# Development of New Experimental NMR Strategy of Cysteine Protease Inhibitors Screening: Toward Enhanced Drug Discovery

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## Supporting information.

### 1.General information

All chemicals, starting materials, and solvents employed in this study were acquired from Sigma-Aldrich or Alfa Aesar and utilized without additional purification. The progress of the reactions was tracked through thin-layer chromatography (TLC) on aluminum sheets coated with Merck 60 F254 silica gel (thickness 0.2 mm), and the detection was conducted under a UV lamp set at either 254 or 365 nm. Column chromatography was carried out using silica gel 60 (230–400 mesh, 0.040–0.063 mm). The melting points (m.p. [°C]) of the samples were measured using open capillary tubes, utilizing the BioCoteThermo scientific digital melting point IA9200 and remain unadjusted. NMR spectra were acquired on Jeol 600 MHz spectrometers at room temperature, and the samples dissolved in an appropriate deuterated solvent. $^{1}H$ and $^{13}C$ chemical shifts were reported relative to residual solvent peaks. Multiplicities are denoted as s (singlet), d (doublet), t (triplet), q (quartet), p (pentet), m (multiplet), and br (broad signal). Coupling constants (J) are reported in hertz (Hz). The infrared spectra of compounds were recorded at room temperature on a Thermo Scientific Nicolet IS50 FT-IR. For high-resolution mass spectra we used Q Exactive Plus Orbitrap LC-MS-MS system.

### 1.2 Synthesis

Products 2a and 2b were synthesized as described in the literature[1,2].

Intermediate 3 and 5 were obtained by applying the procedure reported by Khalid Boujdi *et al*.[3] and were then used in the next step without further purification.

***Synthesis procedure of Product 4, 6 and 8:***

To a solution of EA (0.32mmol) in dichloromethane were added EDC.HCl (0.36 mmol), HOBt (0.36mmol), and TEA (0.65 mmol) in 0°C. This solution was then stirred at this temperature during

30 min. Afterwards, the corresponding amine intermediate **3**, **5** or **7** (0.32mmol) was added and the resulting mixture was then allowed to move to room temperature and stirred overnight. After extraction with DCM, the combined organic phases are washed with a saturated sodium chloride solution, dried over magnesium sulfate, filtered and then concentrated under pressure. The residue obtained is purified by column chromatography eluting with a mixture AcOEt / DCM (1/4 (v/v)) to give the expected product as white solid.

**Product 4: 2-(2,3-dichloro-4-(2-methylenebutanoyl)phenoxy)-N-(1-methyl-1H-indazol-5-yl)acetamide**: White solid; Yield 44 %; m.p: 174-176 °C. $^{1}$H NMR ($CDCl_3$, 600 MHz), δ 7.98 (s, 1H), 7.66 (dd, *J* = 8.1, 1.0 Hz, 1H), 7.50 (dt, *J* = 7.5, 1.0 Hz, 1H), 7.25 (d, *J* = 8.5 Hz, 1H), 7.19-7.13 (m, 1H), 6.97 (d, *J* = 8.5 Hz, 1H), 5.97 (s, 1H), 5.60 (s, 1H), 4.82 (s, 2H), 4.36 (s, 1H), 4.27 (s, 3H), 2.47 (t, *J* = 7.4 Hz, 2H), 1.15 (t, *J* = 7.4 Hz, 3H). $^{13}$C NMR ($CDCl_3$, 151 MHz), δ 195.52, 166.41, 156.88, 150.28, 135.04, 134.82, 133.22, 131.85, 129.07, 127.48, 126.97, 124.55, 121.13, 120.64, 118.98, 111.09, 68.41, 49.25, 38.47, 23.47, 12.47. IR (neat): v = 3395 (NH), 1663, 1652 (C=O) cm$^{-1}$. HRMS (+ESI) calcd for $C_{21}H_{19}Cl_2N_3O_3$+H1 432.0803, found 432.0647. HPLC purity: 97 % (MOS-1 HYERSIL 250x4.6 mmI.D column, 50% MeCN/$H_2O$, 254 nm, 23 °C $t_R$ = 7.6 min).

**Product6**: **2-(2,3-dichloro-4-(2-methylenebutanoyl)phenoxy)-N-(2-methyl-2H-indazol-7-yl)acetamide:** White solid; Yield 46 %; m.p: 175-177 °C. $^{1}$H NMR ($CDCl_3$, 600 MHz), δ 7.98 (s, 1H), 7.66 (dd, *J* = 8.1, 1.0 Hz, 1H), 7.50 (dt, *J* = 7.5, 1.0 Hz, 1H), 7.25 (d, *J* = 8.5 Hz, 1H), 7.19-7.13 (m, 1H), 6.97 (d, *J* = 8.5 Hz, 1H), 5.97 (s, 1H), 5.60 (s, 1H), 4.82 (s, 2H), 4.36 (s, 1H), 4.27 (s, 3H), 2.47 (t, *J* = 7.4 Hz, 2H), 1.15 (t, *J* = 7.4 Hz, 3H). $^{13}$C NMR ($CDCl_3$, 151 MHz), δ 195.52, 166.41, 156.88, 150.28, 135.04, 134.82, 133.22, 131.85, 129.07, 127.48, 126.97, 124.55, 121.13, 120.64, 118.98, 111.09, 68.41, 49.25, 38.47, 23.47, 12.47. IR (neat): v = 3395 (NH), 1663, 1652 (C=O) cm$^{-1}$. HRMS (+ESI) calcd for $C_{21}H_{19}Cl_2N_3O_3$+H1 432.0803, found 432.0647. HPLC purity: 98 % (MOS-1 HYERSIL 250x4.6 mmI.D column, 50% MeCN/$H_2O$, 254 nm, 23 °C $t_R$ = 7.9 min).

**Product 8: N-((1H-indol-6-yl)methyl)-2-(2,3-dichloro-4-(2-methylenebutanoyl)phenoxy)acetamide:** White solid, Yield: 35%. m.p. 189-190 °C. $^{1}$H NMR ($CDCl_3$, 600 MHz) δ 8.44 (s, 1H), 7.60 (d, *J* = 8.1 Hz, 1H), 7.25-7.23 (m, 1H), 7.20 (d, *J* = 8.5 Hz, 1H), 7.09 (s, 1H), 7.04 (dd, *J* = 8.1, 1.5 Hz, 1H), 6.94 (s, 1H), 6.90 (d, *J* = 8.6 Hz, 1H), 6.53 (ddd, *J* = 3.1, 2.0, 1.0 Hz, 1H), 5.98 (t, *J* = 1.5 Hz, 1H), 5.60 (s, 1H), 4.68 (s, 2H), 4.66 (d, *J* = 5.9 Hz, 2H), 2.48 (q, *J* = 7.4 Hz, 2H), 1.16 (t, *J* = 7.4 Hz, 3H). $^{13}$C NMR ($CDCl_3$, 151 MHz) δ 199.73, 196.35, 166.90, 154.89, 150.45, 136.25, 134.54, 131.86, 131.51, 129.65, 127.78, 127.44, 125.24, 121.38, 120.03, 111.31, 110.16, 102.77, 68.67, 43.91, 23.70, 12.73. IR (neat):*v* = 3359 (NH), 1633 (C=O) cm$^{-1}$. HRMS (+ESI) *m/z*: $[M+H]^+$calculated for $C_{22}H_{21}Cl_2N_2O_3$: 431,0851, found, 431,0860 . HPLC purity: 98% (MOS-1 HYERSIL 250x4.6 mmI.D column, 50% MeCN/$H_2O$, 254 nm, 23 °C, $t_R$ = 18.9 min).

