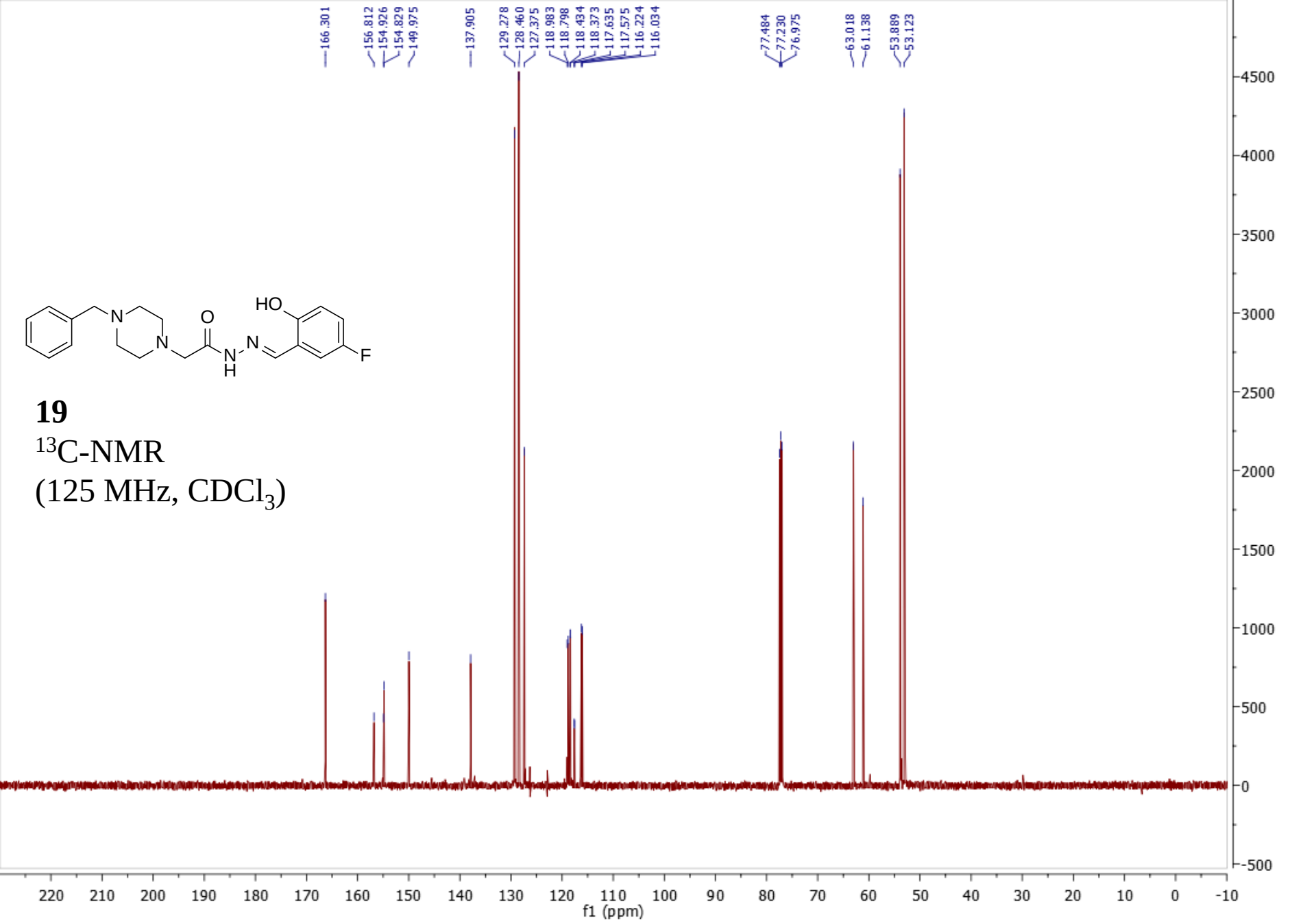
(470 MHz, $CDCl_3$)



19

^{1}H-NMR

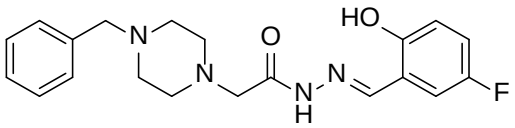
(500 MHz, $CDCl_3$)



19

^{13}C-NMR

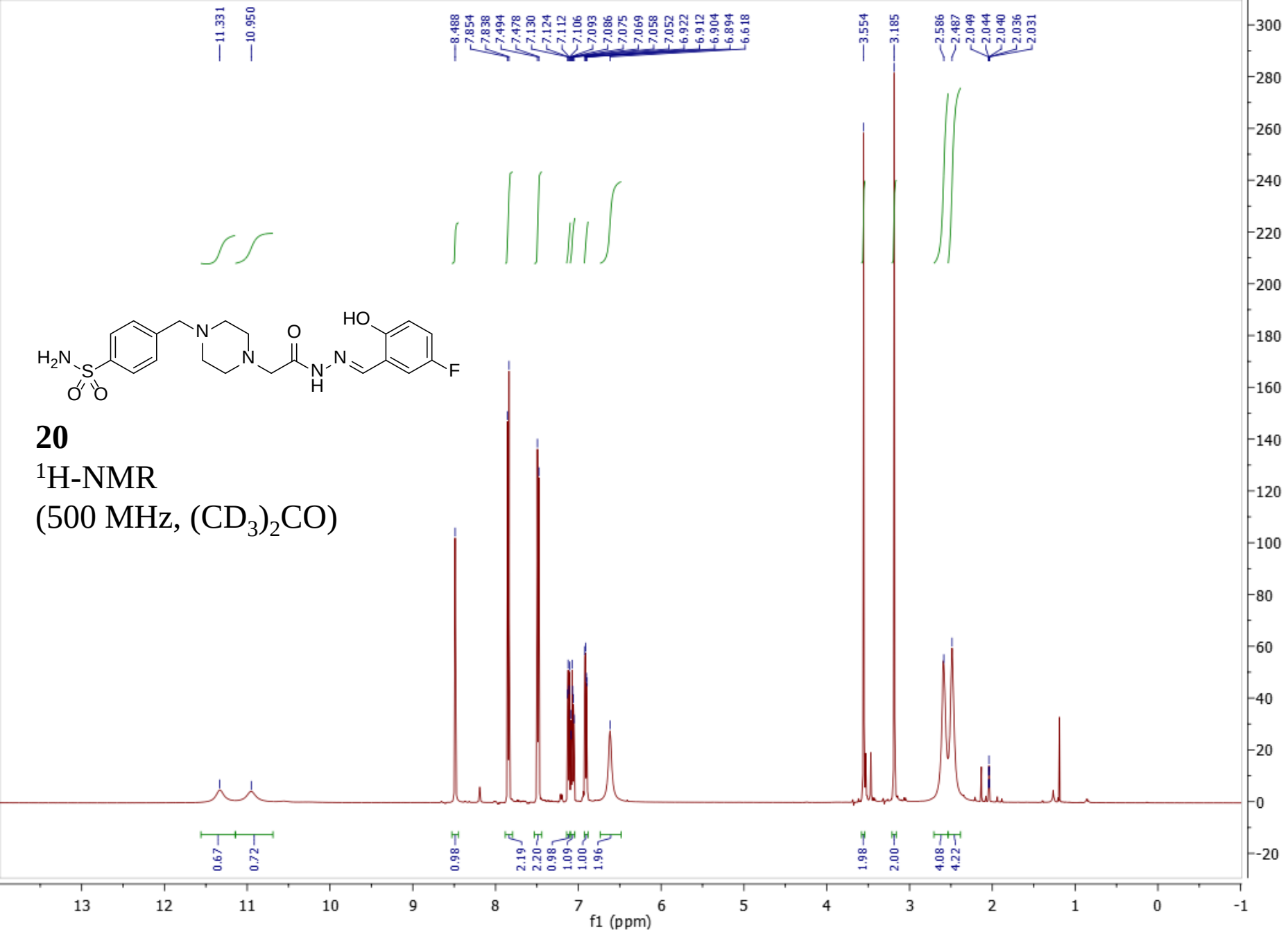
(125 MHz, $CDCl_3$)



19

^{19}F-NMR

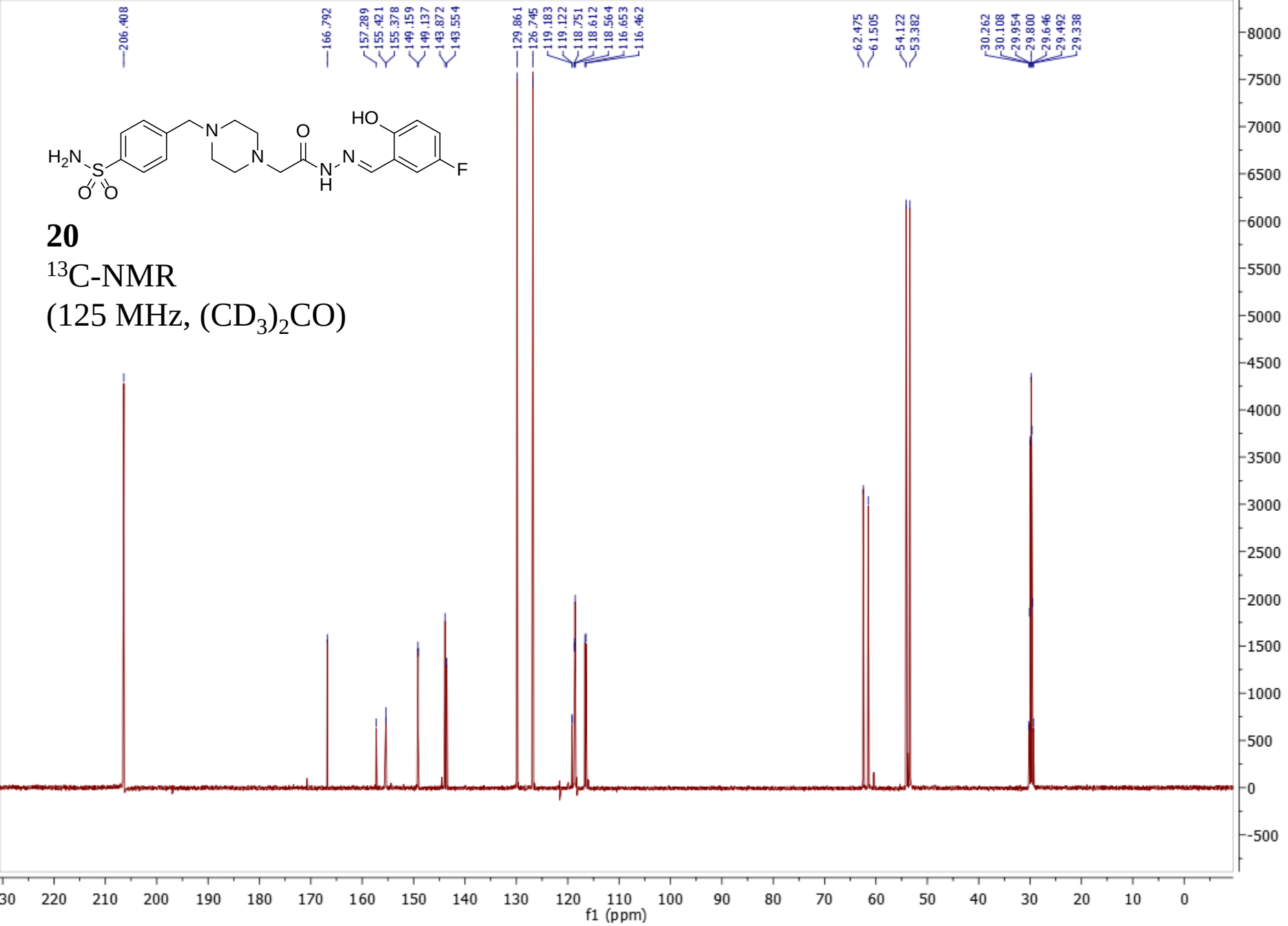
(470 MHz, $CDCl_3$)



20

^{1}H-NMR

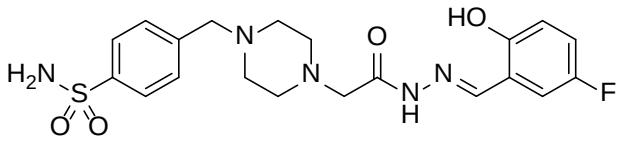
(500 MHz, $(CD_3)_2CO$)



20

^{13}C-NMR

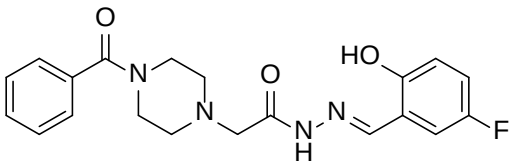
(125 MHz, $(CD_3)_2CO$)



20

^{19}F-NMR

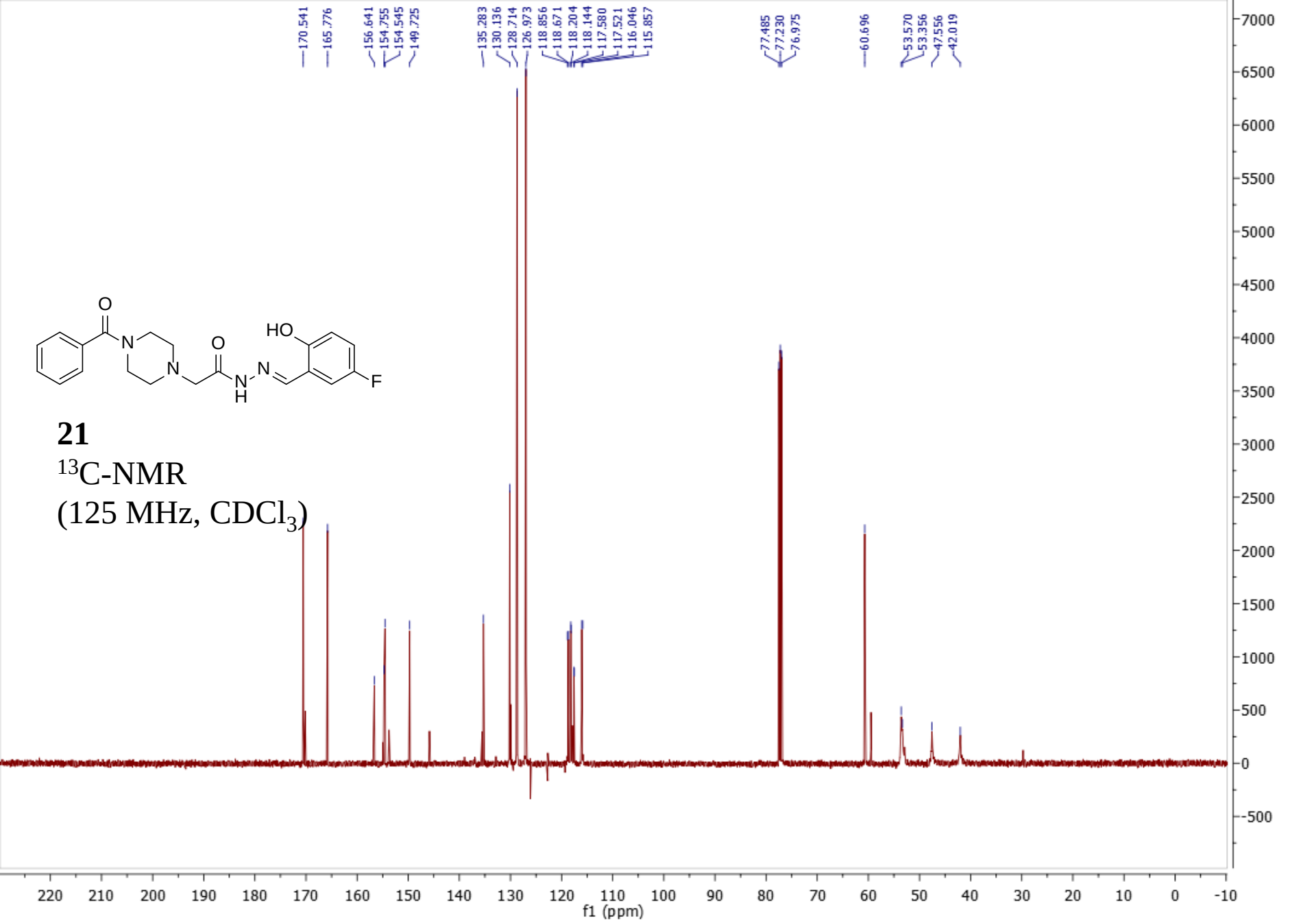
(125 MHz, $(CD_3)_2CO$)



21

^{1}H-NMR

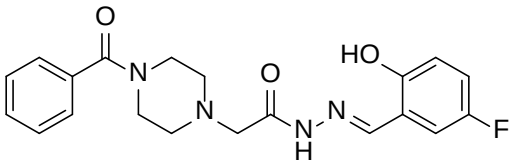
(500 MHz, $CDCl_3$)



