

Supplementary Material

Synthesis and biological evaluation of (*E*)-cinnamic acid, (*E*)-2-styrylthiazole and (*E*)-2-[2-(naphthalen-1-yl)vinyl]thiazole derivatives

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SUPPORTING INFORMATION I – Bioassay Methods

1.. Anti-cancer (cytotoxicity) bioassays

1.1. Resazurin-based *in vitro* cytotoxicity test using HeLa cells

The screening was conducted using multi-well plates which are suited for HeLa cells in the log phase of growth with final cell density > 10 cells/cm. Each experiment normally includes a blank control, containing medium without the cells.^{28,45} Non-contaminated HeLa cells (6.57 x 10⁵ cells per well) in media were allowed to grow in the incubator under an atmosphere of 5% CO₂ at 37 °C for 24 h. To each well was dispensed 200 μL of HeLa culture, containing 6.57 x 10⁵ cells under LabEAir laminar flow hood (Vivid Air, South Africa); 20 μL of resazurin dye (Sigma TOX-8) and test compound (50 μL) were added, which were then incubated in the presence of 5% CO₂ at 37 °C for 24 hours in a shaker, to enhance the distribution of the dye. The absorbance of each well was measured with Bio-tek Power Wave X fluorometer (Beijing, China), and increases in fluorescence was monitored at a wavelength of 590 nm, using an excitation wavelength of 560 nm.⁴⁶

2. Anti-cancer (cytotoxicity) xCELLigence RTCA assays using SH-SY5Y cells

2.1. SH-SY5Y cell culturing (thawing) and sub-culturing

Dulbecco's modified eagle medium (DMEM) supplemented with 1% (v/v) L-glutamine (2 mM), 10% (v/v) Fetal Calf Serum (FCS) and 1% (v/v) of PBS (penicillin-streptomycin-amphotericin) was prepared and warmed at 37°C in a humidified atmosphere under 5% CO₂.^{47,48} The SH-SY5Y culture vessel, containing complete growth medium, was equally warmed to 37 °C in the incubator for 2 minutes to thaw the cells. The surfaces of all the vials were decontaminated by spraying with 70% (v/v) ethanol, and subsequent steps were carried out under a strict aseptic condition in a laminar flow cabinet. The cells were aseptically transferred to a 15 mL centrifuge tube, containing 9 mL of DMEM, centrifuged at 1200 rpm at 4 °C for 2 minutes. The cell pellets were re-suspended in the DMEM in a T25 flask and incubated at 37°C under 5% CO₂ and the cells were fed every 4 days by replacing the medium with a fresh DMEM.^{47,48}

The SH-SY5Y cells were sub-cultured from a T25 flask at a passage of 75% confluence into a T75 flask, in a ratio of 1:2-1:5. The floating cells were discarded with the spent medium, adherent cells rinsed with 3 mL of PBS, followed by the addition of 1 mL of trypsin solution, and were then placed in the incubator under 5% CO₂ at 37°C for 2 minutes until the cells were detached. Fresh medium (1 mL) was then added, aspirated and centrifuged at 2000 rpm at 4 °C for 2 minutes. The cell pellets were re-suspended in the medium, transferred into a new T75 flask, incubated under 5% CO₂ at 37 °C and new medium was added after 4 days.^{47,48}

2.2. Cytotoxicity assay using xCELLigence RTCA SP instrument

The optimal seeding concentration for the proliferation experiments of the SH-SY5Y was first determined.⁴⁸ Prior to seeding of the SH-SY5Y cells to each well, 100 µl of the medium (DMEM) was added to each well, and scanned by the xCelligence RTCA system to determine the baseline values.⁴⁸ Ten thousand (1 x 10⁴) SH-SY5Y cells in 50 µl culture medium (DMEM-Ham supplemented with 1% L-glutamine (2 mM), 10% Fetal Calf Serum (FCS) and 1% PBS) were seeded into each well of the microelectronic censored E-Plate 96, and incubated at 37°C under 5% CO₂ in a humidified atmosphere.^{47,48} The cultures proliferation, attachment, and spreading of the cells were monitored every 30 minutes for 24 hours by the xCELLigence, after seeding. The cells at the log growth phase were exposed in duplicate to 50 µL of different concentrations of the test compounds in the medium, incubated at 37°C under 5% CO₂ at relative humidity of up to 98%, and scanned every 15 minutes for 24 hours.⁴⁸ The controls wells

received either SH-SY5Y cells (normal cell growth), medium only, medium-DMSO at a concentration of 0.20% (v/v) or blank (PBS solvent). All experiments were run for a total of 48 h.^{48,49}