***Synthesis procedure of Product 9.***

**(2,3-Dichloro-4-hydroxyphenyl)butan-1-one (1b)**[4]

Anisole **1a** (2.0 g, 11.36 mmol, 1 equiv.) and butyryl chloride (1.45 g, 13.63 mmol, 1.5 equiv.) were dissolved in 50 mL of absolute DCM, and the mixture was cooled to 0 °C. A solution of $AlCl_3$ (2.27 g, 17.04 mmol, 1.5 equiv.) in 25 mL of DCM was added dropwise within 30 min, and the mixture was stirred for 16 hours. The mixture was concentrated in vacuum to dryness then dissolved in

75 mL of DCM. This procedure was repeated twice. An additional amount of 1.5 equiv. of $AlCl_3$ was added, and the mixture was heated under reflux for 6 hours. The mixture was placed in an ice bath and acidified with concentrated HCl to pH = 1. After, tartaric acid was added for the complexation of aluminum until the solution was cleared up. The reaction mixture was diluted with EtOAc (2 x 20 mL), washed with $H_2O$ and KOH solution (10 %). The combined organic extracts were dried over $MgSO_4$ and concentrated to dryness. The crude residue was purified by flash chromatography, using DCM as eluent, to furnish **1b.**

Yield: 77%. White solid. $^1$H NMR ($CDCl_3$, 400 MHz) δ 7.41 (d, *J*= 9.0 Hz, 1H), 7.02 (d, *J*= 9.0 Hz, 1H), 6.02 (s, 1H), 2.92 (t, *J* = 2.4 Hz, 2H), 1.76 (h, *J*= 7.4 Hz, 2H), 1.00 (t, *J*= 7.4 Hz, 3H).

**1-(2,3-Dichloro-4-(prop-2-yn-1-yloxy)phenyl)butan-1-one (1c)**[5]

Phenol **1b** (1.5 g, 6.46 mmol, 1 equiv.), potassium tert-butoxide (1.45 g, 12.92 mmol, 2 equiv.), and a catalytic amount of KI (0.10 g, 0.64 mmol, 0.1 equiv.) were dissolved in THF. Then, an amount of ethyl bromoacetate (1.3 g, 7.75 mmol, 1.2 equiv.) was added slowly and the mixture was heated to 60 °C for 24 hours. The solution was poured into $H_2O$, acidified to pH = 1 with concentrated HCl, and extracted with EtOAc (2 x 20 mL). The organic layers were washed with 10 % KOH, $H_2O$, and brine then dried over $MgSO_4$. The crude product was purified by flash chromatography, using DCM/EtOAc, 4:1 (v/v) as eluent, to furnish 1c.

Yield: 80% from 3. White solid. $^1$H NMR ($CDCl_3$, 600 MHz) δ7.32 (d, *J* = 8.7 Hz, 1H), 6.75 (d, *J* = 8.6 Hz, 1H), 4.74 (s, 2H), 4.26 (q, *J* = 7.1 Hz, 2H), 2.87 (t, *J* = 7.3 Hz, 2H), 1.70 (h, *J* = 7.4 Hz, 2H), 1.28 (t, *J* = 7.2 Hz, 3H), 0.95 (t, *J* = 7.4 Hz, 3H).

**2-(4-butyryl-2,3-dichlorophenoxy)acetic acid (1d**)[6]

To a solution of ester **1c** (0.2 g, 0.63 mmol, 1 equiv.) in absolute ethanol (6 mL), KOH (0.07 g, 1.25 mmol, 2 equiv.) in 2 mL of $H_2O$, was added dropwise at 0 °C then left stirring at room temperature until completion of the reaction. The mixture was brought back to 0 °C, then HCl (2M) was added until pH = 1. The mixture was extracted with $Et_2O$ (2 x 20 mL), the combined organic layers were dried over $MgSO_4$, filtered and evaporated to dryness to give **8** that was used without any further purification in the next step.

Yield: 90%. White solid. $^1$H NMR ($CDCl_3$, 600 MHz) δ 7.35 (d, *J* = 8.7 Hz, 1H), 6.80 (d, *J* = 8.7 Hz, 1H), 4.81 (s, 2H), 2.90 (t, *J* = 7.4 Hz, 2H), 1.72 (h, *J* = 7.4 Hz, 2H), 0.97 (t, *J* = 7.4 Hz, 3H).

**N-((1*H*-indol-5-yl)methyl)-2-(4-butyryl-2,3-dichlorophenoxy)acetamide (9).**

In a 10 mL flask, a mixture of EDCI.HCl (0.054 g, 0.35 mmol, 1.1 equiv.), HOBt (0.048 g, 0.35 mmol, 1.1 equiv.), $Et_3N$ (0.066 g, 0.66 mmol, 2 equiv.) and **1d** (0.33 mmol, 1 equiv.) in DMF (5 mL) were sired at 0 °C for 1 hour. Then, an amount of 5-(aminomethyl) indole **(7**) (0.33 mmol, 1 equiv.) was added. The reaction mixture was stirred overnight at room temperature. This solution was extracted with EtOAc (2 x 20 mL), washed with a saturated solution of NaCl, dried over $MgSO_4$, filtered and then concentrated under pressure. The residue obtained was purified by column chromatography using DCM/EtOAc, 4:1 (v/v) as eluent, to give **9** in 20 %.

Yield: 20 %**.** White solid. m.p. 176.-177 °C. $^1$H NMR (THF-d8, 600 MHz,) δ 10.13 (s, 1H), 7.54 (br. s, 1H), 7.45 (dd, *J* = 8.4, 5.9 Hz, 2H), 7.30 (s, 1H), 7.17 (t, *J* = 2.8 Hz, 1H), 7.09 (d, *J* = 8.7 Hz, 1H), 6.94 (d, *J* = 8.1 Hz, 1H), 6.37 (d, *J* = 2.7 Hz, 1H), 4.66 (s, 2H), 4.52 (d, *J* = 5.9 Hz, 2H), 2.85 (t, J = 7.5 Hz, 2H), 1.67 (q, *J* = 7.5 Hz, 2H), 0.95 (t, *J* = 7.5 Hz, 3H).$^{13}$C NMR (THF-d8, 151 MHz,) δ 204.48, 200.81, 166.92, 157.22, 137.55, 135.41, 133.01, 128.60, 128.49, 125.56, 120.96, 120.05, 112.69, 111.26, 102.19, 69.69, 45.14, 44.14, 30.41, 18.51, 14.02. IR (neat): v = 3318 (NH), 1662 (C=O) cm$^{-1}$. HRMS (+ESI) m/z: [M+H]+ calculated for $C_{21}H_{21}Cl_2N_3O_3$: 419,0929, found: 419,0910.HPLC purity: 99% (MOS-1 HYERSIL 250x4.6 mmI.D column, 50% MeCN/$H_2O$, 254 nm, 23 °C, $t_R$ = 17.0 min).