21

^{13}C-NMR

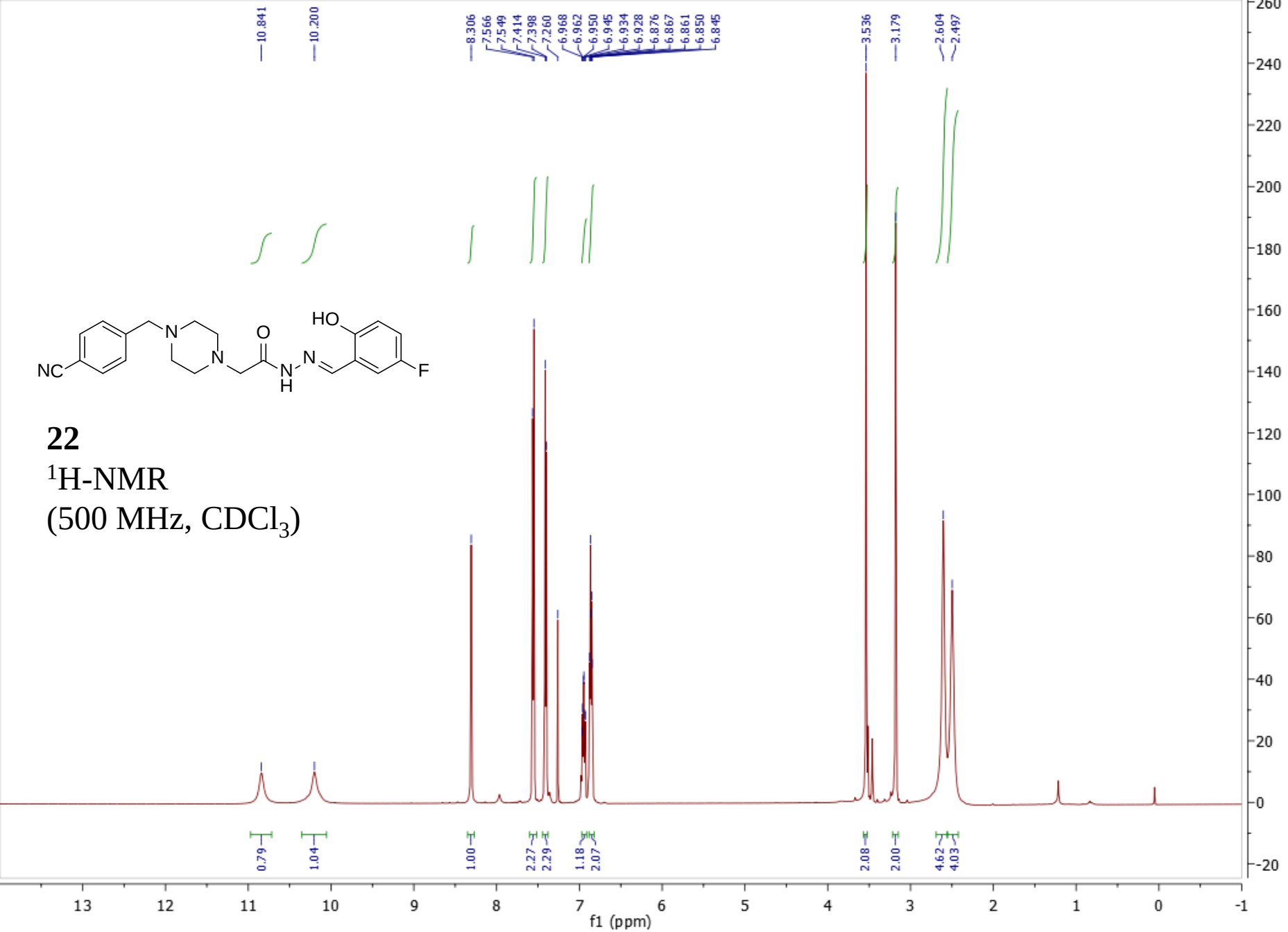
(125 MHz, $CDCl_3$)



21

^{19}F-NMR

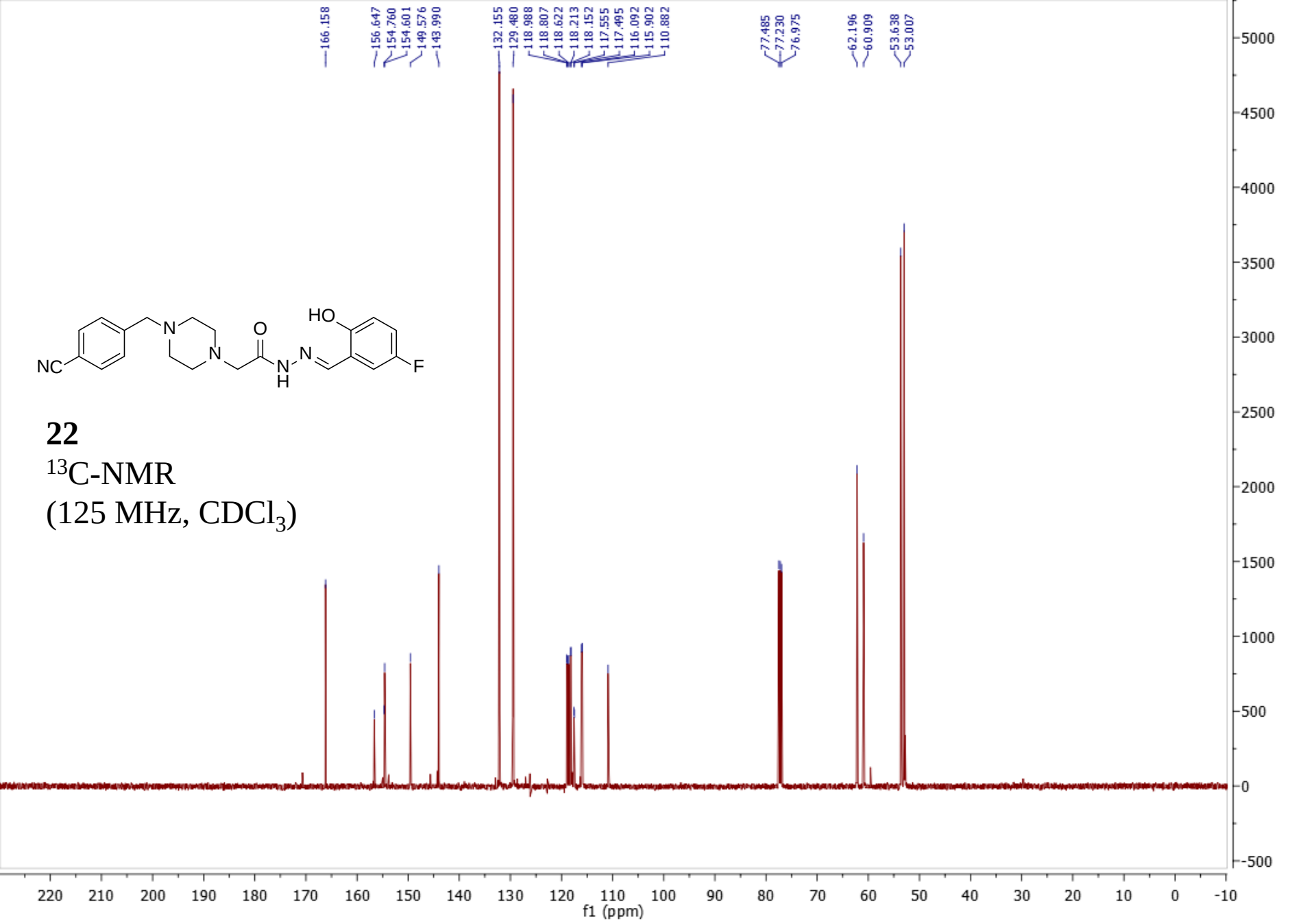
(470 MHz, $CDCl_3$)



22

^{1}H-NMR

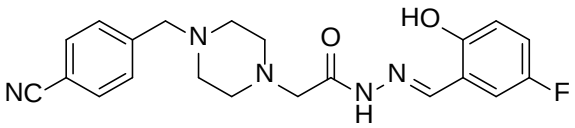
(500 MHz, $CDCl_3$)



22

^{13}C-NMR

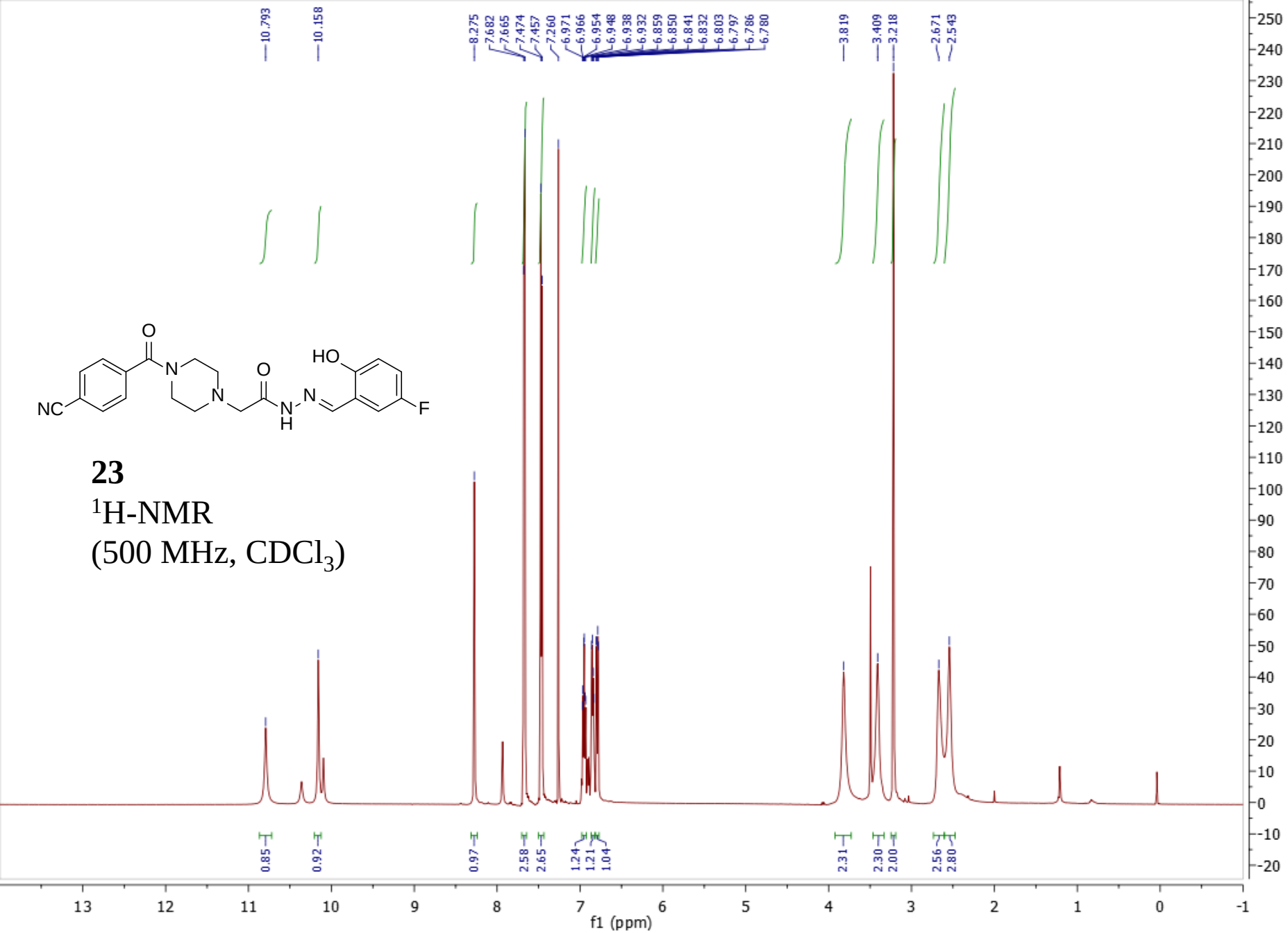
(125 MHz, $CDCl_3$)



22

^{19}F-NMR

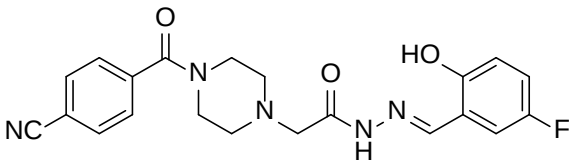
(470 MHz, $CDCl_3$)



23

^{1}H-NMR

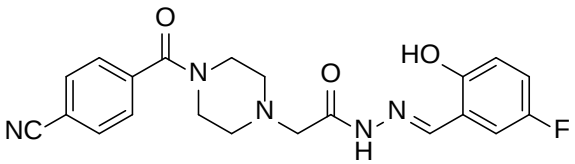
(500 MHz, $CDCl_3$)



23

^{13}C-NMR

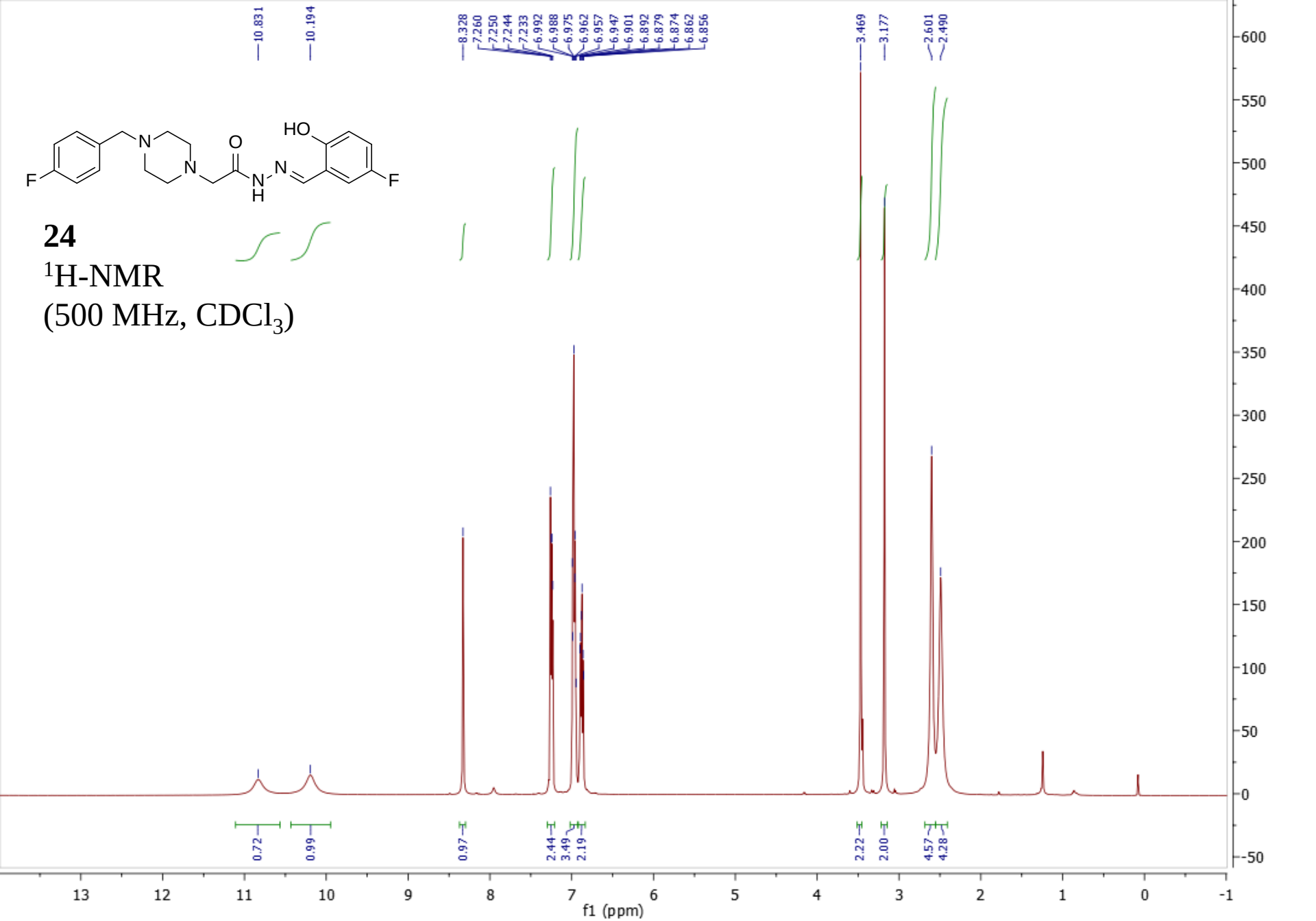
(125 MHz, $CDCl_3$)

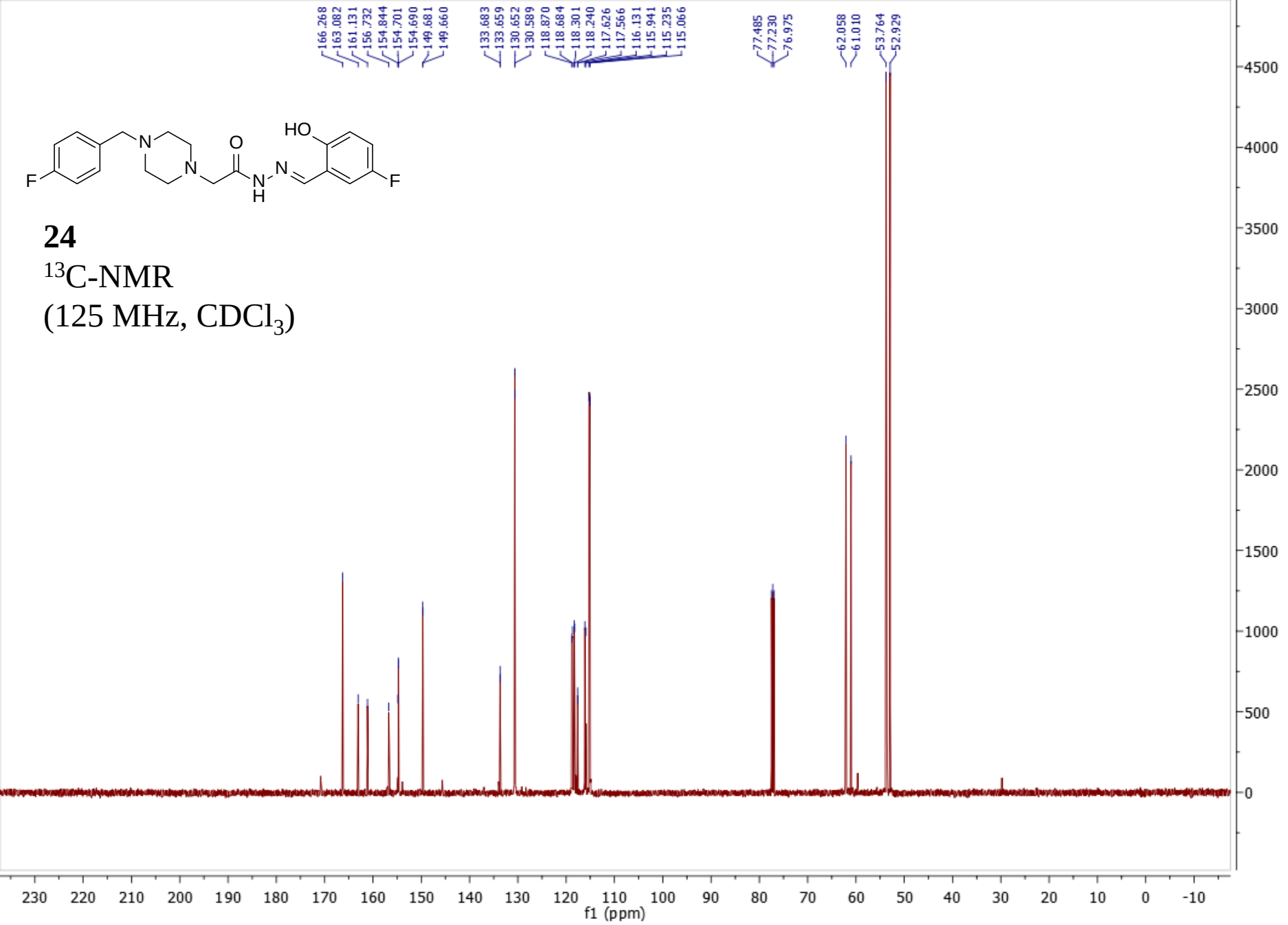


23

^{19}F-NMR

(470 MHz, $CDCl_3$)