2.3. Statistical and data analysis of xCelligence RTCA data

The calculations were automatically computed by the RTCA-integrated software of the xCELLigence RTCA system.⁵⁰ The RTCA software also performs a non-linear regression analysis of selected sigmoidal dose-response for each test compound in comparison to the experimental data points, and calculates the linear or logarithmic half maximum inhibitory concentrations (IC₅₀ or log IC₅₀) at a given time point that produce 50% reduction of cell index (CI), relative to the SH-SY5Y control CI (100%).⁴⁹ All data have been generated by the RTCA software, and are presented as mean (mmol/L) ± SEM (standard error of mean).⁴⁸ The cytotoxic effects of the test compounds were evaluated by plotting a dose response curve (DRC) of the Cell index (CI) at a time point against the linear- or Log-concentration.⁴⁸

3. Antimalarial bioassay

3.1. Procedure for antimalarial resasurin-based PfLDH bioassay

3.1.1. Preparation of solutions

- i) **Malstat solution:** Triton X-100 (400 µL), L-lactic acid (4 g), Trizma base (1.32 g) and acetylpyridine adenine dinucleotide; APAD (22 mg) were dissolved in H₂O, and the pH was adjusted to 9 using a basic Tris Buffer (Tris-C).
- ii) **NBT/PES solution:** Nitro blue tetrazolium salt (160 mg) and phenazineethosulphate (8 mg) were dissolved in H₂O (100 mL). The bottle was covered with aluminium foil, and the solution was stored at 4 °C. The solution was used within a month of storage.

3.1.2. Antimalarial whole cell PfLDH-based inhibition assay

Giensa stain is a mixture of malstat and NBT/PES solutions. Malstat solution was prepared by mixing triton X-100 (400 µL), L-lactic acid (4 g), Trizma base (1.32 g) and acetylpyridine adenine dinucleotide; APAD (22 mg) were dissolved in H₂O, and the pH was adjusted to 9 using a basic Tris Buffer (Tris-C). Also, NBT/PES solution was obtained from Nitro blue tetrazolium salt (160 mg) and phenazineethosulphate (8 mg) were dissolved in H₂O (100 mL), the bottle was covered with aluminium foil, and the solution was stored at 4 °C. The two solutions were used within a month of storage.

3.1.3. Antimalarial PfLDH-based single concentration screening

The solutions of all of the test compounds (20 mM in DMSO) were stored in a deep freezer at -40 °C prior to the screening. Stock solutions were diluted with the media (blood plasma / haemolysed red blood cells) to obtain a concentration of 20 μM that was used for the test. Chloroquine (CQ) was used as a positive control (reference or standard antimalarial drug). A 100 μL of each compound in triplicate was placed in a 96 well plate, and control wells contained only the media (blank). The parasite culture was removed from the T75 flask, and transferred into an Eppendorff tube, then centrifuged at 2000 rpm for 5 minutes (Eppendorff AG centrifuge 5810R, New Jersey, U.S.A). The supernatant was discarded, and the culture pellets were retained at the bottom of the EPPENDORFF tube. Blood smear viable cell count assays were performed using Giemsa stain to determine the percentage parasitemia and 2% haematocrit, 2% parasitemia suspension was made in a 10 μL culture media.⁴⁵

The parasite viability of a test sample = $\frac{\text{Abs test compound}}{\text{Abs positive control}} \times 100$

