Design and synthesis new dihydropyrimidine derivatives with cytotoxic effect as dual EGFR/VEGFR-2 inhibitors

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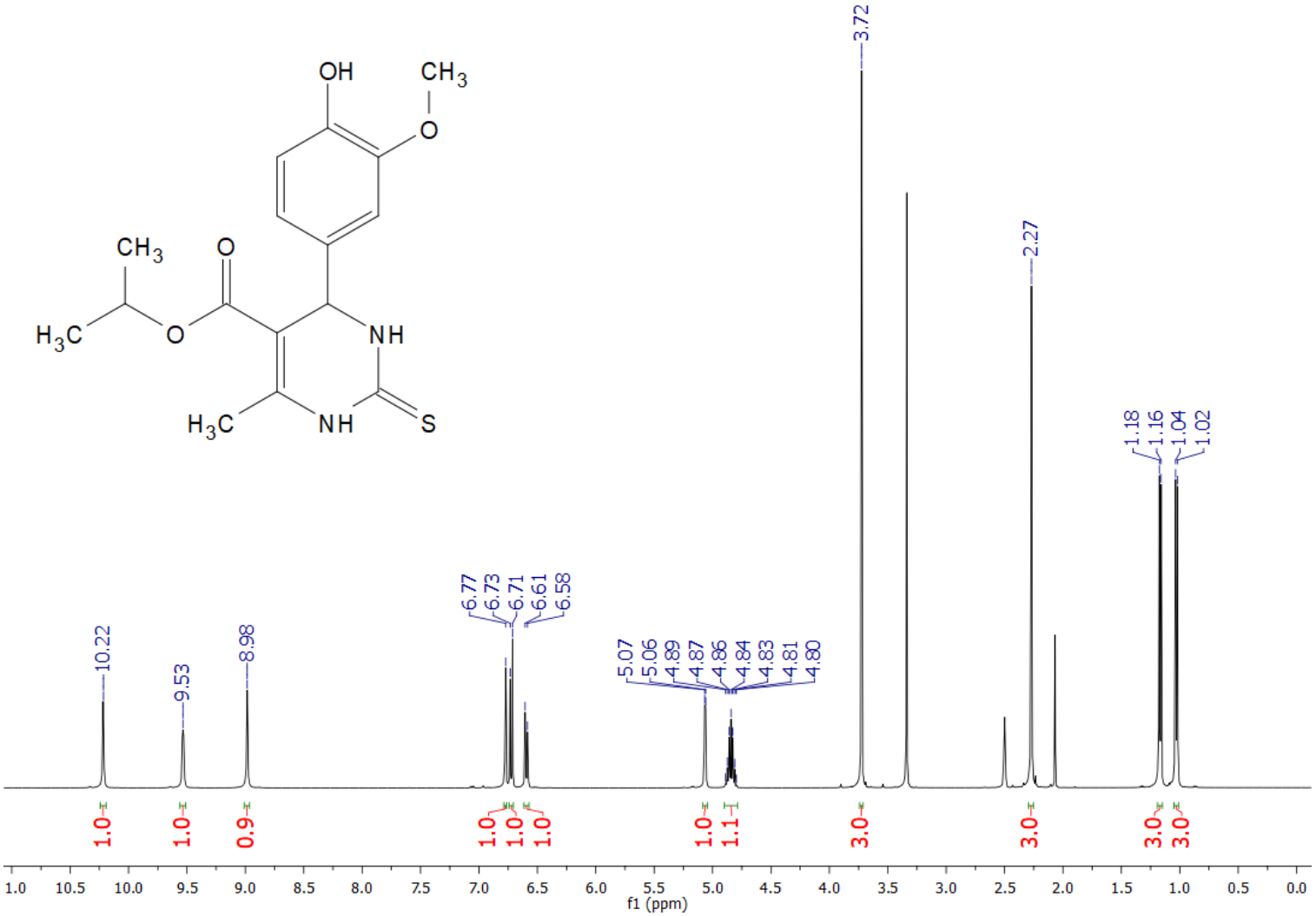


Figure S1: ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of **4**

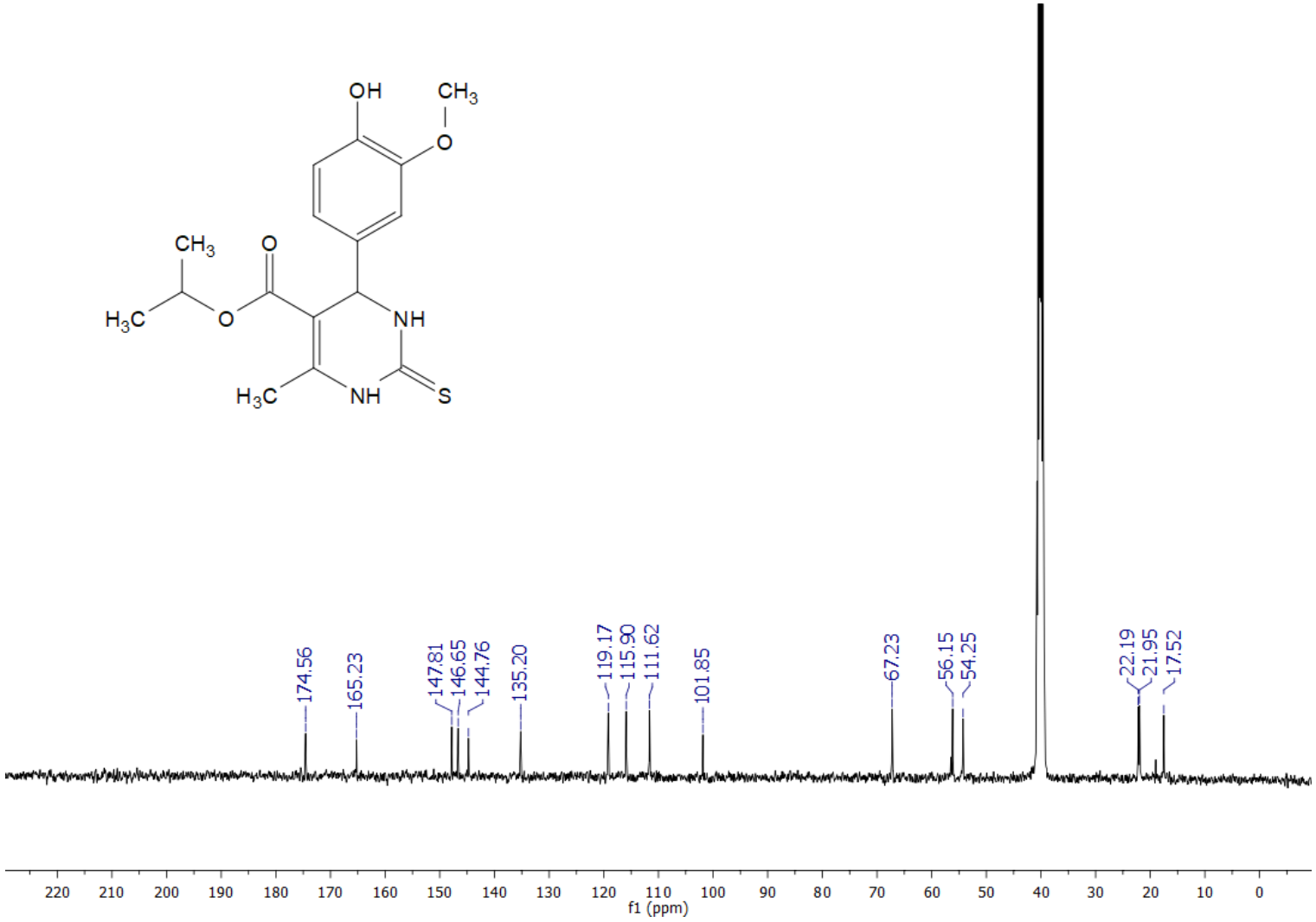
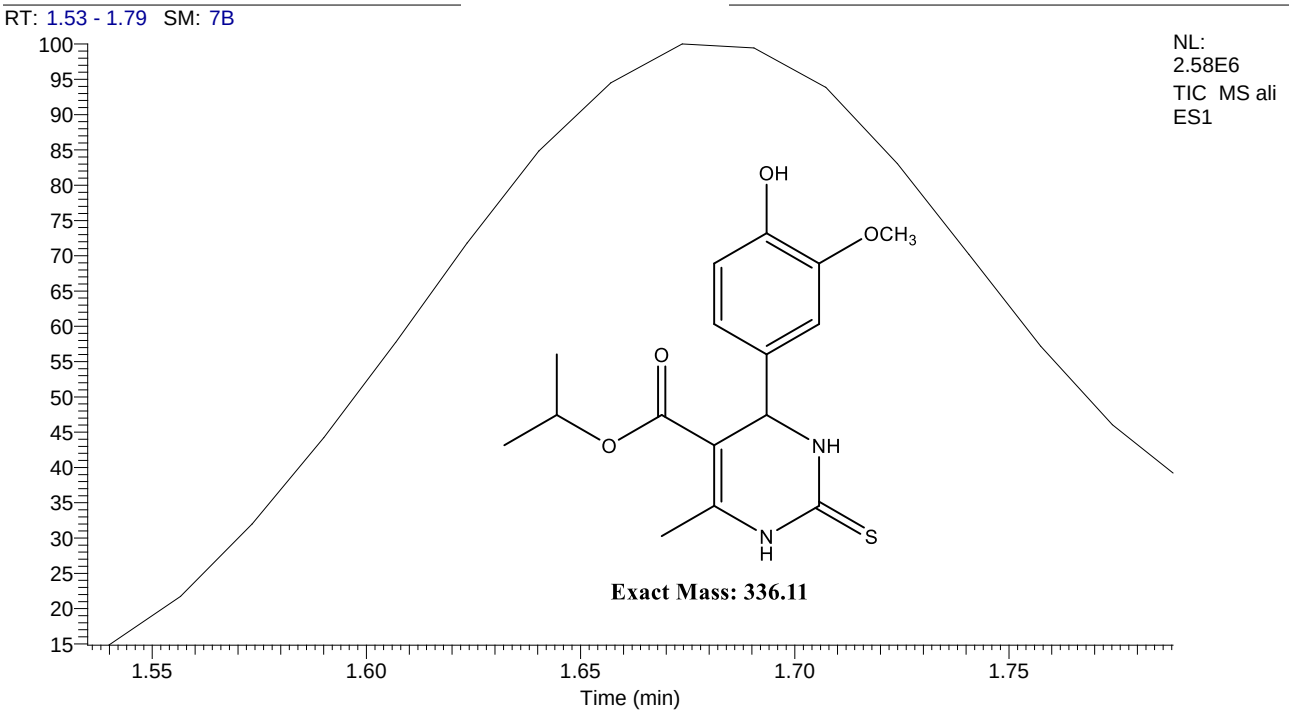


Figure S2: ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of **4**



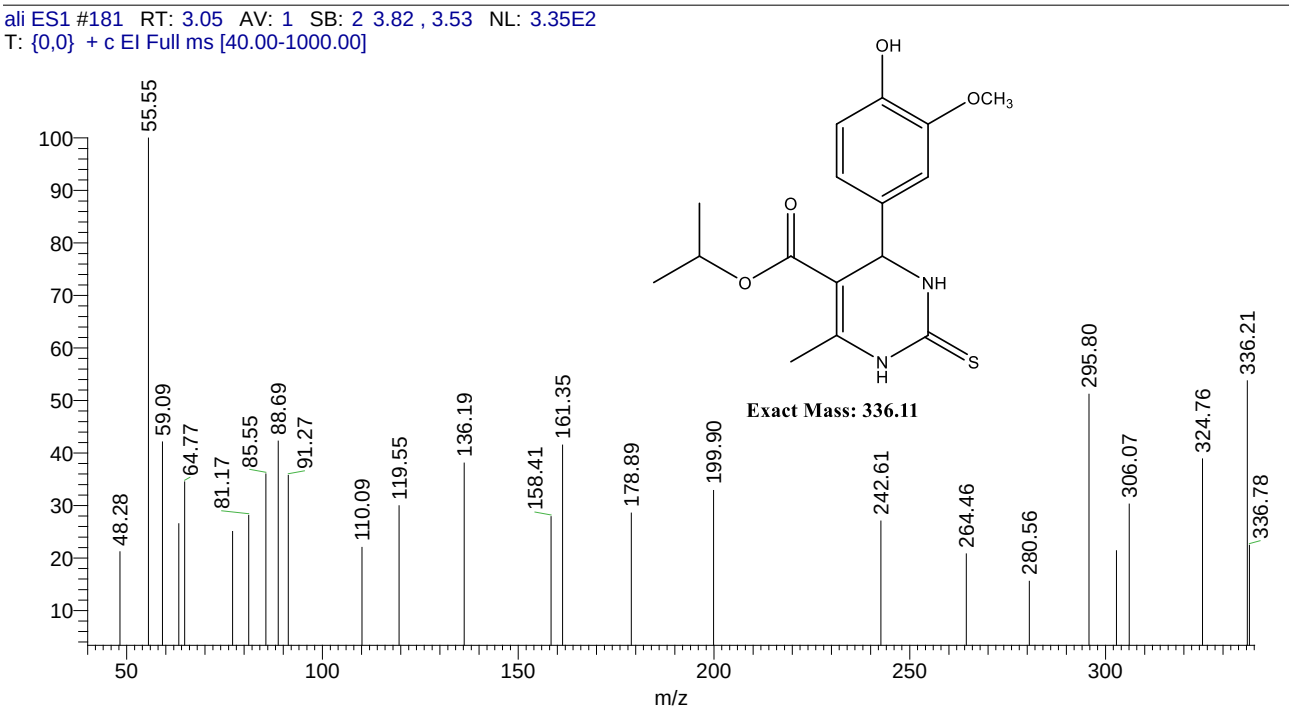


Figure S3: Mass spectrum of **4**

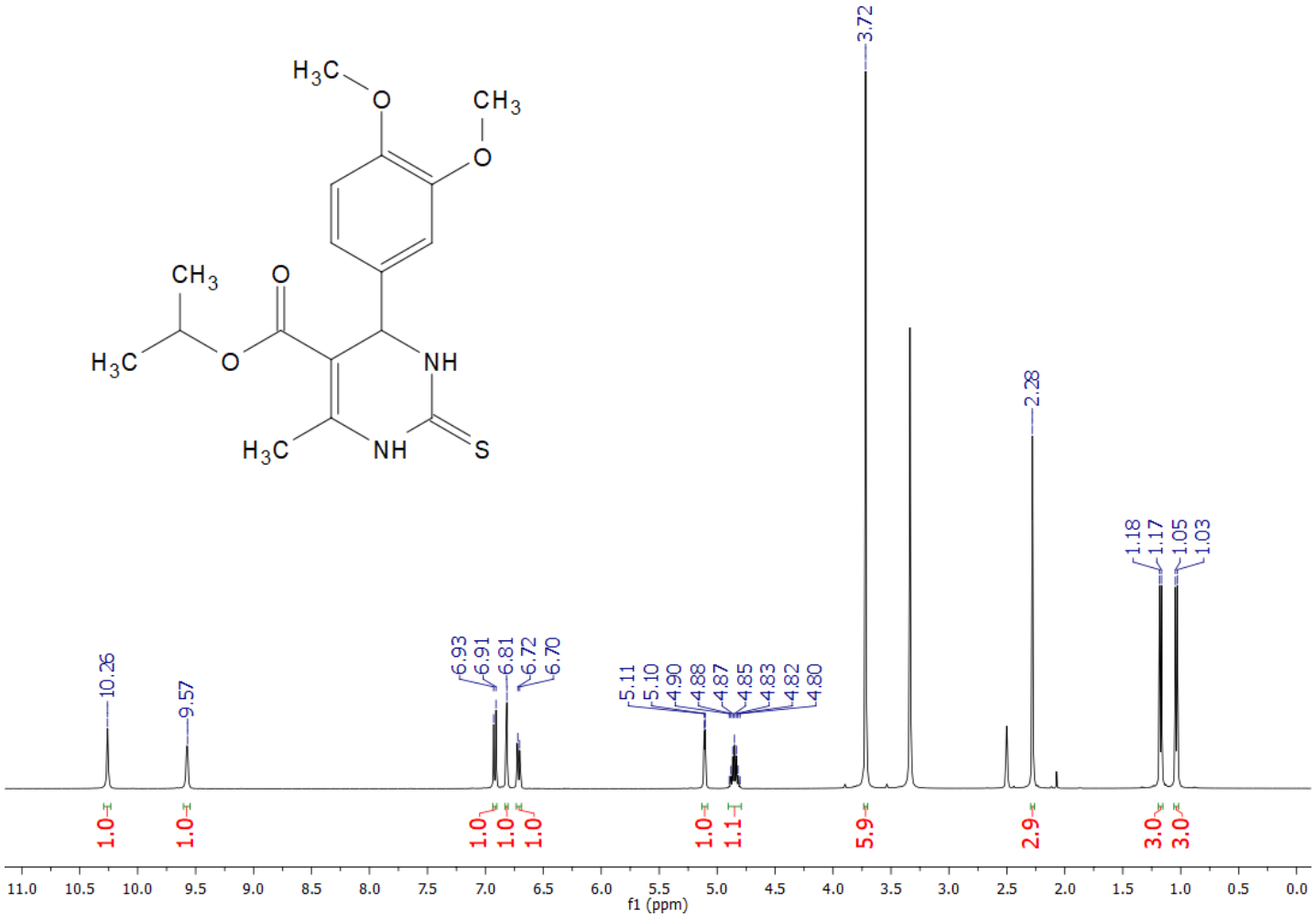


Figure S4: ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of **5**

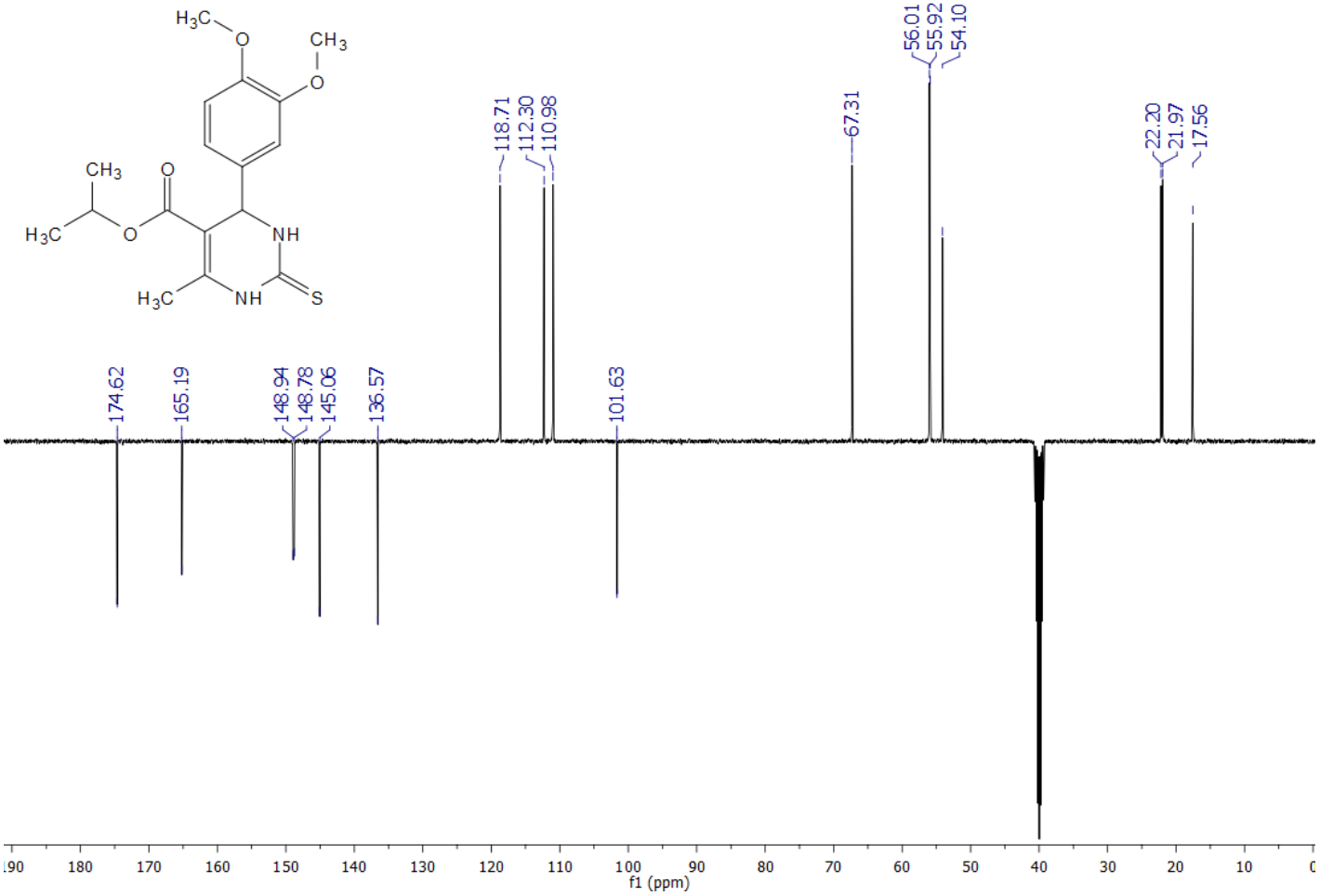
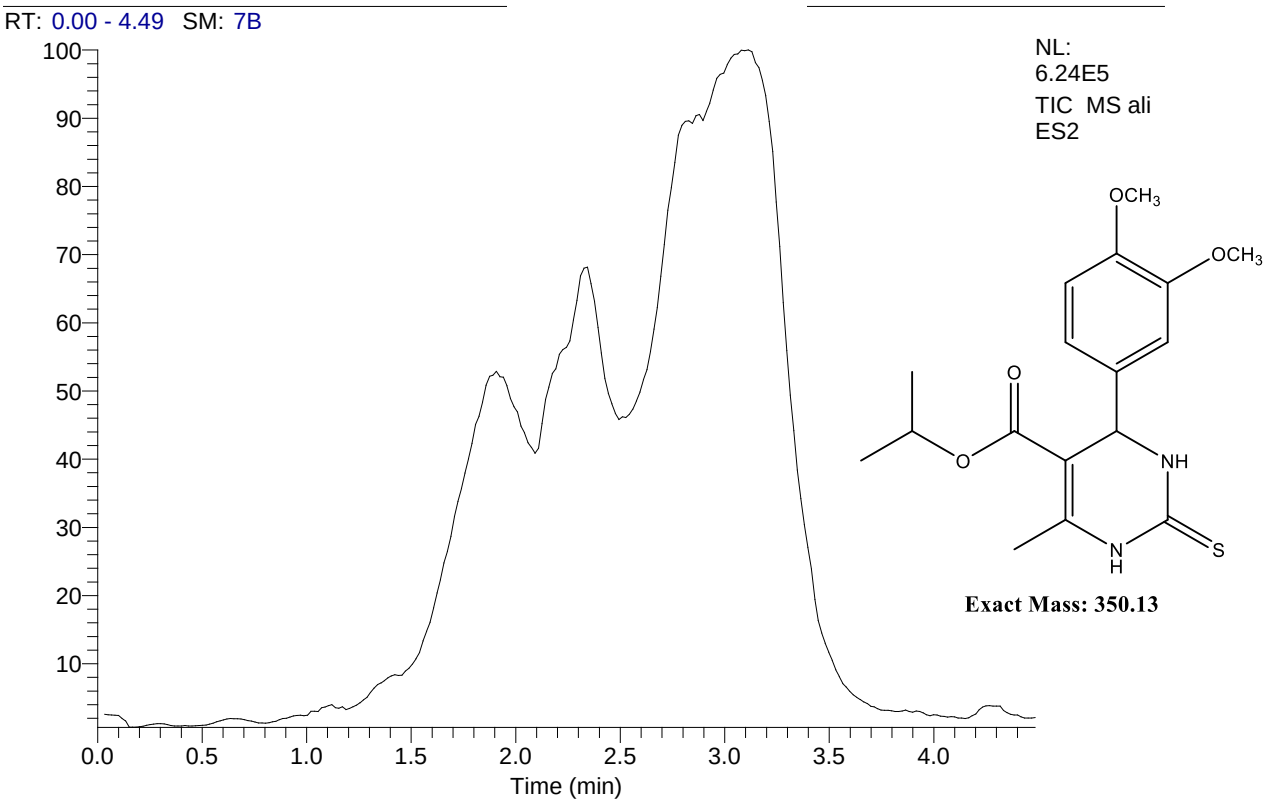