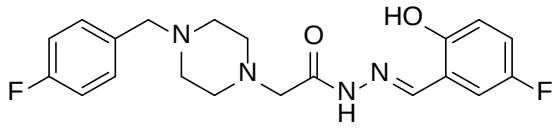

10.831
10.194
8.328
7.260
7.250
7.244
7.233
6.992
6.988
6.975
6.962
6.957
6.947
6.901
6.892
6.879
6.874
6.862
6.856
3.469
3.177
2.601
2.490
HO
O
N
N
N
H
F
F
24
1H-NMR
(500 MHz, CDCl3)
0.72
0.99
0.97
2.44
3.49
2.19
2.22
2.00
4.57
4.28
f1 (ppm)



24

^{13}C-NMR

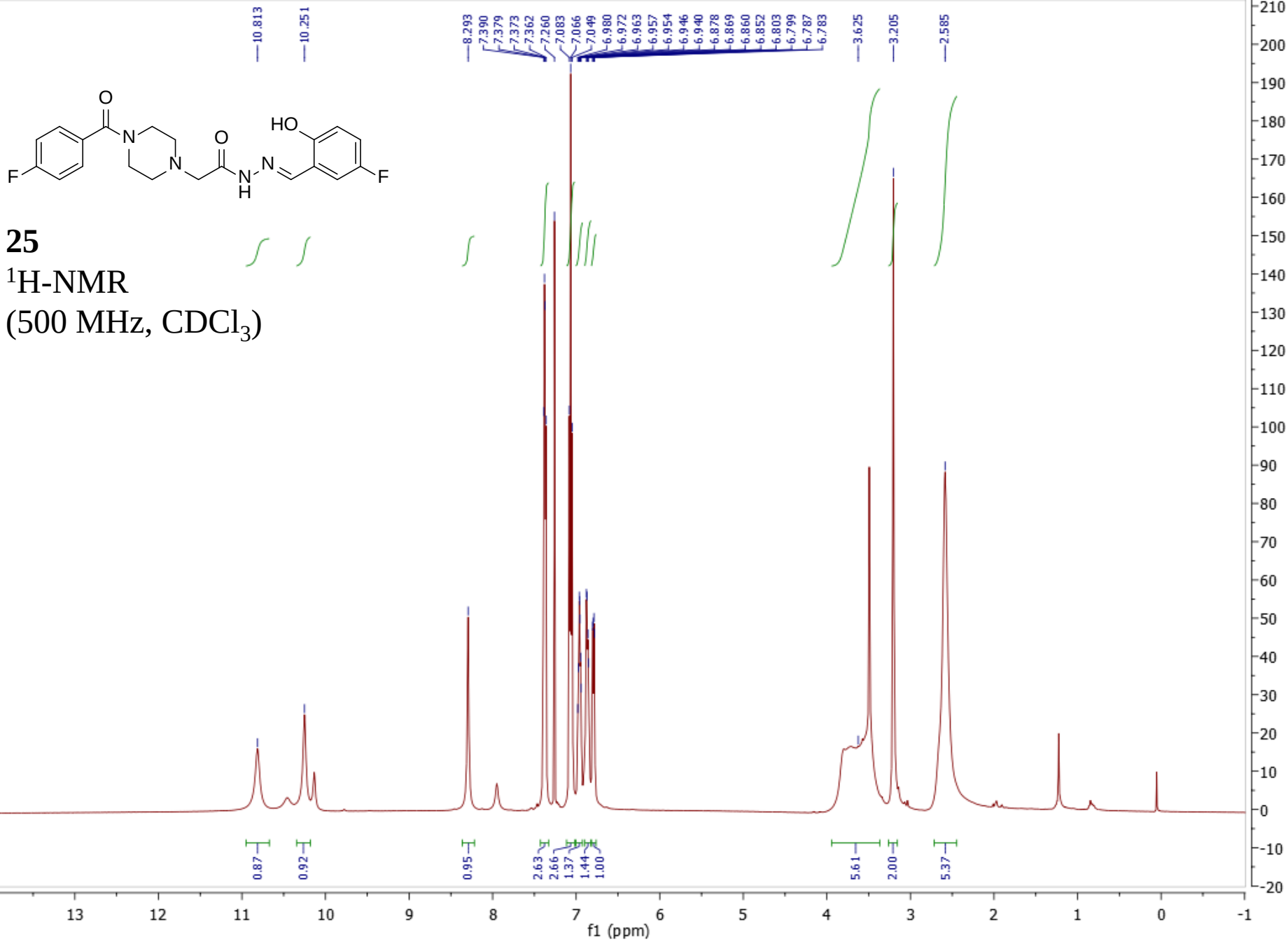
(125 MHz, $CDCl_3$)



24

^{19}F-NMR

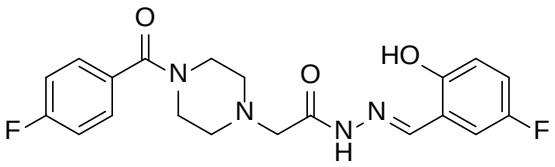
(470 MHz, $CDCl_3$)



25

^{1}H-NMR

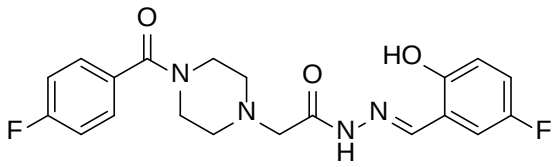
(500 MHz, $CDCl_3$)



25

^{13}C-NMR

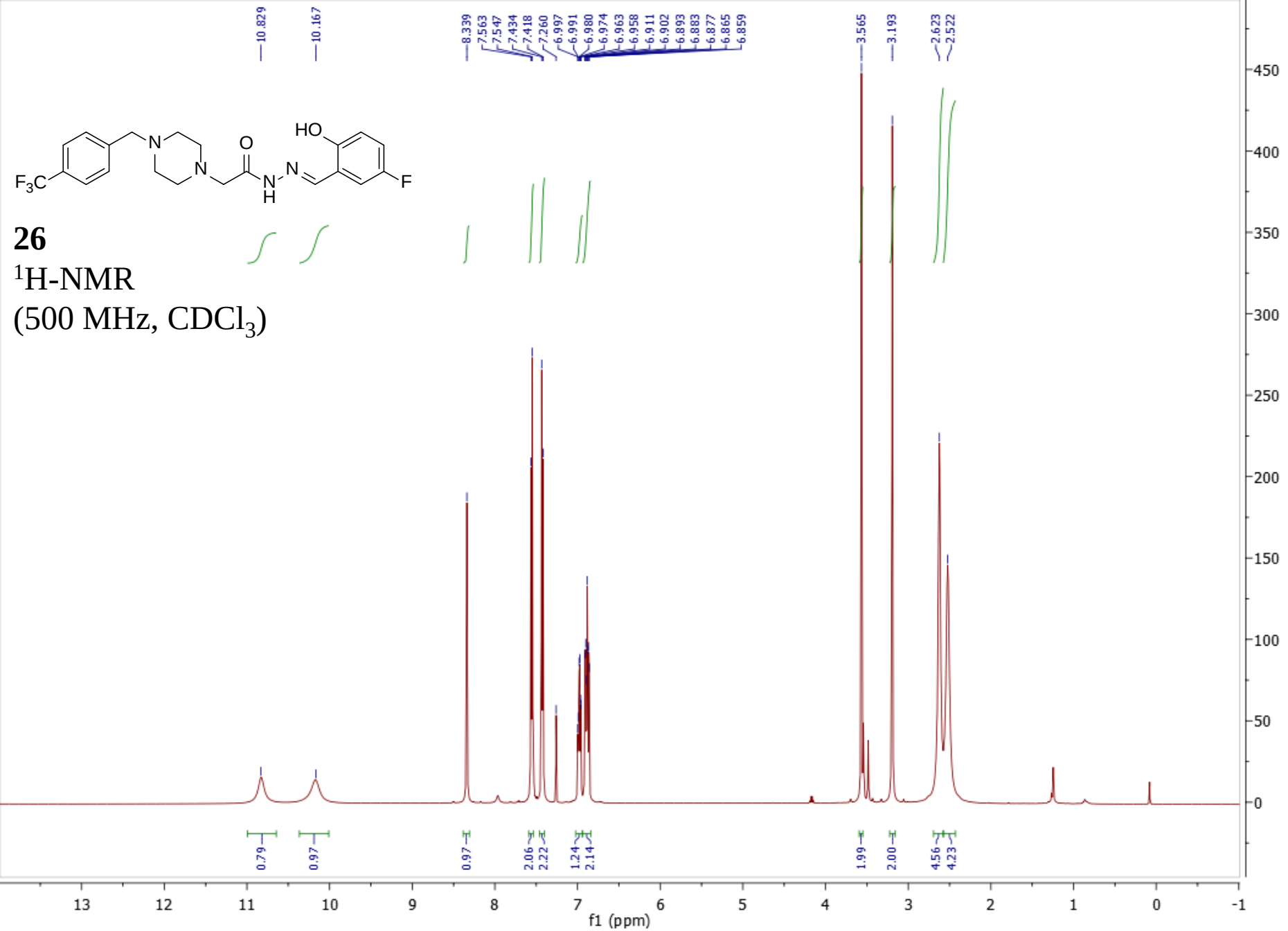
(125 MHz, $CDCl_3$)

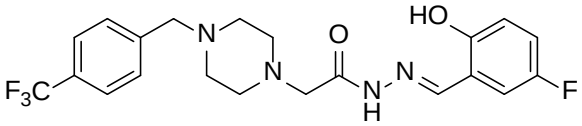


25

^{19}F-NMR

(470 MHz, $CDCl_3$)

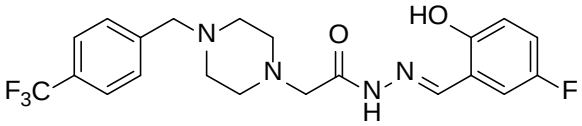

10.829
10.167
8.339
7.563
7.547
7.434
7.418
7.260
6.997
6.991
6.980
6.974
6.963
6.958
6.911
6.902
6.893
6.883
6.877
6.865
6.859
3.565
3.193
2.623
2.522
HO
N
O
N
N
N
H
F3C
F
26
1H-NMR
(500 MHz, CDCl3)
0.79
0.97
0.97
2.06
2.22
1.24
2.14
1.99
2.00
4.56
4.23
f1 (ppm)



26

^{13}C-NMR

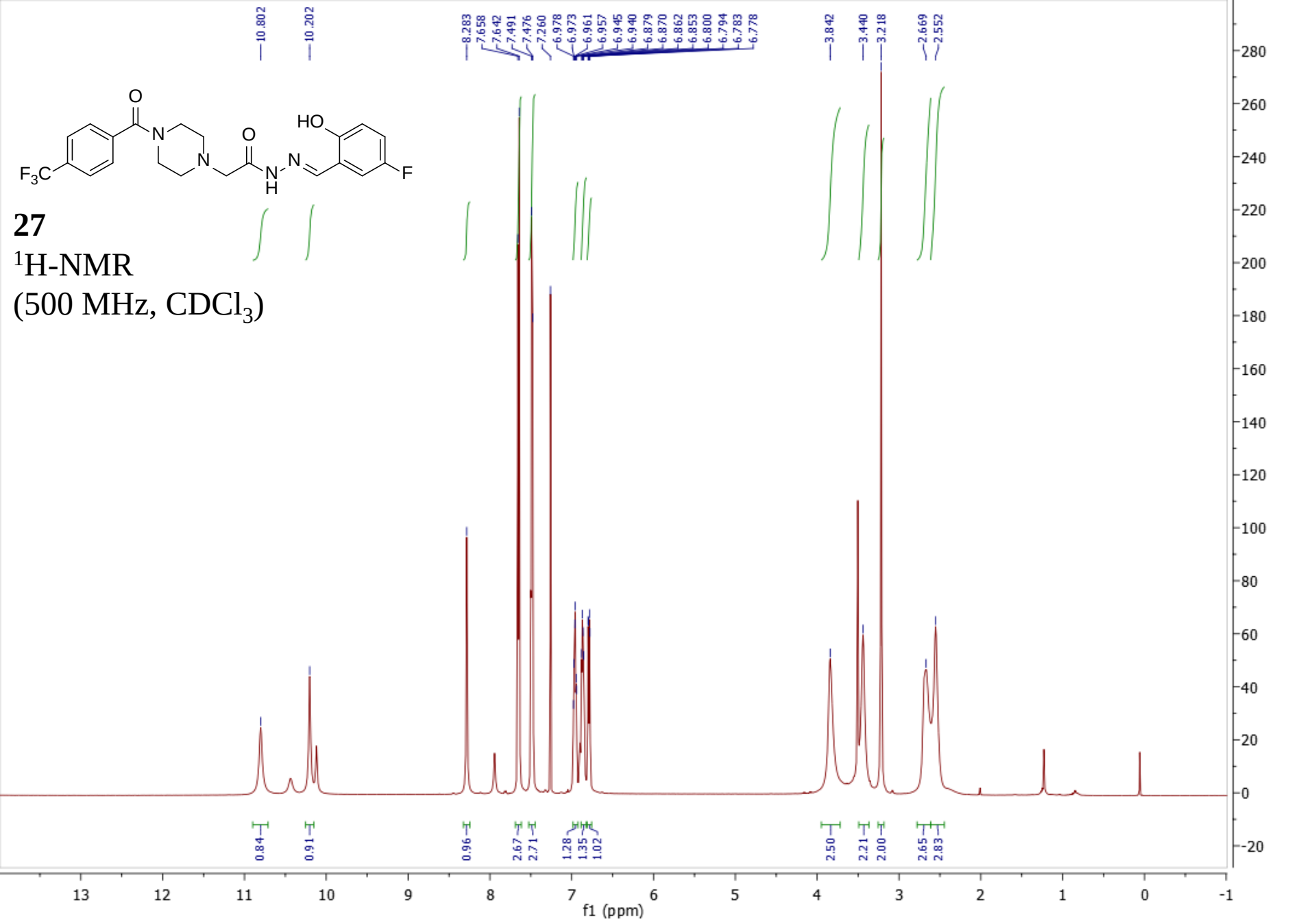
(125 MHz, $CDCl_3$)



26

^{19}F-NMR

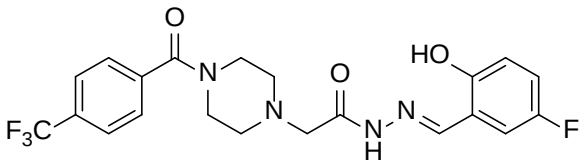
(470 MHz, $CDCl_3$)



27

^{1}H-NMR

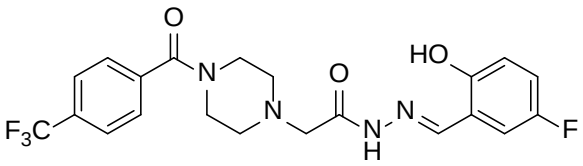
(500 MHz, $CDCl_3$)



27

^{13}C-NMR

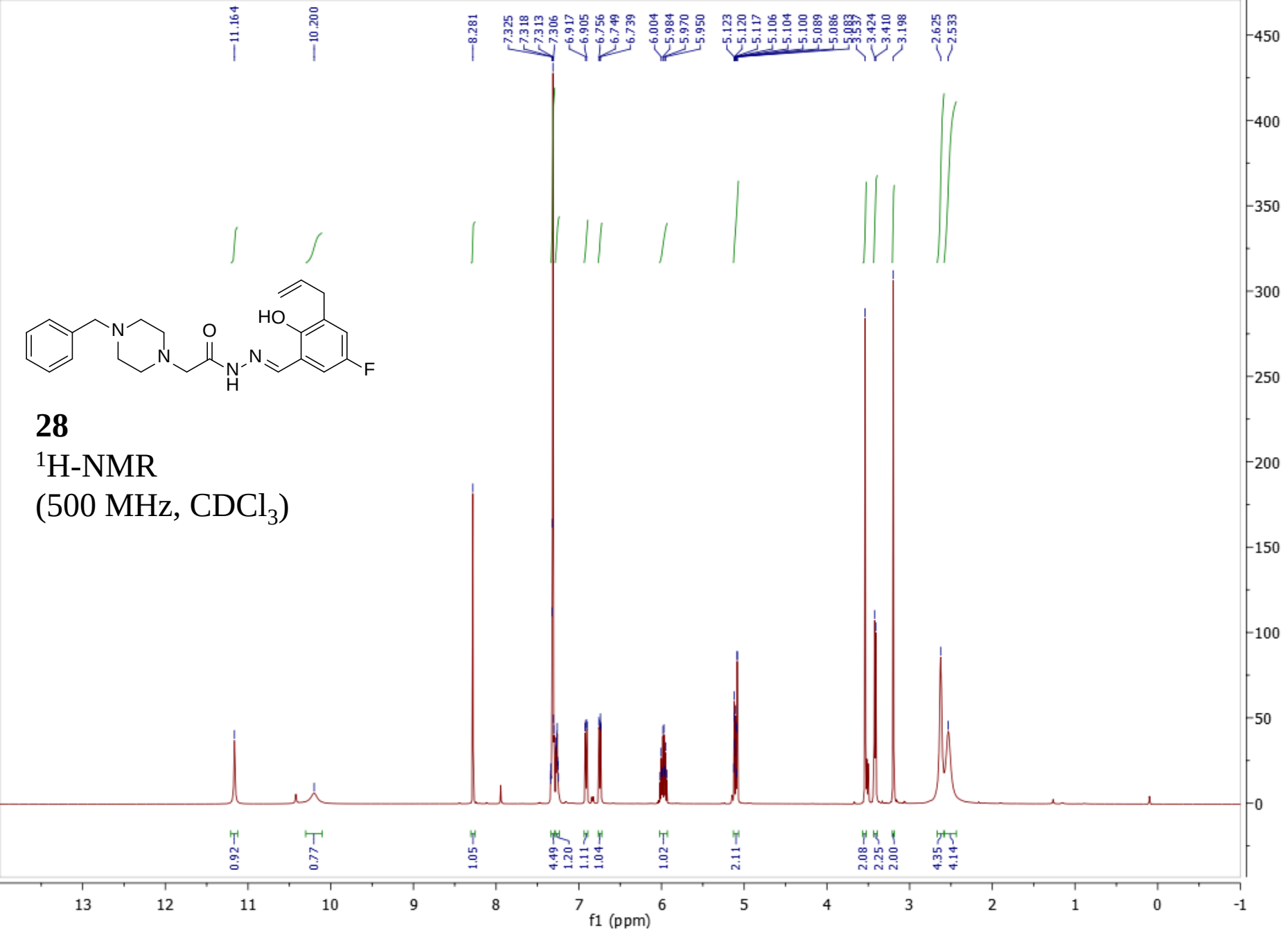
(125 MHz, $CDCl_3$)



