Supplementary Information

Ferulic and *p*-Coumaric Acid Derivatives as Dual EGFR-VEGFR2 Inhibitors: Design, Semi-Synthesis and Biological Investigations

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Supplementary Information

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3. **Material and methods**

3.1 chemistry

3.1.1. Instrument

On a Stuart melting point apparatus (SMP10), the uncorrected melting points of all materials were measured. On Pye-Unicam SP-3-300 infrared spectrophotometer (KBr disks), the IR spectra were captured and expressed in wave number (cm^{-1}). On a Bruker Avance III NMR spectrometer, 400 MHz, 1H-NMR spectra were run. At the School of Pharmacy, Ain Shams University, the ^{13}C-NMR spectra (δ, ppm) were conducted at 100 MHz and 125 MHz on a BRUKER NMR spectrometer (BRUKER, Manufacturing & Engineering Inc., Anaheim, CA, USA). TMS was utilized in deuterated dimethyl sulfoxide as an internal standard (DMSO-d6). Ppm is used to quote

chemical shits (δ). These acronyms are used: s for singlet; d for doublet; and m for multiplet. All values for the coupling constant (J) are given in hertz. On a Shimadzu GCMSQP-1000EX mass spectrometer, the mass spectra were captured at a 70 eV energy level.The reactions were monitored by thin-layer chromatography (TLC) using TLC sheets coated with UV fluorescent silica gel Merck 60 F254 plates and were visualized using a UV lamp and different solvents as the mobile phase.

3.1.2. Chemical and reagents

p-Comaric acid, ferulic acid, ethyl-2-chloroacetate, hydrazine hydrate , benzaldehyde, *m*-nitro benzaldehyde, anisaldehyde and 3,4,5 trimethoxy benzaldehyde were purchased from Aldrich (USA). Solvents and other reagents were of pure grade and used without further purification.

2-Ethoxy-2-oxoethyl (*E*)-3-(4-(2-ethoxy-2-oxoethoxy)-3-substituted phenyl) acrylate (1a-b)

p-Coumaric acid/ ferulic acid (0.1 mol) was dissolved in *N,N*-dimethylformamide (DMF 100 ml) and mixed with anhydrous potassium carbonate (0.16 mol). Ethyl 2-chloroacetate (0.1 mol) was added, and the mixture refluxed for 12 h. The reaction mixture was cooled and poured into crushed ice. The crude product was filtered and recrystallized from ethanol [1].

2-Ethoxy-2-oxoethyl (*E*)-3-(4-(2-ethoxy-2-oxoethoxy)phenyl)acrylate (1a)

White; Yield 81 %; M.p.; 118-120 °C; IR (cm^{-1}):1690(C=O),1248(C-O-C). EI-MS (m/z, %): 336 (M^+); ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.72 (d, *J*= 8 Hz, 2H, Ar-H), 7.69 (d, *J*= 16 Hz, 1H, CH=CH), 6.99 (d, *J*= 8 Hz, 2H, Ar-H), 6.60 (d, *J*= 16 Hz,1H, CH=CH), 4.86 (s, 2H, O-CH_2), 4.76 (s, 2H, COO-CH_2), 4.18 (q, *J*= 12 Hz, 4H, 2xCH_2 CH_3), 1.22 (t, *J*= 8 Hz,6H, 2xCH_2-CH_3). Anal.Calcd. for $C_{17}H_{20}O_7$: C, 60.71; H, 5.99; O, 33.30. Found: C,60.94 ; H,6.08.

2-Ethoxy-2-oxoethyl(E)-3-(4-(2-ethoxy-2-oxoethoxy)-3 methoxyphenyl)acrylate (1b)

White; Yield 81 %; M.p.; 88-90 °C; IR (cm^{-1}):1614(C=O),1275(C-O-C) EI-MS (m/z, %): 366 (M^+); ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.67 (d, *J*= 16 Hz, 1H, CH=CH), 7.46 (s, 1H, Ar-H), 7.26 (d, *J*= 8 Hz, 1H, Ar-H), 6.91 (d, *J*= 8 Hz, 1H, Ar-H), 6.68 (d, *J*= 16 Hz,1H, CH=CH), 4.84 (s, 2H, O-CH_2), 4.77 (s, 2H, COO-CH_2), 4.21-4.14 (m, 4H, 2xCH_2 CH_3),3.85

(s, 3H, OC<u>H</u>$_3$), 1.22 (t, *J*= 16 Hz,6H, 2xCH$_2$-<u>CH</u>$_3$). Anal.Calcd. for $C_{18}H_{22}O_8$: C, 59.01; H, 6.05; O, 34.94. Found: C,59.23; H,6.18.

2-Hydrazinyl-2-oxoethyl (*E*)-3-(4-(2-hydrazinyl-2-oxoethoxy)-3-substituted phenyl)acrylate (2a-b)

A mixture of compound **1a-b** (0.01 mol) and hydrazine hydrate (0.02 mol) in methanol was refluxed in a water bath for 9 h. After cooling the separated solid was washed with water and dried to give a crude product which were obtained after recrystallization from ethanol[2].

2-Hydrazinyl-2-oxoethyl(E)-3-(4-(2-hydrazinyl-2-oxoethoxy)phenyl)acrylate (2a)

White; Yield 89 %; M.p.; 176-178 °C; IR (cm^{-1}):3451-3429 (NH$_2$), 3296 (NH), 1615(C=O),1259(C-O-C) EI-MS (m/z, %): 308 (M^+); ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 9.38 (s, 2H, 2xNH), 7.68 (d, *J*= 8 Hz, 2H, Ar-<u>H</u>),7.62 (d, *J*= 16 Hz, 1H, C<u>H</u>=CH), 7.00 (d, *J*= 8 Hz, 2H, Ar-<u>H</u>), 6.51 (d, *J*= 16 Hz,1H, CH=C<u>H</u>), 4.55 (s, 2H, COO-<u>CH</u>$_2$), 4.35 (s, 4H, 2xNH$_2$), 3.72 (s, 2H, O-<u>CH</u>$_2$). Anal.Calcd. for $C_{13}H_{16}N_4O_5$: C, 50.65; H, 5.23; N, 18.17; O, 25.95. Found: C,50.83; H,5.44; N,18.40 .

2-Hydrazinyl-2-oxoethyl(E)-3-(4-(2-hydrazinyl-2-oxoethoxy)-3-methoxyphenyl) acrylate (2b)

Pale yellow; Yield 91 %; M.p.; 145-147 °C; IR (cm^{-1}):3201-3067 (NH$_2$), 2970(NH), 1657(C=O), 1264 (C-O-C). EI-MS (m/z, %): 338 (M^+); ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 9.20 (s, 2H, 2xNH), 7.60 (d, *J*= 16 Hz, 1H, C<u>H</u>=CH), 7.39 (s,1H, Ar-<u>H</u>), 7.23 (d, *J*= 8 Hz, 1H, Ar-<u>H</u>), 6.93 (d, *J*= 8 Hz, 1H, Ar-<u>H</u>), 6.59 (d, *J*= 16 Hz,1H, CH=C<u>H</u>), 4.52 (s, 2H, COO-<u>CH</u>$_2$), 4.34 (s, 4H, 2xNH$_2$),3.84 (s, 3H. OC<u>H</u>$_3$), 3.72 (s, 2H, O-<u>CH</u>$_2$). Anal.Calcd. for: $C_{14}H_{18}N_4O_6$:C, 49.70; H, 5.36; N, 16.56; O, 28.37. Found: C,49.94; H,5.48; N,16.73 .

General procedure for the synthesis of target compounds (3a-h)

A mixture of compound **2a-b** (0.01 mol) and appropriate aromatic aldehyde (0.01 mol) in the presence of trace amount of glacial acetic acid was refluxed for 5 h. (monitored by TLC) in methanol (20 mL). The solvent was removed under reduced pressure and the product was recrystallized from chloroform[3].

3.2.4 *In vivo* studies

3.2.4.1 Animals and tumor cell lines

Adult female Swiss albino mice purchased from Theodor Bilharzia Research Institute, Giza, Egypt, with an average body weight of (18-23) g were used. Mice were housed under constant conditions of 12 h light/dark cycle in a temperature-controlled environment under conditions of controlled humidity (22 ± 2 °C), with free access to standard laboratory mice food and water.

Solid Ehrlich carcinoma (SEC) cells were obtained from the National Cancer Institute (Cairo University, Egypt). The tumor cell line was proliferated in mice through serial intraperitoneal (I.P.) transplantation of a volume of 0.2 mL physiological saline containing 1×10^6 viable cells for 24 h. SEC cells were collected 7 days after I.P. implantation. The harvested cells were diluted with saline to obtain a concentration of 5×10^6 viable SEC cells/mL. A volume of 0.2 mL saline contains 1×10^6 SEC cells that were I.P. implanted into each normal mouse. SEC cells (1×10^6 tumor cells/mouse) were implanted subcutaneously into the right thigh of the hind limb.

The experimental animals were randomly divided into four groups. Group 1 served as the normal saline control. Group 2 served as the SEC control (1×10^6 cells/mouse). Group 3 served as the compound **3f**-treated group (6 mg/kg B.Wt., I.P.). Group 4 received the standard anti-cancer drug of Sorafenib (6 mg/kg BW, I.P.) and is considered the reference control. Body weight and survival were recorded daily until the 24th day in both treated and control groups. At the end of the experiment, the anesthetized animals were then sacrificed for the examination of the antitumor activity examination.

3.2.4.2. Antitumor potentiality

It includes tumor volume, weight, and tumor inhibition ratio (TIR%). Time interval measurements of tumor volume using a digital Vernier caliper (Tricle Brand, Shanghai, China). Measure tumor length and width using clipper and then calculate tumor volume using formulations V = (L x W x W)/2, where V is tumor volume, W is tumor width, L is tumor length. While TIR% was calculated according to the following equation $\frac{Tumor\ volume\ (Control) - Tumor\ volume\ (treated)}{Tumor\ volume\ (control)} x\ 100$.

3.2.4.3. Histological examinations

Solid ascites tissue samples are usually collected and fixed in neutral formalin. After fixation, the specimens undergo dehydration and clearing processes. The tissues are then embedded in paraffin wax to provide support for sectioning into thin slices of approximately 4-5 micrometers. These sections are stained with Hematoxylin and Eosin (H&E) to assess general tissue structure. The slides are then examined blindly under a light microscope to evaluate histopathological features and associated pathological changes according to Frajacomo, de Souza Padilha.

3.2.4.4. Immunohistochemical preparation

Sections (4 μm) were deparaffinized, rehydrated, and endogenous peroxidase activity was quenched using 3% hydrogen peroxide. Heat-induced epitope retrieval was performed in either citrate buffer (pH 6.0) or Tris-EDTA (pH 9.0) for 20 min at 95–100 °C, depending on the target antigen. After cooling and PBS washing, nonspecific binding was blocked with a commercial protein blocking solution. Sections were incubated with anti-BCL-2 (clone 124, 1:100, CST), anti-cleaved caspase-3 (Asp175, 1:100, CST), and anti-EGFR (clone 31G7, 1:50, Thermo Fisher). Detection was achieved with a polymer-based HRP/DAB system (Dako EnVision™ K5007) and hematoxylin counterstaining. Stained slides were examined under light microscopy, and five representative high-power fields (×400) per case were analyzed. For objective quantification, digital images were processed in ImageJ. Values from five fields per case were averaged for analysis.

Refernces

1 N. Manju, B. Kalluraya, K. Byrappa, M. Abdoh, *IUCrData* **2016**, *1*, 10.1107/S2414314616015947.
2 S. Kumar, P. Kumar, N. Sati, *Journal of Pharmacy & Bioallied Sciences* **2012**, *4*, 246.
3 R. Elrayess, N. Ghareb, M. Azab, M. Said, *Life Science Journal* **2020**, *10*, 1784-1793.