Figure S5: ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of **5**



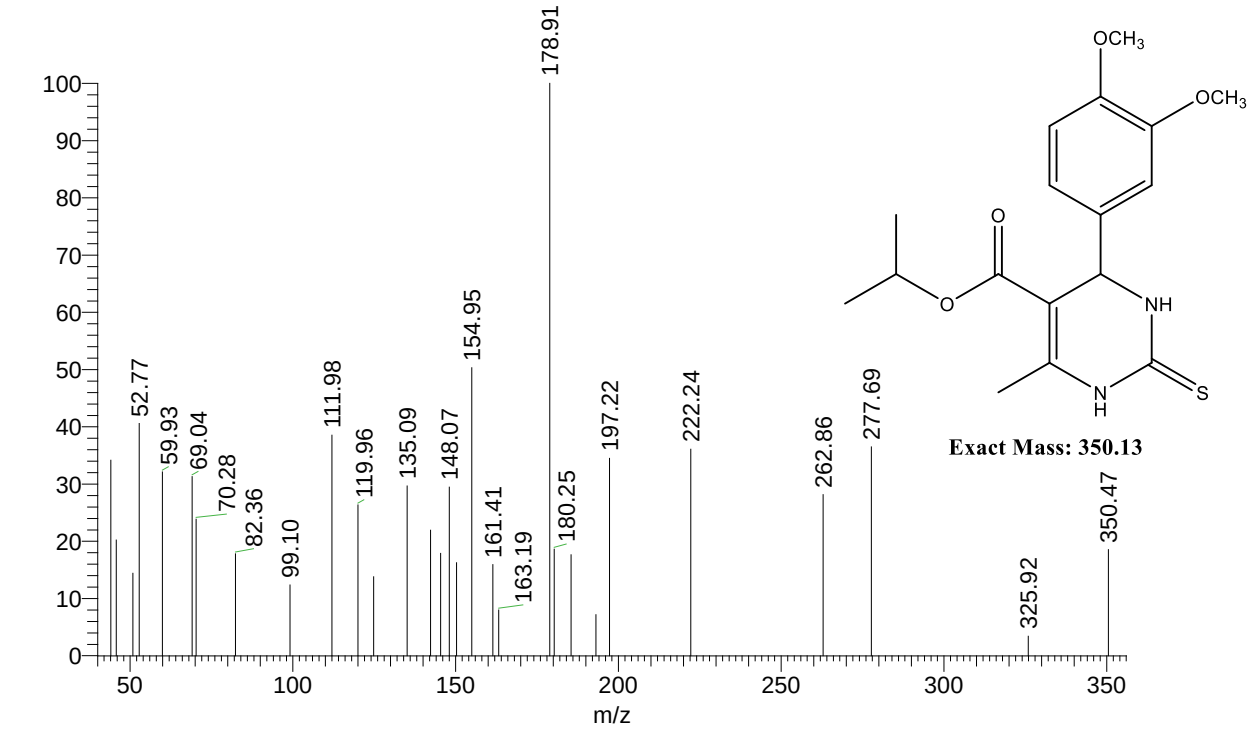


Figure S6: Mass spectrum of **5**

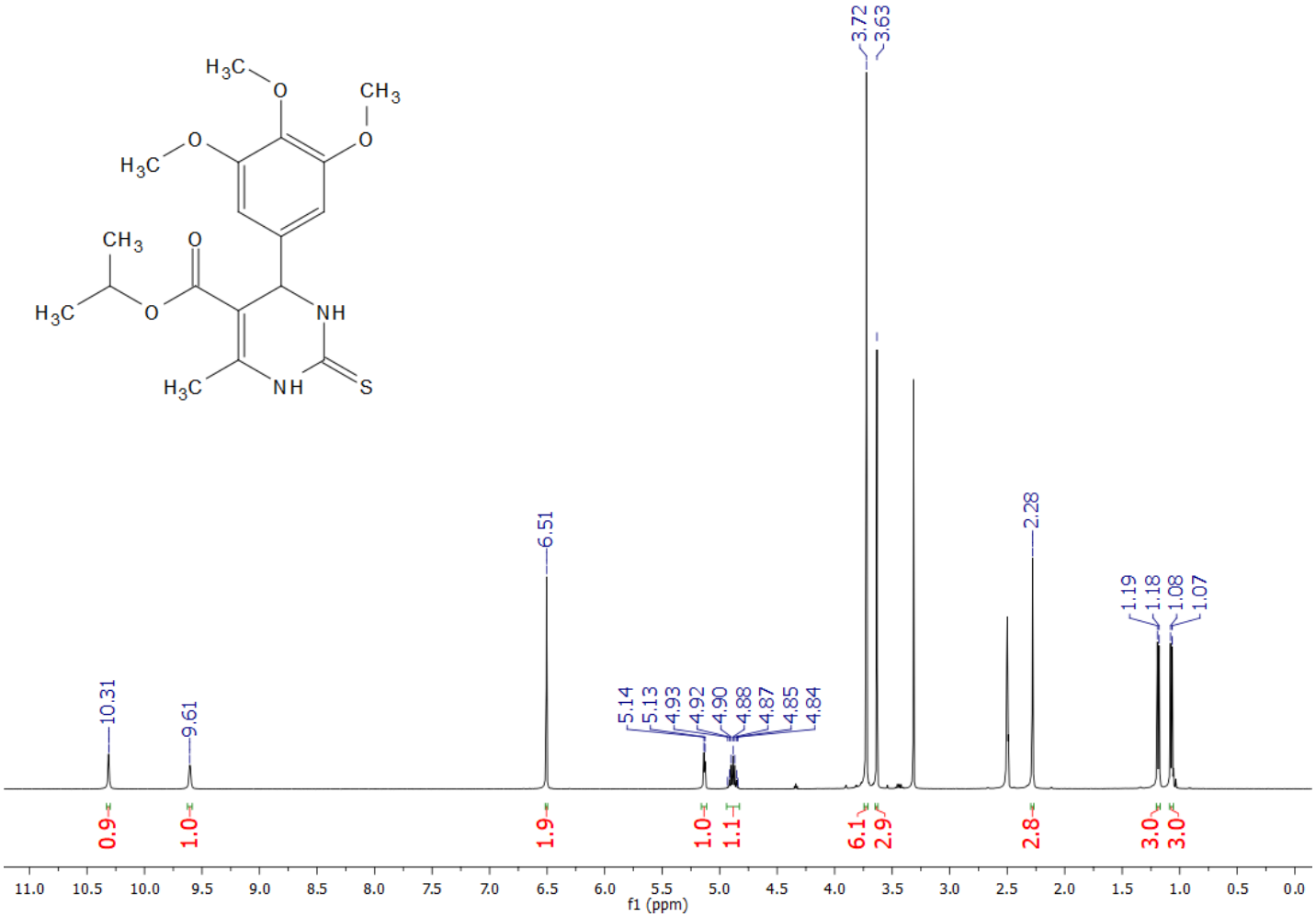


Figure S7: ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of **6**

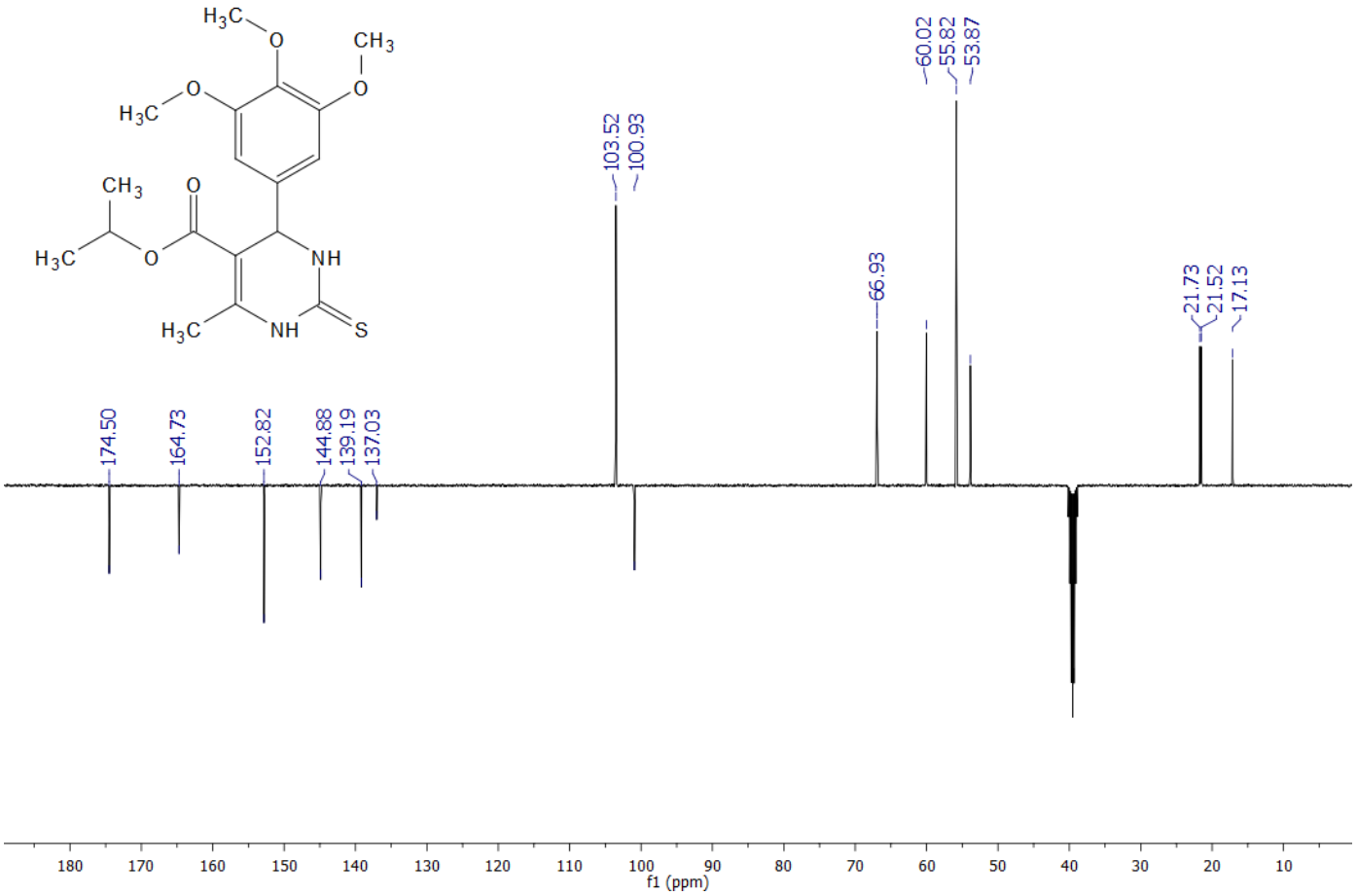
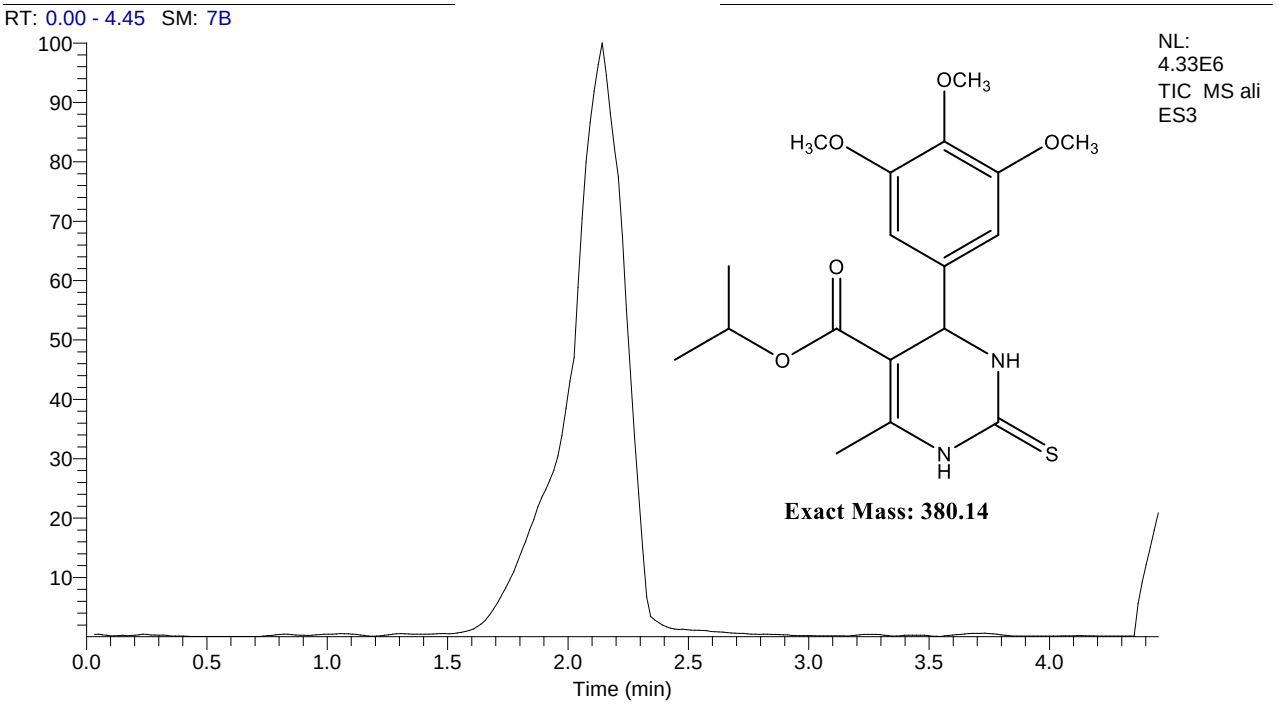


