Supporting Information

Comprehensive *In silico* Screening of the Antiviral Potentialities of A New Humulene Glucoside from *Asteriscus hierochunticus* against SARS-CoV-2

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S.1. Materials and Methods

S.1.1. General

Bruker Avance III 500 MHz spectrometer was used to acquire 1D and 2D NMR spectra information with samples dissolved in DMSO-d_6 [^{13}C and ^{1}H NMR data at 125 and 500 MHz, respectively]. Chemical shift values were recorded in ppm and referenced to the residual protons of solvent (DMSO). Mass spectra were acquired on an Agilent Technologies 6200 series mass spectrometer. IR spectra were measured on Agilent Cary 630 FTIR spectrometer (Agilent Technologies, USA). Optical rotations were measured by AUTOPOL IV Automatic Polarimeter (Rudolph, Hackettstown, NJ, USA). Isolation and purification of all compounds were performed by column chromatography (CC), over normal silica gel (32–63 μ, Dynamic adsorbents Inc.), and reversed-phase C-18 silica (Polar bond, J. T. Baker). Precoated silica gel F_{254} aluminium sheet (0.25 mm, Sorbtent Tech.) or Silica 60 RP–18 F_{254}aluminum sheet (20 × 20 cm, Merck) was used for TLC analysis. Spots were visualized by observing under UV-254 and 365 nm light and by spraying with 1% vanillin (Sigma) in conc. H_2SO_4-EtOH (1:9) followed by heating with a heat gun. All isolation and purification procedures were done by using analytical grade solvents (Fischer chemicals). Positive control drugs include standard antileishmanial agents Pentamidine and amphotericin-B (Sigma-Aldrich, St Louis, MO); Chloroquine and artemisinin (Sigma-Aldrich, MO) for antimalarial assay.

S.1.2. Plant material

A. hierochunticus (whole plant) was collected from the Mediterranean Coastal area of Egypt and identified in the Department of Botany, Faculty of Science, Mansoura University, Egypt, and a voucher specimen with Taxonomic number AH-14-PD was deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University, Egypt.

S.1..3. Extraction and Isolation

Dried and grounded whole plant of *A. hierochunticus (*500 g) was macerated with methanol (98 %) by percolation (4L × 4 × 20h) at room temperature. The filtrate was evaporated *in vacuo* at 40 ºC with a crude extract yield of 35 g (7 %). About 33 g of the extract was mixed with 30 g RP-18 silica gel, grounded to a fine powder, sieved and applied to a VLC over RP-18 silica (30 cm × 3.5 cm, 500 g). The column was eluted with gradients of H_2O/MeOH (90:10 – 0:100) to give 29 fractions (1-29). Fraction 9 (800 mg) was chromatographed over normal silica gel and eluted with gradients of solvents mixture of EtOAc: $CHCl_3$:MeOH:H_2O (15:8:4:1; 10:6:4:1; 0:8:2:0.25; 0:7:3:0.5; MeOH 100%) to obtain 5 fractions (9A-9E). Fraction 9C (67.7mg) was fractionated with $CHCl_3$: MeOH (10:1) [CC size 2cm x 35cm, normal SiO_2, vol., 15 mL each] to give six fractions coded 9C1-9C6. Repeated purification of 9C6 (19.0) mg yielded compound **1** (13.6 mg).

S.1.4. (-)-(2Z,6E,9E) 8α-hydroxy-2,6,9-humulatrien-1(12)-olide.

White needles: $[\alpha]^{25}_D$ = -78.6 (*c* 0.1, CH_3OH).IR υ max 3391, 1748 cm^{-1}. HRESIMS: *m/z*433.1783 $[M+Na]^+$ (calcd for $C_{21}H_{30}O_8Na$ 433.1838). ^{1}H NMR (500 MHz DMSO-d_6): δ_H4.62 (s, H-1), 7.27 (s, H-2), 2.38 (dd, *J*= 5.7, 10.4 Hz, H-4), 2.05(br. d, *J* = 12.8 Hz, H-5), 2.66(tdd, *J* =12.8, 10.8, 7.1 Hz, H-5), 5.07 (br. s, H-6), 4.79 (d, *J*= 8.1 Hz, H-8), 5.58 (dd, *J*= 16.1, 8.1 Hz, H-9), 5.22 (d, *J*= 16.1 Hz, H-10), 1.48 (s, 3H, H_3-13), 1.17 (s, 3H, H_3-14), 1.06 (s, 3H, H_3-15), 3.86 (d, *J* = 7.8 Hz, H-1'), 2.96 (overlapped, H-2'), 3.09 (t, *J* = 8.9 Hz, H-3'), 3.02 (td, *J* = 8.9, 3.0 Hz, H-4'), 2.95 (m, H-5'), 3.40 (dt, *J*= 11.8, 5.7 Hz, H-6'a), 3.63 (ddd, *J*= 11.8, 6.1, 2.1 Hz, H-6'b) (Table S1). ^{13}C NMR (125 MHz, DMSO-d_6): δ_C 87.5 (C-1), 149.4 (C-2), 132.6 (C-3), 24.9 (C-4), 22.8 (C-5), 126.0 (C-6), 140.9 (C-7), 83.9 (C-8), 131.6 (C-9), 134.9 (C-10), 40.3 (C-11), 172.6 (C-12), 11.4 (C-13), 25.6 (C-14), 21.4 (C-15), 99.9 (C-1´), 73.4 (C-2´), 76.9 (C-3´), 70.2 (C-4´), 77.1 (C-5´), 61.2 (C-6´).

