# Evolution of a 4-Benzyloxy-benzylamino Chemotype to Provide Efficacious, Potent, and Isoform Selective PPARα Agonists as Leads for Retinal Disorders

Xiaozheng Dou,[†,§] Dinesh Nath,[†,§] Henry Shin,[‡] Elmar Nurmemmedov,[∥] Philip C. Bourne,[§] Jian-Xing Ma,[‡] and Adam S. Duerfeldt*[†,§]

[†]*Institute for Natural Products Applications and Research Technologies and* [§]*Department of Chemistry & Biochemistry, University of Oklahoma, 101 Stephenson Parkway, Stephenson Life Sciences Research Center, Norman, Oklahoma 73019, United States*

[‡]*Department of Physiology, University of Oklahoma Health Sciences Center, 941 Stanton L. Young Boulevard, Oklahoma City, Oklahoma 73104, United States*

[∥]*John Wayne Cancer Institute & Pacific Neuroscience Institute at Providence Saint John's Health Center, 2200 Santa Monica Boulevard, Santa Monica, California 90404, United States*

**Supporting information** –Two-concentration luciferase data, PPARα expression and purification, cellular thermal shift western blots, cytotoxicity, *in silico* metabolic predictions, isothermal titration calorimetry thermograms and curve fits, $^{1}$H and $^{13}$C NMR spectra for final compounds, HPLC traces for select leads, and pharmacokinetic data sets.

*to whom correspondence should be addressed. Department of Chemistry and Biochemistry, University of Oklahoma, 101 Stephenson Parkway, Stephenson Life Sciences Research Center, Norman, Oklahoma 73019. Phone: 1-405-325-2232. Email: adam.duerfeldt@ou.edu.

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## PPARA LUCIFERASE TWO-CONCENTRATION ASSESSMENT

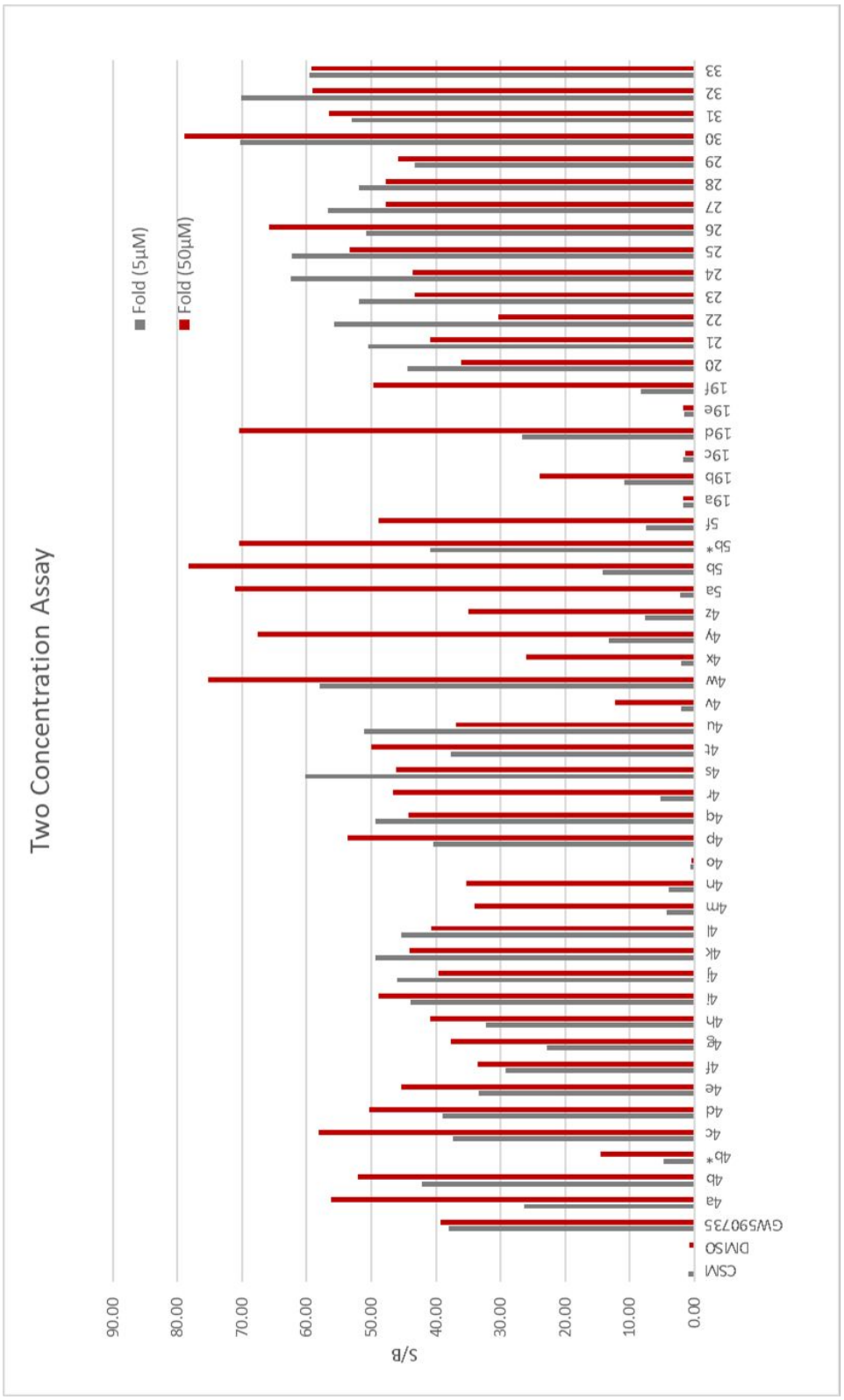



**Figure 1. Initial two-concentration assessment of 4a-4z, 5a, 5b, 5b*, 5f, 20a-20f, and 21-34 for PPARα agonism in a luciferase-based cellular assay (Indigo).** Results presented from a single experiment as mean signal/mean background (S/B). GW590735 was evaluated at 5 µM and 40 µM due to observed cytotoxicity at higher concentrations.

## ISOTHERMAL CALORIMETRY

ITC experiments were performed using a MicroCal PEAQ ITC (MicroCal Inc., Northampton, MA, USA). PPARα was concentrated down to a final concentration of 3.27 mg/mL using an Amicon Ultra-15 centrifugal concentrator (Millipore). Buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) was used to dilute the ligand stock solution (10, 12.5, or 25 mM in DMSO). DMSO was added to the protein solution at the same percentage (0.8-10%) of the ligand solution. Samples were centrifuged before the experiment to eliminate possible aggregates. The protein solution (30-85 μM) was placed in the sample cell, and the ligand solution (2.3-15 times more concentrated than the protein) was loaded into the syringe injector. The detailed experimental parameters are shown below. The titrations involved 19 injections of 2 μL at 150 s intervals or 13 injections of 3 μL at 150 s intervals. The experiment was set at 25 °C and the syringe stirring speed was set at 750 rpm. Titration of ligand at same concentration into buffer with same percentage DMSO were used as a reference control. The thermodynamic data were processed with MicroCal PEAQ-ITC Analysis Software provided by Malvern. The one-site binding model was selected to calculate the value of the dissociation constant ($K_d$), the enthalpy (ΔH), the binding free energy (ΔG), and the entropy (-TΔS).

| Ligand | [PPARα] μM | [Ligand] nM | DMSO% (v/v) |
|---|---|---|---|
| **4a (A91)** | 85 | 0.2 | 0.8% |
| **4b** | 60 | 0.5 | 2% |
| **4u** | 30 | 0.25 | 2% |
| **GW590735** | 80 | 0.5 | 2% |

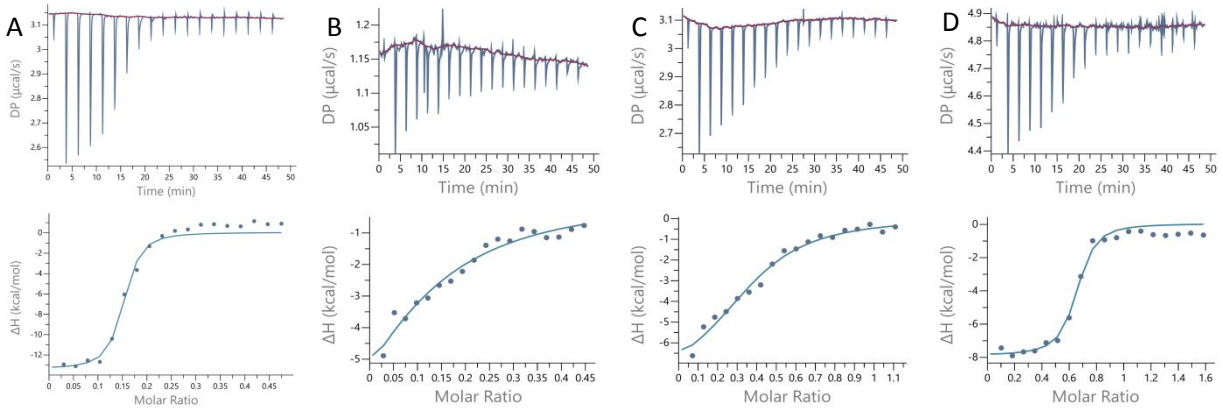



**Figure S2. Isothermal titration calorimetry thermograms of select ligands to PPARα-LBD.** The upper panel shows the raw data of a representative ITC experiment. The lower panel shows the corresponding binding isotherm fit according to the "one binding site" model. A) GW590735; B) compound **4a**; C) compound **4b**; and D) compound **4u**

## PROTEIN EXPRESSION, PURIFICATION

Protein was expressed from the pET28c vector (Novagen) in *Escherichia coli* strain Rosetta™ (DE3) grown in LB media to an OD of 0.6 and then induced with 1 mM IPTG for 20 h at 18 °C. Cells were harvested and resuspended in Buffer A (20 mM Tris, 150 mM NaCl, 5% glycerol, 5 mM imidazole, pH 8.0) at 5 ml/g pellet weight. Cultures were lysed using an Emulsiflex C-3 homogenizer (Avestin), and the lysate clarified by centrifugation (22,000 × g, 40 min). The supernatant was loaded onto a 5ml HisTrap FF Crude column (GE Life Sciences) and eluted with a linear gradient of 5-500 mM imidazole over 20 column volumes. Fractions containing PPARα were identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis. The His-Tag was removed by thrombin cleavage (10 U thrombin/mg) dialyzed overnight at 4 °C against buffer B (20 mM Tris, 150 mM NaCl, 5% glycerol, pH 8.0). The uncleaved protein was removed by reloading the sample onto the 5 ml HisTrap FF Crude column (GE Life Sciences). The flow through was concentrated to a final volume of 5 ml using an Amicon Ultra-15 centrifugal concentrator (Millipore),

and loaded on to a HiLoad Superdex 200PG size exclusion chromatography column (GE Life Sciences) pre-equilibrated with Buffer C (20 mM HEPES, 150 mM NaCl, pH 7.4).

## CETSA DATA

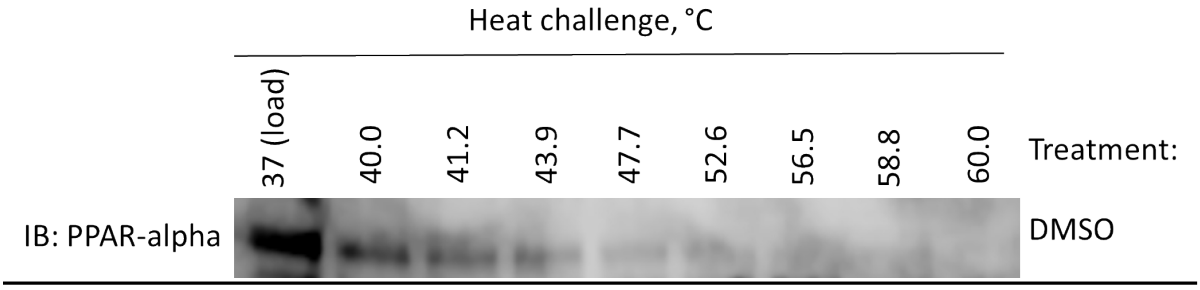



**Figure S3. Melting profile of hPPARα.** MIO-M1 cells were subjected to a heat gradient to determine the thermal melting profile of hPPARα. Band intensity of remaining stable protein at each temperature was quantified, and subsequently the temperature of aggregation ($T_{agg}50$ nd $T_{agg}75$) was determined as 40.8 °C and 45.5 °C, respectively.

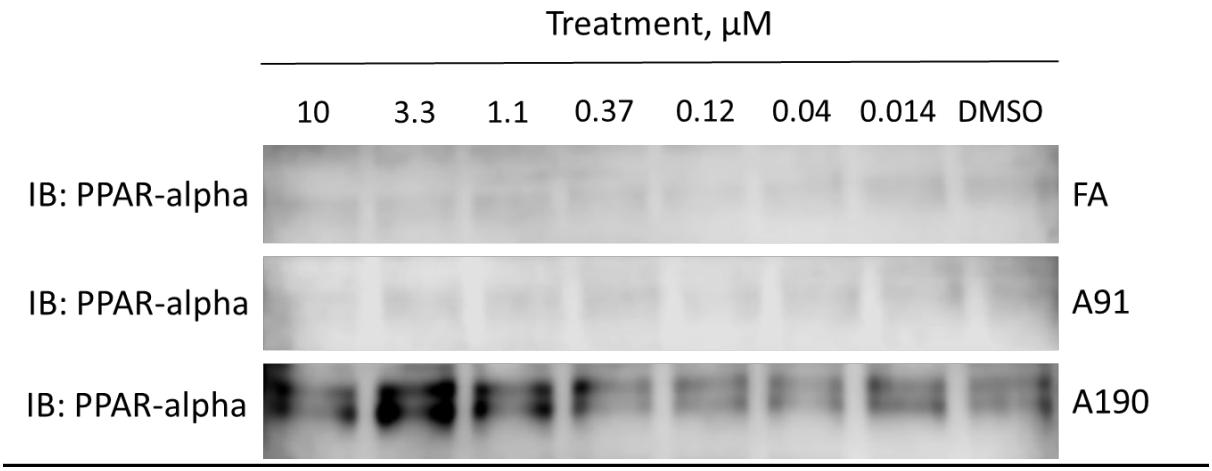



**Figure S4. Dose-response CETSA at 45.5 °C.** MIO-M1 cells treated with increasing doses of inhibitors were subjected to heat challenge at $T_{agg}75$ of 45.5 °C. Band intensity of remaining stabilized protein at each dose was quantified, and subsequently the $EC_{50}$ for each inhibitor was determined.

## LDH Cytotoxicity Data

661W mouse photoreceptor cells were cultured on 96-well plate (6700 cells per well) with varying concentrations of DMSO, GW7647, or A91 (0 to 200 µM) to induce cytotoxicity. Following overnight incubation with the compounds, culture media from the 96-well plate was collected for the LDH quantification assay, which performed following the manufacturer's instructions (Thermo Scientific, #88953). In short, 50 µL of culture medium was mixed and incubated with 50 µL of Reaction Mixture at 25 °C for 30 minutes. 50 µL of Stop Solution was added and the absorbance was measured at 490 nm and 680 nm. Cell death (percentage of maximum LDH release control) was then determined following the manufacturer's protocols.

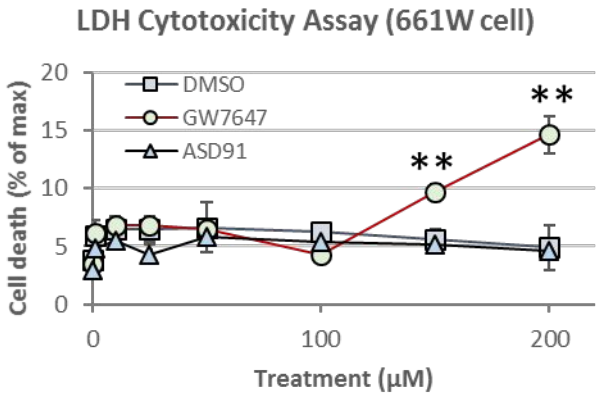



**Figure S5. Cytotoxicity of A91 in a retinal epithelium photoreceptor cell-line 661W.** LDH activities were measured to determine dose-dependent cytotoxicity of ASD091. No significant LDH activities were detected in all concentrations of ASD091 tested, whereas GW7647 began to induce cell death 150 µM (mean ± SD; *n=3*). ***P*<0.01.

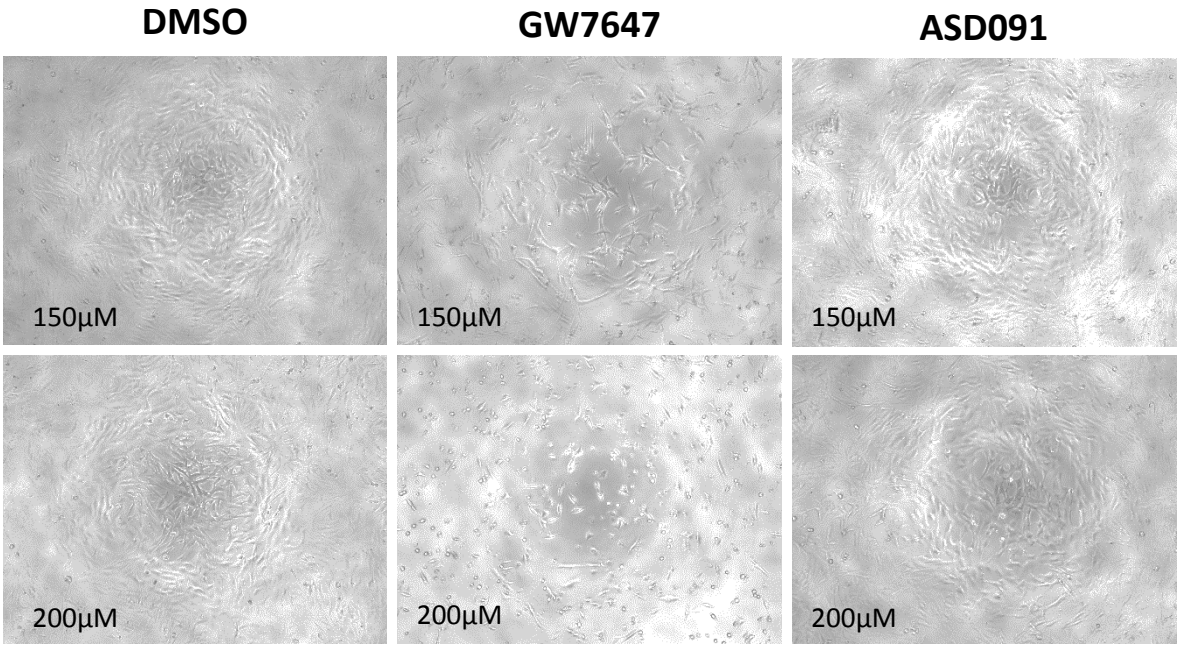



**Figure S6. Cellular morphology after compound treatment under light microscopy.** Unlike GW7647, ASD091-treated cells did not exhibit any obvious signs of cellular morphology changes at all concentrations tested.

## *In silico* Metabolic Predictions

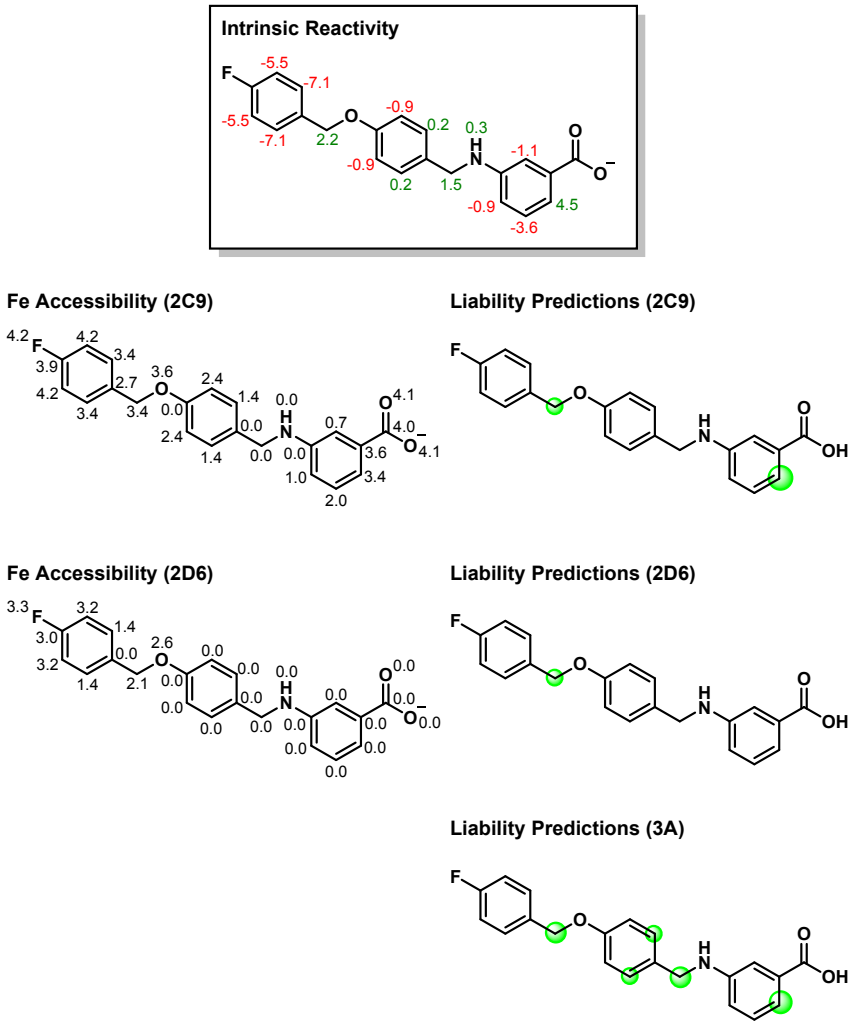
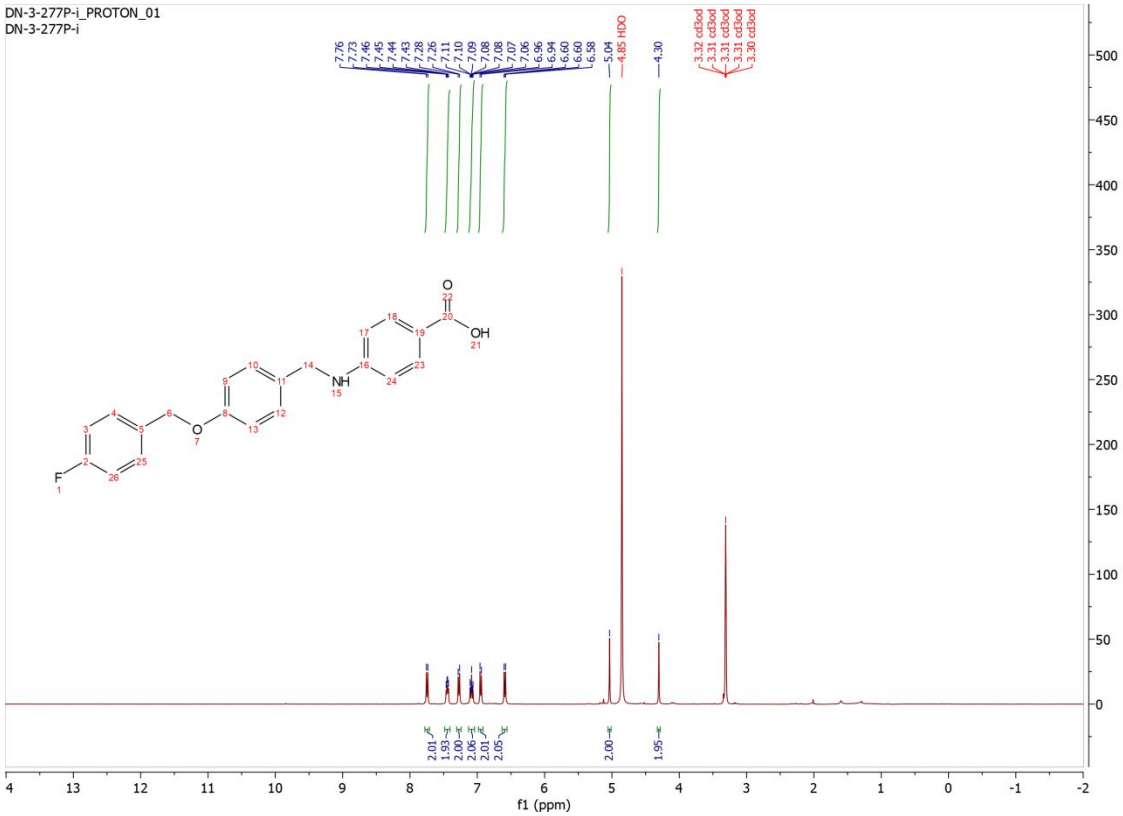



**Figure S7.** ***In silico*** **metabolic predictions determined by Schrodinger's P450 Site of Metabolism Module.** Predictions calculated for **4b** against 2C9, 2D6, and 3A4 CYPs. Components are described as transcribed directly from the manufacturer's manual. **Intrinsic Reactivity** = atoms are labeled with the intrinsic reactivity calculated with Hammett and Taft methodology. Positive values are predicted to be more reactive, negative values are predicted to be less reactive. **Fe Accessibility** = atoms are labeled with accessibility to the iron. This is defined as the natural logarithm of the number of poses for the atom in which the atom was within 5 Å of Fe. Larger values indicate greater accessibility. **Liability Predictions** = linear combination of the accessibility and the intrinsic reactivity. Results are displayed as green circles, in which the radius is proportional to the score. Larger circles mean higher reactivity.