27

^{19}F-NMR

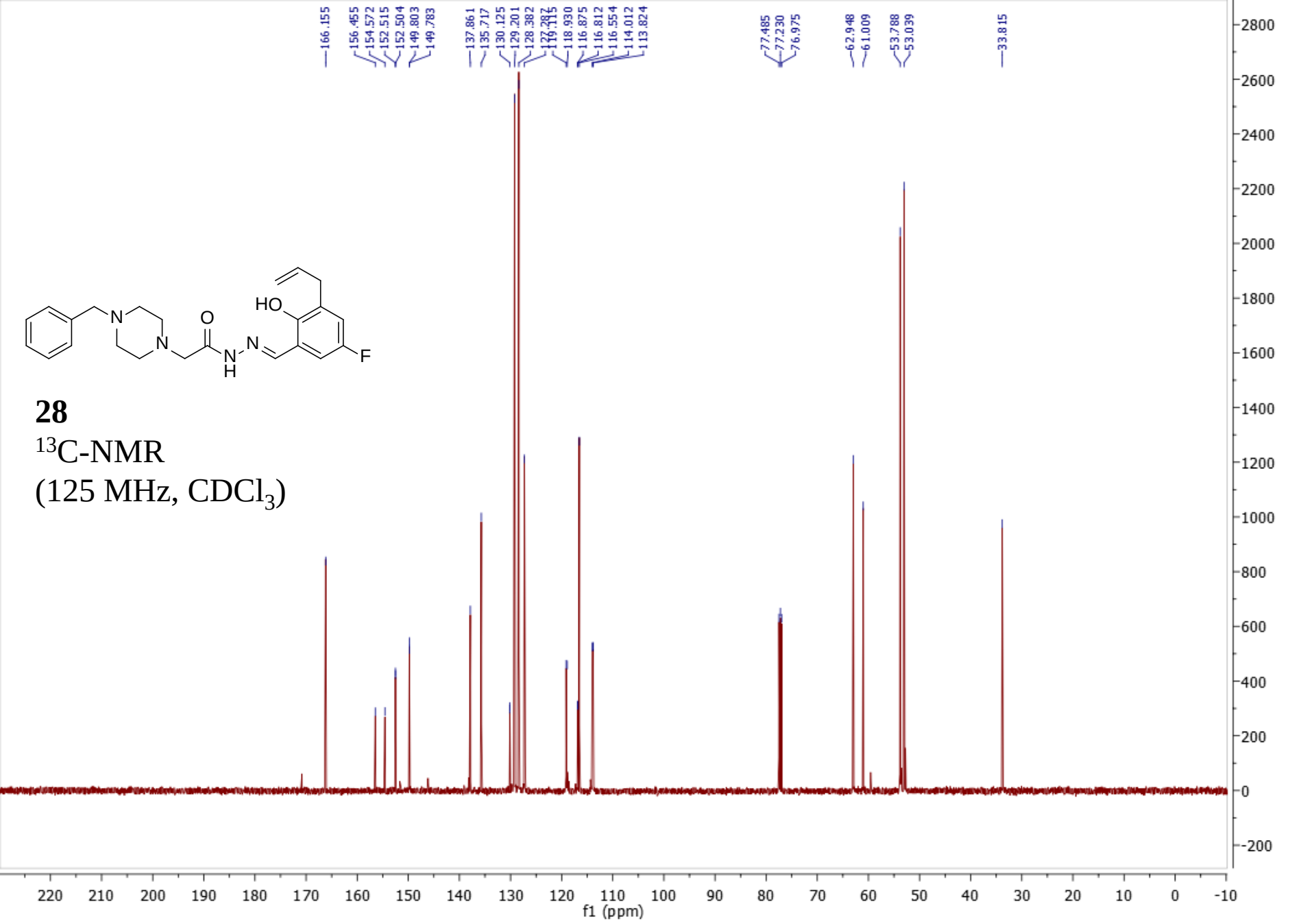
(470 MHz, $CDCl_3$)



28

^{1}H-NMR

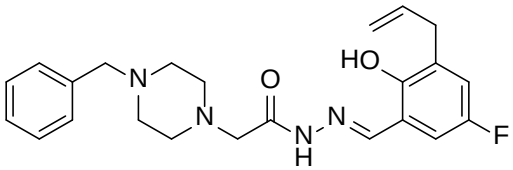
(500 MHz, $CDCl_3$)



28

^{13}C-NMR

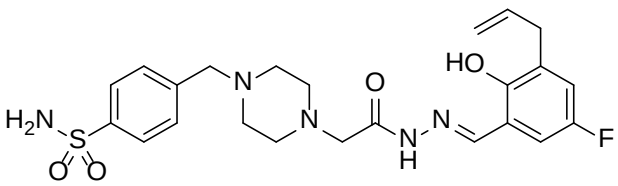
(125 MHz, $CDCl_3$)



28

^{19}F-NMR

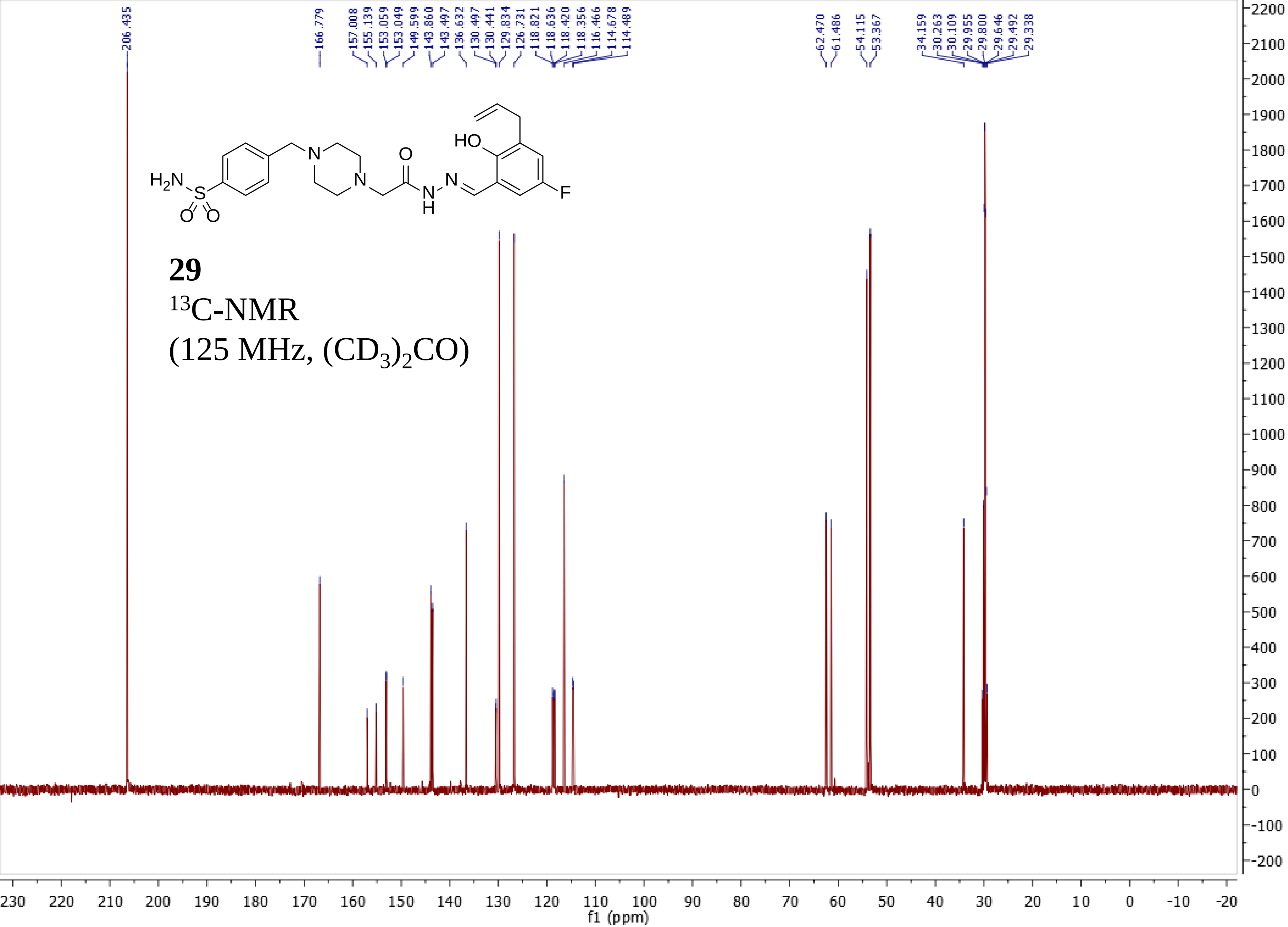
(470 MHz, $CDCl_3$)

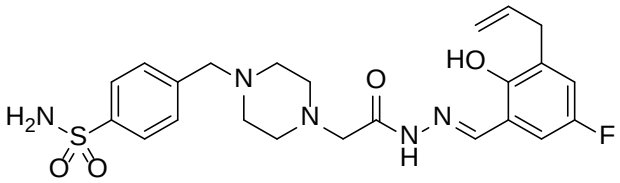


29

^{1}H-NMR

(500 MHz, $(CD_3)_2CO$)

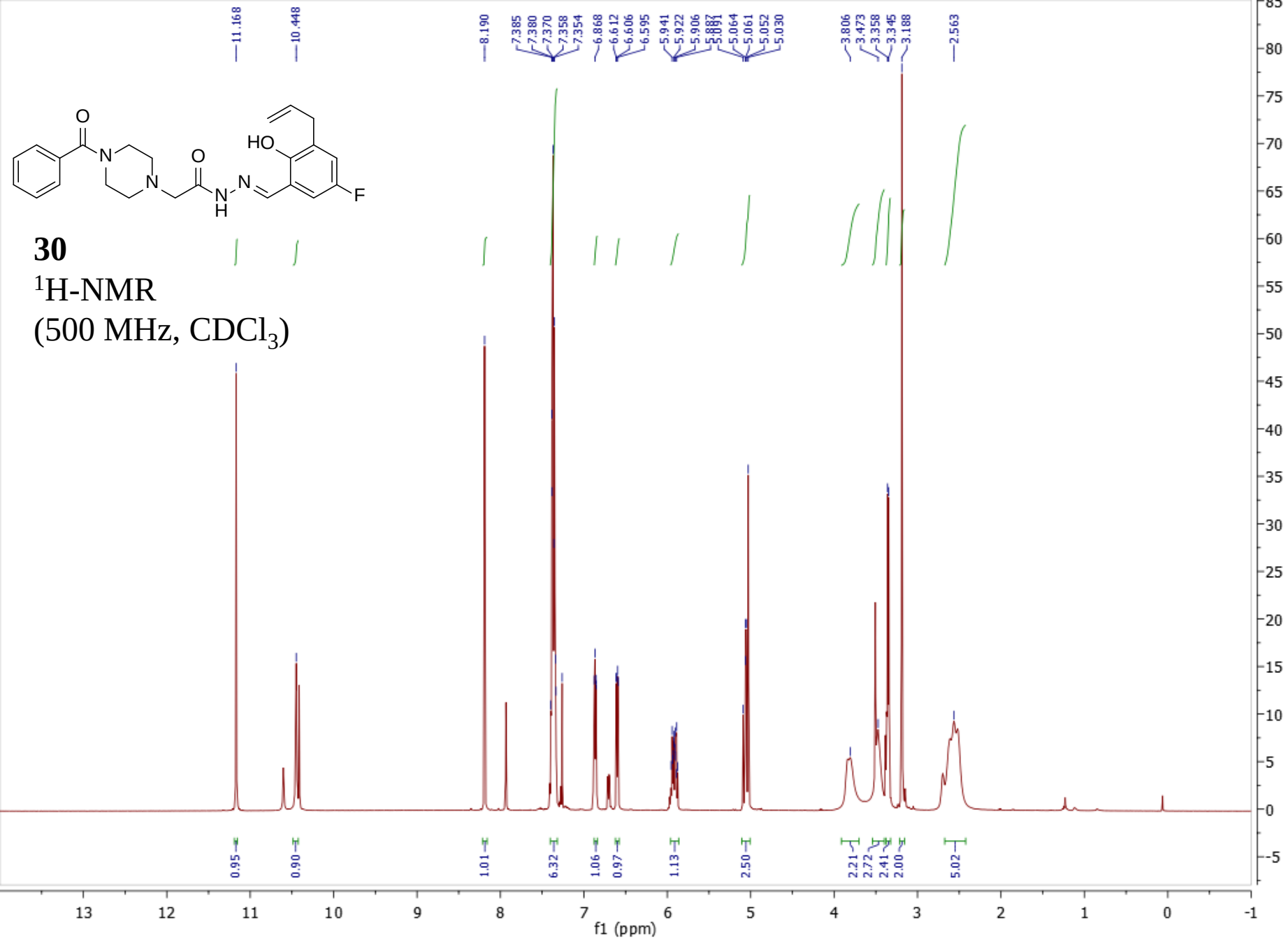

206.435
166.779
157.008
155.139
153.059
153.049
149.599
143.860
143.497
136.632
130.497
130.441
129.834
126.731
118.821
118.636
118.420
118.356
116.466
114.678
114.489
62.470
61.486
54.115
53.367
34.159
30.263
30.109
29.955
29.800
29.646
29.492
29.338
H2N
S
O
O
N
N
O
N
H
N
HO
F
29
13C-NMR
(125 MHz, (CD3)2CO)
f1 (ppm)

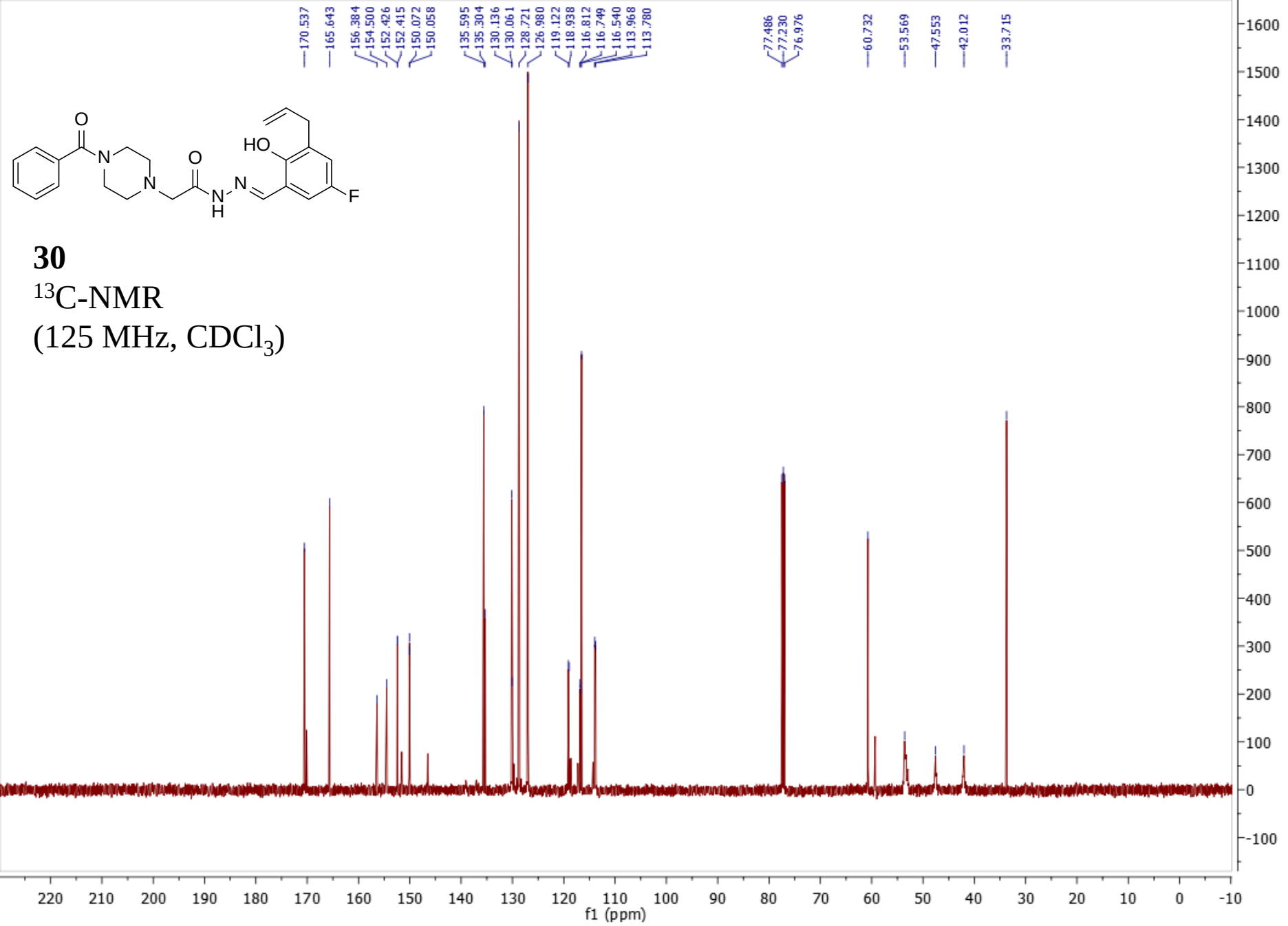


29

^{19}F-NMR

(470 MHz, $(CD_3)_2CO$)