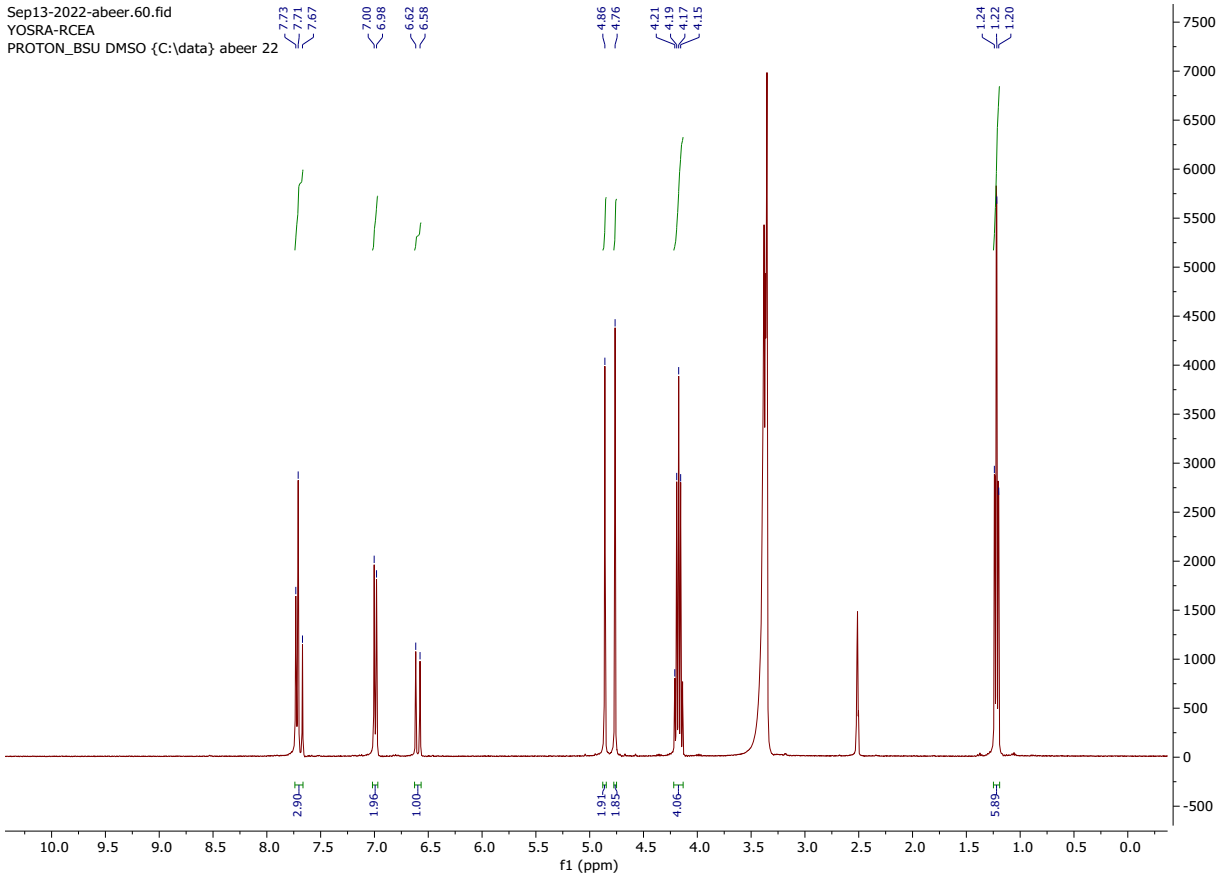


Fig. S1. ^{1}H-NMR of compound 1a

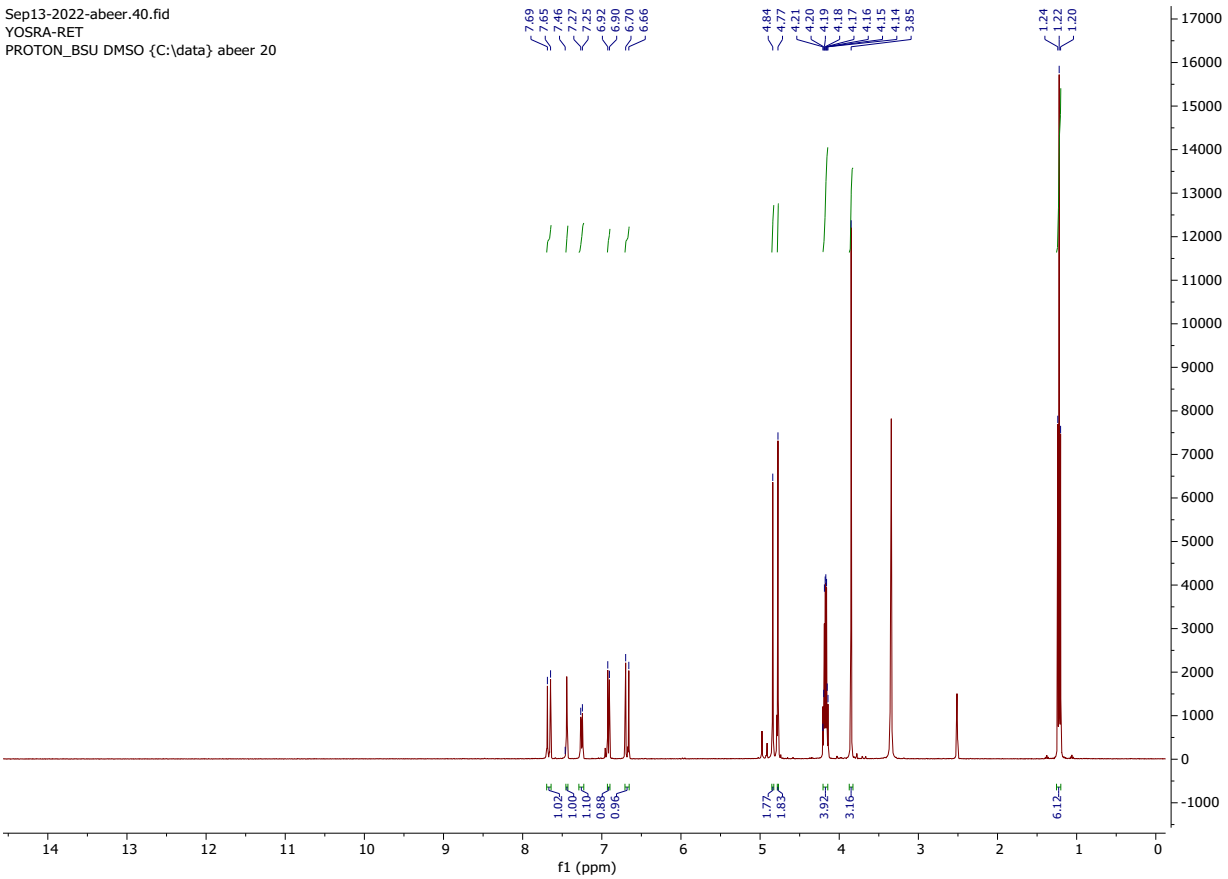


Fig. S2. ^{1}H-NMR of compound 1b

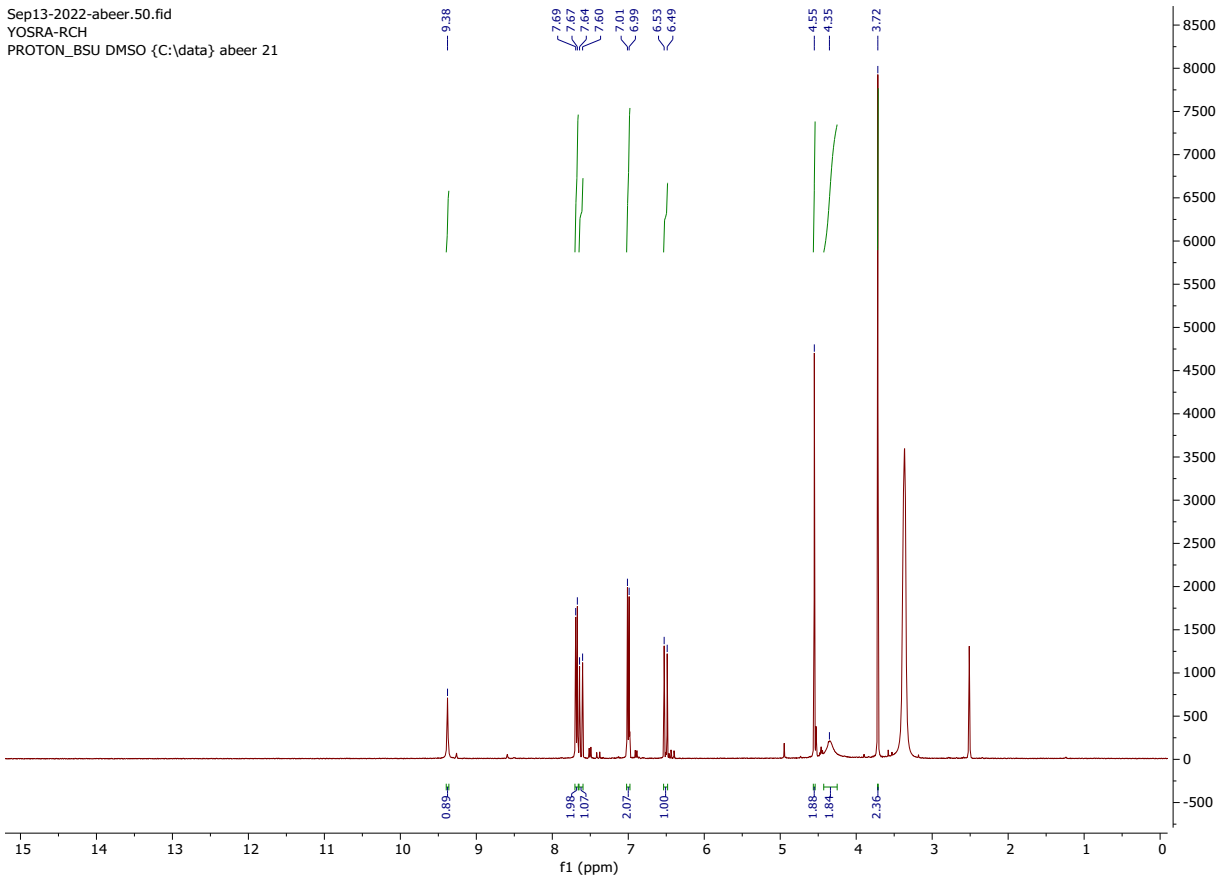


Fig. S3. ^{1}H-NMR of compound 2a

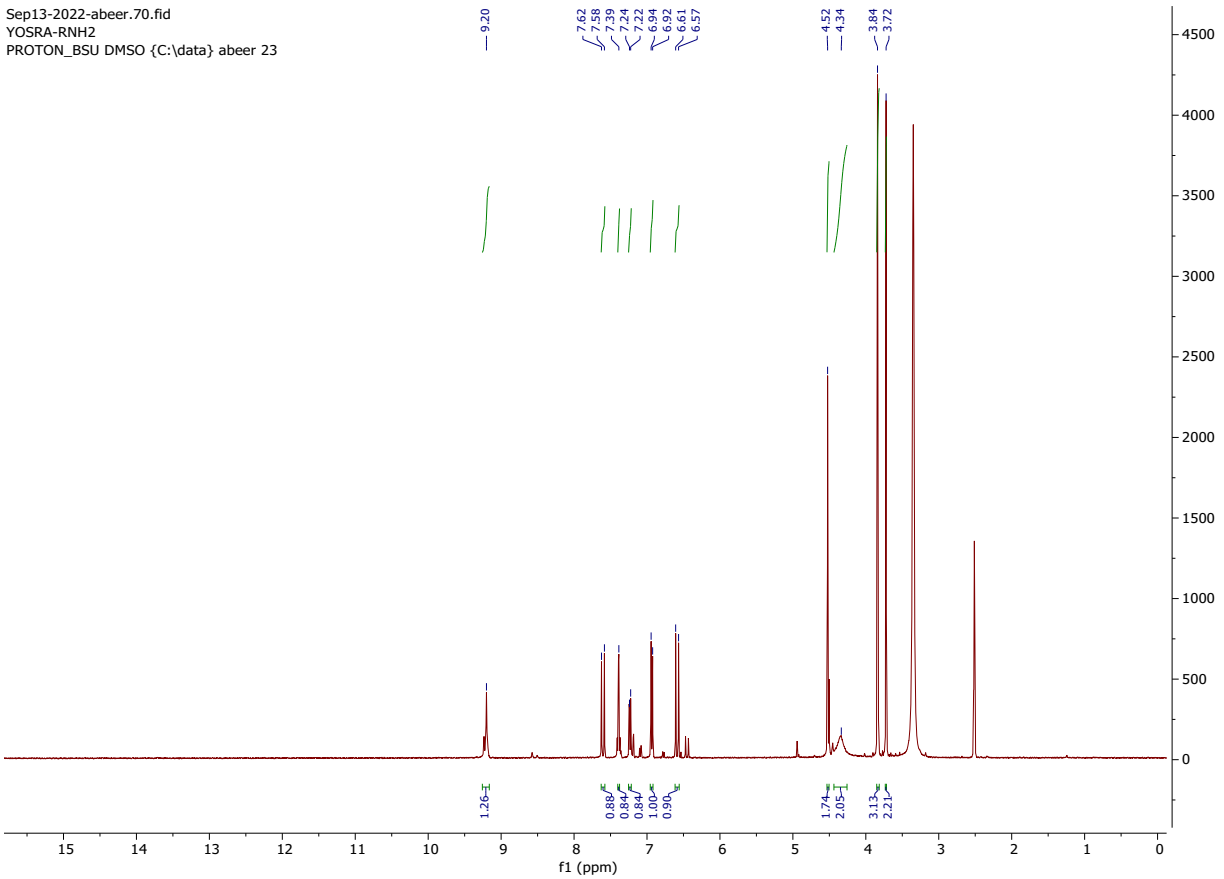


Fig. S4. ^{1}H-NMR of compound 2b

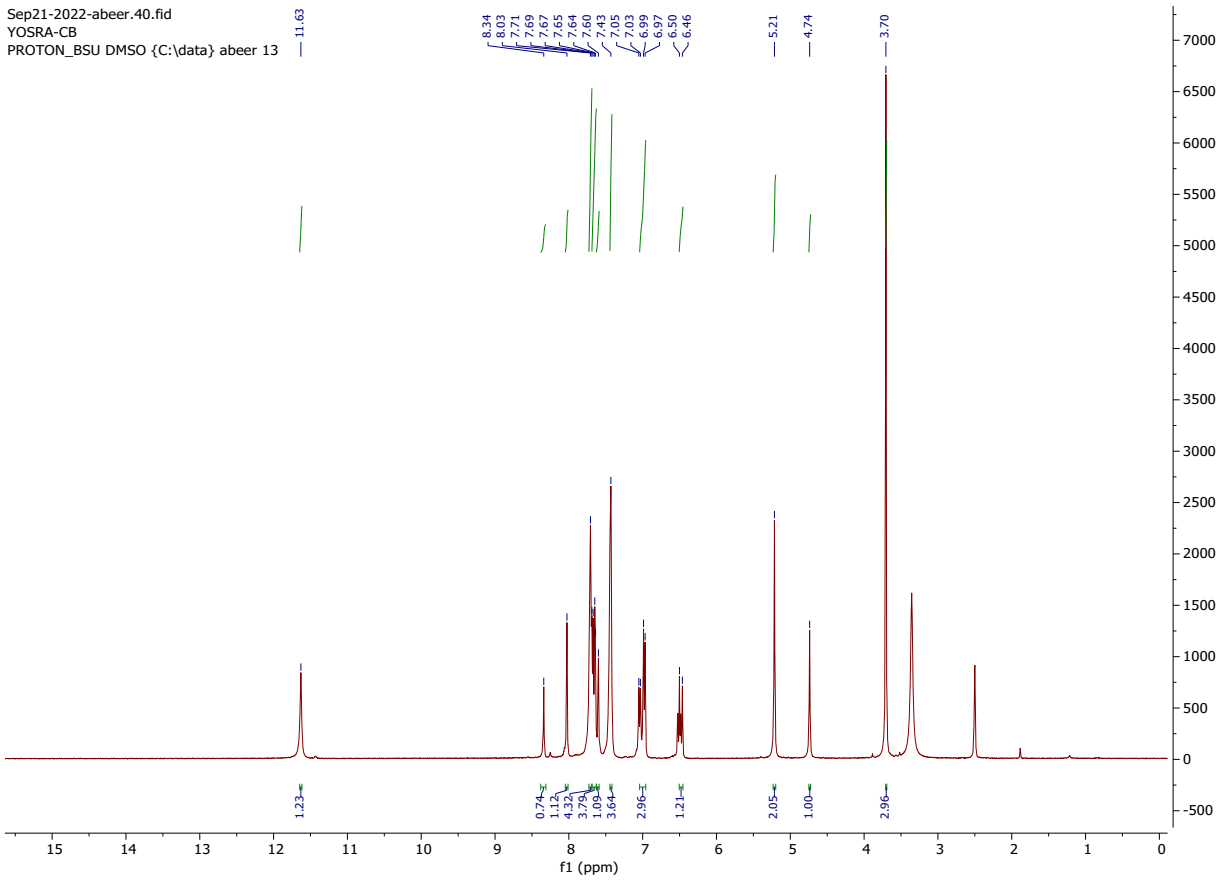


Fig. S5. ^{1}H-NMR of compound 3a

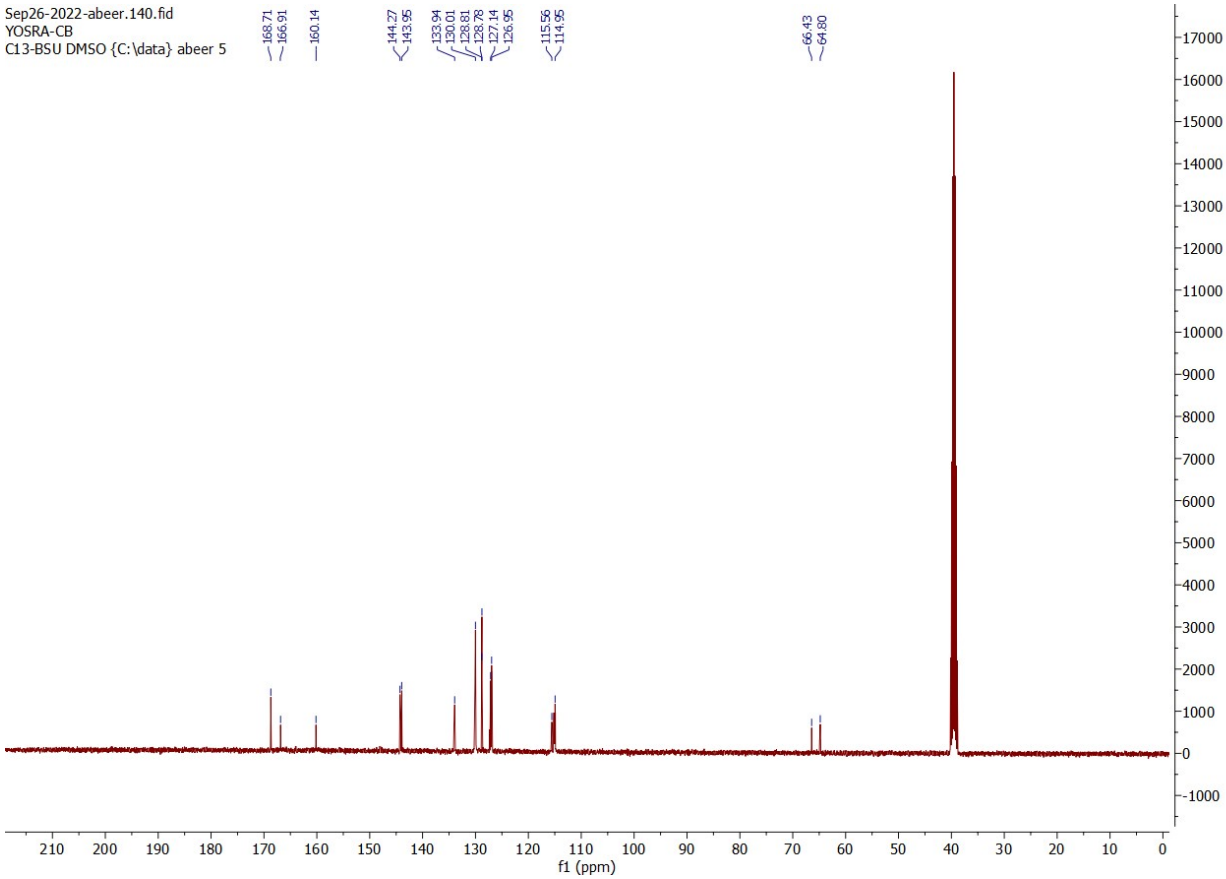


Fig. S6. ^{13}C-NMR of compound 3a

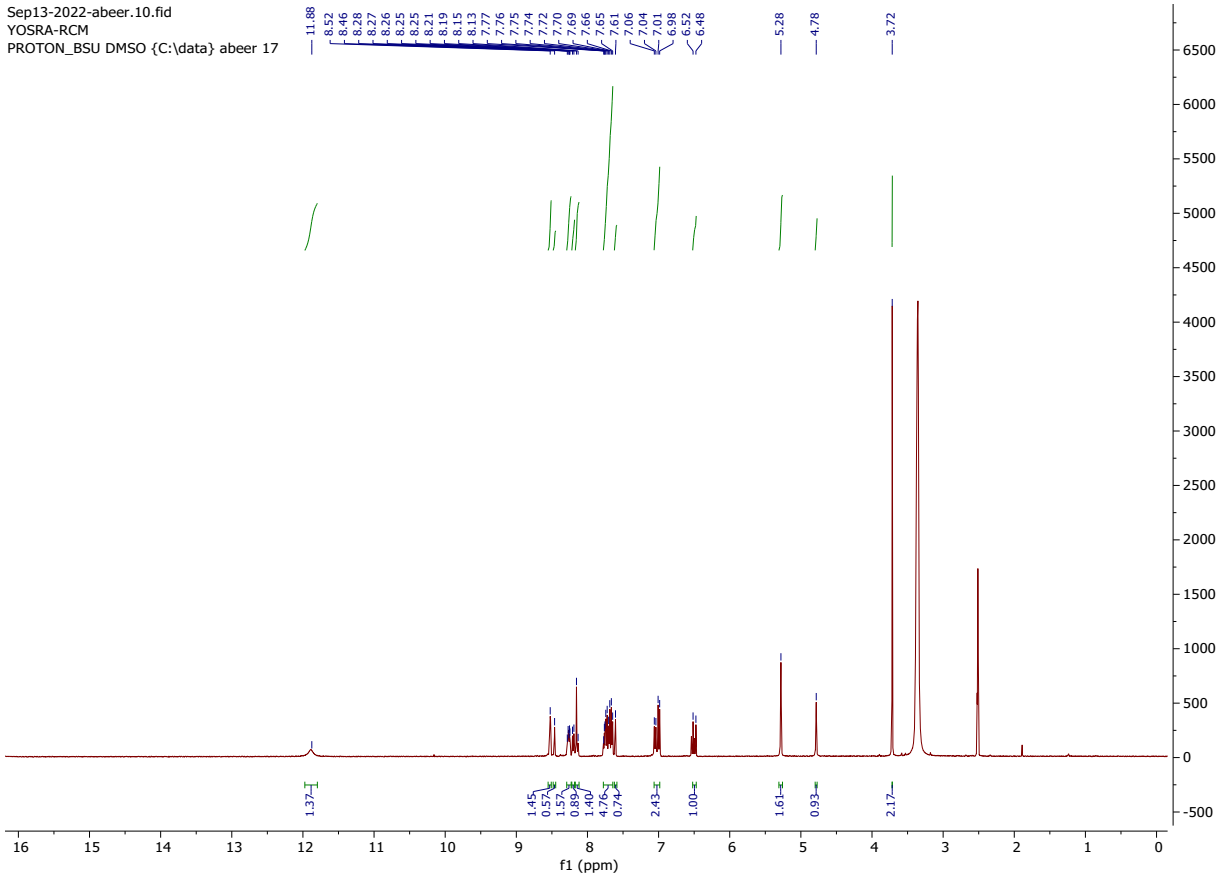