Figure S8: ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of **6**



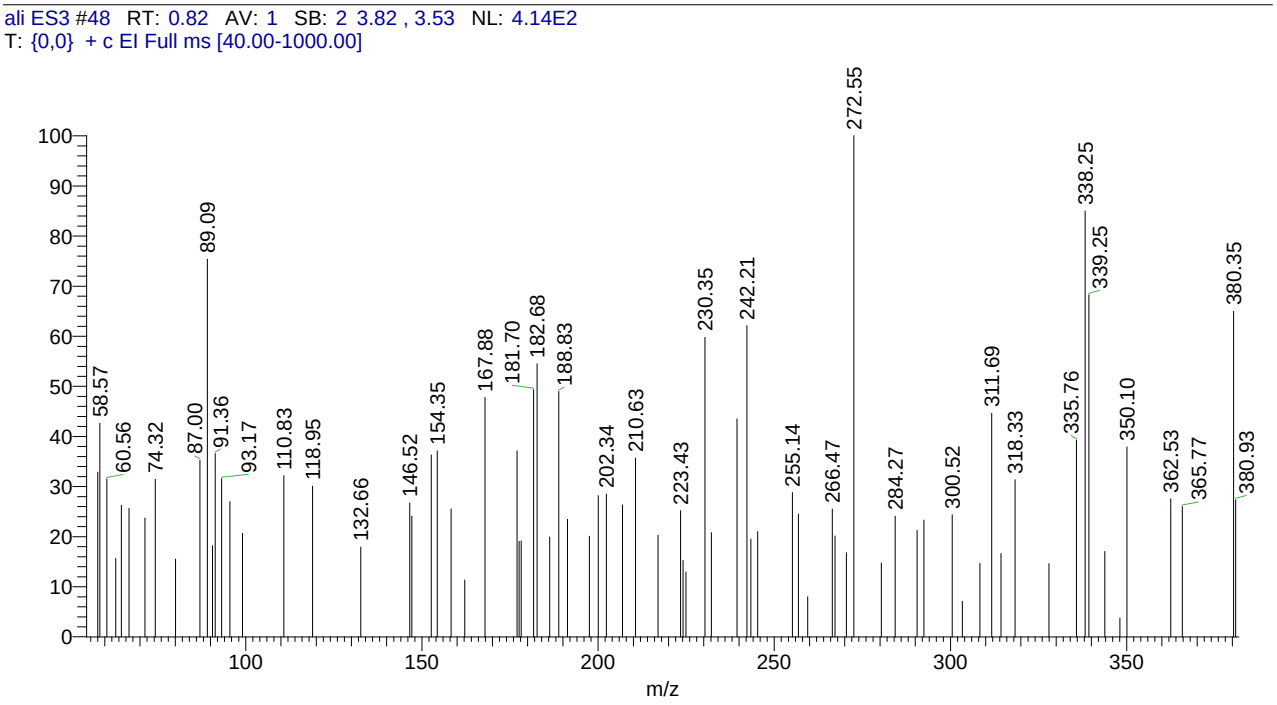


Figure S9: Mass spectrum of **6**

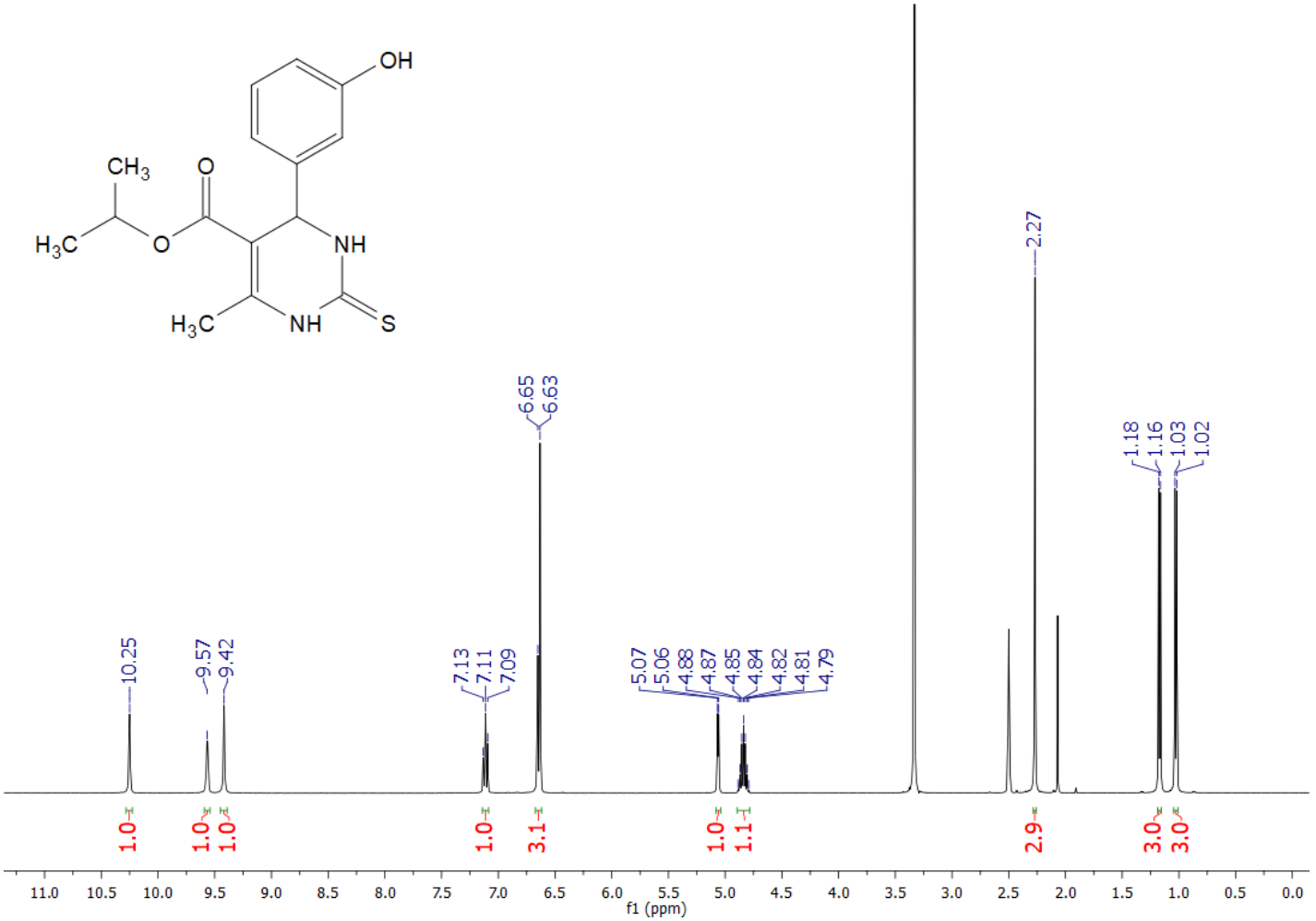


Figure S10: ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of **7**

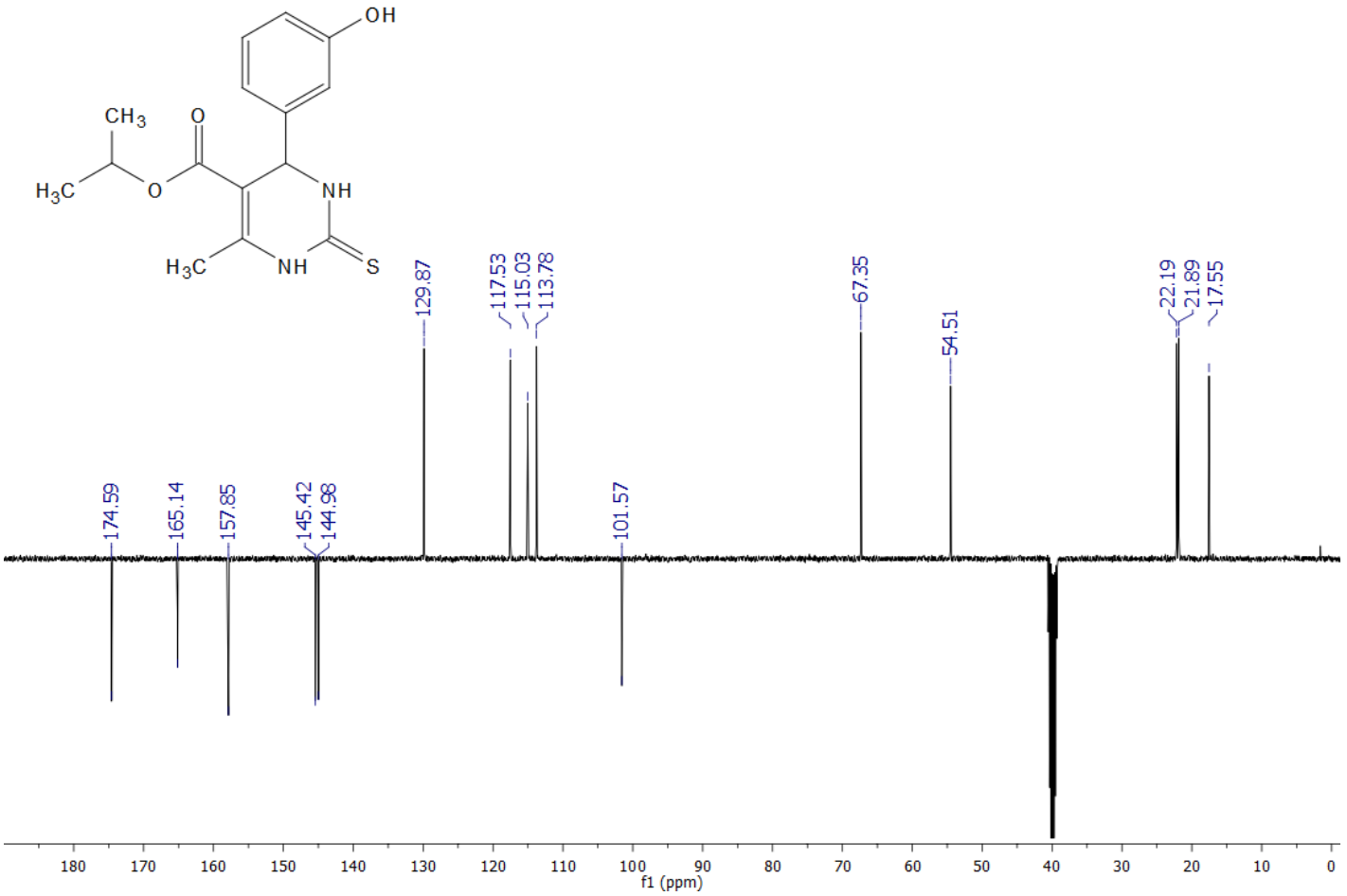
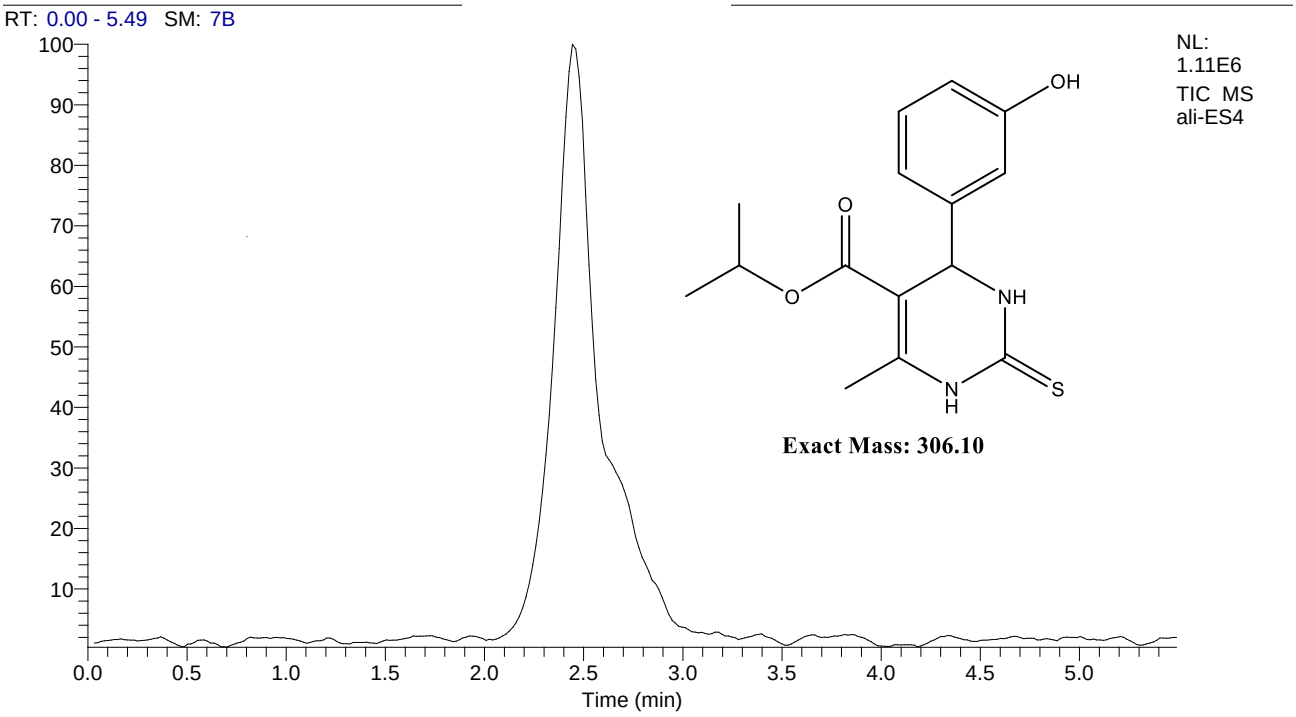


Figure S11: ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of **7**



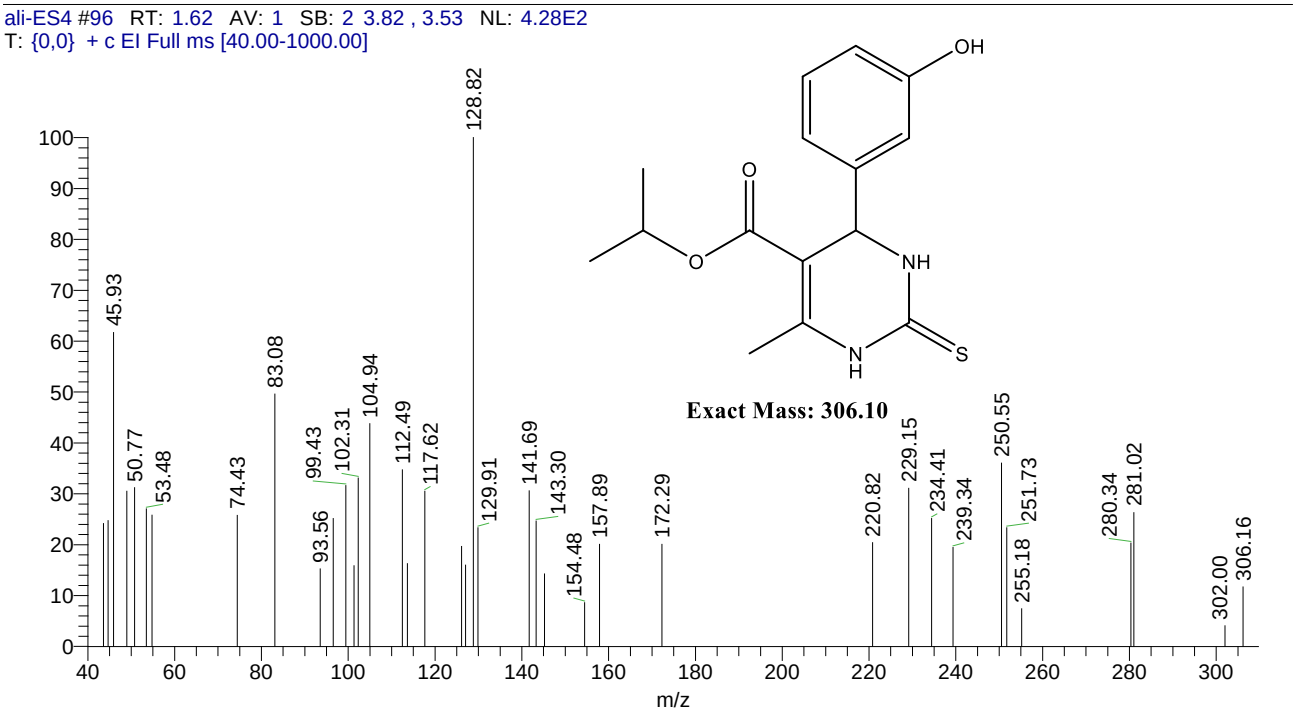


Figure S12: Mass spectrum of **7**

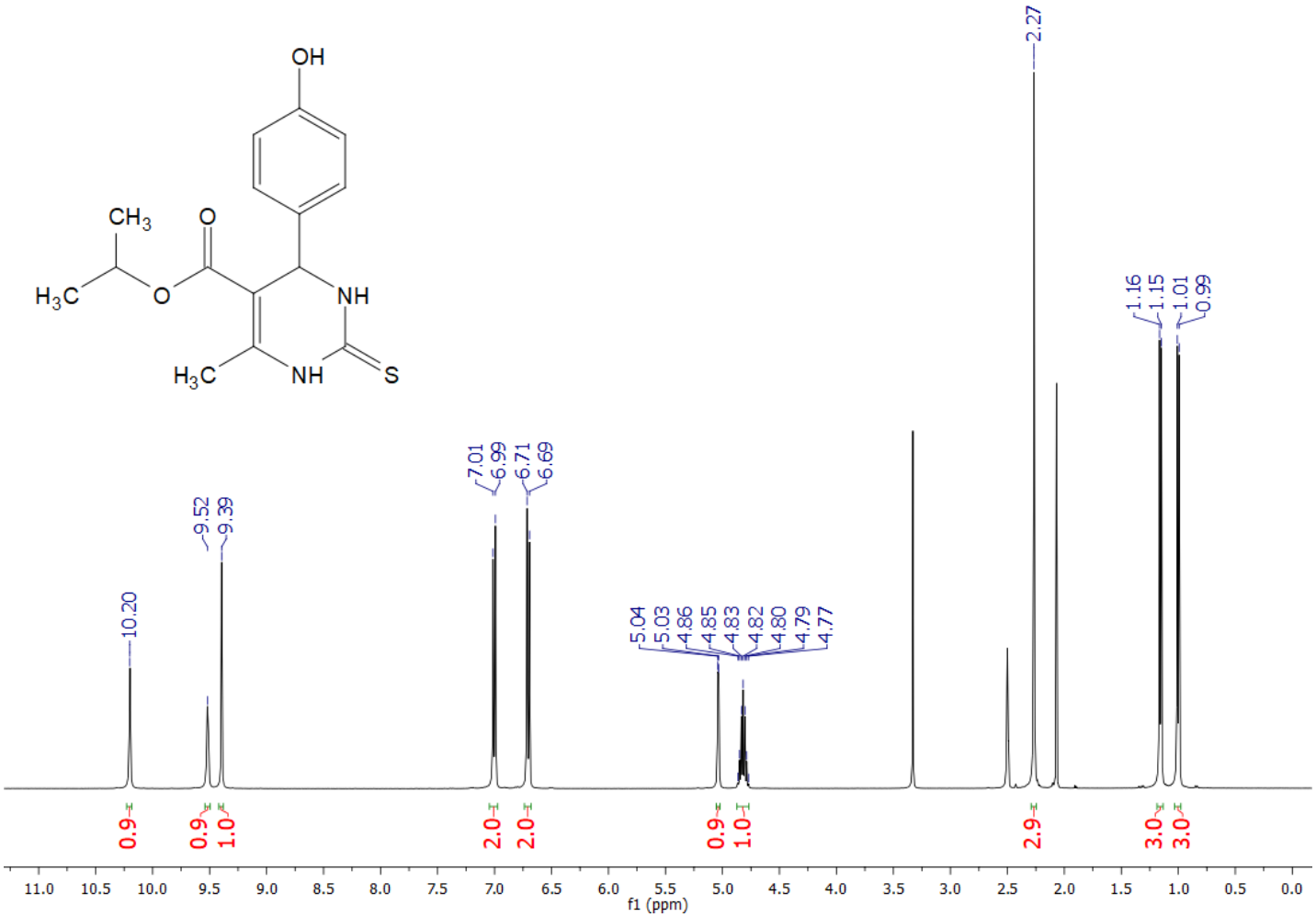


Figure S13: ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of **8**

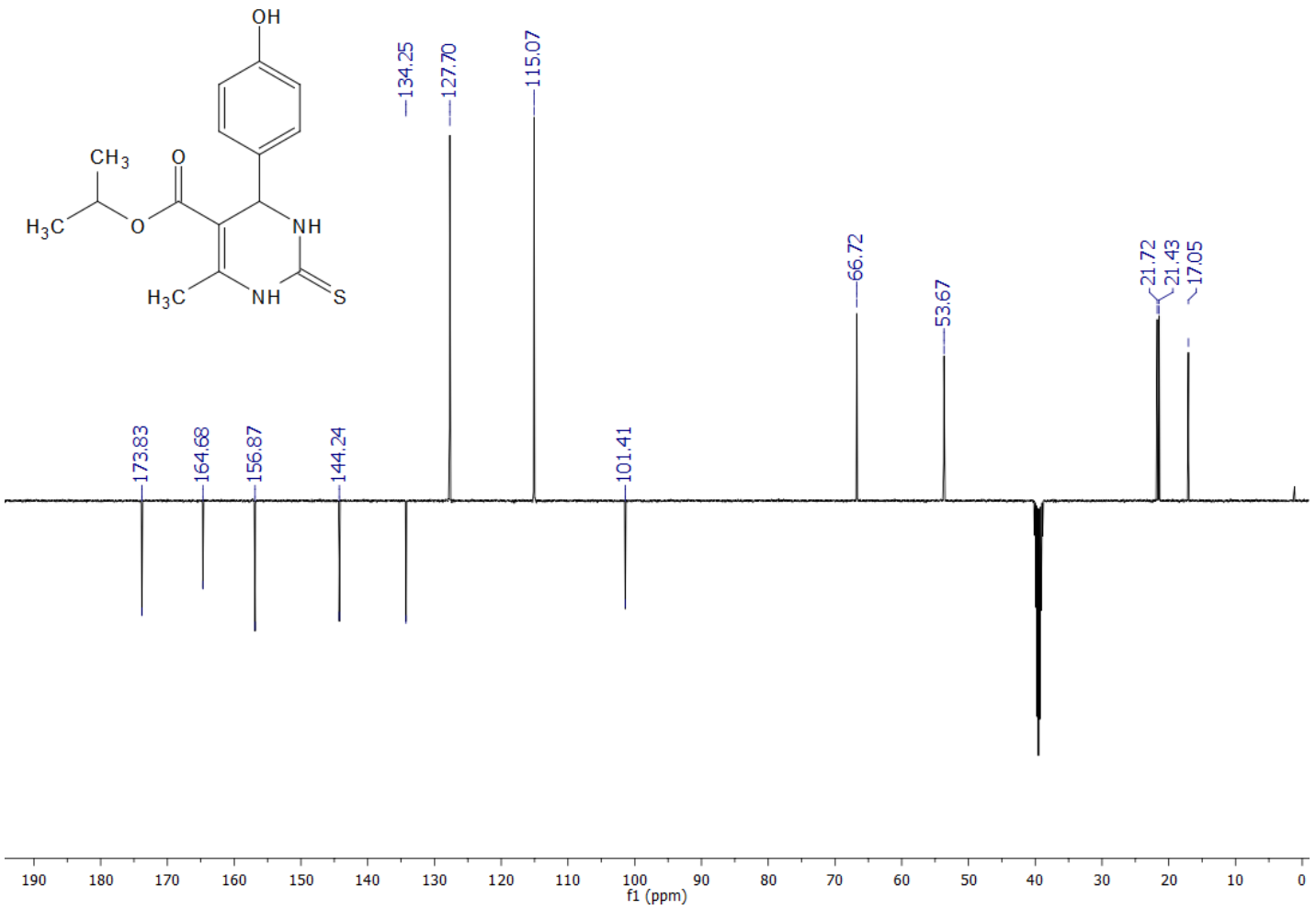
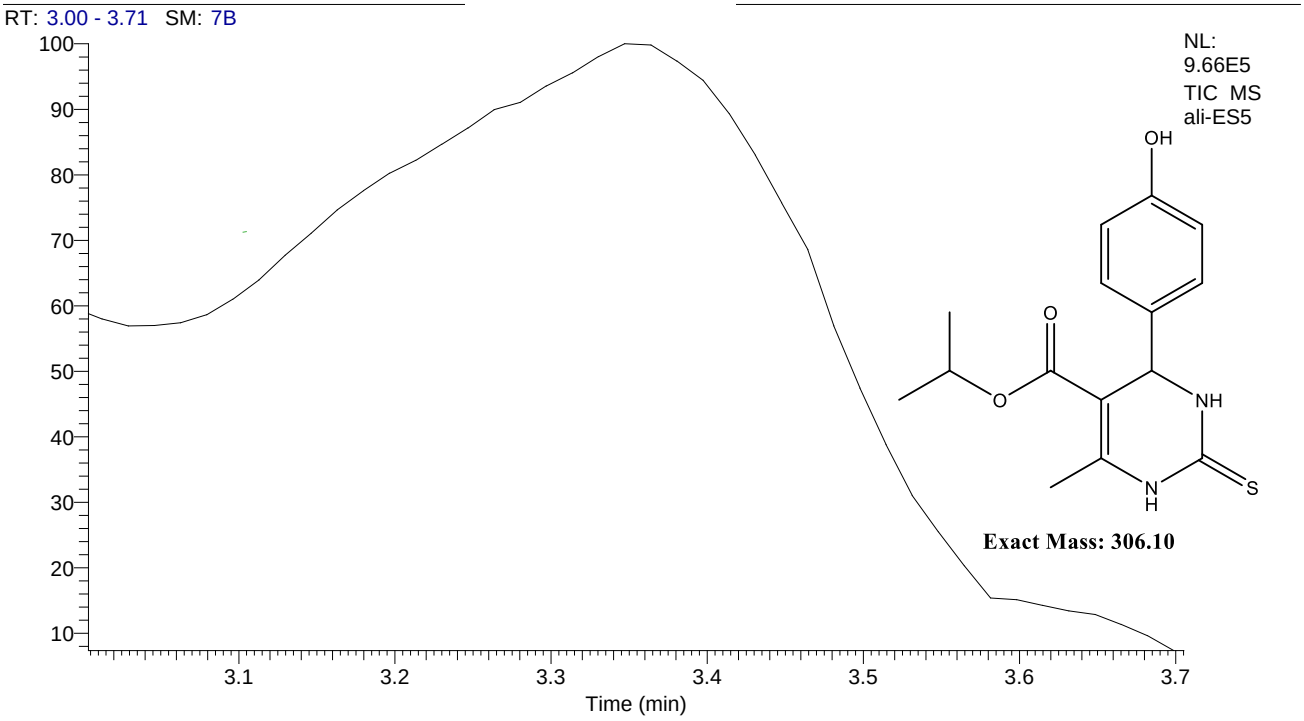