## FINAL COMPOUND $^{1}H$ and $^{13}C$ NMR SPECTRA

4b

4b*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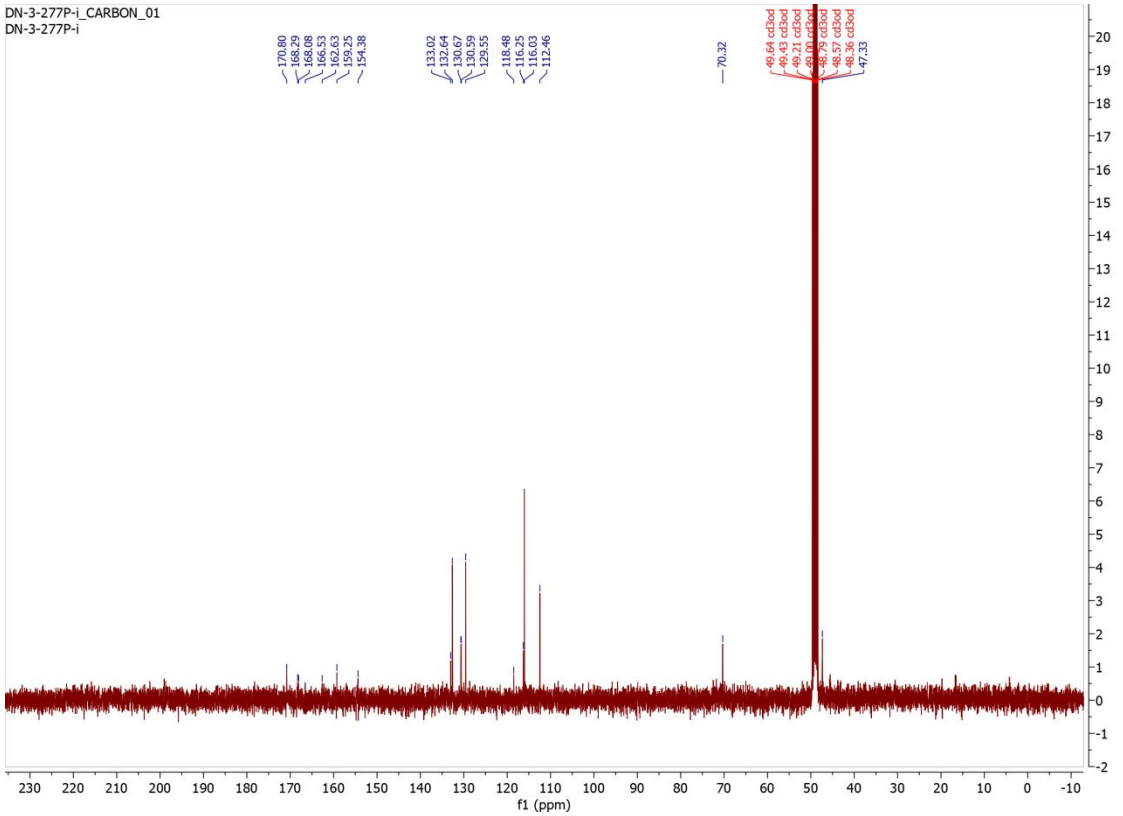
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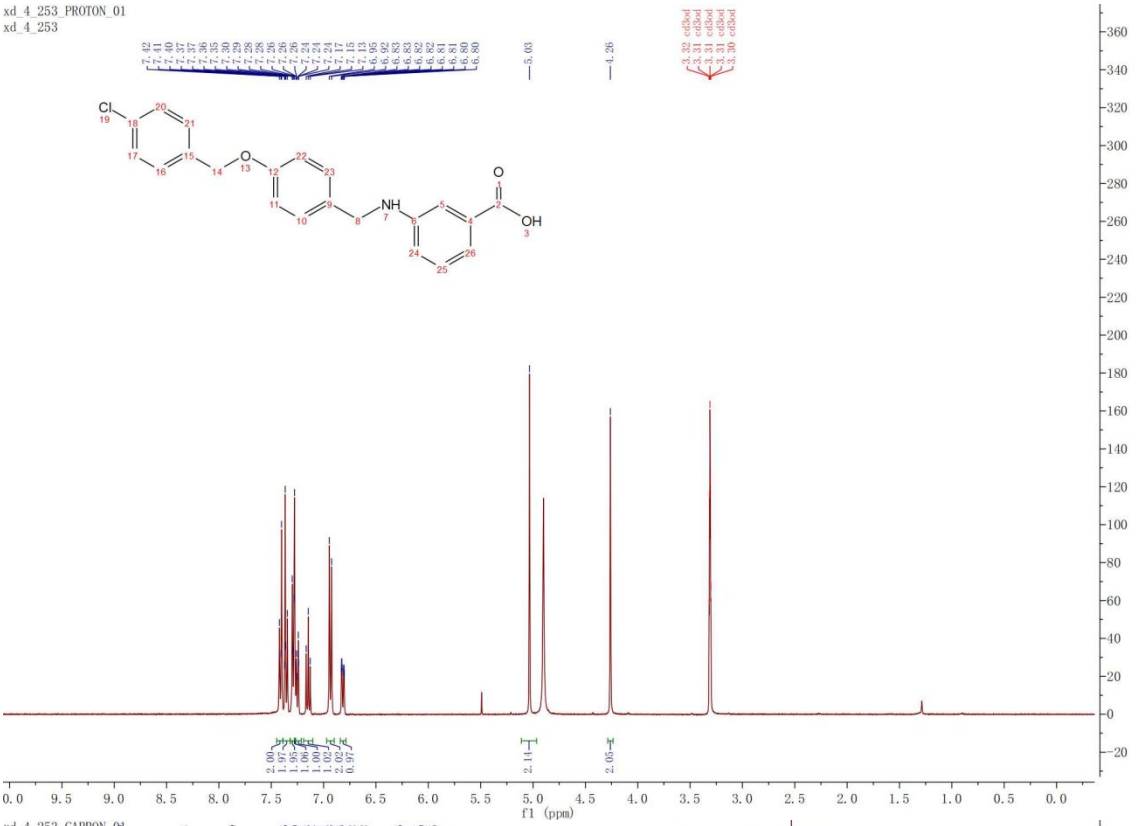
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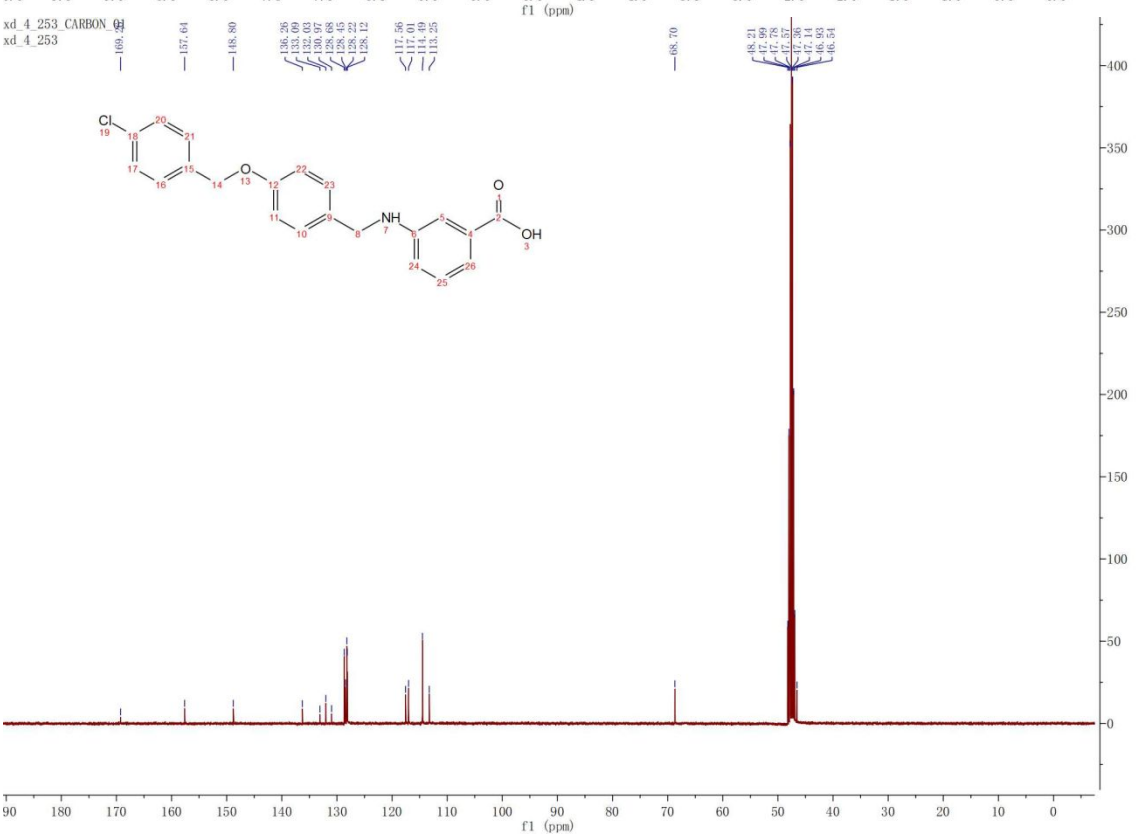
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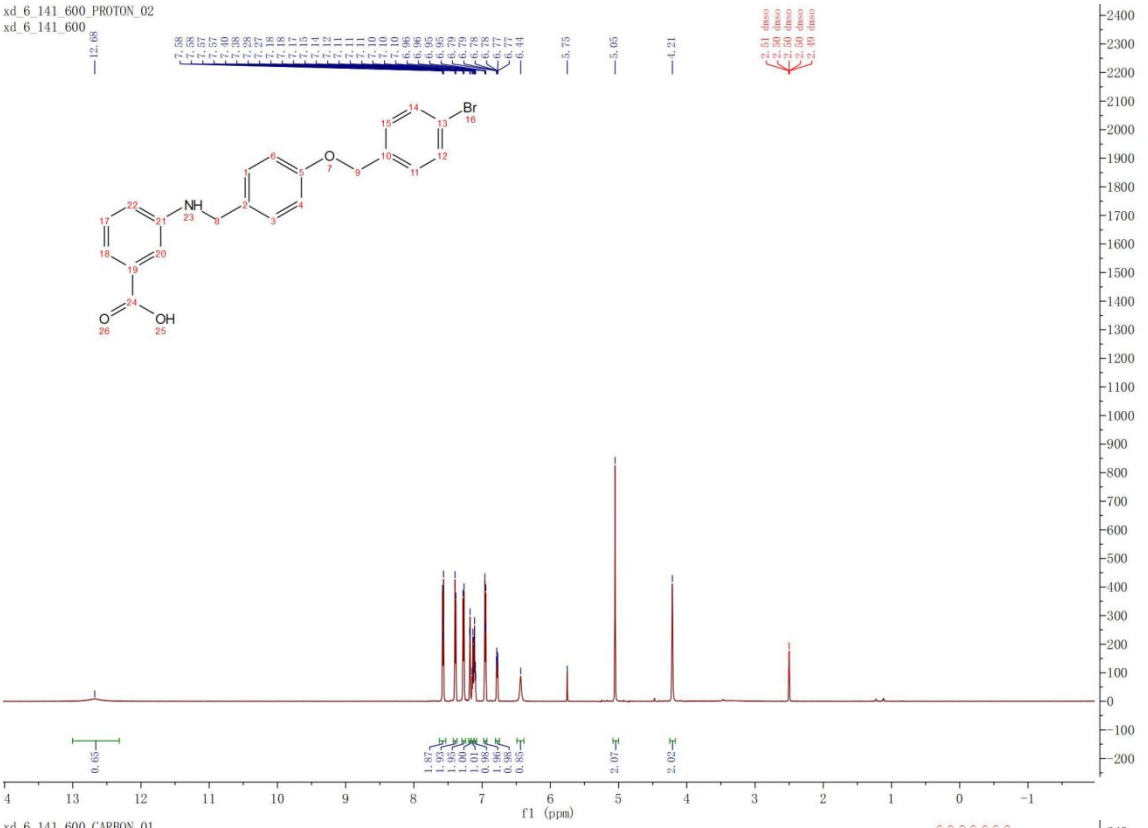
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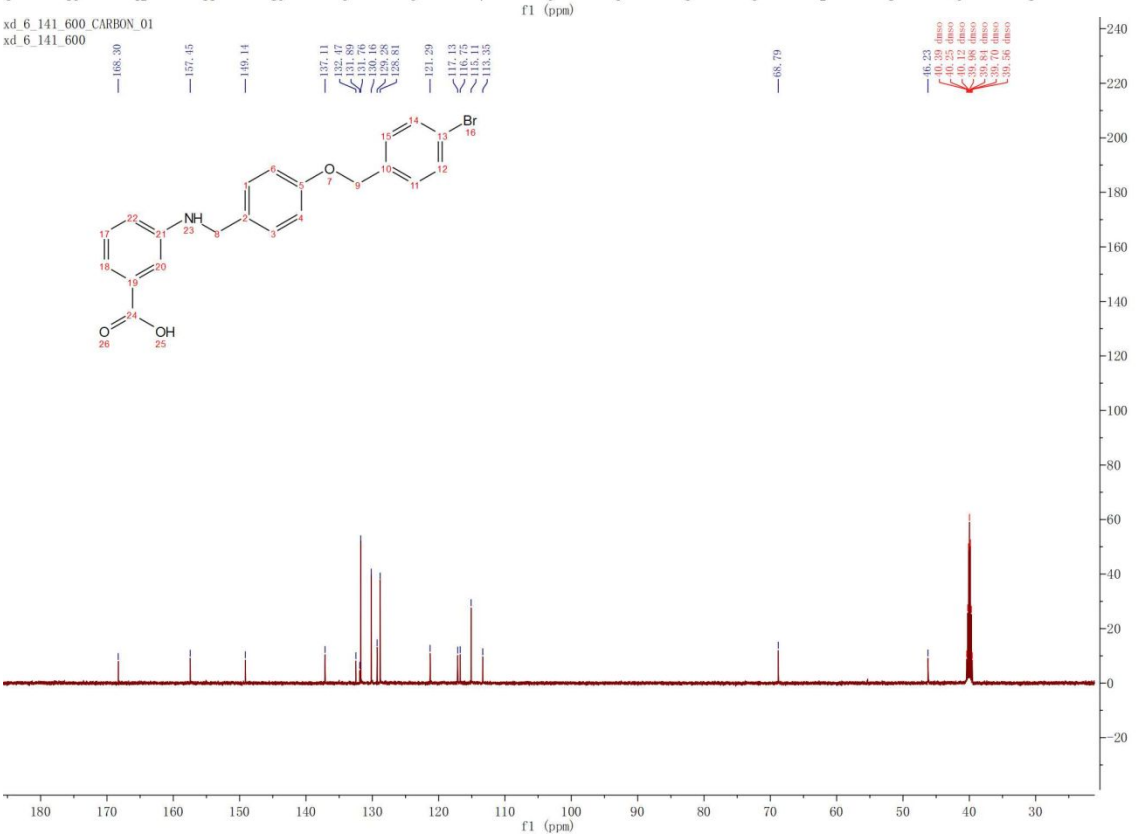
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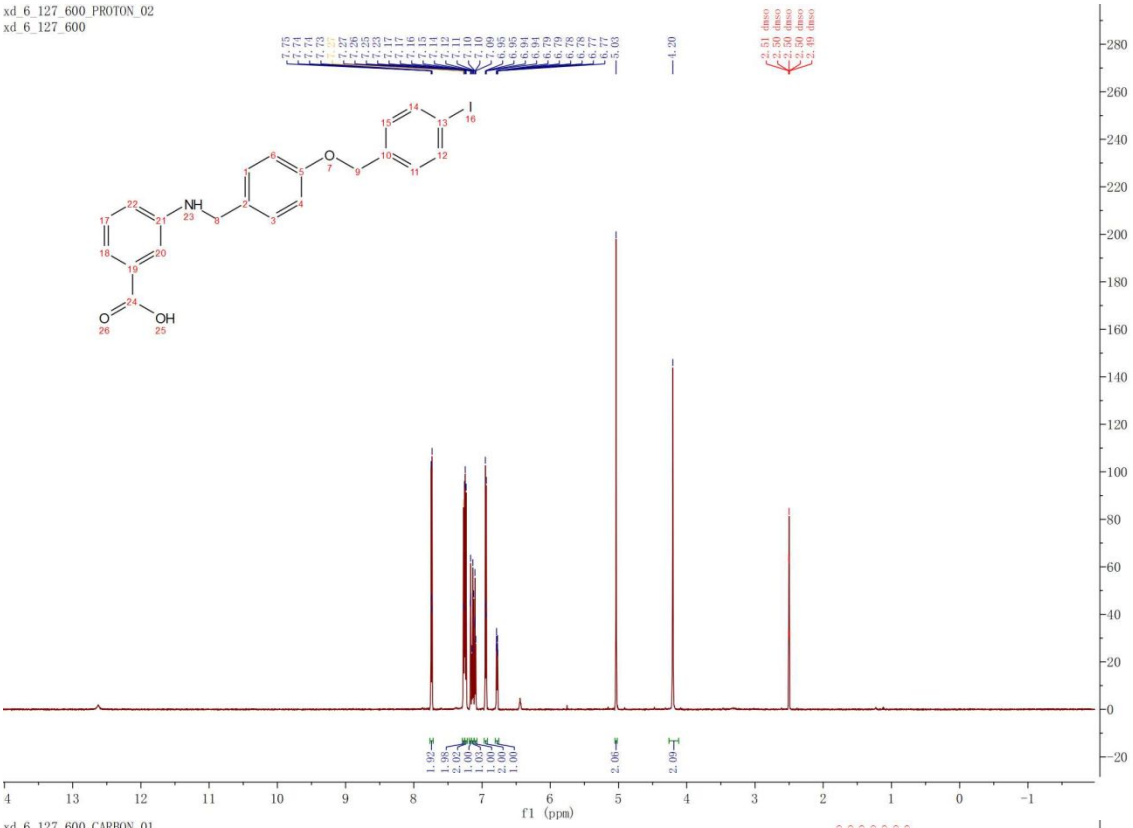
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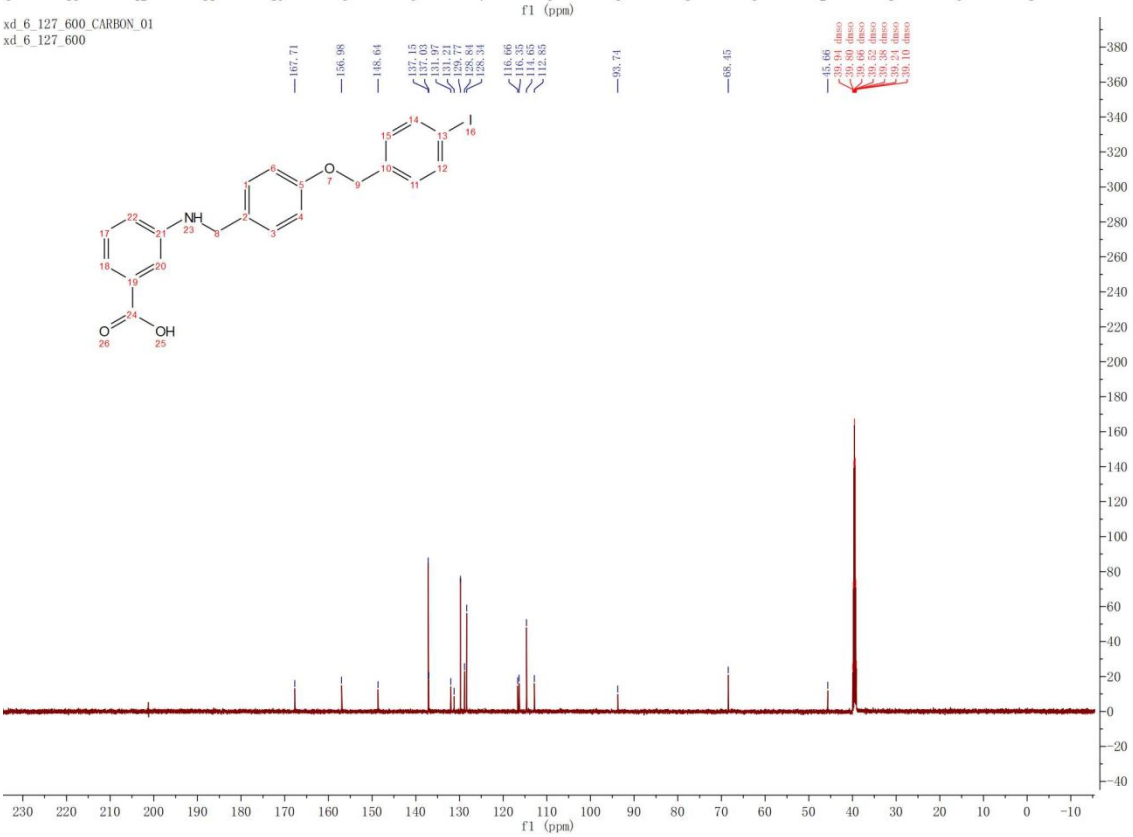
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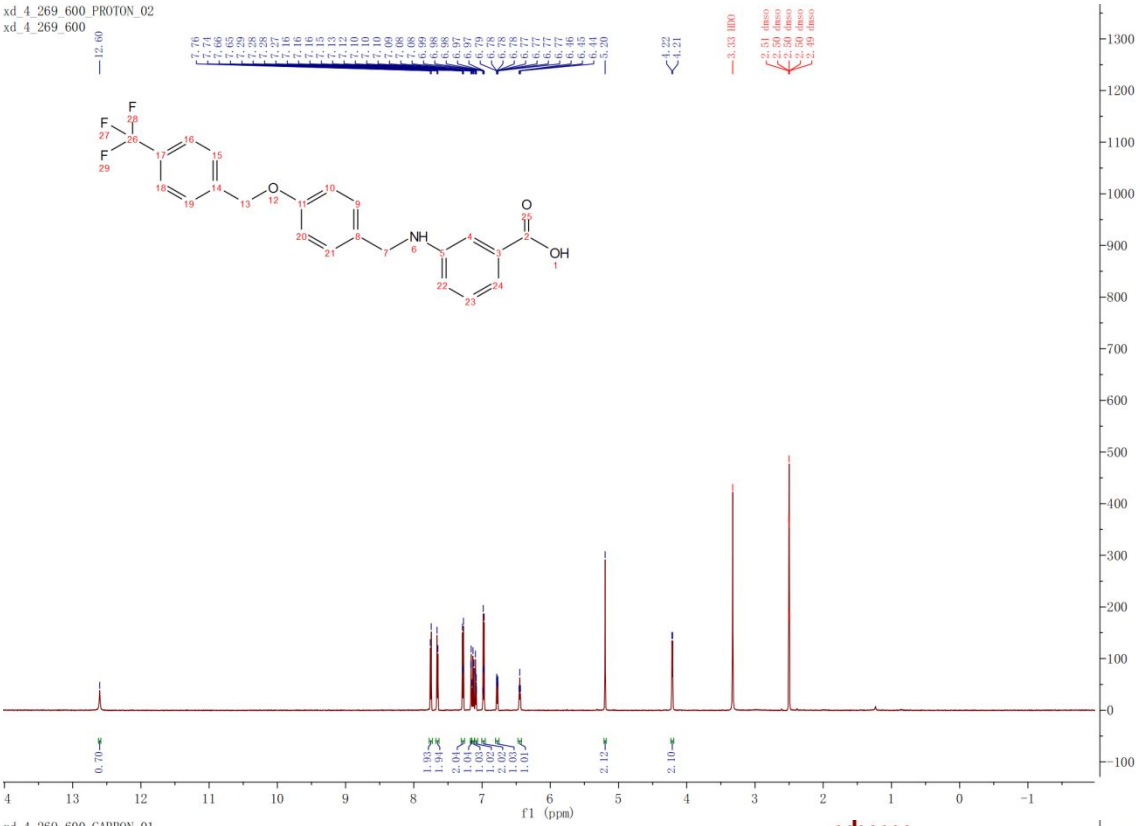
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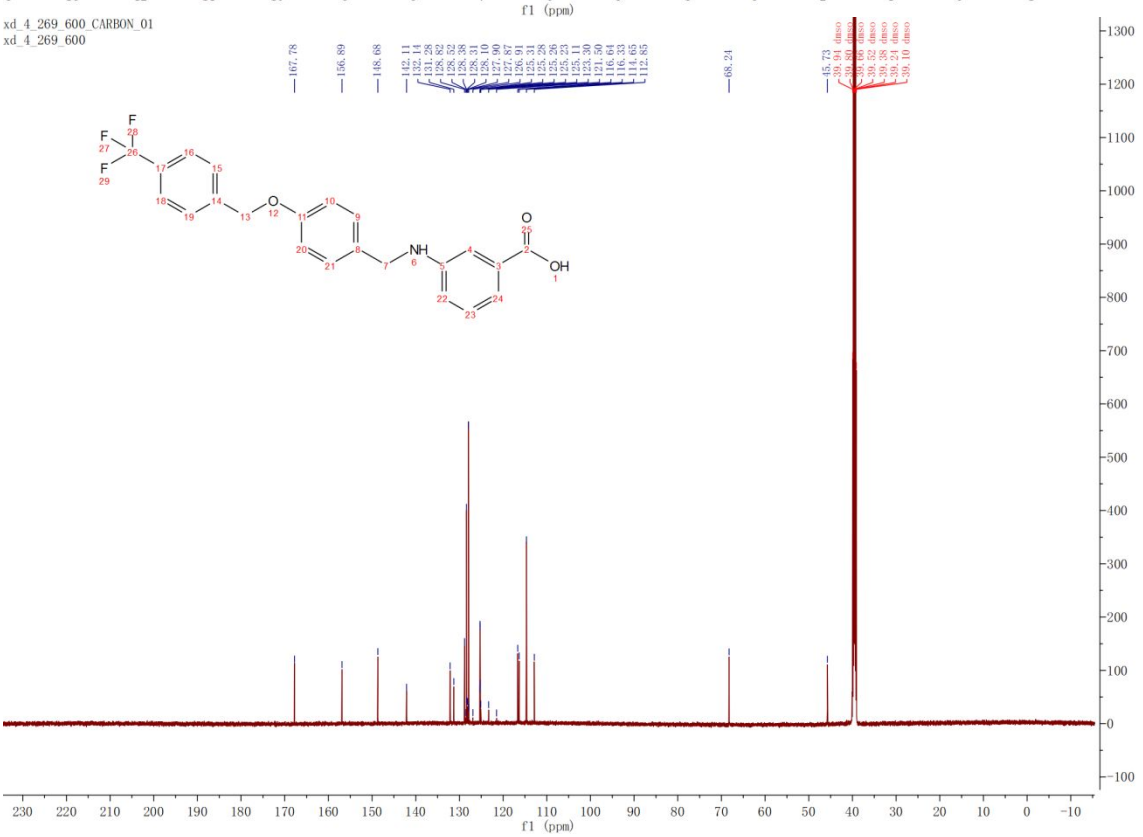
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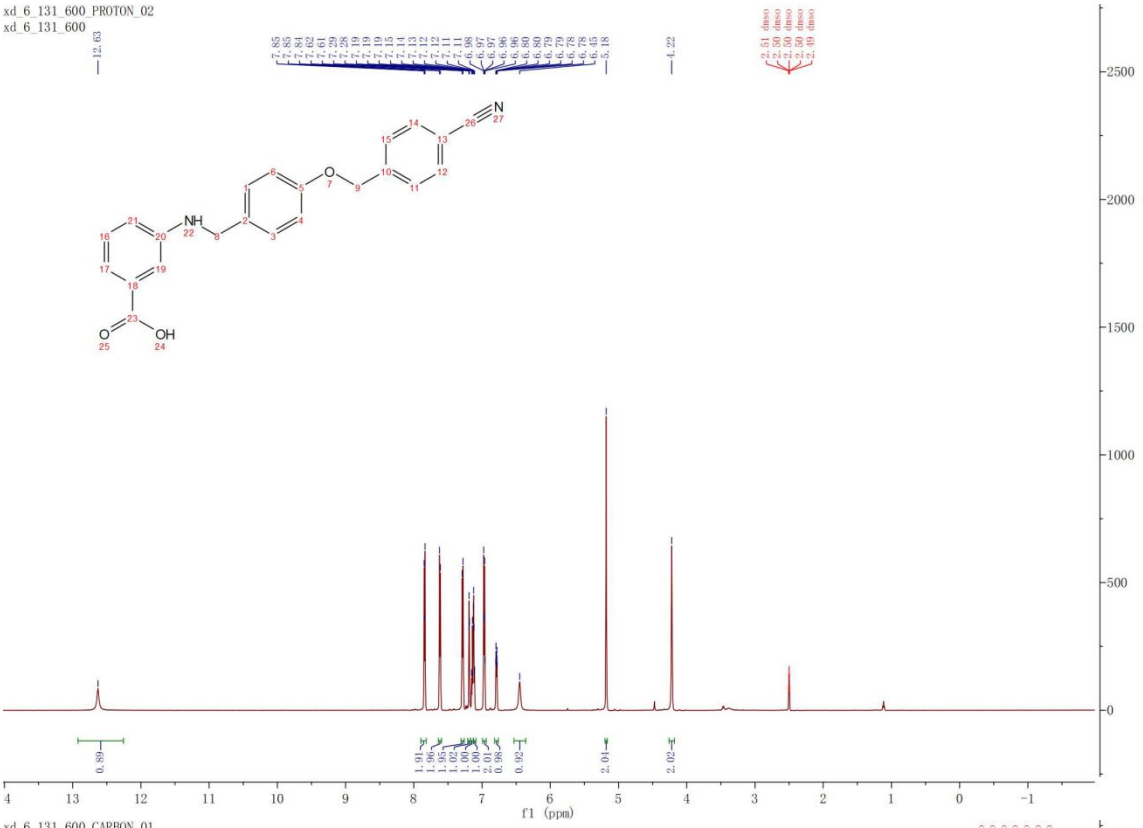
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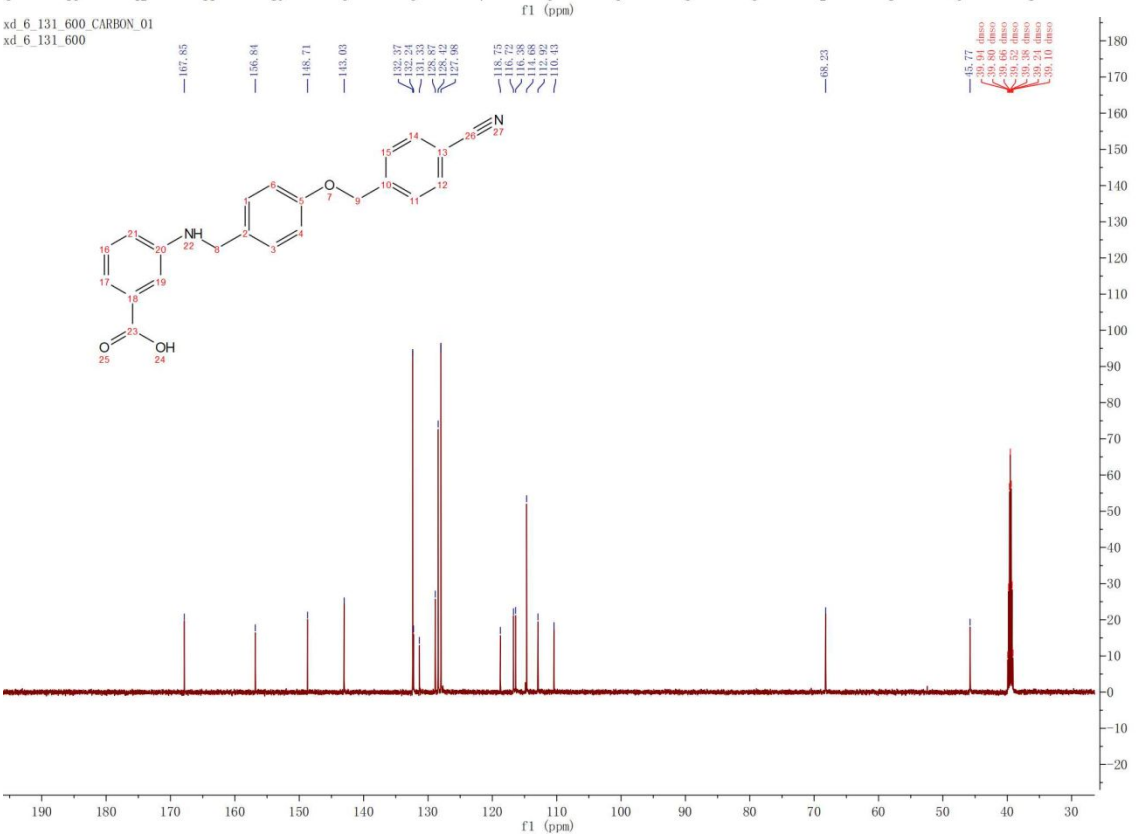
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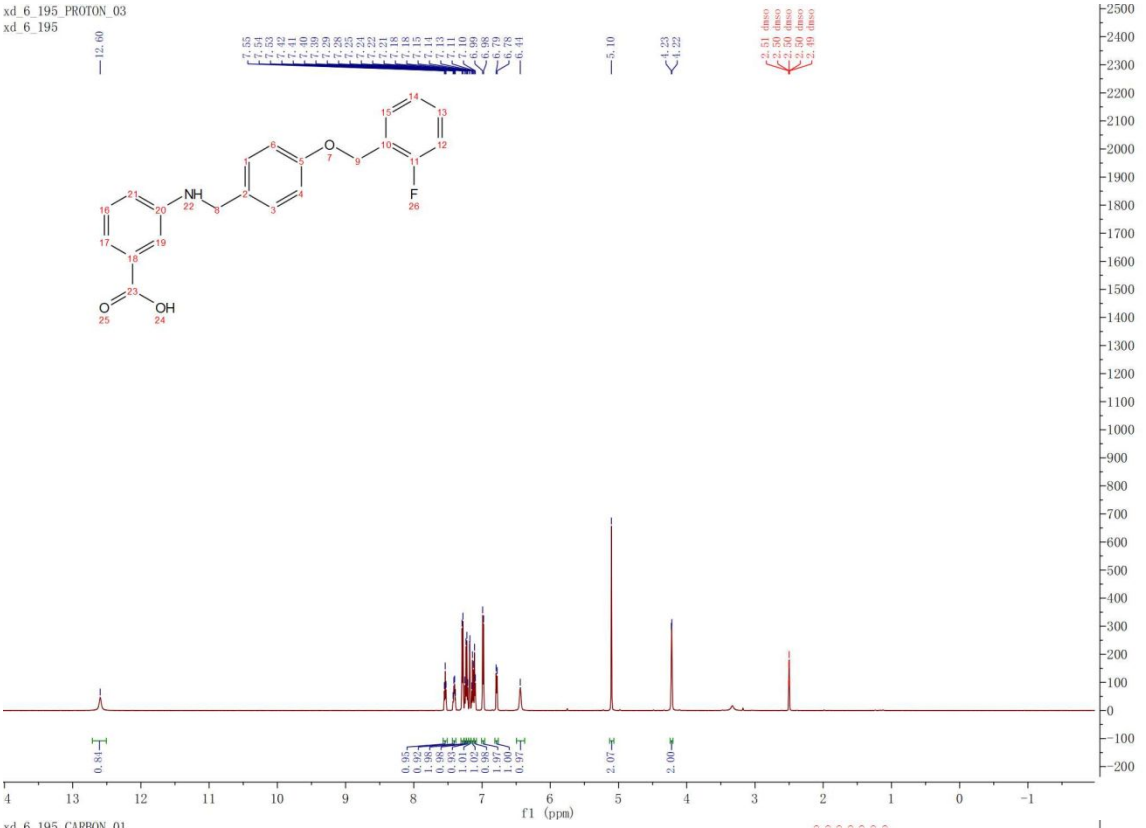
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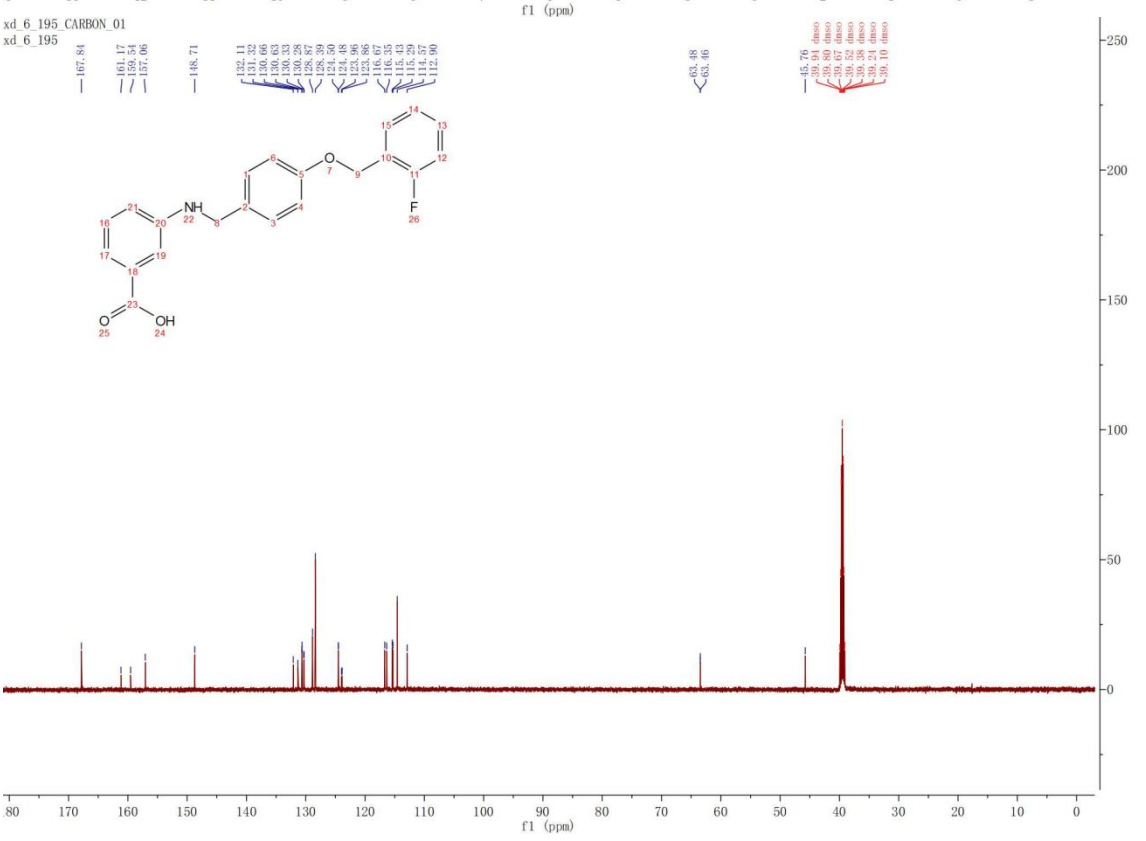
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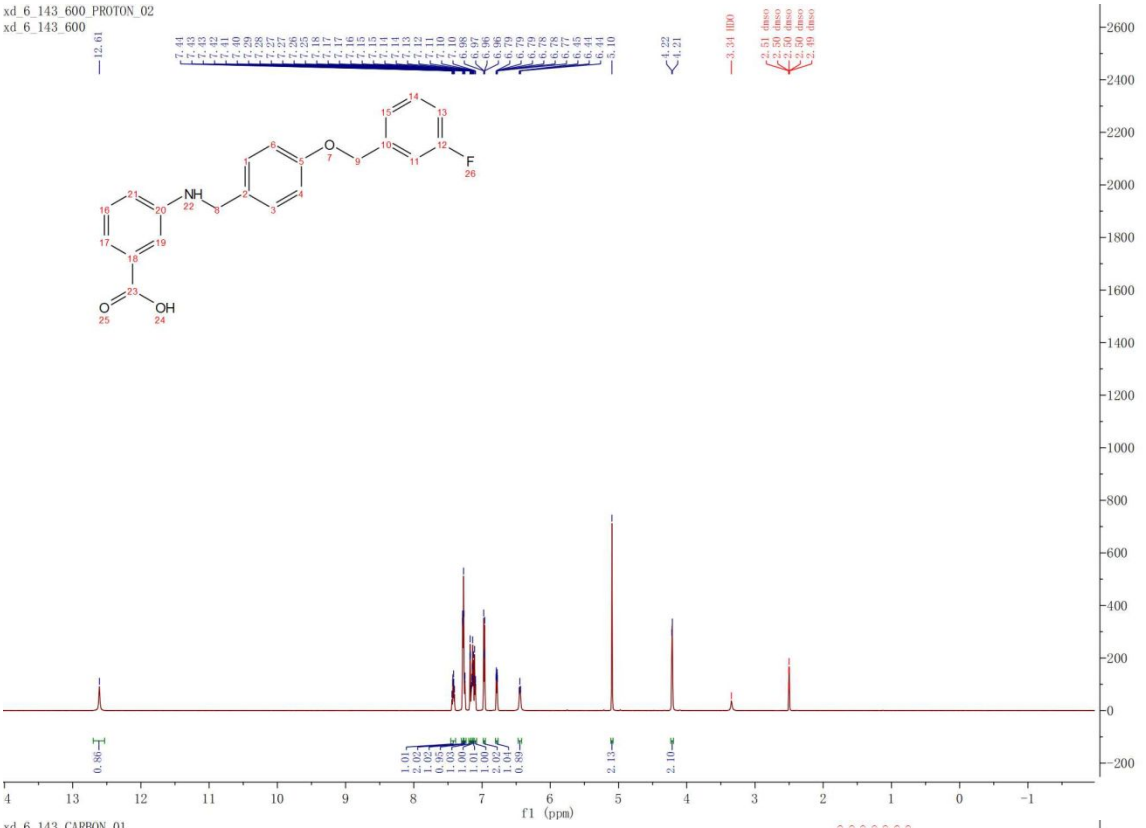
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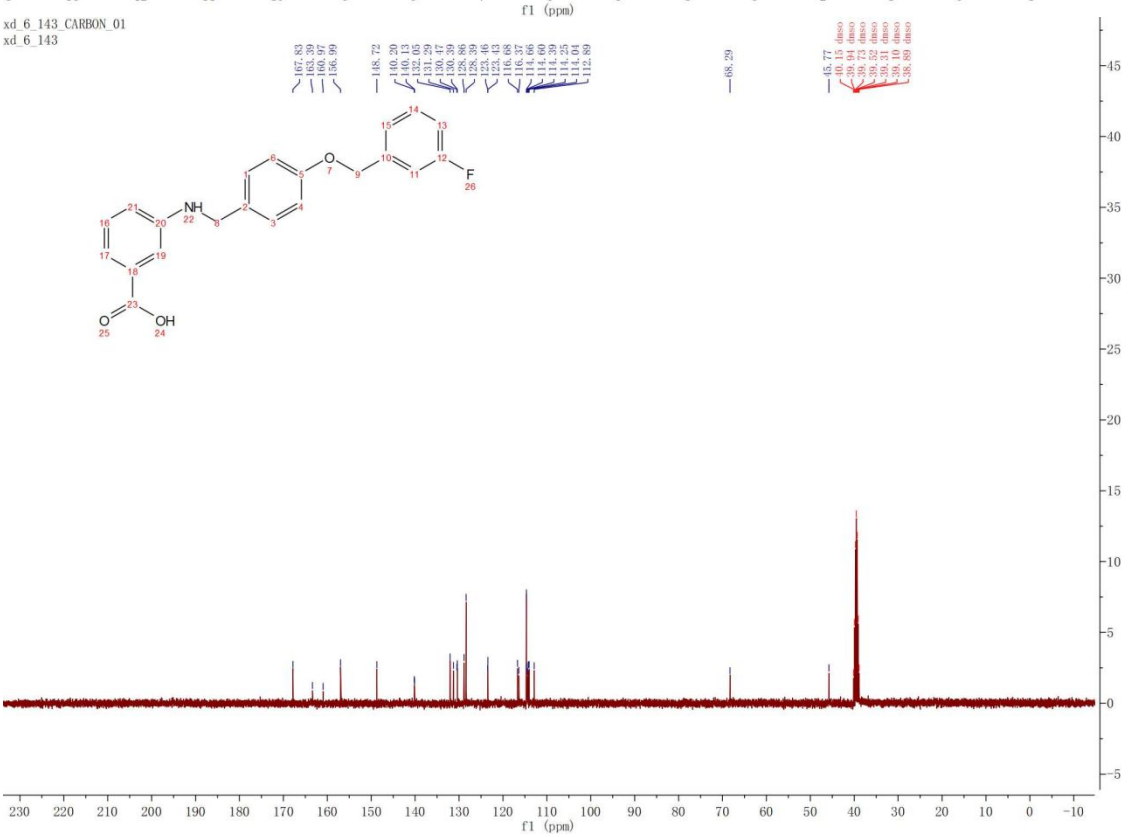
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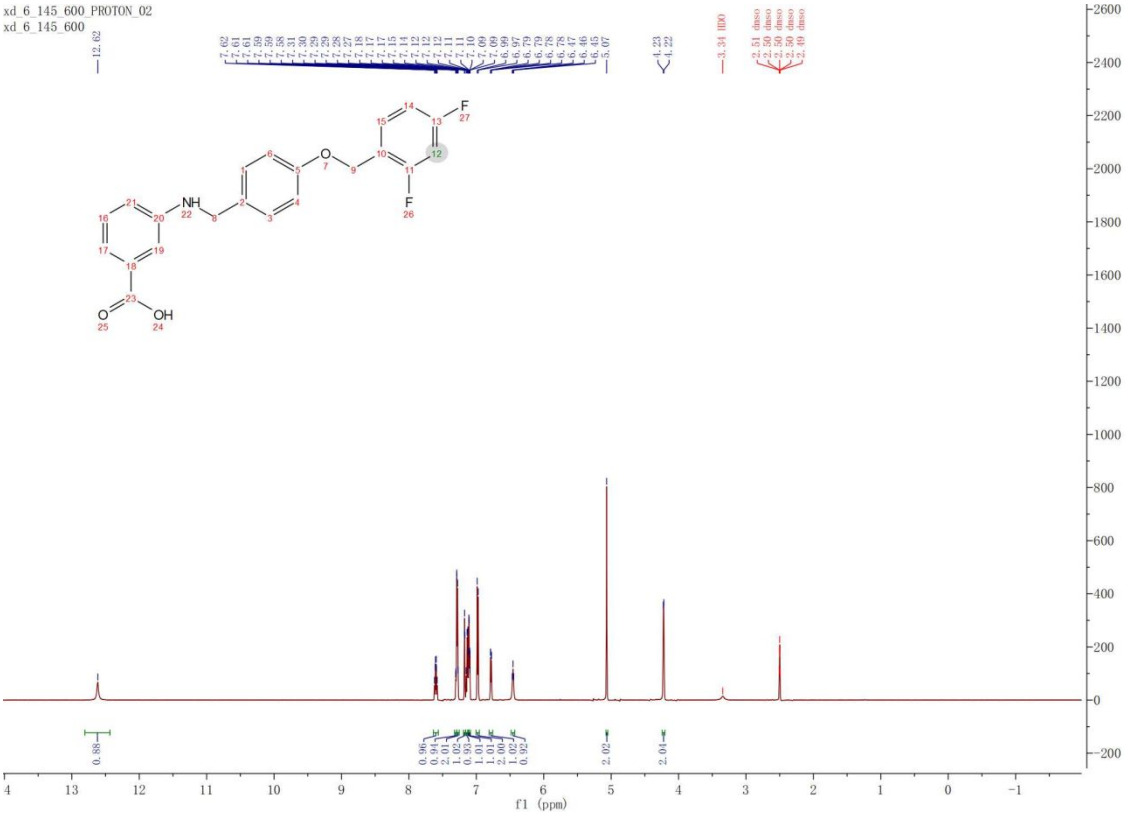
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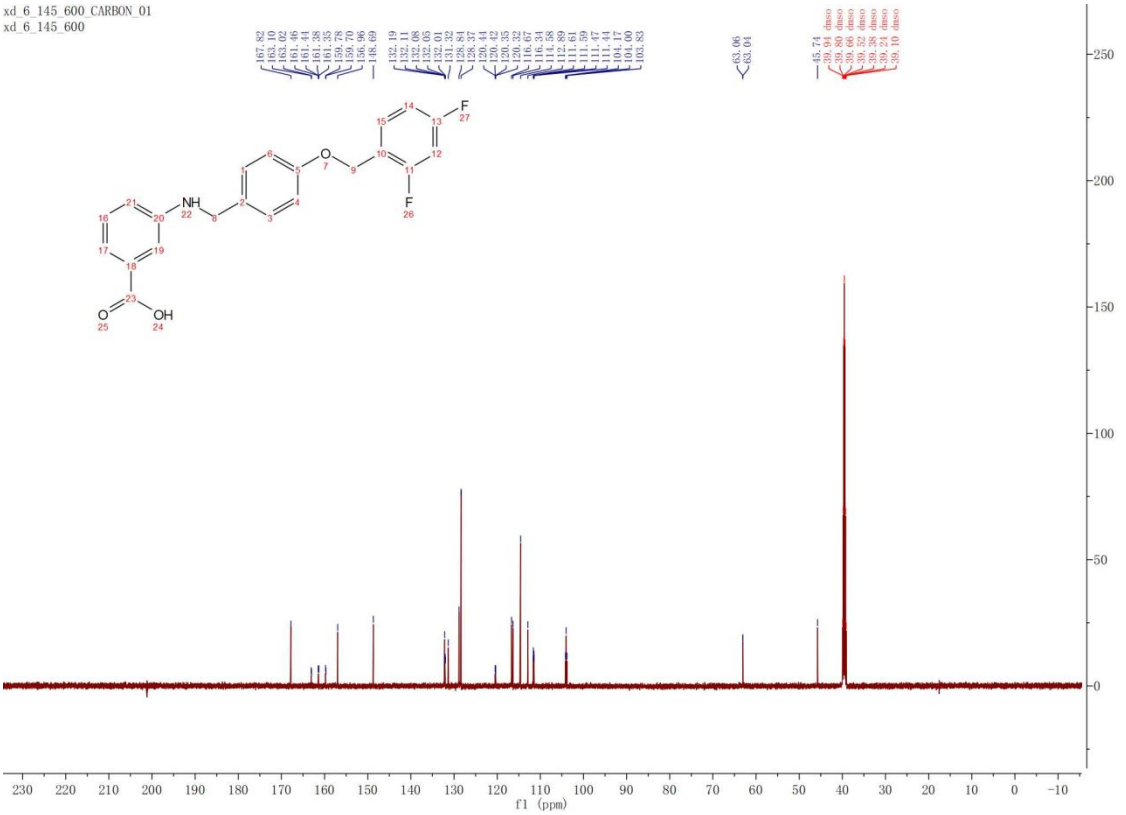
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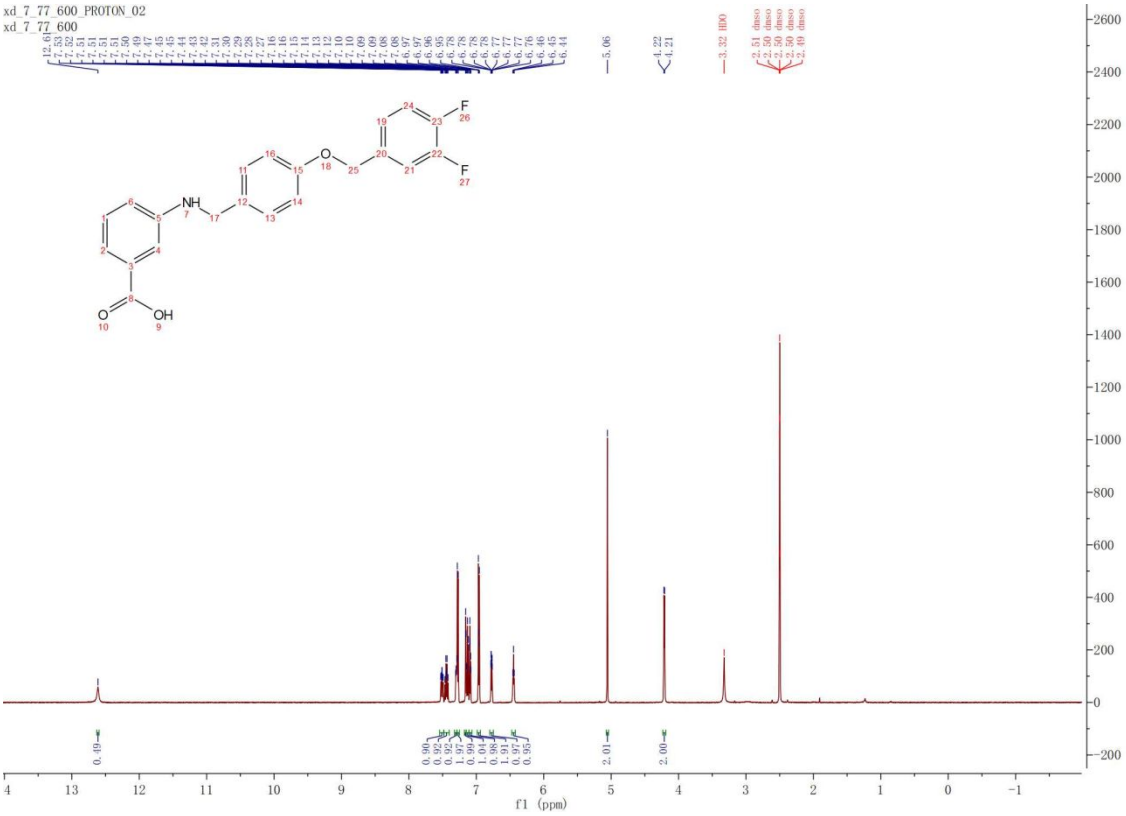
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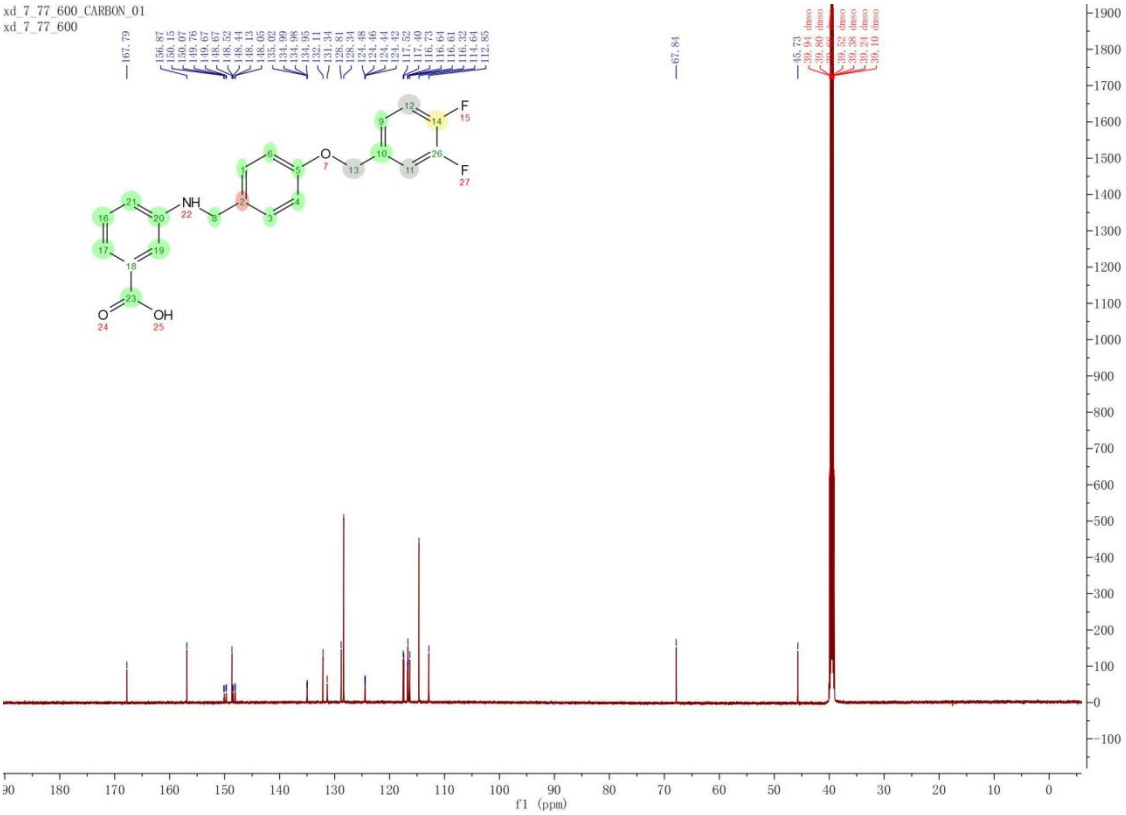
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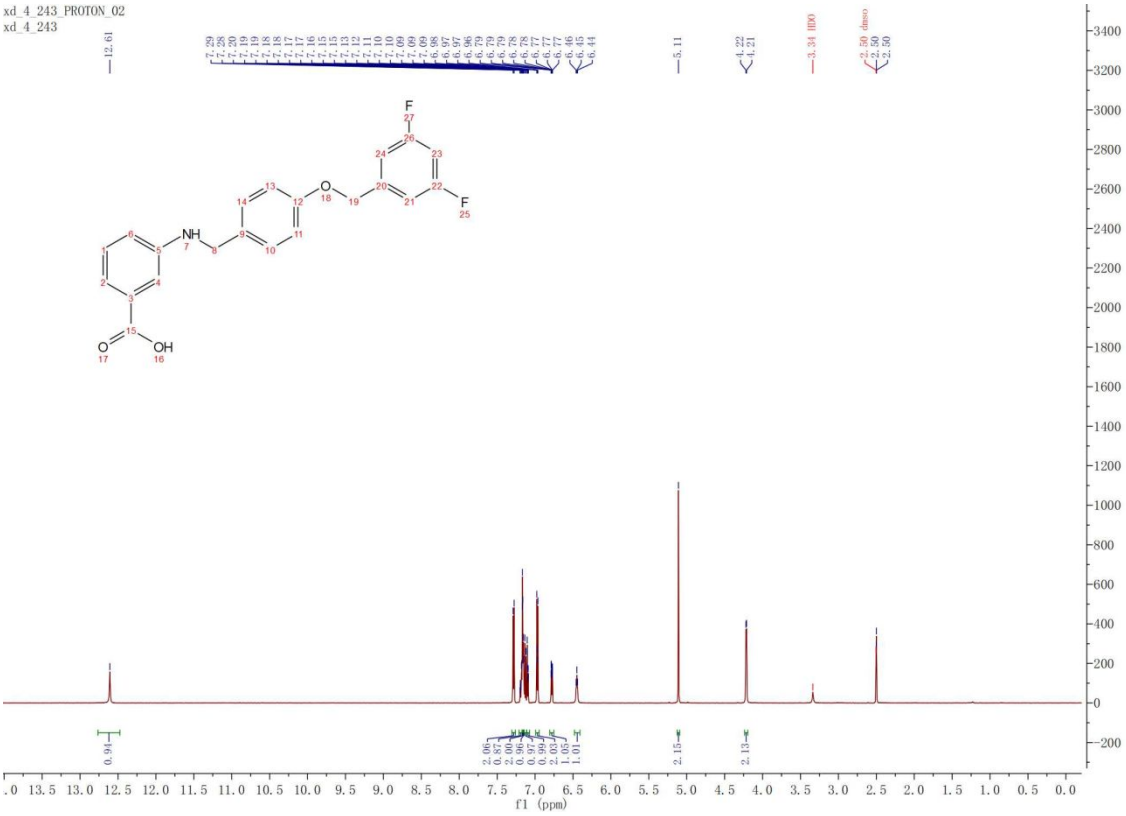
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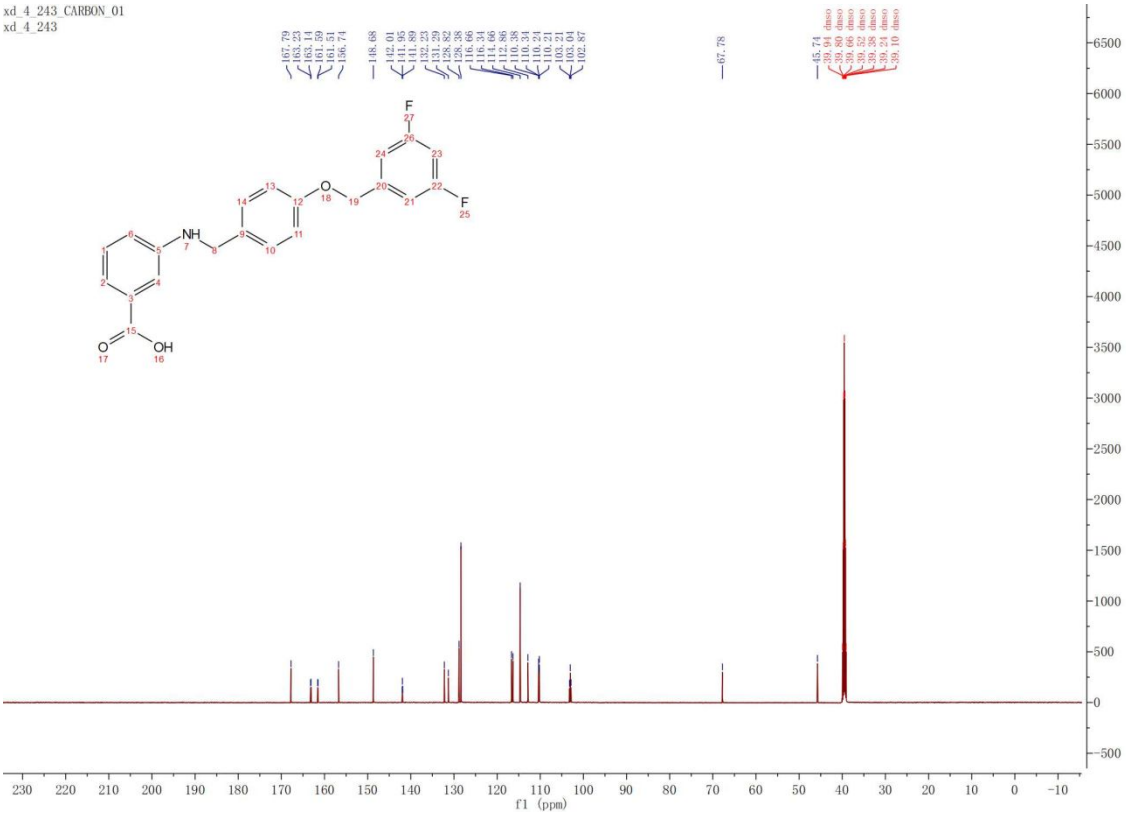
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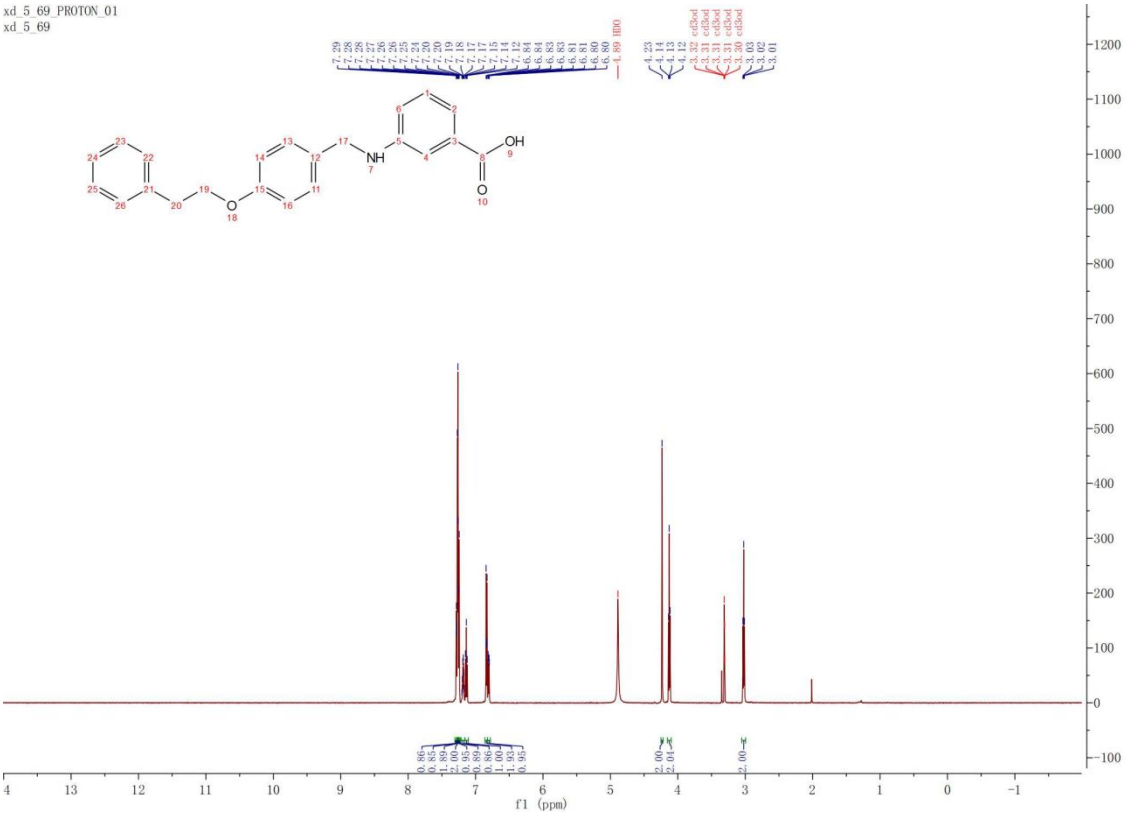