## Figures

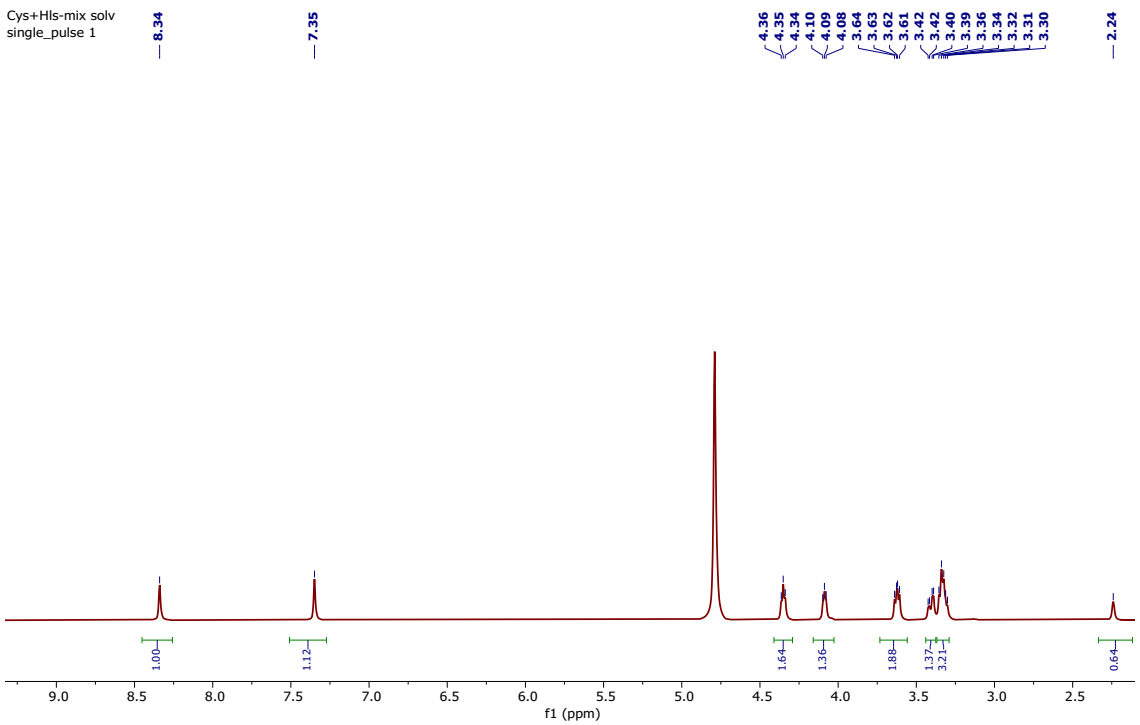


***Figure s1.****$^1$H NMR spectrum of cysteine and histidine mixture in $D_2O$.*

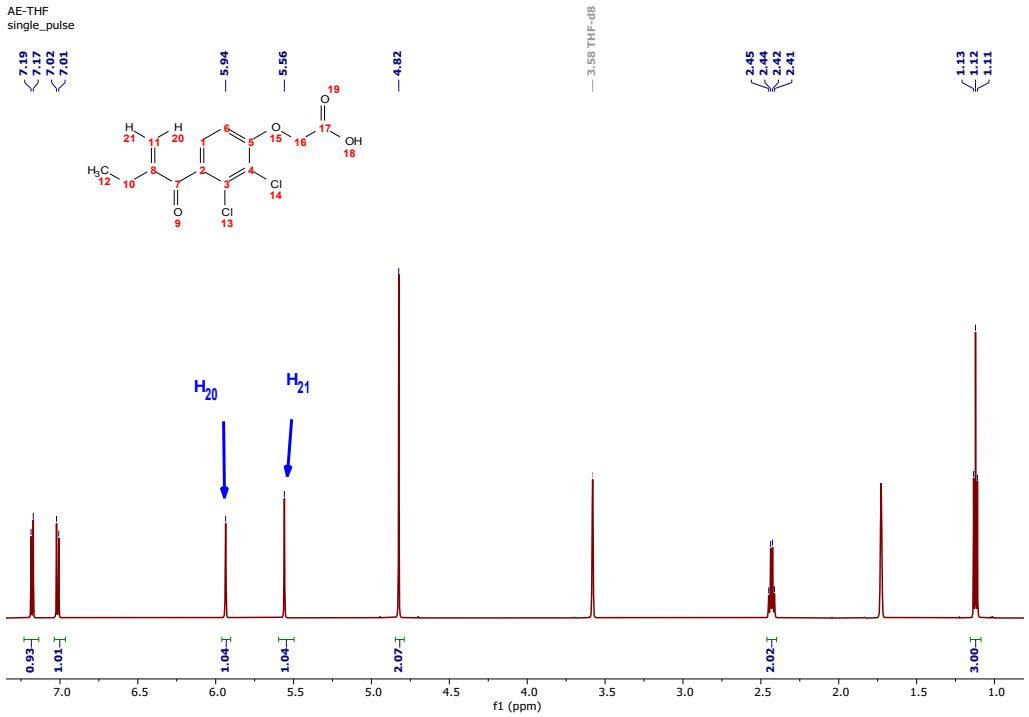


***Figure s2.*** *$^1$H NMR spectrum of **EA** in THF-$d_6$.*

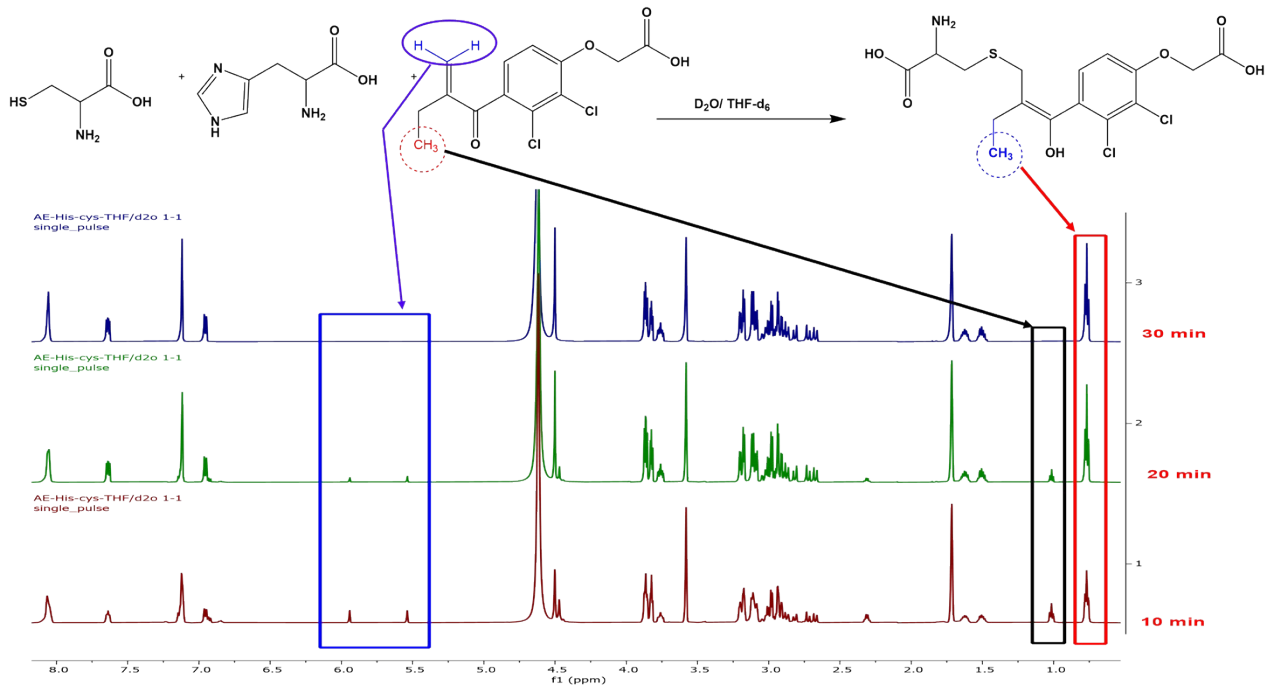


***Figure S3****. Evolution of the reaction of **EA** with cysteine in presence of histidine in THF-$d_6$/$D_2O$ in time*