Figure S14: ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of **8**



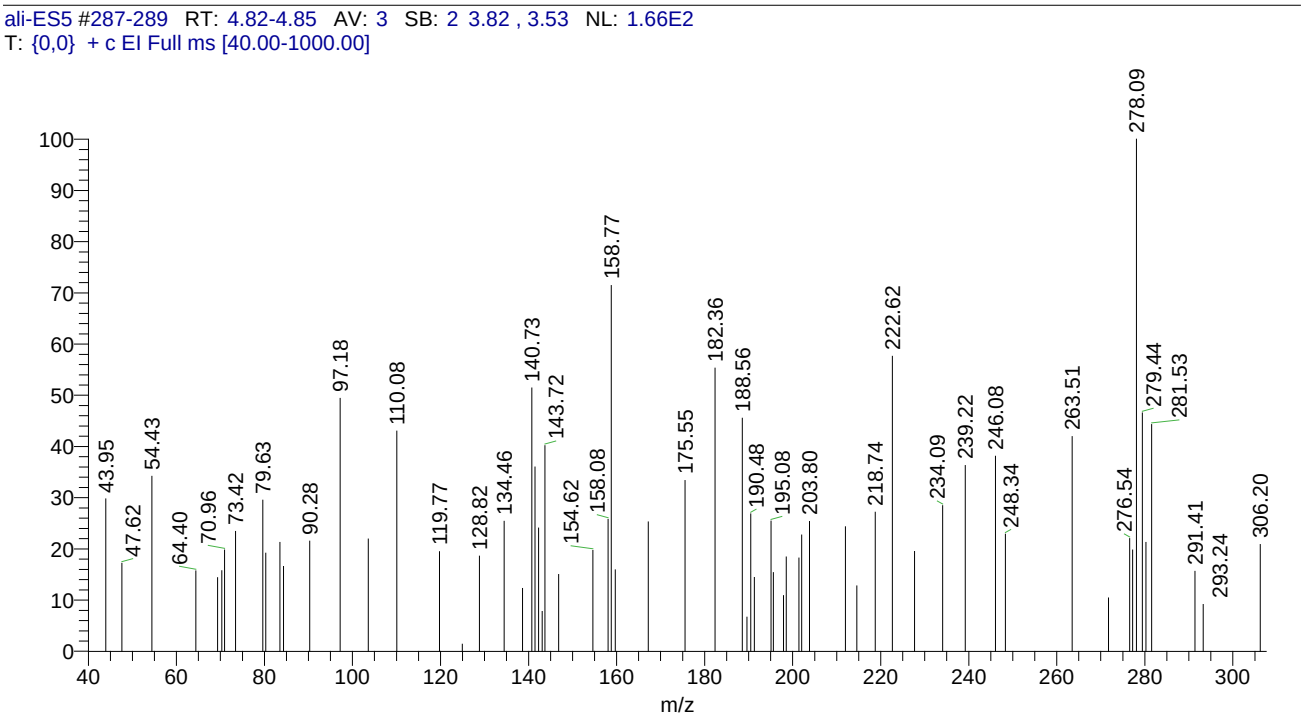


Figure S15: Mass spectrum of **8**

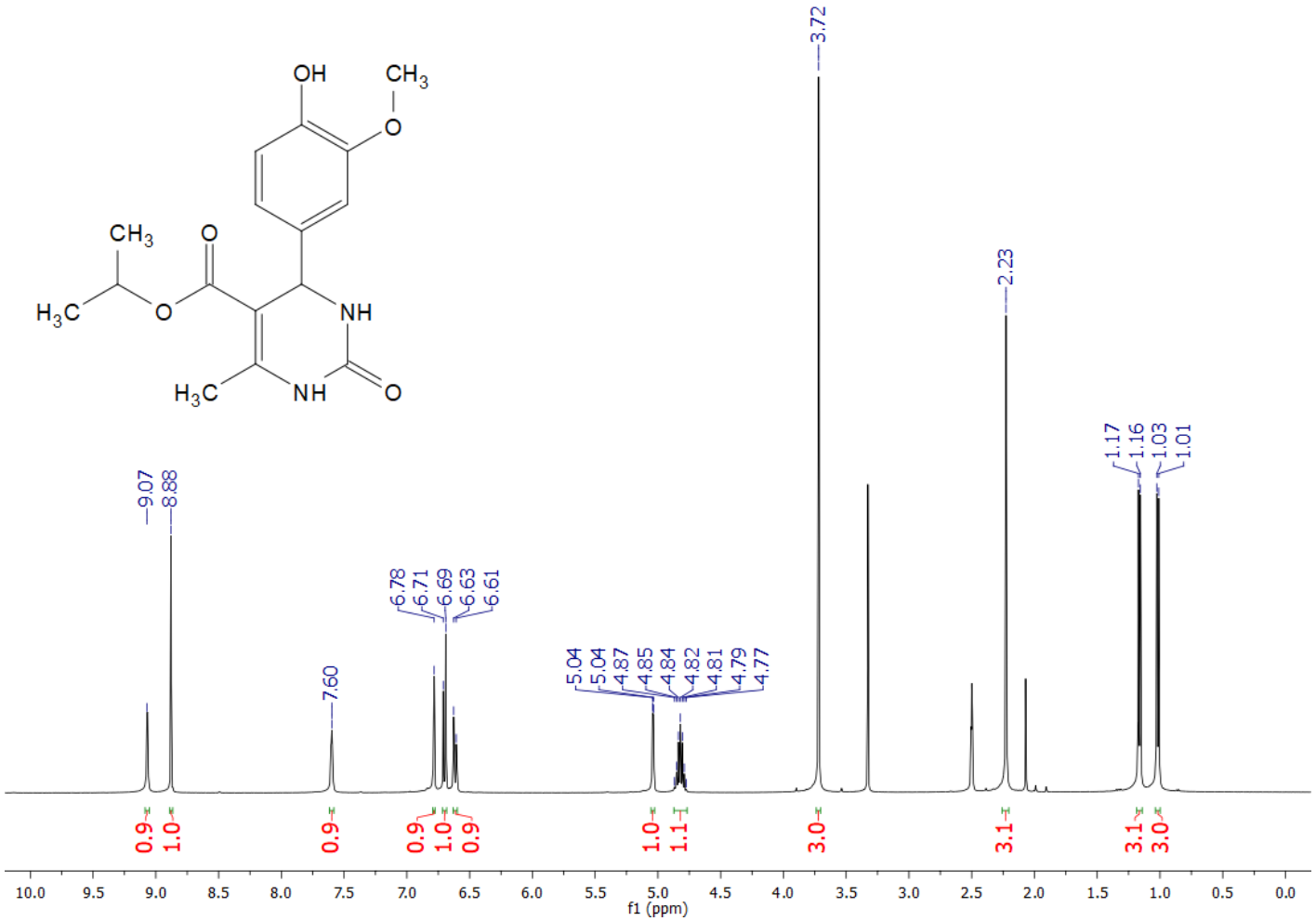


Figure S16: ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of **9**

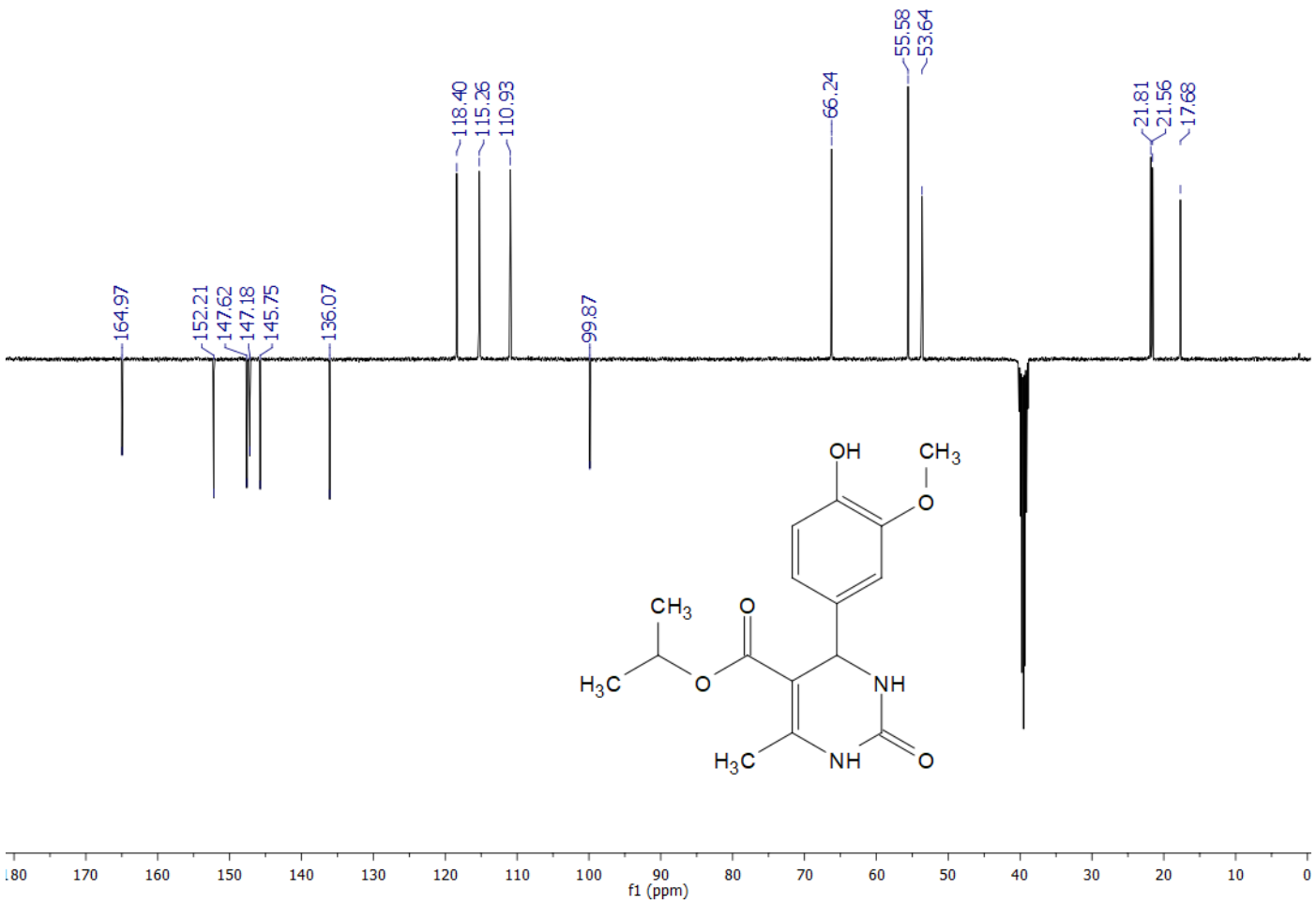


Figure S17: ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of **9**

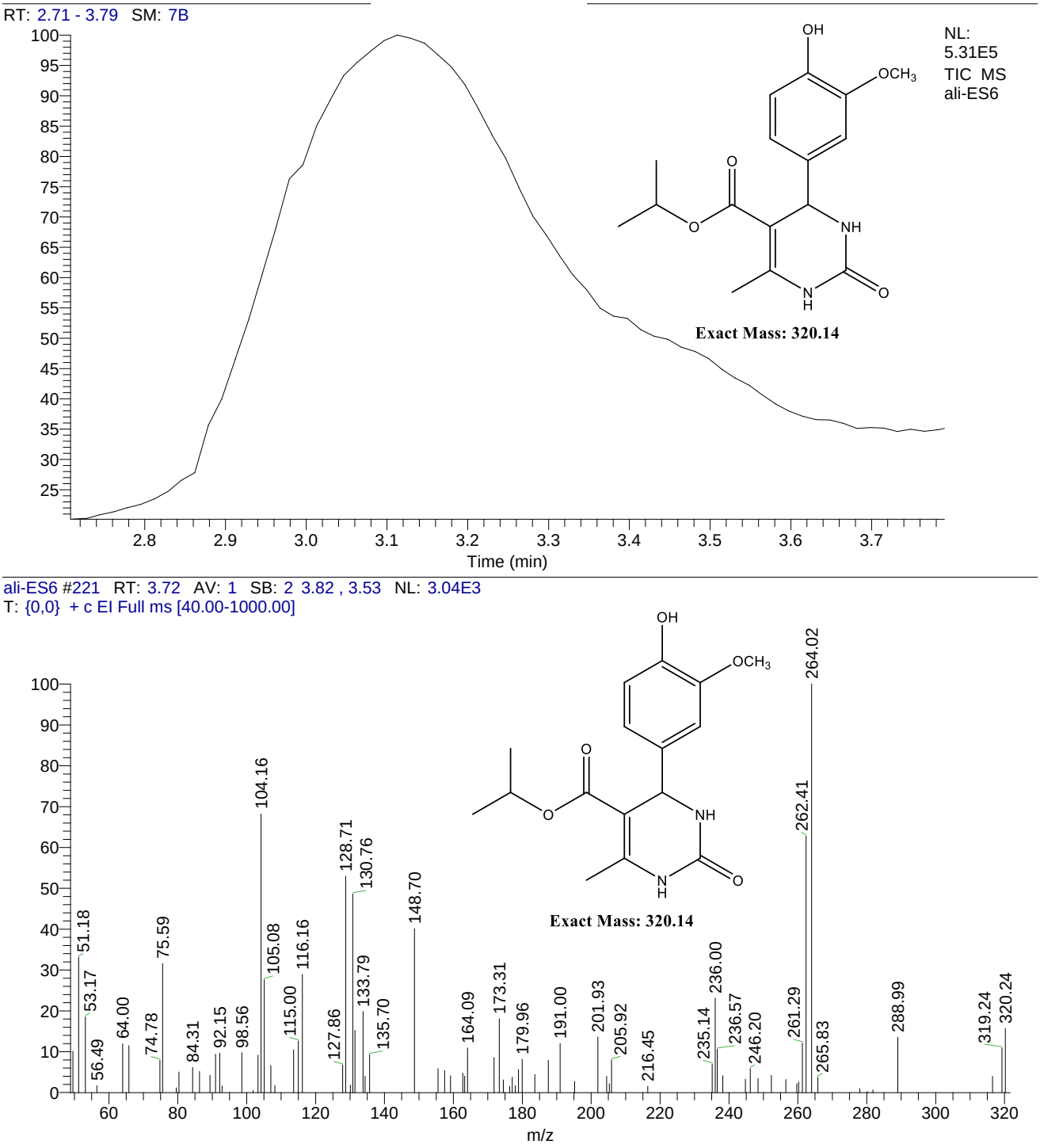


Figure S18: Mass spectrum of **9**

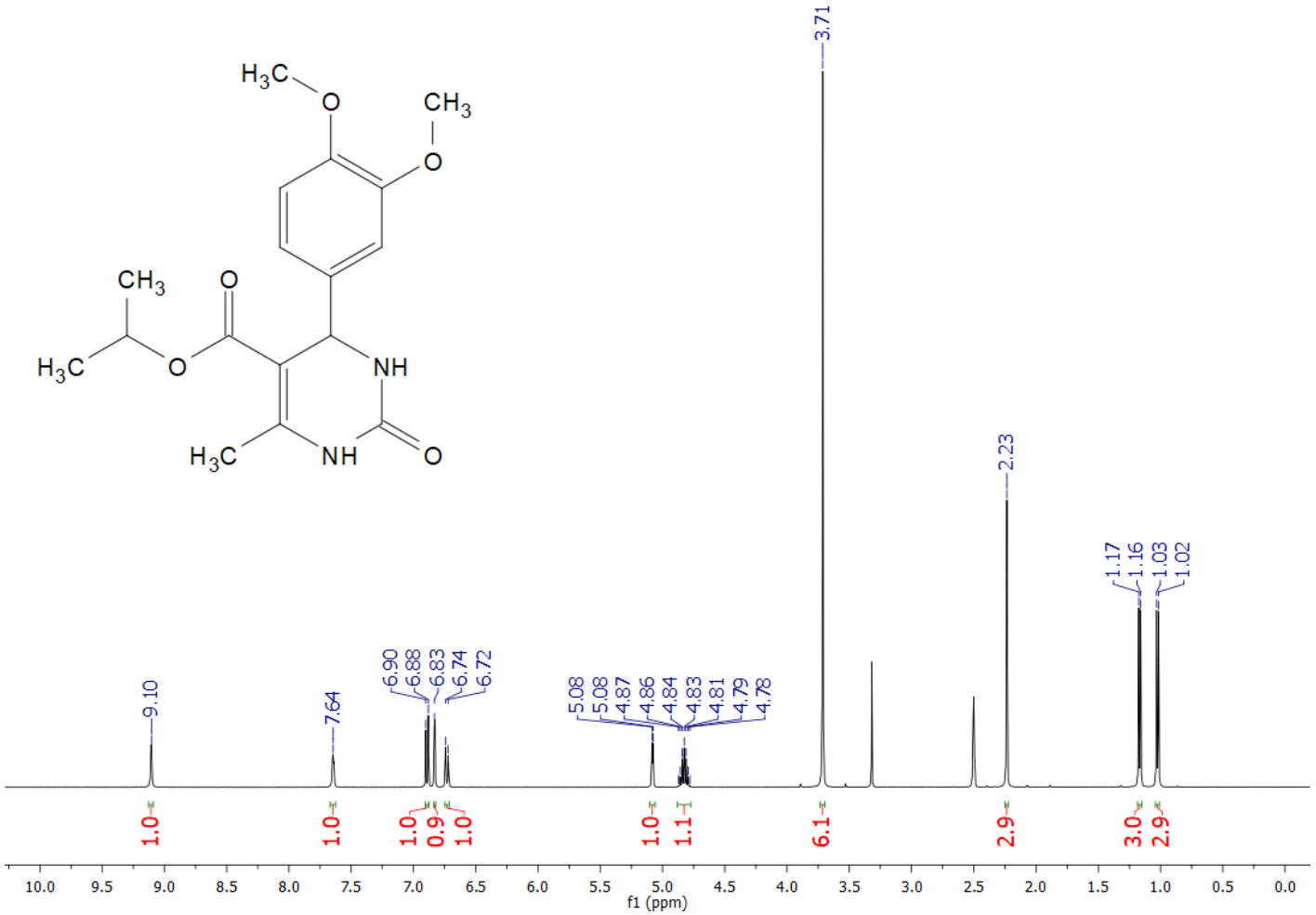


Figure S19: ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of **10**

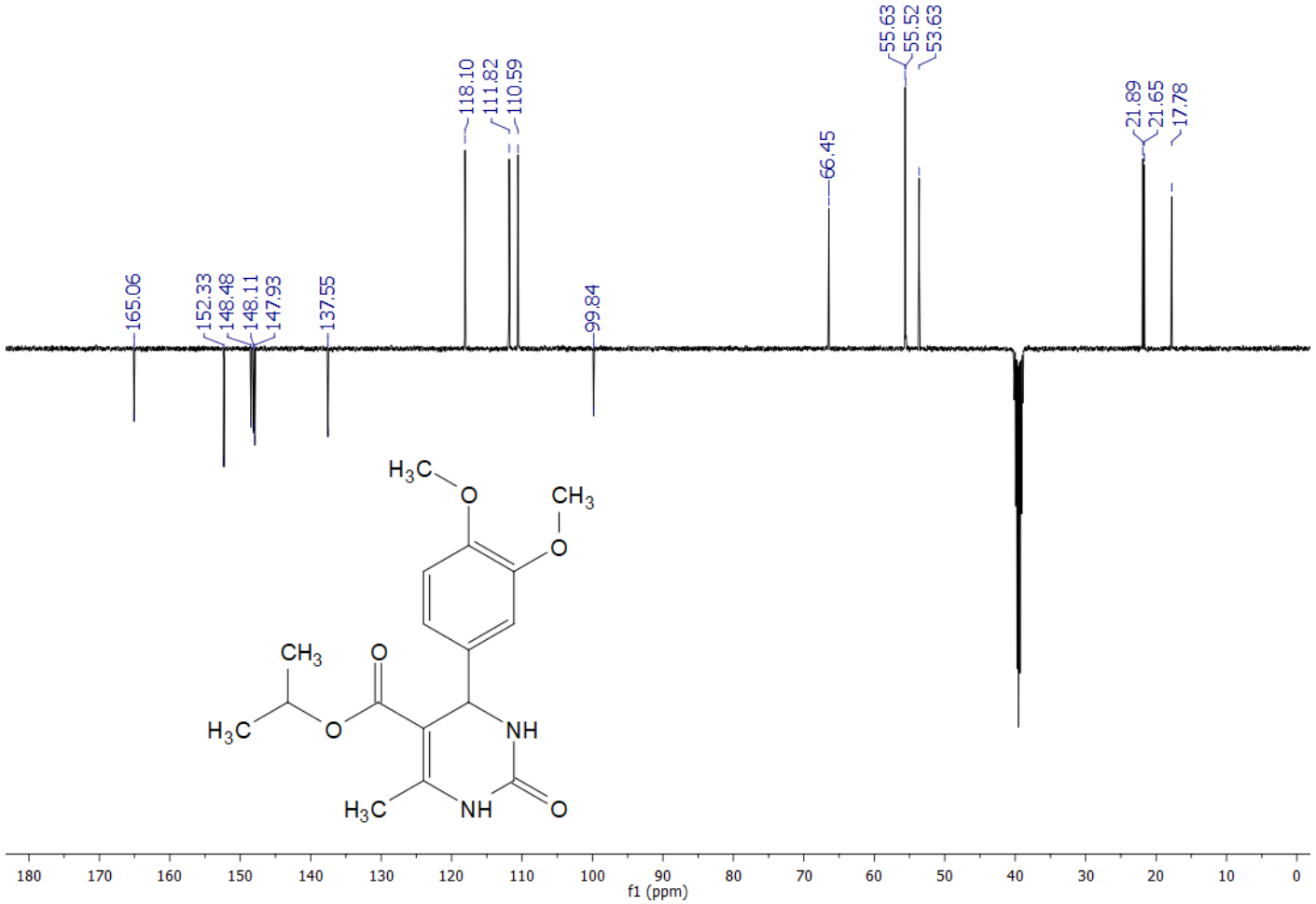
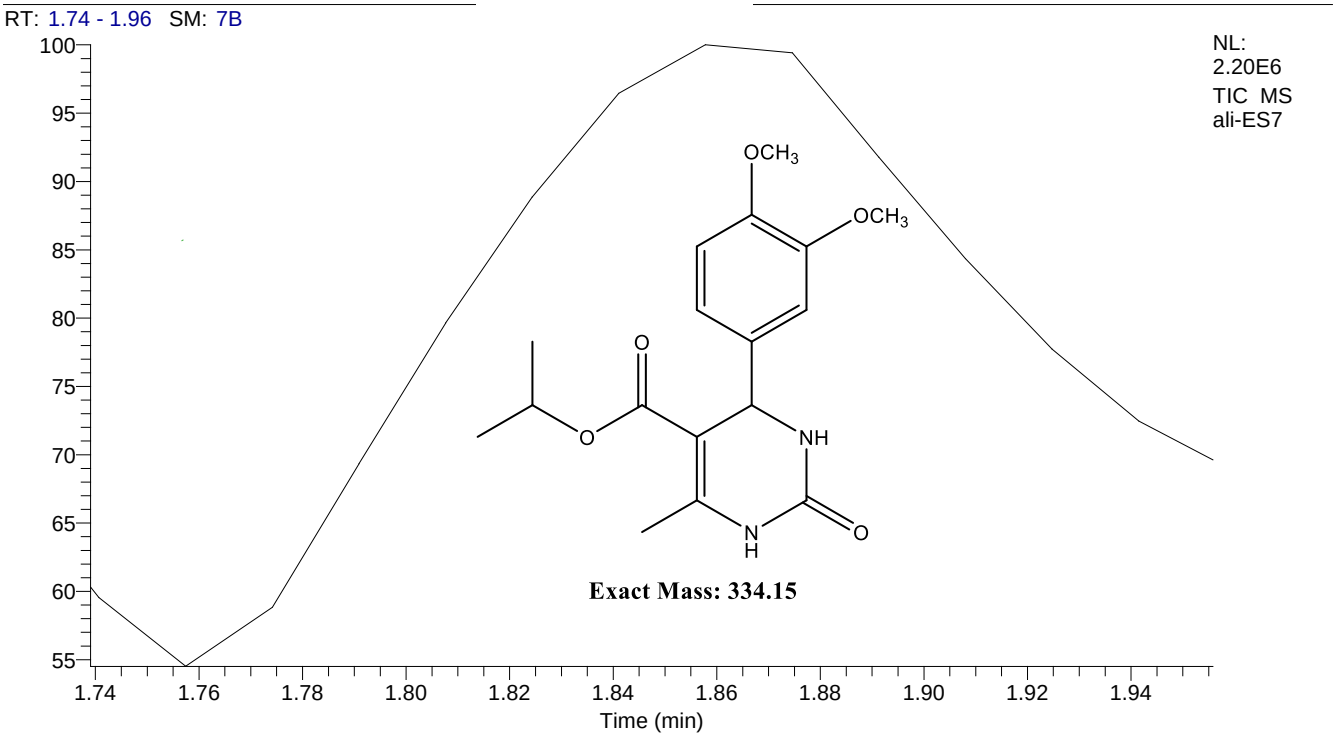