S.1.5. In silico studies

S.1.5.1. Conformational Search for humulene-glucoside 1

Conformational Search for humulene-glucoside 1 was carried out using the LowModeMD Search protocol using MOE2014. This protocol is a short molecular dynamics simulation using velocities with little kinetic energy on the high-frequency vibrational modes. At first, humulene-glucoside 1 was drawn using ChemBioDraw Ultra 14.0 and saved as SDF format. Then, the saved file was opened using MOE software. Then the forcefield of MMFF94X was selected. From the compute panel, the conformational research was run. The output data base was save and the best conformer was selected for further studies [1].

S.1.5.2. Molecular docking

The crystal structures of the target proteins: i) COVID-19 main protease (M^{pro}) (PDB ID:**6**lu7, resolution: 2.16 Å), ii) non-structural protein (nsp10) (PDB ID: 6W4H, resolution: 1.80 Å), iii) RNA-dependent RNA polymerase (PDB ID: 7BV2, resolution: 2.50Å), and iv) SARS-CoV-2 helicase (PDB ID: 5RMM, resolution: 2.20 Å) weredownloaded from Protein Data Bank (http://www.pdb.org). Molecular Operating Environment (MOE) was used for the docking analysis [2]. In these studies, the free energies, and binding modes of the tested molecules against target proteins were determined. At first, the water molecules were removed from the crystal structures of target proteins, retaining only one chain which is essential for binding. The Co-crystallized ligands were used as reference ligands. Then, the protein structures were protonated and the hydrogen atoms were hidden. Next, the energy was energy minimized by applying MMFF94x force field. After that, the binding pockets of each protein were defined [3,4].

The structures of the examined compound and the co-crystallized ligands were drawn using ChemBioDraw Ultra 14.0 and saved as SDF formats. Then, the saved files were opened using MOE software and 3D structures were protonated. Next, the energy of the molecules was minimized applying MMFF94x force field. Validation processes were performed for each target receptor by running the docking process for only the co-crystallized ligand. low RMSD values between docked and crystal conformations indicate valid performance [5,6]. The docking procedures were carried out utilizing a default protocol. In each case, 30 docked structures were generated using genetic algorithm searches. The output from of MOE software was further analyzed and visualized using Discovery Studio 4.0 software [6-9].

S.1.5.3. ADMET

ADMET descriptors (absorption, distribution, metabolism, excretion, and toxicity) of the compounds were determined using Discovery studio 4.0. At first, the CHARMM force field was applied then the tested compounds were prepared and minimized according to the preparation of small molecule protocol. Then ADMET descriptors protocol was applied to carry out these studies [5,8].

S.1.5.4. In silico toxicity

The toxicity parameters were calculated using Discovery studio 4.0. Simeprevir was used as a reference drug. At first, the CHARMM force field was applied then the compounds were prepared and minimized according to the preparation of small molecule protocol. Then different parameters were calculated from the toxicity prediction (extensible) protocol.

S.1.6. Antileishmanial assay

Compound (**1**) and standard antileishmanial agents (positive controls) were evaluated against *L. donovani* promastigote, *L. donovani* axenic amastigote, and *L. donovani* intracellular amastigote according to the Alamar Blue colorimetric assay which measures quantitatively the proliferation of various cell lines (humans, animals, and bacteria) [10]. The assay was done on a culture of *L. donovani* promastigotes (2×10^5 cells/mL) in a 96-well microplate into which test sample solutions were added diluted appropriately to a final concentration of 10 μg/mL. The plates were subsequently incubated for 72 hours at 26°C, and parasite growth was determined. Included in the assay as positive control agents were Pentamidine and amphotericin B used as standard antileishmanial agents. The IC_{50} values of test compounds and standard antileishmanial agents were computed from the growth-inhibition curve.