Fig. S7. ^{1}H-NMR of compound 3b

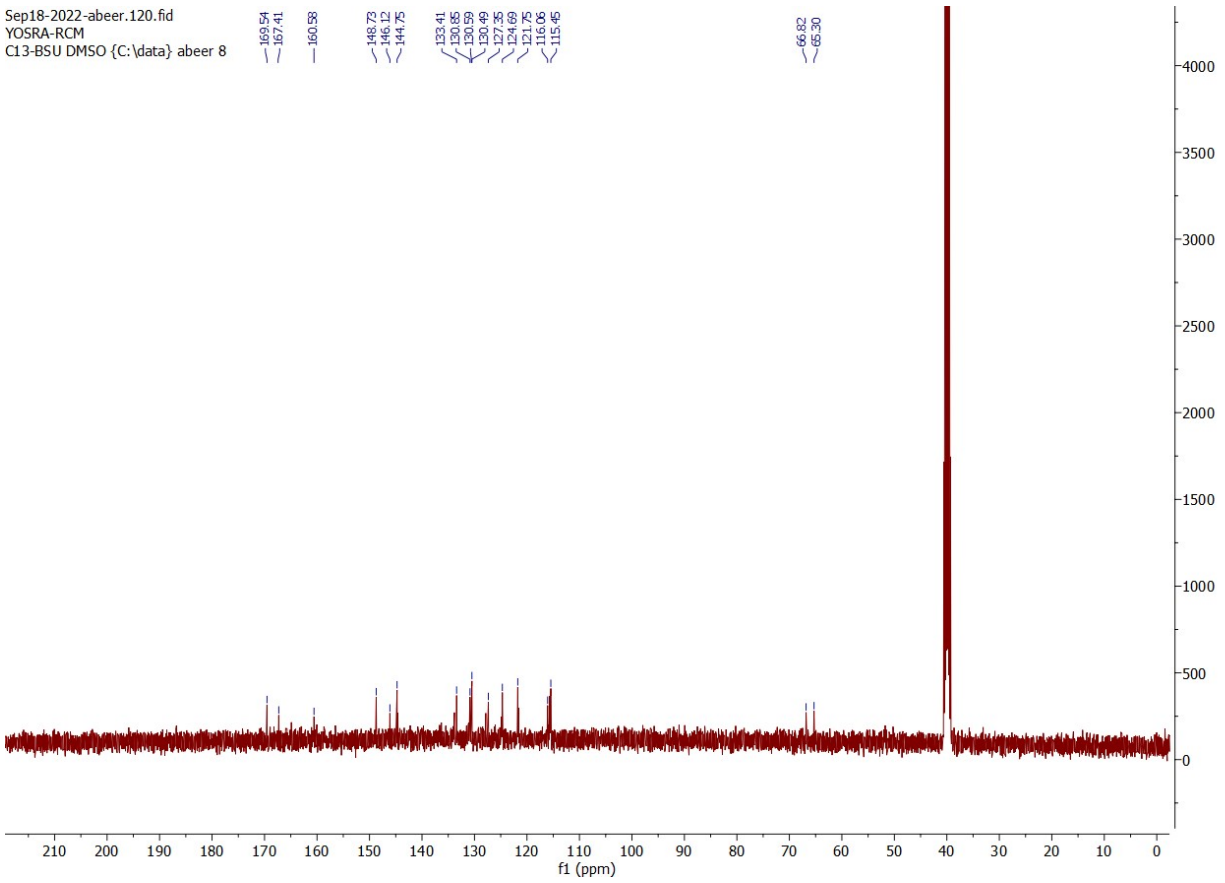


Fig. S8. ^{13}C-NMR of compound 3b

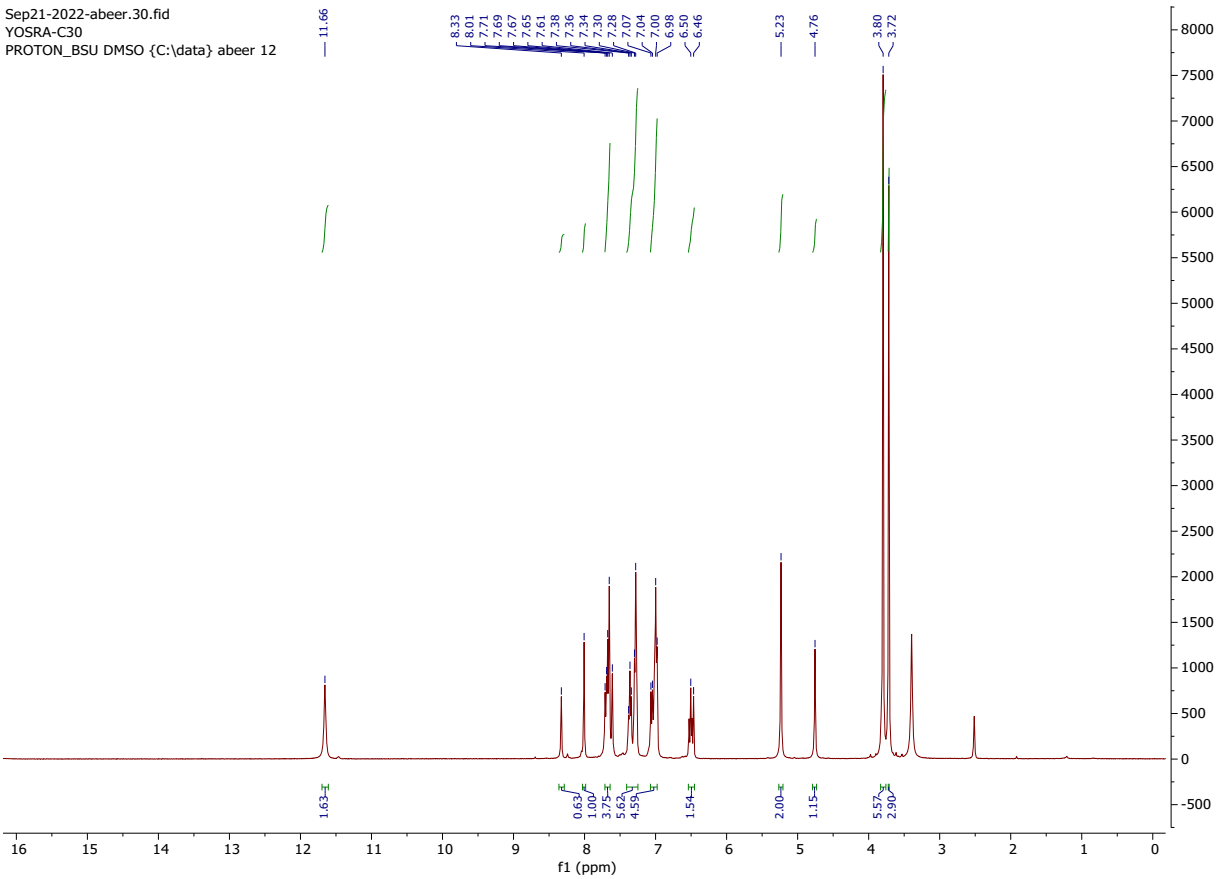


Fig. S9. ^{1}H-NMR of compound 3c

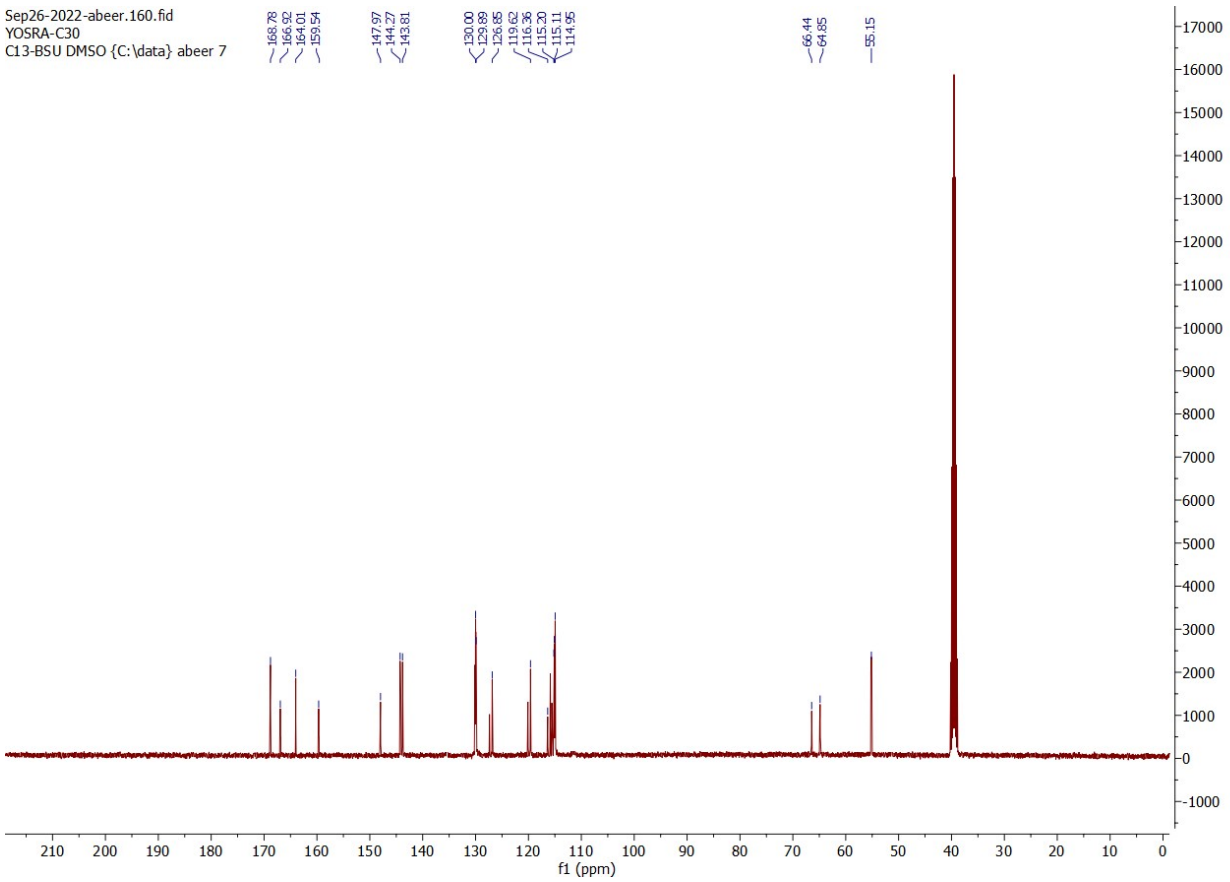


Fig. S10. ^{13}C-NMR of compound 3c

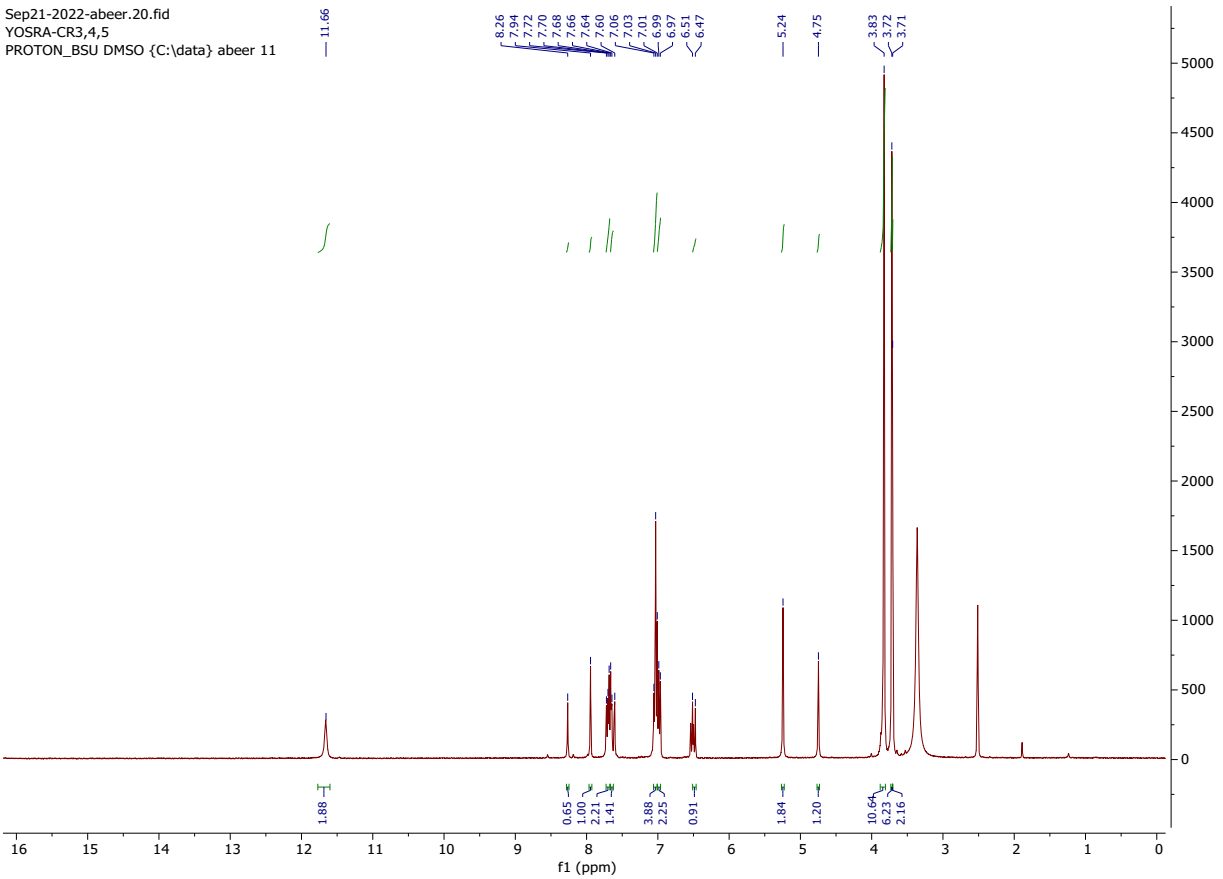


Fig. S11. ^{1}H-NMR of compound 3d

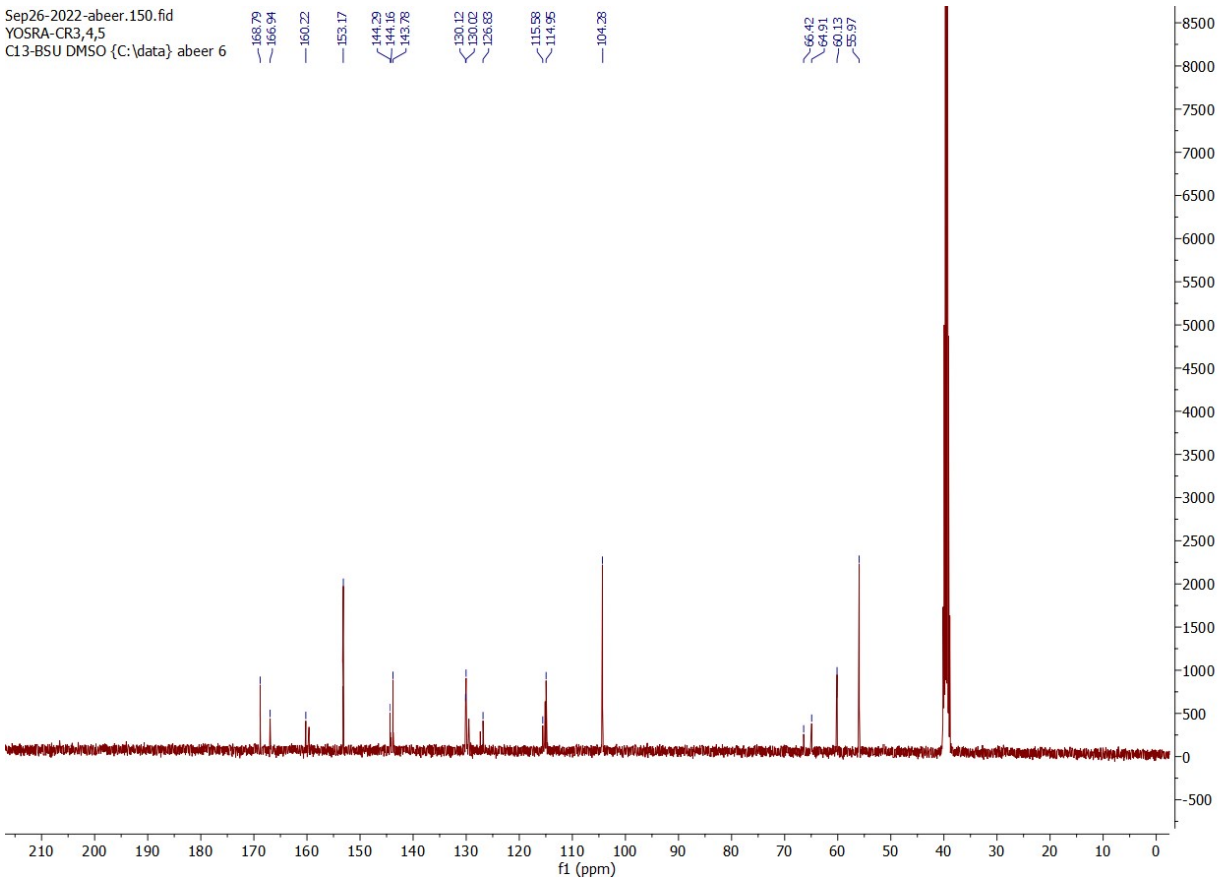


Fig. S12. ^{13}C-NMR of compound 3d

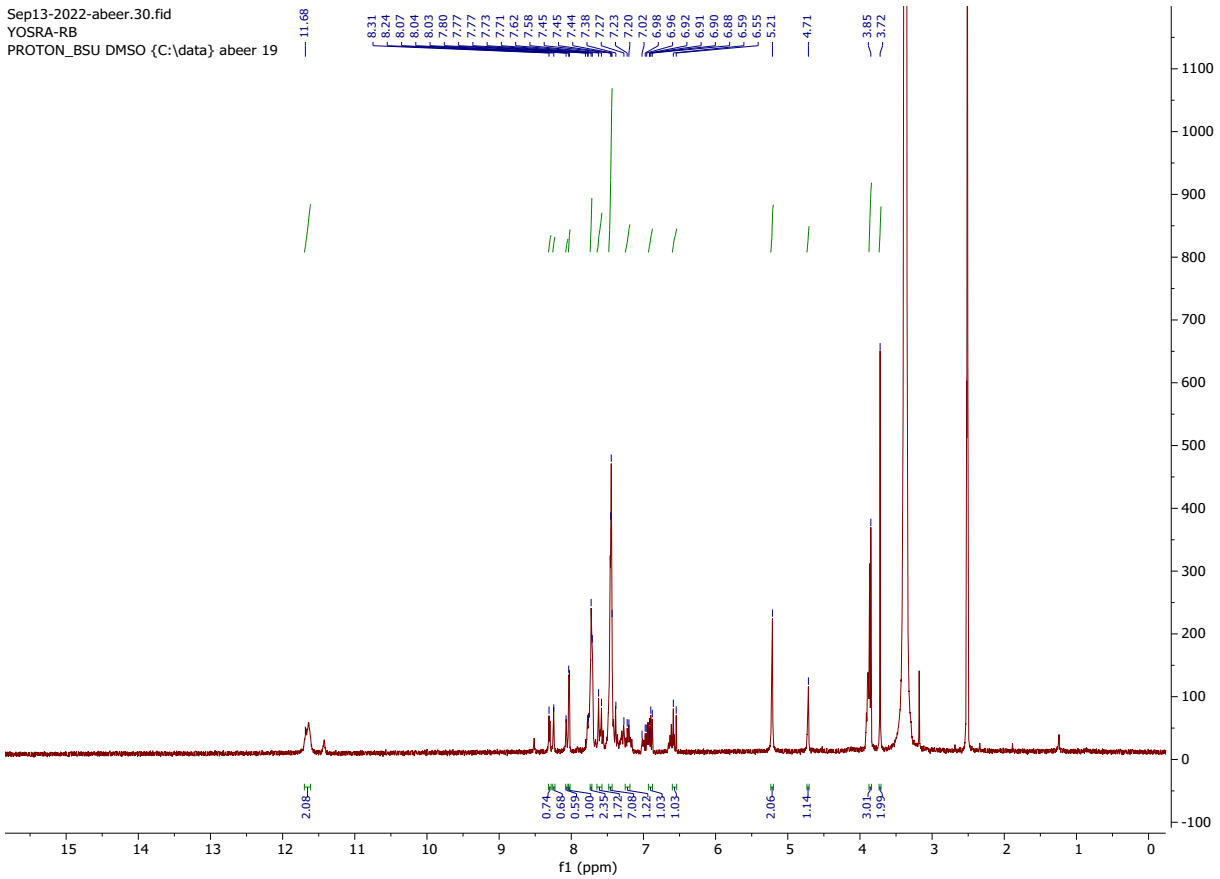


Fig. S13. ^{1}H-NMR of compound 3e

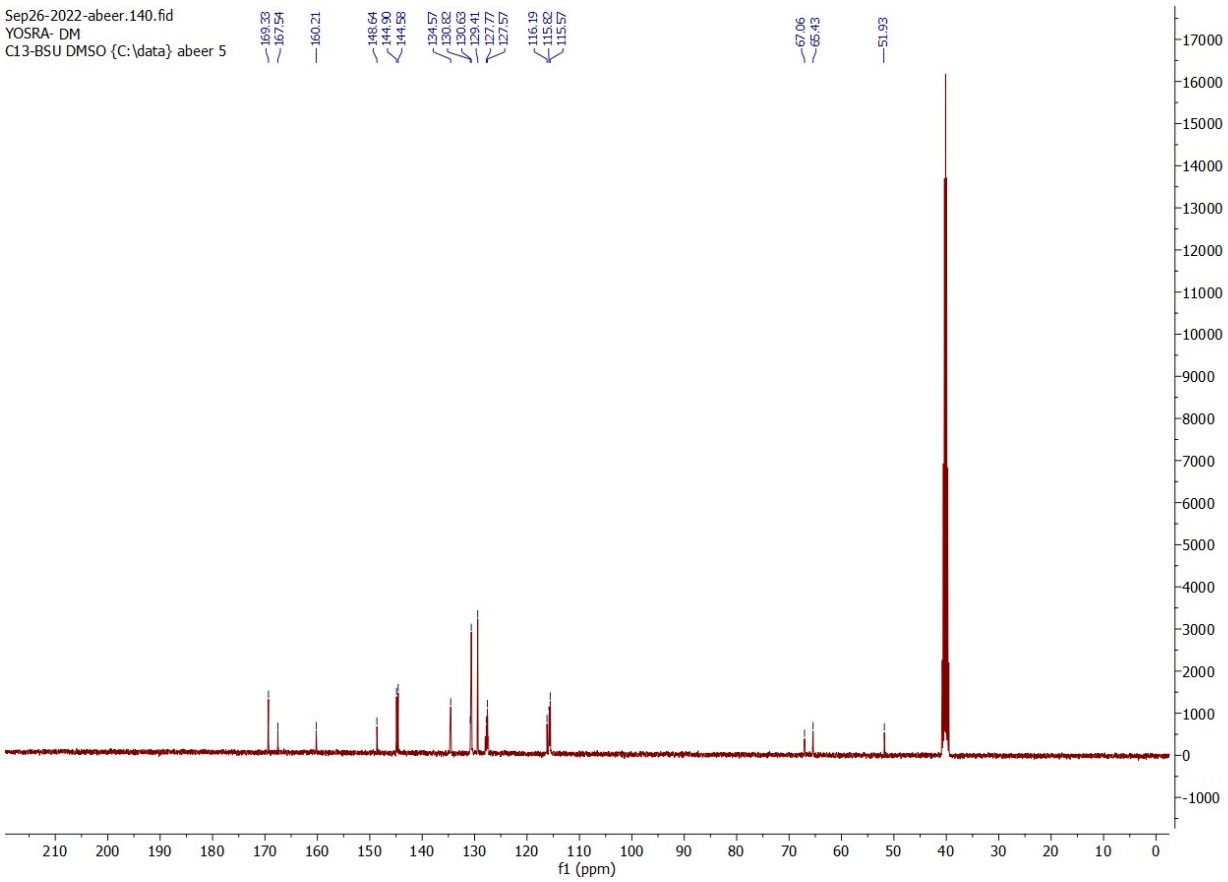