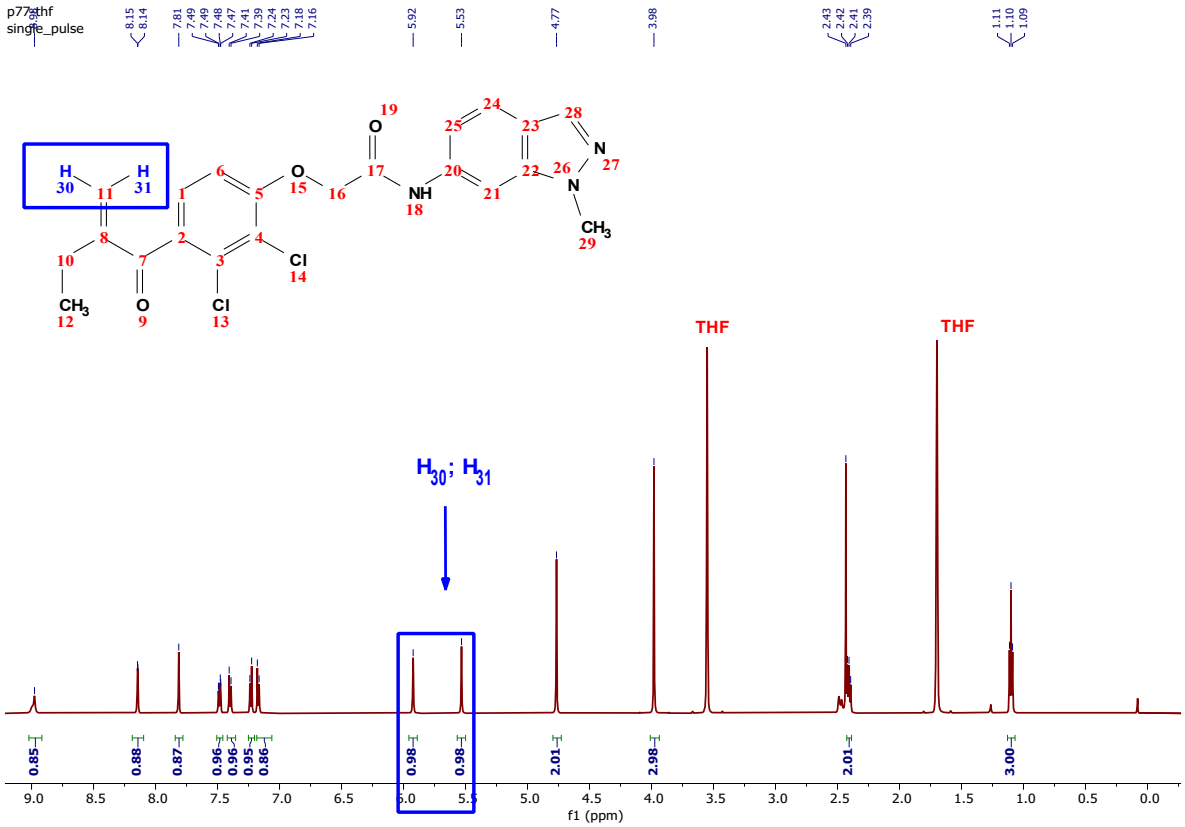


***Figure s4.*** *$^1$H NMR spectrum of **4** in THF-$d_6$.*

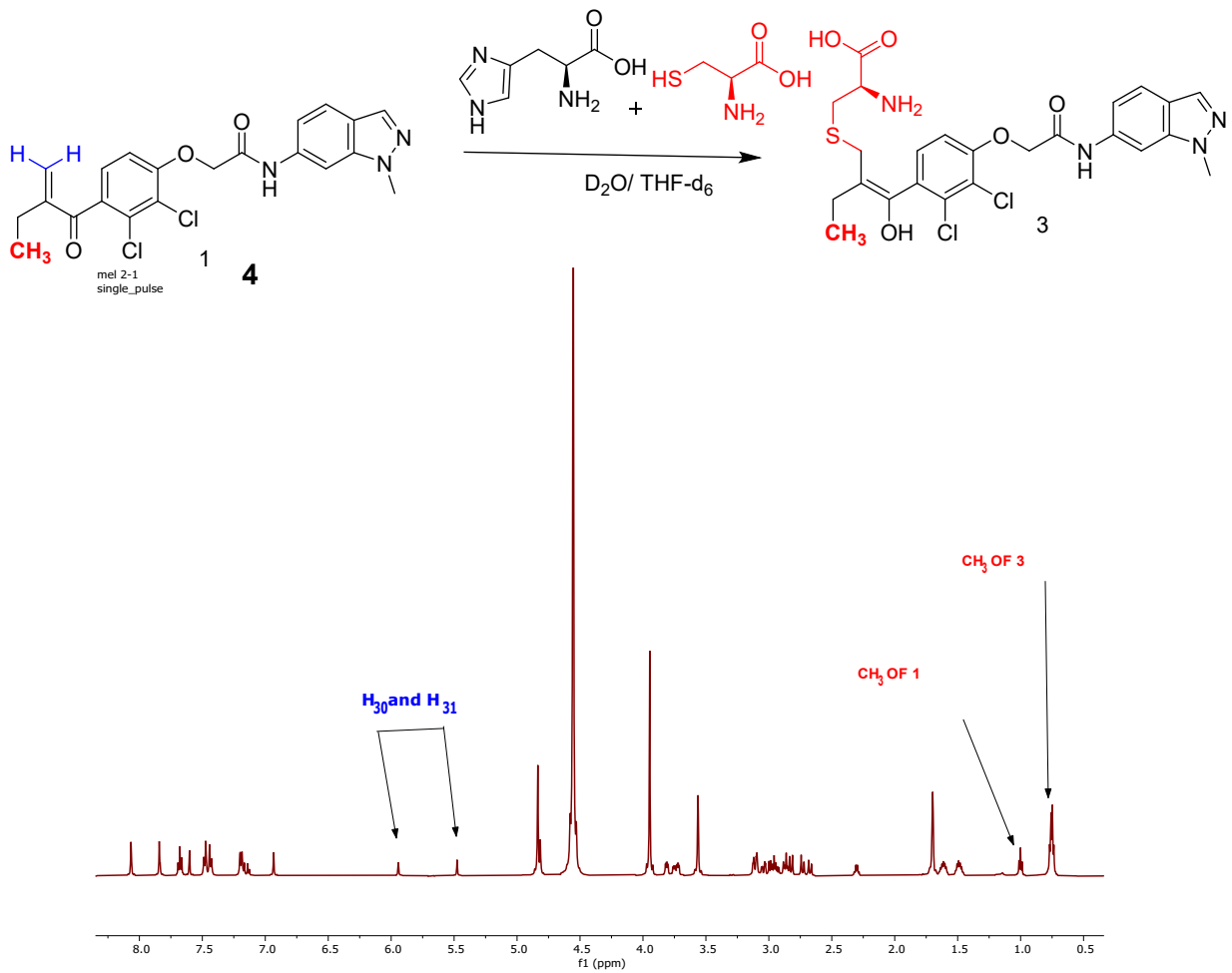


***Figure S5.*** *Reaction of 2 equiv. of* ***4*** *with cysteine in presence