S.1.7. In vitro macrophage amastigote assay

This was done according to the parasite-rescue, and transformation assay described [10,11]. In this assay, differentiated THP1 cells (human acute monocytic leukemia cell line) are infected *in vitro* with *Leishmania donovani* and the efficacy of the pure compounds and standard antileishmanial agents determined. The THP1 culture was prepared from a four-day-old cell culture (not more than 10^6 cells/mL) and diluted with 10% heat-inactivated fetal bovine serum (FBS) to give a cell count of $2.5x10^5$ cells/mL in RPMI-1640. The cells previously diluted with DMSO were seeded into a 96-well microplate to which phorbol 12-myristate 13-acetate (PMA) at a final concentration of 25 ng/ml was added. Cells suspension (200 μL) with cells count of 5×10^4 cells were dispensed into a clear flat-bottom 96-well plate and incubated overnight in a 37 °C, 5% CO_2 incubator for the differentiation of the cells. After the incubation period, PMA-treated THP1 cells were carefully washed twice with serum-free RPMI-1640 medium. The medium was replaced with 200 μl (5×10^5 cells/mL) of the diluted *L. donovani* promastigotes culture harvested at the stationary phase (metacyclic infective stage) and suspended in RPMI-1640 medium with 2% FBS at a density of 2.5×10^6 cells/mL and further incubated in a 5% CO_2 incubator at 37°C for at least 24 hours to allow infection of macrophages with the Leishmania parasites. After 24 hours the non-adherent macrophages and unattached Leishmania promastigotes were washed off with serum-free RPMI-1640 medium. The infected macrophages were then incubated with different concentrations of standard antileishmanial drugs (pentamidine and amphotericin B) or the test compounds for 48 hours at 37°C and 5% CO_2 in 200 μL RMPI1640 medium and 2% FBS. The experiment was repeated for uninfected THP1 cells, infected cells without drugs or test compounds. After the incubation period, the cultures were washed off with serum-free RPMI-1640 and treated with 20 μL of 0.05% sodium dodecyl sulfate in RPMI-1640 medium for 30 seconds to release amastigotes from the infected macrophages. Furthermore, 180 μL of RPMI-1640 medium with 10% FBS was added to each well and incubated at 26°C for 48 hours to allow the transformation of the rescued amastigotes to promastigotes. To each well containing transformed promastigotes, 20 μL of Alamar blue was added and the plates incubated at 26°C for 24 hours and read on a Fluostar Galaxy fluorimeter (BMG Lab Technologies) at 544 nm excitation, 590nm emission wavelengths. Each compound was tested in duplicate at six concentrations, and the dose-response curves (percent growth vs. concentration of the drug or test compound) were prepared with ExcelFit and IC_{50}/IC_{90} values computed.

S.1.8. Antitrypanosomal assay

This assay was carried out according to a method previously described [12]. This assay uses 2 days old culture of *T. brucei* in the exponential phase was diluted with IMDM to a concentration of 5 x 10^3 parasites/mL in a clear 96 well microplates. Compound (**1)** and standards (Pentamidine and αdifluoromethylornithine (DFMO) at final concentrations of (10 – 0.4 µg/ml) were prepared in an IMDM medium. Each well received 4 µL of diluted test compounds and 196 µL of the culture to achieve a total culture volume of 200 µl. The plates were incubated at 37 °C in 5 % CO_2 for 48 h, after which 10 µl Alamar Blue (AbD Serotec, catalog number BUF012B) was added to each well, and the plates incubated overnight. A Fluostar Galaxy fluorometer (BMG LabTechnologies) was used to measure the standard fluorescence at 544 nm excitation, 590 nm emission wavelengths. The half-maximal concentration IC_{50} and IC_{90} values were computed from the dose-response growth inhibition curve by XLfit version 5.2.2.

S.1.9. Cytotoxicity assay

The cytotoxicity of compound (**1)** was also tested against transformed human monocytic (THP1) cells. The assay method previously described by Jain et al was adopted. In this experiment, a 4 days old culture of THP1 cells in the experimental phase diluted with RPMI medium to 2.5X 10^5 cells/mL was used. To achieve the parasite cells transformation to the adherent macrophages, Phorbol 12-myristate 13-acetate (PMA) was added to the culture at a concentration of 25 ng/mL. The THP1 cell culture treated with PMA was seeded into 96 well plates with 200 µl culture (2.5X 10^5 cells/mL) in each well and incubated overnight at 37 °C in a 5 % CO_2 incubator. The medium in plates with THP1 cells was replaced with a fresh medium. The test compounds and standards were diluted with RPMI medium in separate plates were added to these plates and further incubated in a 5 % CO_2 incubator at 37 °C for 48 h. After the incubation period, 10 µL of Alamar Blue solution (AbD Serotec, catalog number BUF012B) was added to each well and the plates were incubated further overnight. Again, standard fluorescence was measured on a Fluostar Galaxy fluorometer (BMG LabTechnologies) at 544 nm excitation, 590 nm emission wavelengths. The half-maximal concentration IC_{50} and IC_{90} values were computed from the dose-response growth inhibition curve by XLfit version 5.2.2 .