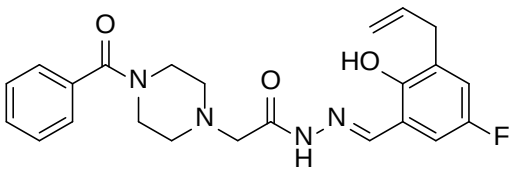

11.168
10.448
8.190
7.385
7.380
7.370
7.358
7.354
6.868
6.612
6.606
6.595
5.941
5.922
5.906
5.064
5.061
5.052
5.030
3.806
3.473
3.358
3.345
3.188
2.563
HO
F
30
1H-NMR
(500 MHz, CDCl3)
0.95
0.90
1.01
6.32
1.06
0.97
1.13
2.50
2.21
2.72
2.41
2.00
5.02
f1 (ppm)



30

^{13}C-NMR

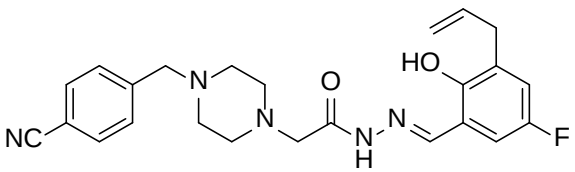
(125 MHz, $CDCl_3$)



30

^{19}F-NMR

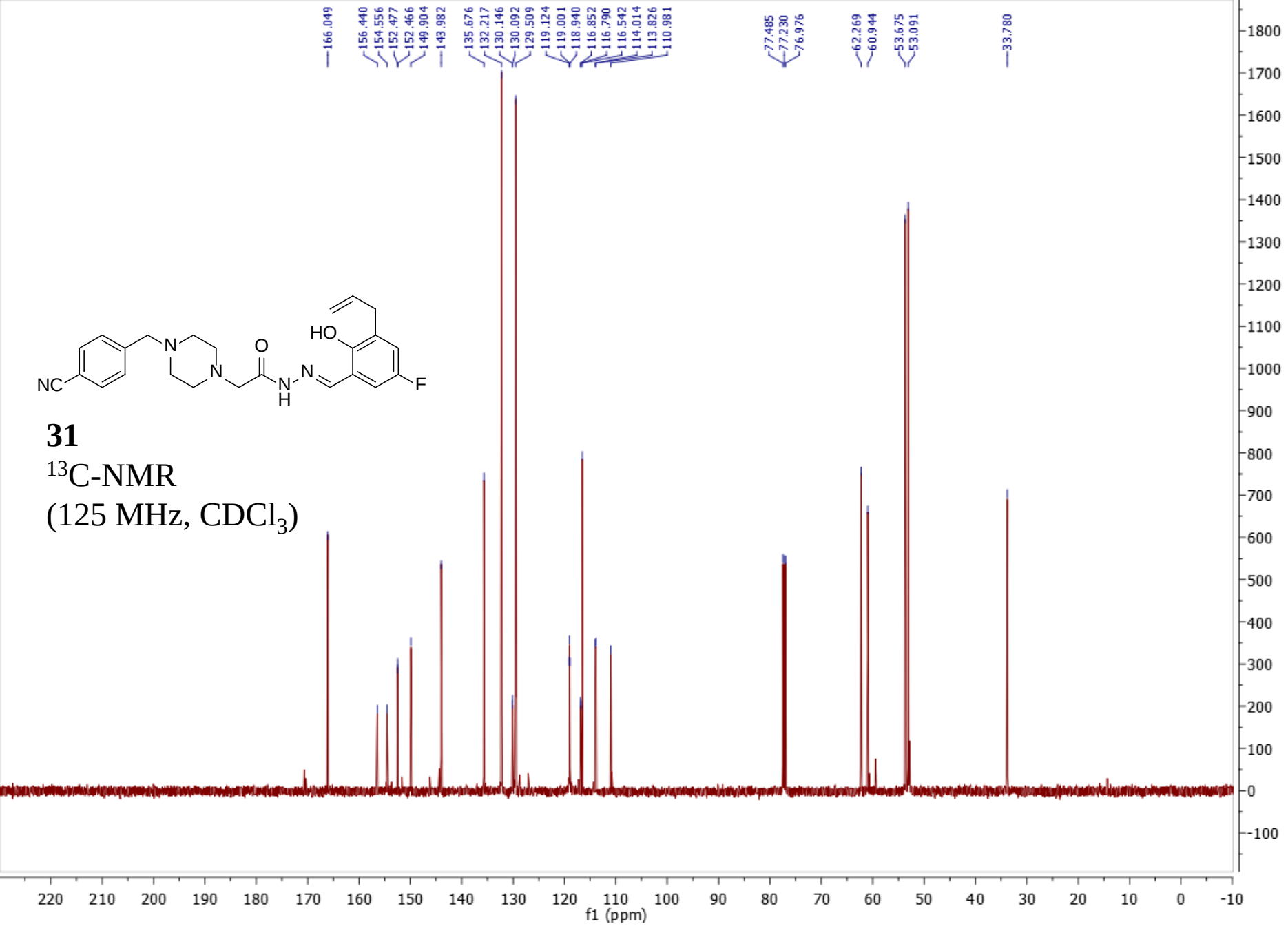
(470 MHz, $CDCl_3$)



31

^{1}H-NMR

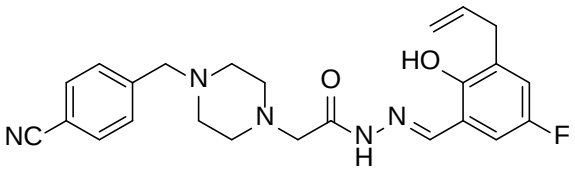
(500 MHz, $CDCl_3$)



31

^{13}C-NMR

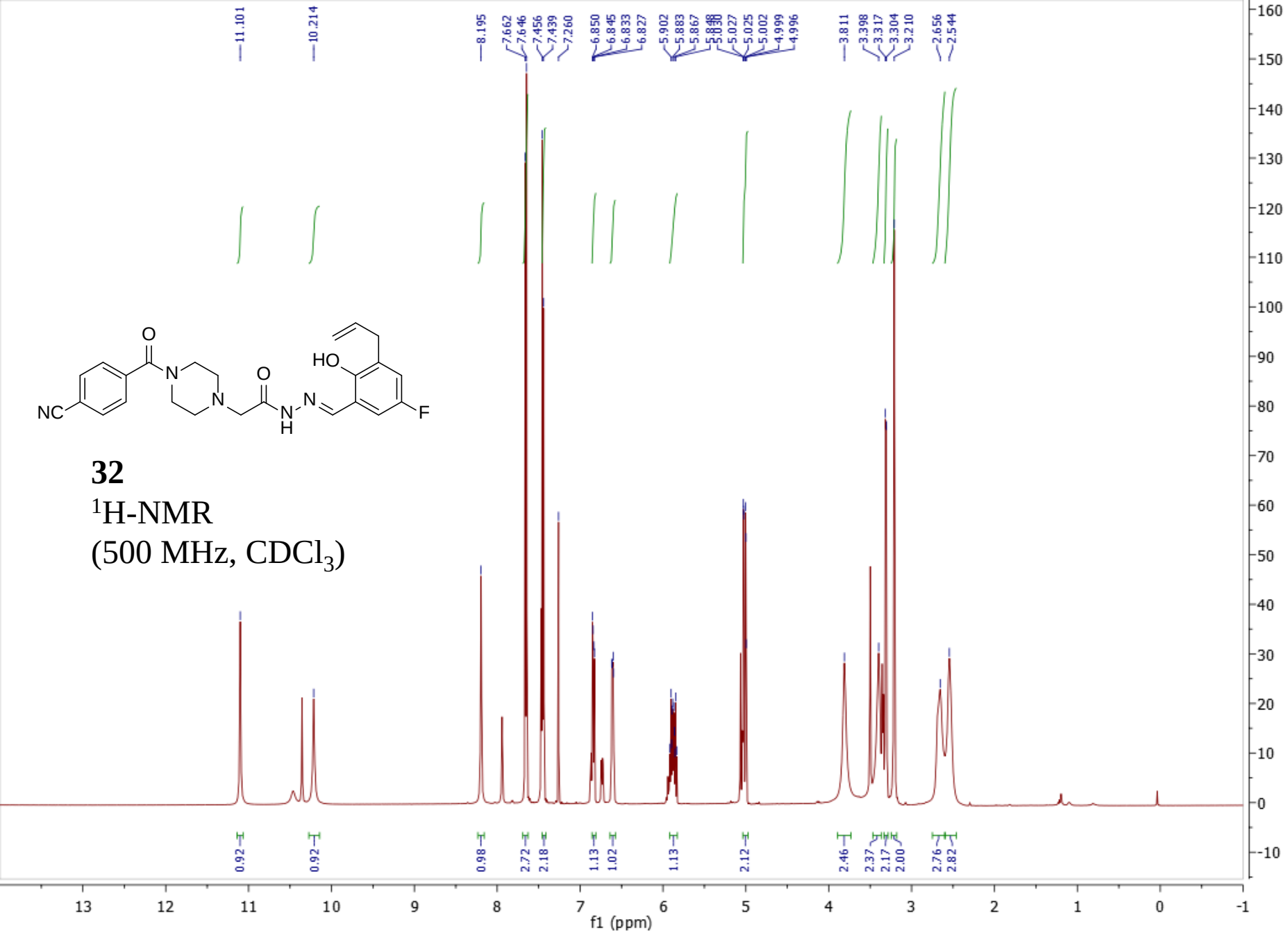
(125 MHz, $CDCl_3$)



31

^{19}F-NMR

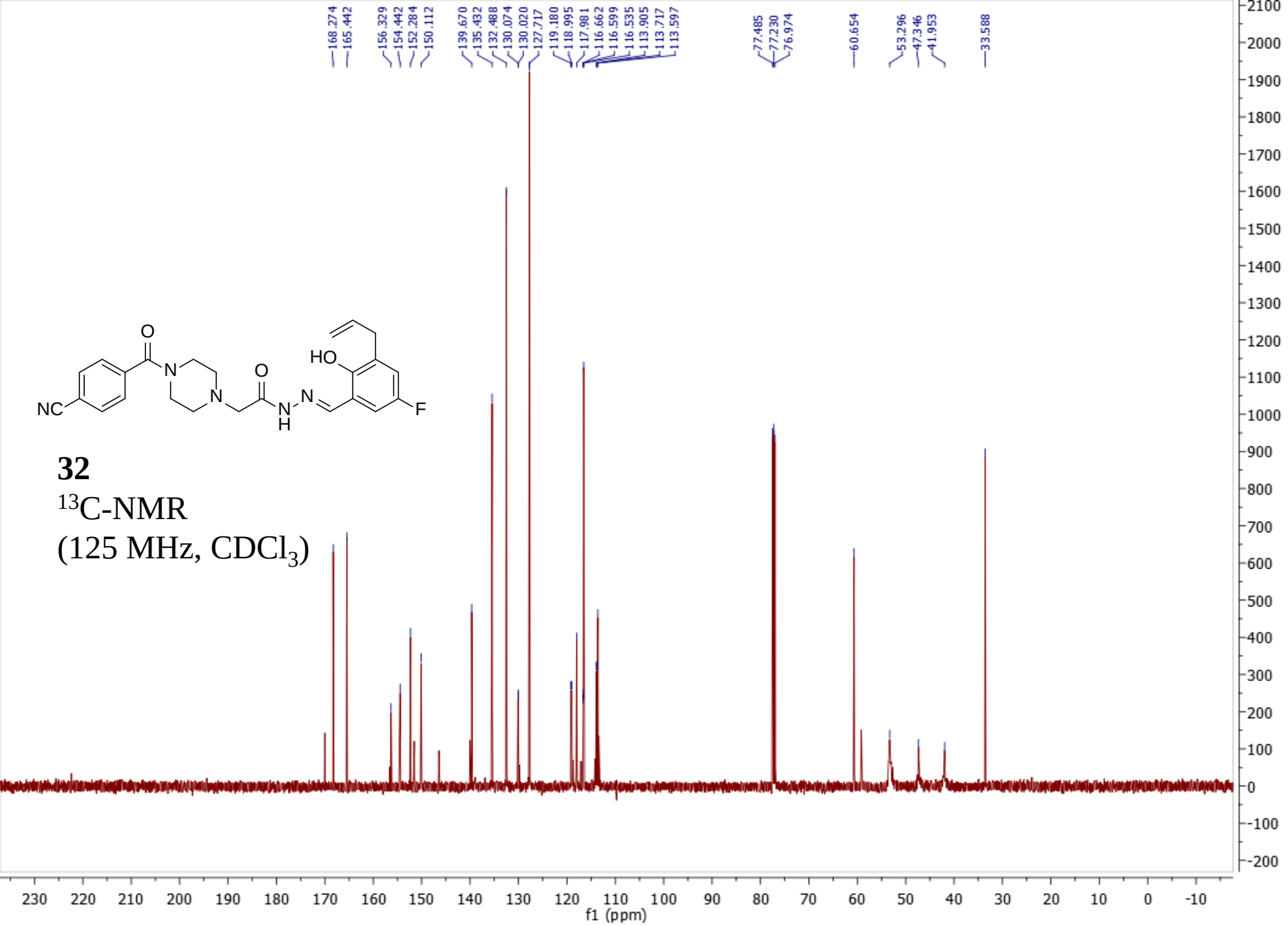
(470 MHz, $CDCl_3$)



32

^{1}H-NMR

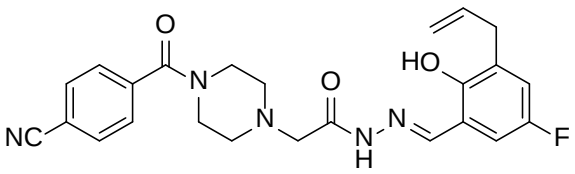
(500 MHz, $CDCl_3$)



32

^{13}C-NMR

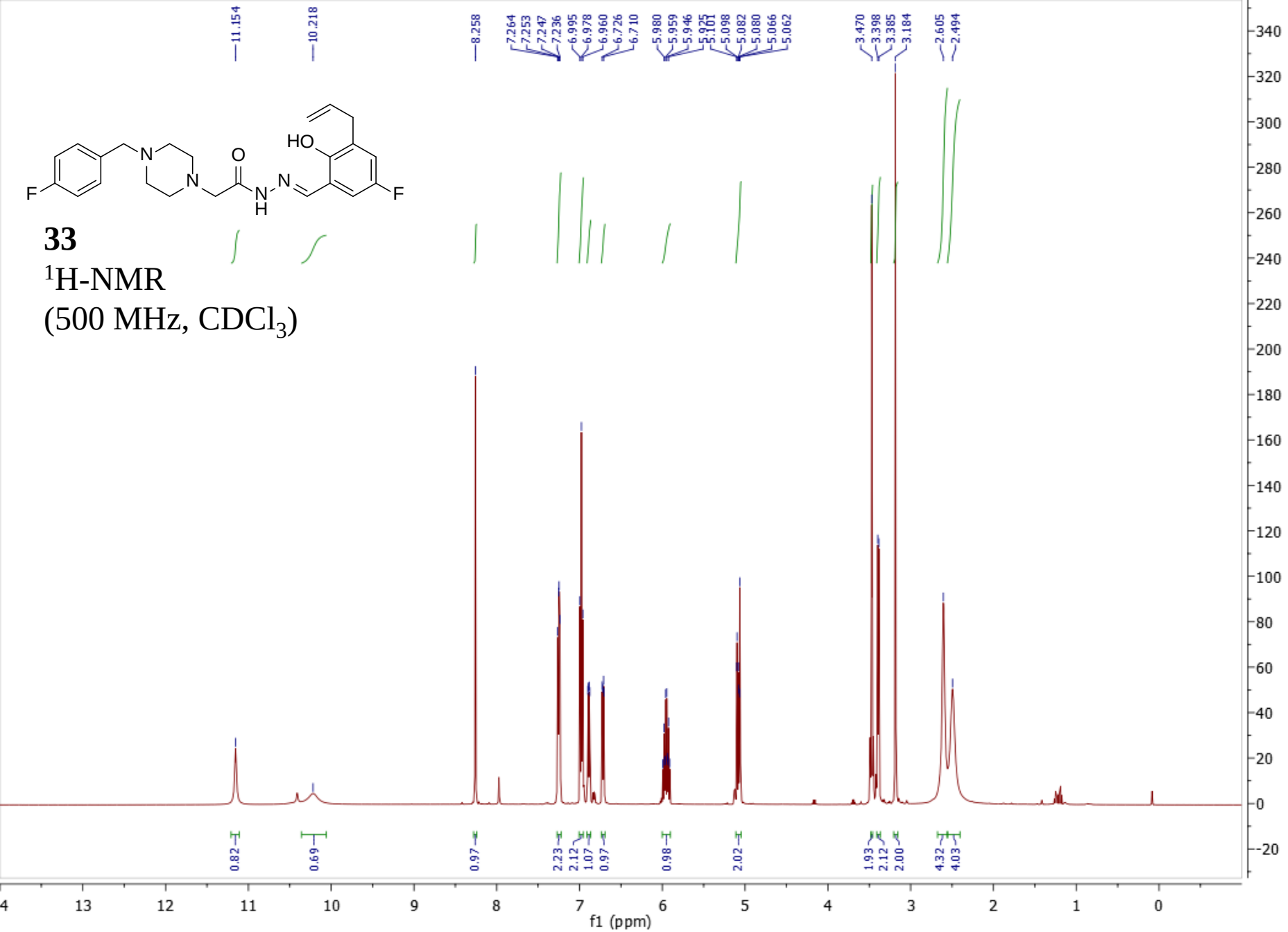
(125 MHz, $CDCl_3$)

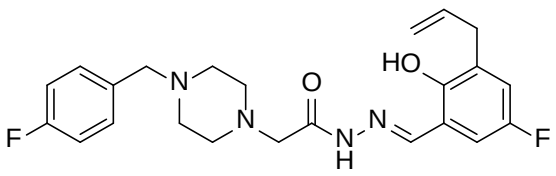


32

^{19}F-NMR

(470 MHz, $CDCl_3$)