Figure S20: ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of **10**



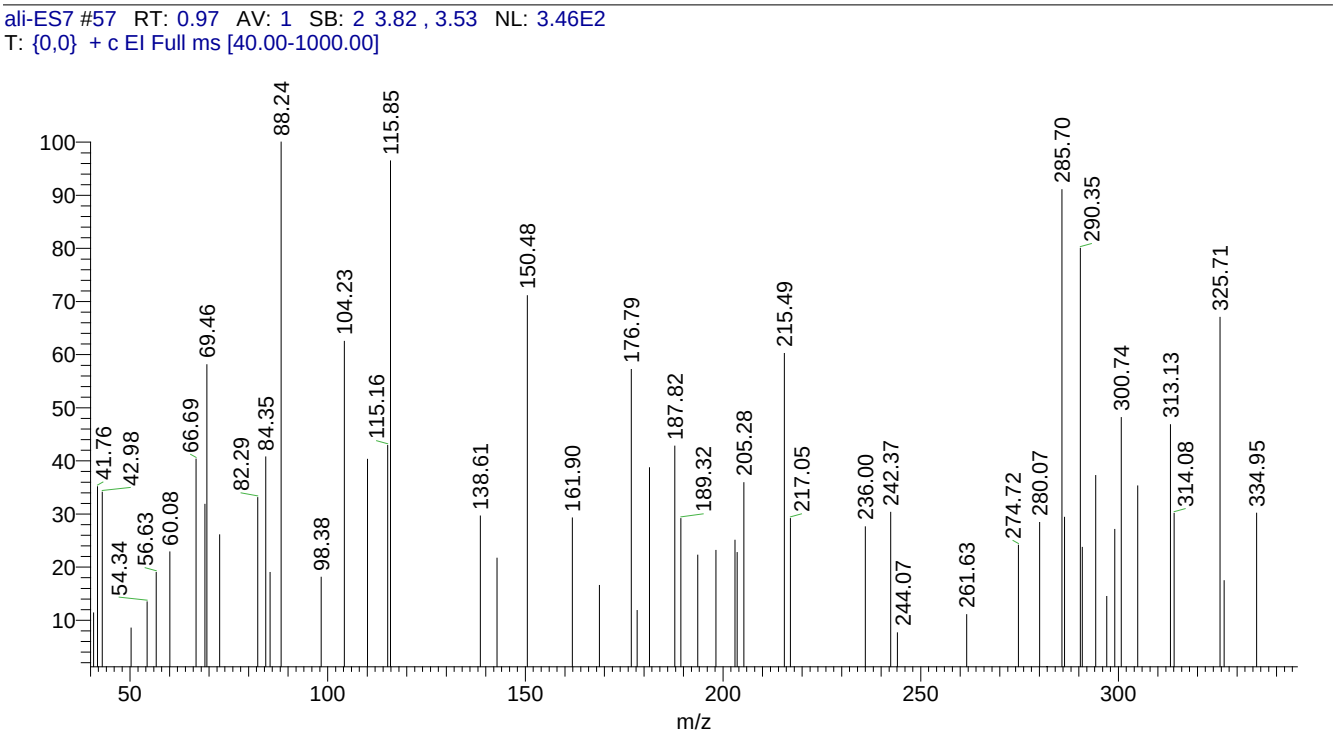


Figure S21: Mass spectrum of **10**

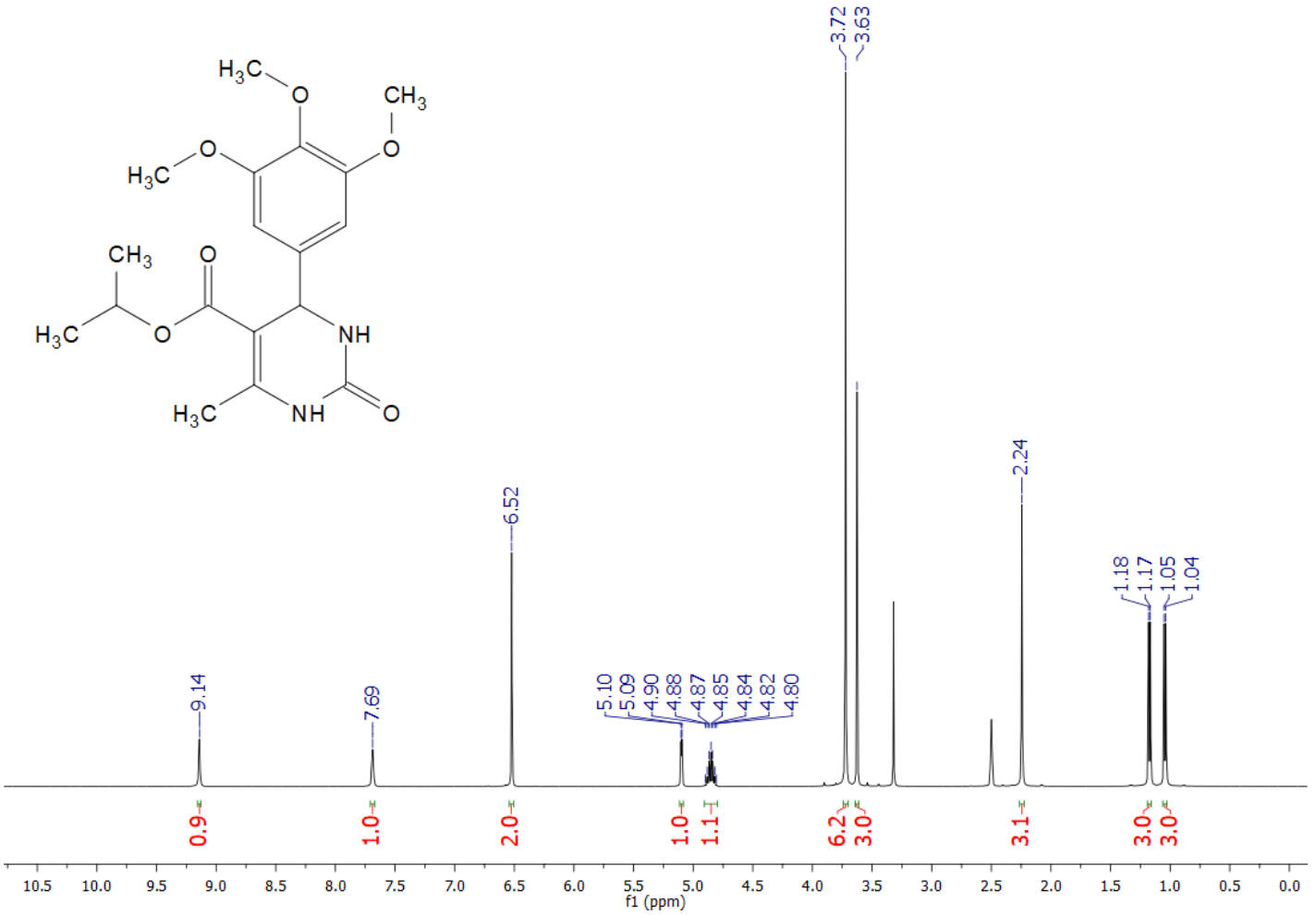


Figure S22: ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of **11**

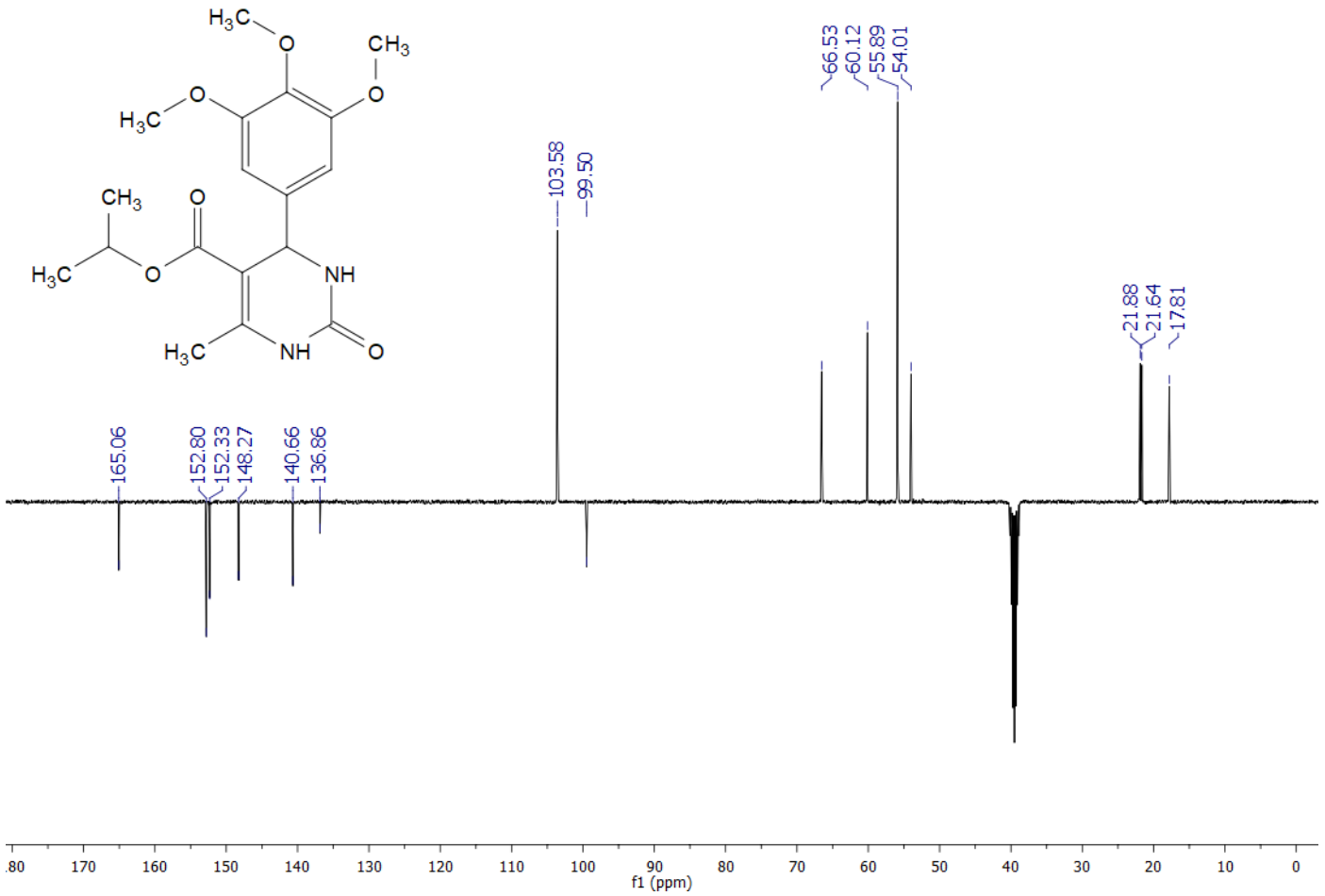
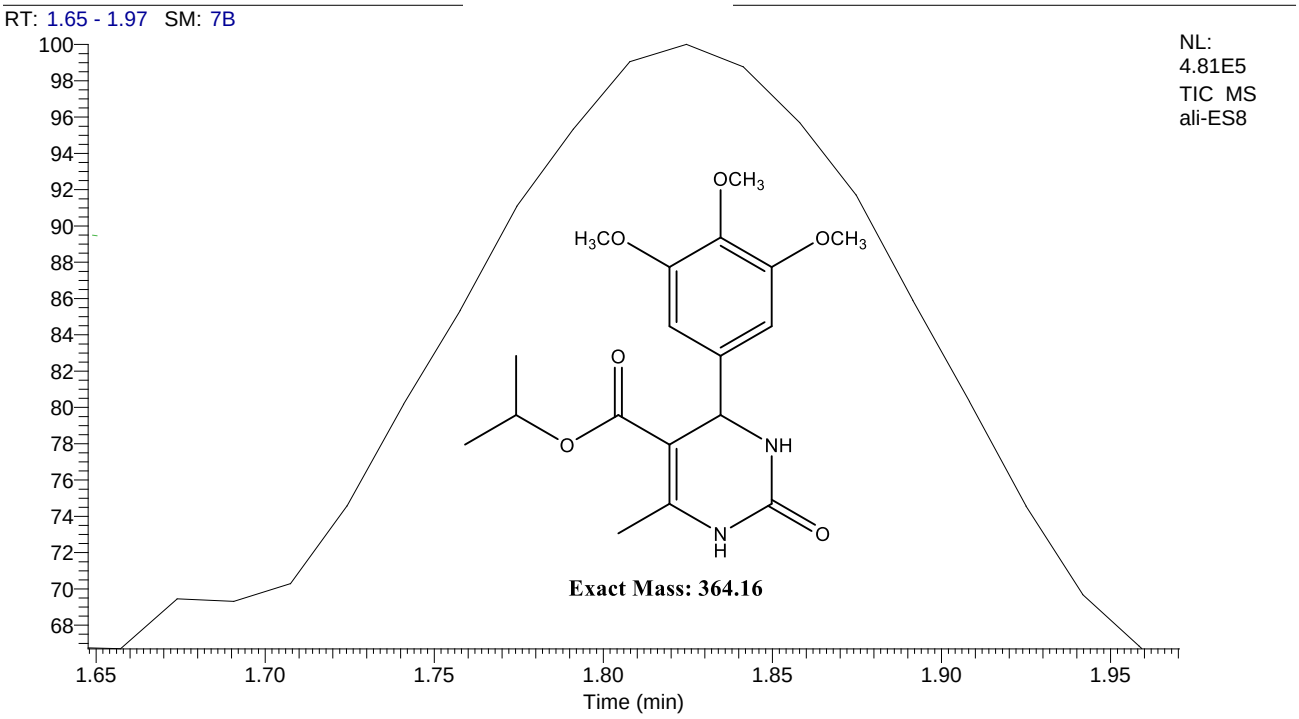