S.1.10. Antimalarial Assay

The *in vitro* antiplasmodial activity of (**1**) was measured by a colorimetric assay that determines the parasite's lactate dehydrogenase (pLDH) activity [13]. Included in this assay are two strains of *Plasmodium falciparum* (Sierra Leone D6 [chloroquine-sensitive] and Indochina W2 [chloroquine-resistant]) obtained from the Walter Reed Army Institute of Research, Silver Spring, MD. The effects of the test compound on plasmodial LDH activity were determined using Malstat reagent (Flow Inc, Portland, OR). DMSO (0.25 %) and chloroquine/artemisinin were included in each assay which serves as a vehicle and positive control drugs, respectively. The selectivity indices (SI) were computed by measuring the cytotoxicity of the test compounds against Vero cell lines (monkey fibroblast).

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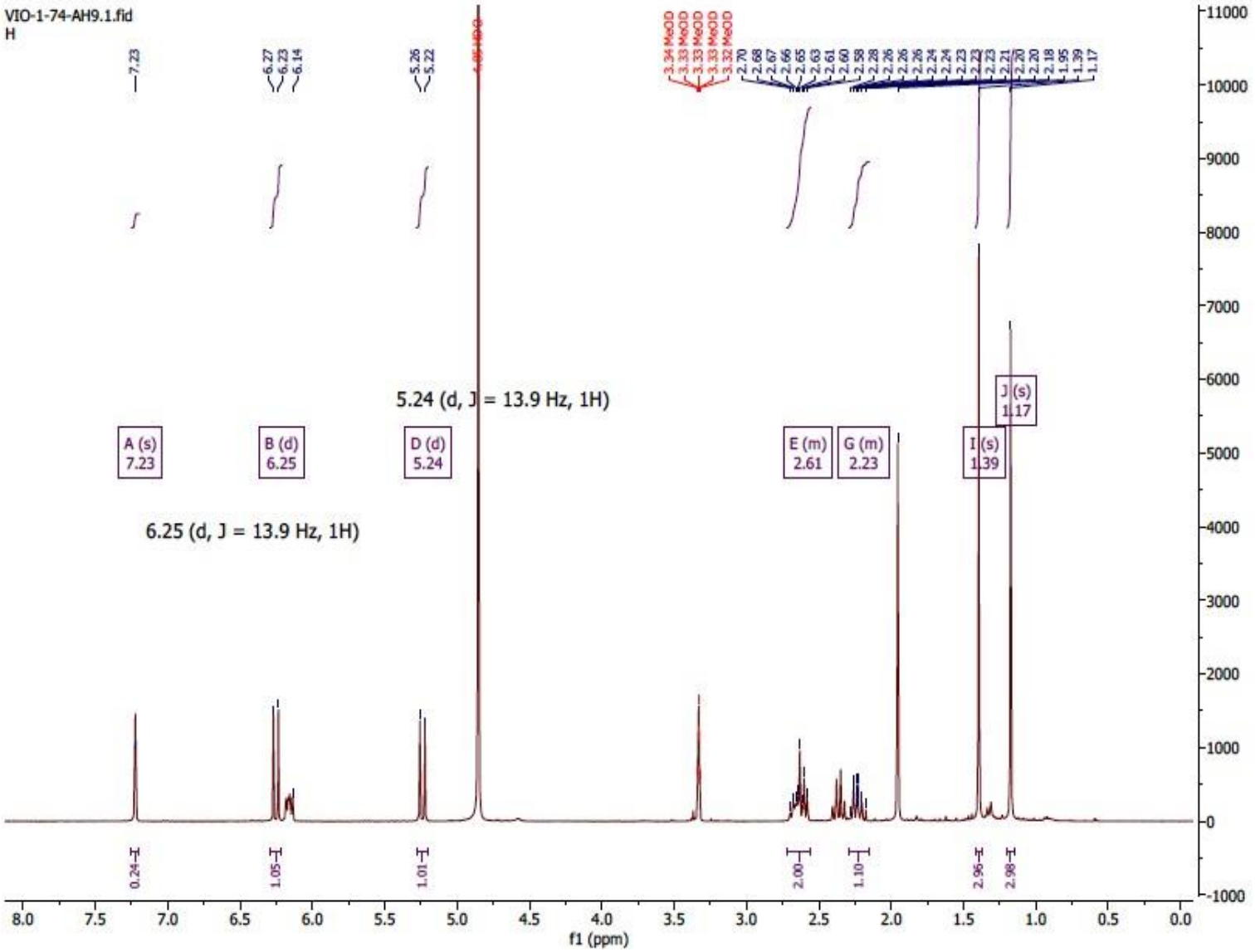
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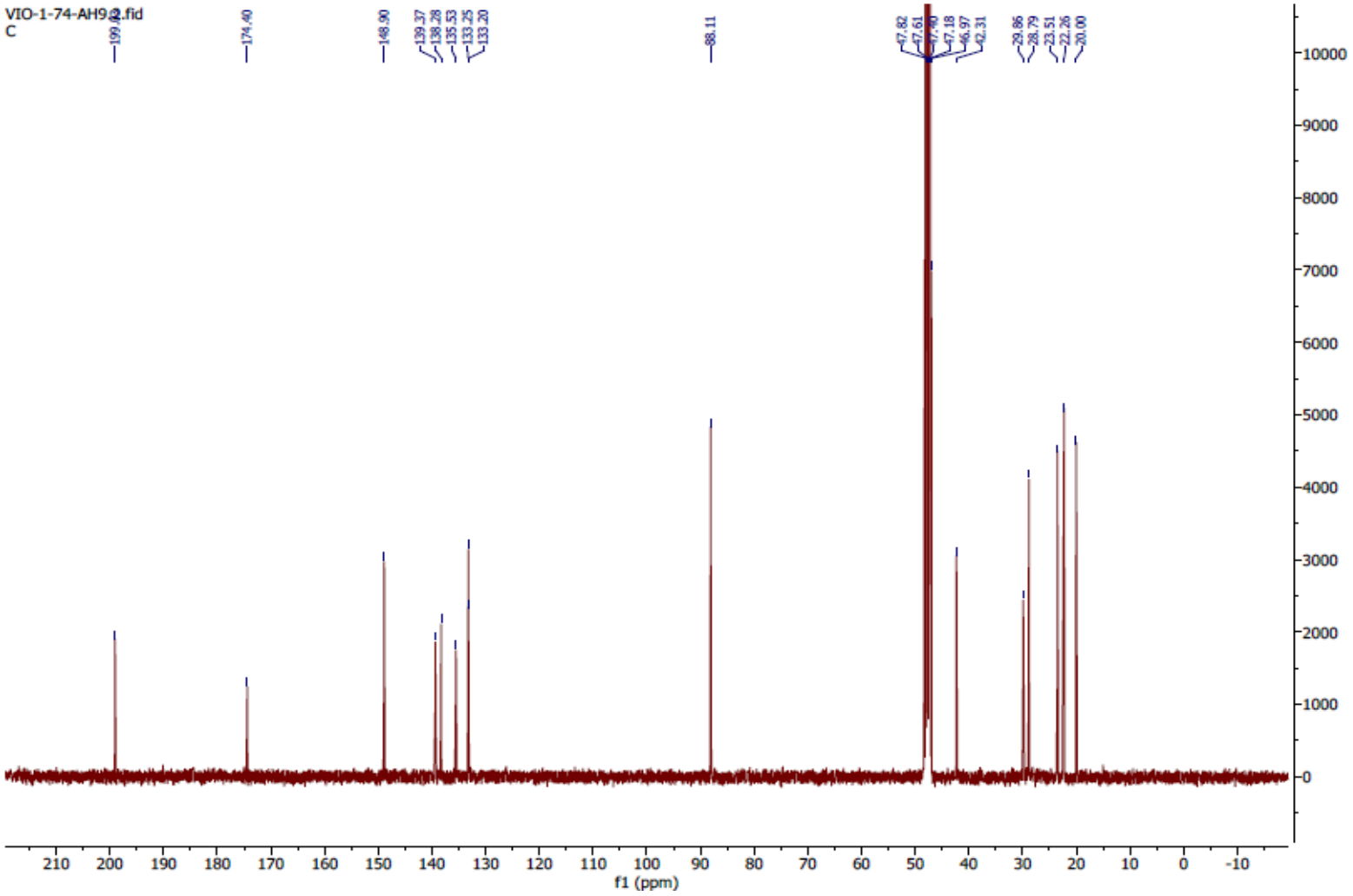
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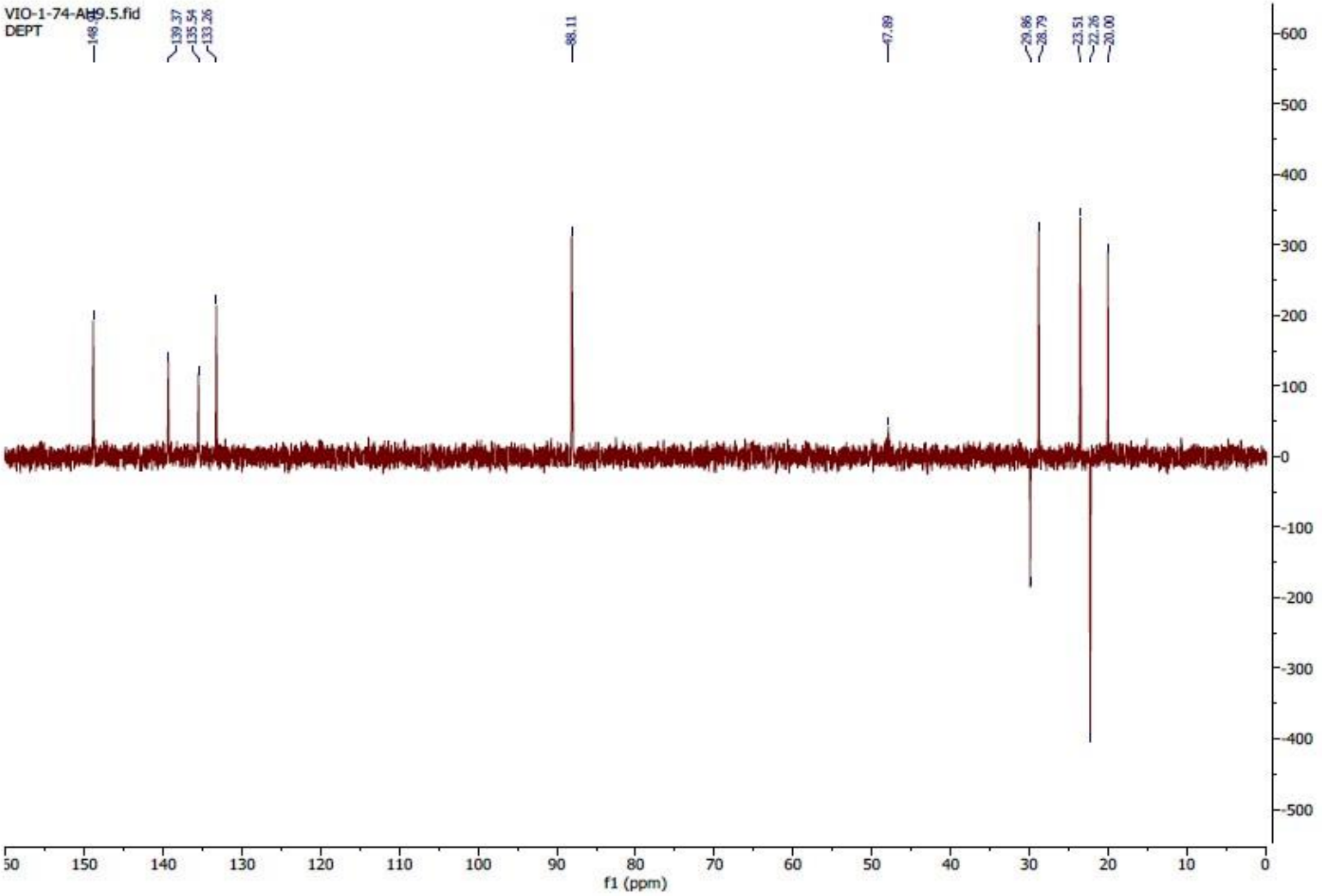
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