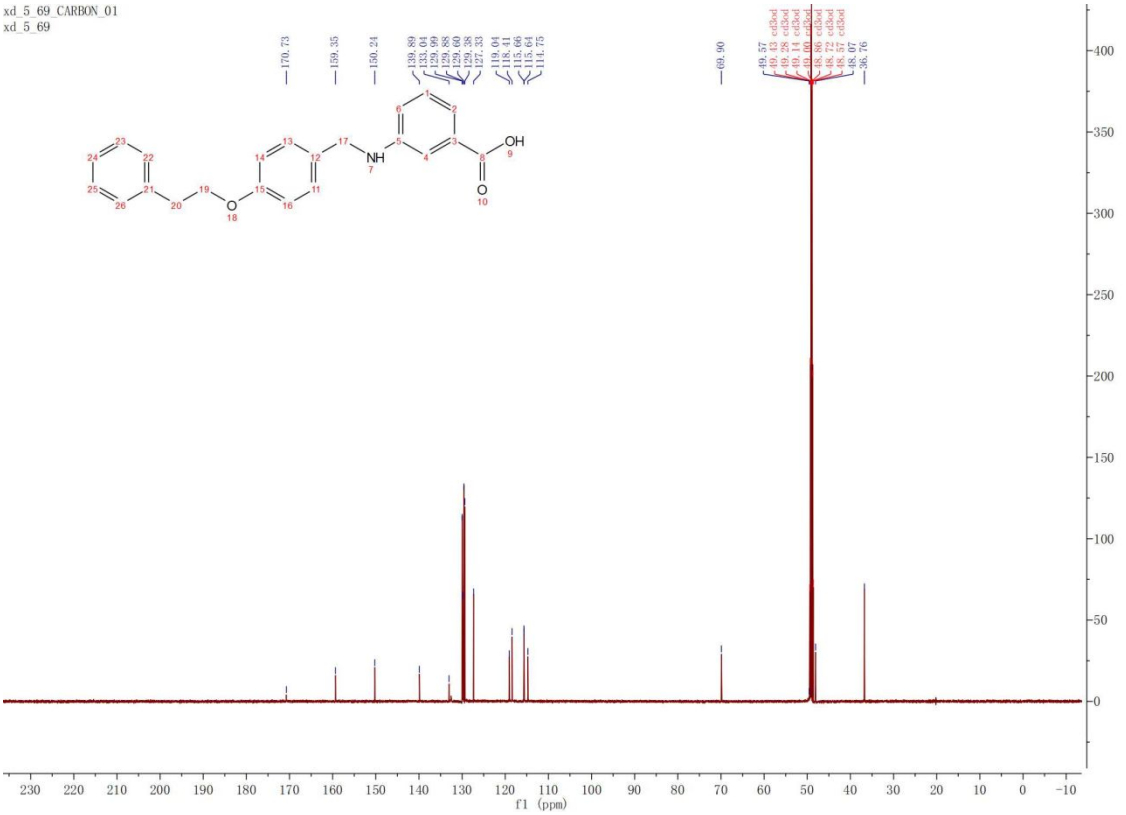

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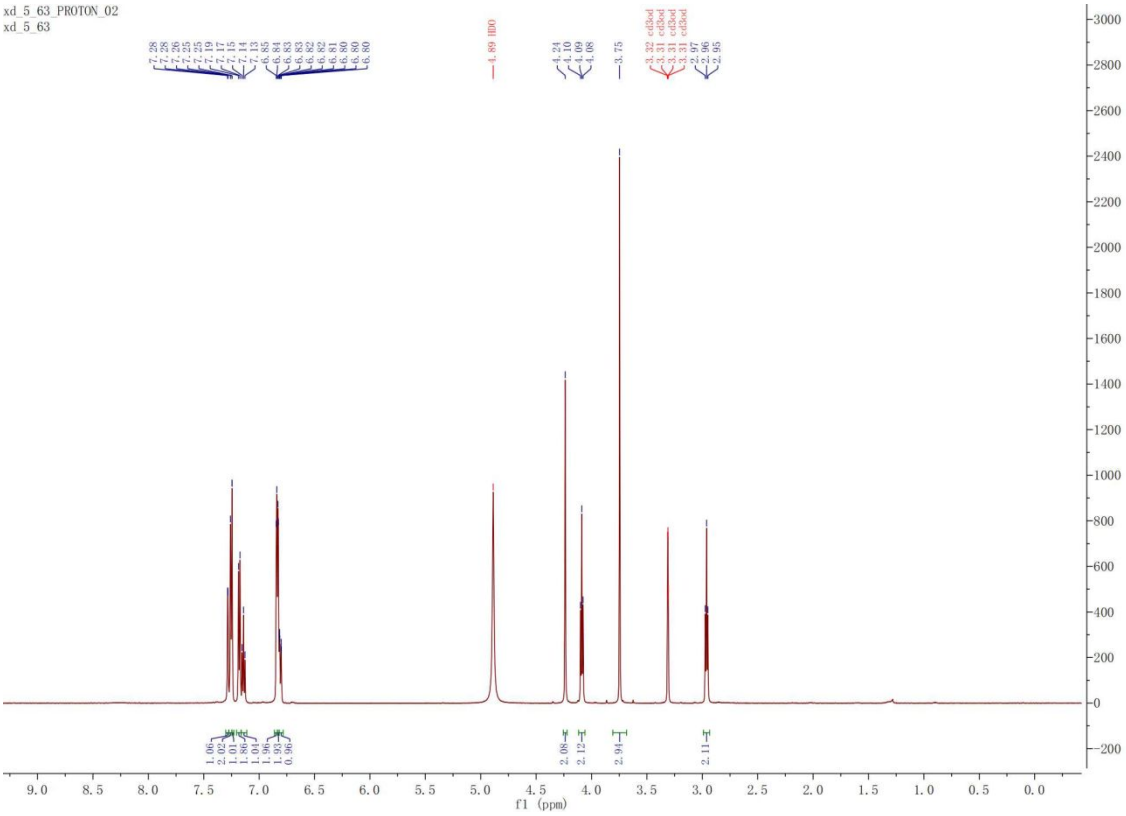
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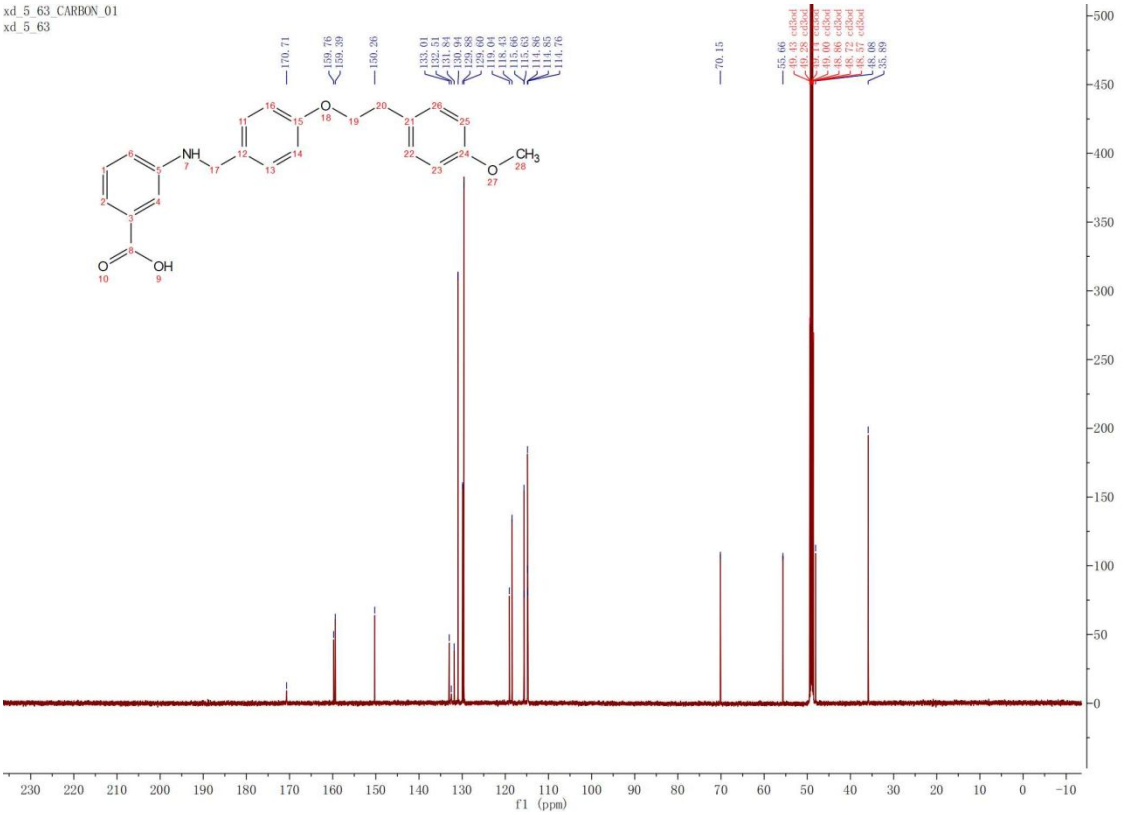
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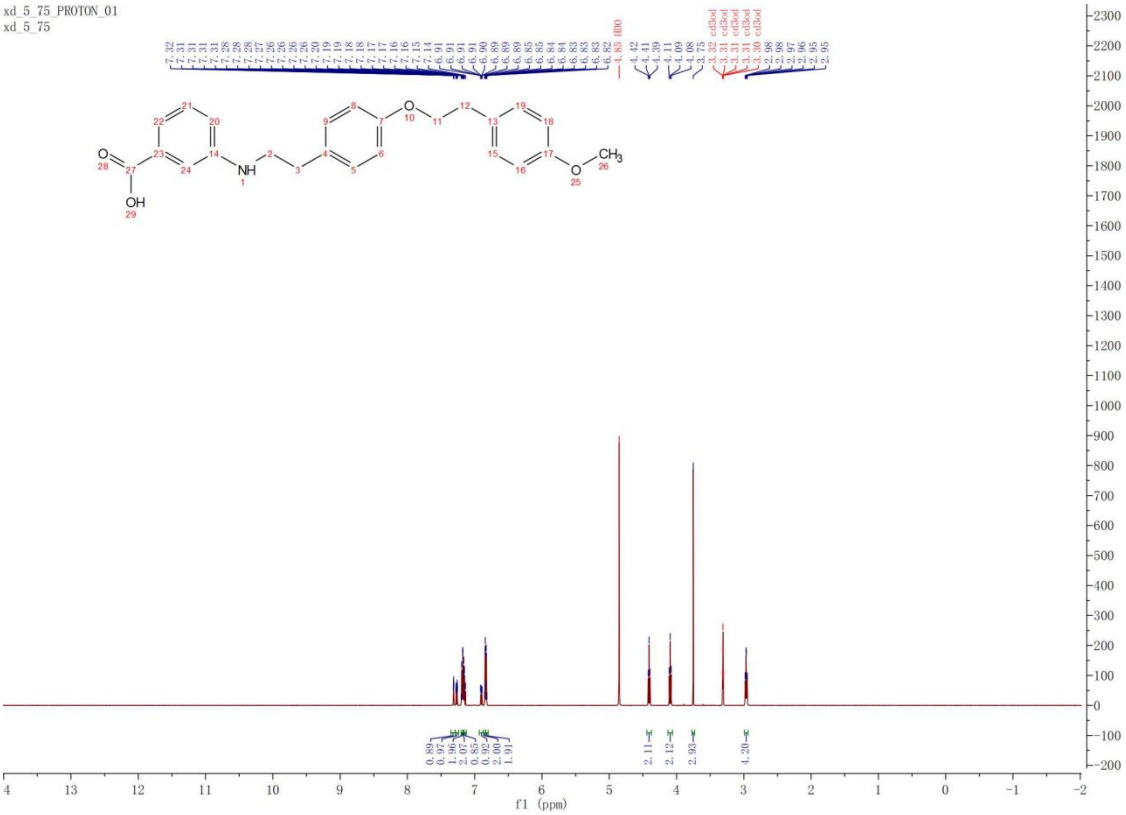
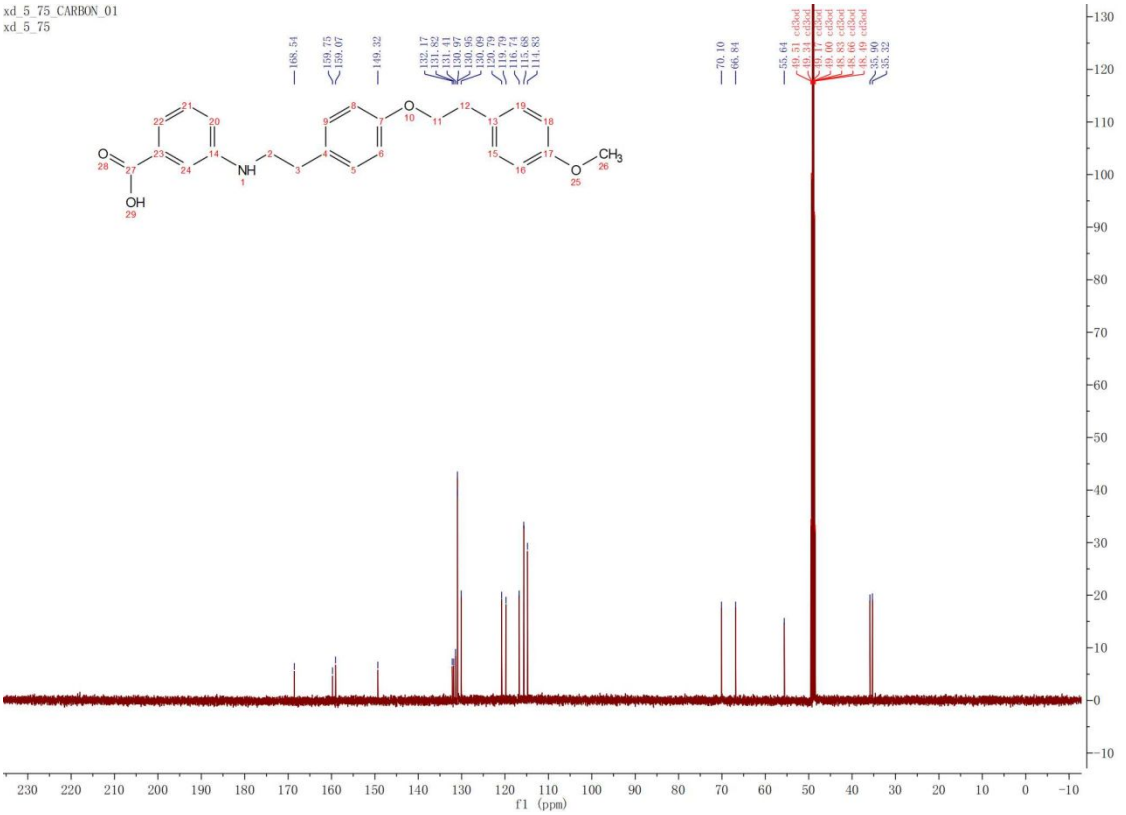
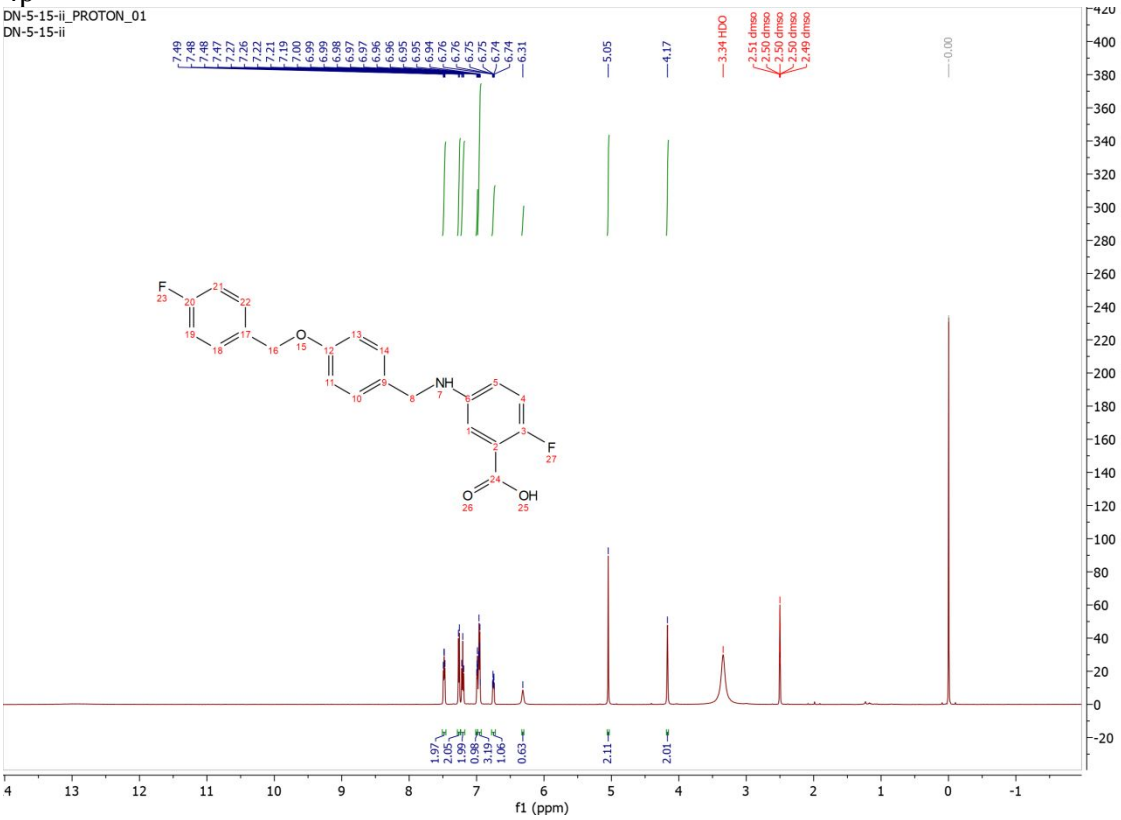
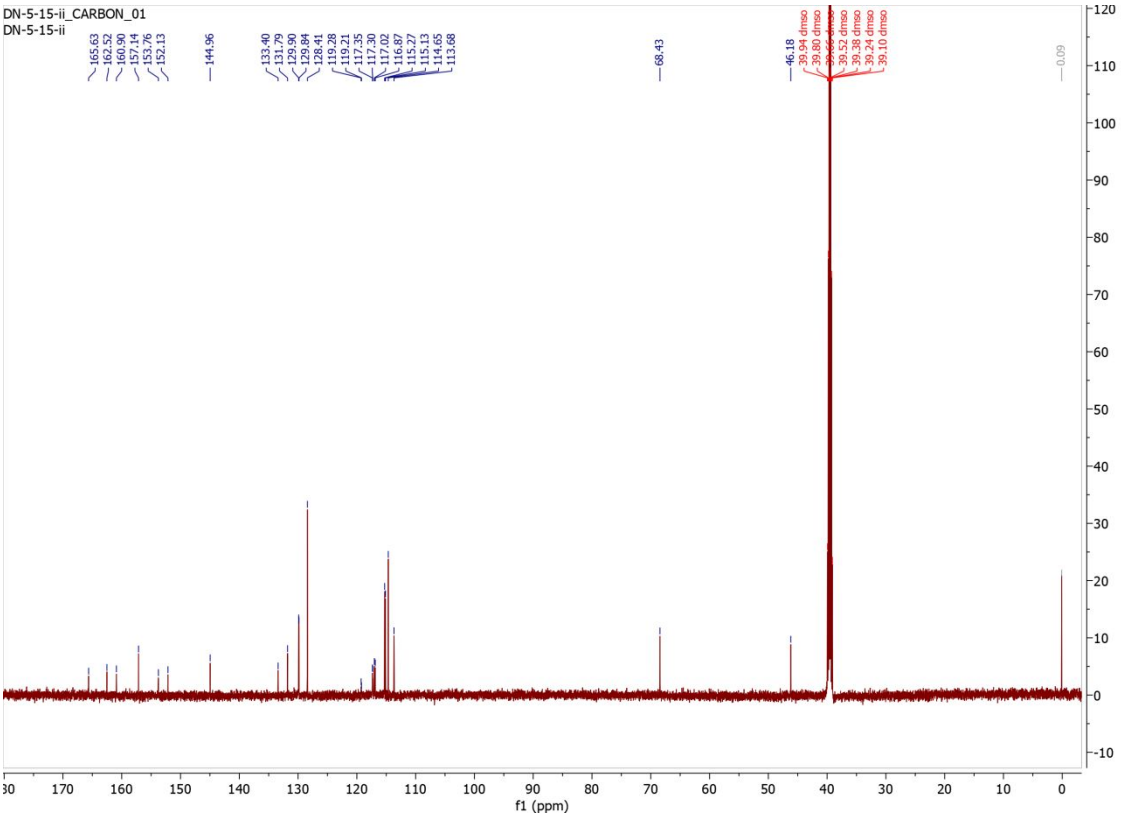