Fig. S14. ^{13}C-NMR of compound 3e

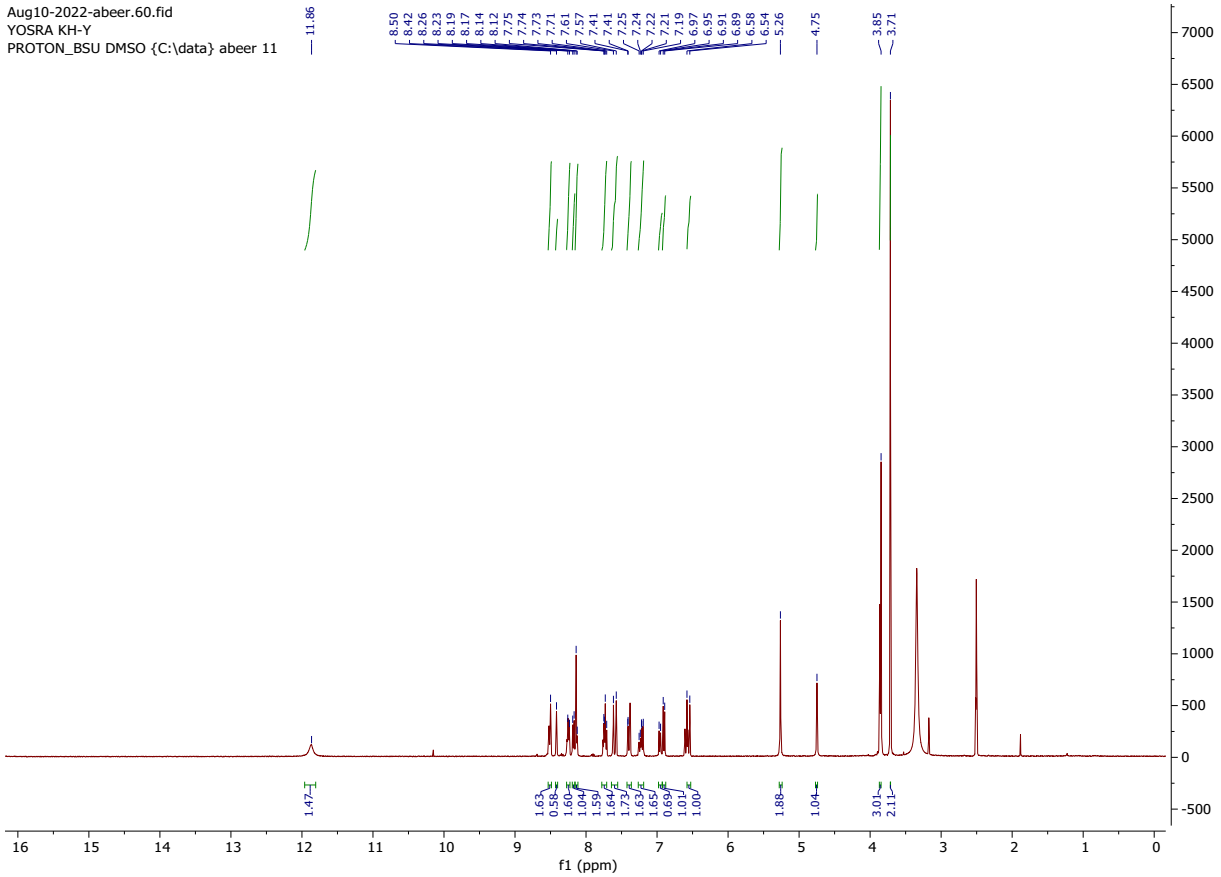


Fig. S15. ^{1}H-NMR of compound 3f

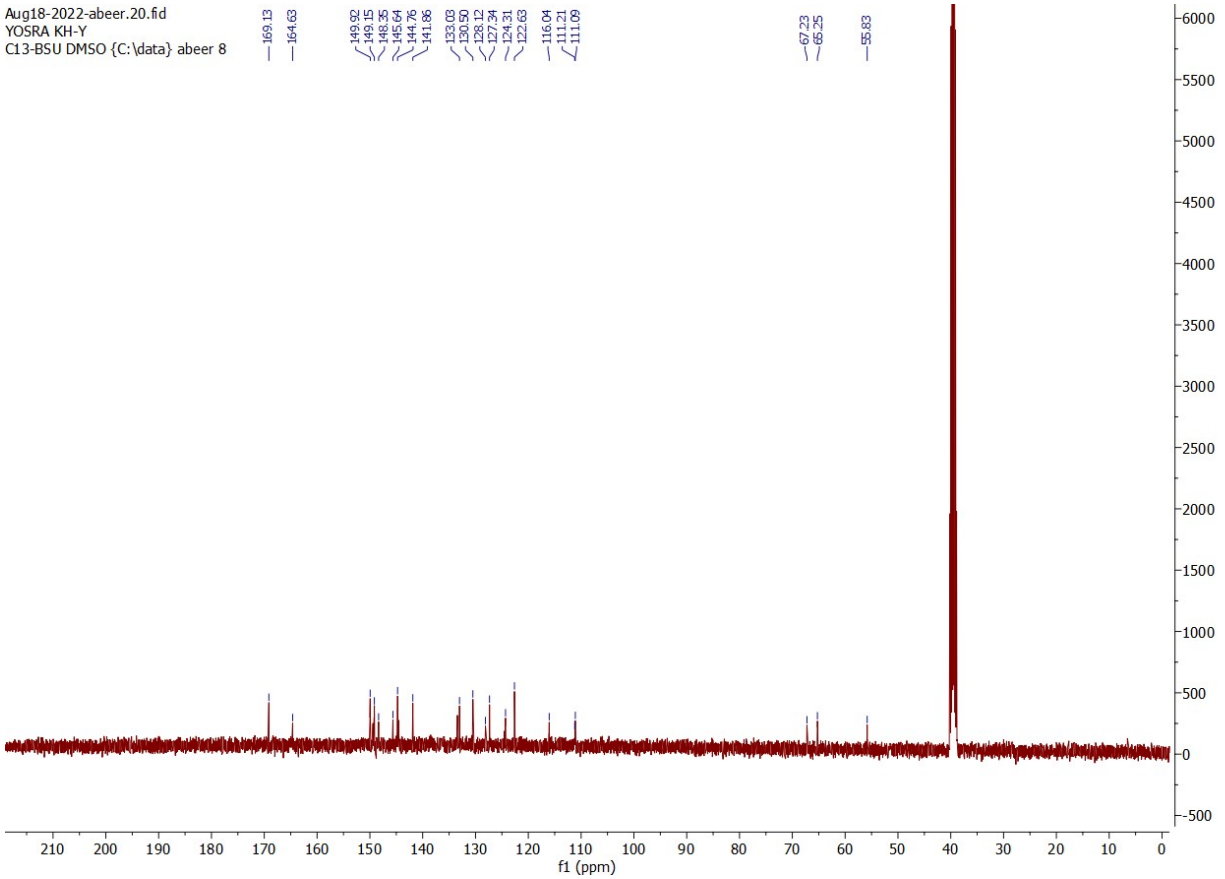


Fig. S16. ^{13}C-NMR of compound 3f

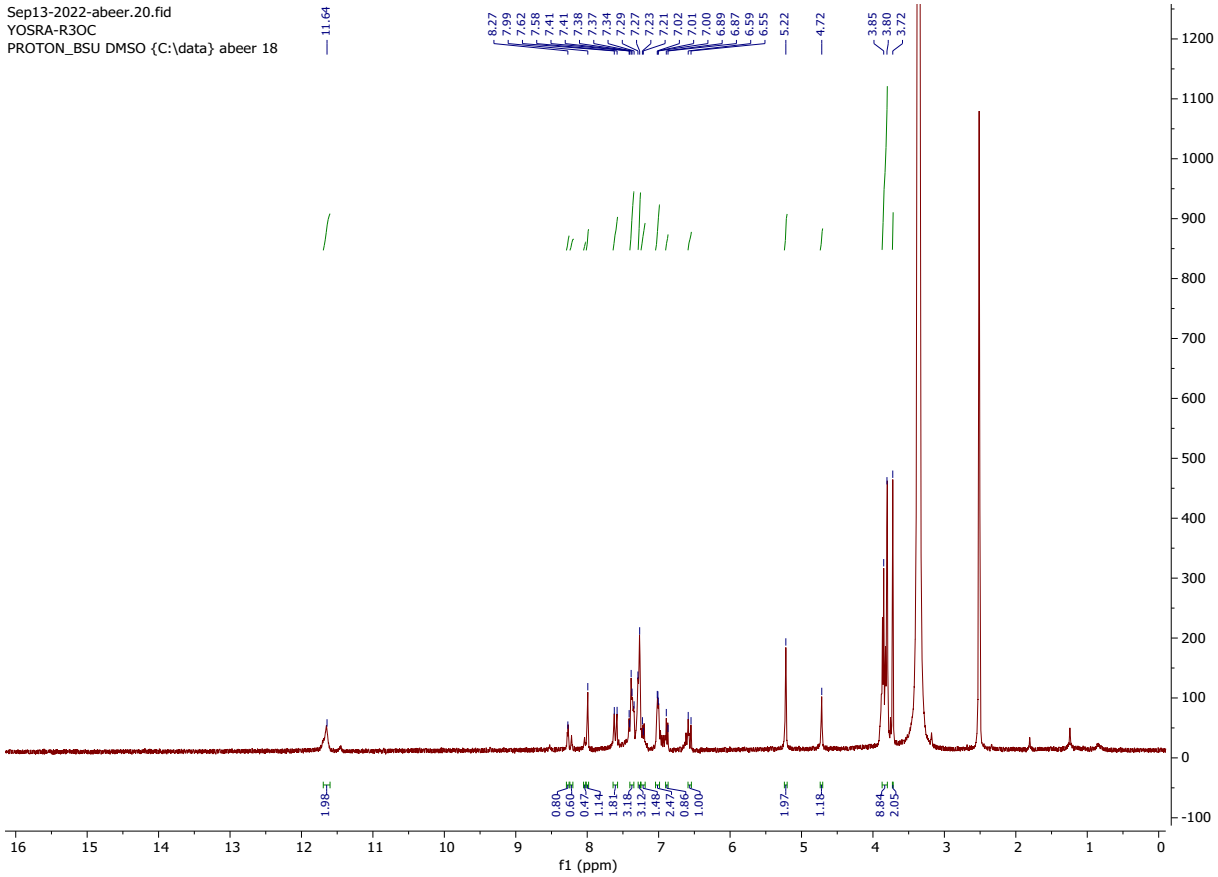


Fig. S17. ^{1}H-NMR of compound 3g

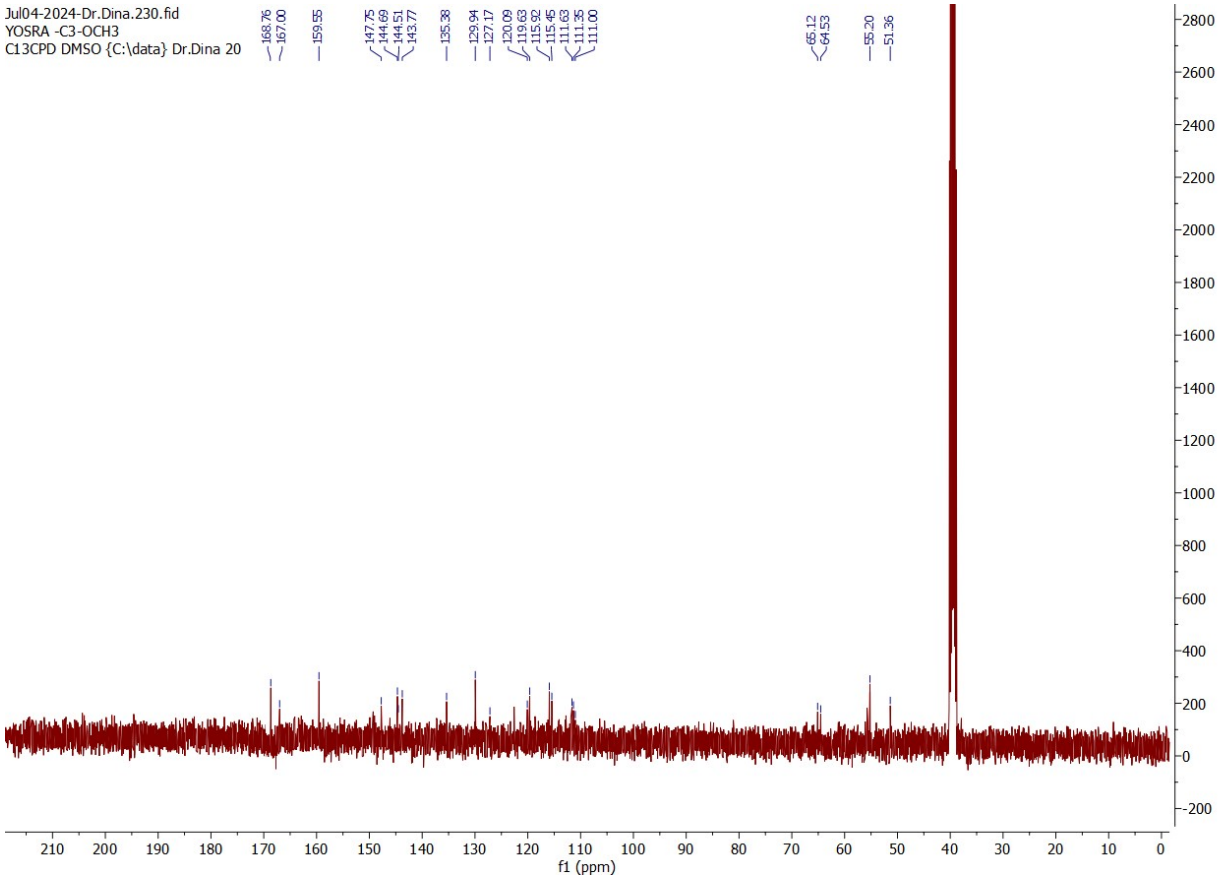


Fig. S18. ^{13}C-NMR of compound 3g

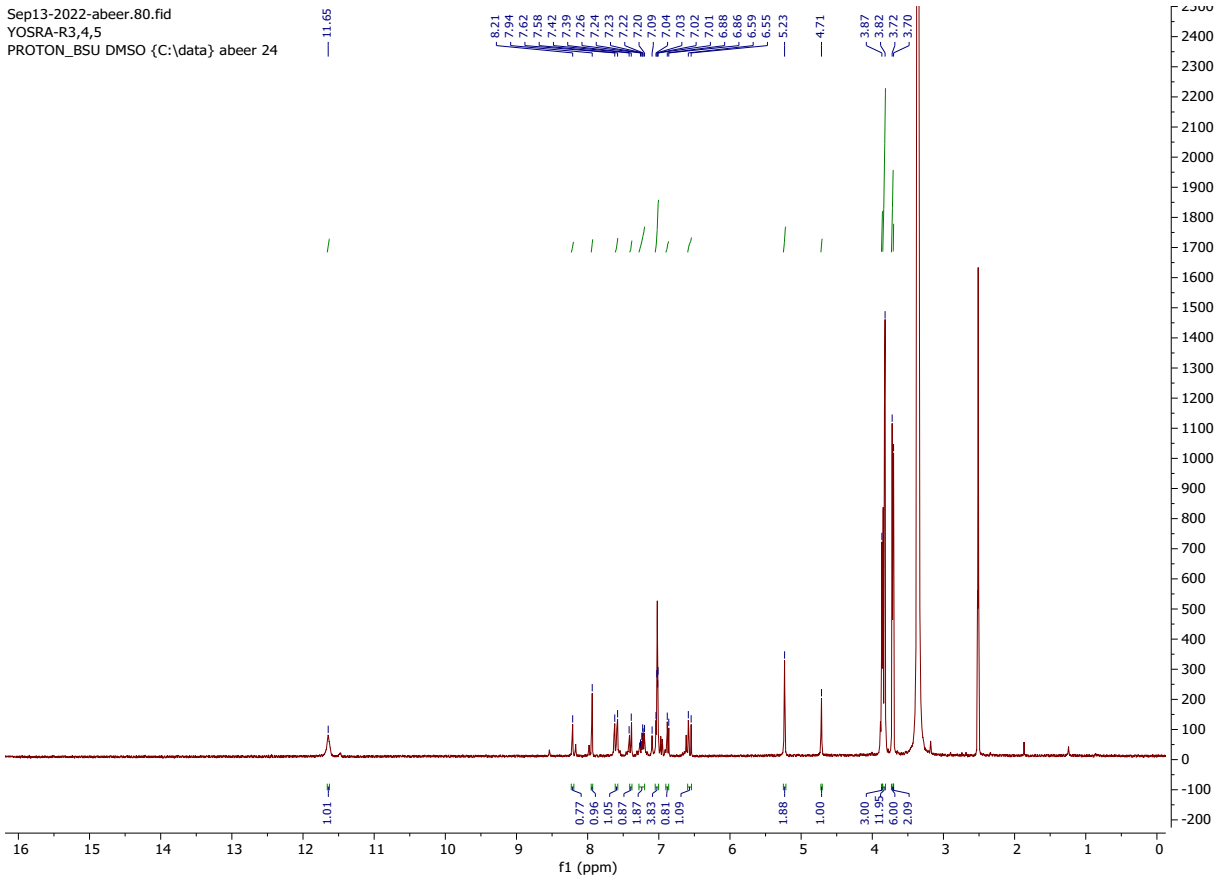


Fig. S19. ^{1}H-NMR of compound 3h

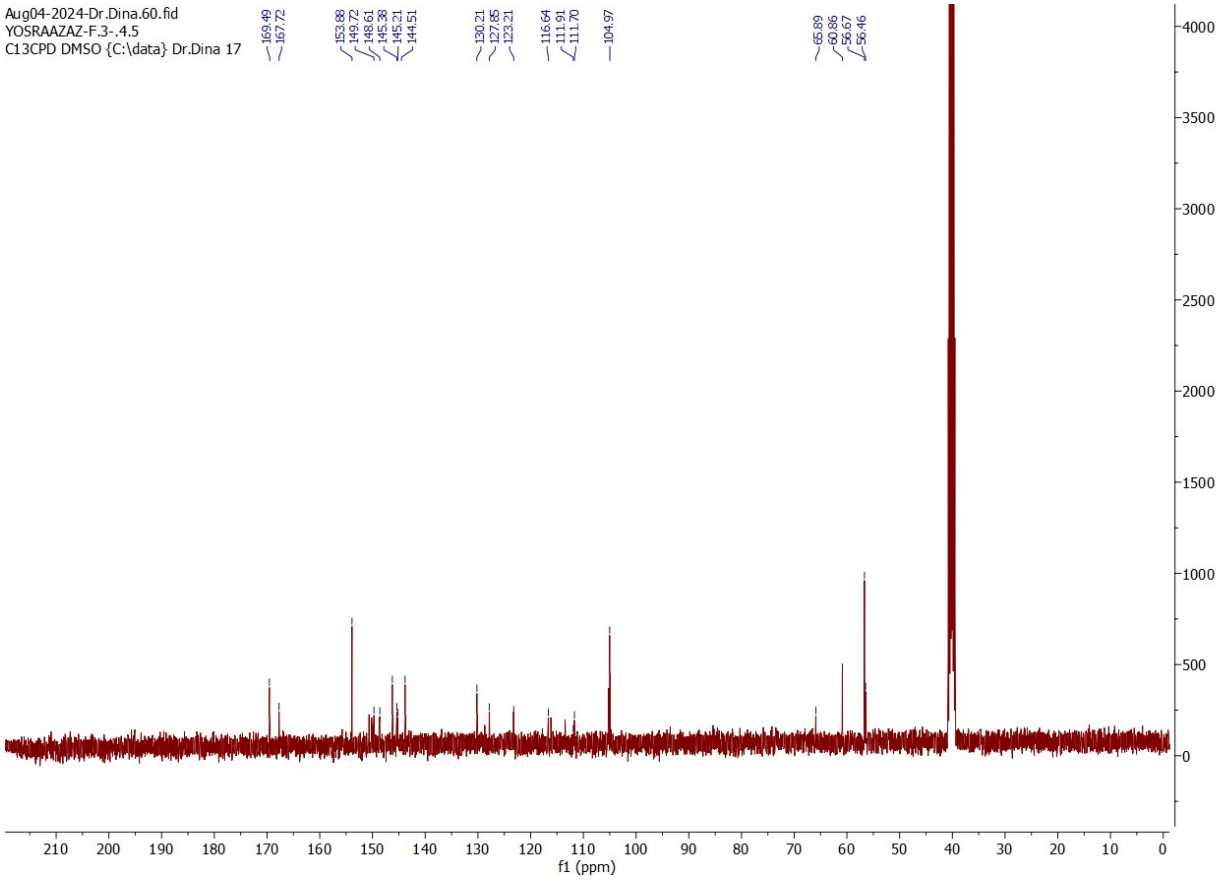
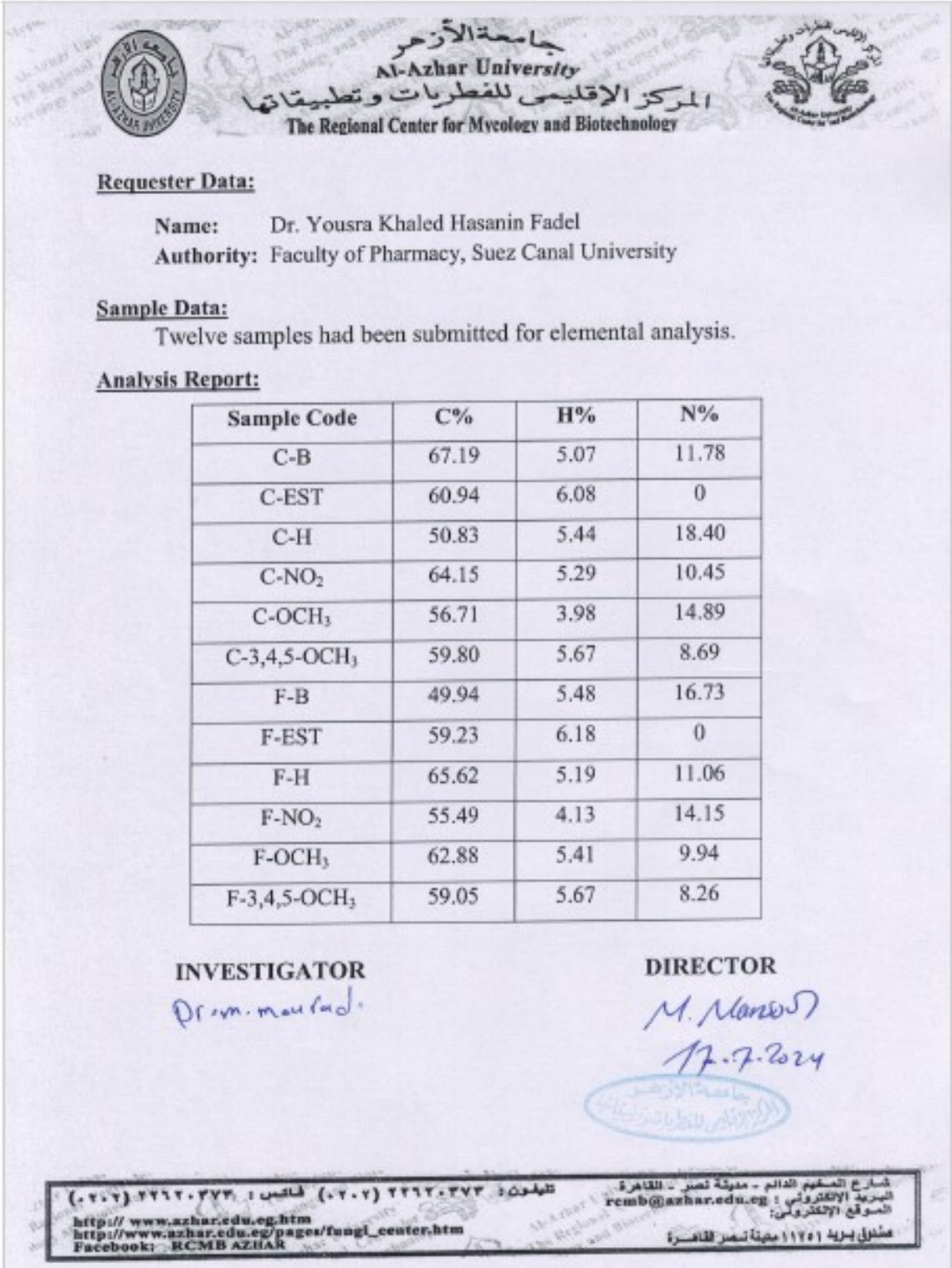


Fig. S20. ^{13}C-NMR of compound 3h

Supplementary Information

Sample code	C-B	C-EST	C-H	C-NO_2	C-OCH_3	C-3,4,5-OCH_3	F-B	F-EST	F-H	F-NO_2	F-OCH_3	F-3,4,5-OCH_3
Paper code	3a	1a	2a	3b	3c	3d	3e	1b	2b	3f	3g	h



جامعة الأزهر
Al-Azhar University
المركز الإقليمي للفطريات وتطبيقاتها
The Regional Center for Mycology and Biotechnology

Requester Data:

Name: Dr. Yousra Khaled Hasanin Fadel
Authority: Faculty of Pharmacy, Suez Canal University

Sample Data:

Twelve samples had been submitted for elemental analysis.