4. In vitro anti-mycobacterial green fluorescent protein microplate assay (GFPMA)

4.1. Anti-tuberculosis green fluorescent protein microplate assay (GFPMA)

MIC90 and MIC99, which are the concentration of the thiazole-based compounds, inhibit 90% and 99% of the growth of the virulent *M. tuberculosis* H37Rv (*Mtb* H37Rv), were determined using the GFPMA in a microplate-based fluorometric assay. A stock culture of *Mtb* H37Rv was grown to observed density at 600 nm (*i.e.*, OD600) of 0.6-0.7 in the Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween-80, 0.2% glycerol, and albumin-NaCl-glucose (ADC) complex. Culture dilutions were made in the medium (1:500) and 50 μL was dispensed into each microwell of a 96-well plate (row 2-12). In order to determine the MIC90 and MIC99, the test compounds were dissolved in DMSO to make stock solutions of 12.8 mM, and serial dilutions was done up to a final concentration of 640 μM. Each compound (100 μL) was added to the first row of the wells of the 96-well plate, and two fold serial dilutions were made, to provide ten dilutions of each compound (160-0.078 μM). Rifampicin was used for positive controls, while 5% DMSO and the Middlebrook 7H9-based media were employed as negative controls. The plates were incubated for 14 days at 37 °C, and the MIC90 and MIC99 values were read using an inverted fluorometric plate reader on Day 7 and Day 14, post-inoculation. The lowest test and reference drug concentrations that inhibit the growth of more than 90% and

99% of the *Mtb* H37Rv at day 7, scored on a scale of 10, were considered as the MIC90 and MIC99 values respectively.⁵¹

5. Antibacterial disc susceptibility assay

5.1. Preparation of extraction solutions and nutrient media

- i) Saline water: 0.9% NaCl (normal saline)
- ii) Peptone water: 0.5% peptone
- iii) Ringer solution (0.3% w/v): 2.979 g of solute containing NaCl (2.251 g), NaHCO₃ (0.503 g), KCl (0.105 g) and CaCl₂ (0.120 g) was dissolved in a litre of distilled H₂O.
- iv) Cetrimide agar: A 46 g of cetrimide (*i.e.*, peptone: 20.5 g, MgCl₂, K₂SO₄ and cetrimide: 13.6 g) from BioLab / Merck was suspended in double distilled water (1L) and glycerol (10 mL) was added. The suspension was autoclaved at 121⁰ C for 15 minutes, cooled to 45-50⁰ C and poured into the sterile 90 mm petri-dishes (Spellbound Labs, Port Elizabeth, South Africa).
- v) Nutrient agar: A 31 g of nutrient agar (*i. e.*, meat extract = 1 g, yeast extract = 2 g, peptone = 5 g, NaCl = 8 g and agar = 15 g) from BOIOLAB was suspended in double distilled water (1 L), boiled whilst stirring until the agar completely dissolved and autoclaved using a [vertical N-class autoclave steriliser \(REXMED RAU-530](#), Rexall industries co Ltd., Taiwan) at 121⁰C for 15 minutes, cooled to 45-50⁰ C and poured into the sterile 90 mm petri dishes (Spellbound Labs, Port Elizabeth, South Africa).

5.2. Extraction procedure for *Pseudomonas aeruginosa* from soil samples

Each soil sample, collected in Grahamstown, was measured as 5 g, in triplicate, using a top loading balance [Zeiss West Germany (Pty) Ltd., Germany] into sterile flasks, and placed under a laminar flow hood. The extraction solutions (50 mL), consisting of peptone water (0.5% w/v), normal saline (0.9% w/v) and RINGER solution (0.3% w/v), were added to the flasks, where saline solution served as the negative control.^{28,29,45} The samples were mixed properly for 2 min at 15 sec intervals, serial dilutions (10⁻¹) of each of the samples prepared, and 200 µl of both the undiluted and diluted solutions were spread plated on cetrimide agar (90 mm in diameter). The plates were allowed to dry under LabEAir laminar flow hood (Vivid Air, South Africa), for ten seconds, incubated at 37⁰C for 24 hours and observed under the UV lamp (Syngene, Division of Synoptic Ltd.) at 366 nm for colonies with blue fluorescence. A pure colony was selected, then propagated twice on nutrient agar, and incubated using a Labcon^(R) LTIM low temperature incubator (California, USA) at 37⁰C for 24 hours to obtain colonies with the same morphology, before the antibiotic assays were conducted.^{52,53}