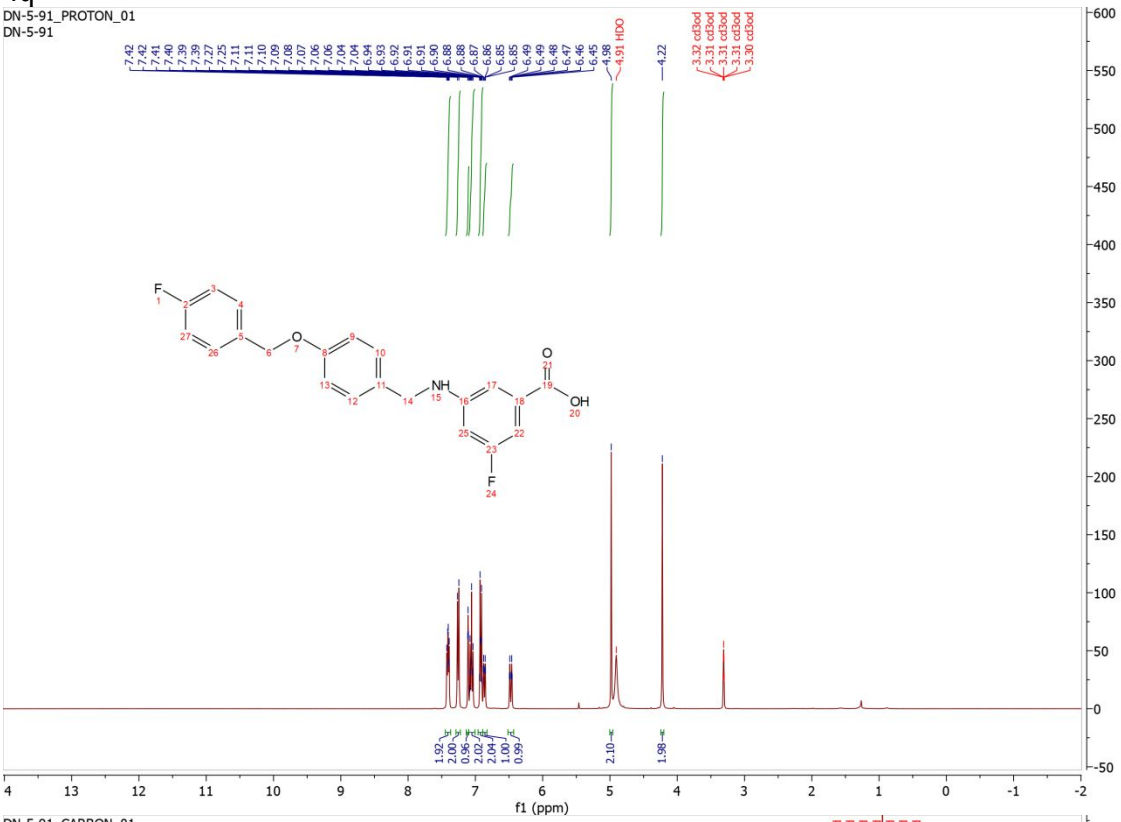



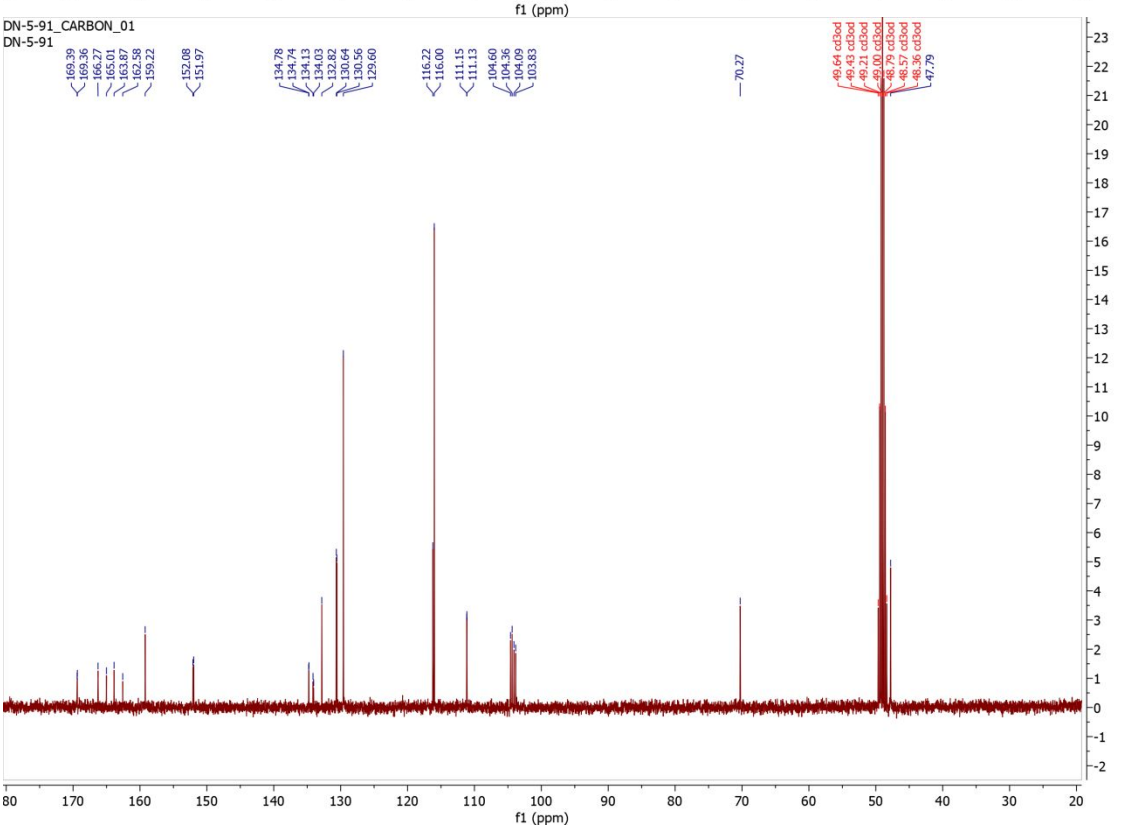
4p


DN-5-15-ii_PROTON_01
DN-5-15-ii
7.49
7.48
7.48
7.47
7.27
7.26
7.22
7.21
7.19
7.00
6.99
6.99
6.98
6.97
6.97
6.96
6.96
6.95
6.95
6.94
6.76
6.76
6.75
6.75
6.74
6.74
6.31
5.05
4.17
3.34 HDO
2.51 dmso
2.50 dmso
2.50 dmso
2.50 dmso
2.49 dmso
0.00
F
O
NH
F
O
OH
1.97
2.05
1.99
0.98
3.19
1.06
0.63
2.11
2.01
f1 (ppm)

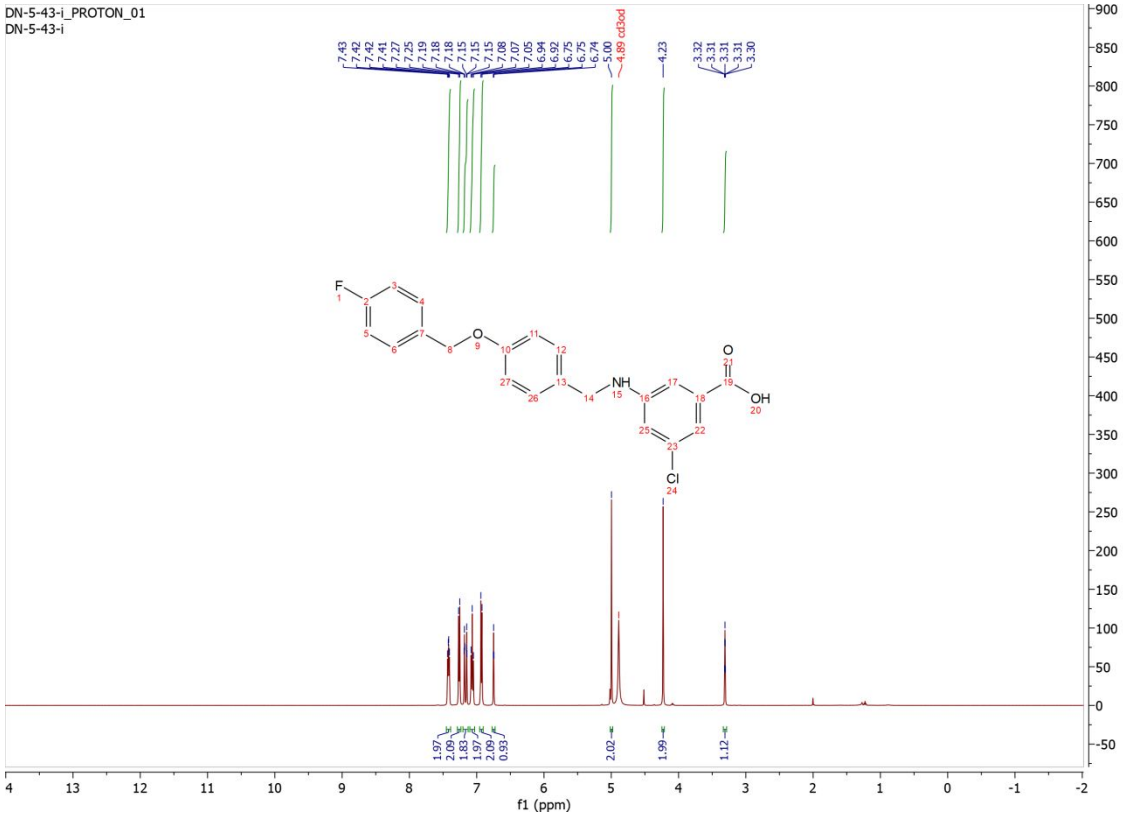

DN-5-15-ii_CARBON_01
DN-5-15-ii
165.63
162.52
160.90
157.14
153.76
152.13
144.96
133.40
131.79
129.90
129.84
128.41
119.28
119.21
117.35
117.30
117.02
116.87
115.27
115.13
114.65
113.68
68.43
46.18
39.94 dmso
39.80 dmso
39.66 dmso
39.52 dmso
39.38 dmso
39.24 dmso
39.10 dmso
0.09
f1 (ppm)

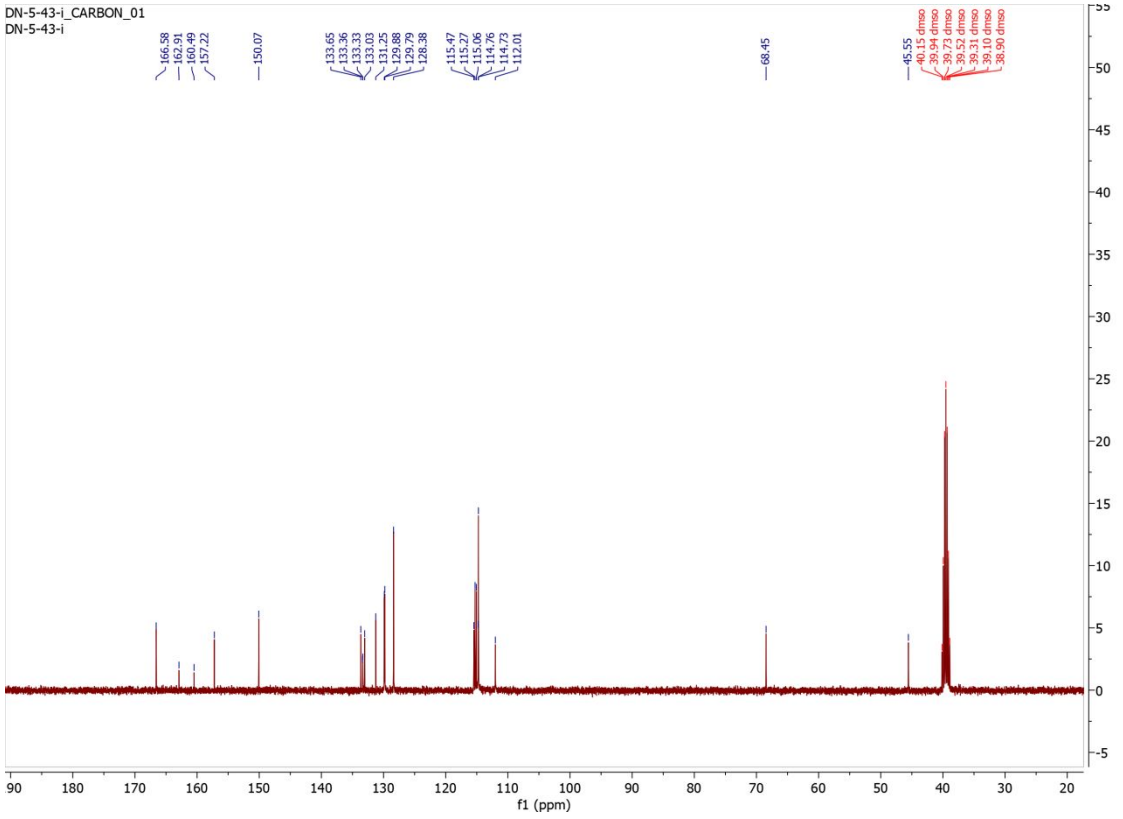
4q


DN-5-91_PROTON_01
DN-5-91
4.91 HDO
4.22
f1 (ppm)

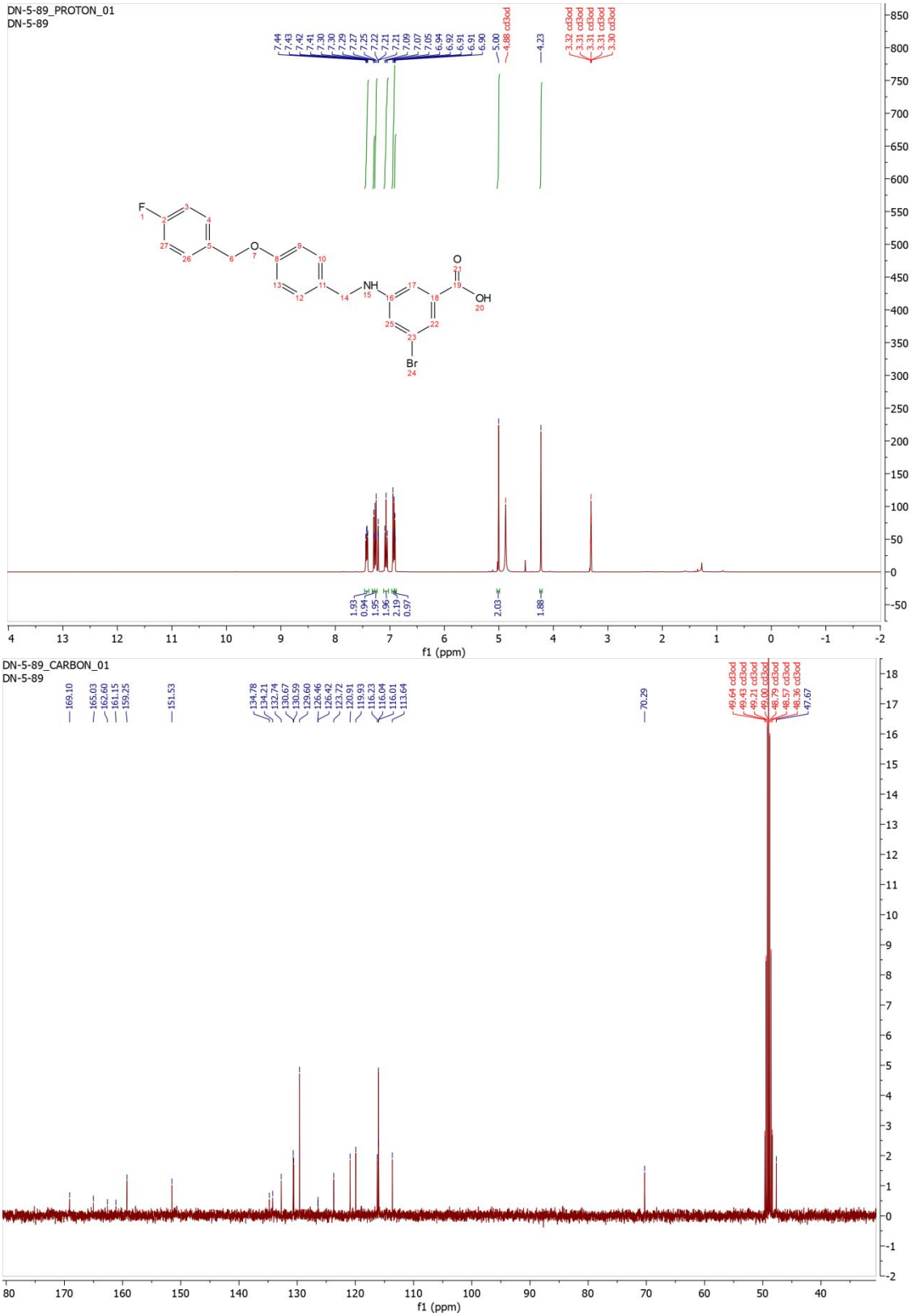

DN-5-91_CARBON_01
DN-5-91
f1 (ppm)

4r

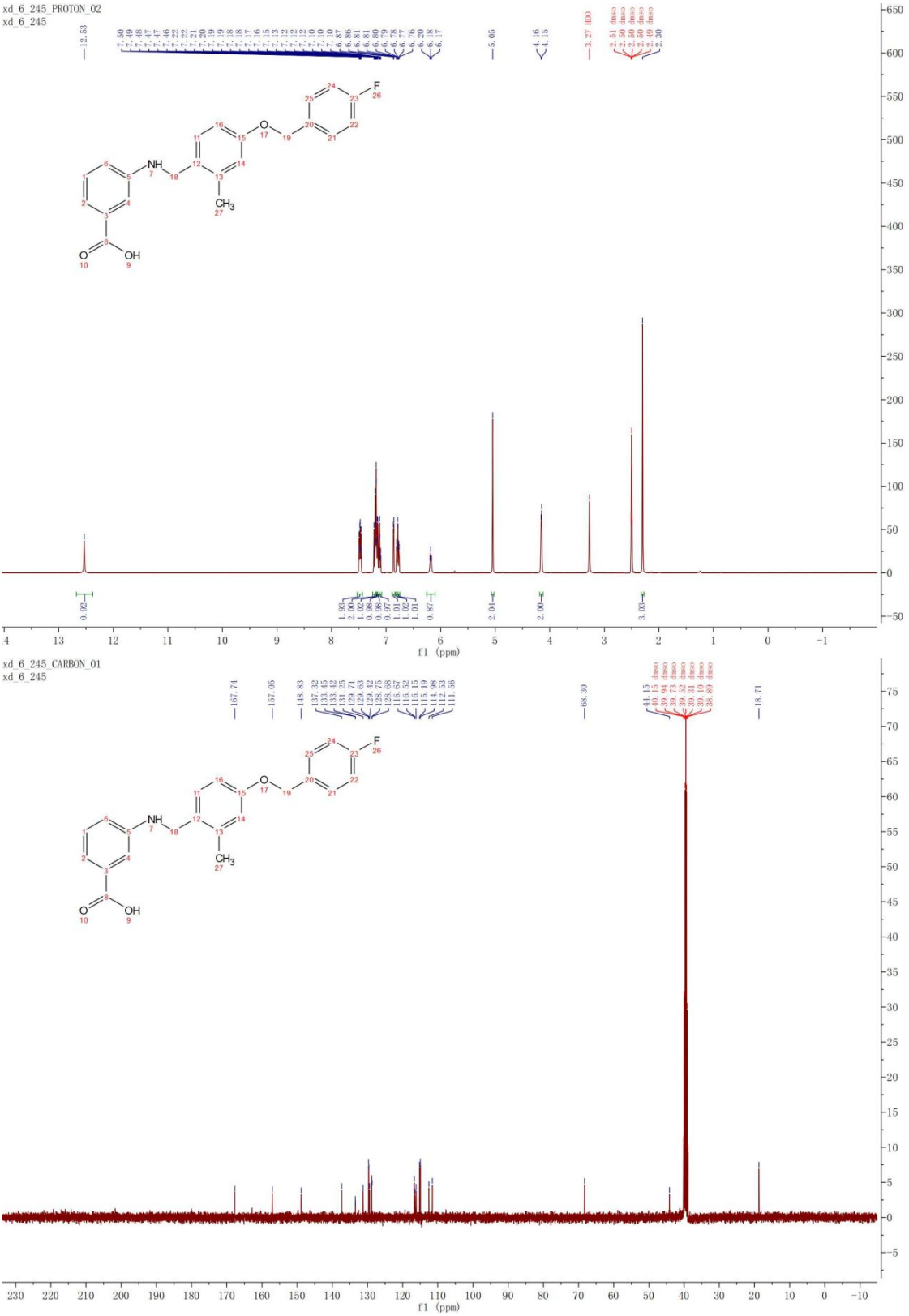

DN-5-43-i_PROTON_01
DN-5-43-i
f1 (ppm)


DN-5-43-i_CARBON_01
DN-5-43-i
f1 (ppm)

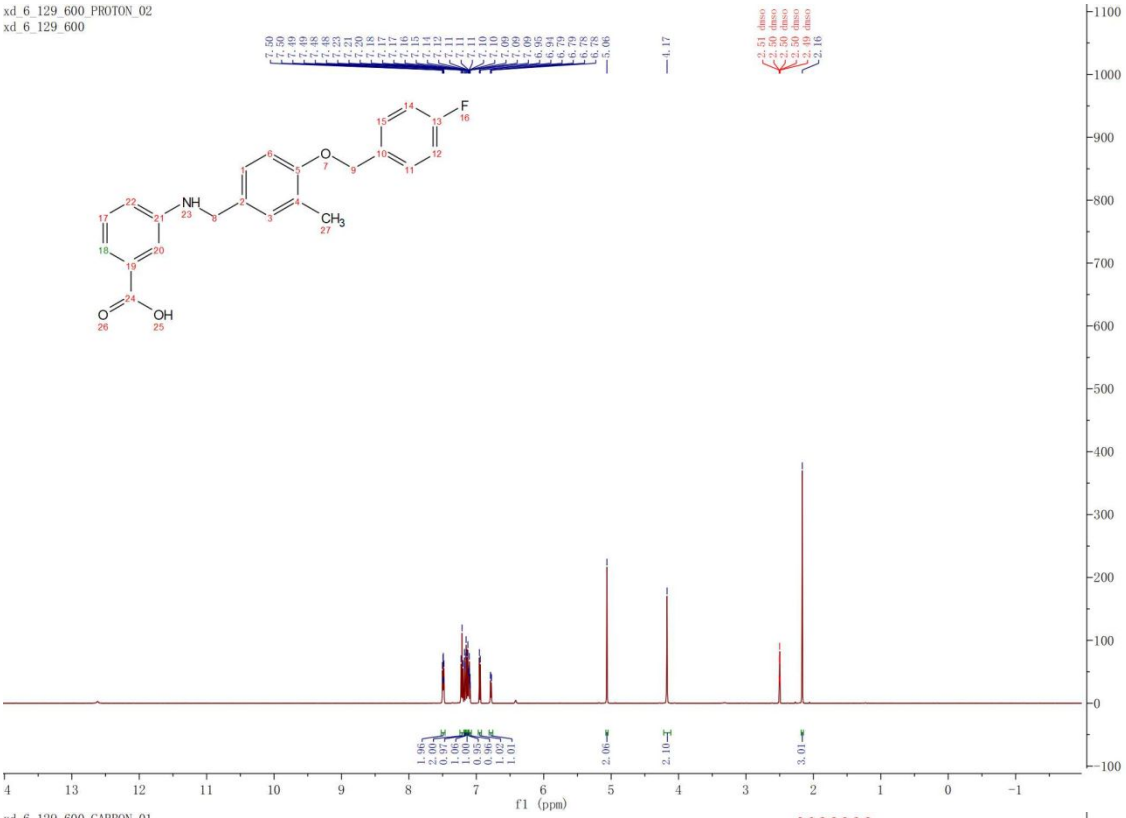
4s

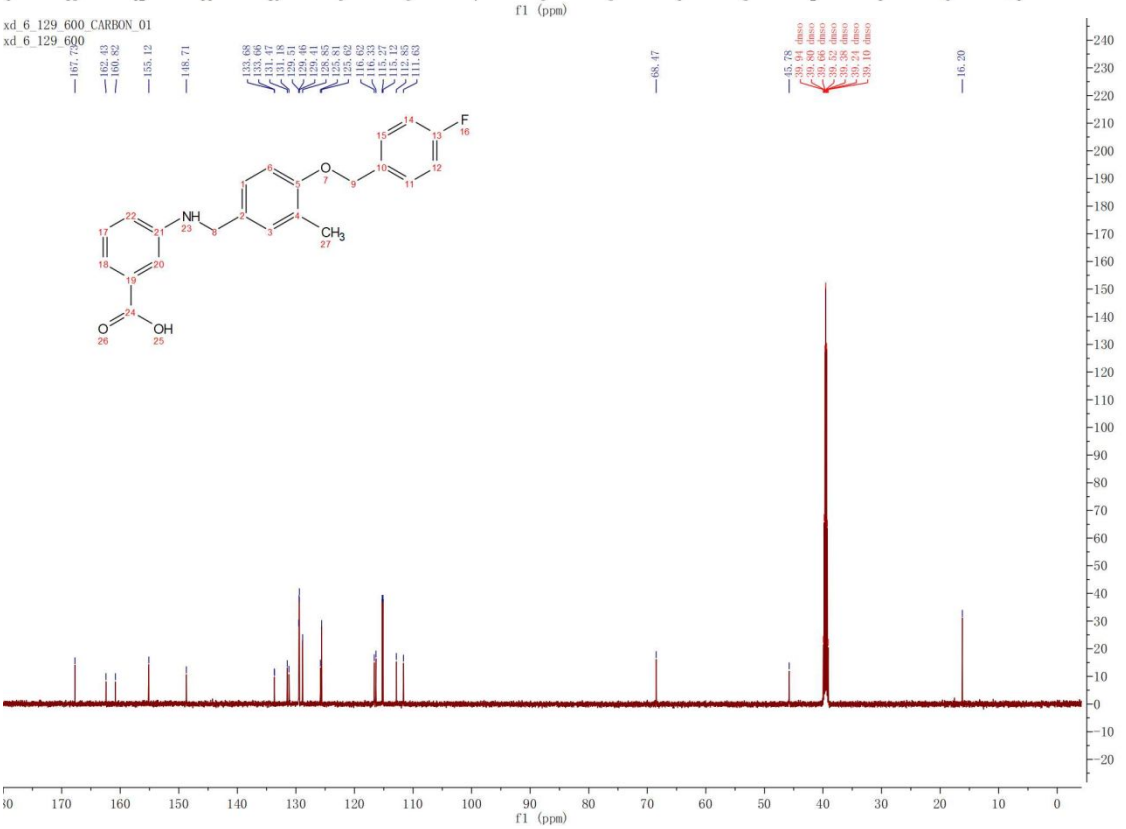


4t

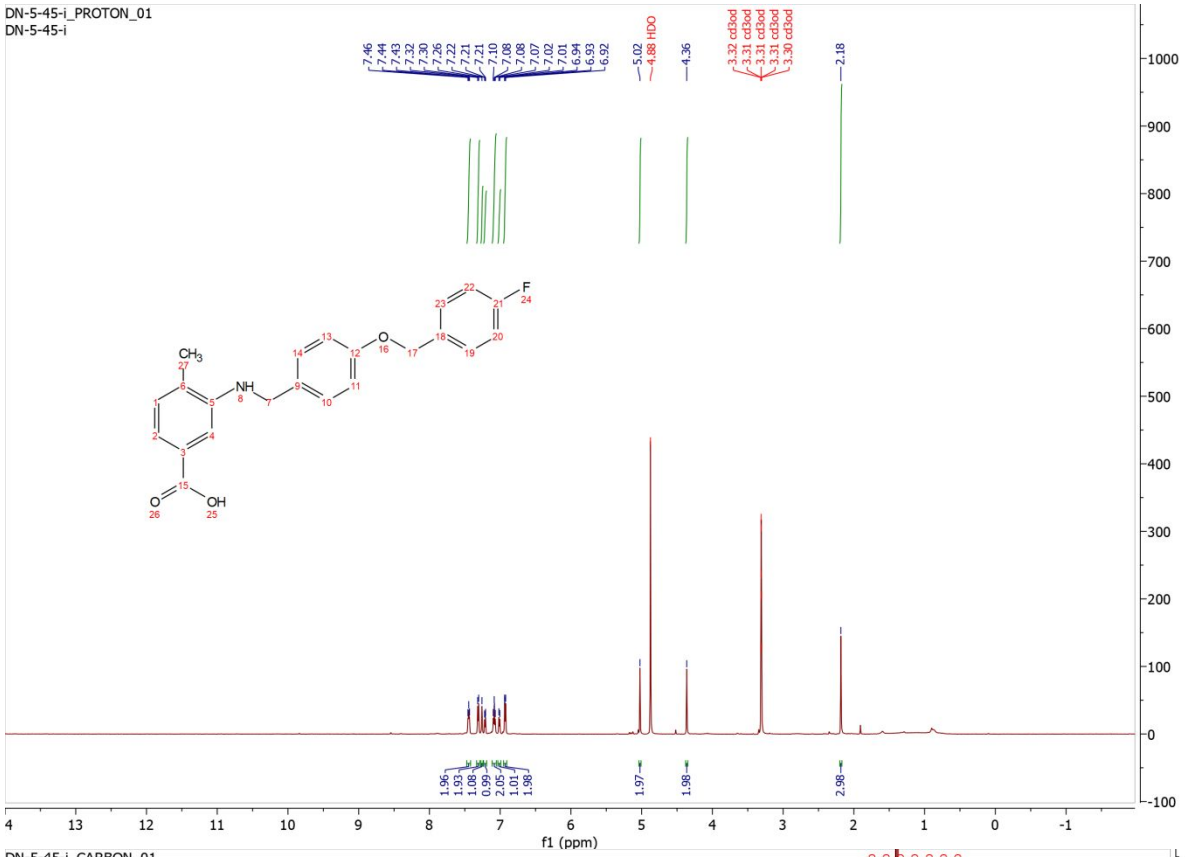

xd_6_245_PROTON_02
xd_6_245
xd_6_245_CARBON_01
xd_6_245
f1 (ppm)
f1 (ppm)

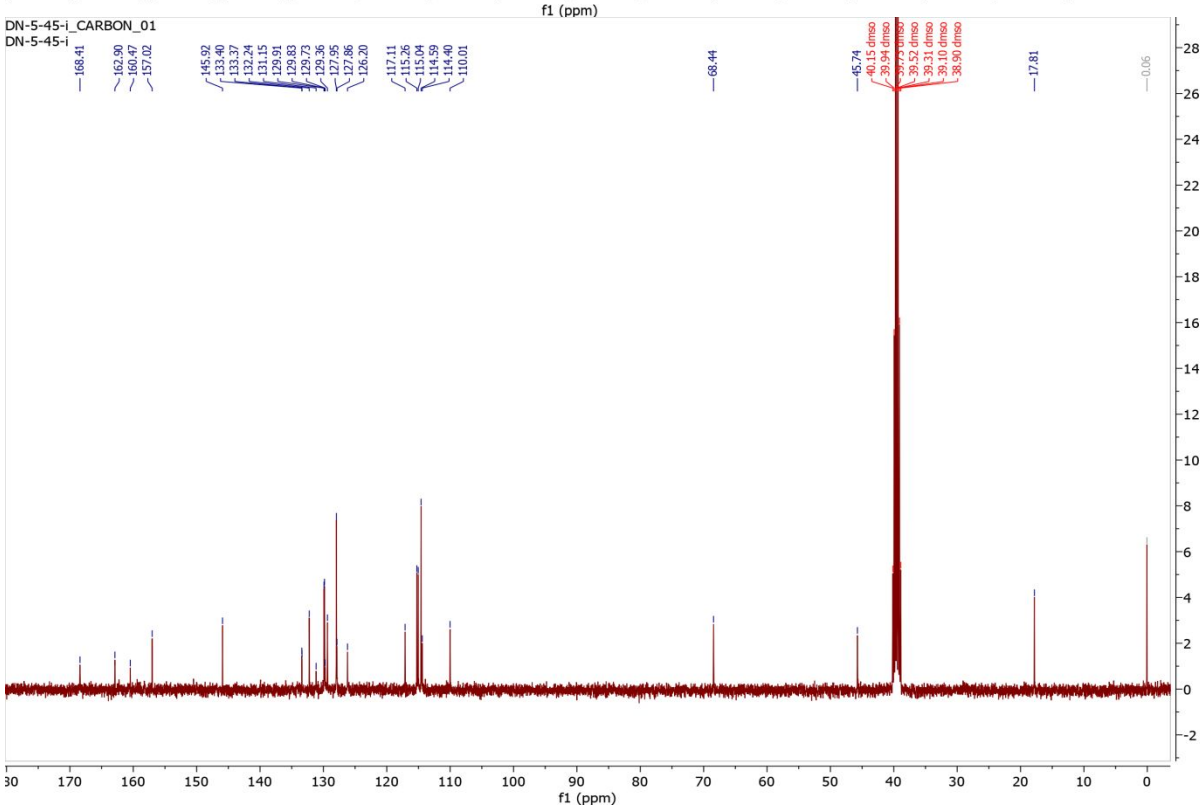
4u


xd_6_129_600_PROTON_02
xd_6_129_600
f1 (ppm)

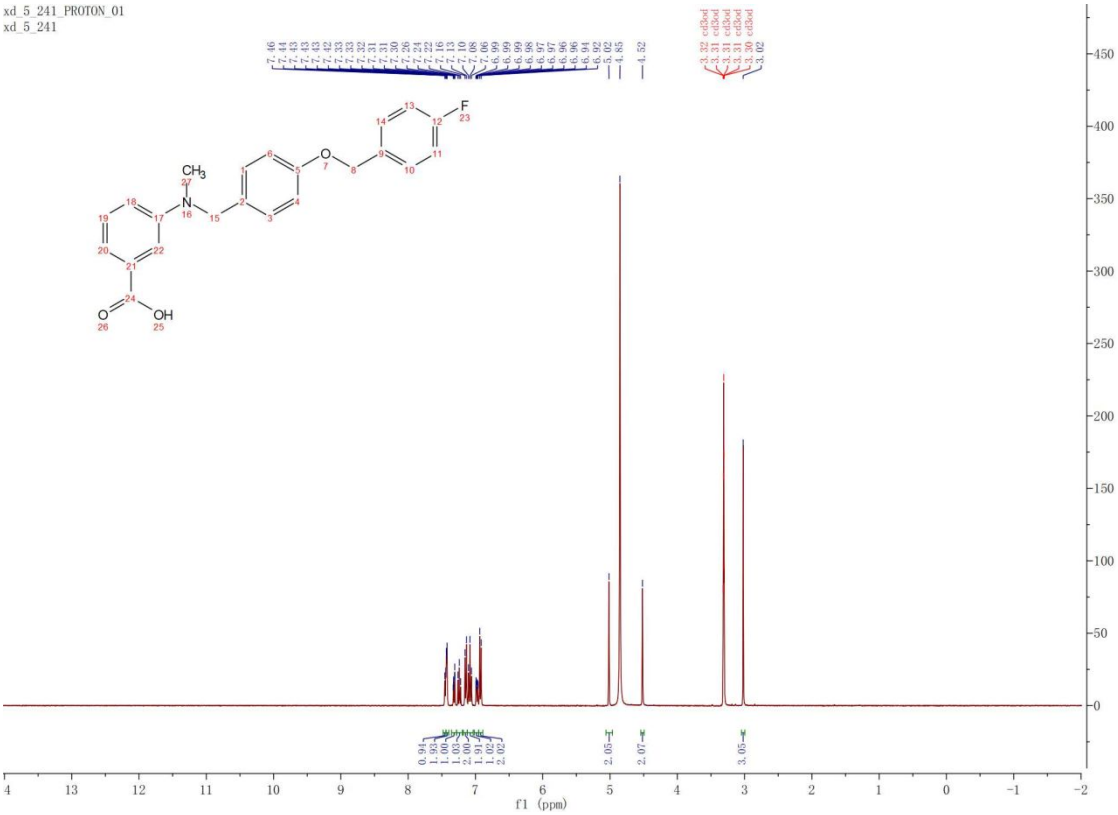

xd_6_129_600_CARBON_01
xd_6_129_600
f1 (ppm)

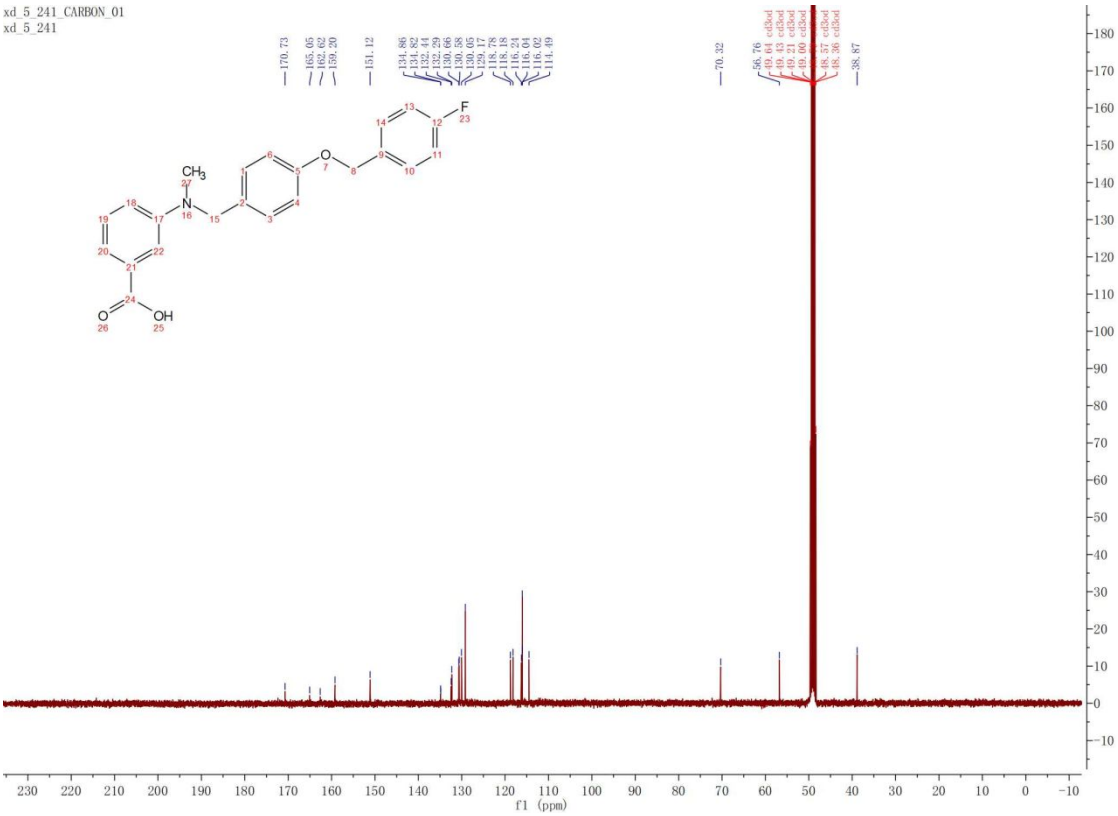
4v


DN-5-45-i_PROTON_01
DN-5-45-i
7.46
7.44
7.43
7.32
7.30
7.26
7.22
7.21
7.21
7.10
7.08
7.08
7.07
7.02
7.01
6.94
6.93
6.92
5.02
4.88 HDO
4.36
3.32 cd3od
3.31 cd3od
3.31 cd3od
3.31 cd3od
3.30 cd3od
2.18
1.96
1.93
1.08
0.99
2.05
1.01
1.98
1.97
1.98
2.98
f1 (ppm)