of histidine in THF-$d_6$/$D_2O$.*

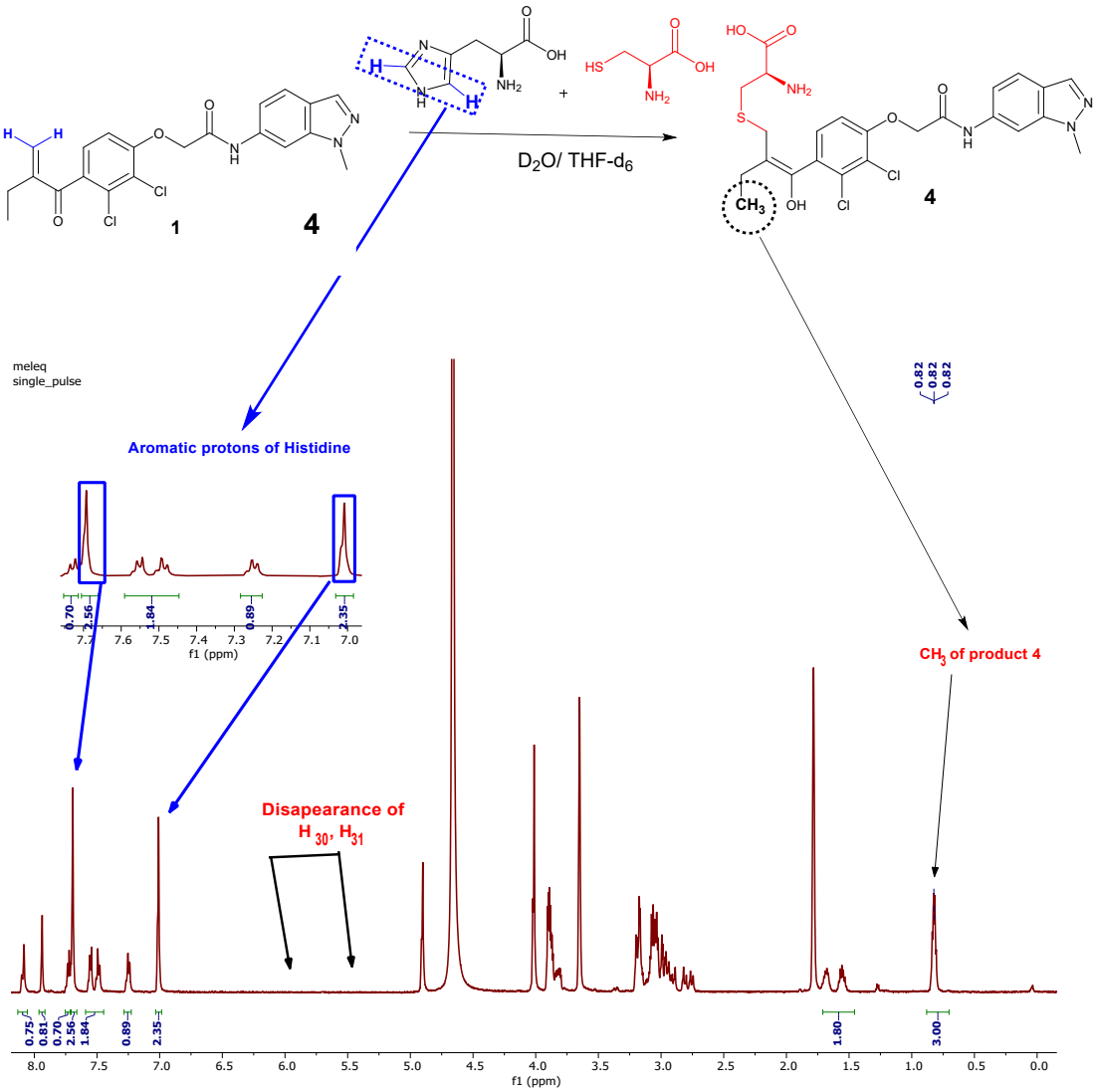


***Figure S6***. *Instantaneous reaction of **4** with cysteine in presence of histidine in THF-$d_6$/$D_2O$ in stochiometric conditions.*