5.3. Disc diffusion antibacterial susceptibility test

5.3.1. Inoculation of test plates

The inoculums of *P. aeruginosa* were prepared from the nutrient agar culture, with at least three to five well-isolated colonies with the same morphology, and aseptically transferred into a sterile test tube containing 5 mL of 0.9% w/v saline solution.⁵⁴ Then a sterile cotton swap was dipped into the inoculums and rotated firmly, several times, against the upper inside wall of the tube to express excess fluid. The entire surface of nutrient agar was streaked three times, turning the plate at 60° between streaking to obtain even inoculation.⁵⁴ The lid was left ajar for about 5 minutes to allow for any surface moisture to be absorbed before applying the test compound-impregnated discs.⁵⁴

5.3.2. Disc impregnation and inoculation of agar plates with test compounds and controls

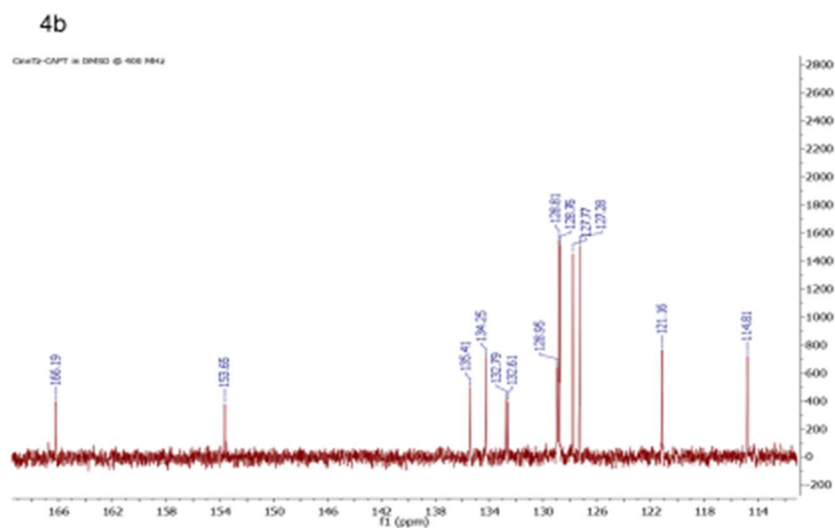
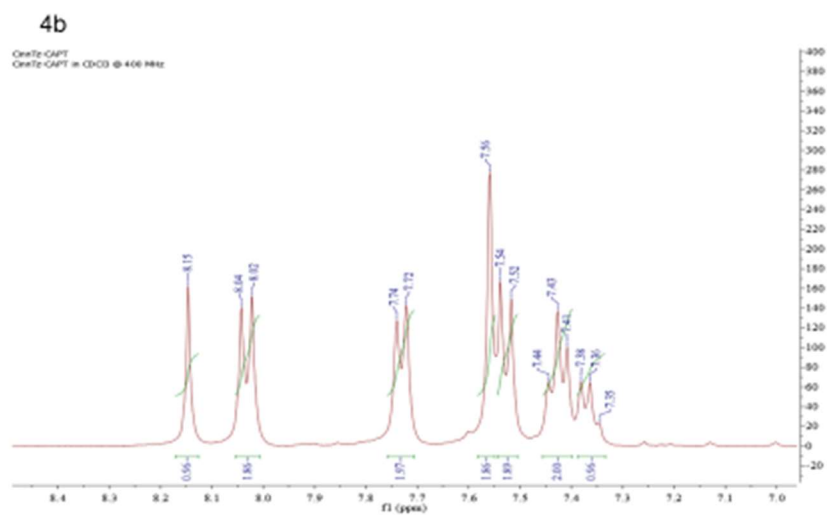
Aliquot solutions of the test compounds (2000 µM) were made from a stock solution (10 mM), using methanol or methylene chloride, depending on the solubility of each compound.⁵⁵ Further serial dilution of the 2000 µM aliquot solutions were made to obtain 1000 µM, 100 µM, 10 µM and 1 µM solutions.⁵² The aliquot and diluted solutions (10 µL) were impregnated aseptically under a laminar flow hood on previously sterilised discs (8 mm diameter) made from polytetrafluoroethylene (PTFE) papers, and the residual solvent from methanol or methylene chloride was allowed to evaporate. This procedure was repeated with PTFE-impregnated solvents (methanol and methylene chloride), which were used as the negative controls (blank), while streptomycin (10 µg) and ampicillin (25 µg) mast-discs from Mast Diagnostics, Mast Group Ltd., Merseyside, UK, served as the positive controls (or reference drugs).^{56,57} Test compound discs were aseptically dispensed onto the surface of the inoculated agar plates (150 mm in diameter), and pressed down to ensure complete contact with the agar surface. Each nutrient agar plate accommodated eight discs, including five discs of the test compounds at different concentrations, (10 -2000 µL), two antibiotic controls (ampicillin and streptomycin) and the blank (solvent-impregnated disc). The plates were inverted and incubated at 37 °C for 24 hours.^{56,57}