Analysis Report:

Sample Code	C%	H%	N%
C-B	67.19	5.07	11.78
C-EST	60.94	6.08	0
C-H	50.83	5.44	18.40
C-NO_2	64.15	5.29	10.45
C-OCH_3	56.71	3.98	14.89
C-3,4,5-OCH_3	59.80	5.67	8.69
F-B	49.94	5.48	16.73
F-EST	59.23	6.18	0
F-H	65.62	5.19	11.06
F-NO_2	55.49	4.13	14.15
F-OCH_3	62.88	5.41	9.94
F-3,4,5-OCH_3	59.05	5.67	8.26

INVESTIGATOR Prof. m. mourad.

DIRECTOR M. Mansour 17.7.2024

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Facebook: RCMB AZHAR
تليفون: ٢٢٦٢٠٣٧٣ (٠٢٠٢) فاكس: ٢٢٦٢٠٣٧٣ (٠٢٠٢)
مشارل بريد ١١٧٥١ مدينة نصر القاهرة

Figure S21. Elemental analysis of synthesized compounds 1a-b, 2a-b and 3a-h

Figure S22. Mass spectrum of compound 1a

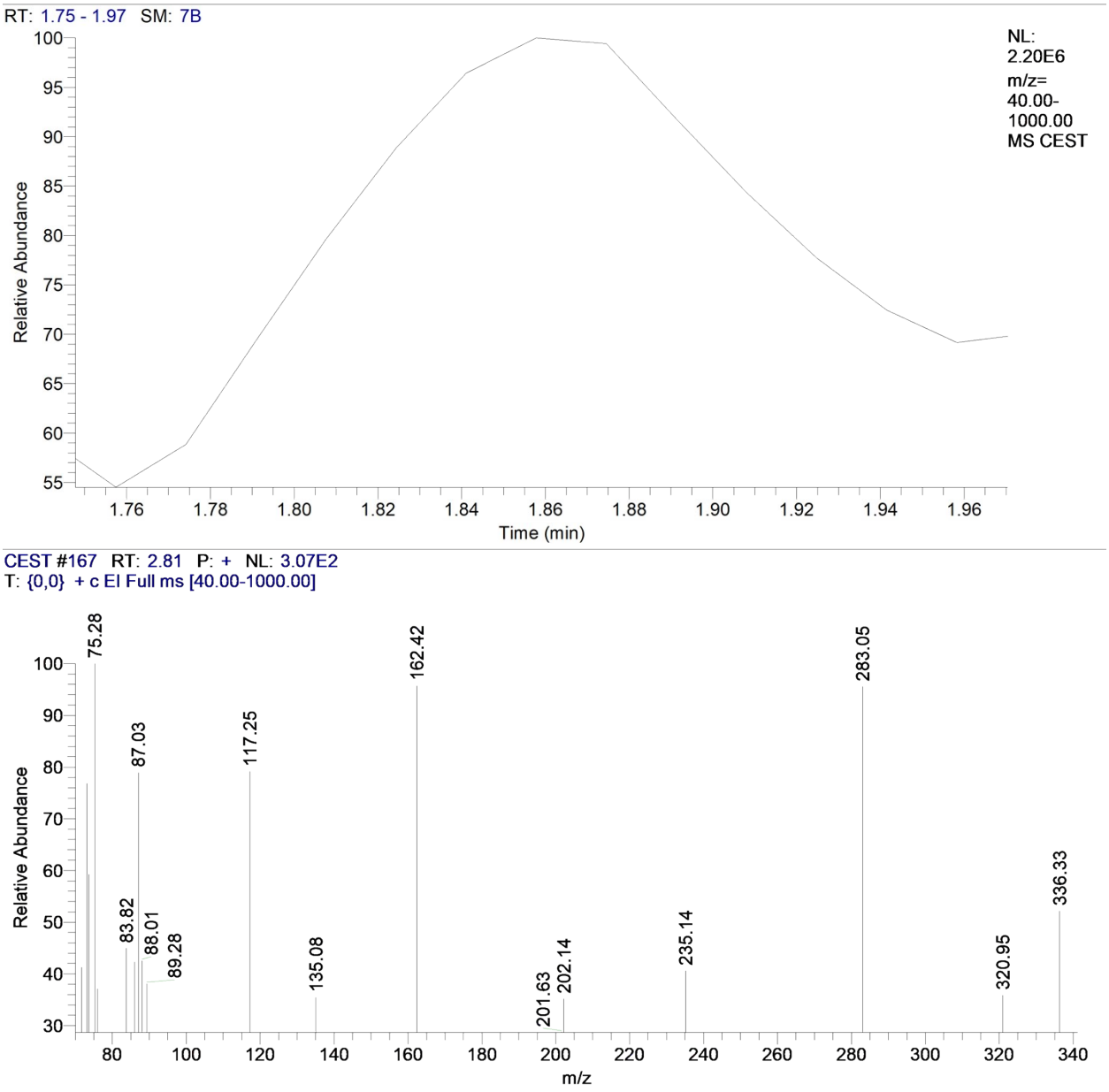
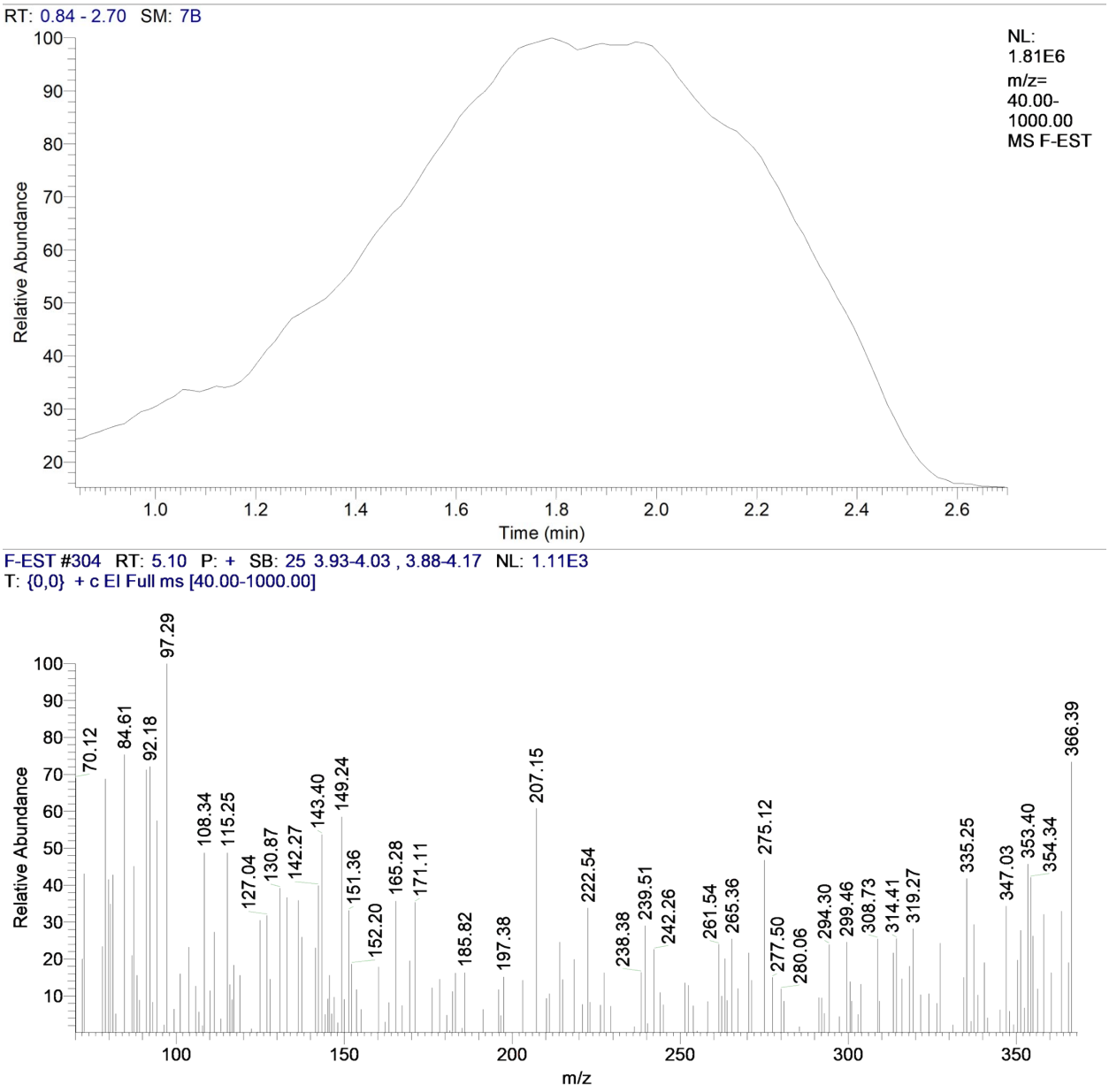