Figure S23: ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of **11**



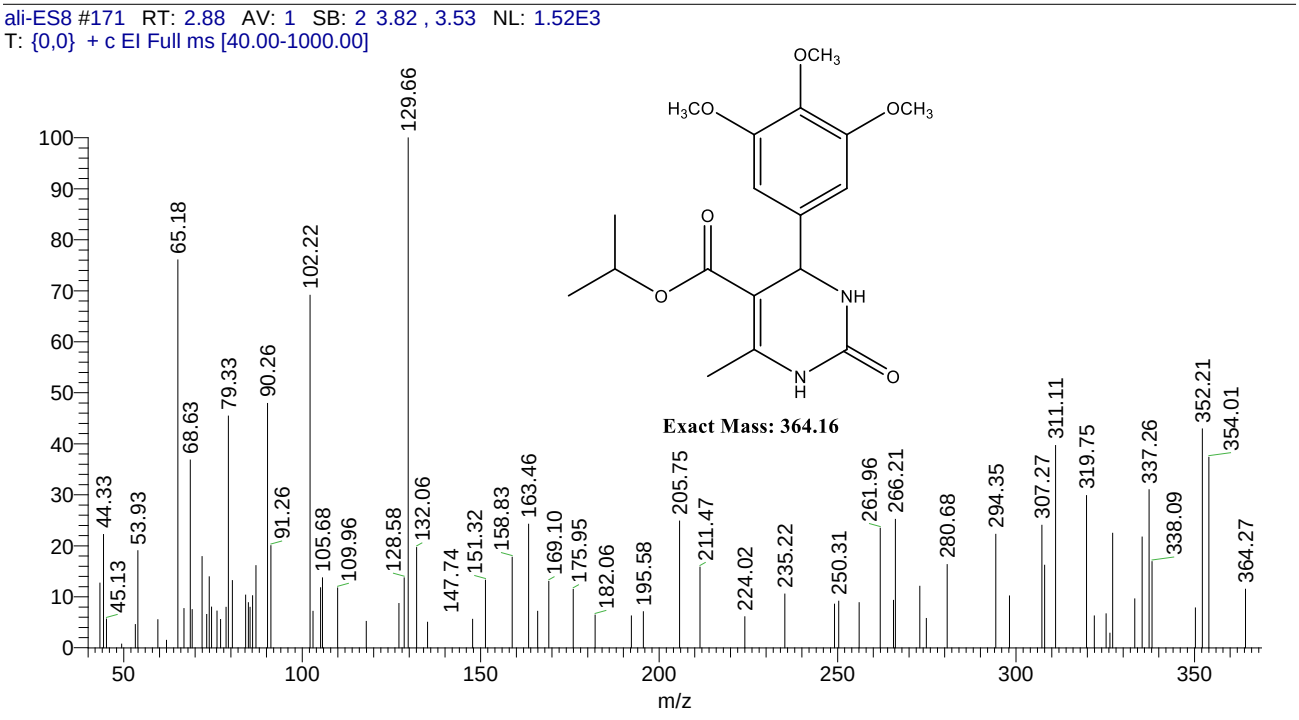


Figure S24: Mass spectrum of **11**

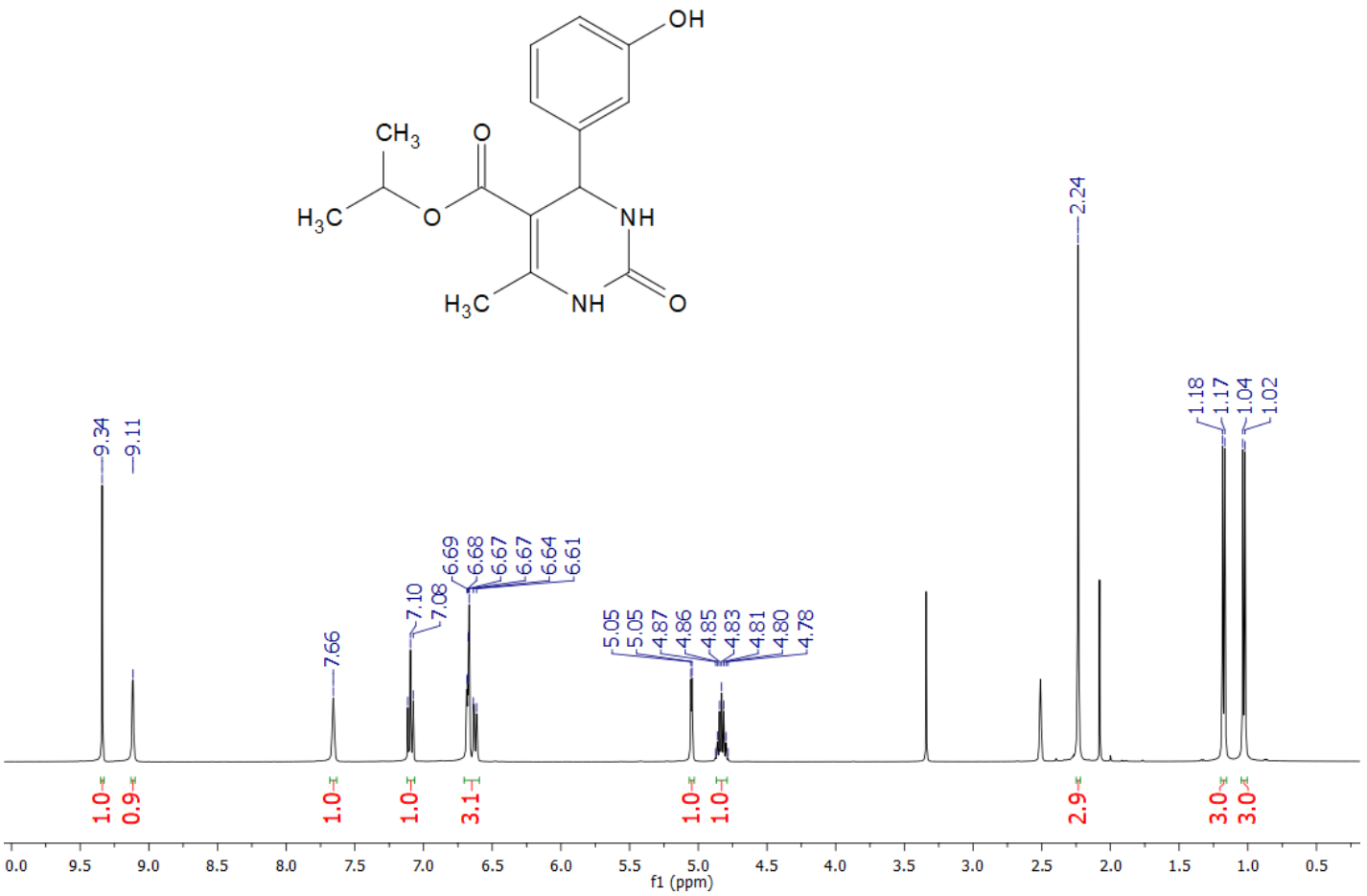


Figure S25: ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of **12**

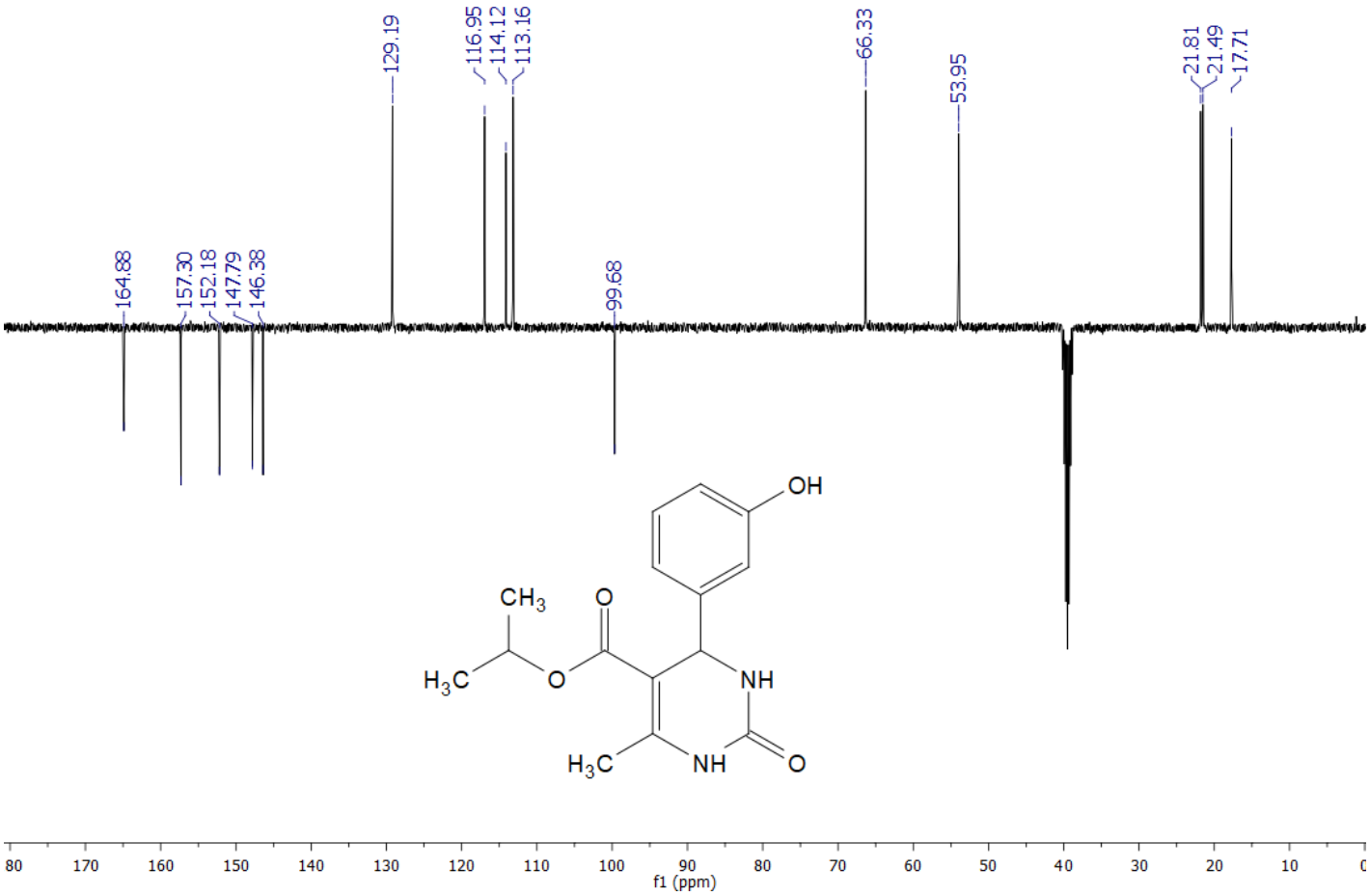
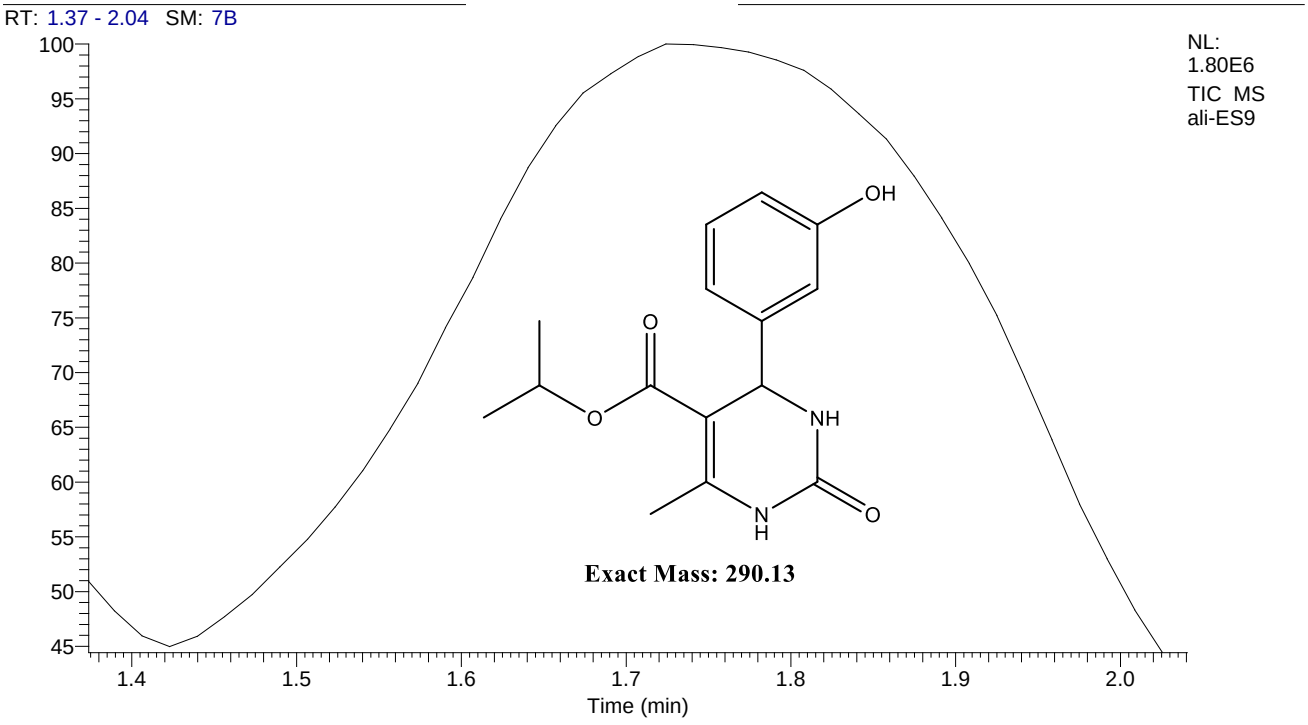


Figure S26: ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of **12**



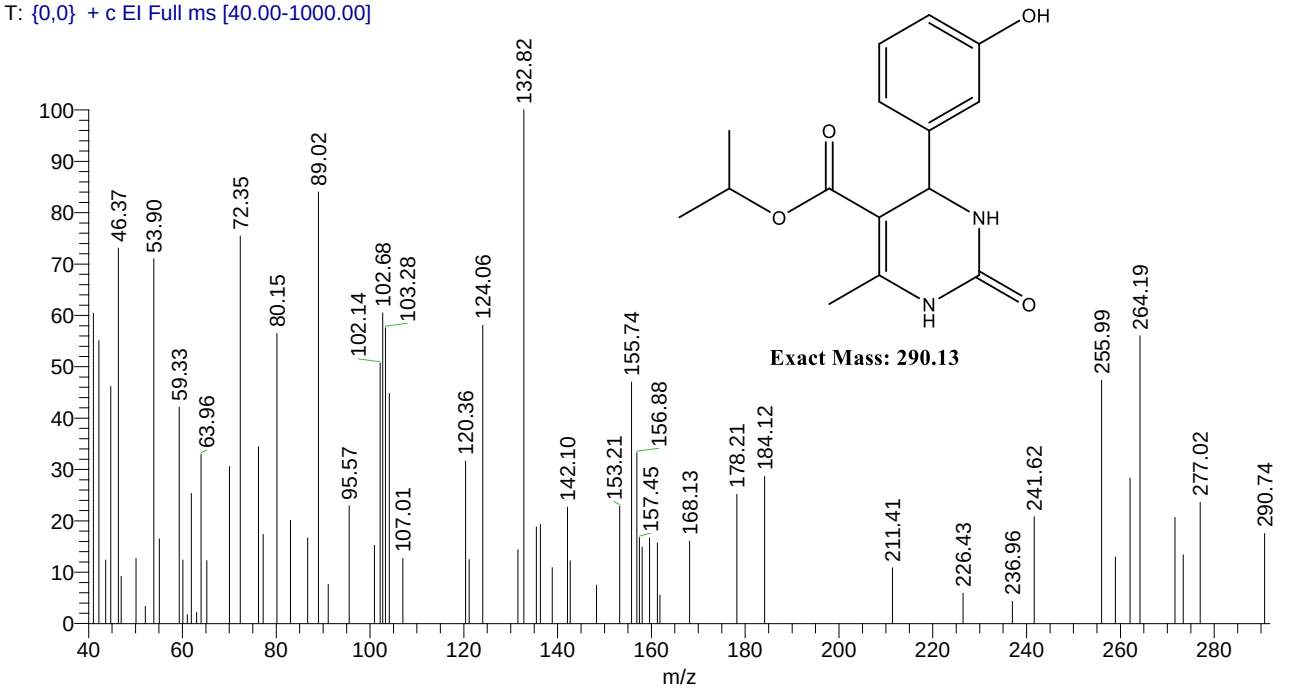


Figure S27: Mass spectrum of **12**

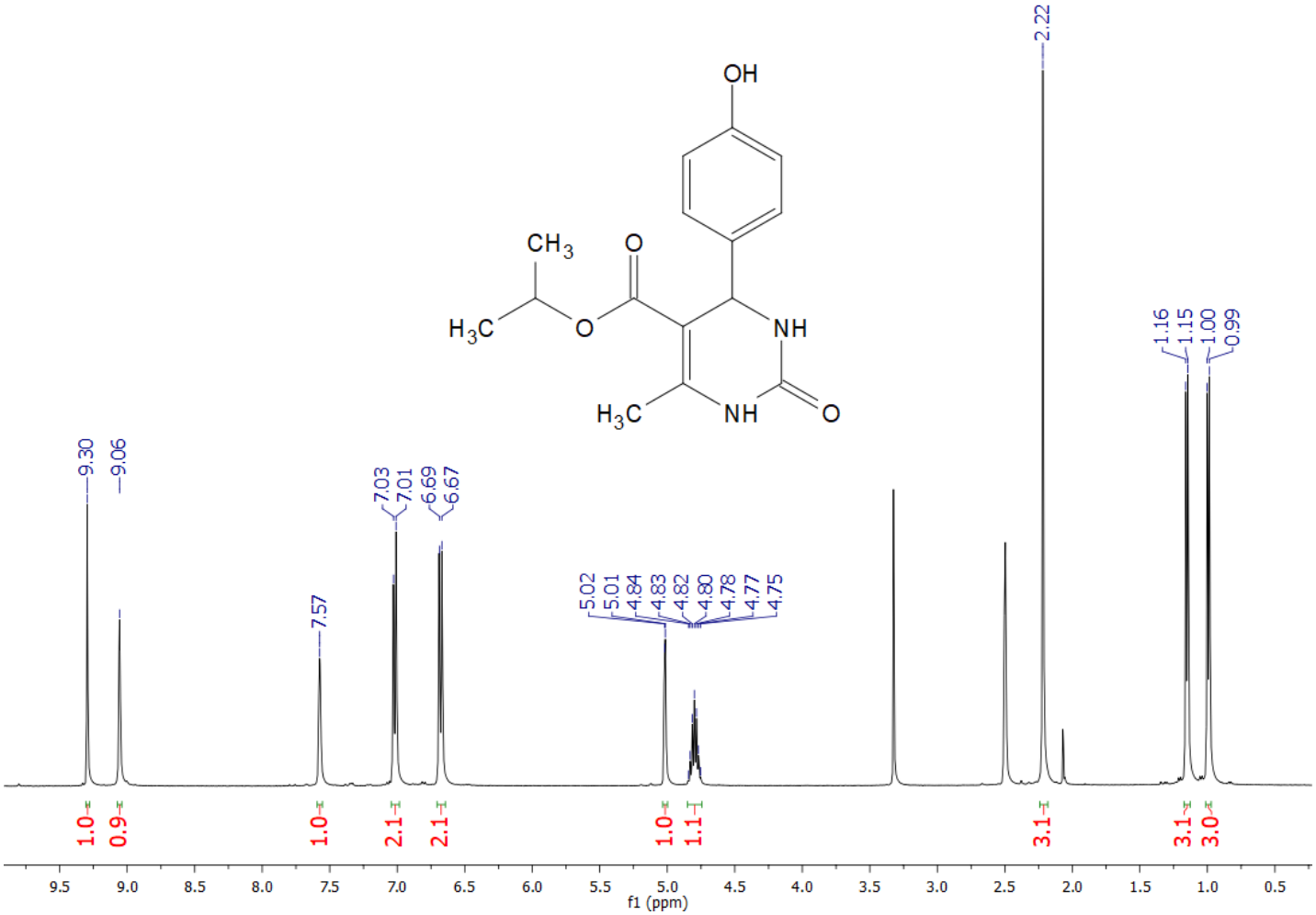


Figure S28: ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of **13**

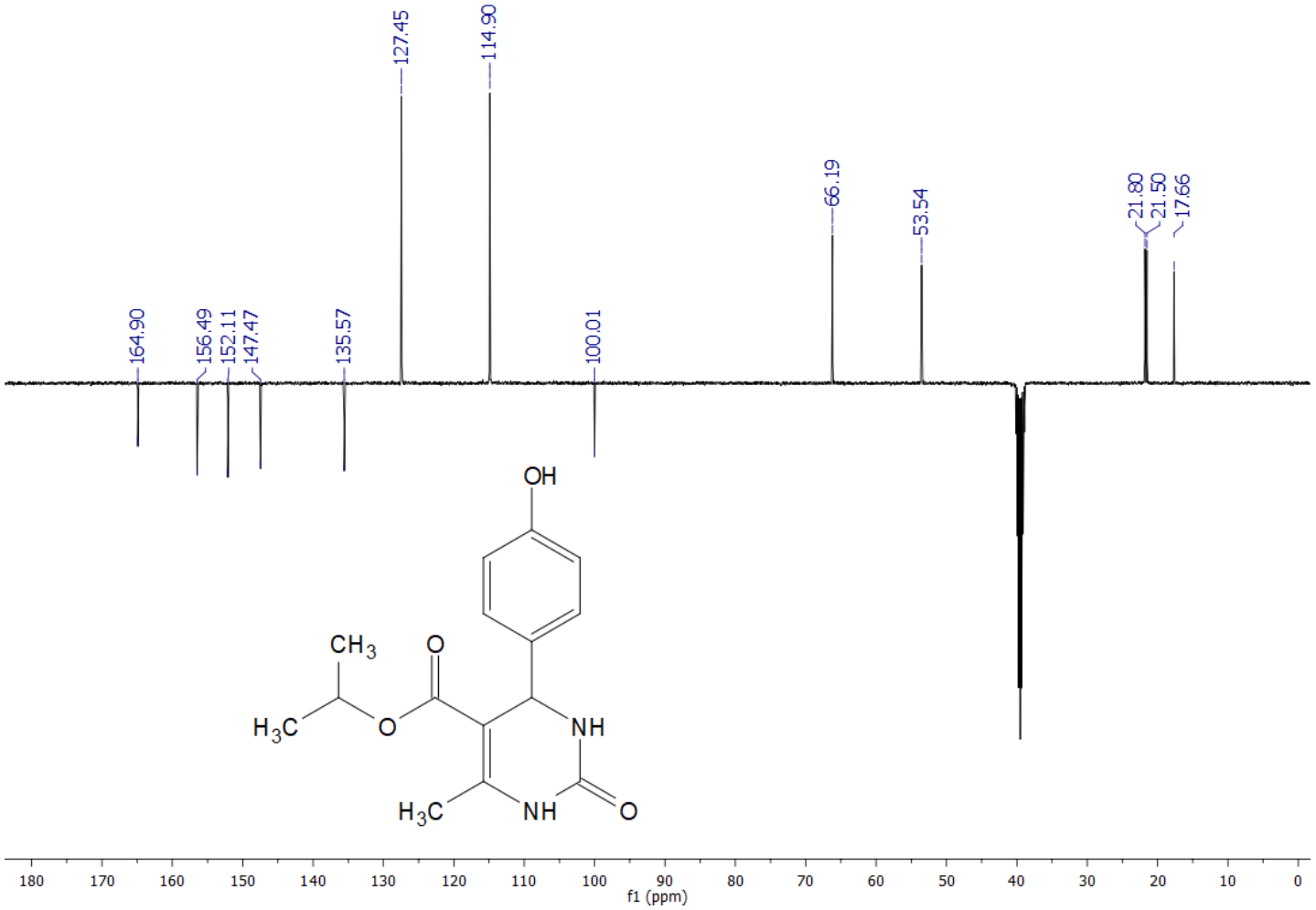
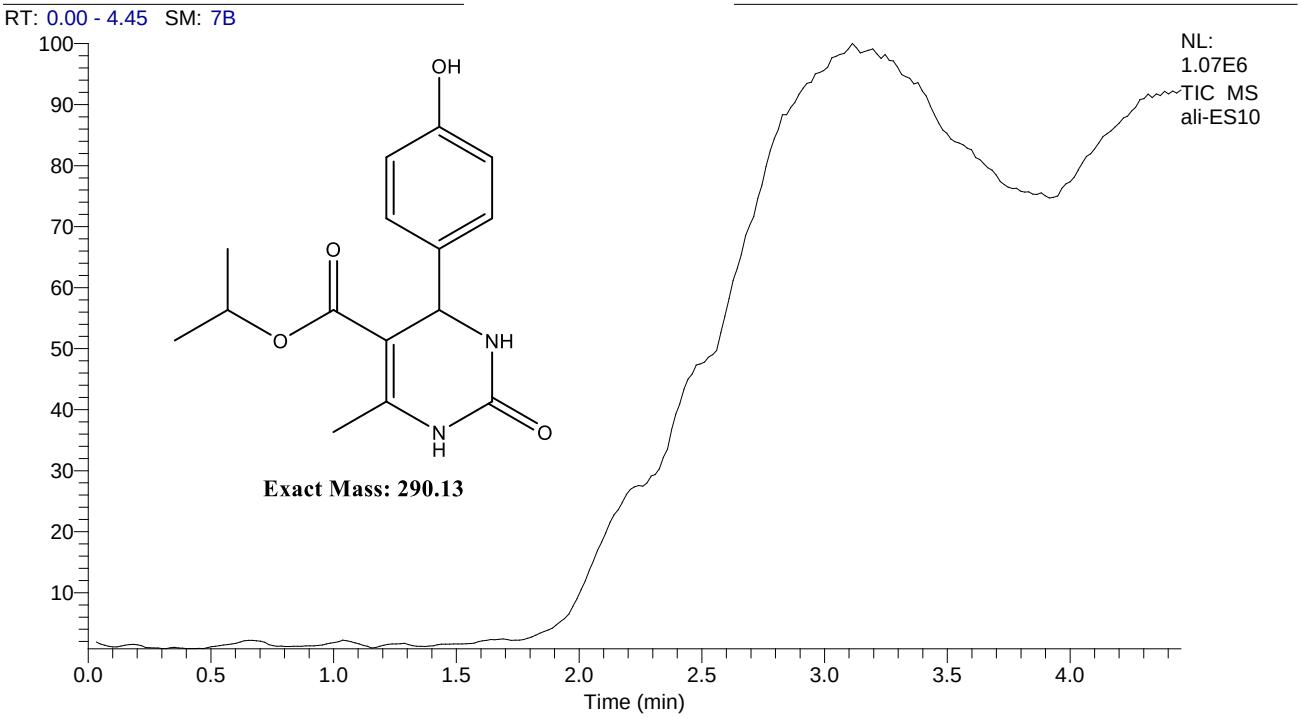