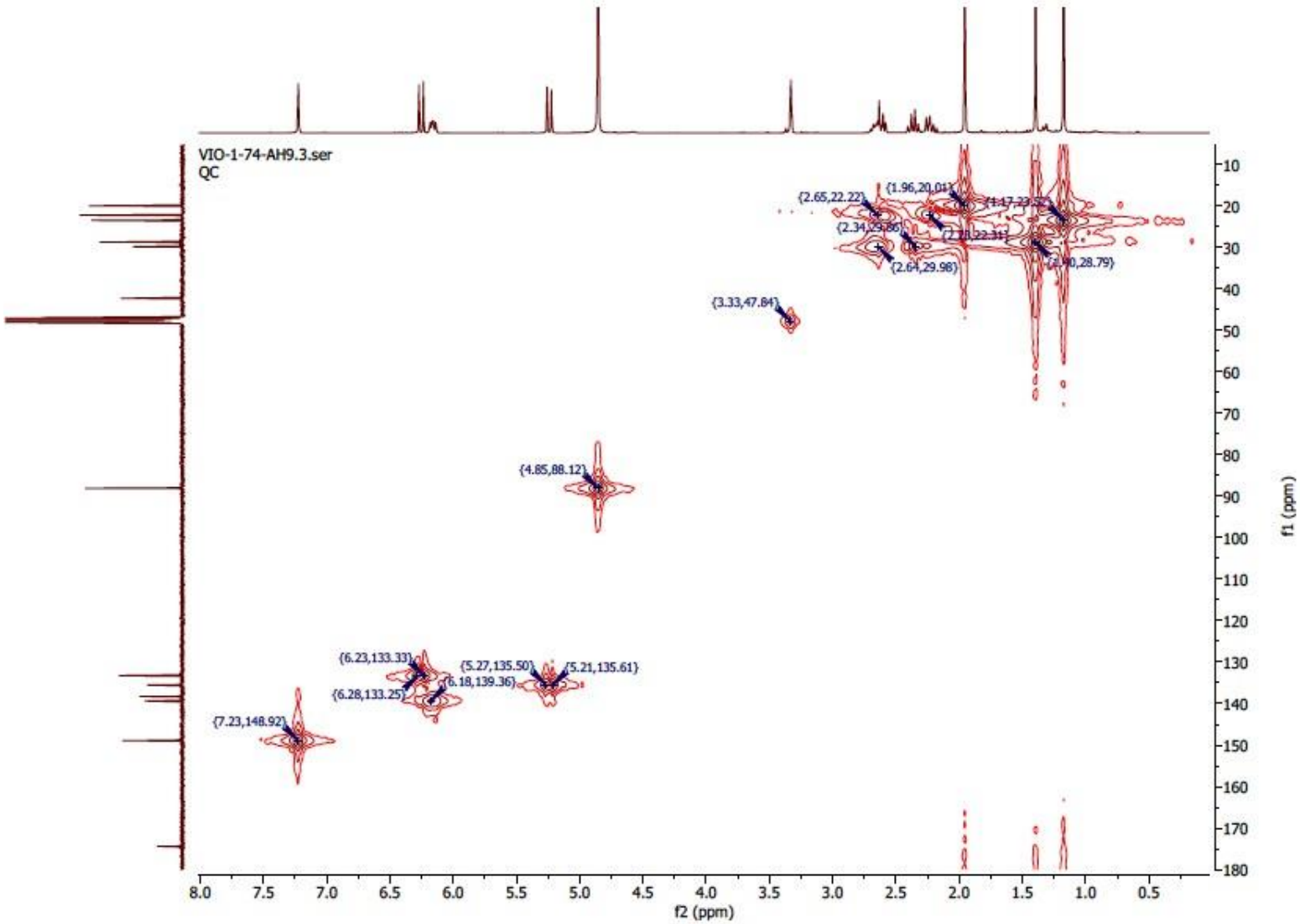
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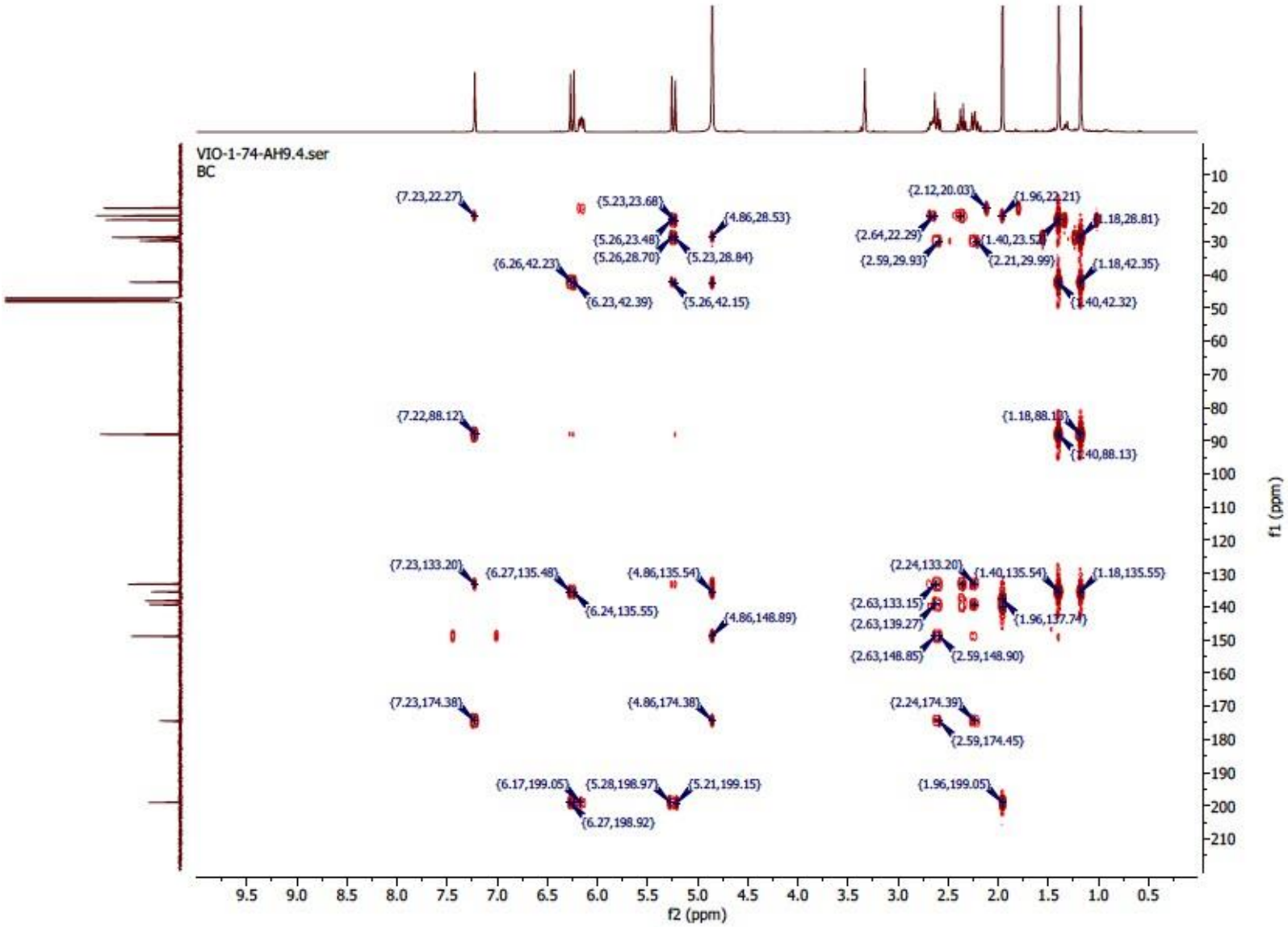
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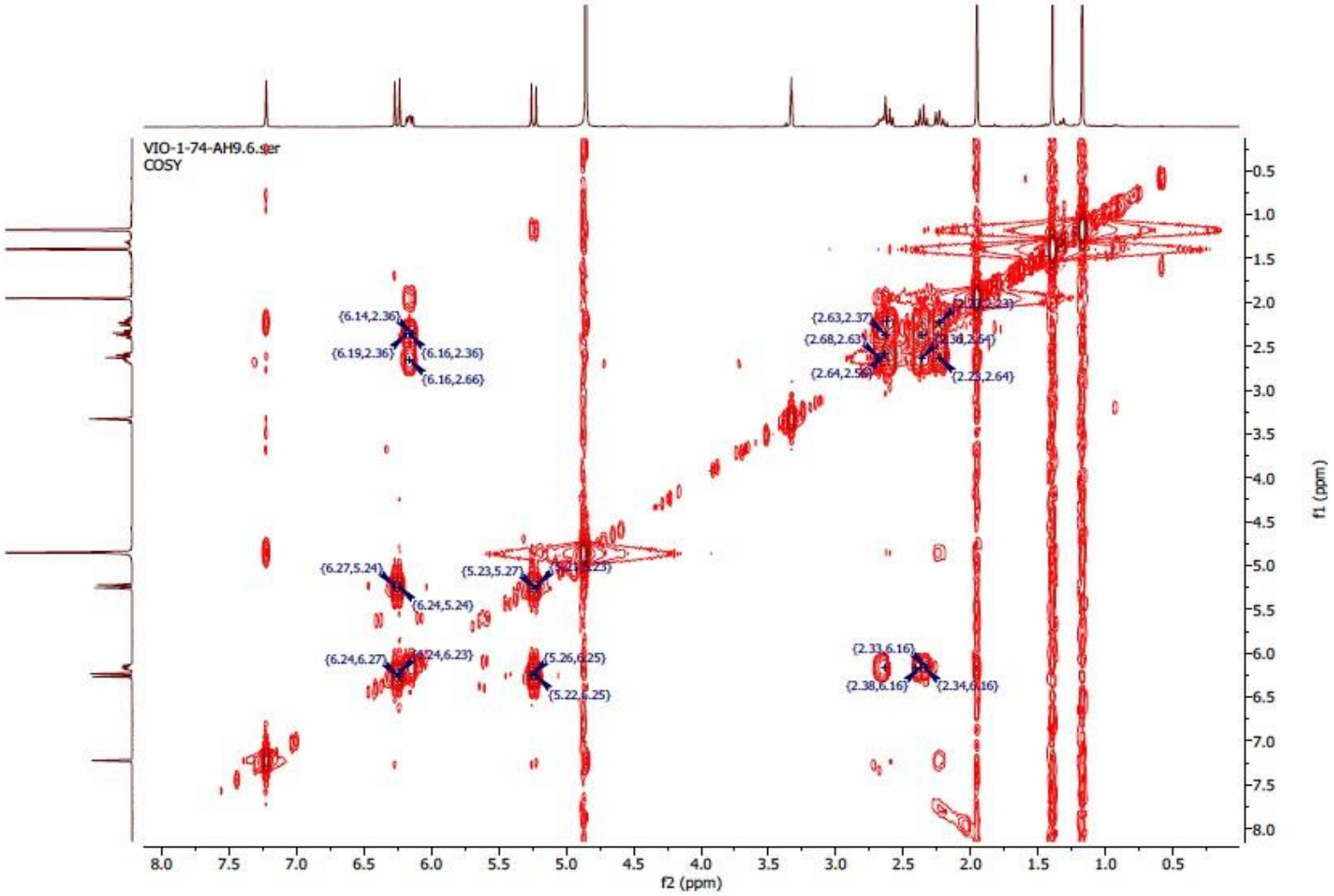
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