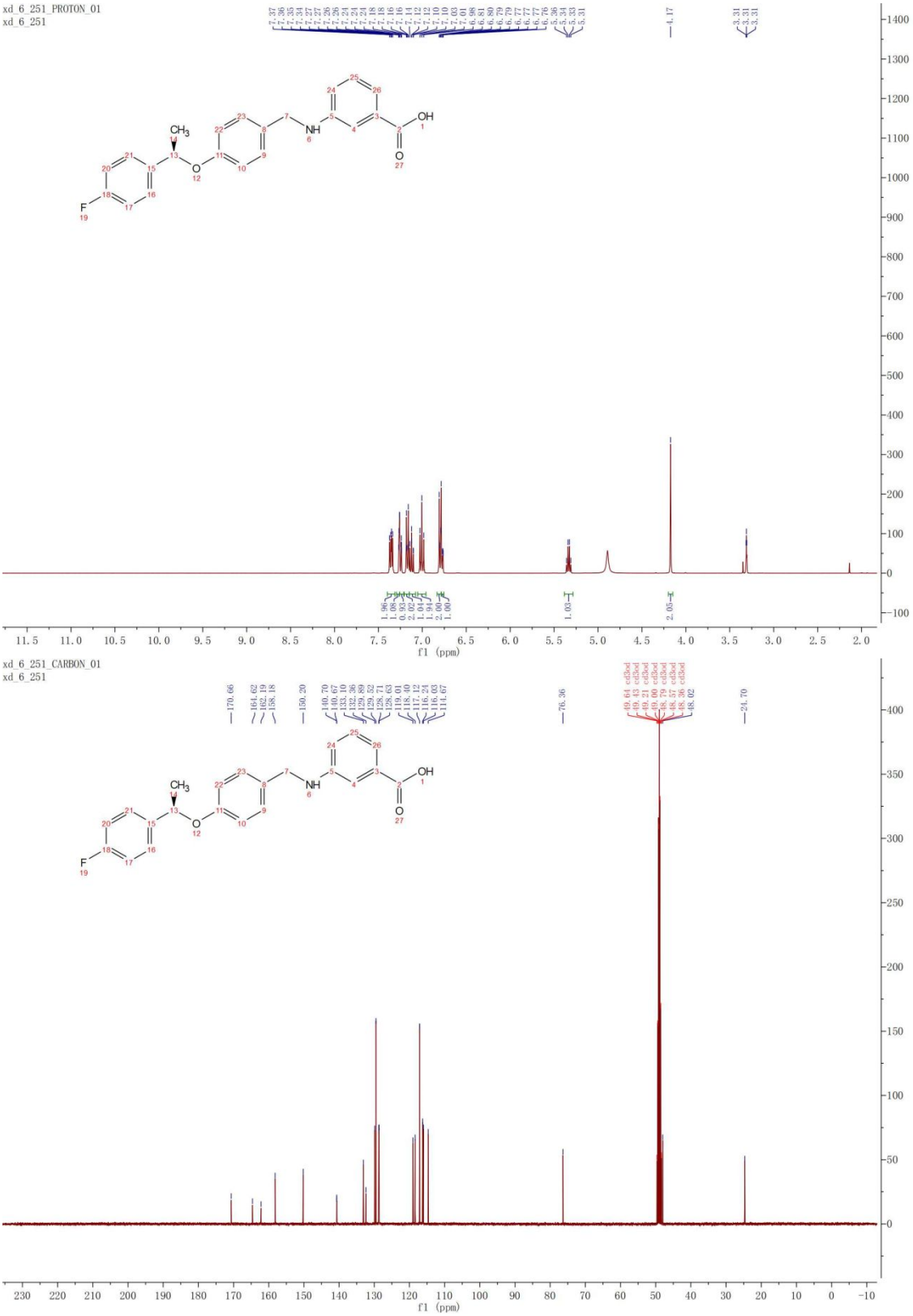

DN-5-45-i_CARBON_01
DN-5-45-i
168.41
162.90
160.47
157.02
145.92
133.40
133.37
132.24
131.15
129.91
129.83
129.73
129.36
127.95
127.86
126.20
117.11
115.26
115.04
114.59
114.40
110.01
68.44
45.74
40.15 dmso
39.94 dmso
39.52 dmso
39.31 dmso
39.10 dmso
38.90 dmso
17.81
0.06
f1 (ppm)

4w

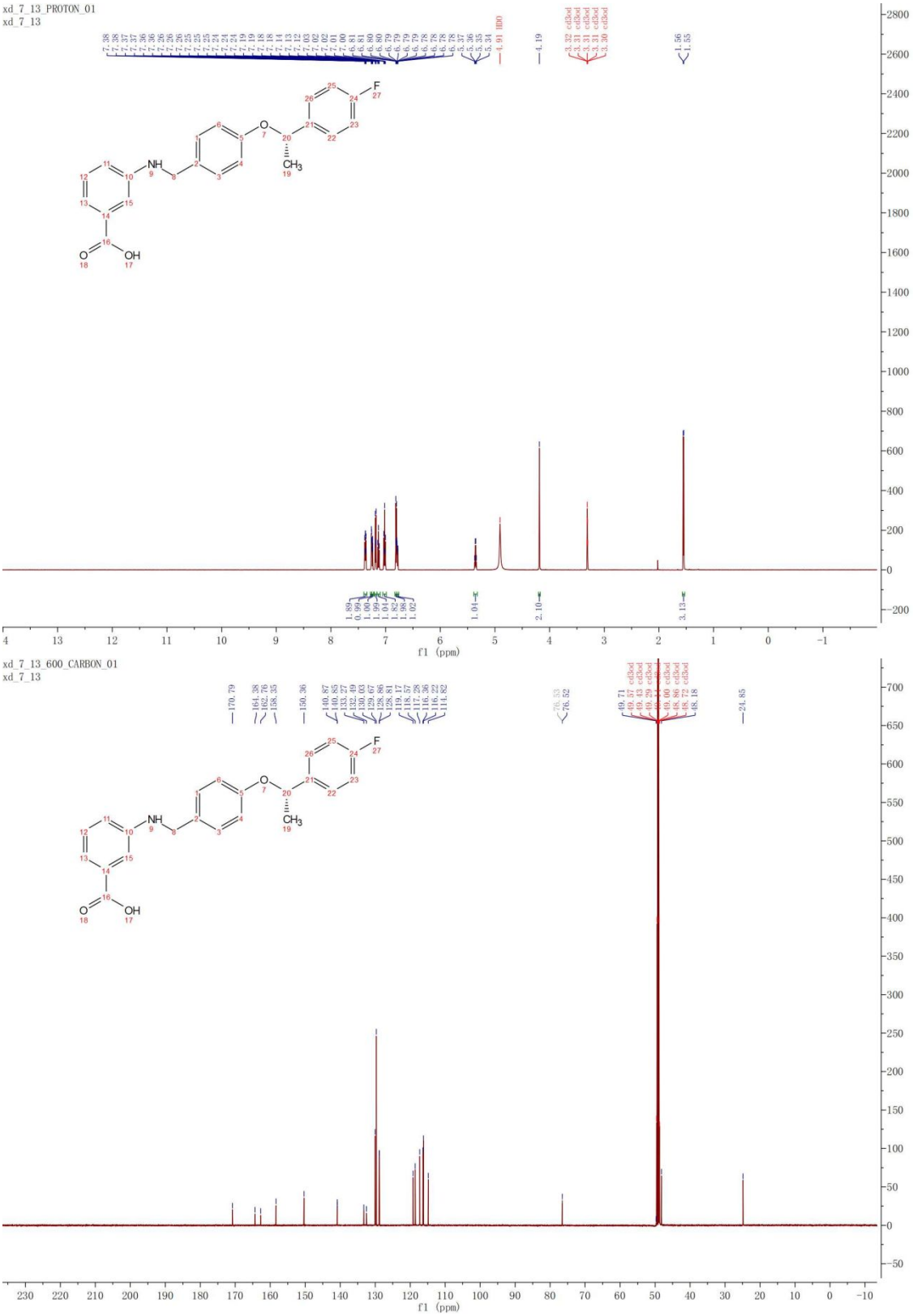

xd_5_241_PROTON_01
xd_5_241
f1 (ppm)


xd_5_241_CARBON_01
xd_5_241
f1 (ppm)

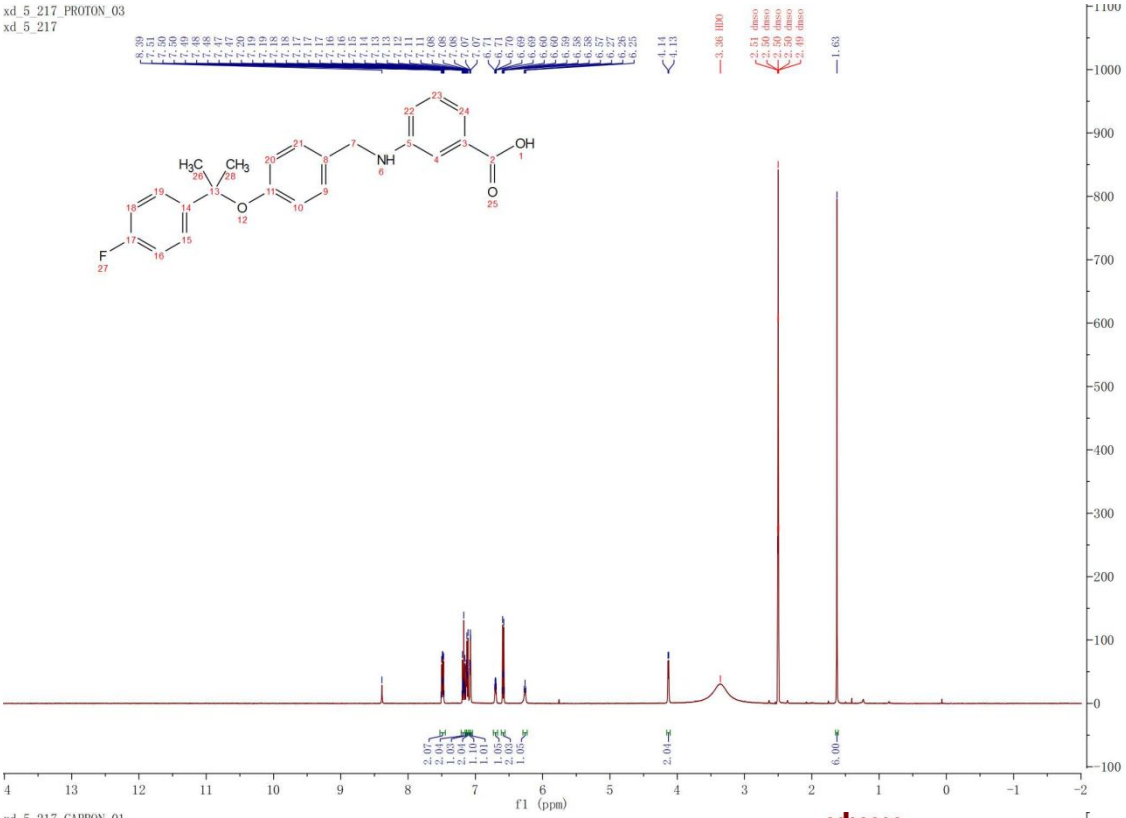
4x

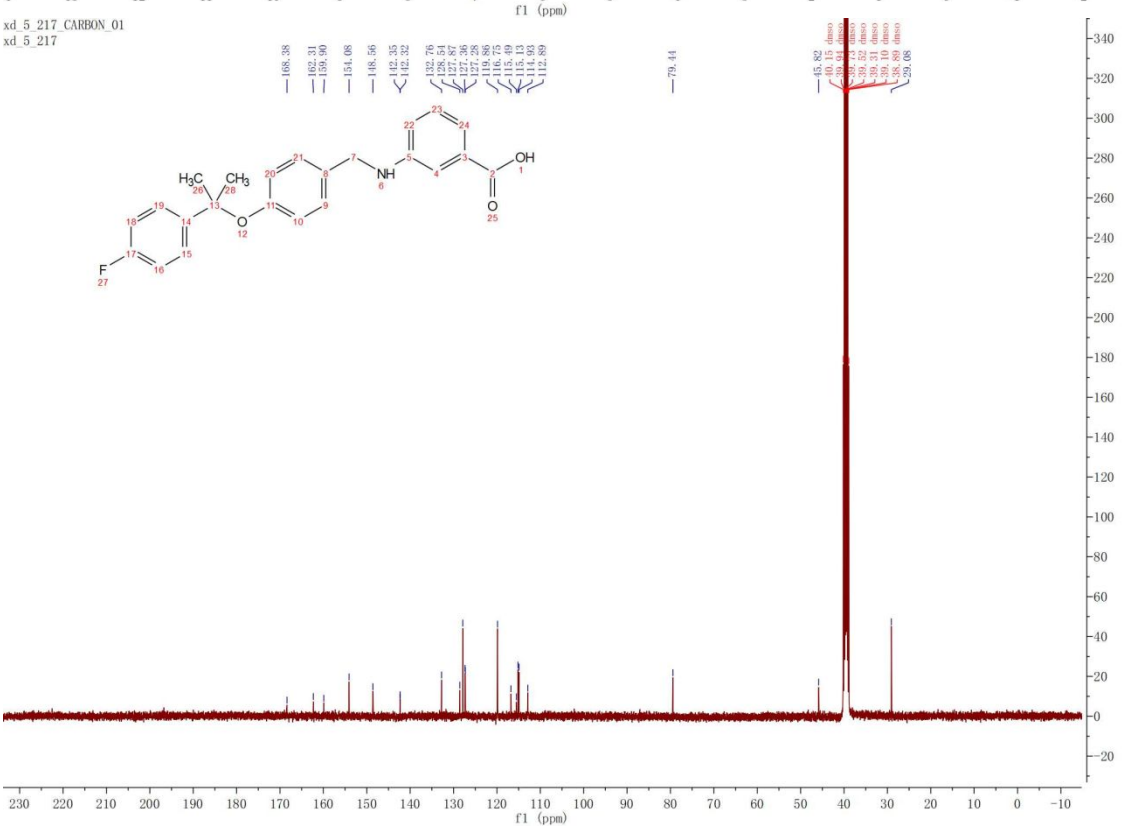

xd_6_251_PROTON_01
xd_6_251
f1 (ppm)
xd_6_251_CARBON_01
xd_6_251
f1 (ppm)

4y

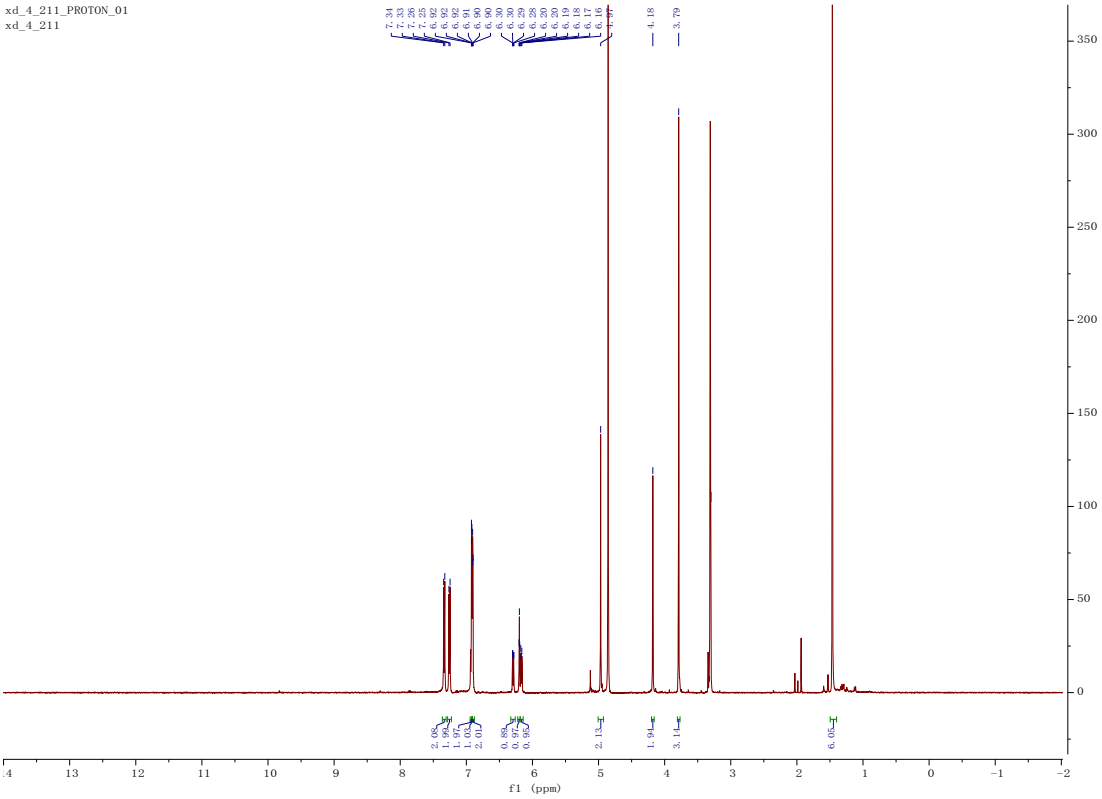


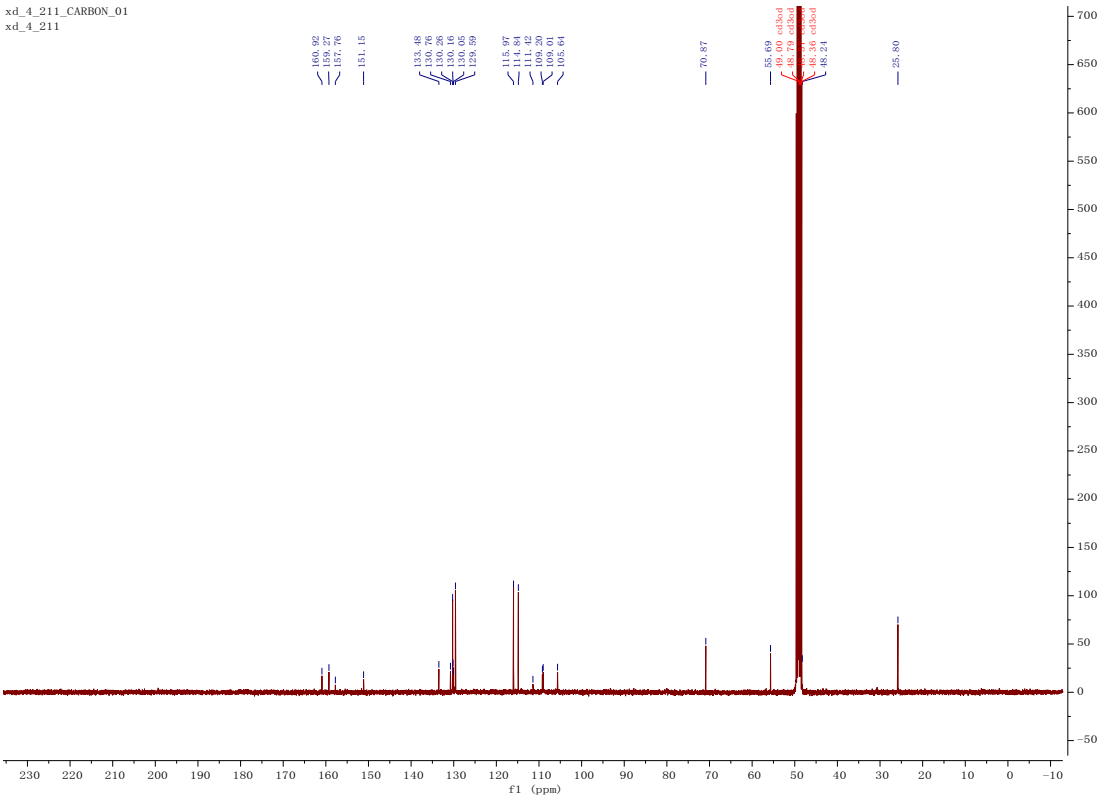
4z


xd_5_217_PROTON_03
xd_5_217
f1 (ppm)

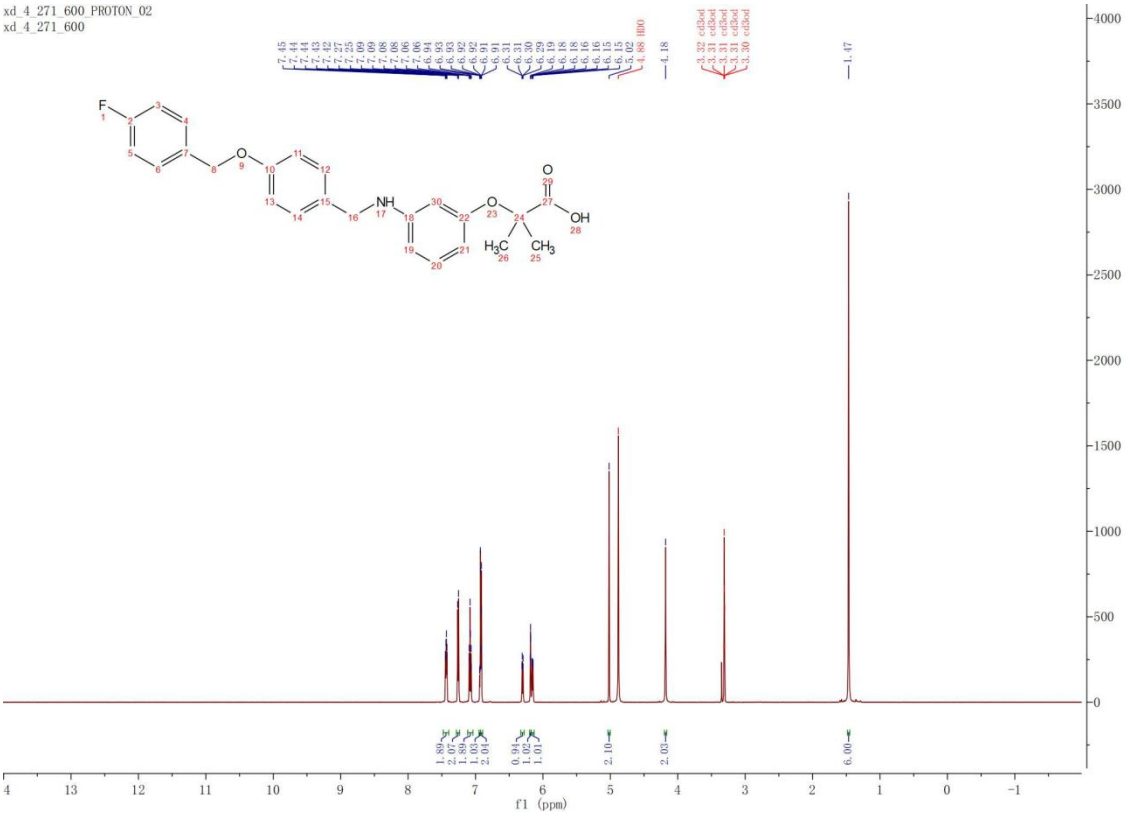

xd_5_217_CARBON_01
xd_5_217
f1 (ppm)

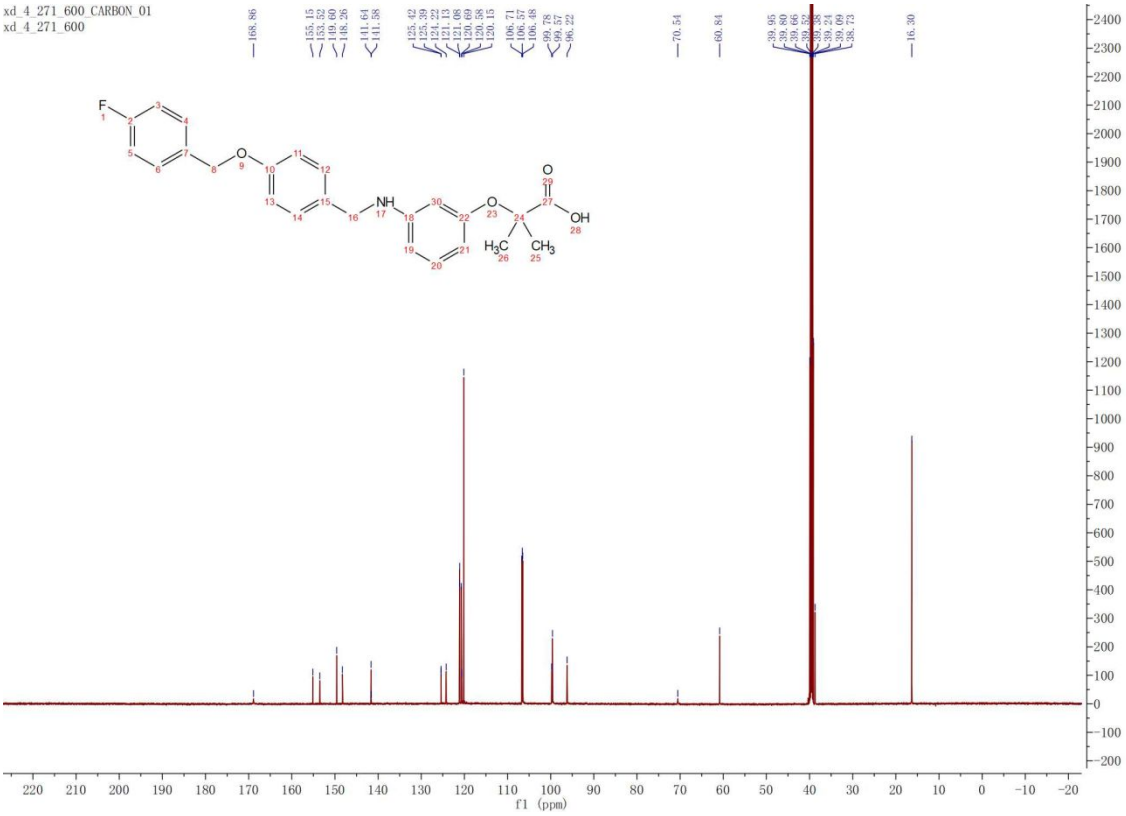
5a


xd_4_211_PROTON_01
xd_4_211
f1 (ppm)

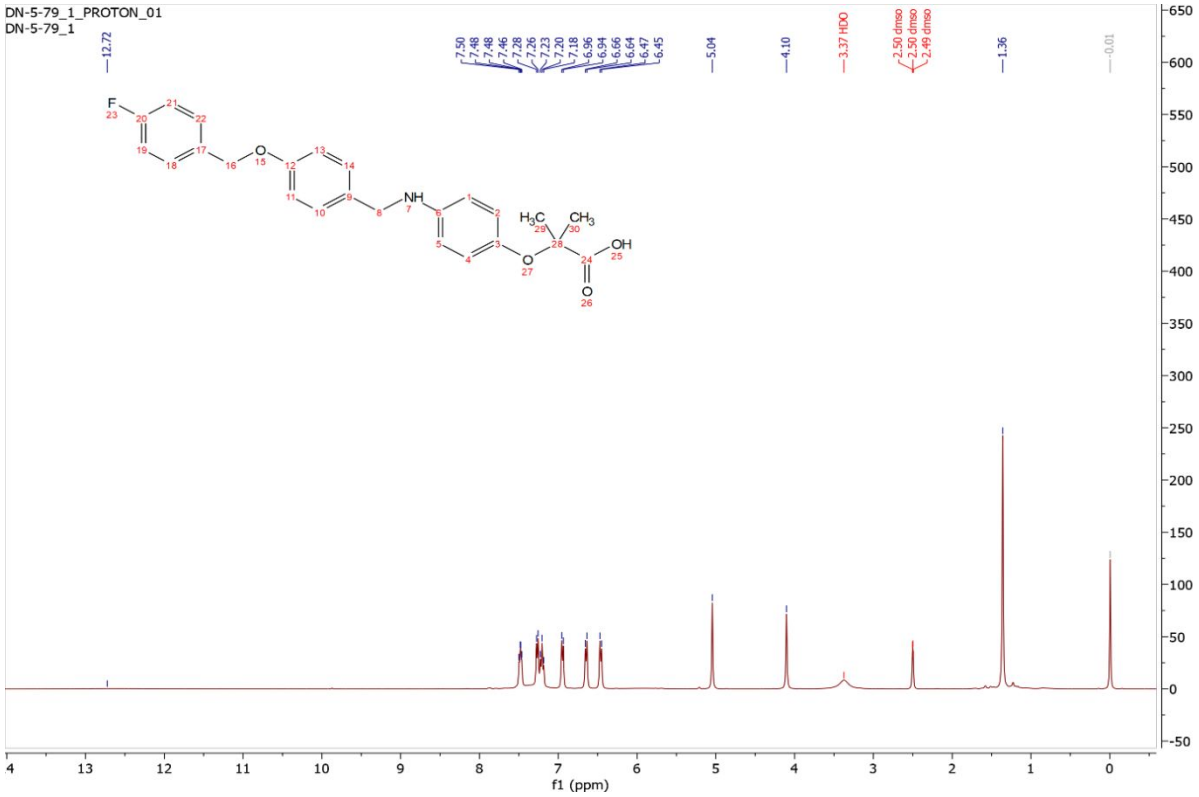

xd_4_211_CARBON_01
xd_4_211
f1 (ppm)

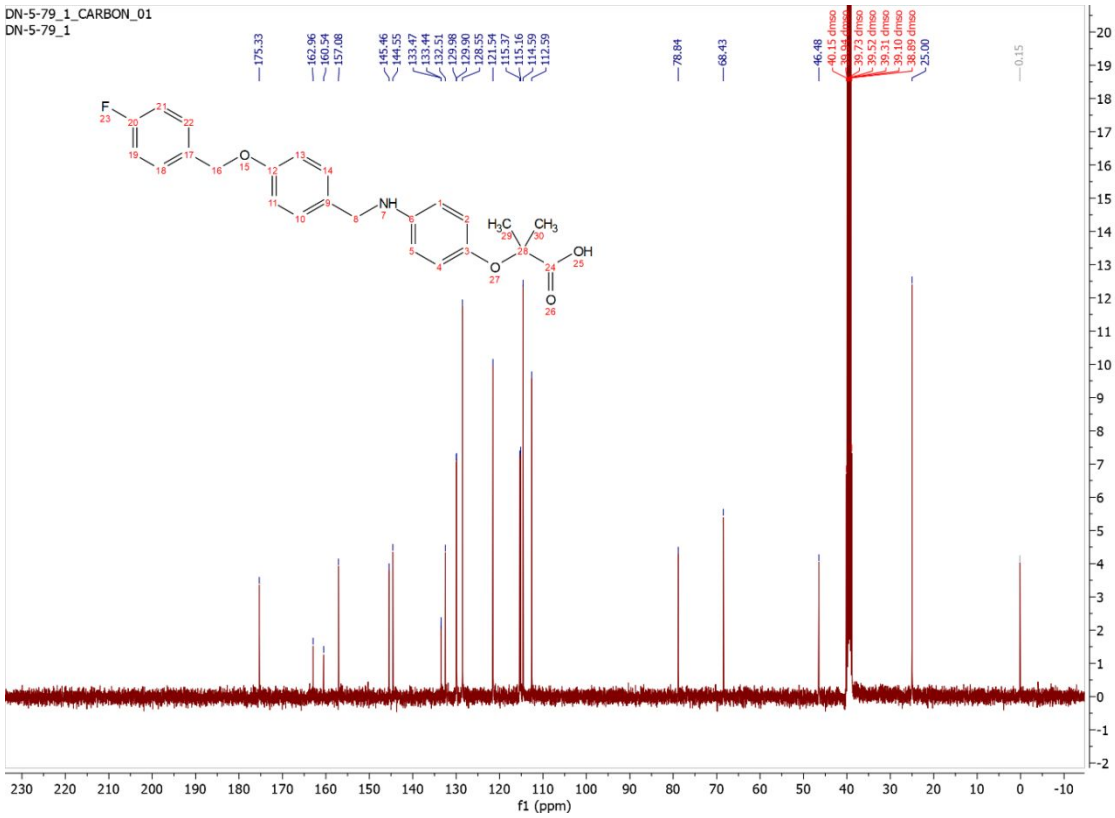
5b


xd_4_271_600_PROTON_02
xd_4_271_600
f1 (ppm)

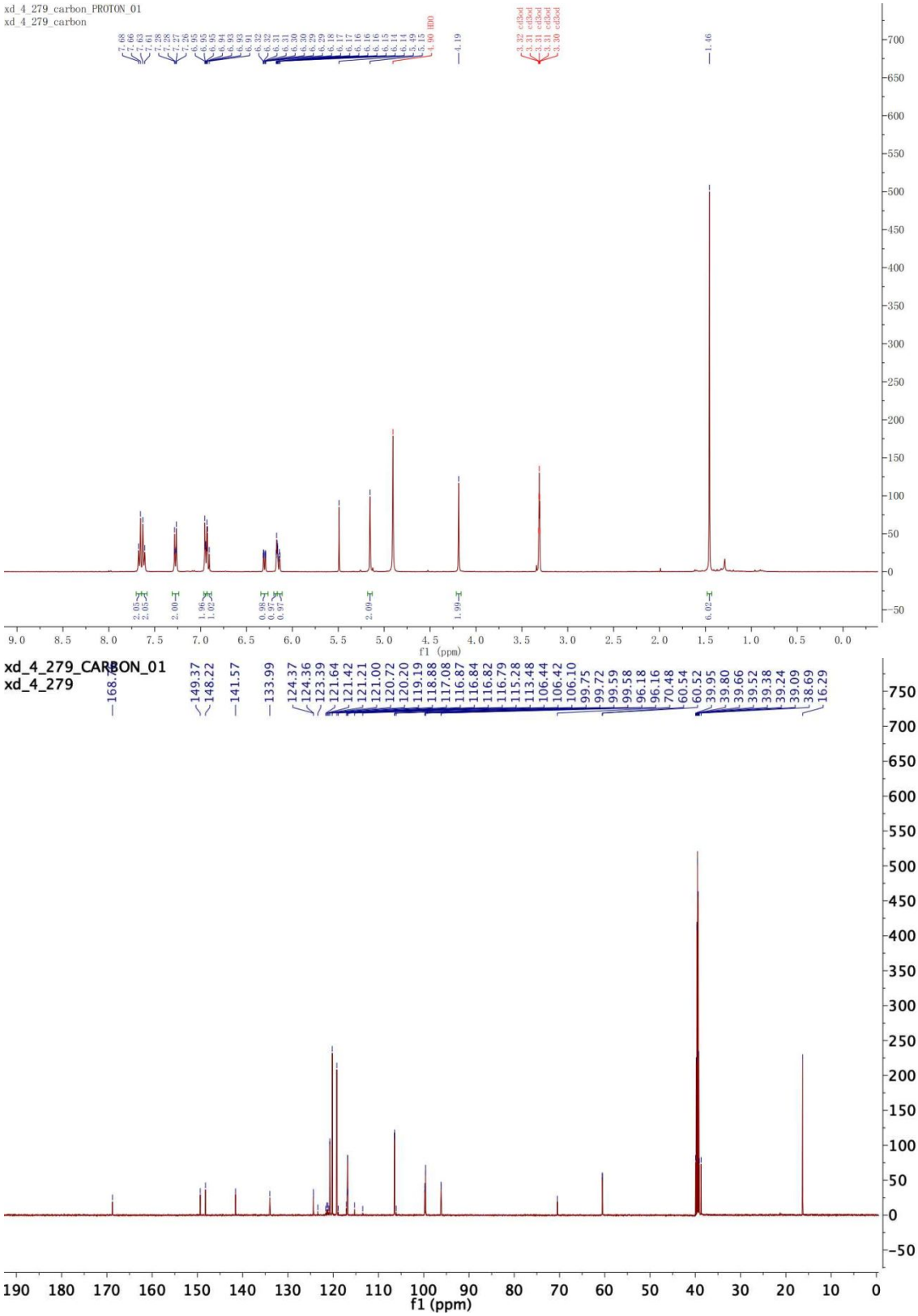

xd_4_271_600_CARBON_01
xd_4_271_600
f1 (ppm)

5b*

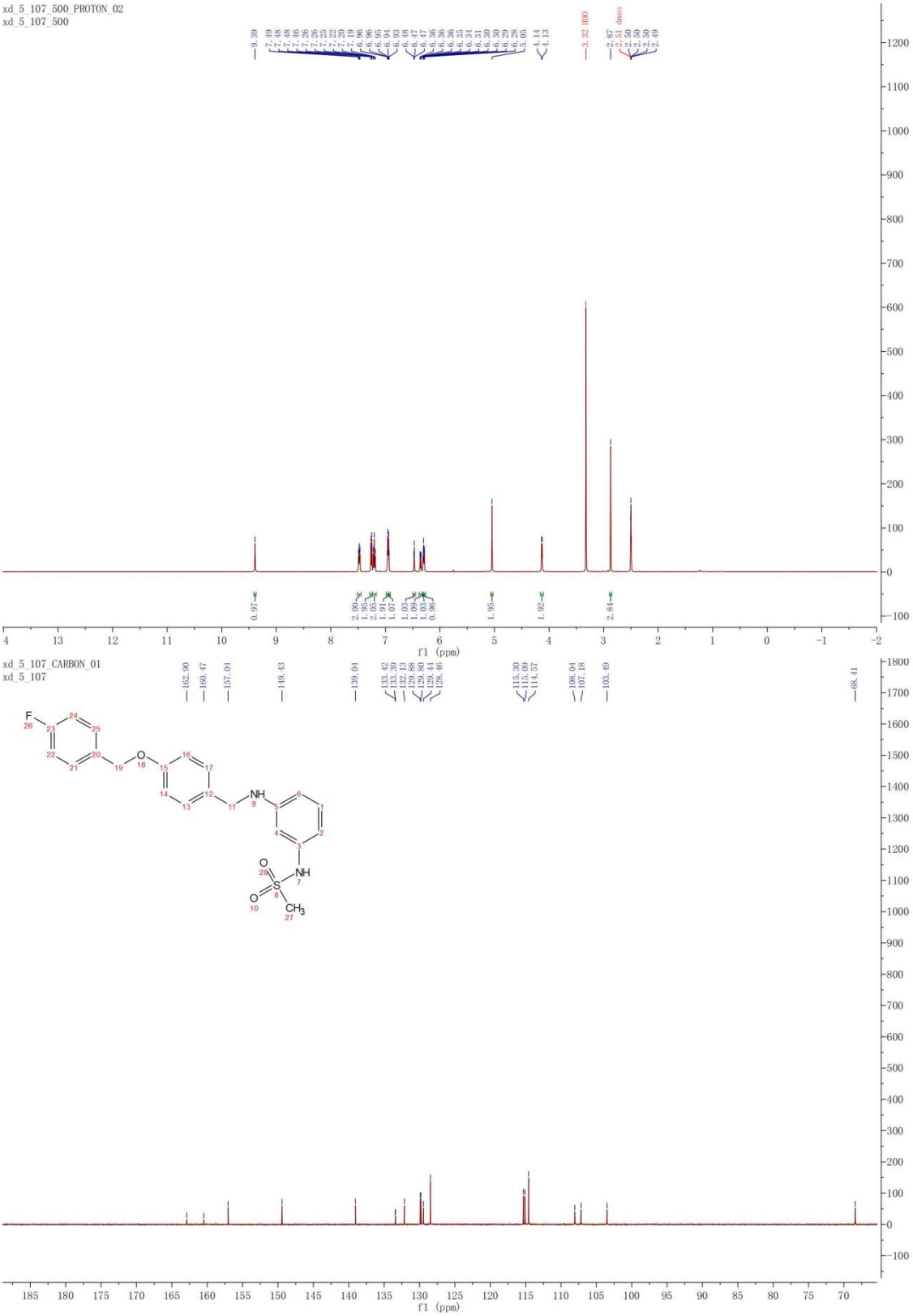

DN-5-79_1_PROTON_01
DN-5-79_1
12.72
7.50
7.48
7.48
7.46
7.28
7.26
7.23
7.20
7.18
6.96
6.94
6.66
6.64
6.47
6.45
5.04
4.10
3.37 HDO
2.50 dmso
2.50 dmso
2.49 dmso
1.36
0.01
f1 (ppm)