Figure S29: ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of **13**



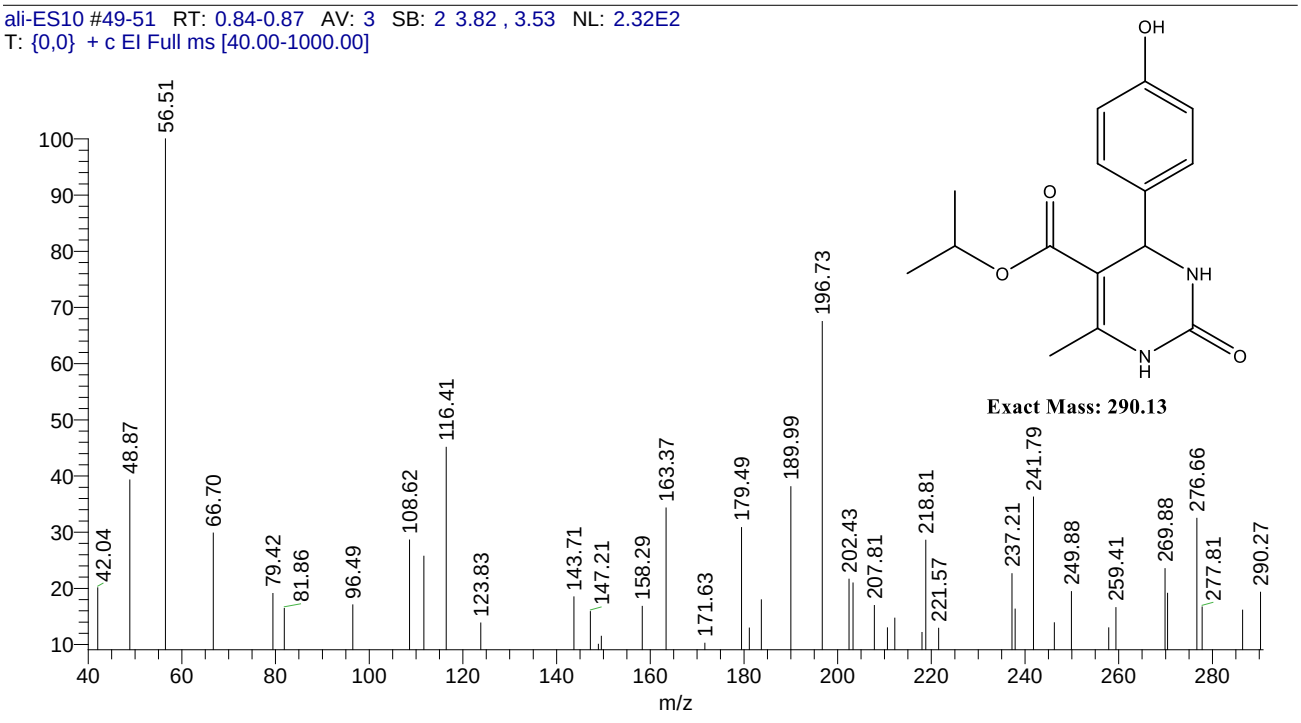


Figure S30: Mass spectrum of **13**

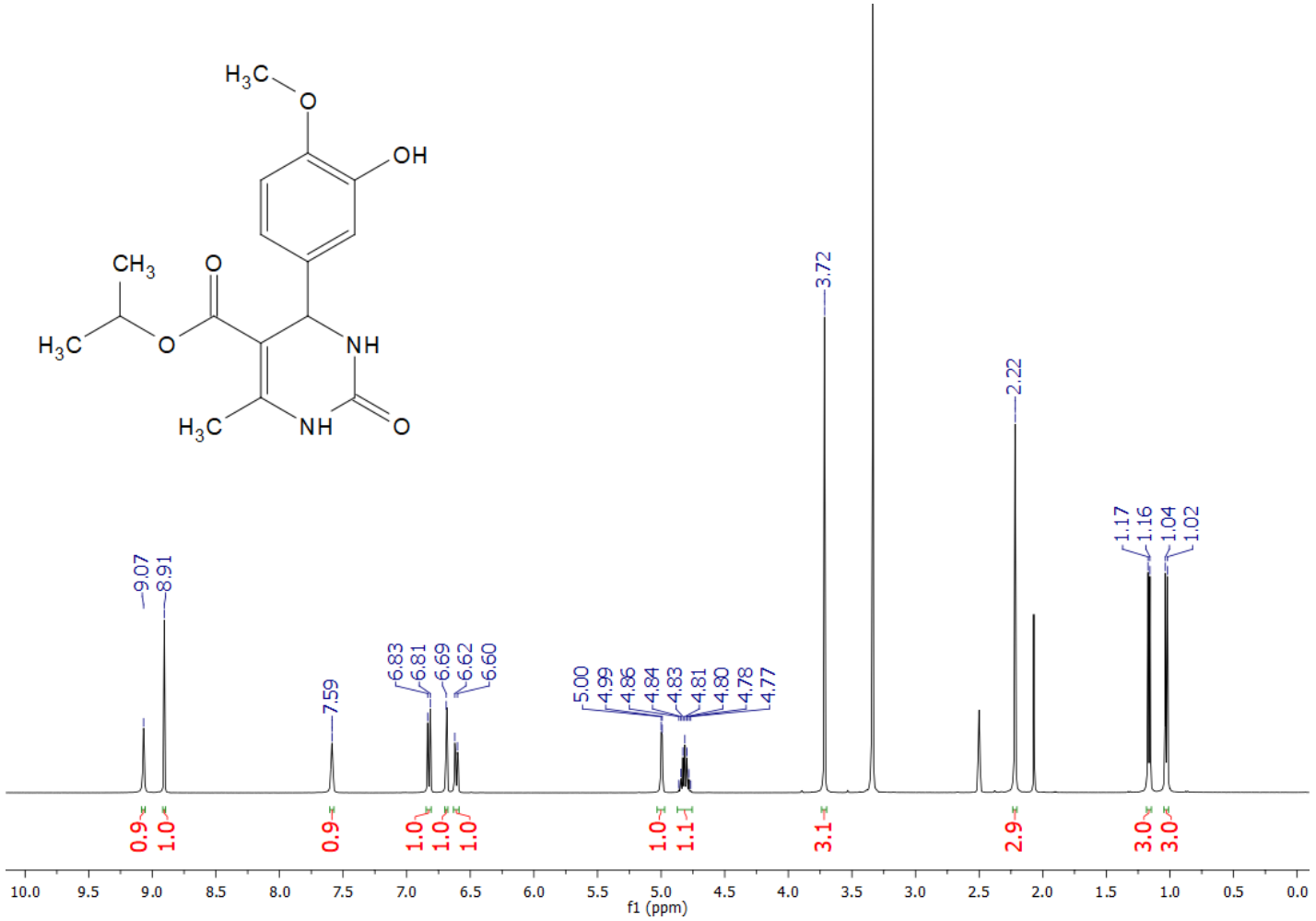


Figure S31: ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of **14**

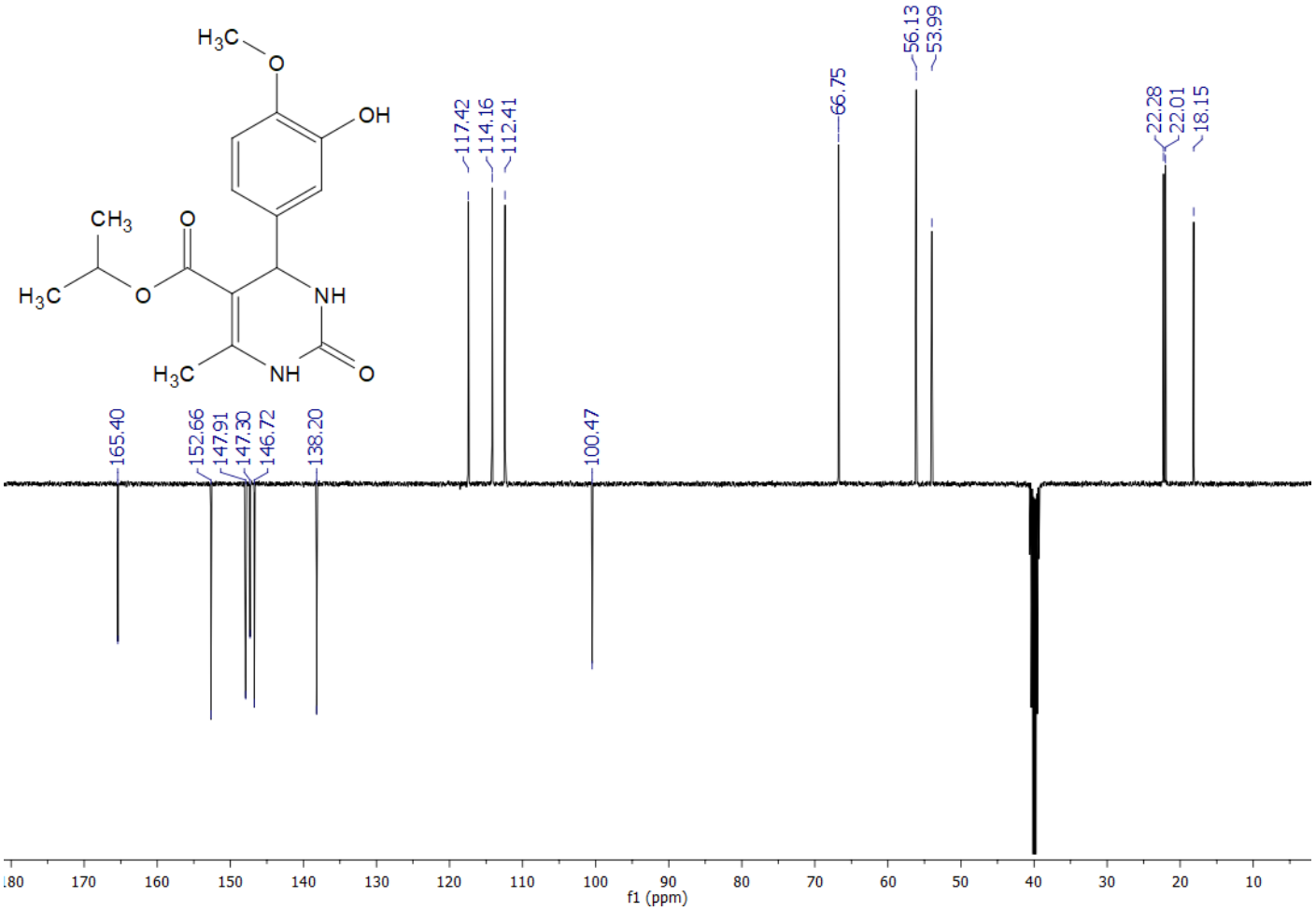
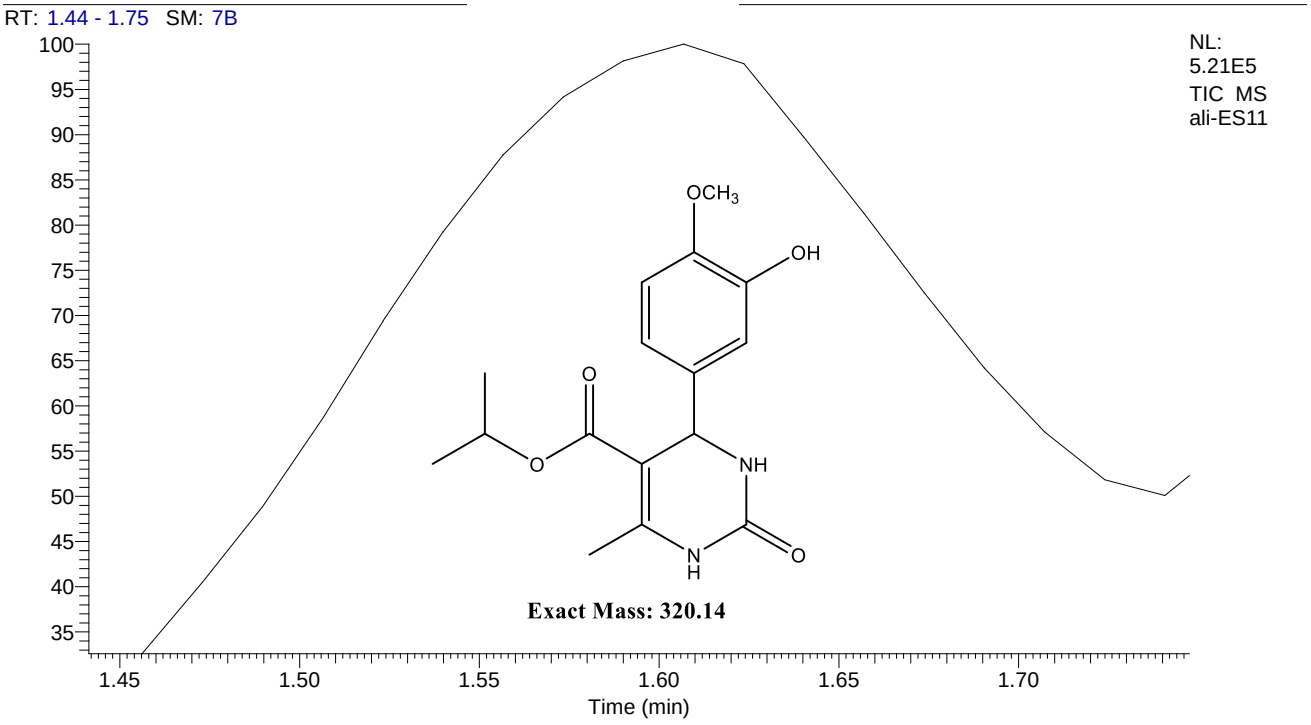