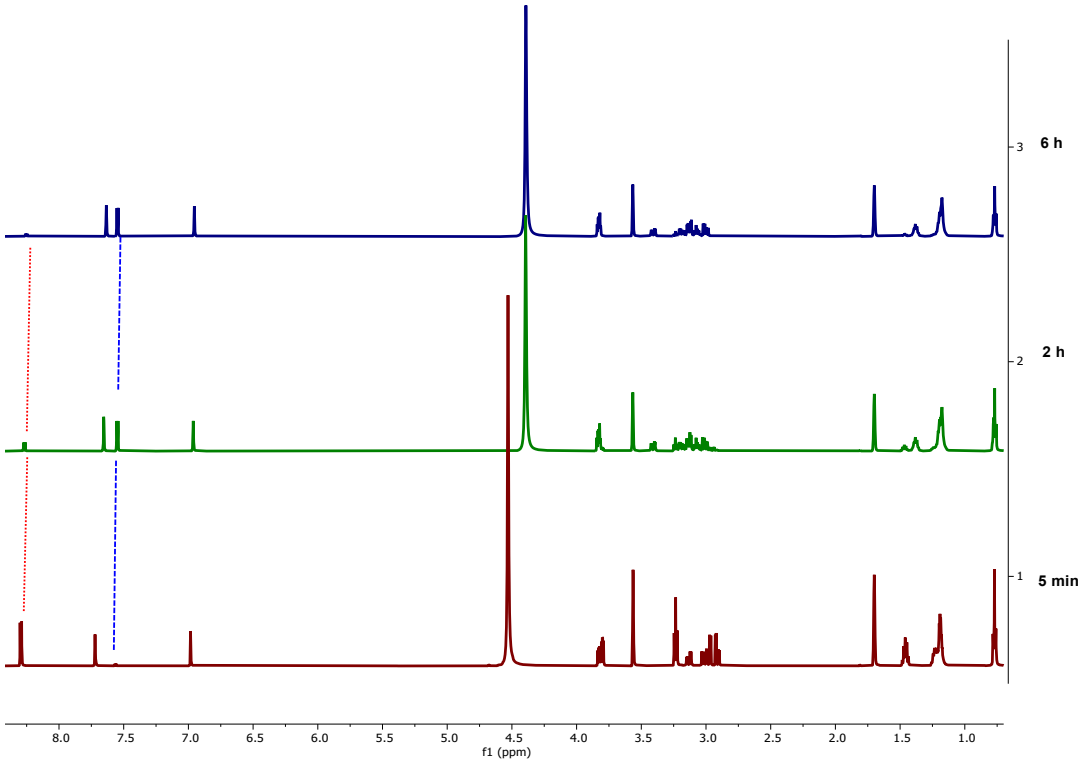


***Figure S7**. Evolution of the reaction of Carmofur with cysteine in presence of histidine in THF-$d_6$/$D_2O$.*

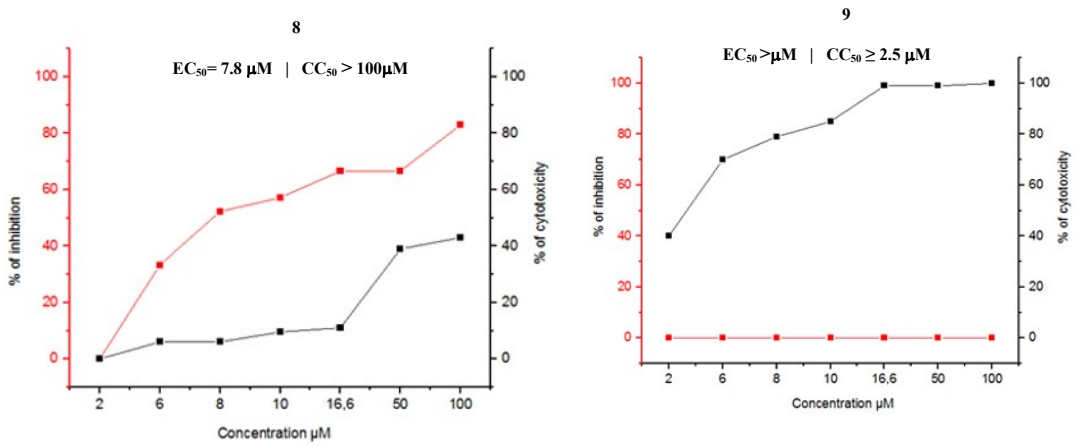


***Figure S8**. $EC_{50}$ and $CC_{50}$ of compounds **8** and **9**. The red line represents the inhibition capacity and the black line represents cytotoxicity.*

A

| mode | affinity (kcal/mol) | dist from best mode rmsd l.b. | rmsd u.b. |
|---|---|---|---|
| 1 | -6.0 | 0.000 | 0.000 |
| 2 | -5.8 | 1.208 | 1.879 |
| 3 | -5.8 | 0.743 | 1.010 |
| 4 | -5.6 | 1.355 | 1.845 |
| 5 | -5.5 | 1.714 | 6.404 |
| 6 | -5.5 | 1.761 | 6.507 |
| 7 | -5.4 | 2.750 | 3.826 |
| 8 | -5.4 | 2.482 | 6.746 |
| 9 | -5.4 | 2.351 | 6.765 |