Figure S23. Mass spectrum of compound 1b



Supplementary Information

Figure S24. Mass spectrum of compound 2a

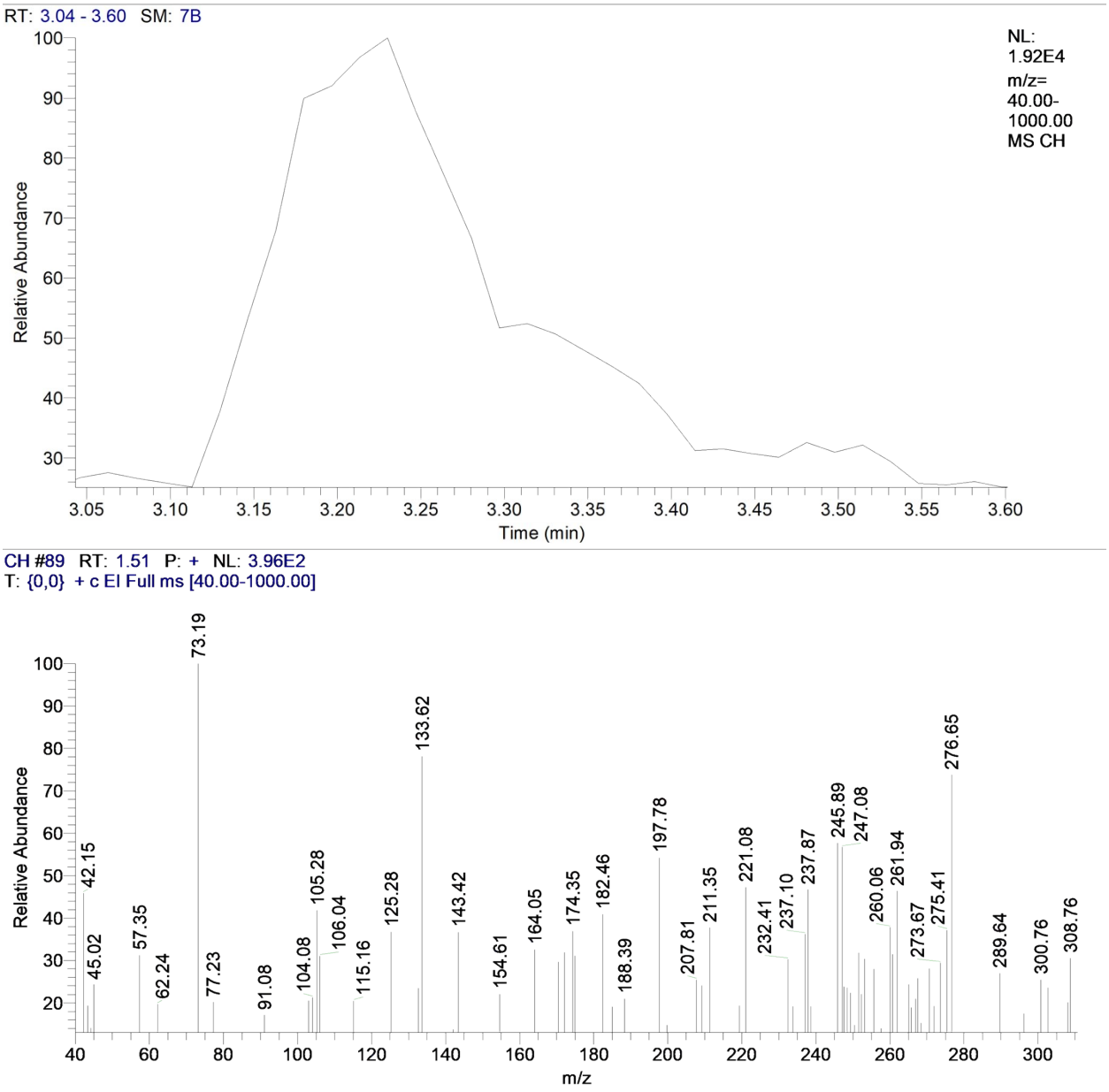


Figure S25. Mass spectrum of compound 2b

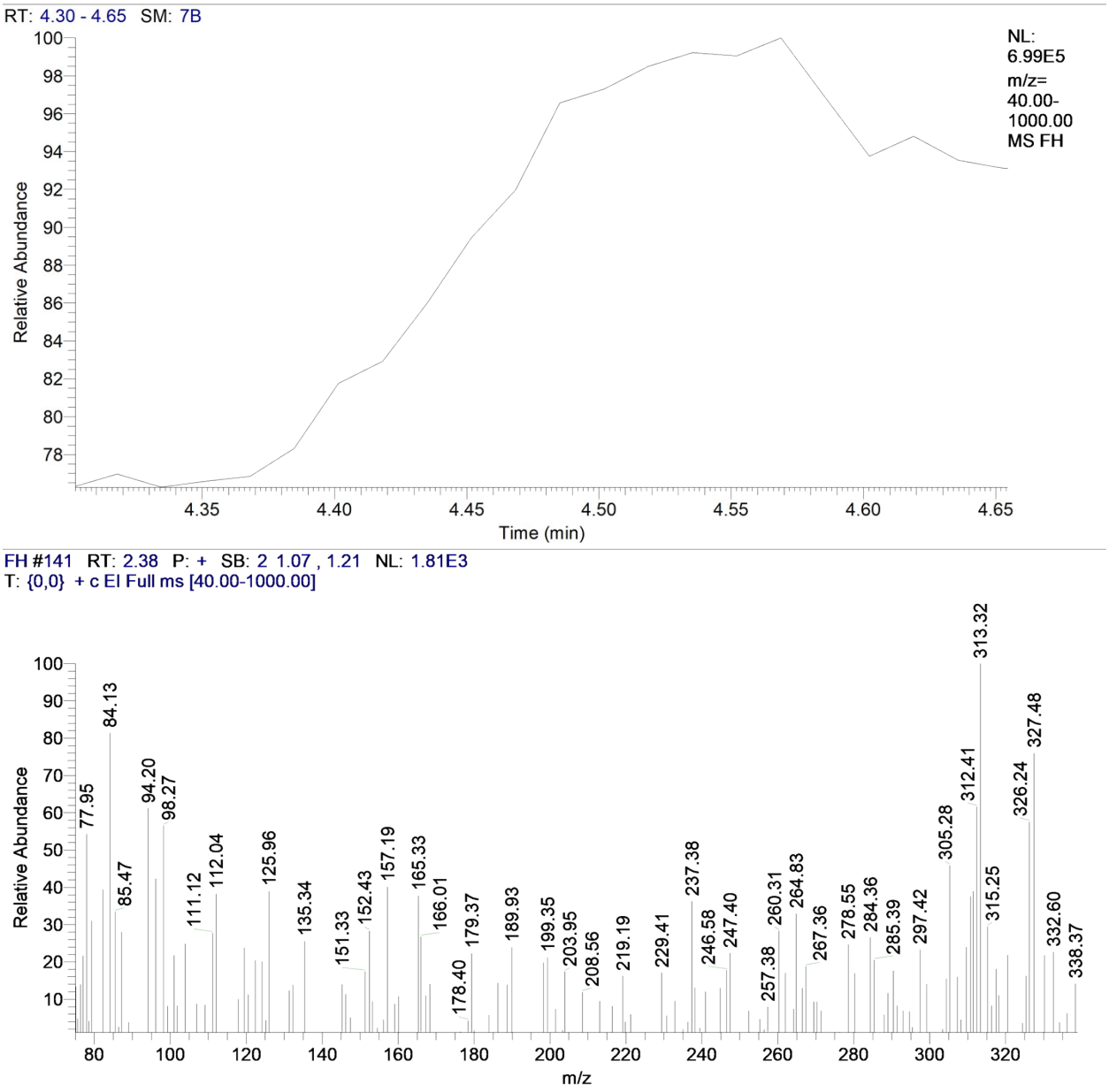


Figure S26. Mass spectrum of compound 3a

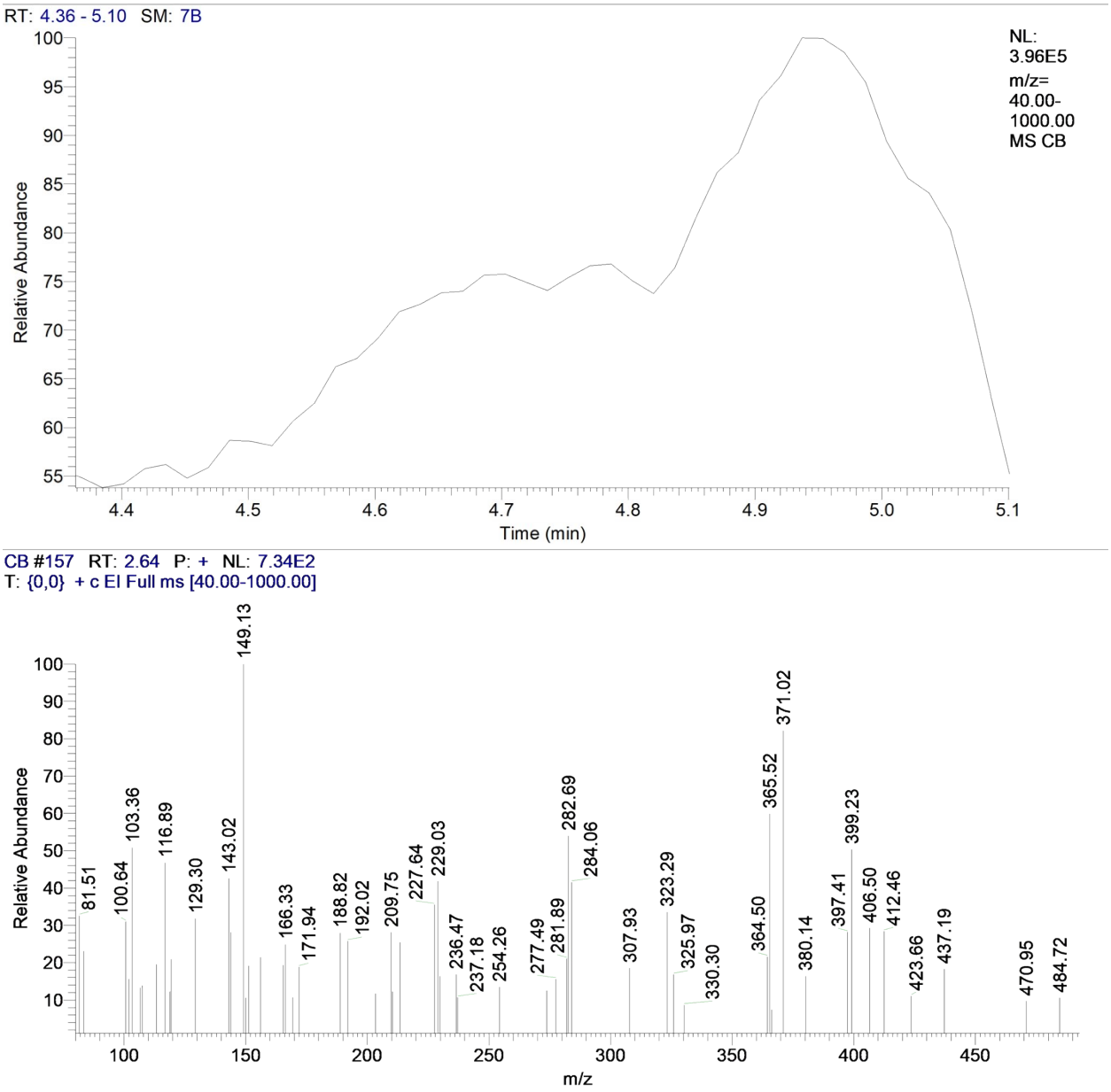


Figure S27. Mass spectrum of compound 3b

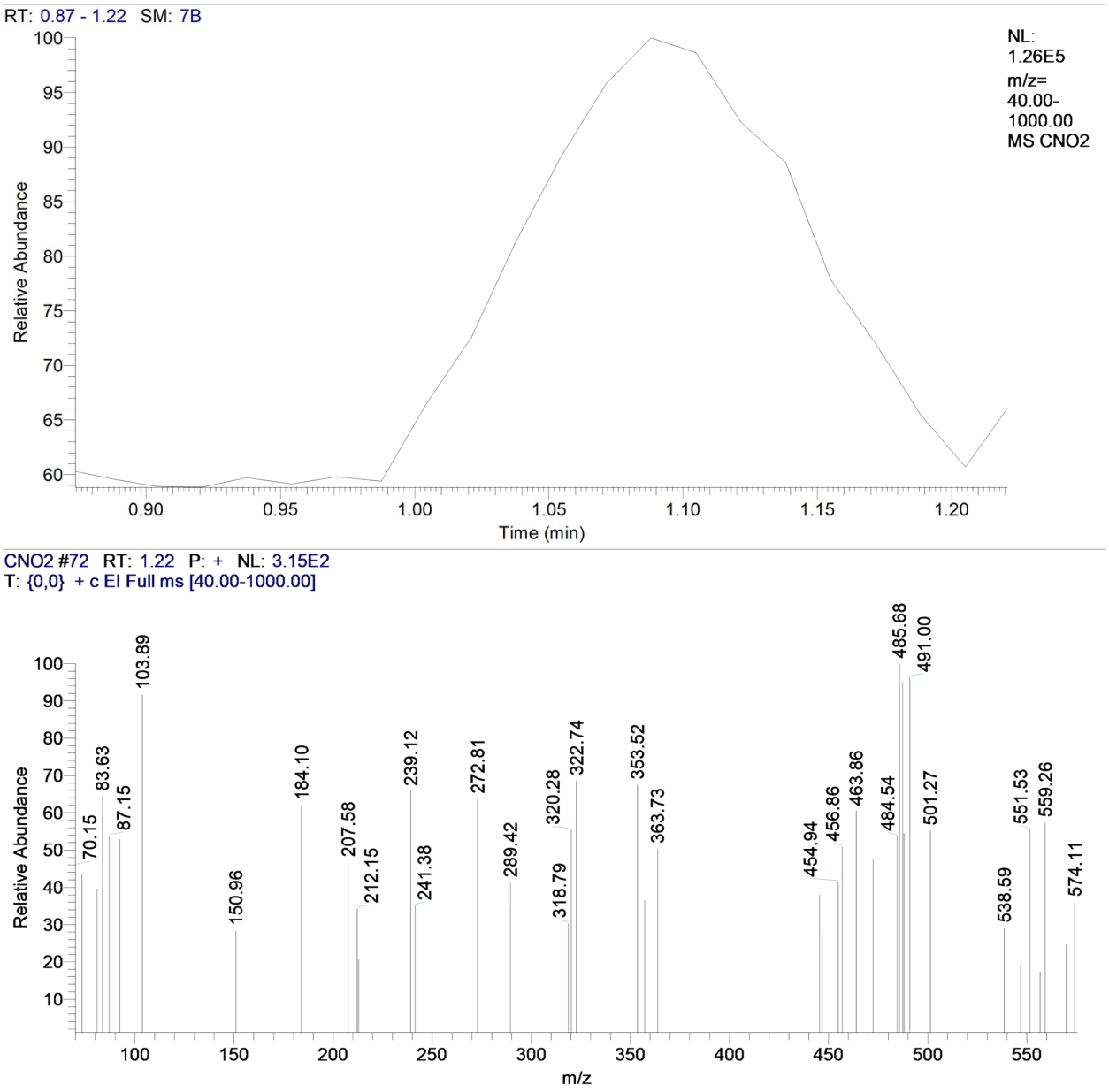


Figure S28. Mass spectrum of compound 3c

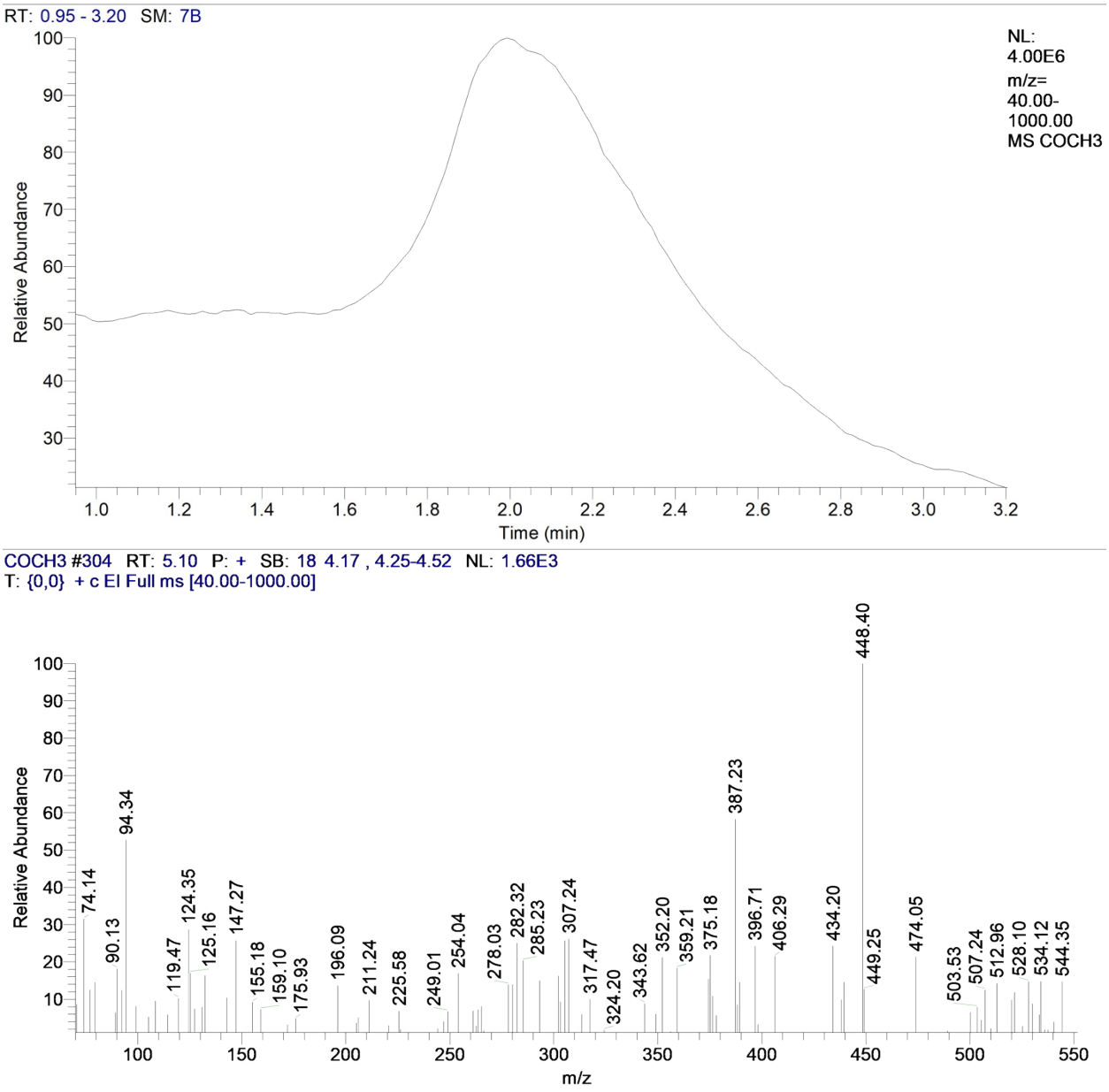


Figure S29. Mass spectrum of compound 3d

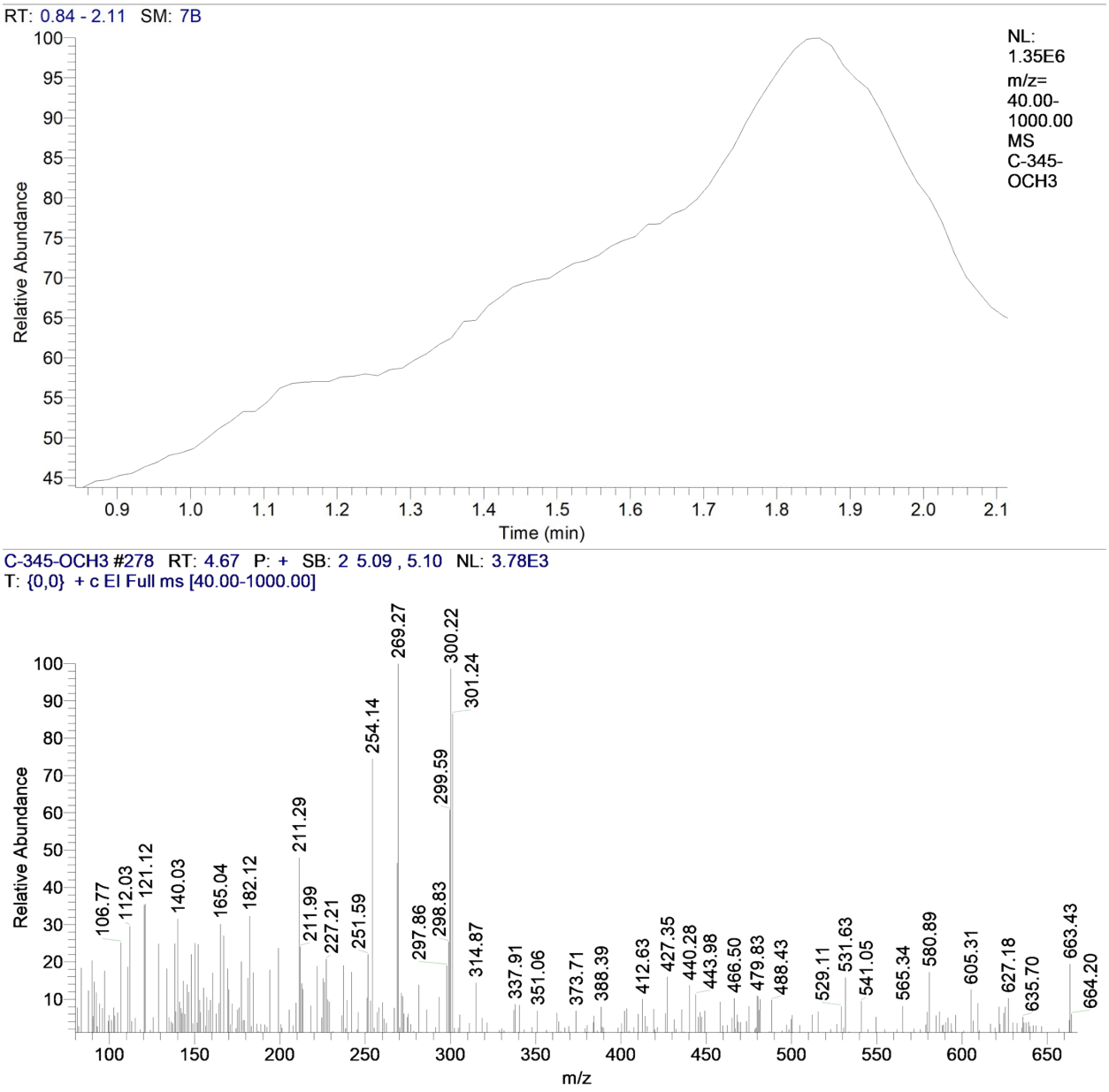