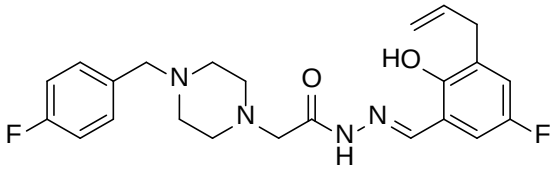

11.154
10.218
8.258
7.264
7.253
7.247
7.236
6.995
6.978
6.960
6.726
6.710
5.980
5.959
5.946
5.101
5.098
5.082
5.080
5.066
5.062
3.470
3.398
3.385
3.184
2.605
2.494
HO
O
N
N
N
H
F
F
33
1H-NMR
(500 MHz, CDCl3)
0.82
0.69
0.97
2.23
2.12
1.07
0.97
0.98
2.02
1.93
2.12
2.00
4.32
4.03
f1 (ppm)



33

^{13}C-NMR

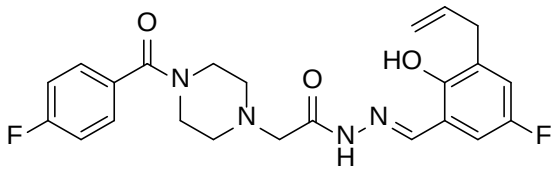
(125 MHz, $CDCl_3$)



33

^{19}F-NMR

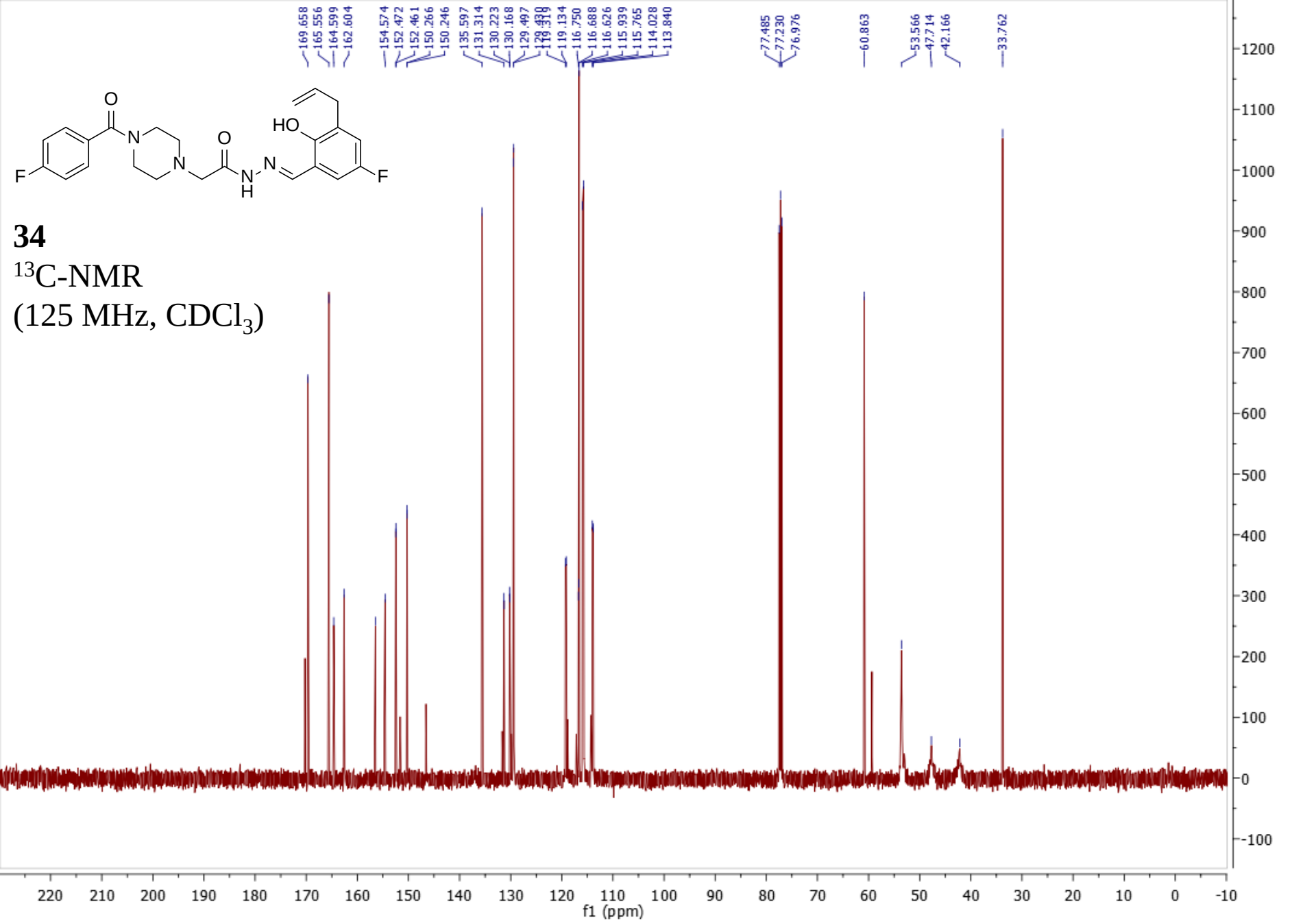
(470 MHz, $CDCl_3$)

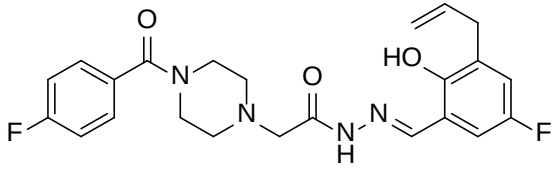


34

^{1}H-NMR

(500 MHz, $CDCl_3$)

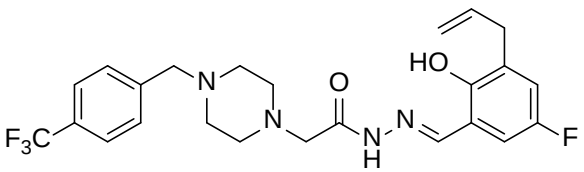

O
N
N
O
N
H
N
HO
F
F
34
13C-NMR
(125 MHz, CDCl3)
169.658
165.556
164.599
162.604
154.574
152.472
152.461
150.266
150.246
135.597
131.314
130.223
130.168
129.497
119.134
116.750
116.688
116.626
115.939
115.765
114.028
113.840
77.485
77.230
76.976
60.863
53.566
47.714
42.166
33.762
f1 (ppm)



34

^{19}F-NMR

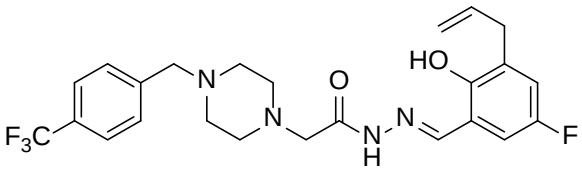
(470 MHz, $CDCl_3$)



35

^{1}H-NMR

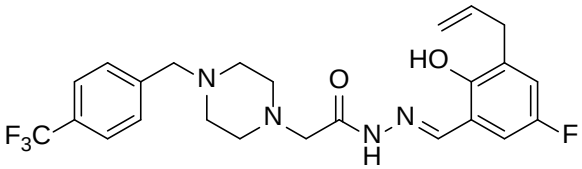
(500 MHz, $CDCl_3$)



35

^{13}C-NMR

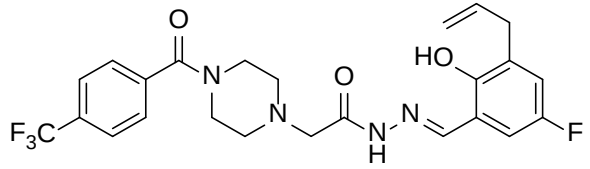
(125 MHz, $CDCl_3$)



35

^{19}F-NMR

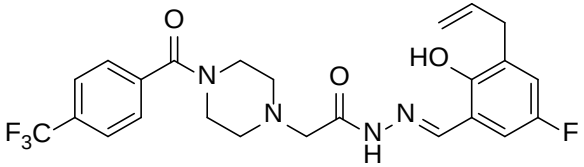
(470 MHz, $CDCl_3$)



36

^{1}H-NMR

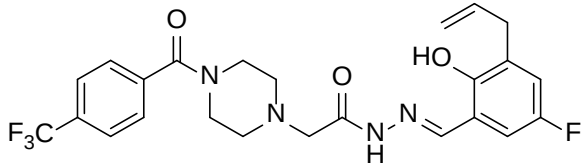
(500 MHz, $CDCl_3$)



36

^{13}C-NMR

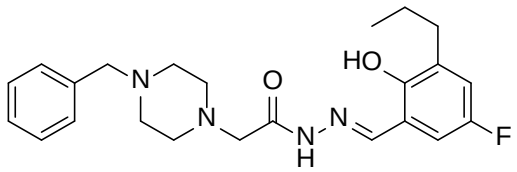
(125 MHz, $CDCl_3$)



36

^{19}F-NMR

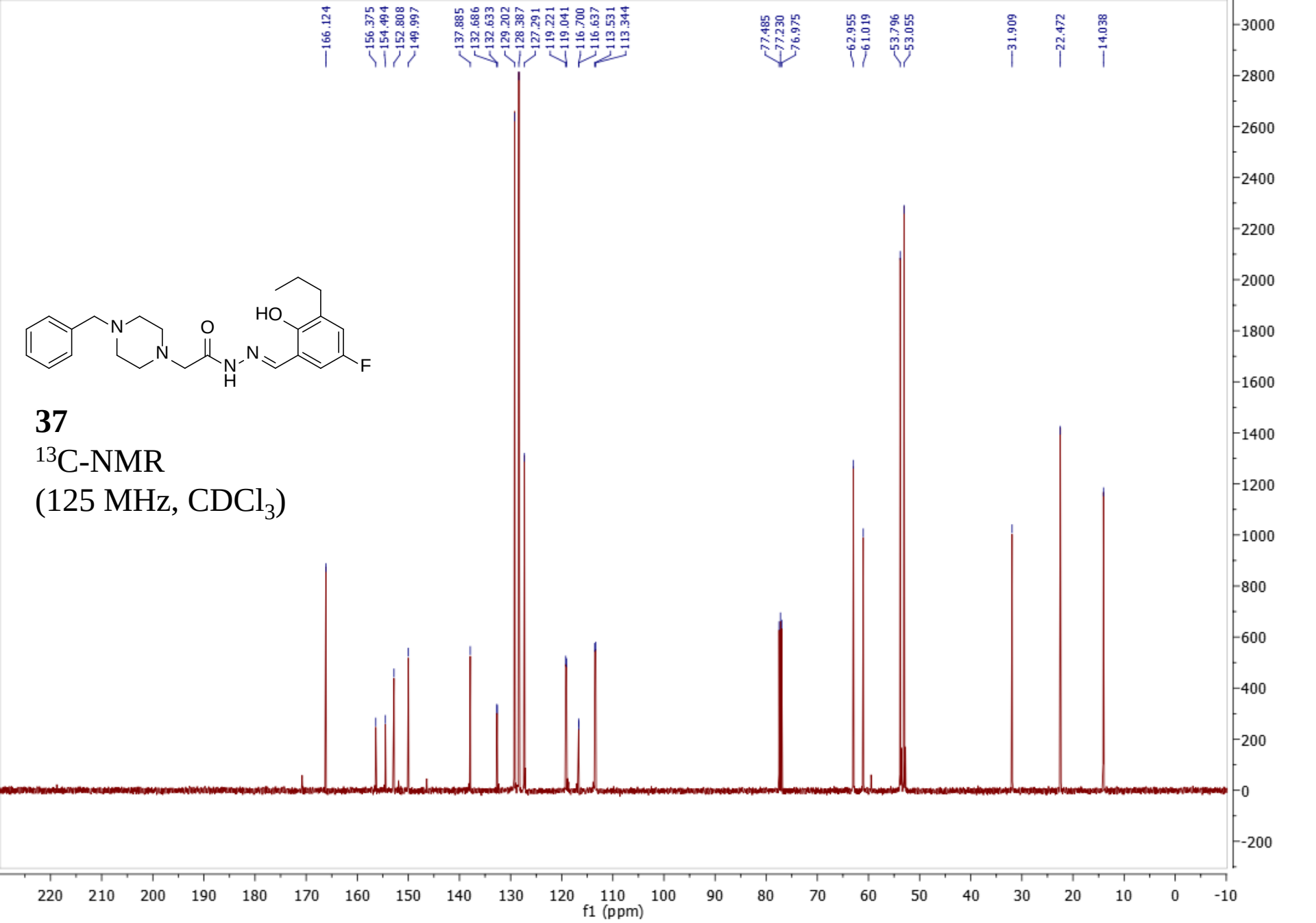
(470 MHz, $CDCl_3$)



37

^{1}H-NMR

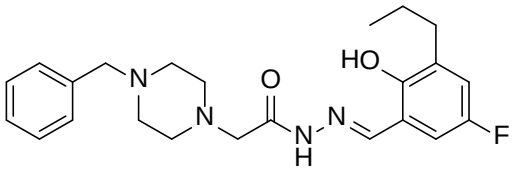
(500 MHz, $CDCl_3$)



37

^{13}C-NMR

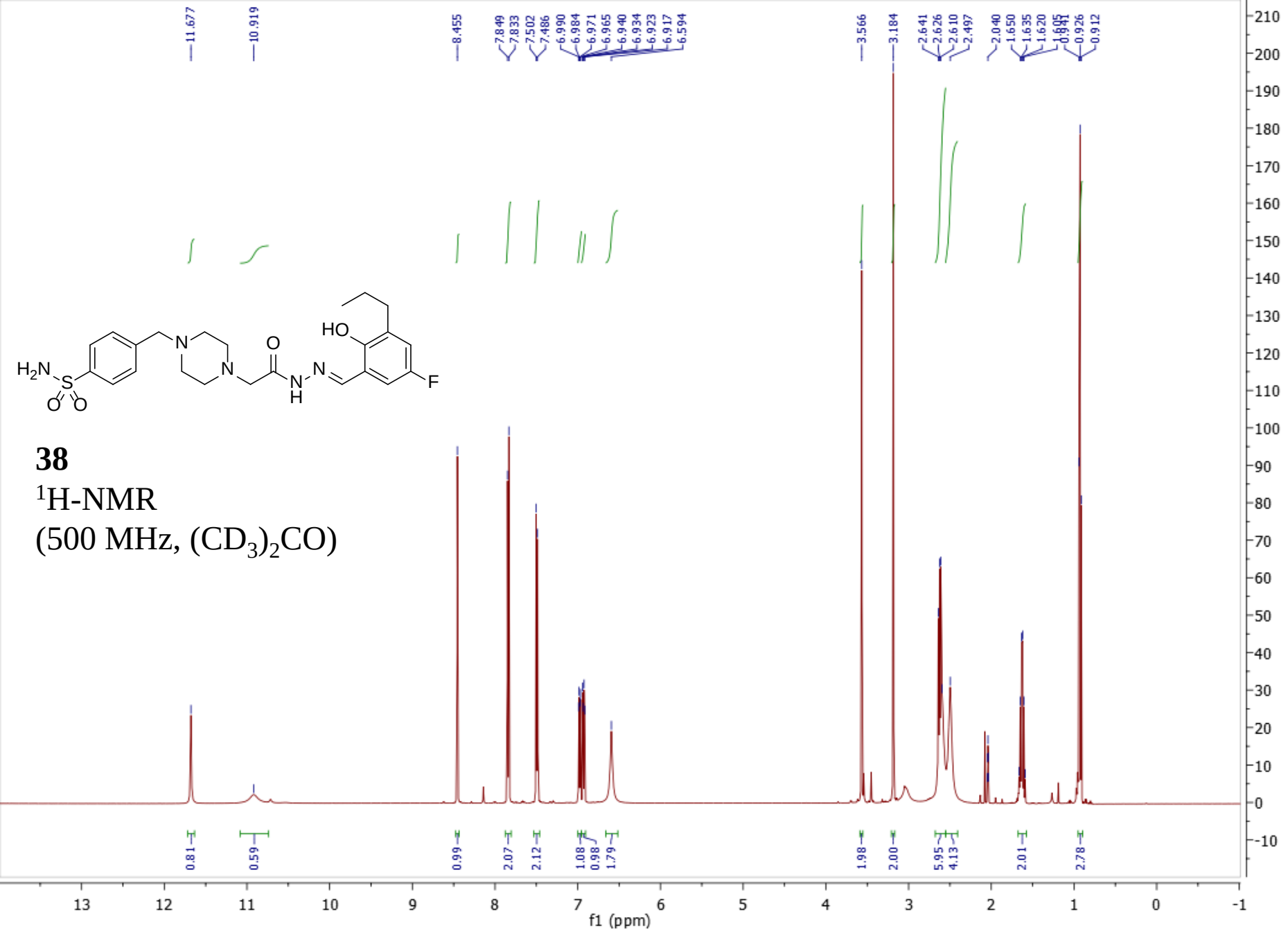
(125 MHz, $CDCl_3$)