B

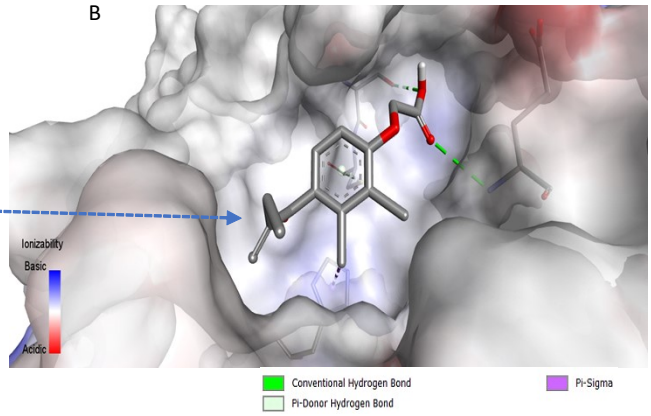


***Figure S9**: Etacrynic Acid docking results against M$^{pro}$ (A); Etacrynic compound inside M$^{pro}$ pocket (B).*

## Spectral data of compounds:

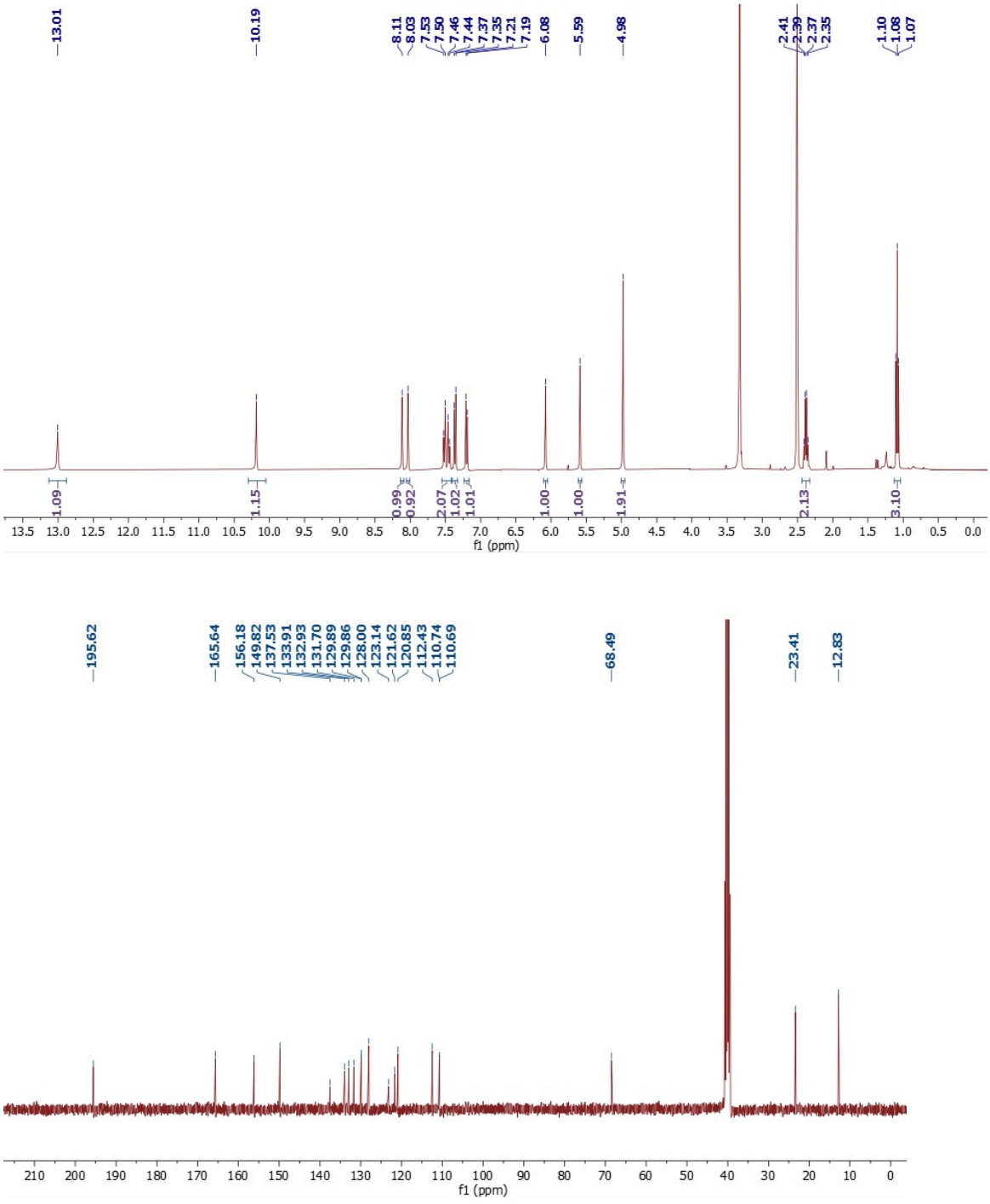


**Figure S10: $^{1}$H (600 MHz) and $^{13}$C (151 MHz) NMR spectra of 4.**

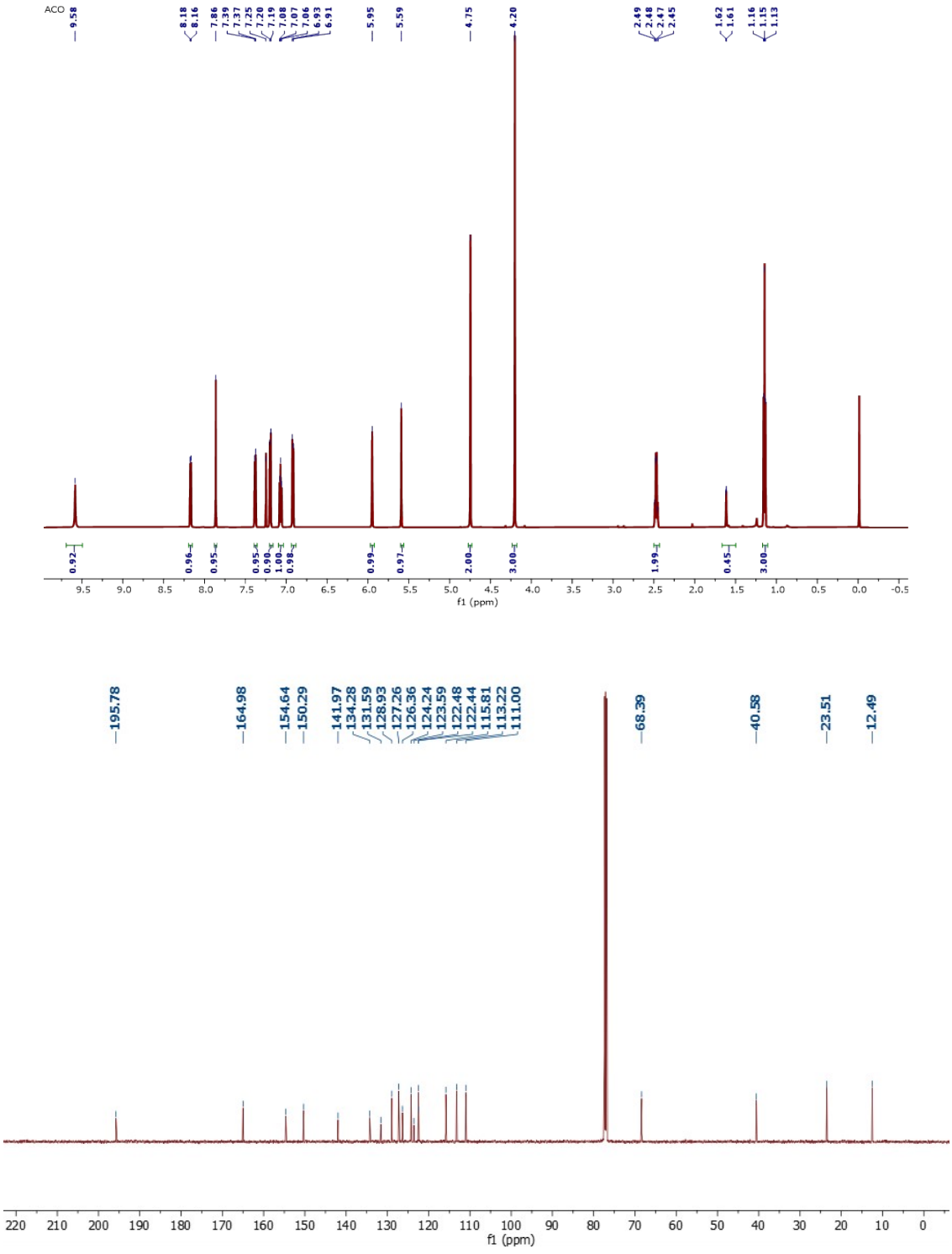