Figure S30. Mass spectrum of compound 3e

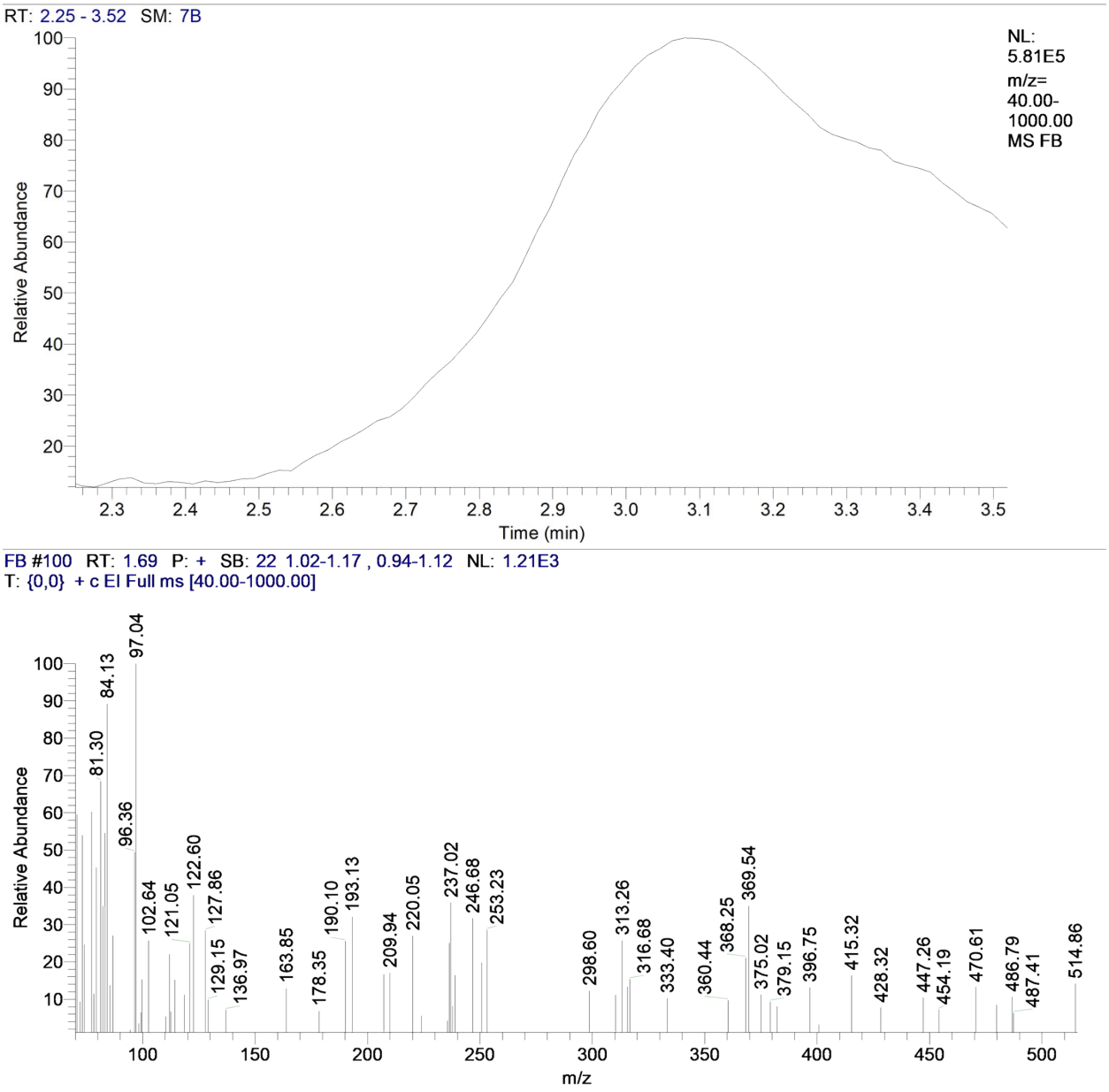


Figure S31. Mass spectrum of compound 3f

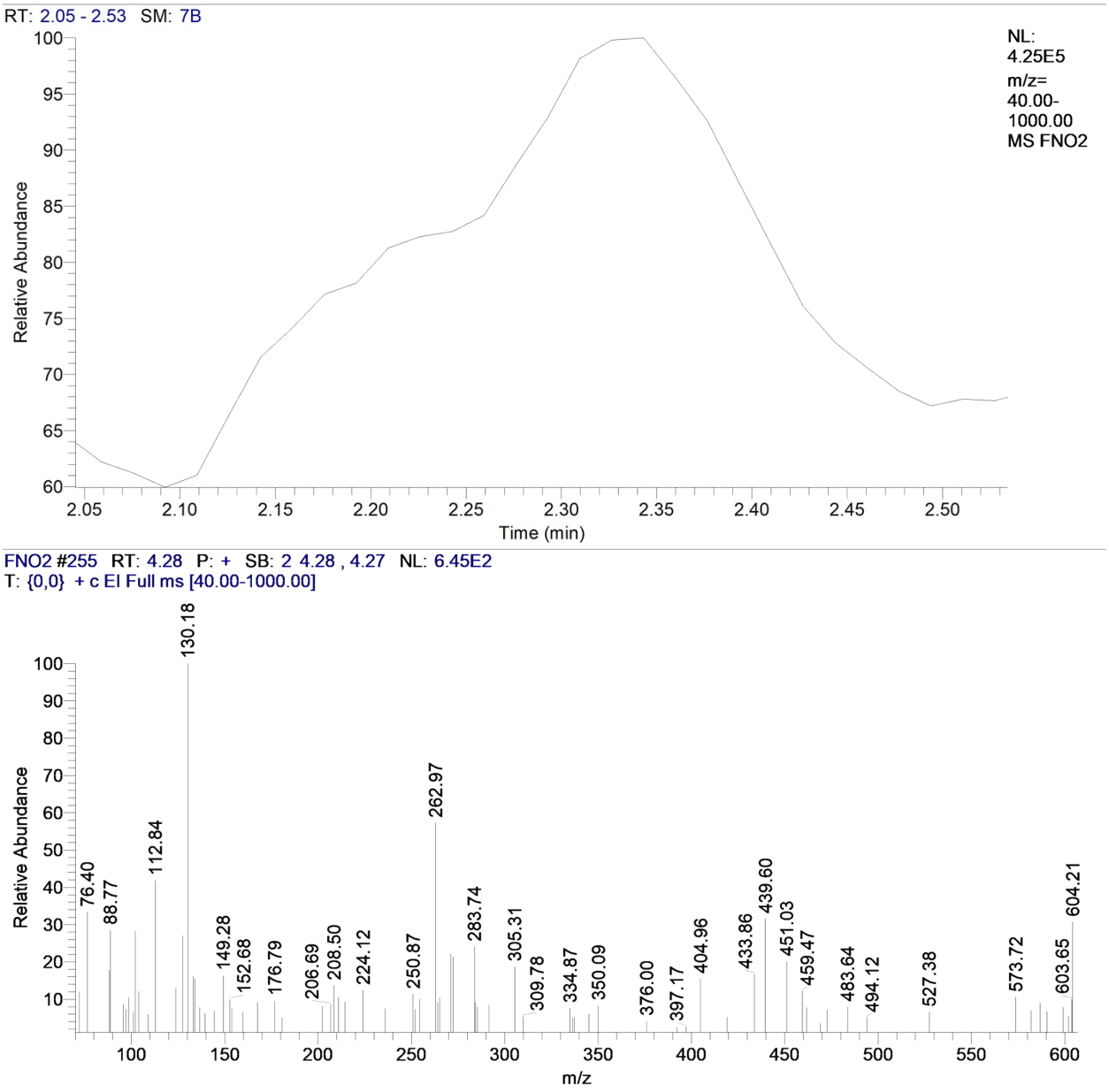


Figure S32. Mass spectrum of compound 3h

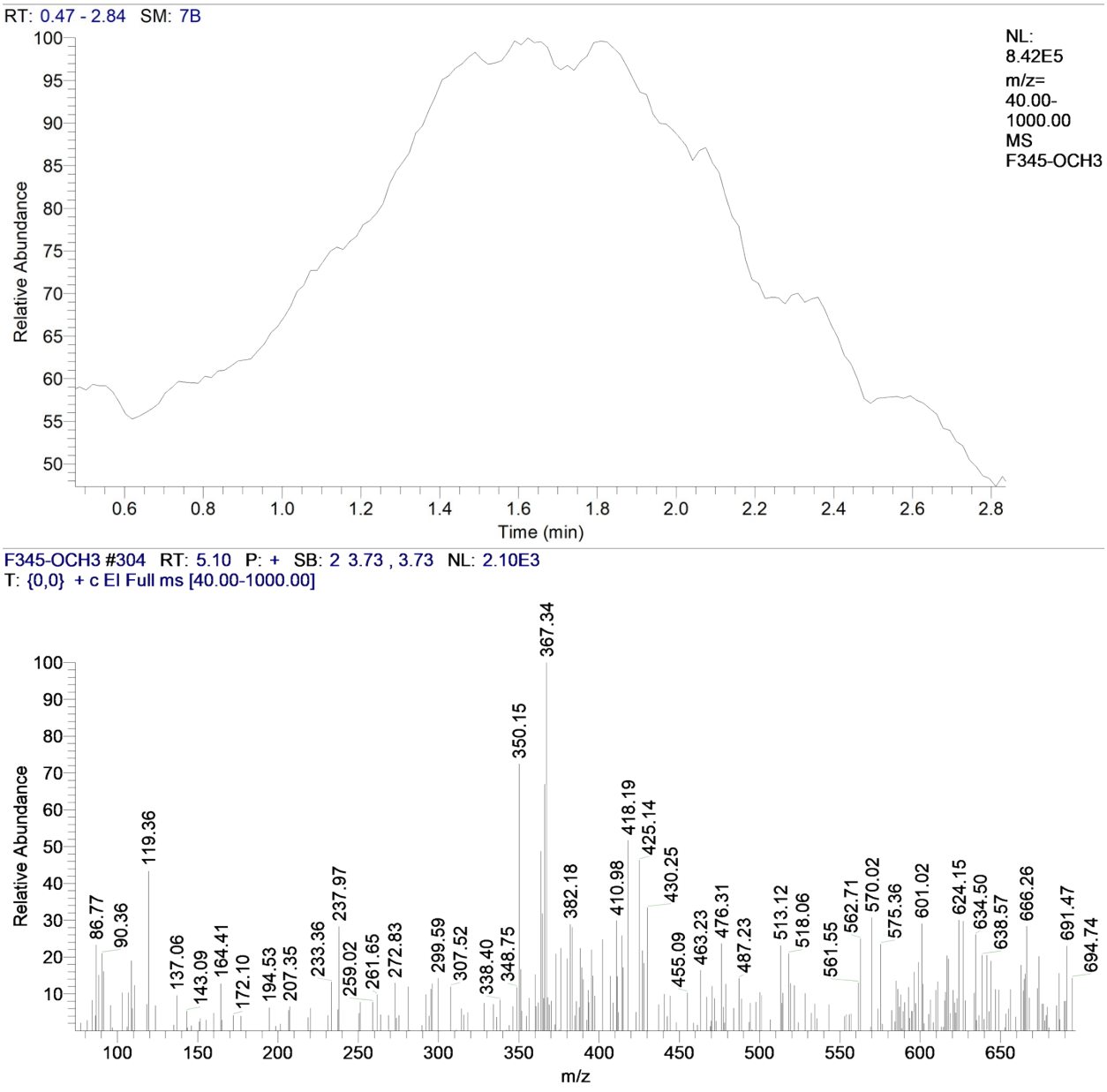
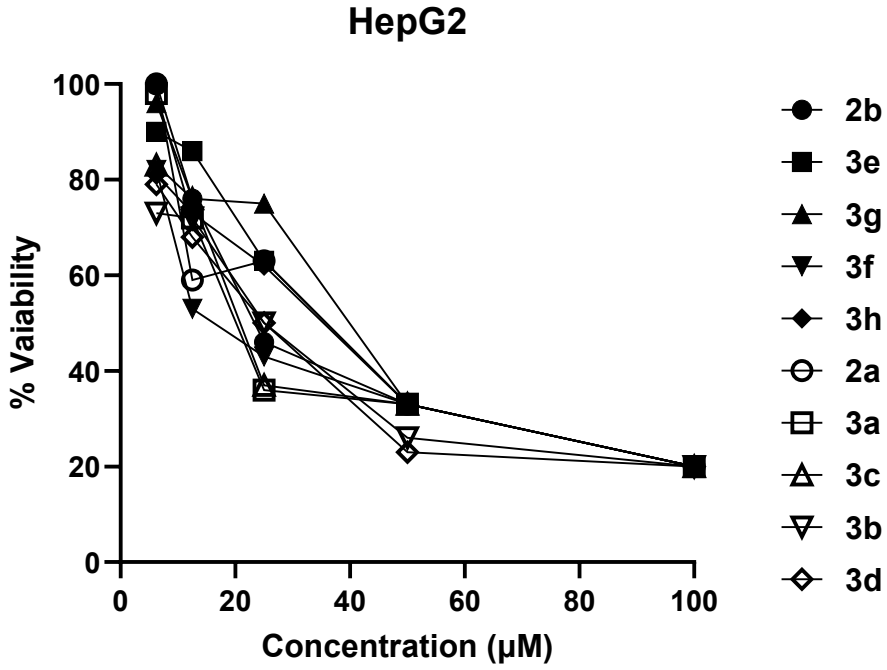
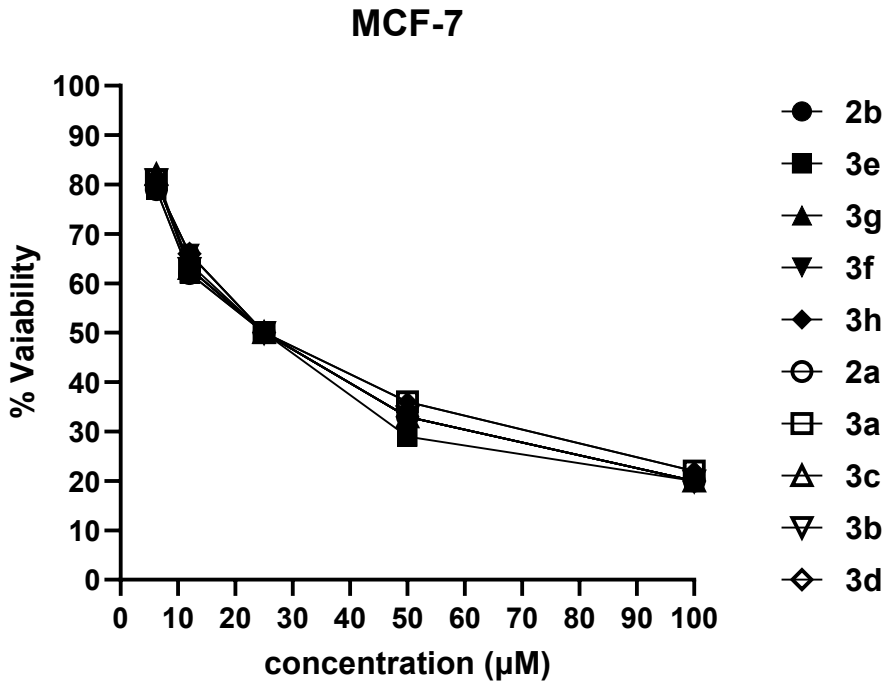
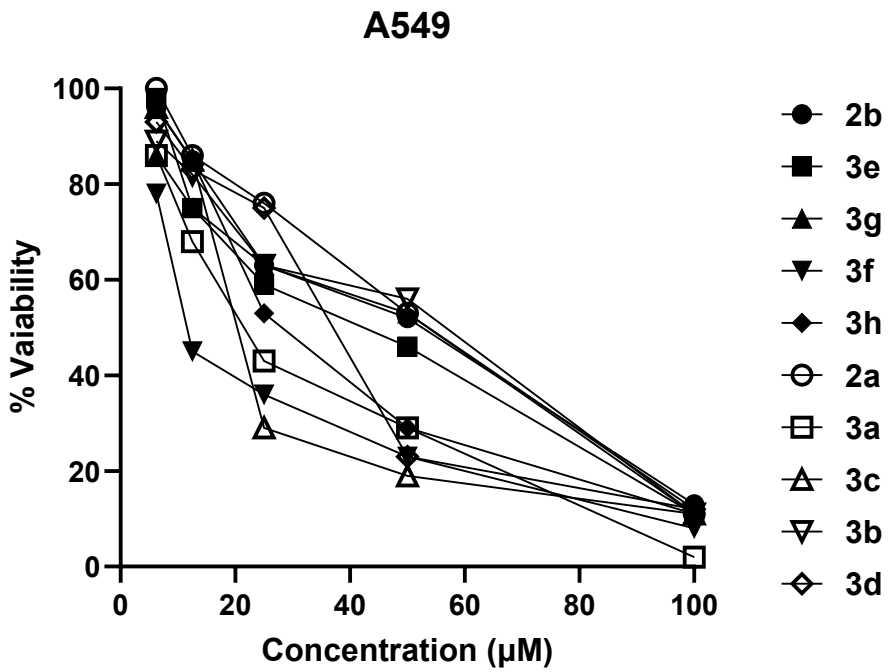


Fig. S33. IC_{50} curves (for MCF-7, HepG2and A594) of the compounds **2a-b** & **3a-h**






A549
% Vaiability
Concentration (μM)
0
20
40
60
80
100
2b
3e
3g
3f
3h
2a
3a
3c
3b
3d