37

^{19}F-NMR

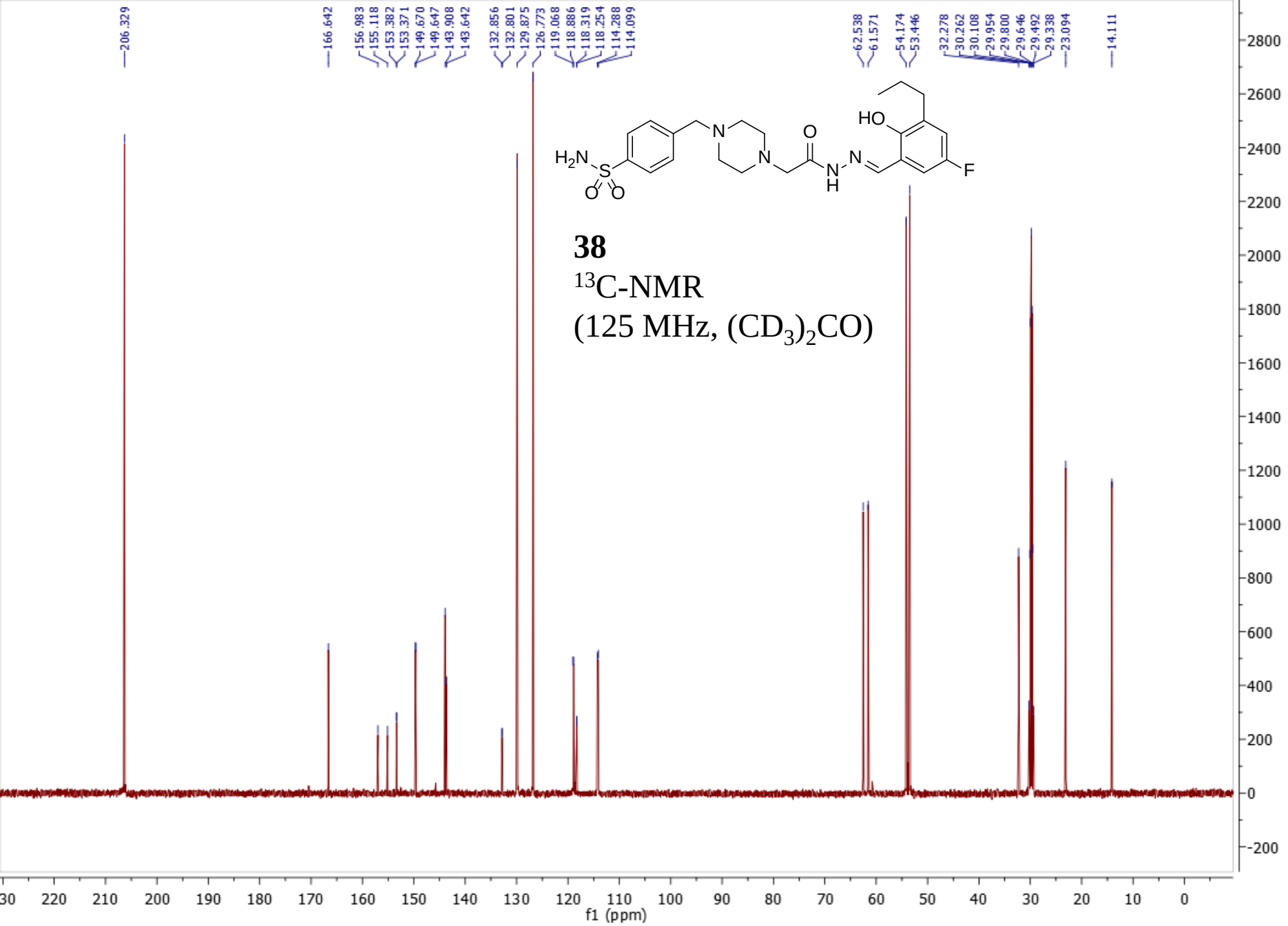
(470 MHz, $CDCl_3$)

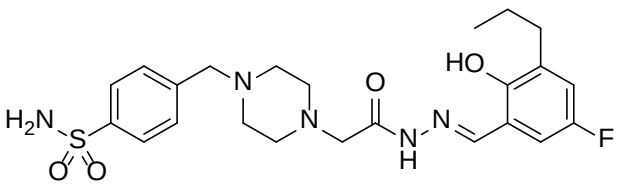


38

^{1}H-NMR

(500 MHz, $(CD_3)_2CO$)

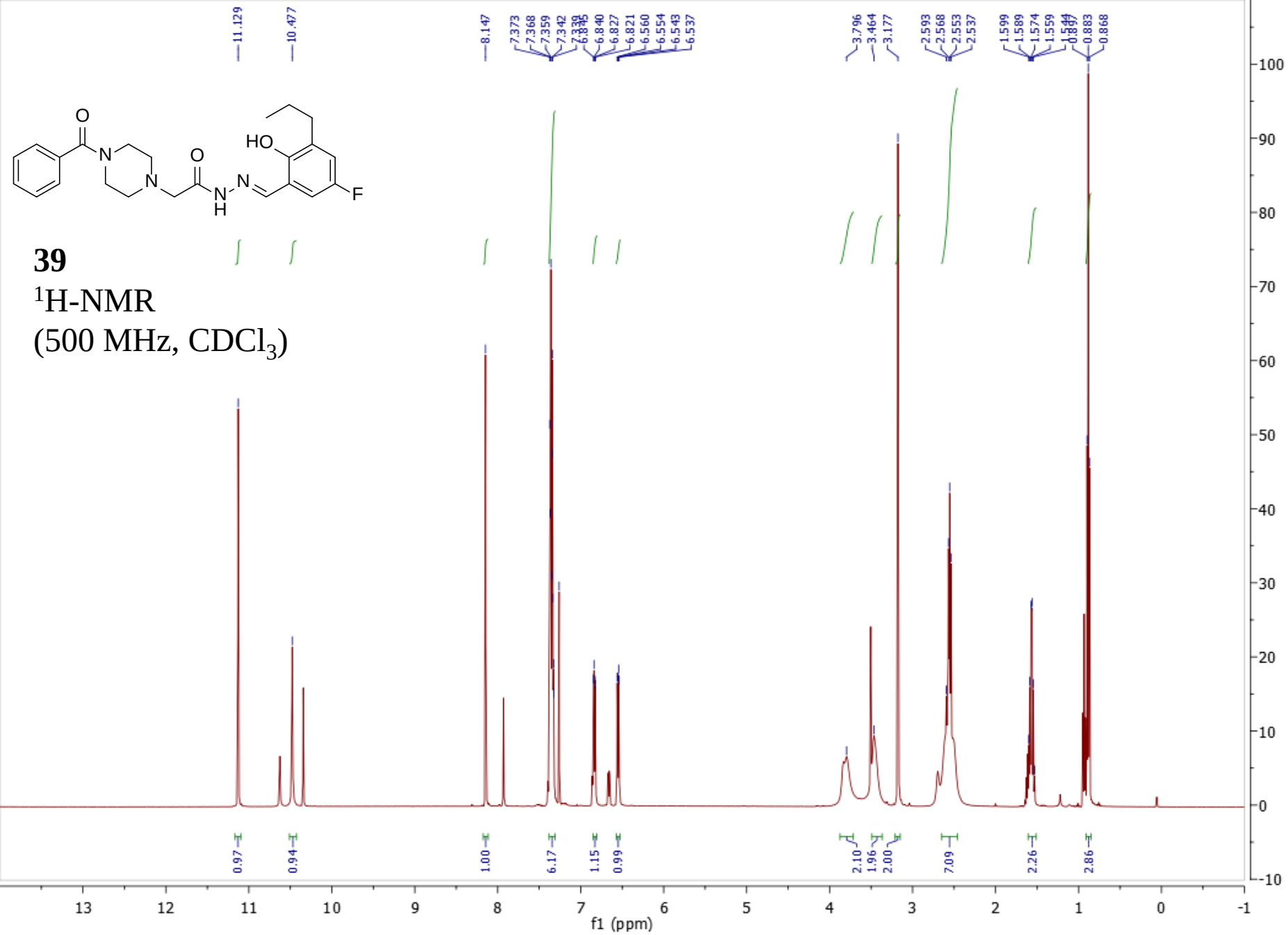

206.329
166.642
156.983
155.118
153.382
153.371
149.670
149.647
143.908
143.642
132.856
132.801
129.875
126.773
119.068
118.886
118.319
118.254
114.288
114.099
62.538
61.571
54.174
53.446
32.278
30.262
30.108
29.954
29.800
29.646
29.492
29.338
23.094
14.111
HO
N
N
O
N
N
H
H2N
S
O
O
F
38
13C-NMR
(125 MHz, (CD3)2CO)
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
30 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)



38

^{19}F-NMR

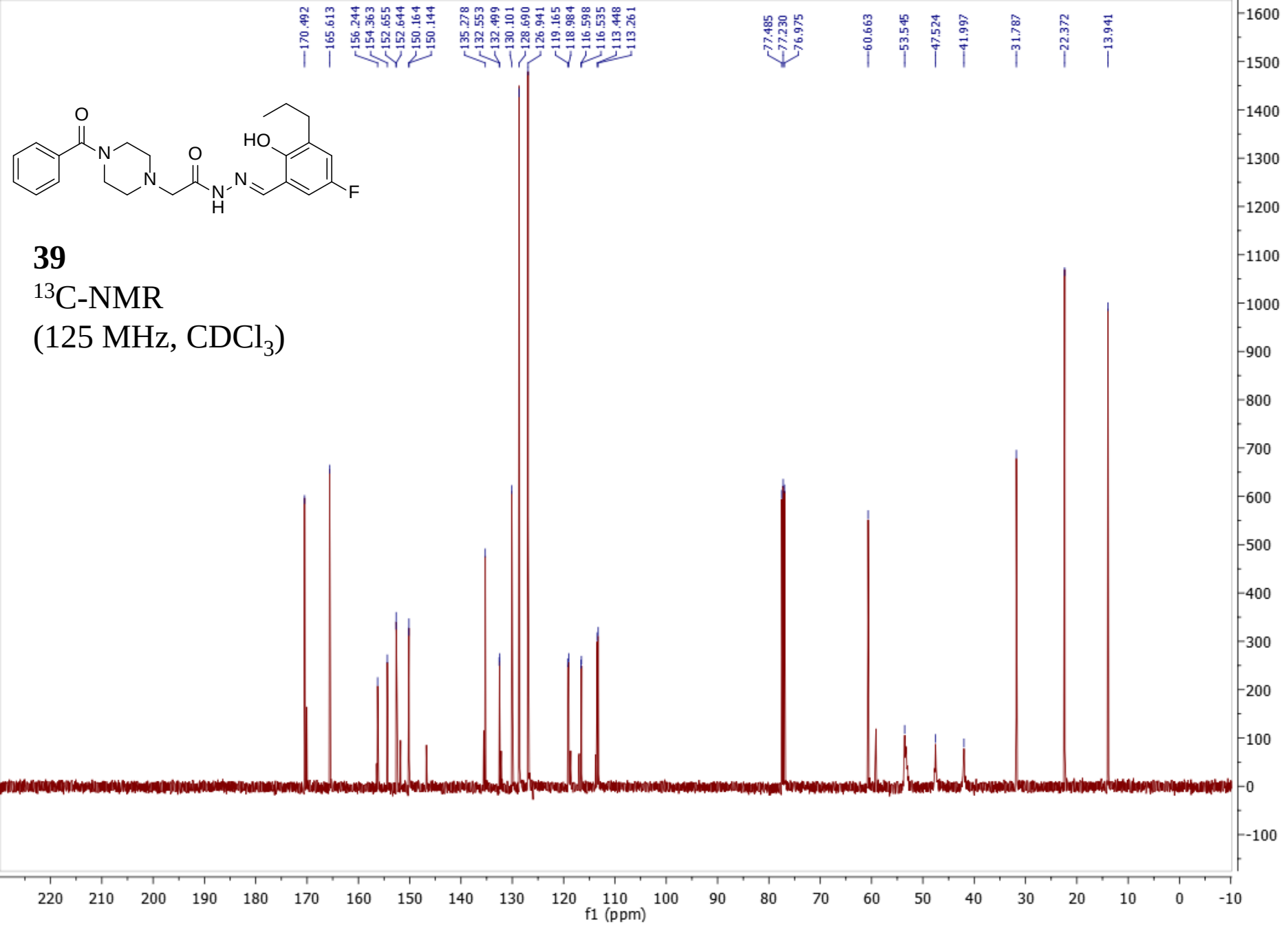
(470 MHz, $(CD_3)_2CO$)



39

^{1}H-NMR

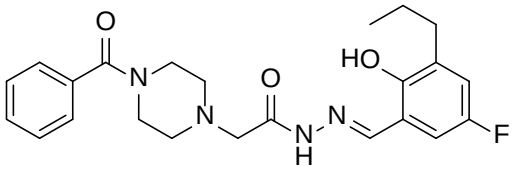
(500 MHz, $CDCl_3$)



39

^{13}C-NMR

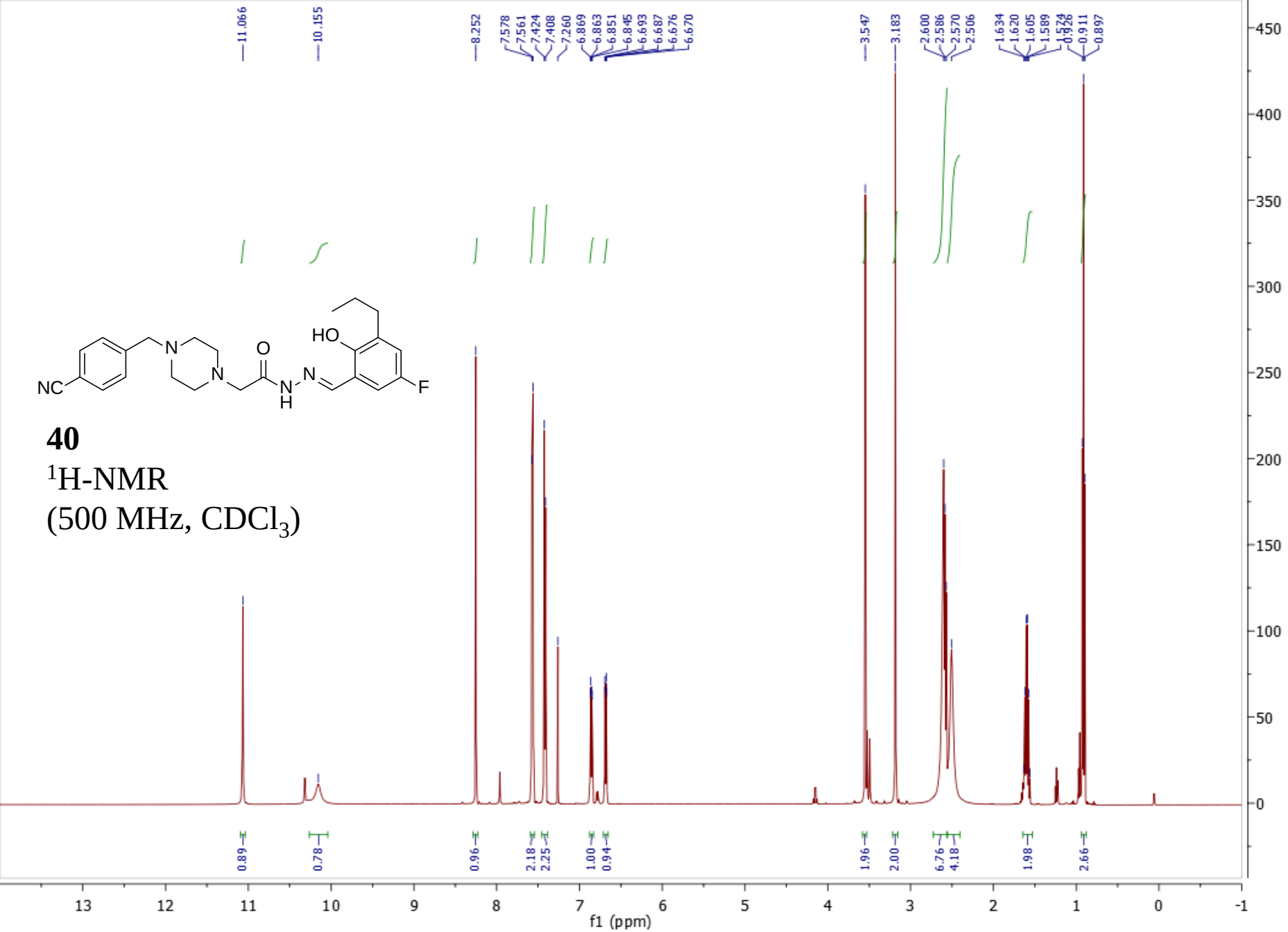
(125 MHz, $CDCl_3$)



39

^{19}F-NMR

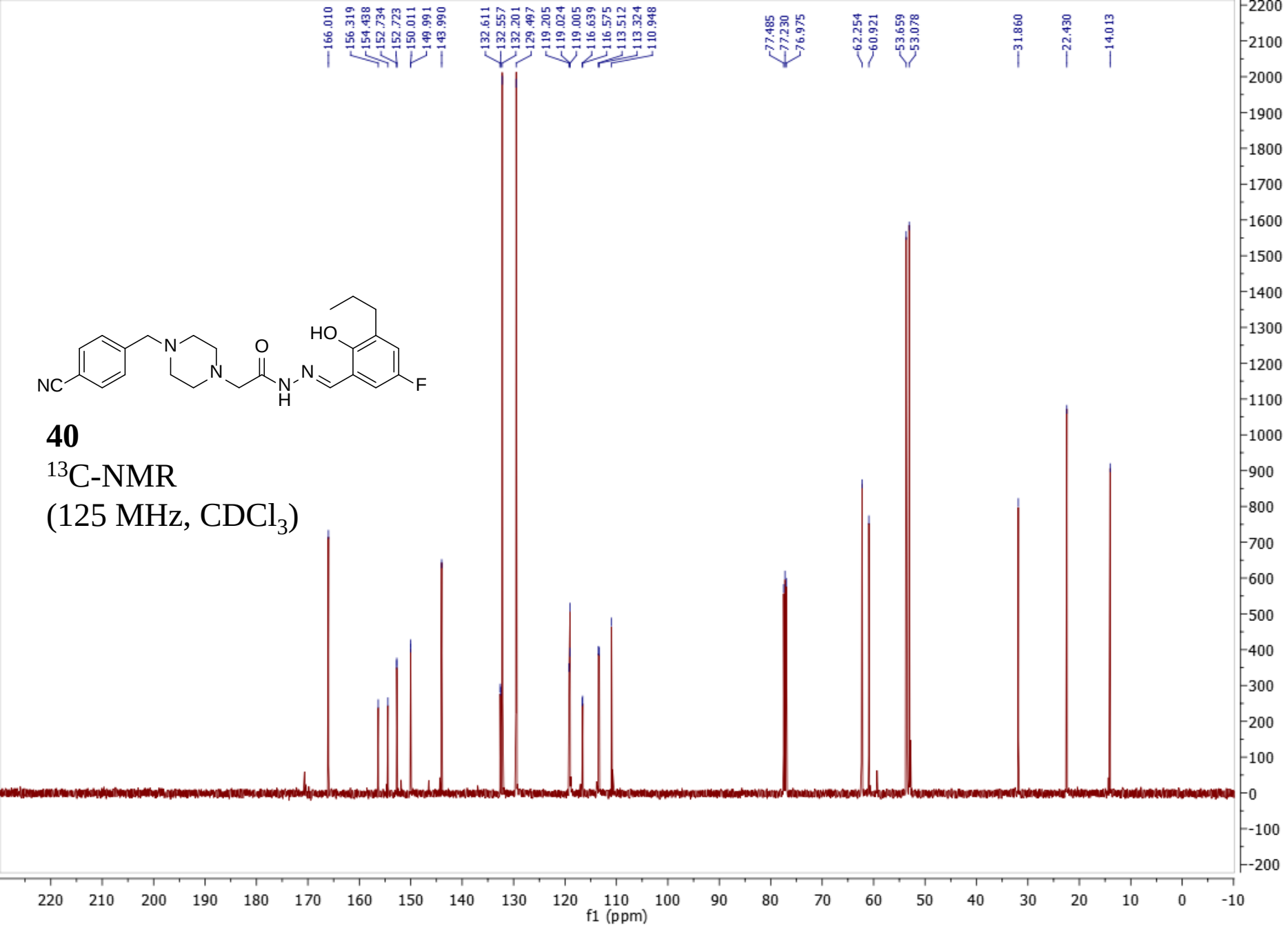
(470 MHz, $CDCl_3$)