DN-5-79_1_CARBON_01
DN-5-79_1
175.33
162.96
160.54
157.08
145.46
144.55
133.47
133.44
132.51
129.98
129.90
128.55
121.54
115.37
115.16
114.59
112.59
78.84
68.43
46.48
40.15 dmso
39.94 dmso
39.73 dmso
39.52 dmso
39.31 dmso
39.10 dmso
38.89 dmso
25.00
0.15
f1 (ppm)

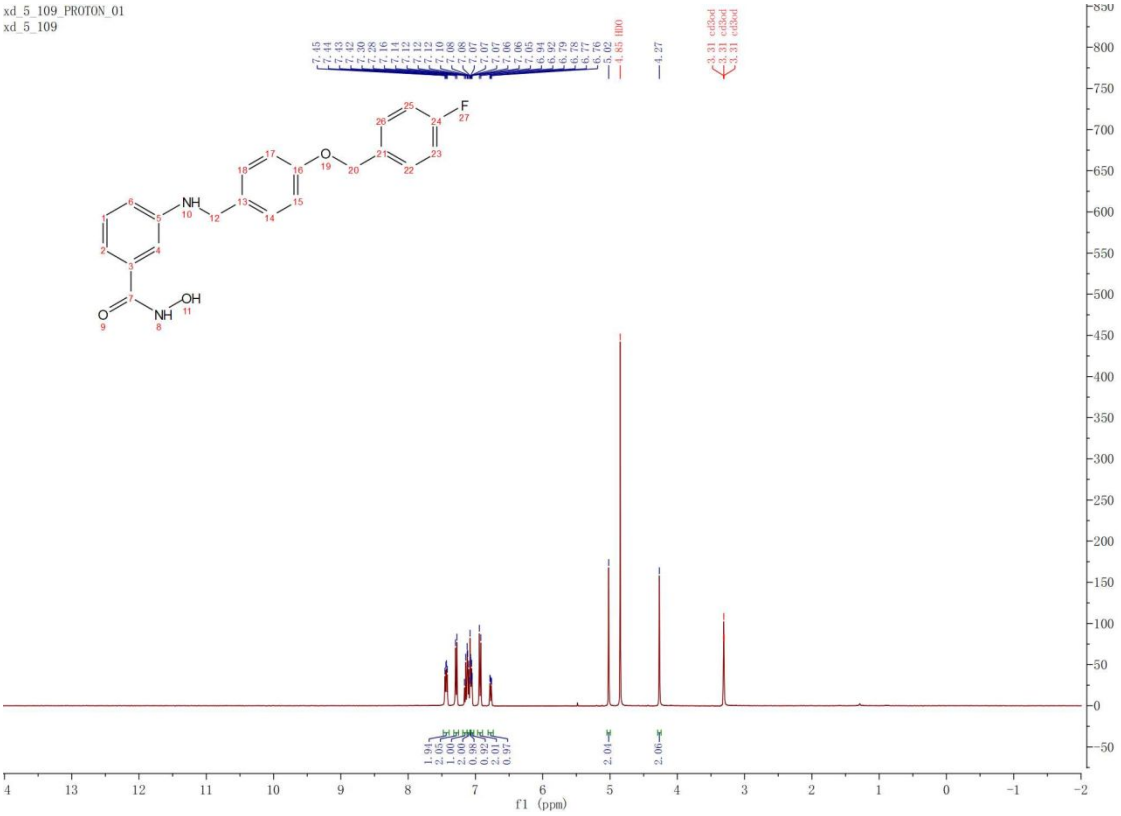
5f

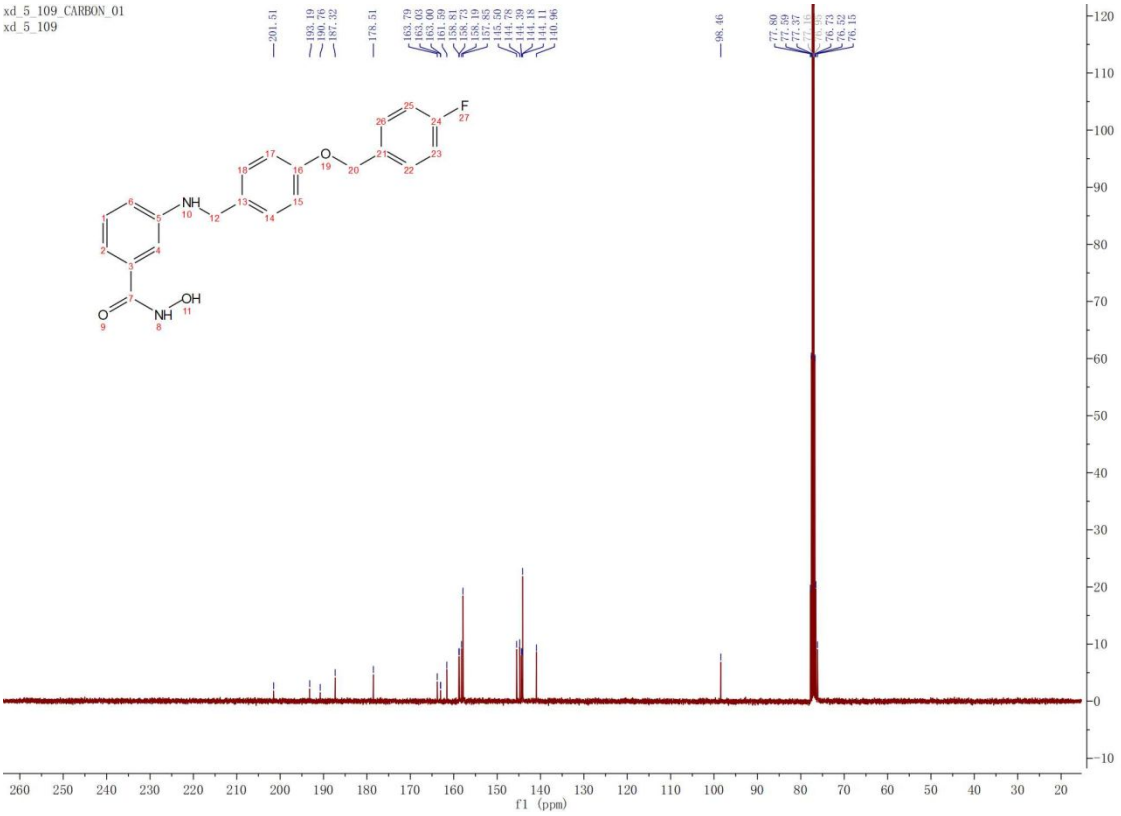

xd_4_279_carbon_PROTON_01
xd_4_279_carbon
xd_4_279_CARBON_01
xd_4_279
f1 (ppm)

19a

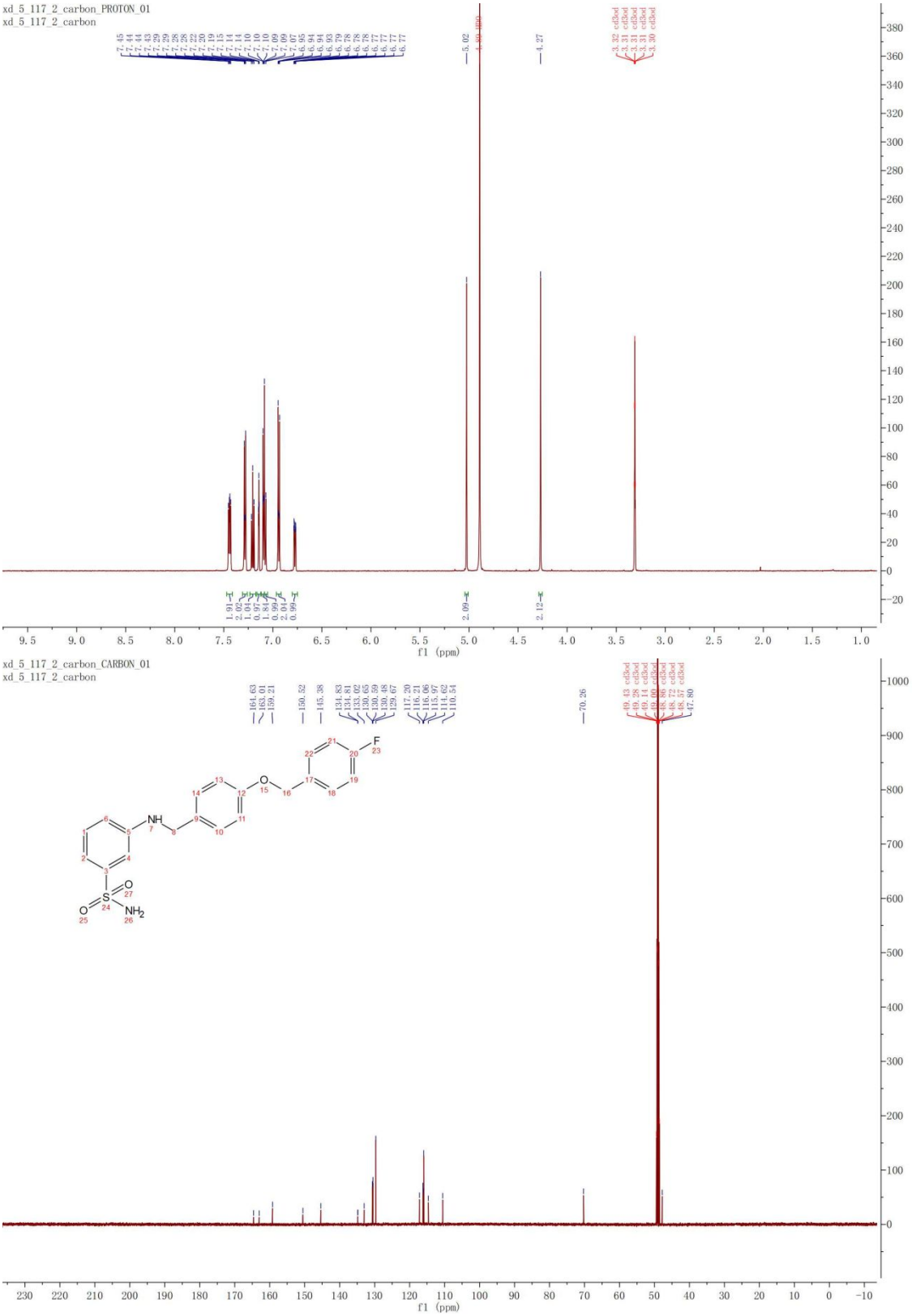

xd_5_107_500_PROTON_02
xd_5_107_500
xd_5_107_CARBON_01
xd_5_107
f1 (ppm)
f1 (ppm)

19b

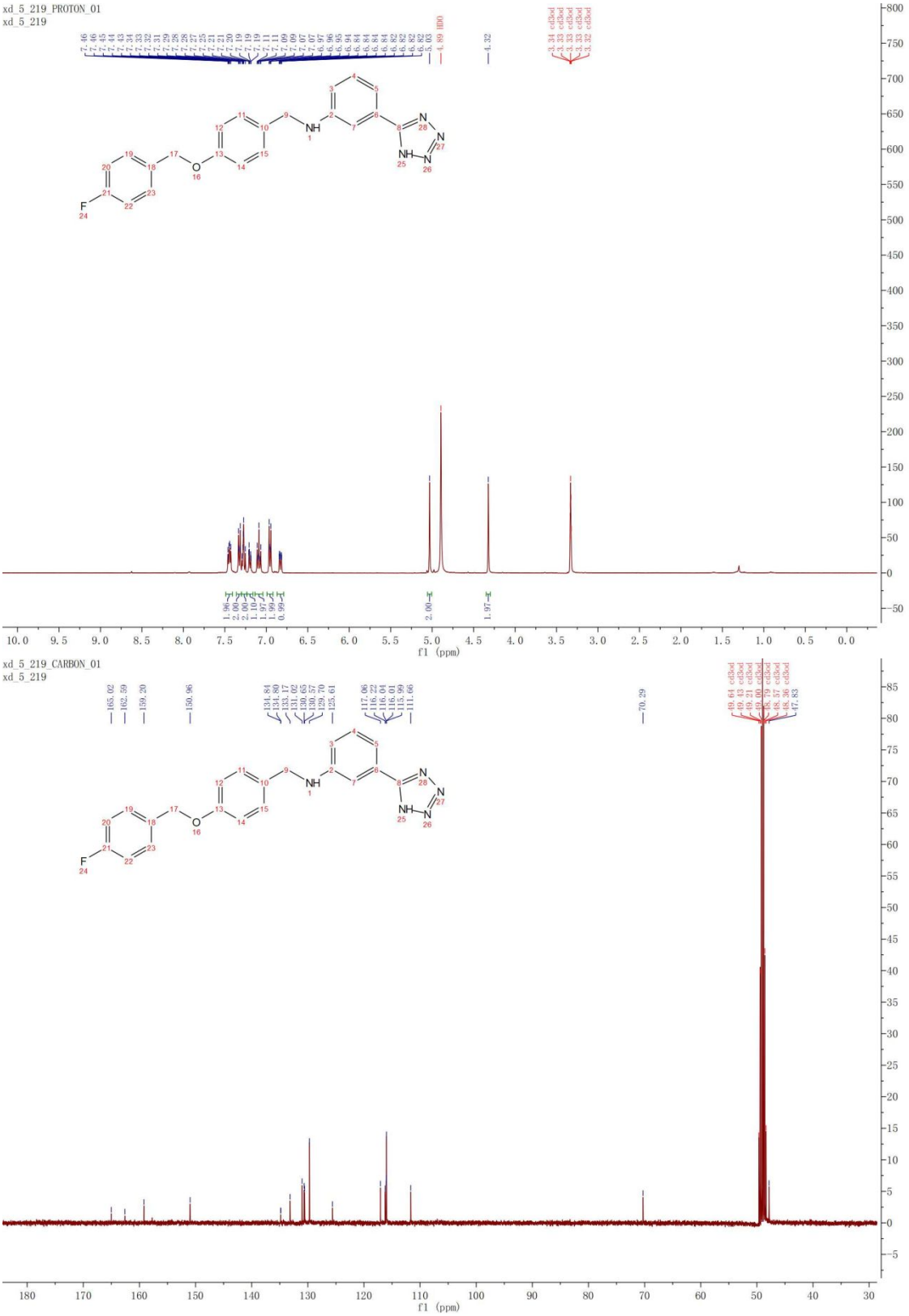

xd_5_109_PROTON_01
xd_5_109
f1 (ppm)


xd_5_109_CARBON_01
xd_5_109
f1 (ppm)

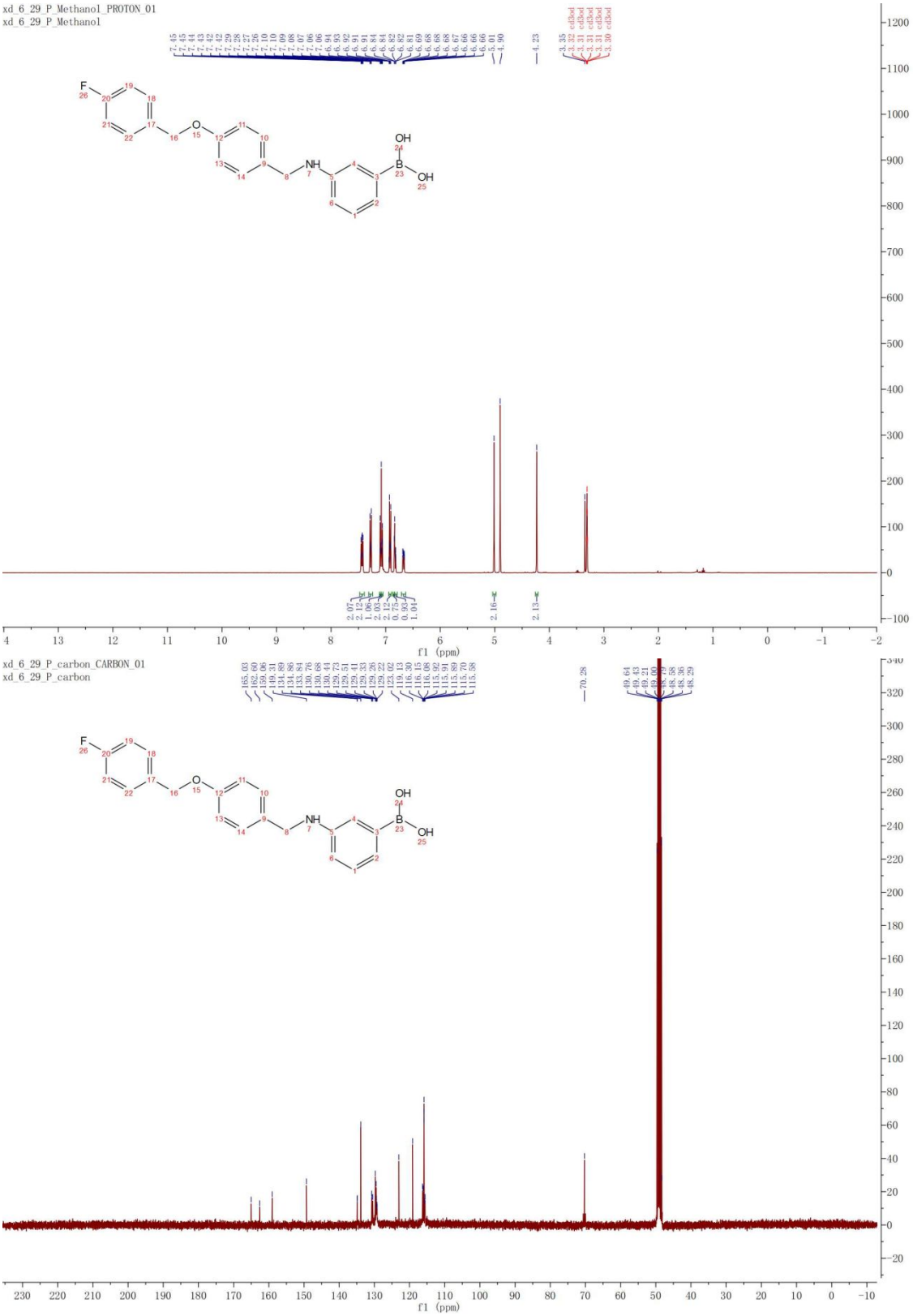
19c


xd_5_117_2_carbon_PROTON_01
xd_5_117_2_carbon
xd_5_117_2_carbon_CARBON_01
xd_5_117_2_carbon
f1 (ppm)
f1 (ppm)

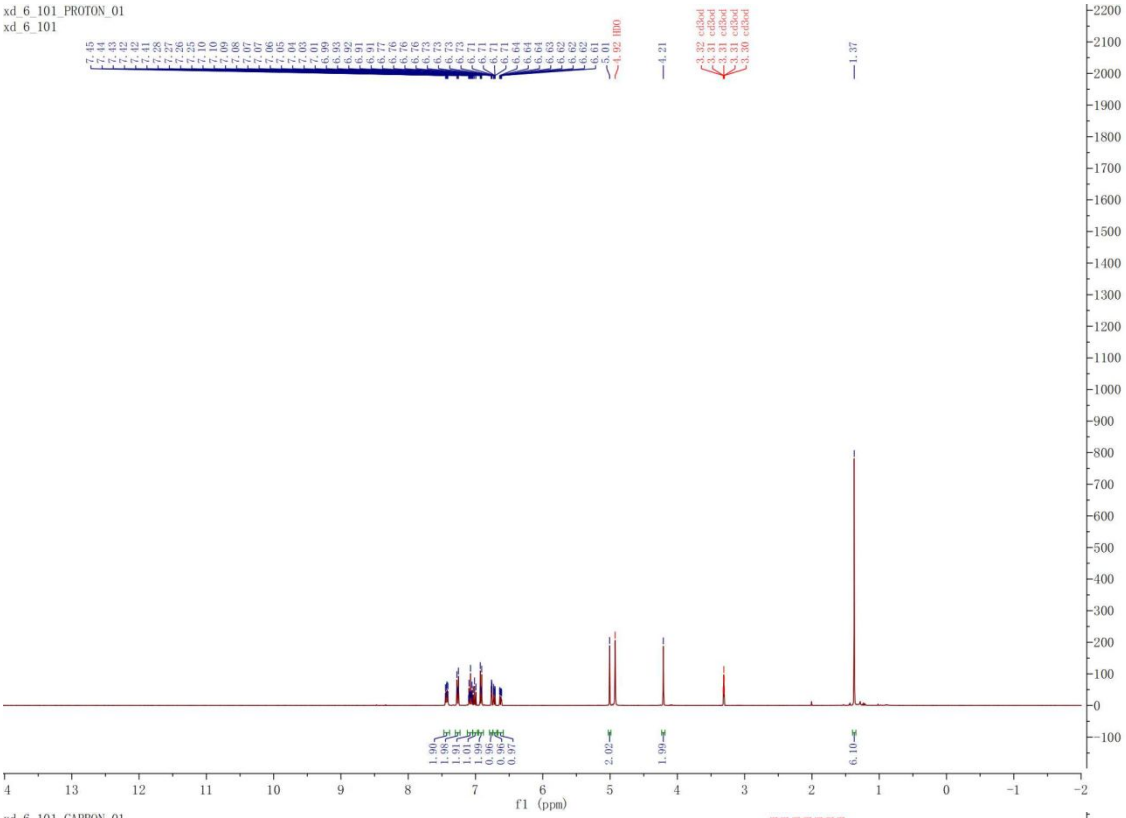
19d

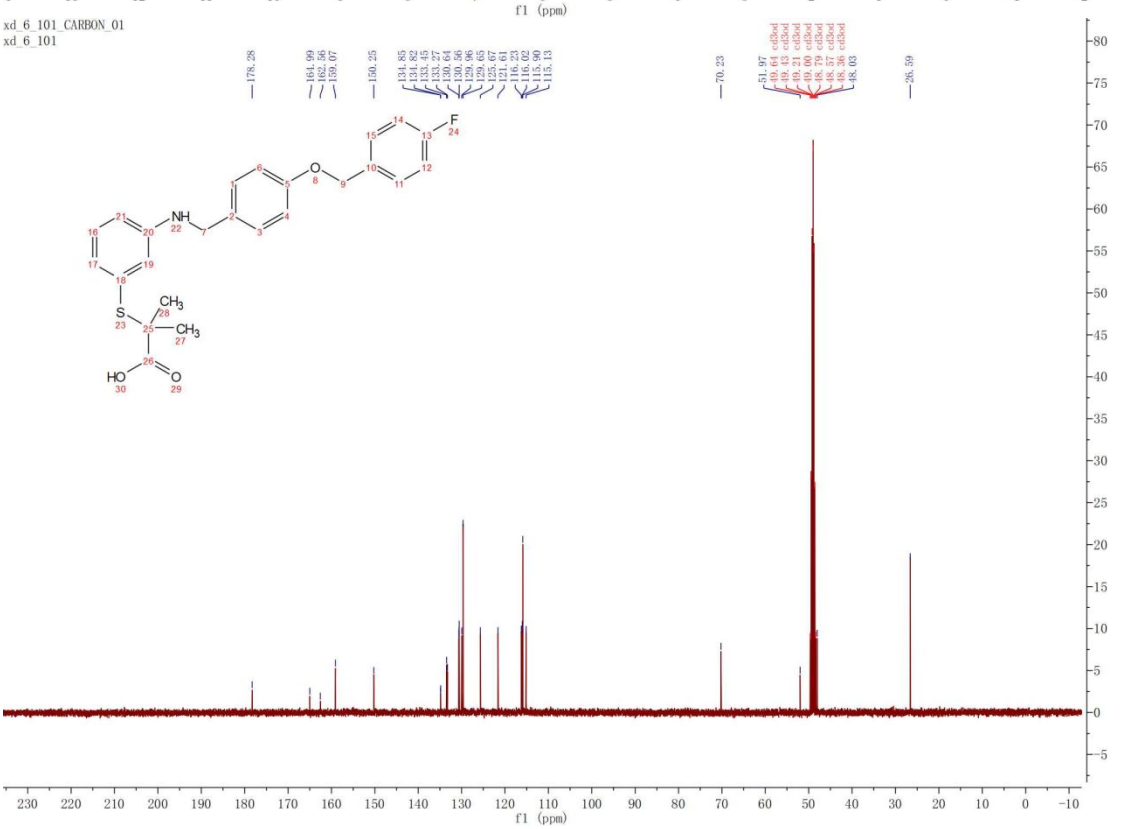

xd_5_219_PROTON_01
xd_5_219
xd_5_219_CARBON_01
xd_5_219
f1 (ppm)
f1 (ppm)

19e

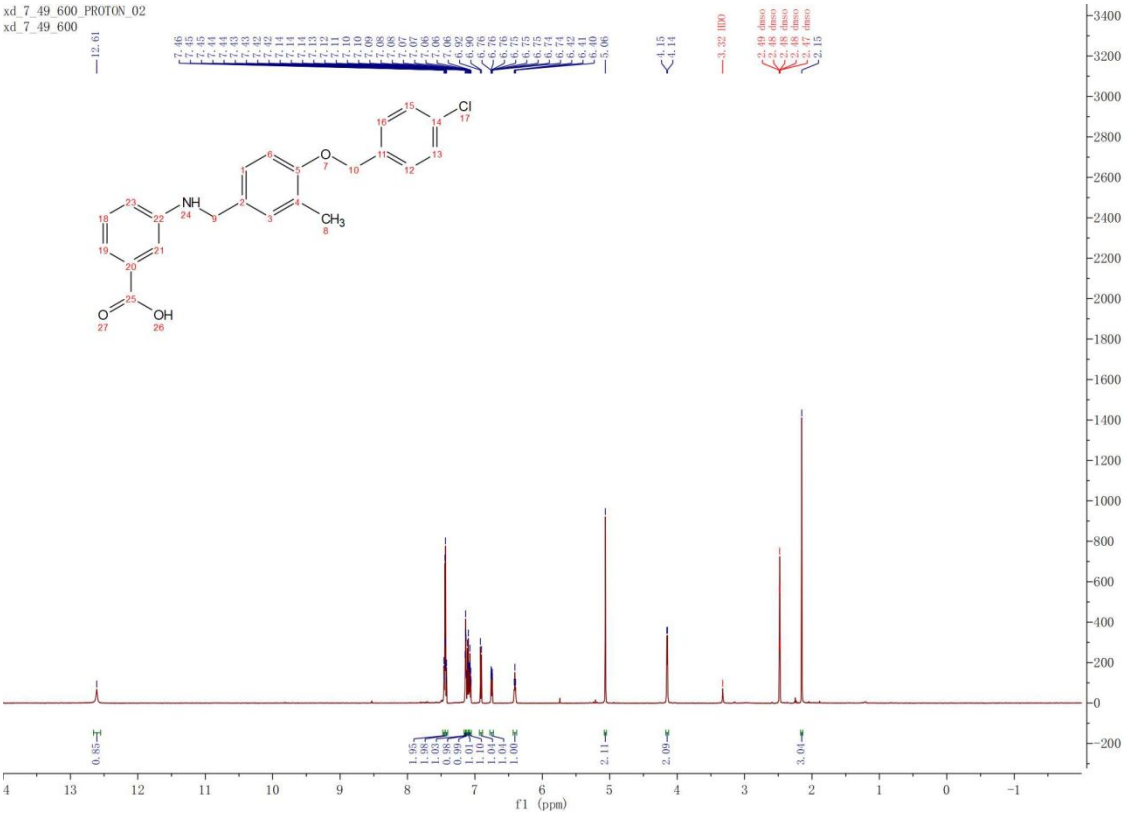

xd_6_29_P_Methanol_PROTON_01
xd_6_29_P_Methanol
xd_6_29_P_carbon_CARBON_01
xd_6_29_P_carbon
f1 (ppm)

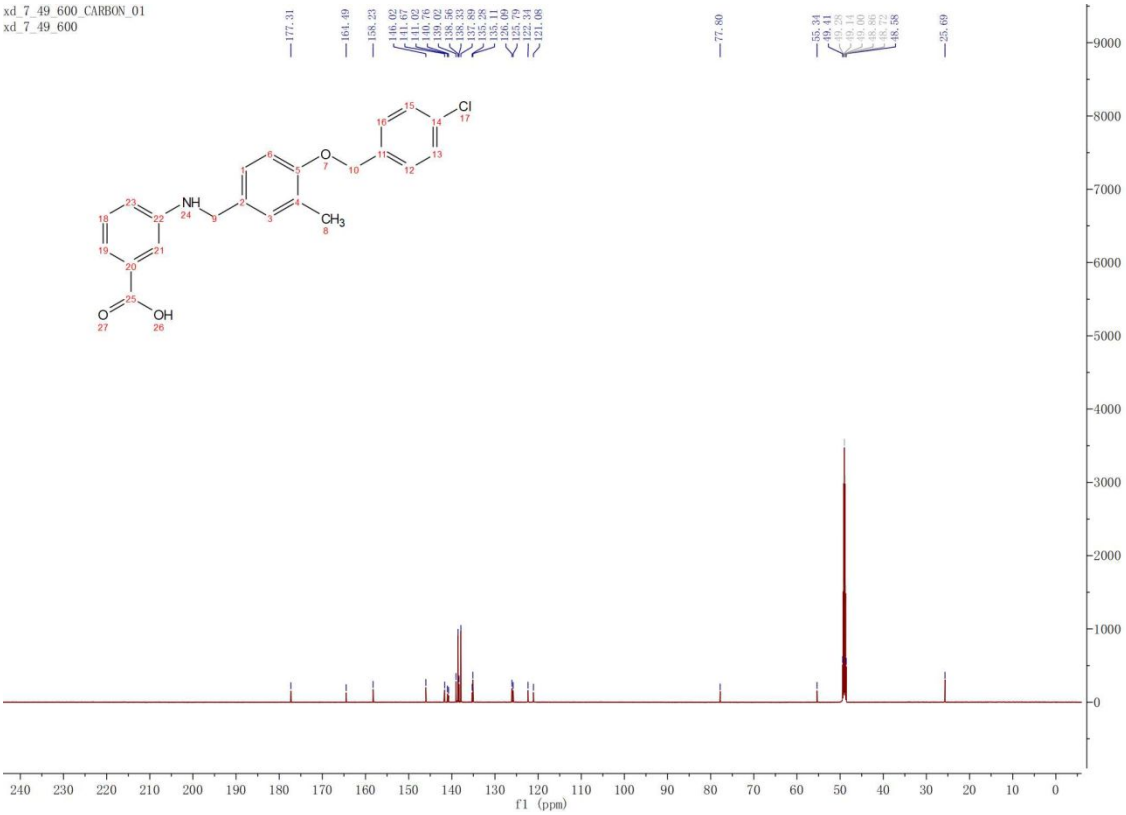
19f


xd_6_101_PROTON_01
xd_6_101
f1 (ppm)

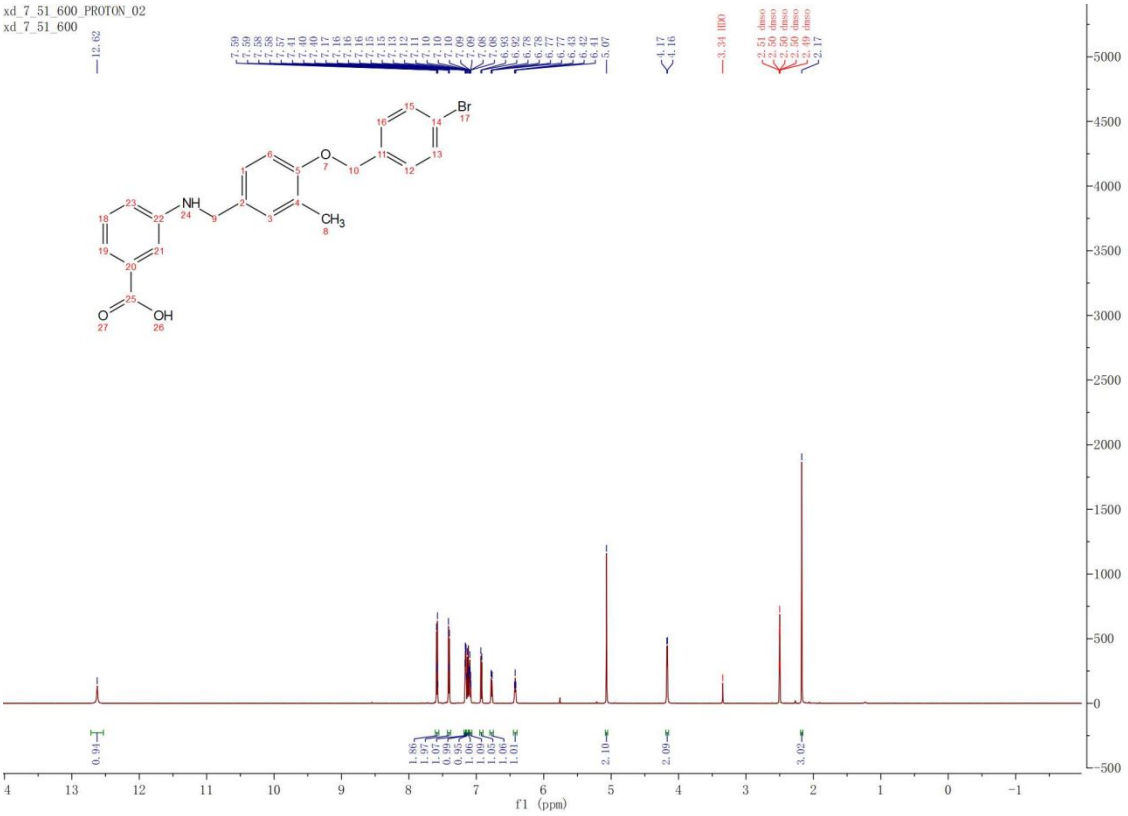

xd_6_101_CARBON_01
xd_6_101
f1 (ppm)

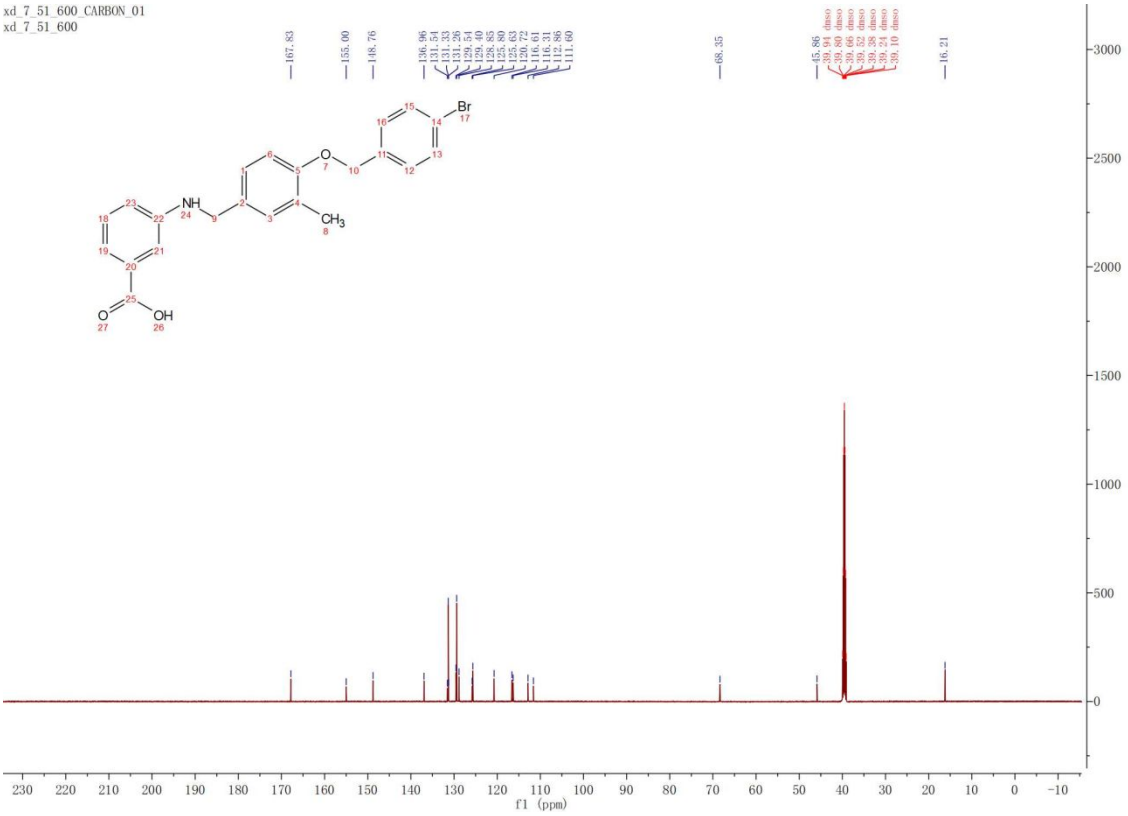
20


xd_7_49_600_PROTON_02
xd_7_49_600
f1 (ppm)