**Figure S11: $^{1}H$ (600 MHz) and $^{13}C$ (151 MHz) NMR spectra of 6.**

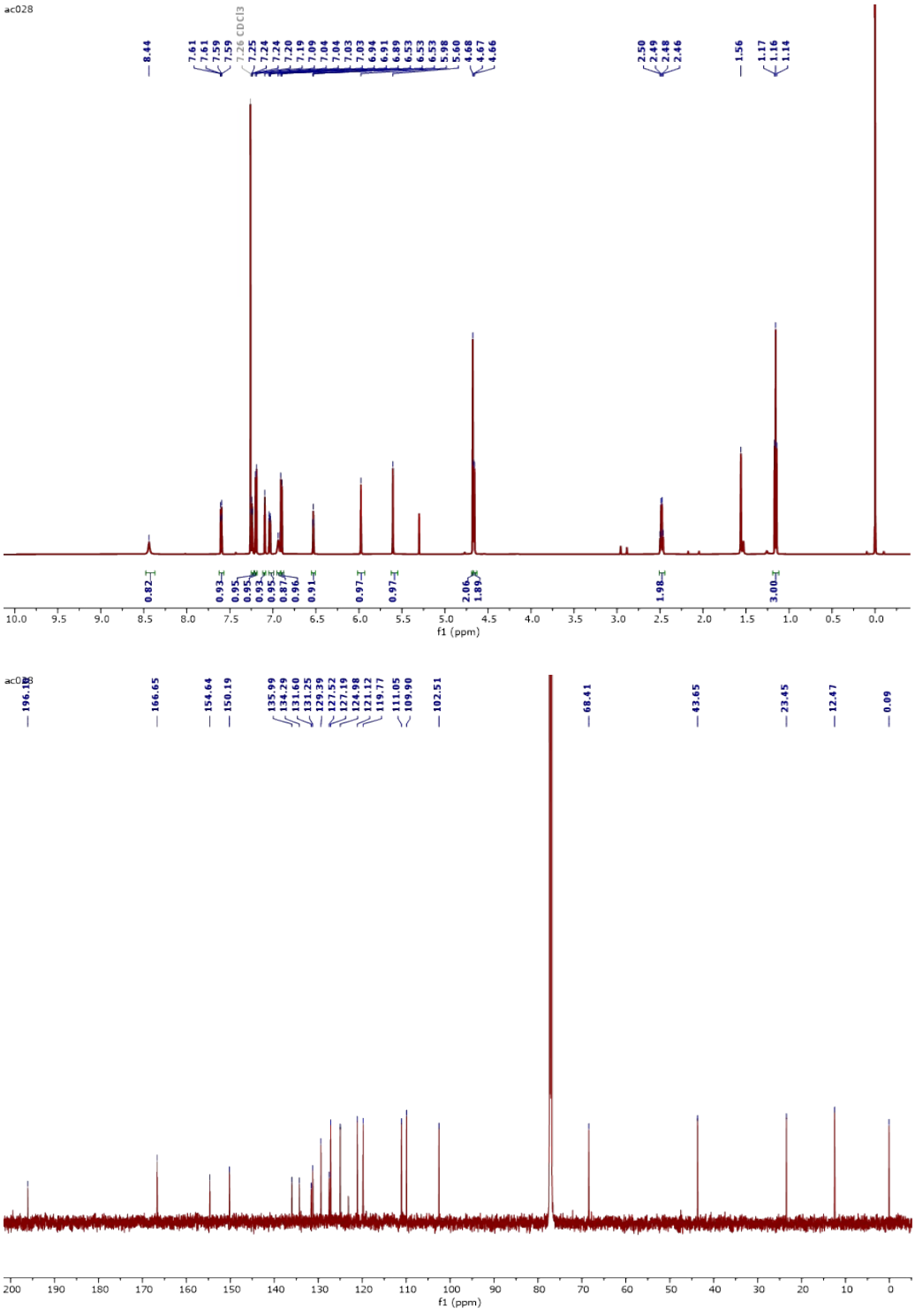


**Figure S12: $^{1}H$ (600 MHz) and $^{13}C$ (151 MHz) NMR spectra of 4d.**

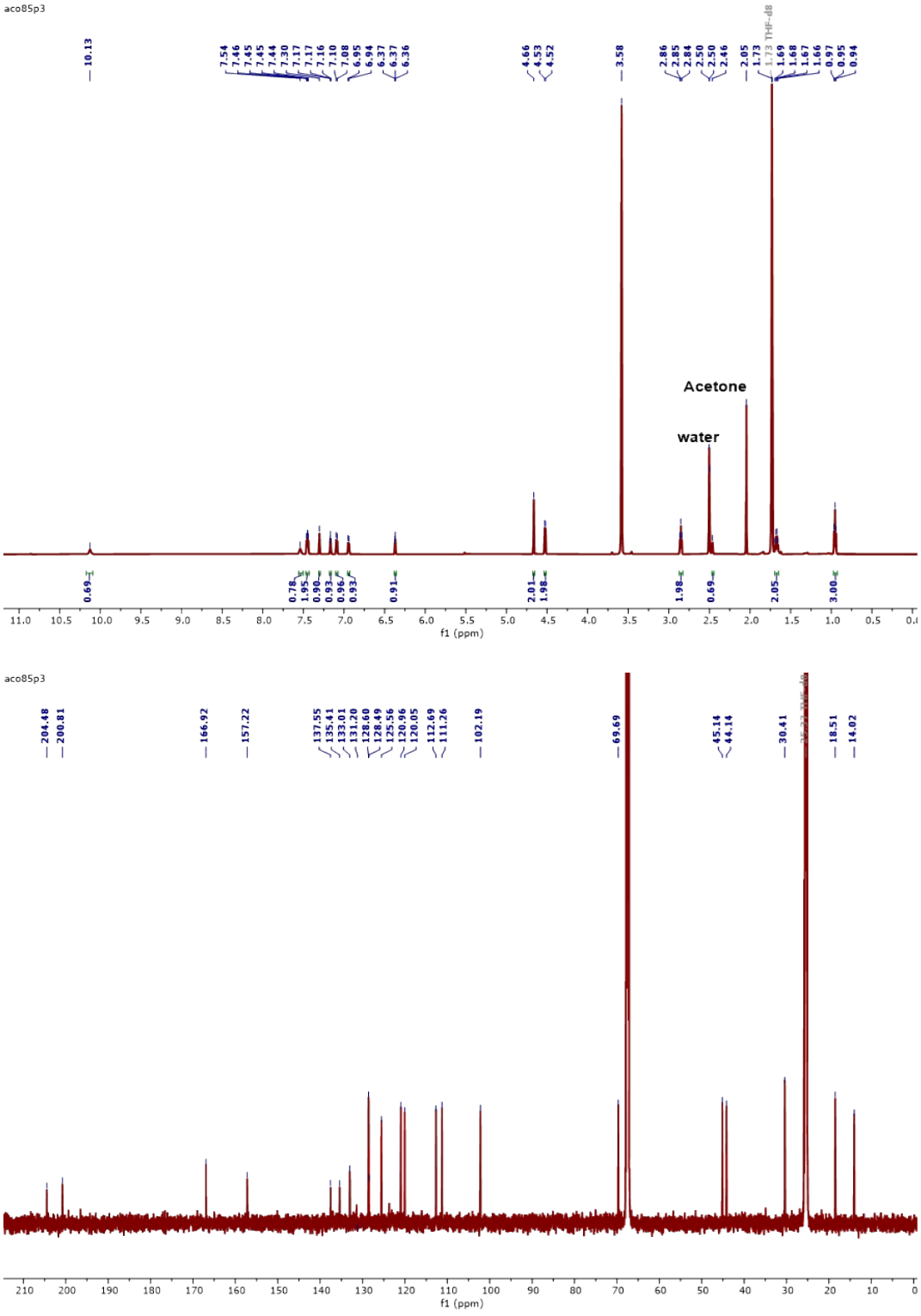


**Figure S13: $^{1}$H (600 MHz) and $^{13}$C (151 MHz) NMR spectra of 9.**

## References


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