40

^{1}H-NMR

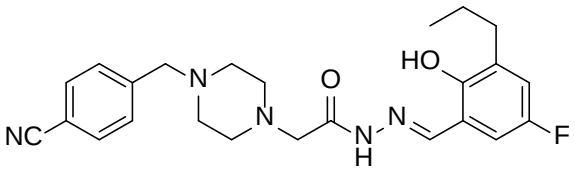
(500 MHz, $CDCl_3$)



40

^{13}C-NMR

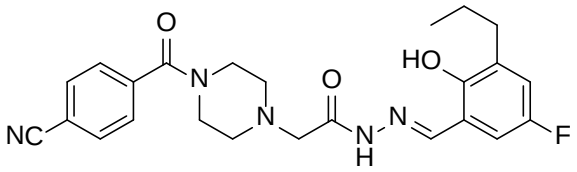
(125 MHz, $CDCl_3$)



40

^{19}F-NMR

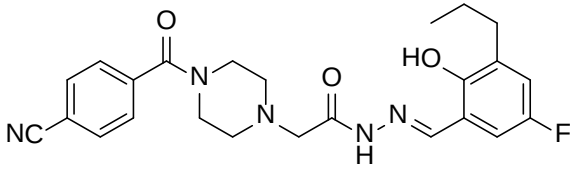
(470 MHz, $CDCl_3$)



41

^{1}H-NMR

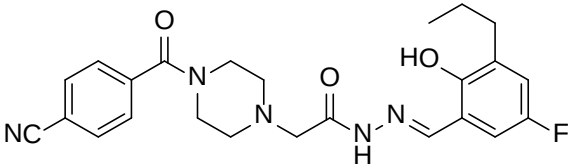
(500 MHz, $CDCl_3$)



41

^{13}C-NMR

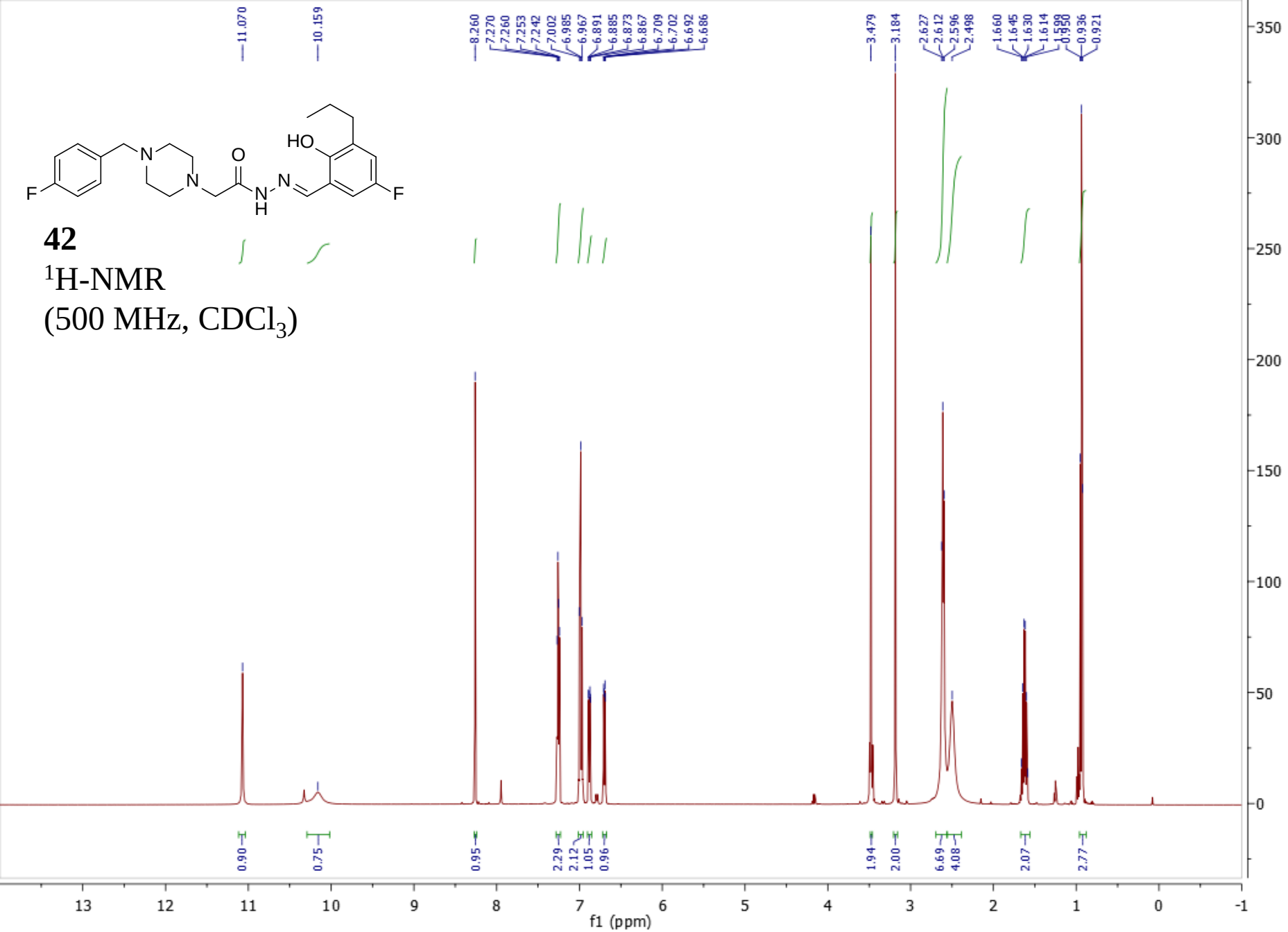
(125 MHz, $CDCl_3$)



41

^{19}F-NMR

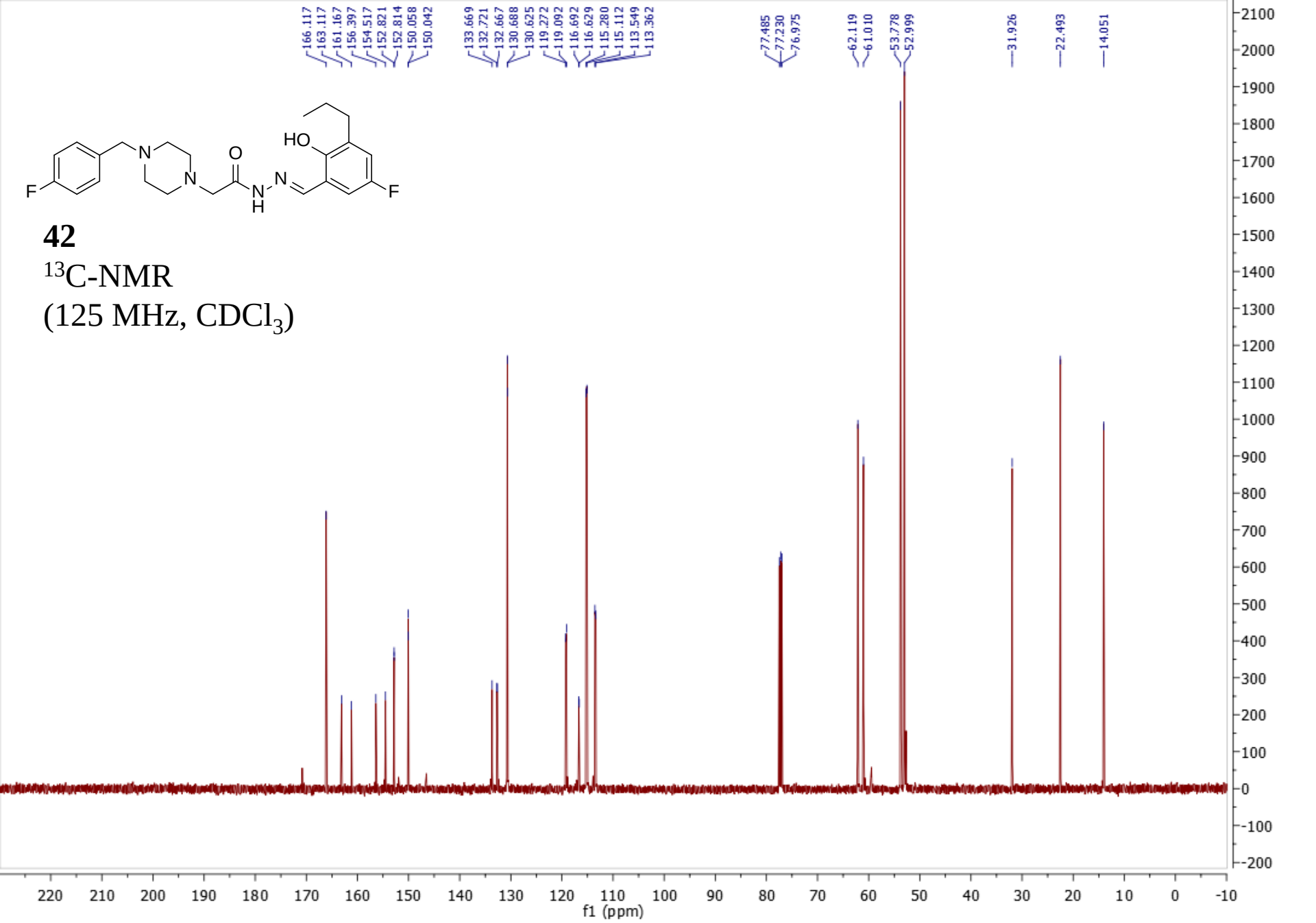
(470 MHz, $CDCl_3$)



42

^{1}H-NMR

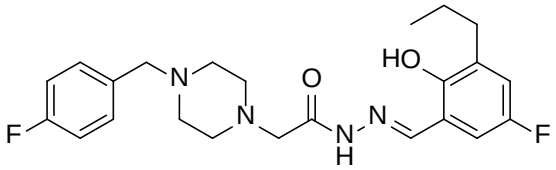
(500 MHz, $CDCl_3$)



42

^{13}C-NMR

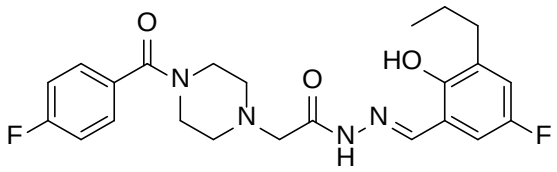
(125 MHz, $CDCl_3$)



42

^{19}F-NMR

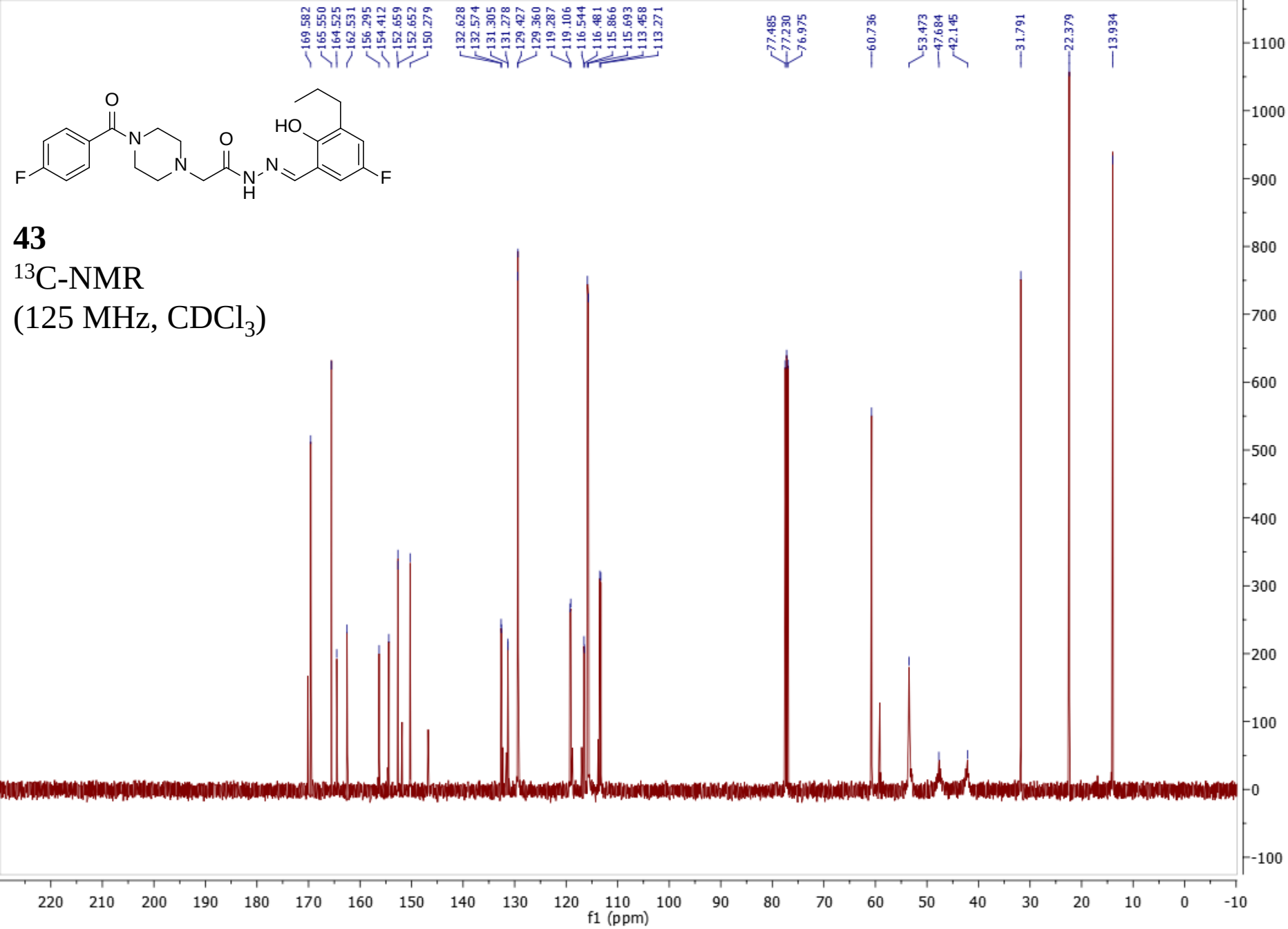
(125 MHz, $CDCl_3$)

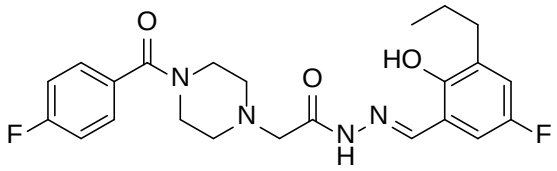


43

^{1}H-NMR

(500 MHz, $CDCl_3$)

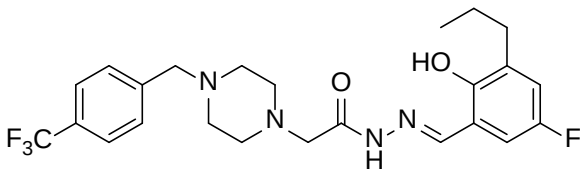

169.582
165.550
164.525
162.531
156.295
154.412
152.659
152.652
150.279
132.628
132.574
131.305
131.278
129.427
129.360
119.287
119.106
116.544
116.481
115.866
115.693
113.458
113.271
77.485
77.230
76.975
60.736
53.473
47.684
42.145
31.791
22.379
13.934
O
N
N
O
N
H
N
HO
F
F
43
13C-NMR
(125 MHz, CDCl3)
f1 (ppm)



43

^{19}F-NMR

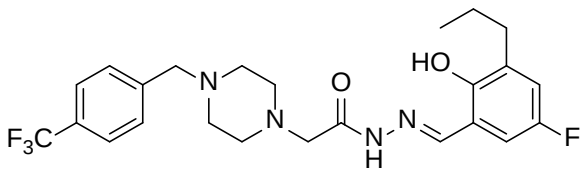
(470 MHz, $CDCl_3$)



44

^{1}H-NMR

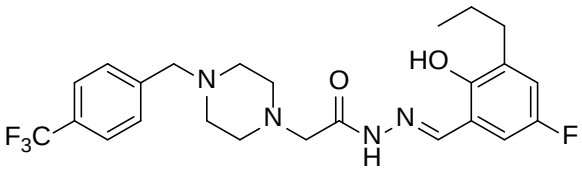
(500 MHz, $CDCl_3$)



44

^{13}C-NMR

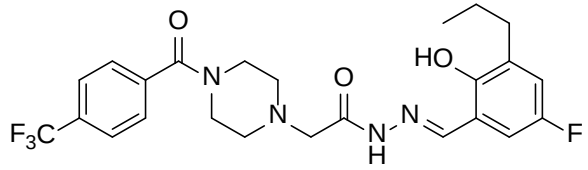
(125 MHz, $CDCl_3$)



44

^{19}F-NMR

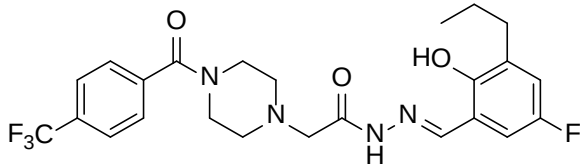
(470 MHz, $CDCl_3$)



45

^{1}H-NMR

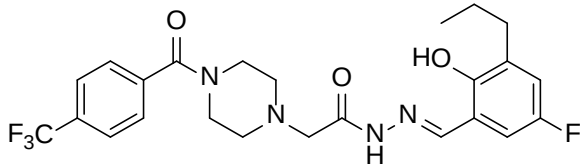
(500 MHz, $CDCl_3$)



45

^{13}C-NMR

(125 MHz, $CDCl_3$)



45

^{19}F-NMR

(470 MHz, $CDCl_3$)