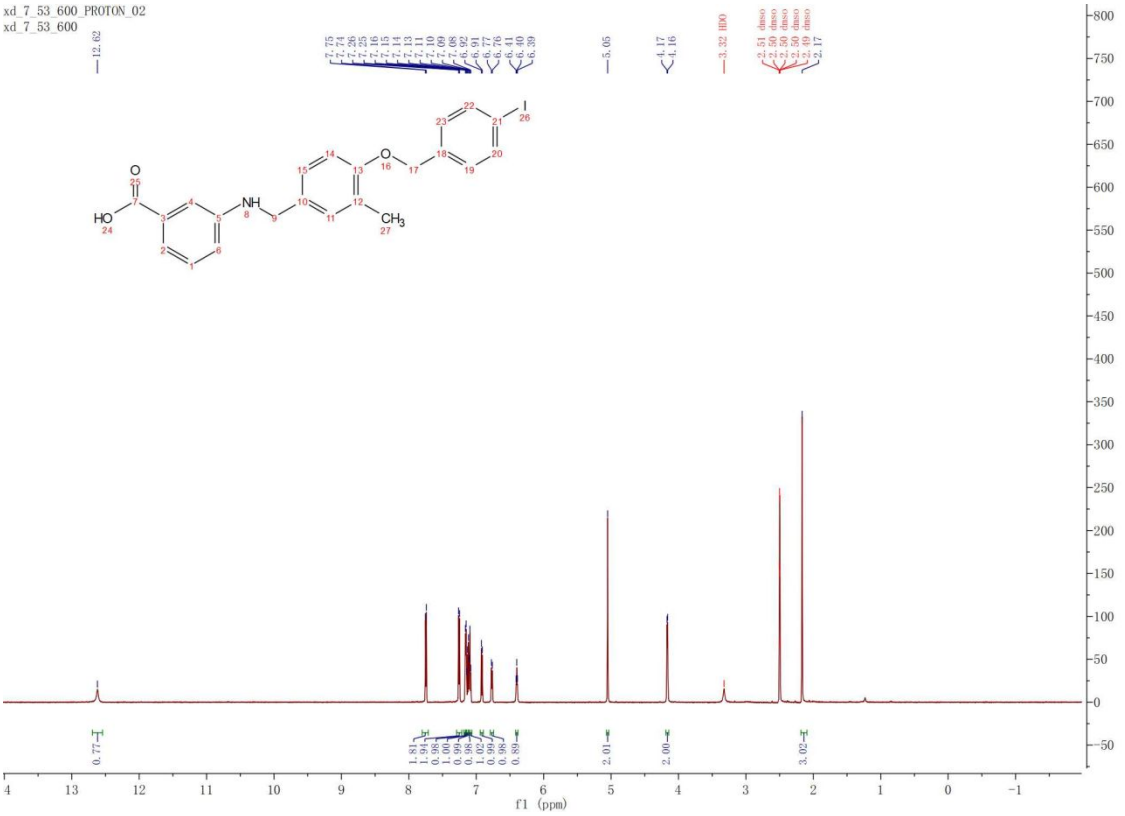

xd_7_49_600_CARBON_01
xd_7_49_600
f1 (ppm)

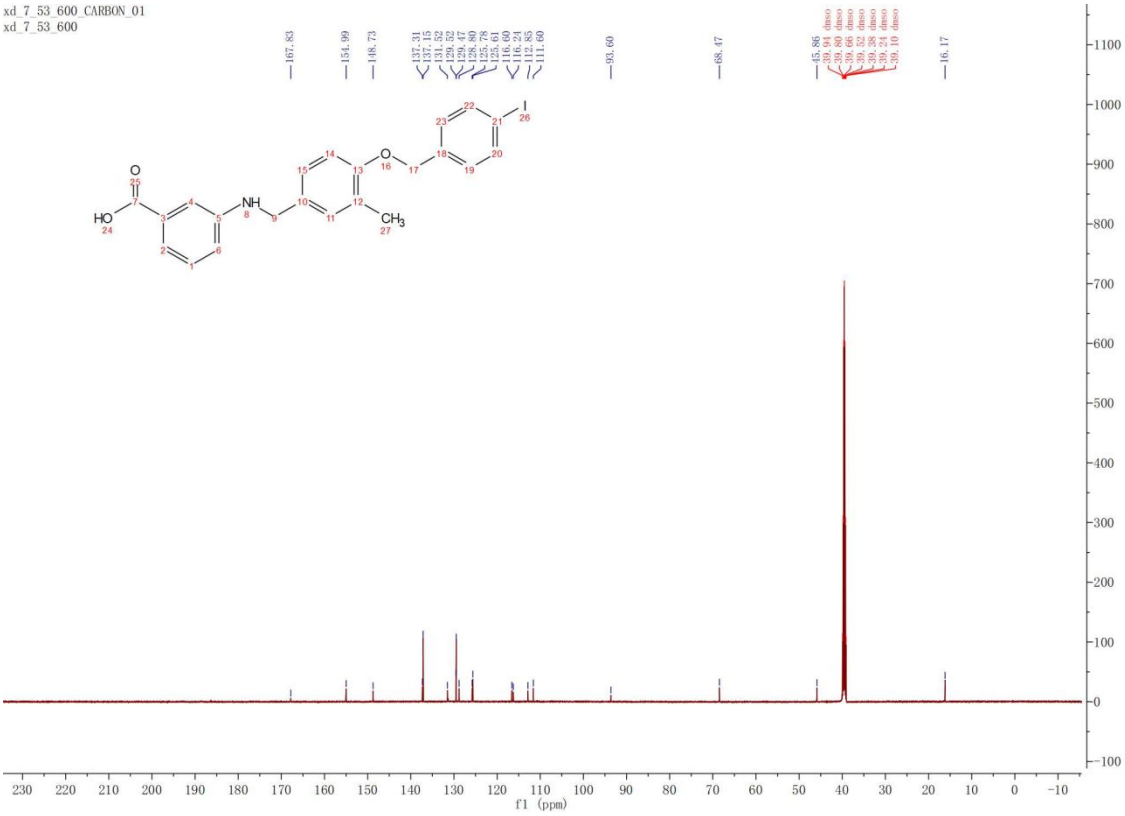
21


xd_7_51_600_PROTON_02
xd_7_51_600
f1 (ppm)

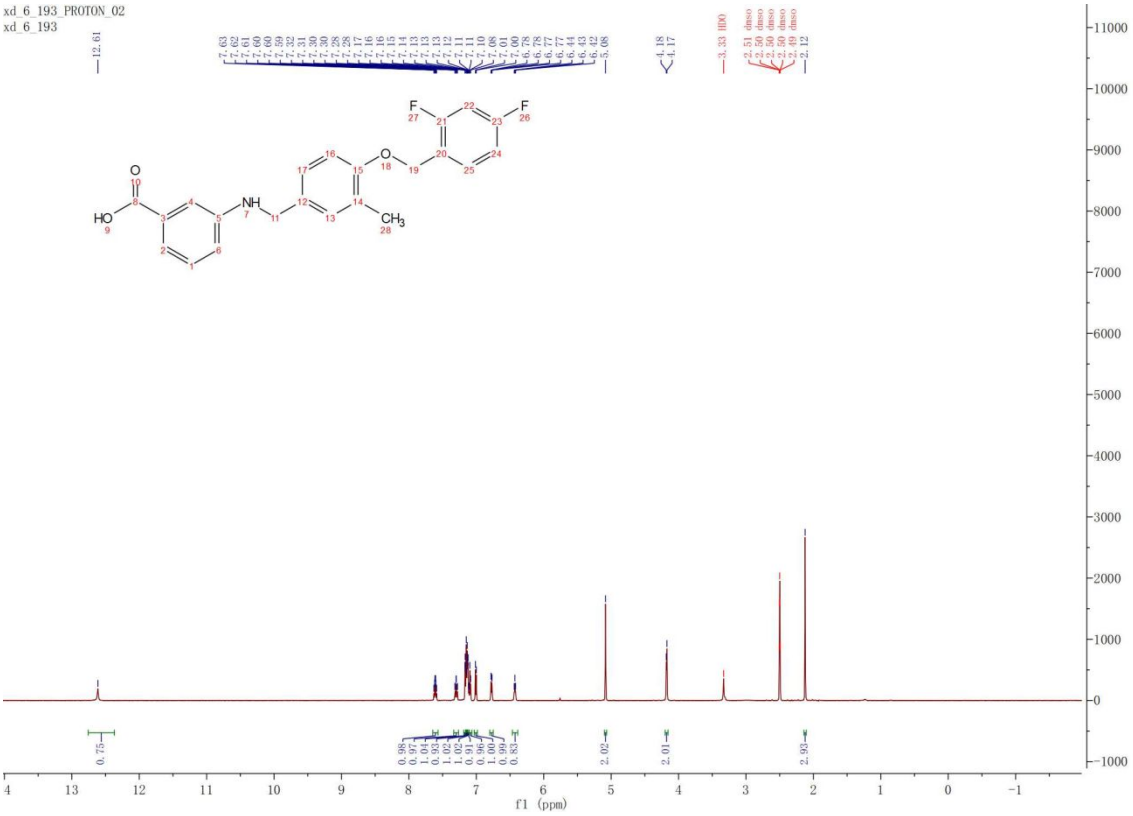

xd_7_51_600_CARBON_01
xd_7_51_600
f1 (ppm)

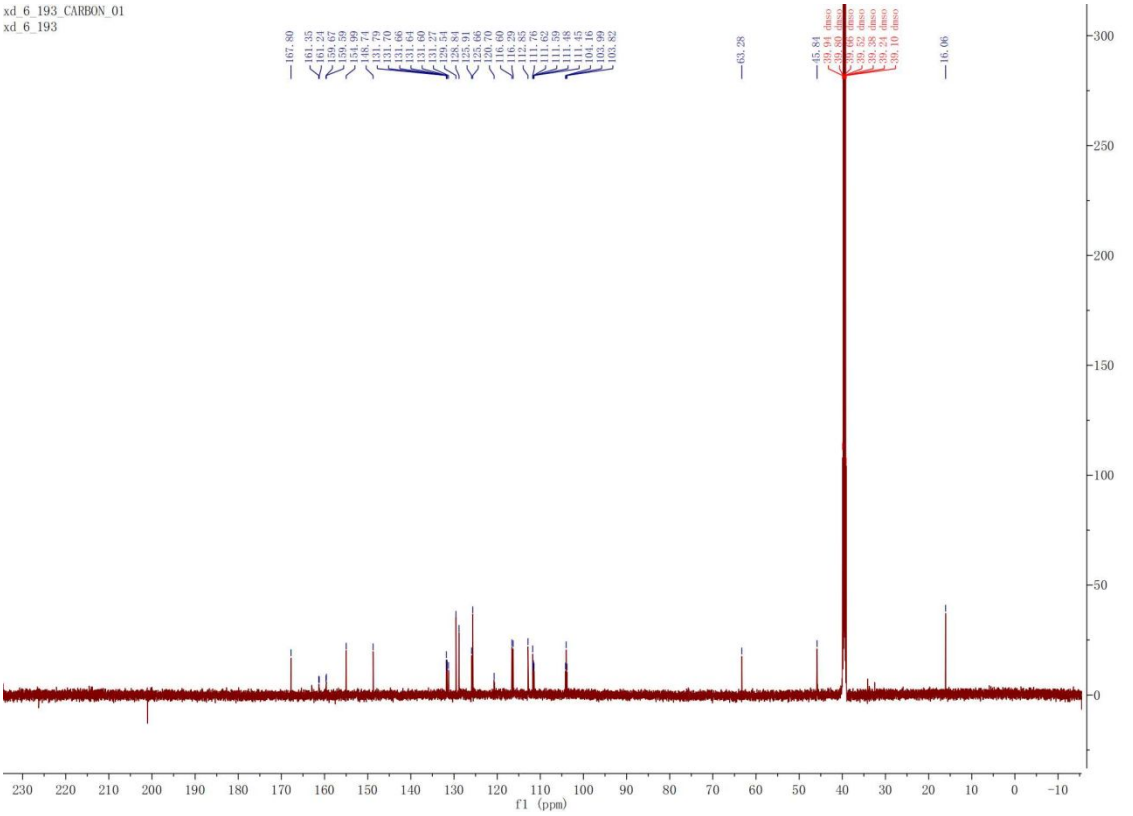
22


xd_7_53_600_PROTON_02
xd_7_53_600
f1 (ppm)

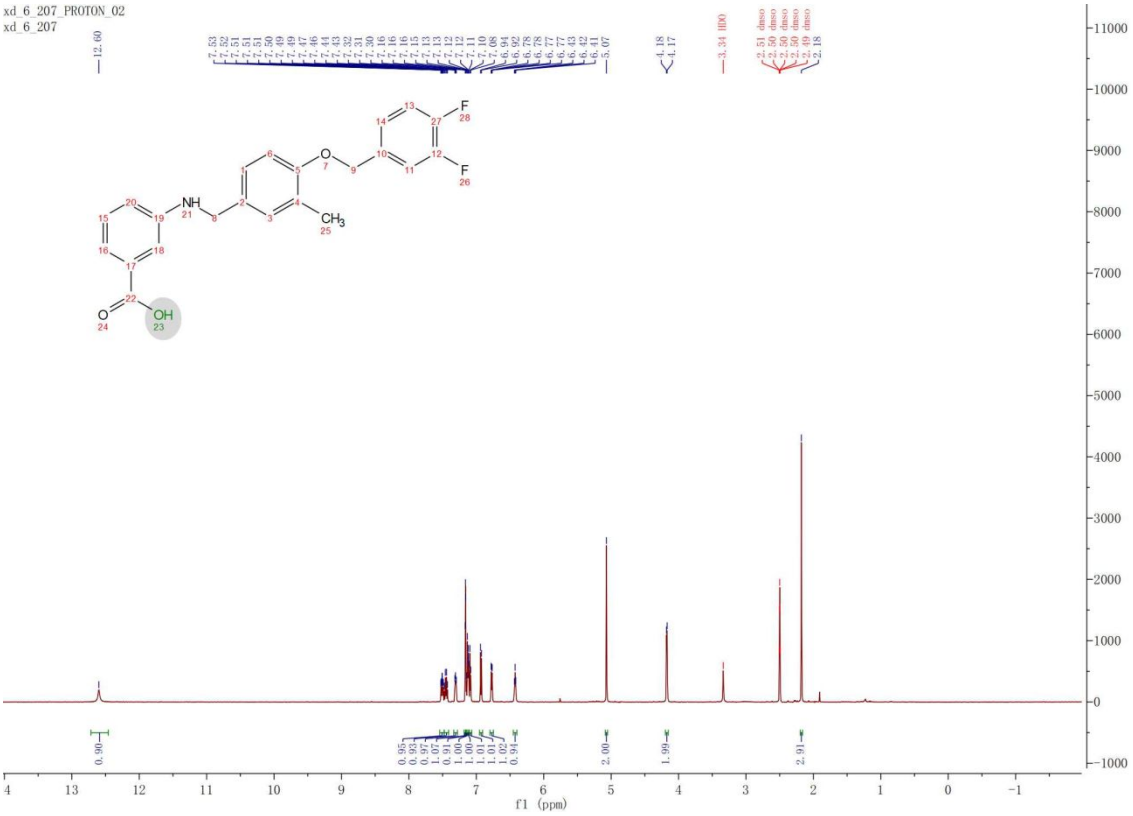

xd_7_53_600_CARBON_01
xd_7_53_600
f1 (ppm)

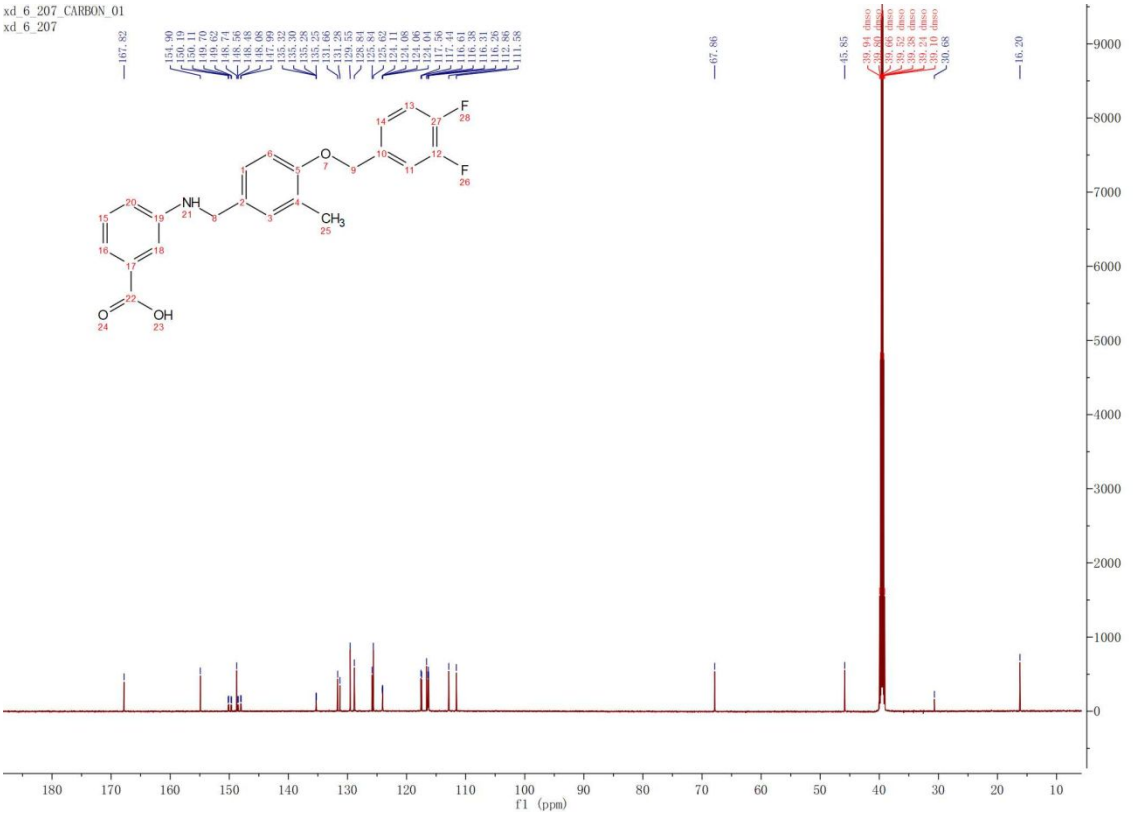
23


xd_6_193_PROTON_02
xd_6_193
f1 (ppm)

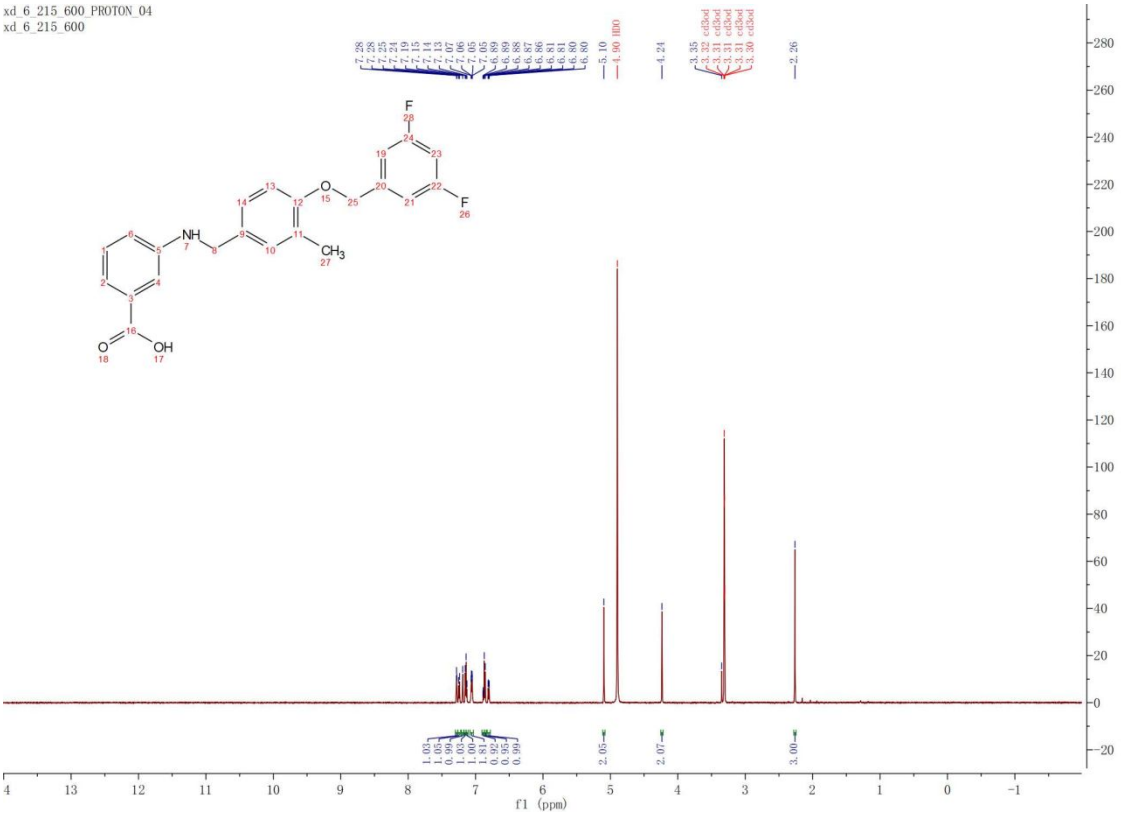

xd_6_193_CARBON_01
xd_6_193
f1 (ppm)

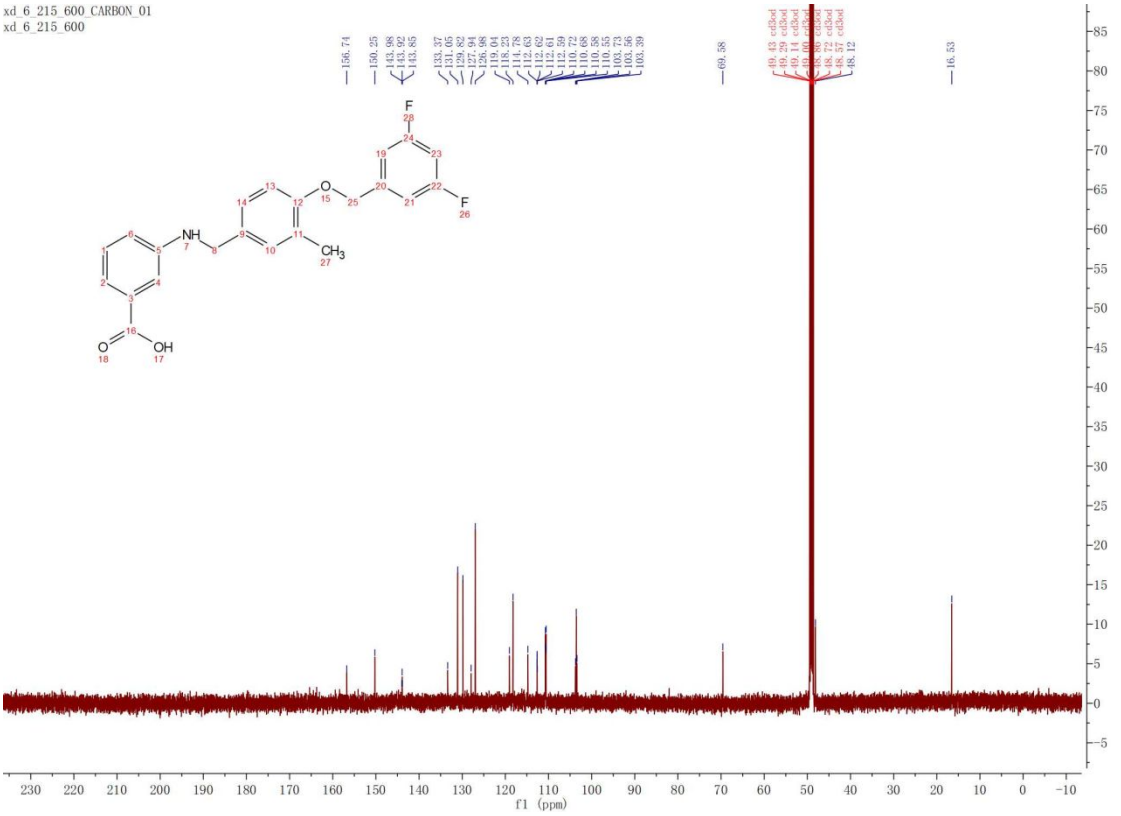
24


xd_6_207_PROTON_02
xd_6_207
f1 (ppm)

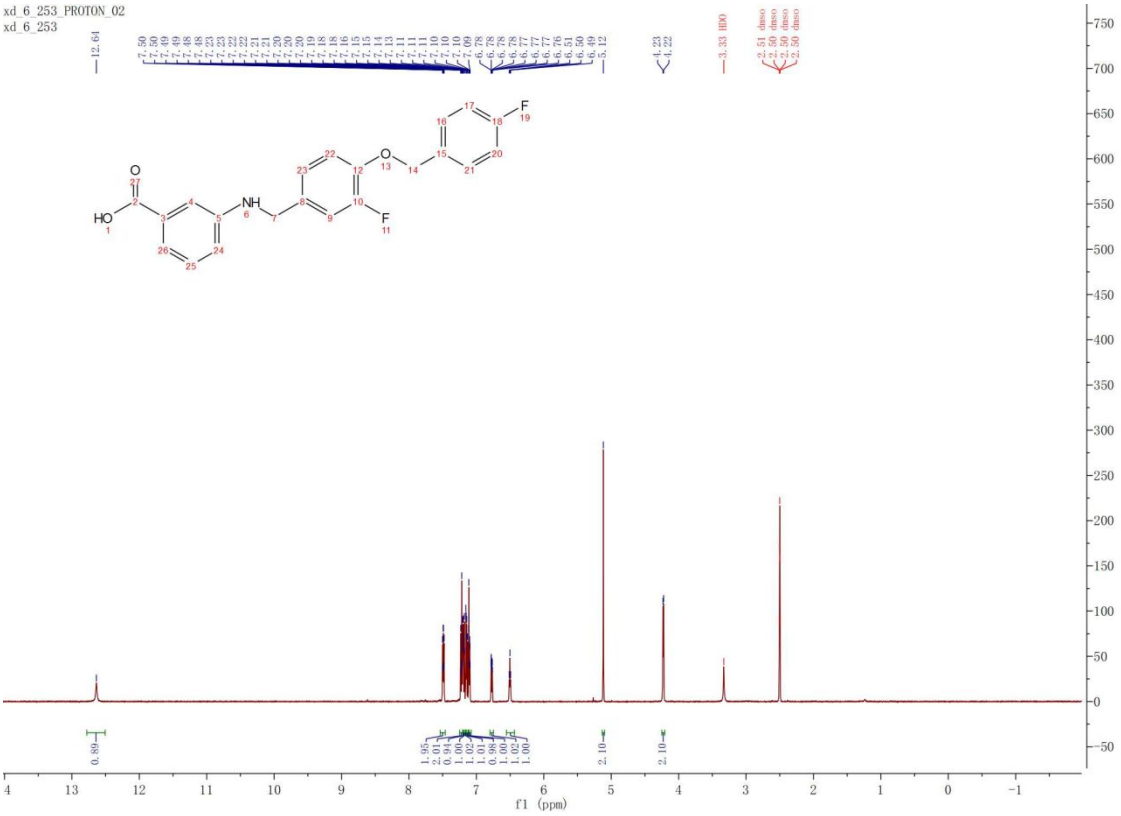

xd_6_207_CARBON_01
xd_6_207
f1 (ppm)

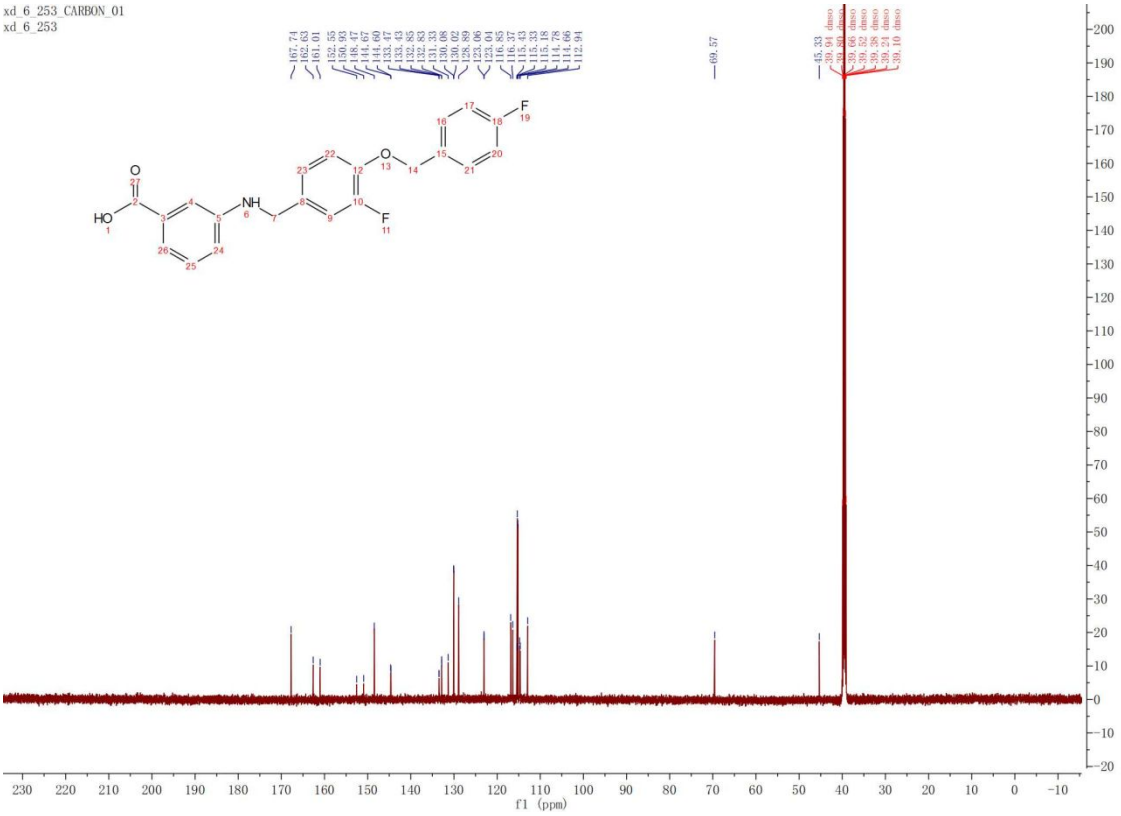
25


xd_6_215_600_PROTON_04
xd_6_215_600
f1 (ppm)

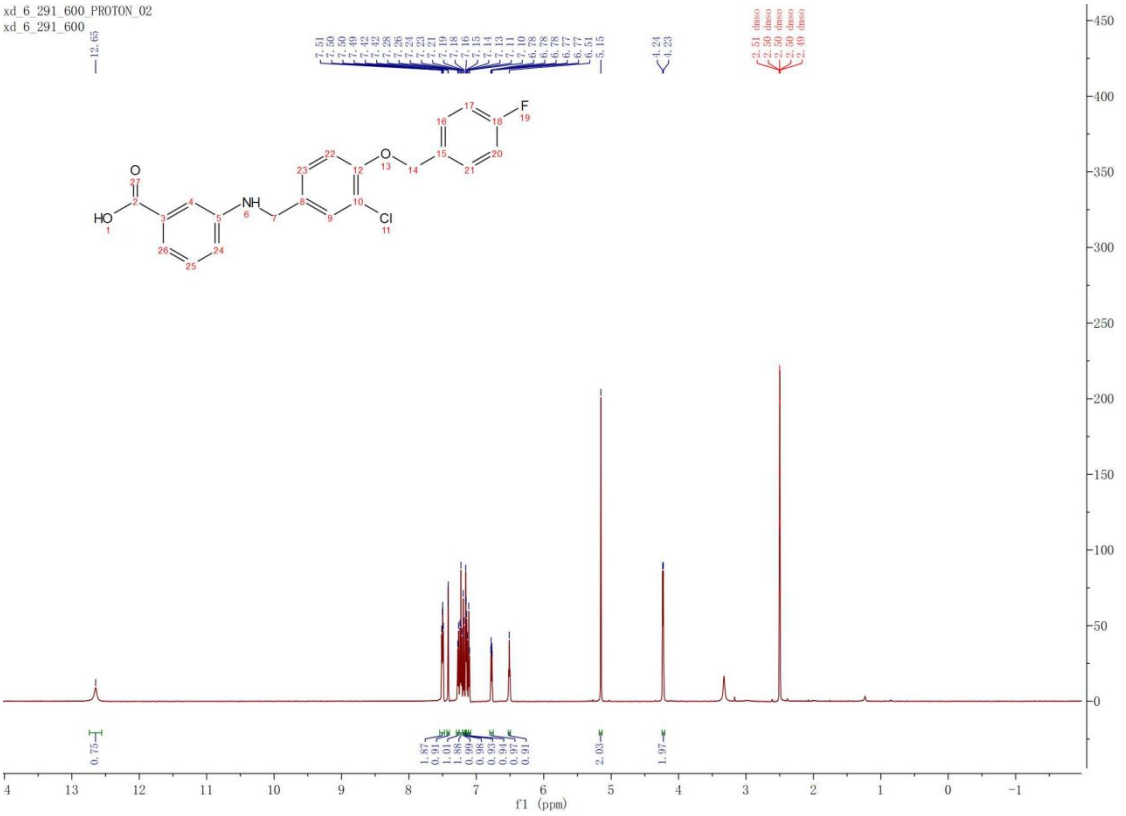

xd_6_215_600_CARBON_01
xd_6_215_600
f1 (ppm)

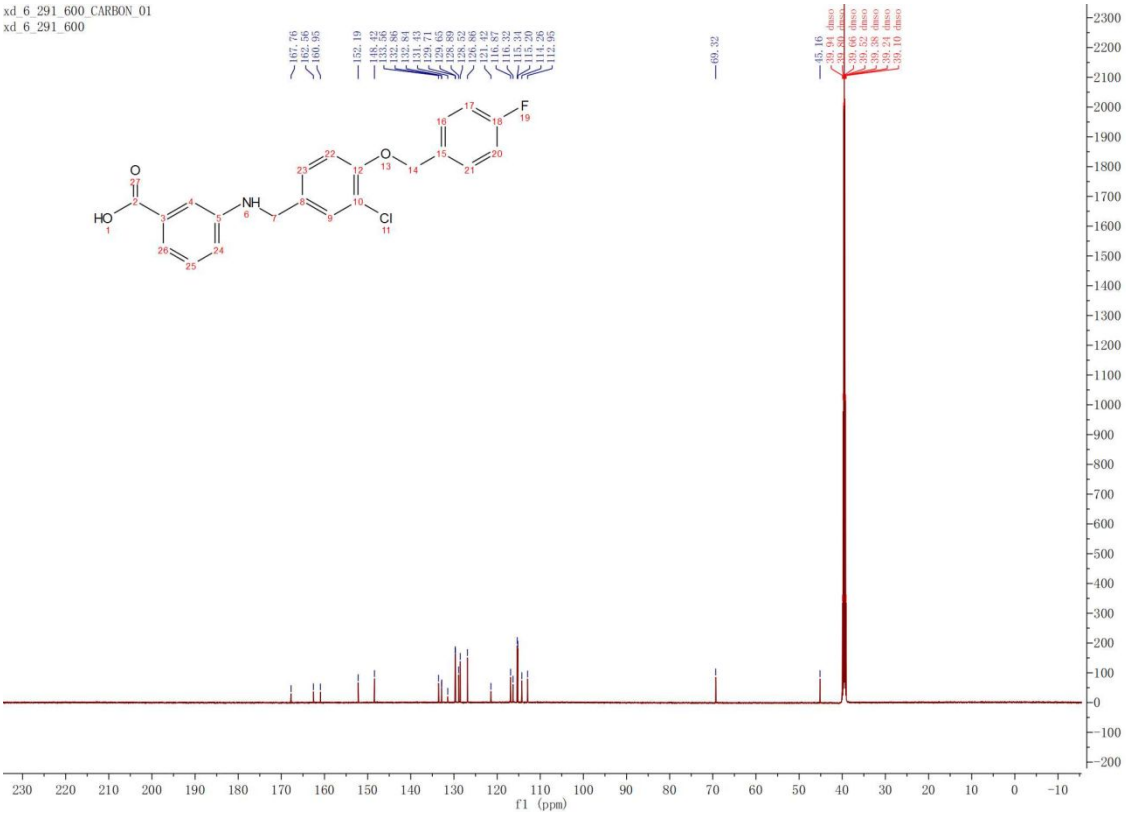
26


xd_6_253_PROTON_02
xd_6_253
f1 (ppm)