Figure S32: ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of **14**



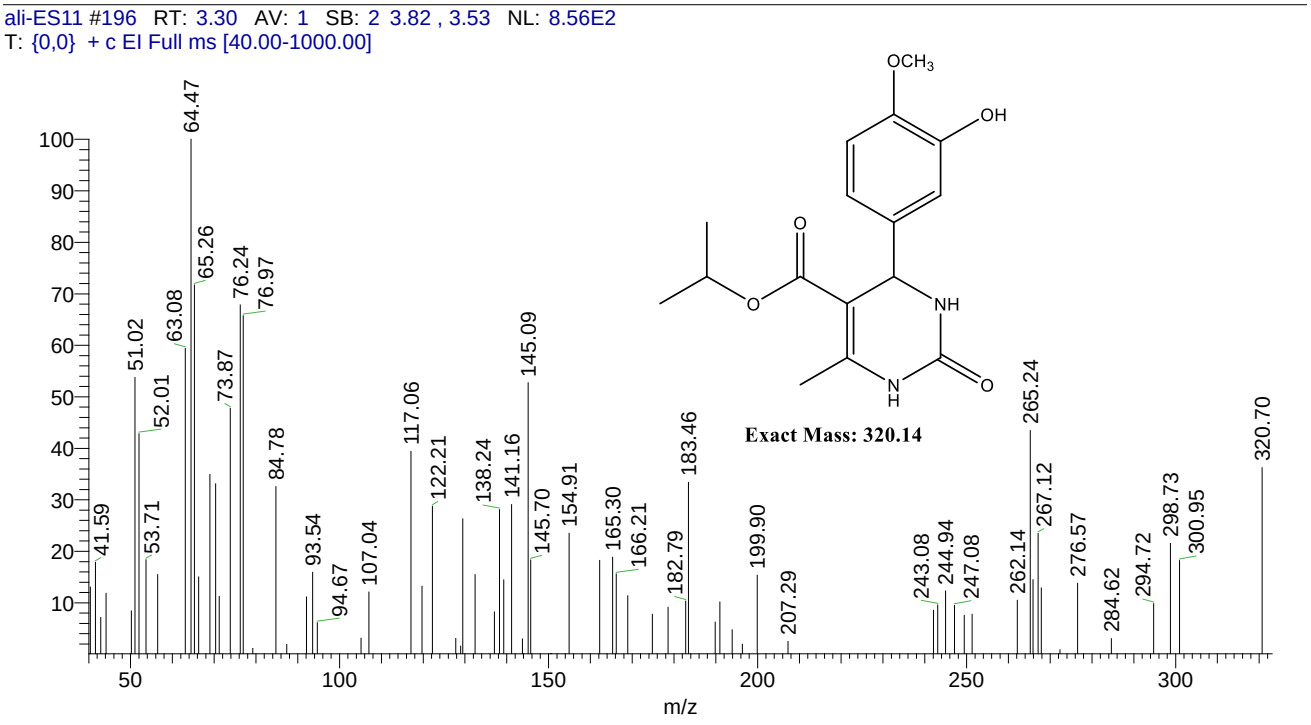


Figure S33: Mass spectrum of **14**

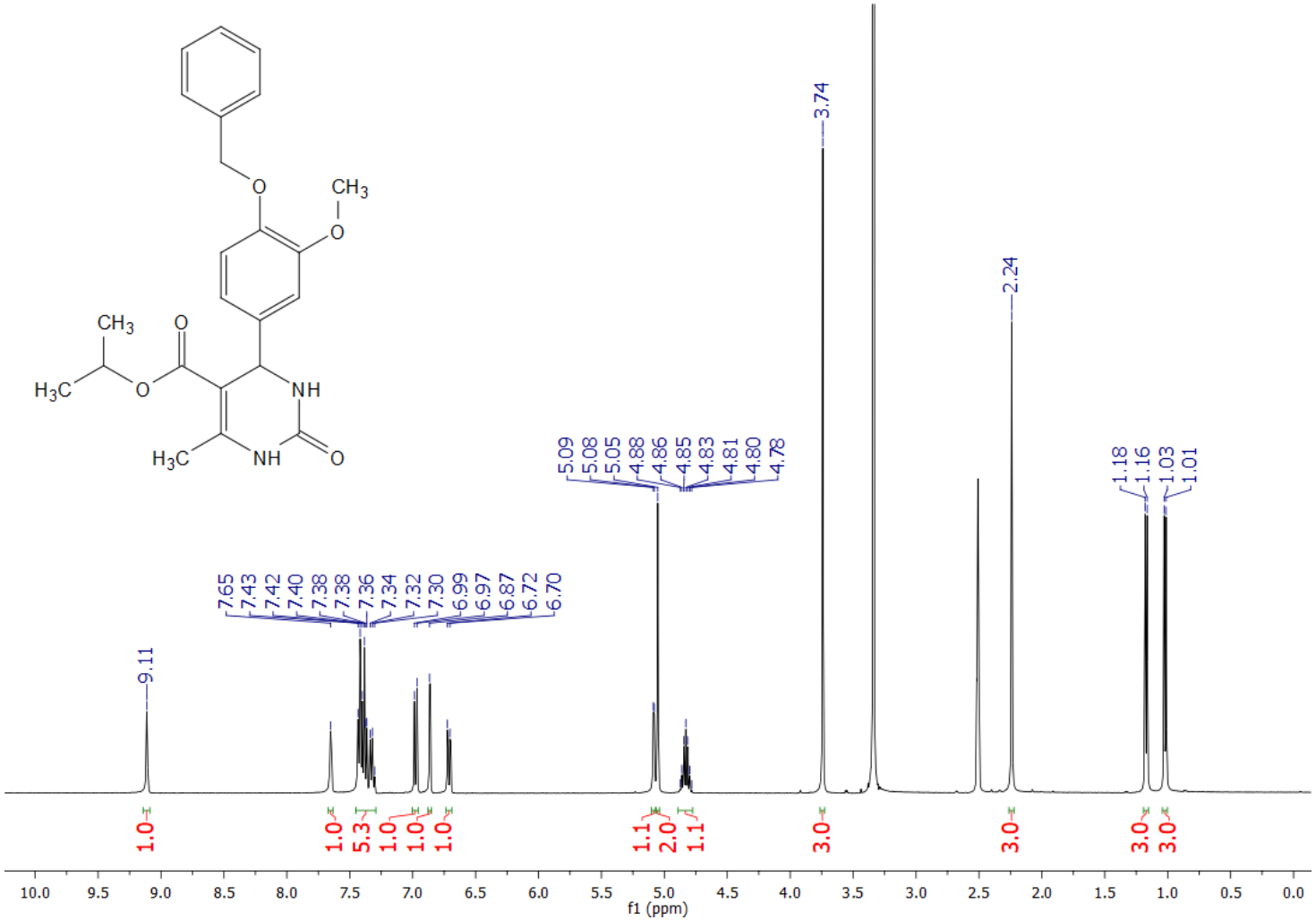


Figure S34: ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of **15**

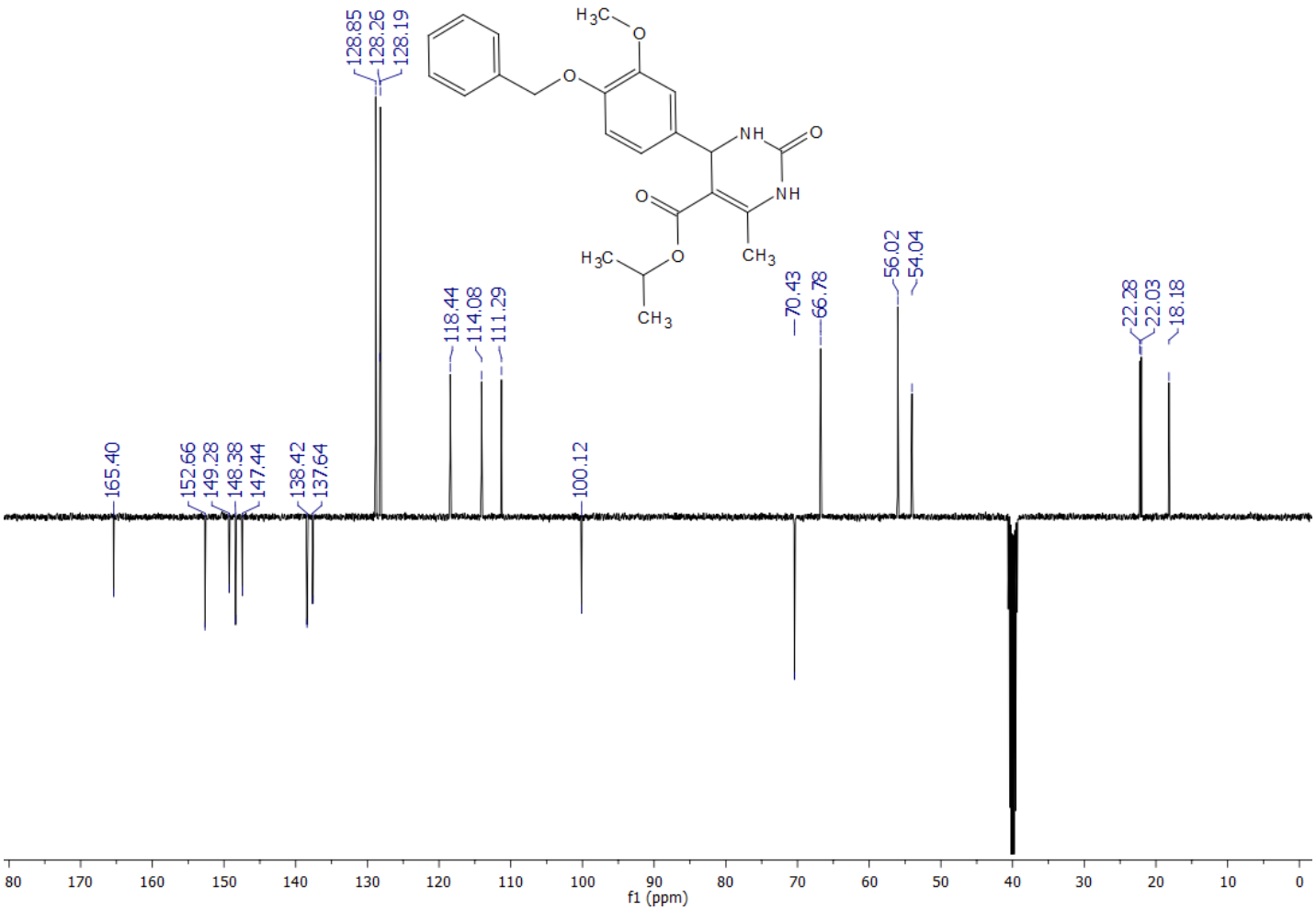
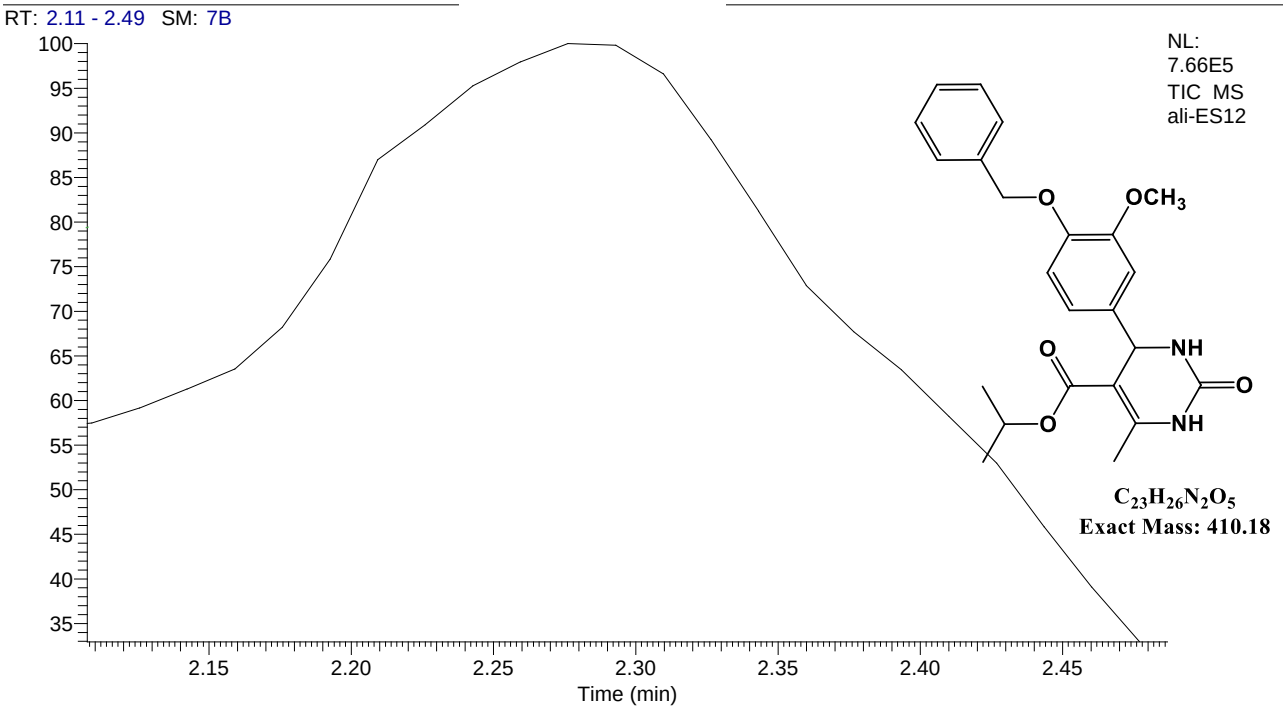


Figure S35: ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of **15**



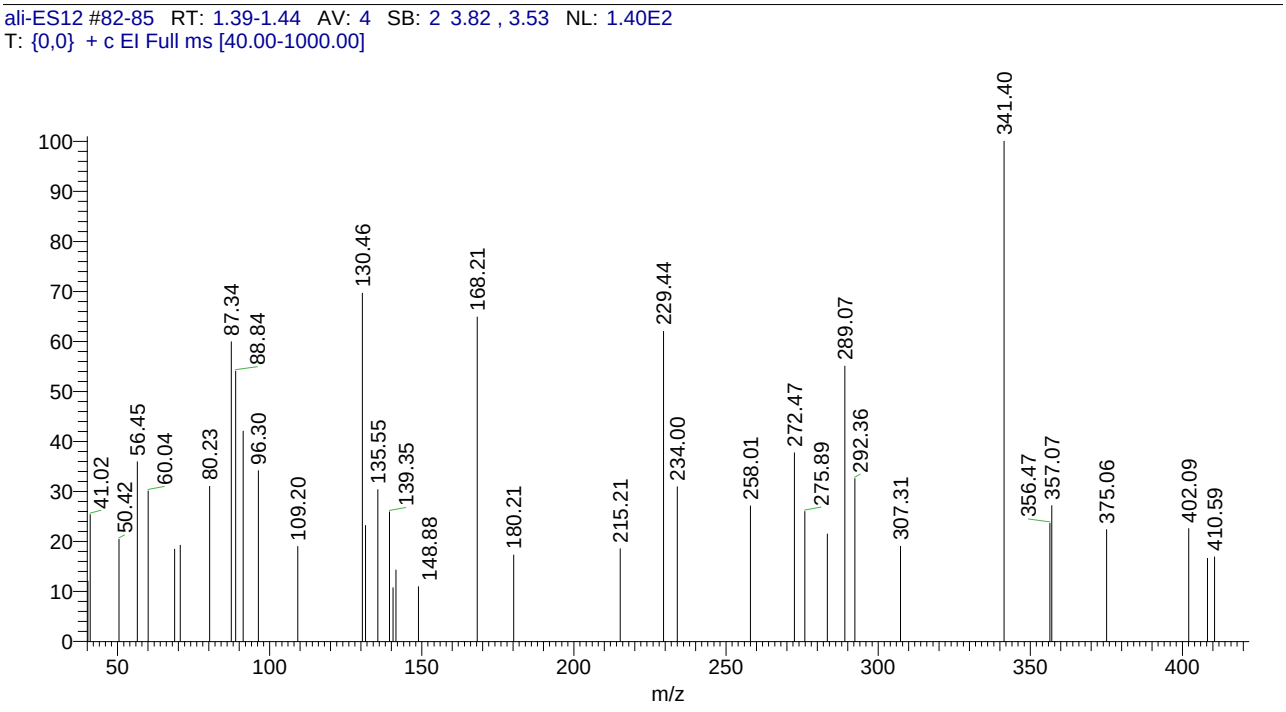


Figure S36: Mass spectrum of **15**

Appendix A

4. EXPERIMENTAL

4.1. Chemistry

Materials and methods

All reagents and solvents were of general purpose or analytical grade and purchased from Sigma-Aldrich Ltd, Fisher Scientific, Fluka and Acros. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker Avance III spectrometer operating at 400, 100 MHz respectively, with Me_4Si as internal standard and DMSO-d_6 as a solvent. Elemental analysis was performed by the regional centre for mycology and biotechnology (Cairo, Egypt). TLC was carried out on precoated silica plates (Keisel gel 60 F_{254}, BDH) using Hexane: Ethyl acetate, 2 : 1, v/v. Compounds were visualised by illumination under UV light (254 nm). Melting points were determined on an electrothermal instrument and are uncorrected. All solvents were dried prior to use and stored over 4 Å molecular sieves, under nitrogen. All the compounds were ≥ 95% pure.

4.2. Biological evaluation

4.2.1. Cytotoxic activity using MTT Assay and evaluation of IC_{50}

4.2.1.1. MTT assay

MTT assay was performed to investigate the effect of the synthesized compounds on mammary epithelial cells (MCF-10A). The cells were propagated in medium consisting of Ham's F-12 medium/ Dulbecco's modified Eagle's medium (DMEM) (1:1) supplemented with 10% foetal calf serum, 2 mM glutamine, insulin (10 μg/mL), hydrocortisone (500 ng/mL) and epidermal growth factor (20 ng/mL). Trypsin ethylenediamine tetra acetic acid (EDTA) was used to passage the cells after every 2-3 days. 96-well flat-bottomed cell culture plates were used to seed the cells at a density

of 104 cells mL-1. The medium was aspirated from all the wells of culture plates after 24 h followed by the addition of synthesized compounds (in 200 µL medium to yield a final concentration of 0.1% (v/v) dimethylsulfoxide) into individual wells of the plates. Four wells were designated to a single compound. The plates were allowed to incubate at 37°C for 96 h. Afterwards, the medium was aspirated and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (0.4 mg/mL) in medium was added to each well and subsequently incubated for 3 h. The medium was aspirated and 150 µL dimethyl sulfoxide (DMSO) was added to each well. The plates were vortexed followed by the measurement of absorbance at 540 nm on a microplate reader. The results were presented as inhibition (%) of proliferation in contrast to controls comprising 0.1% DMSO.

4.2.1.2. Assay for antiproliferative effect

To explore the antiproliferative potential of compounds propidium iodide fluorescence assay was performed using different cell lines such as Panc-1 (pancreas cancer cell line), MCF-7 (breast cancer cell line), HT-29 (colon cancer cell line) and A-549 (epithelial cancer cell line), respectively. To calculate the total nuclear DNA, a fluorescent dye (propidium iodide, PI) is used which can attach to the DNA, thus offering a quick and precise technique. PI cannot pass through the cell membrane and its signal intensity can be considered as directly proportional to quantity of cellular DNA. Cells whose cell membranes are damaged or have changed permeability are counted as dead ones. The assay was performed by seeding the cells of different cell lines at a density of 3000-7500 cells/well (in200µl medium) in culture plates followed by incubation for 24h at 37 °C in humidified 5%CO2/95% air atmospheric conditions. The medium was removed; the compounds were added to the plates at 10 µM concentrations (in 0.1% DMSO) in triplicates, followed by incubation for 48h. DMSO (0.1%) was used as control. After incubation, medium was removed

followed by the addition of PI (25 µl, 50µg/mL in water/medium) to each well of the plates. At -80 °C, the plates were allowed to freeze for 24 h, followed by thawing at 25oC. A fluorometer (Polar-Star BMG Tech) was used to record the readings at excitation and emission wavelengths of 530 and 620 nm for each well. The percentage cytotoxicity of compounds was calculated using the following formula:

$$\% \text{ Cytotoxicity} = \frac{A_C - A_{TC}}{A_C} \times 100$$

Where A*TC*= Absorbance of treated cells and AC= Absorbance of control. Erlotinib was used as positive control in the assay.

4.2.2. EGFR inhibitory assay

Baculoviral expression vectors including pBlueBacHis2B and pFASTBacHTc were used separately to clone 1.6 kb cDNA coding for EGFR cytoplasmic domain (EGFR-CD, amino acids 645–1186). 5′ upstream to the EGFR sequence comprised a sequence that encoded (His)6. Sf-9 cells were infected for 72h for protein expression. The pellets of Sf-9 cells were solubilized in a buffer containing sodium vanadate (100 µM), aprotinin (10 µg/mL), triton (1%), HEPES buffer(50mM), ammonium molybdate (10 µM), benzamidine HCl (16 µg/mL), NaCl (10 mM),leupeptin (10 µg/mL) and pepstatin (10 µg/mL) at 0°C for 20 min at pH 7.4, followed by centrifugation for 20 min. To eliminate the nonspecifically bound material, a Ni-NTA super flow packed column was used to pass through and wash the crude extract supernatant first with 10mM and then with 100 mM imidazole. Histidine-linked proteins were first eluted with 250 and then with 500 mM imidazole subsequent to dialysis against NaCl (50 mM), HEPES (20 mM), glycerol (10%) and 1 µg/mL each of aprotinin, leupeptin and pepstatin for 120 min. The purification was

performed either at 4 °C or on ice. To record autophosphorylation level, EGFR kinase assay was carried out on the basis of DELFIA/Time-Resolved Fluorometry. The compounds were first dissolved in DMSO absolute, subsequent to dilution to appropriate concentration using HEPES (25 mM) at pH 7.4. Each compound (10 µL) was incubated with recombinant enzyme (10 µL, 5 ng for EGFR, 1:80 dilution in 100 mM HEPES) for 10 min at 25^{o}C, subsequent to the addition of 5X buffer (10 µL, containing 2 mM MnCl2, 100 µM Na3VO4, 20 mM HEPES and 1 mM DTT) and ATP-MgCl2 (20 µL, containing 0.1 mM ATP and 50 mM MgCl2) and incubation for 1h. The negative and positive controls were included in each plate by the incubation of enzyme either with or without ATP-MgCl2. The liquid was removed after incubation and the plates were washed thrice using wash buffer. Europium-tagged antiphosphotyrosine antibody (75 µL, 400 ng) was added to each well followed by incubation of 1h and then washing of the plates using buffer. The enhancement solution was added to each well and the signal was recorded at excitation and emission wavelengths of 340 at 615 nm. The autophosphorylation percentage inhibition by compounds was calculated using the following equation:

$$100\% - [(negative\ control)/(positive\ control) - (negative\ control)]$$

Using the curves of percentage inhibition of eight concentrations of each compound, IC50 was calculated. Majority of signals detected by antiphosphotyrosine antibody were from EGFR because the enzyme preparation contained low impurities.

4.2.3. VEGFR-2 inhibitory assay

Prepare all the solutions using autoclaved, deionized water and analytical grade reagents. Prepare and store all the reagents at room temperature (unless indicated otherwise). EDTA solution (0.5 M, pH 8.0): add 95 mL ultra-pure water to 14.612 g EDTA, adjust the pH to 8.0 using NaOH solution, add 5 mL ultra-pure water; 1×Kinase Assay Buffer: add 25 mL HEPES solution (1 M), 190.175 mg EGTA, 5 mL MgCl2 solution (1 M), 1 mL DTT, 50 μL tween-20, 450 mL ultrapure water, adjust pH to 7.5 and constant volume to 500 mL using ultrapure water; 4×Stop solution (40 mM): Mix 0.8 mL of the foregoing EDTA solution, 1 mL 10×Detection Buffer and 8.2 mL ultra-pure water; 1×Detection Buffer: Mix 1 mL 10×Detection Buffer with 9 mL water; 4×VEGFR Kinase solution (1.74 nM, 521 times diluted, stored on ice): 1.2 μL VEGFR mother liquor (0.909 μM) was added to 624 μL 1×Kinase Assay Buffer and mixed; 4×ULight™-labeled JAK1 (substrate) (200 nM, 25 times diluted): 24 μL ULight™-labeled JAK1 (mother liquor concentration 5 μM) was added to 576 μL 1×Kinase Assay Buffer and mixed; 4×ATP Solution (40 μM, 250 times diluted): add 3 μL ATP solution (10 mM) to 747 μL 1×Kinase Assay Buffer and mixed; 4×Detection Mix (8 nM, 390.6 times diluted): 3 μL Europium- antiphospho-tyrosine antibody (PT66) (3.125 μM) was added to 1169 μL1×Detection Buffer and mixed; 2×substrate/ATP Mix: 560 μL foregoing 4×ULight™-labeled JAK1 and 560 μL 4×ATP solution and mixed (prepared before use). The assays used an ULight-labeled peptide substrate and a Europium-W1024-labeled antiphosphotyrosine antibody. The VEGFR-2 was purchased from Carna Biosciences, Inc. (New York, USA). The 384-well plates were obtained from PerkinElmer. Compounds were dissolved in DMSO and diluted to 11 concentrations at a tripling rate from 2.500 μM to 0.042 nM and added 2.5 μL to 384-well plates. 5 μL 2×VEGFR-2 kinase solution (0.5 nM) was added to 384-well plates homogeneous mixing and pre-reaction at room temperature

for 30 min. Next, 2.5 μL 4×Ultra ULightTM-JAK-1(Tyr1023) Peptide (200 nM)/ATP (40 μM) was added to the corresponding wells of a 384-well plate. Negative control: 2.5 μL/well 4×substrate/ATP mixture and 7.5 μL 1×kinase assay buffer in 384-well plate well. Positive control: 2.5 μL/well 4×substrate/ATP mixture, 2.5 μL/well 1×kinase assay buffer with 16% DMSO, 5 μL/well 2×VEGFR-2 kinase solution was added to the 384-well plate, The final concentration of DMSO in the mixing system was 4%. After incubation at room temperature and dark for 60 min, 5 μL 4×stop solution was added to corresponding wells to react for 5 min and then 5 μL 4×detection mix was added to the corresponding wells of a 384-well plate. The mixture was centrifugally mixed and stayed for 60 min at room temperature for color development. The plate was read using a Envision plate reader. The inhibition rate (%) = (positive well reading-compound well reading)/(positive well reading-negative well reading)×100. The corresponding IC_{50} values were calculated using GraphPad Prism 5.0.

Apoptotic markers assay (Enzyme-linked Immunosorbent assay)

The microplate provided in this kit has been pre-coated with an antibody specific to p53, BAX, caspase 3, caspase 6, BCL-2, and CK 18. Standards or samples are then added to the appropriate microplate wells with a biotin-conjugated antibody specific to p53, BAX, caspase 3, caspase 6, BCL-2, and CK 18. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain p53, BAX, caspase 3, caspase 6, BCL-2, and CK 18, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. The concentration of p53, BAX,

caspase 3, caspase 6, BCL-2, and CK 18 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Average the duplicate readings for each standard, control, and samples and subtract the average zero standard optical density. Construct a standard curve by plotting the mean O.D. and concentration for each standard and draw a best fit curve through the points on the graph or create a standard curve on log-log graph paper with p53, BAX, caspase 3, caspase 6, BCL-2, and CK 18 concentration on the y-axis and absorbance on the x-axis. Using some plot software, for instance, curve expert 1.30, is also recommended. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Procedure

1. Determine wells for diluted standard, blank and sample. Prepare 7 wells for standard, 1 well for blank. Add 100 μL each of dilutions of standard (read Reagent Preparation), blank and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 1 h at 37 oC.

2. Remove the liquid of each well, don't wash.

3. Add 100 μL of Detection Reagent A working solution to each well, cover the wells with the plate sealer and incubate for 1 h at 37 oC.

4. Aspirate the solution and wash with 350 μL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser, or auto washer, and let it sit for 1~2 min. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

5. Add 100 μL of Detection Reagent B working solution to each well, cover the wells with the plate sealer and incubate for 30 min at 37 oC.

6. Repeat the aspiration/wash process for total 5 times as conducted in step 4.

7. Add 90 μL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for 10-20 min at 37 oC (Don't exceed 30 min). Protect from light. The liquid will turn blue by the addition of Substrate Solution.

8. Add 50μL of Stop Solution to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.

9. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

4.3. Molecular Docking

The structures of Human Epidermal Growth Factor Receptor (PDB code: 1M17) and Human Vascular Endothelial Growth Factor Receptor 2 (PDB code: 4ASD) were downloaded from the Protein Data Bank. Chemical structures were drawn and optimized using the molecular editors Marvin Sketch and Avogadro. Preparation of both protein structures was performed using Autodock tools v1.5.7, involving the removal of co-crystallized water molecules and reference compounds (erlotinib and sorafenib), followed by the addition of Kollman charges and polar hydrogens. The grid coordinates for EGFR were set to 39 x 66 x 51 for the x, y, and z axes, respectively, with grid spacing of 0.375 Å, while the grid coordinates for VEGFR-2 were set to −23.756 x −1.152 x −11.701 for the x, y, and z axes, respectively, , with grid spacing of 0.375 Å.

Autodock vina was used for molecular docking and the best docking poses was visualized using Biovia Discovery Studio Visualizer 2024.

Compound	Protein	Binding affinity (kcal/mol)	Amino acids
12	EGFR	-7.0684	Met769, Cys773, Leu694, Leu768, Gly772, Val702, Leu820
	VEGFR-2	-5.6495	Glu885, Cys1045, Phe1047, Leu889, Val898, Val899, Val848, Val916, Leu1035
15	EGFR	8.2422	Met769, Thr830, Gln767, Thr766, Leu820, Met742, Ala719, Val702, Lys721, Leu764
	VEGFR-2	5.8777	Glu885, Ala866, Phe918, Phe1047, Leu889, Val848,

			Lys868, Val916, Leu1035, Cys1045, Leu840
Erlotinib	EGFR	-8.1817	Met769, Leu694, Pro770, Gln767, Leu820, Lys721, Leu764, Ala719
Sorafenib	VEGFR-2	-10.1476	Asp1046, Cys919, Glu885, His1026, Glu917, Ile1044, Leu1035, Phe1047, Leu840, Leu1019, Phe918, Leu889, Val848, Ala866, Lys868, Val899, Val916, Cys1045

4.4. Statistical analysis

Computerized Prism 5 program was used to statistically analyzed data using one-way ANOVA test followed by Tukey's as post ANOVA for multiple comparison at $P \leq .05$. Data were presented as mean ± SEM.