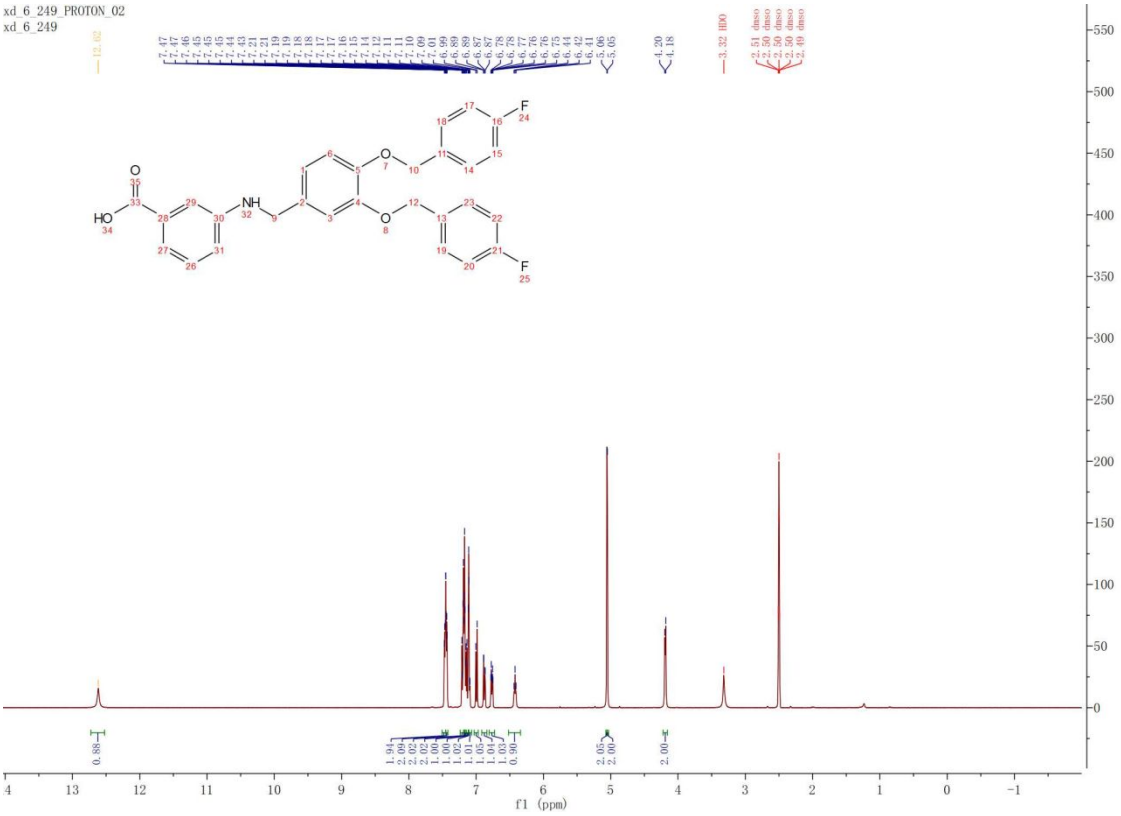

xd_6_253_CARBON_01
xd_6_253
f1 (ppm)

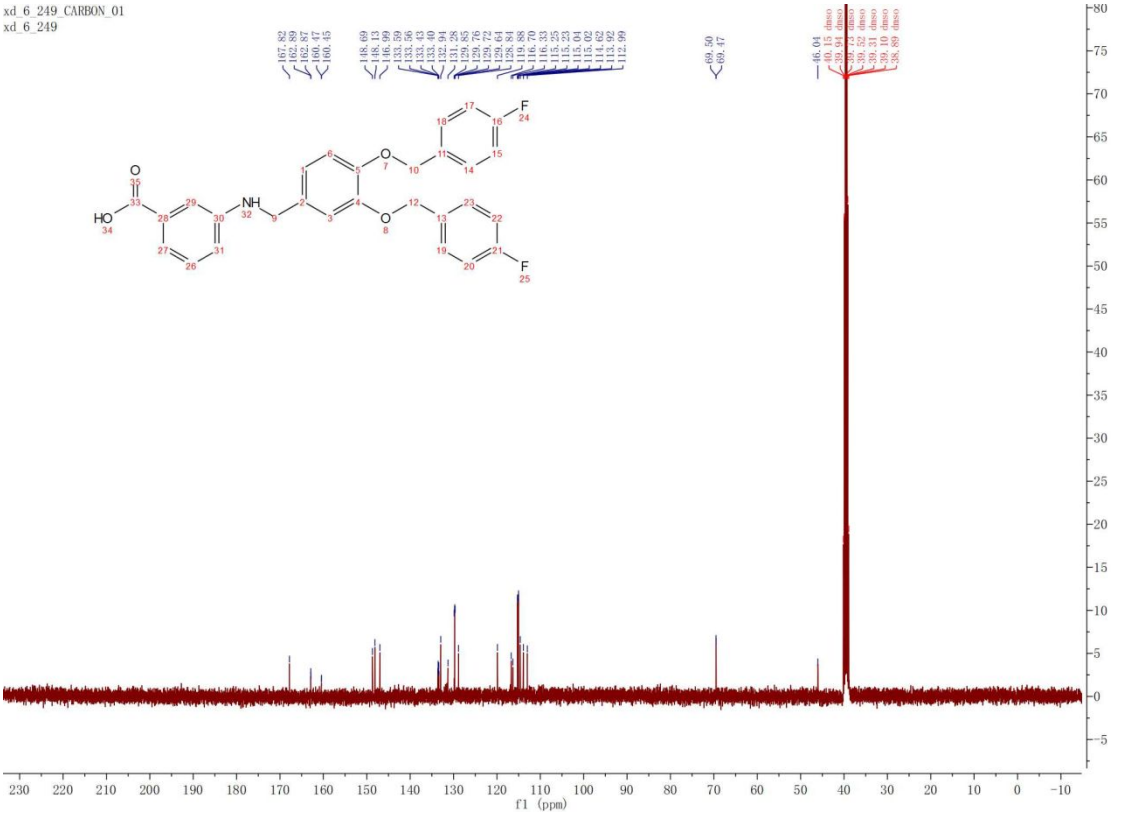
27


xd_6_291_600_PROTON_02
xd_6_291_600
f1 (ppm)

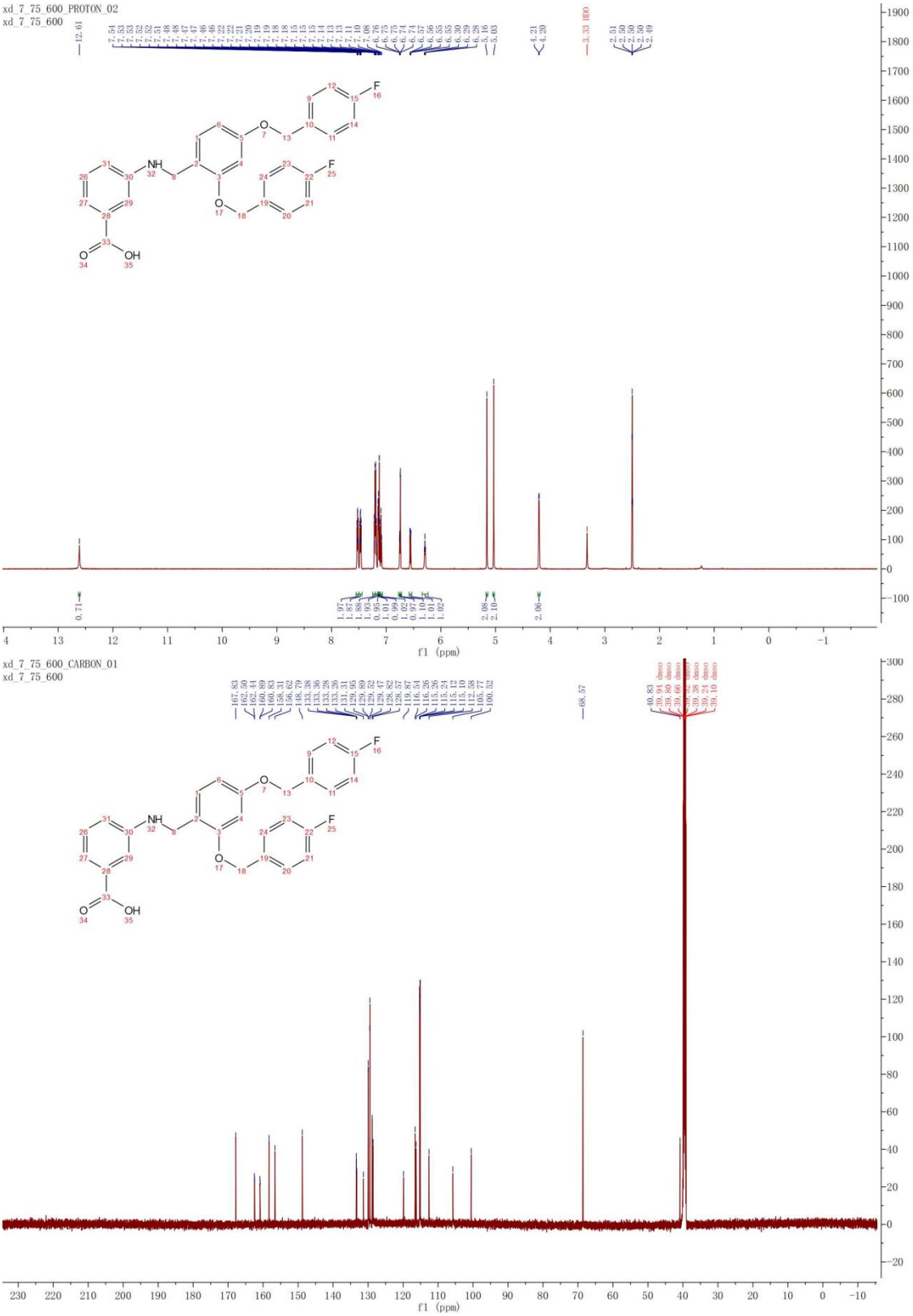

xd_6_291_600_CARBON_01
xd_6_291_600
f1 (ppm)

28

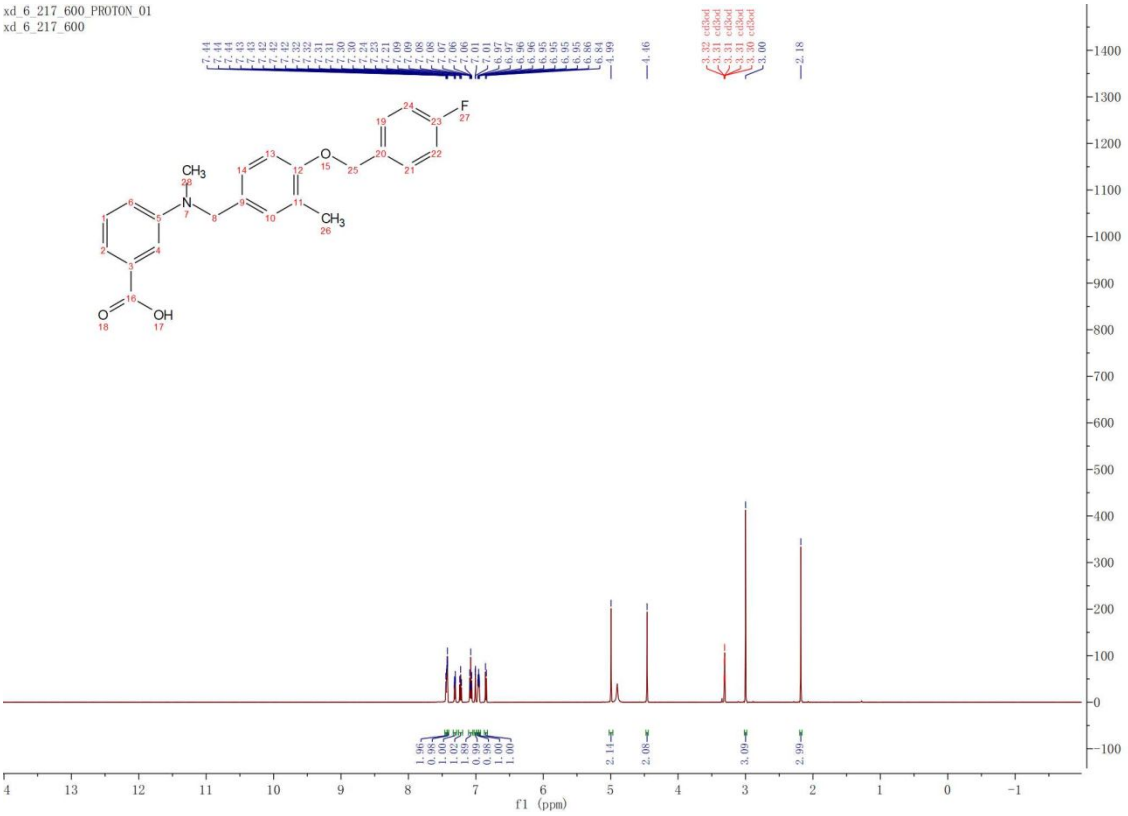

xd_6_249_PROTON_02
xd_6_249
f1 (ppm)

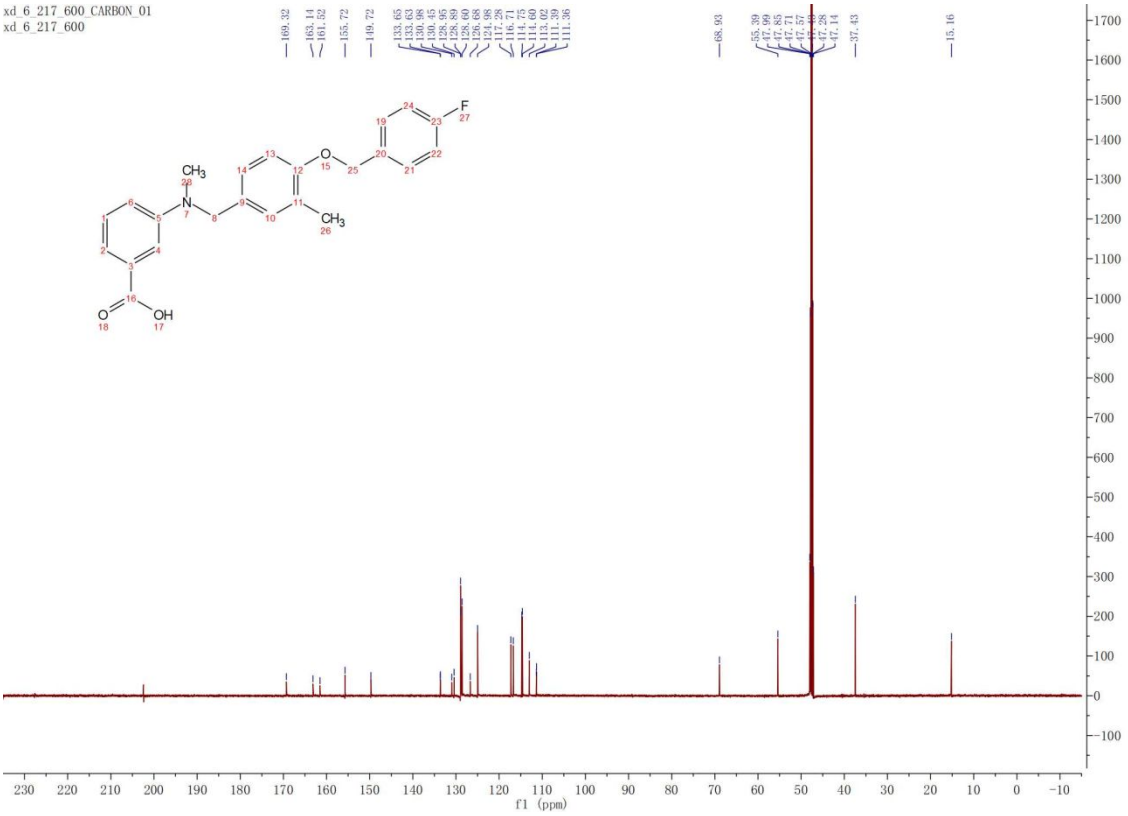

xd_6_249_CARBON_01
xd_6_249
f1 (ppm)

29

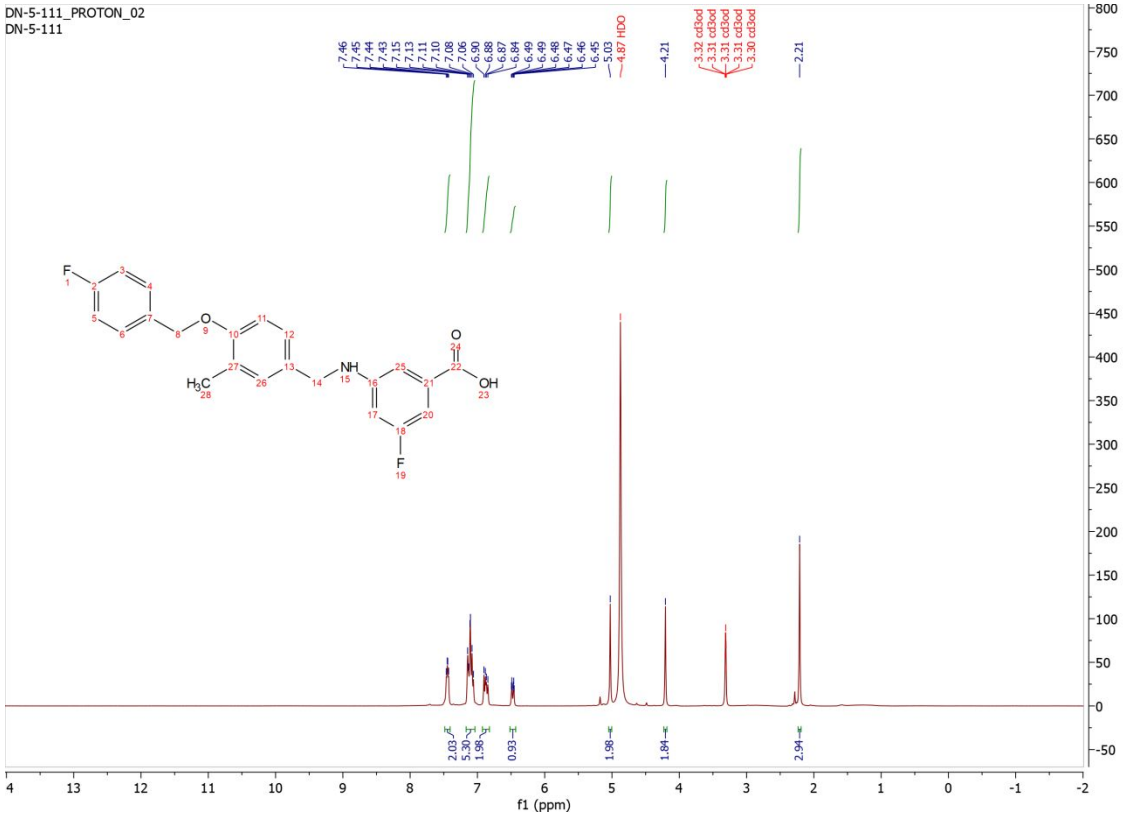

xd_7_75_600_PROTON_02
xd_7_75_600
f1 (ppm)
xd_7_75_600_CARBON_01
xd_7_75_600
f1 (ppm)

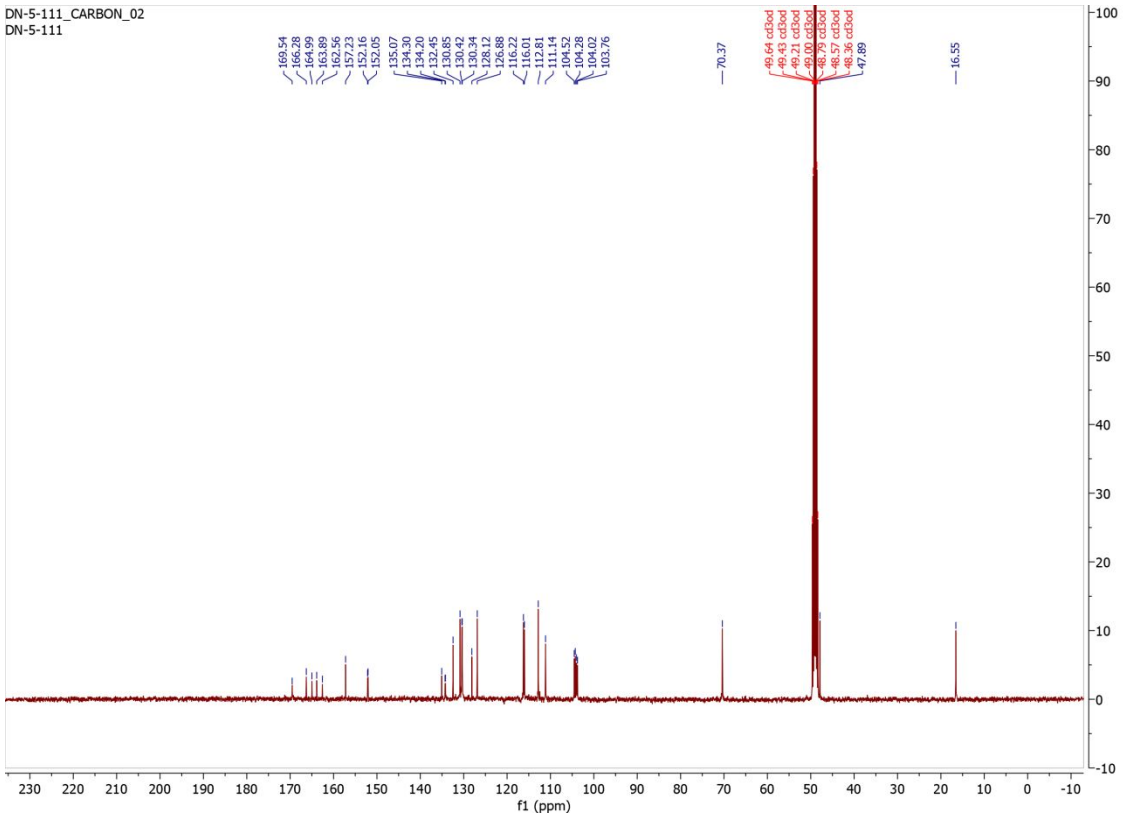
30


xd_6_217_600_PROTON_01
xd_6_217_600
f1 (ppm)

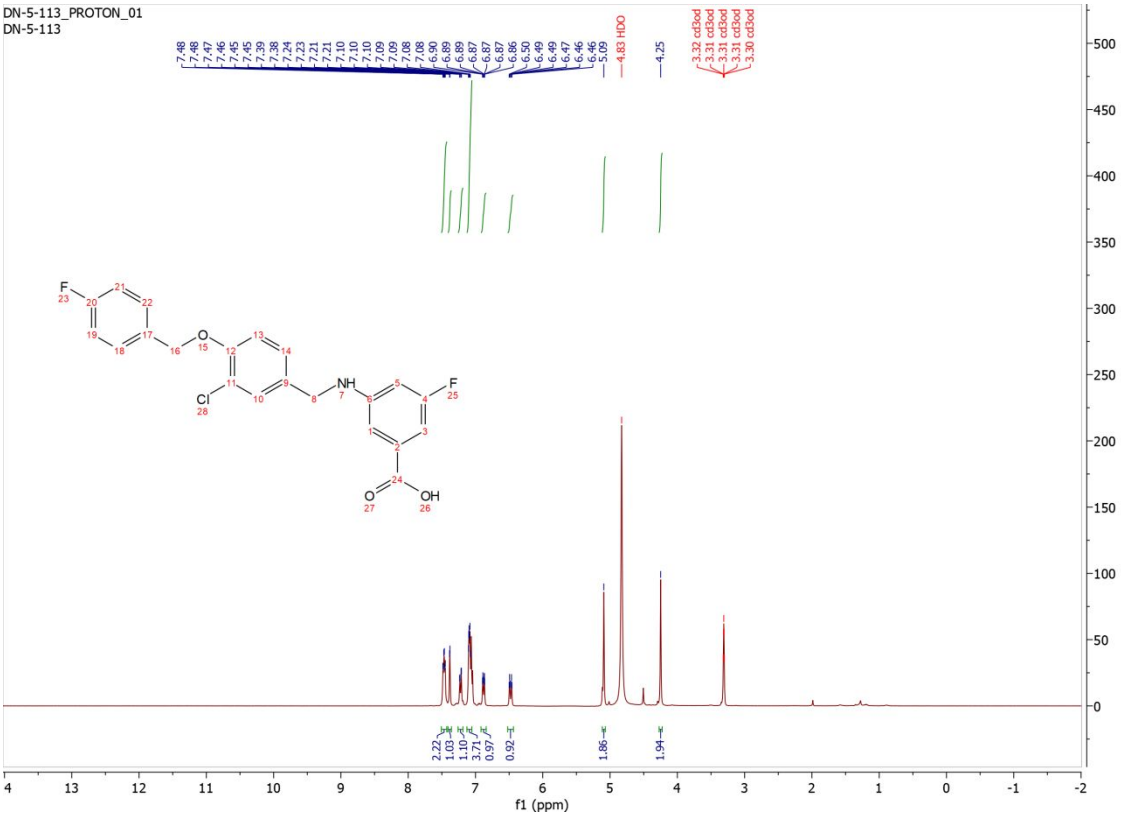

xd_6_217_600_CARBON_01
xd_6_217_600
f1 (ppm)

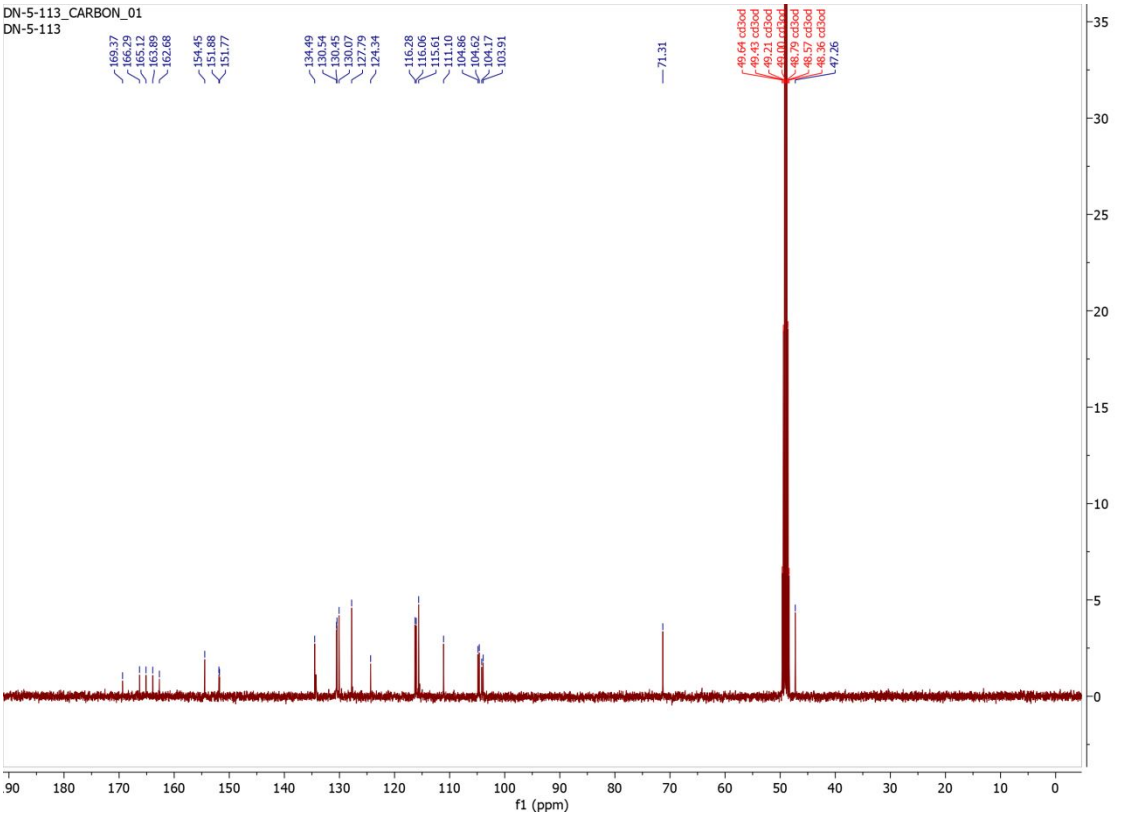
31


DN-5-111_PROTON_02
DN-5-111
7.46
7.45
7.44
7.43
7.15
7.13
7.11
7.10
7.08
7.06
6.90
6.88
6.87
6.84
6.49
6.49
6.48
6.47
6.46
6.45
5.03
4.87 HDO
4.21
3.32 cd3od
3.31 cd3od
3.31 cd3od
3.31 cd3od
3.31 cd3od
3.30 cd3od
2.21
F
O
NH
OH
H3C
2.03
5.30
1.98
0.93
1.98
1.84
2.94
f1 (ppm)


DN-5-111_CARBON_02
DN-5-111
169.54
166.28
164.99
163.89
162.56
157.23
152.16
152.05
135.07
134.30
134.20
132.45
130.85
130.42
130.34
128.12
126.88
116.22
116.01
112.81
111.14
104.52
104.28
104.02
103.76
70.37
49.64 cd3od
49.43 cd3od
49.21 cd3od
49.00 cd3od
48.79 cd3od
48.57 cd3od
48.36 cd3od
47.89
16.55
f1 (ppm)

32

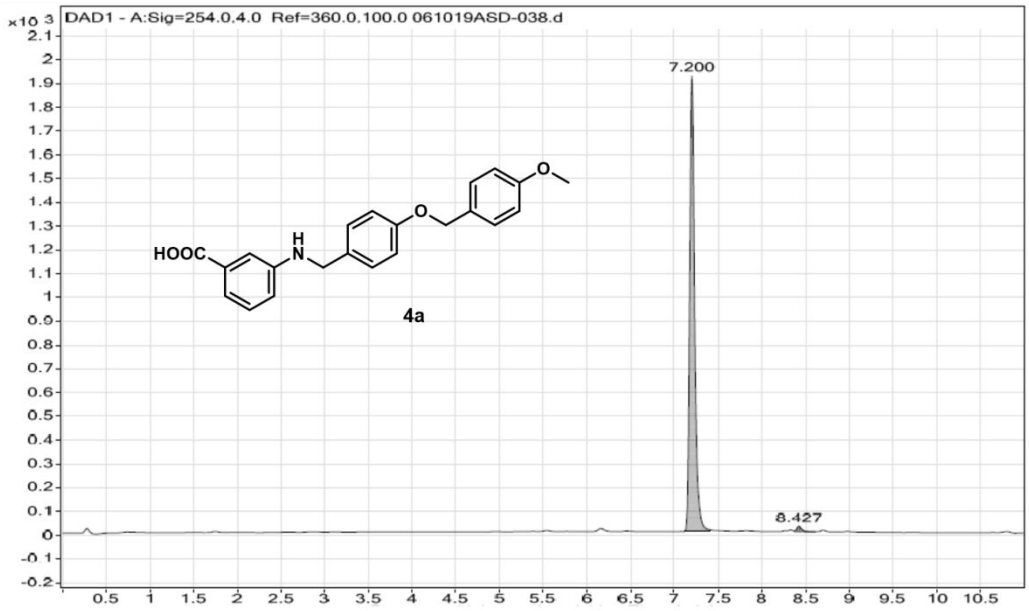

DN-5-113_PROTON_01
DN-5-113
4.83 HDO
4.25
f1 (ppm)


DN-5-113_CARBON_01
DN-5-113
71.31
f1 (ppm)

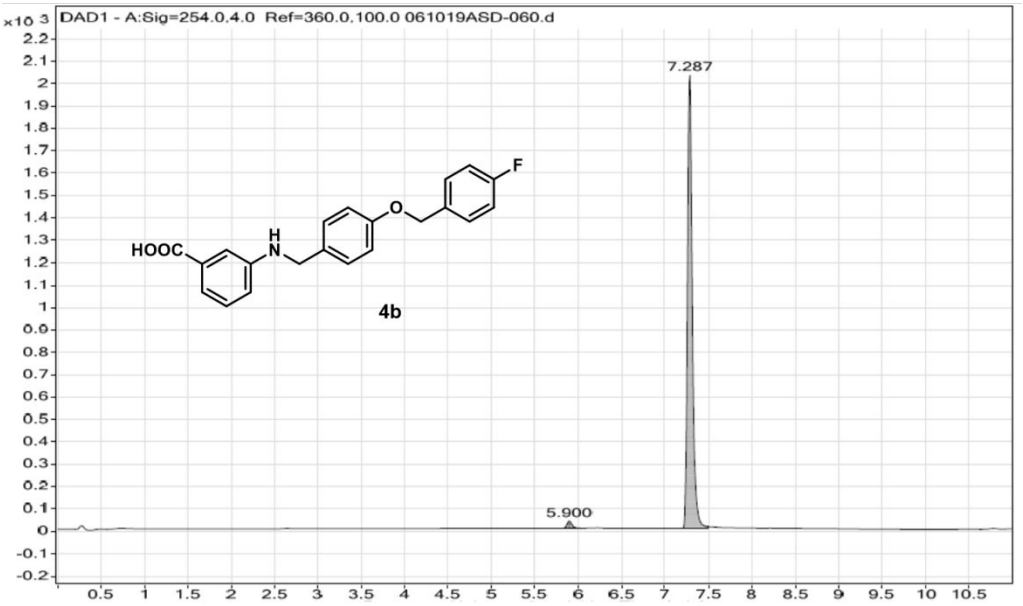
33

## HPLC Traces

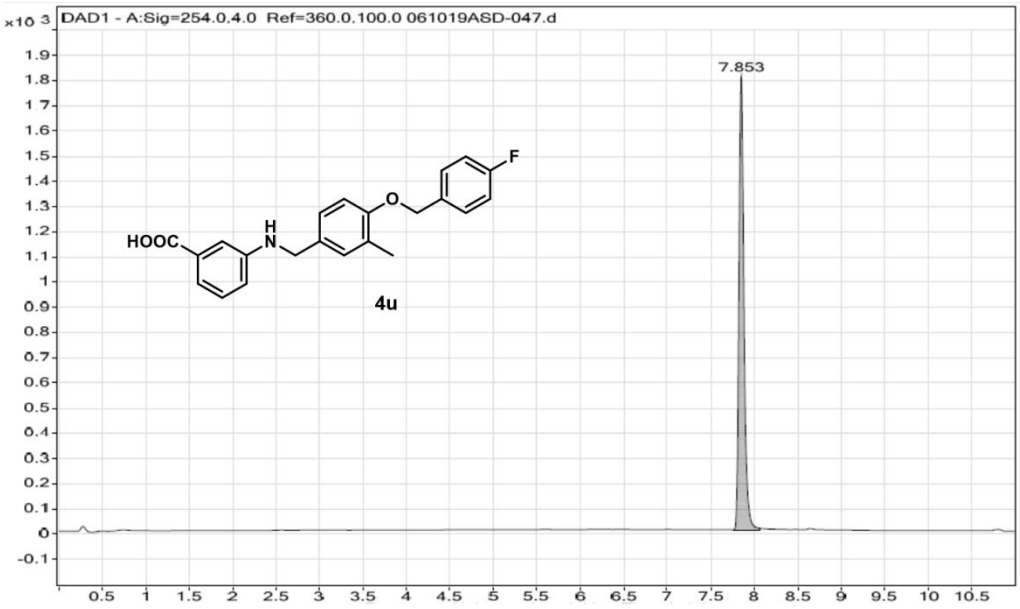
| Peak | RT | Area | Height | Type | Saturated | Width | FWHM | SNR |
|---|---|---|---|---|---|---|---|---|
| **1** | **7.2** | **7611.9** | **1917.3** | | | **0.293** | **0.059** | |
| 2 | 8.427 | 94.93 | 23.43 | | | 0.24 | 0.057 | |



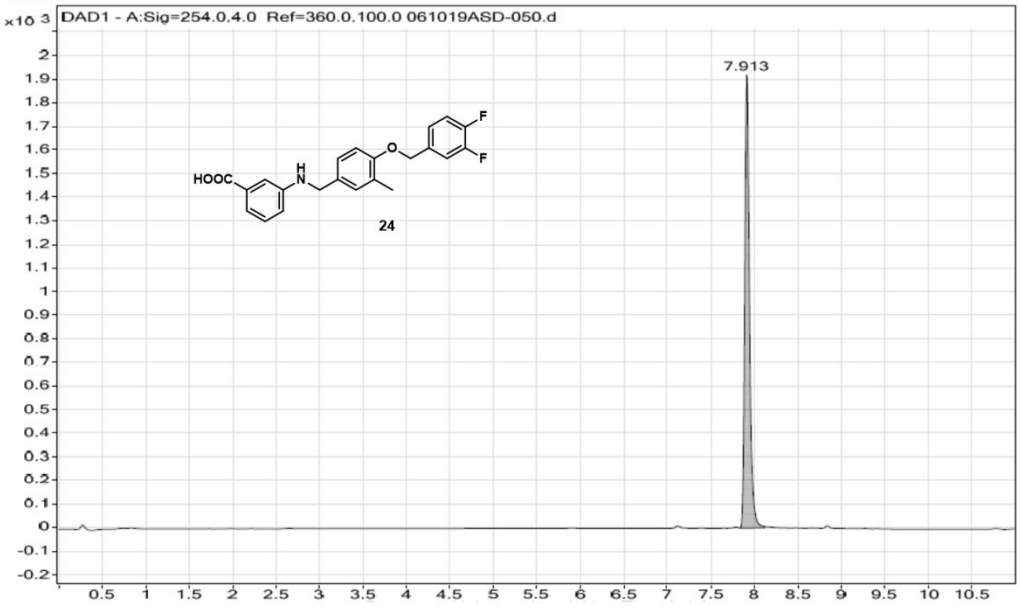

| Peak | RT | Area | Height | Type | Saturated | Width | FWHM | SNR |
|---|---|---|---|---|---|---|---|---|
| **1** | **5.9** | **135.02** | **32.58** | | | **0.247** | **0.061** | |
| 2 | 7.287 | 7833.3 | 2024.17 | | | 0.29 | 0.057 | |



| Peak | RT | Area | Height | Type | Saturated | Width | FWHM | SNR |
|---|---|---|---|---|---|---|---|---|
| 1 | 7.853 | 7214.4 | 1803.97 | | | 0.3 | 0.059 | |




| Peak | RT | Area | Height | Type | Saturated | Width | FWHM | SNR |
|---|---|---|---|---|---|---|---|---|
| 1 | 7.913 | 7258.6 | 1920.86 | | | 0.287 | 0.056 | |



| Peak | RT | Area | Height | Type | Saturated | Width | FWHM | SNR |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.28 | 115.51 | 32.09 | | | 0.189 | 0.054 | |
| 2 | 3.867 | 62.05 | 16.78 | | | 0.193 | 0.052 | |
| 3 | 4.4 | 2570.6 | 805.61 | | | 0.226 | 0.048 | |