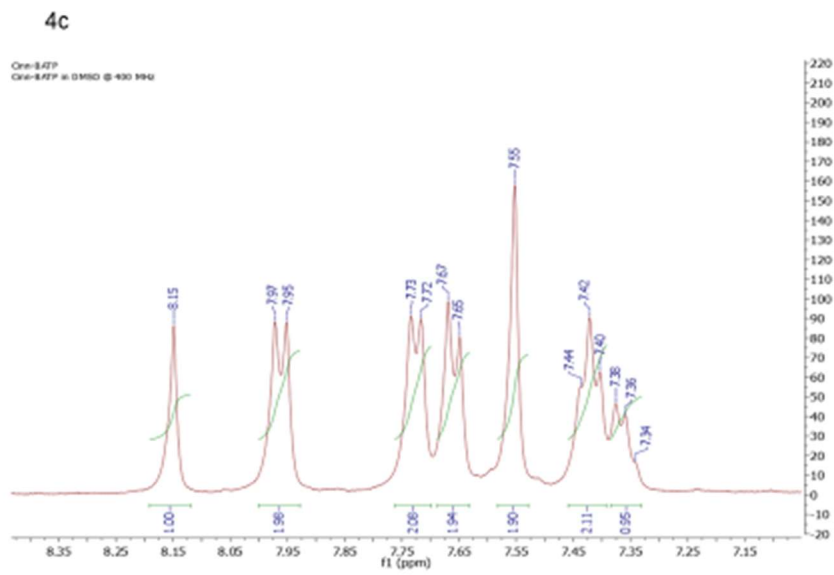
The level of susceptibility of *P. aeruginosa* to each of the test compounds was compared to the reference drugs (*i.e.*, positive controls), which were determined by measuring the diameters (mm) of the cleared zones with no visible growth. The diameter was measured from the centre of the disc to the edge of the zone of inhibition. The cleared zone, produced by a compound compared

to that of the reference drug, represents the level of relative susceptibility of *P. aeruginosa* to the compound at the tested concentration, as shown in Table 1.^{56,57}

References for bioassays

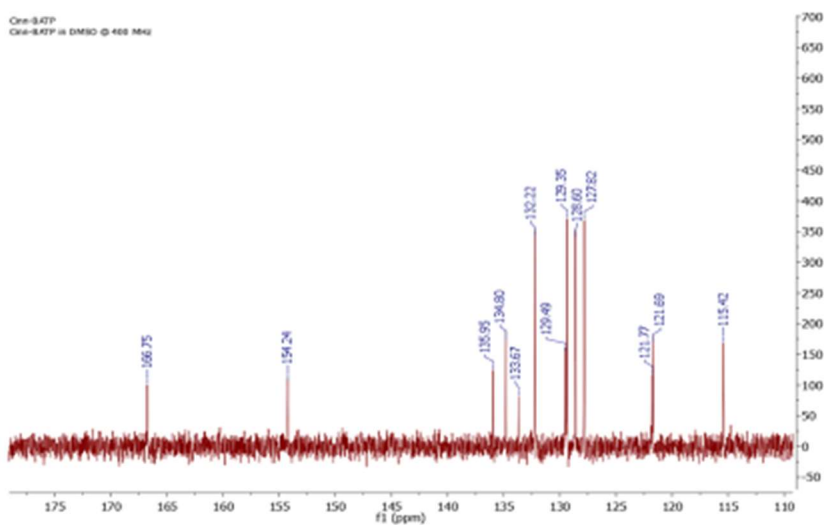
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SUPPORTING INFORMATION II – *NMR spectra*



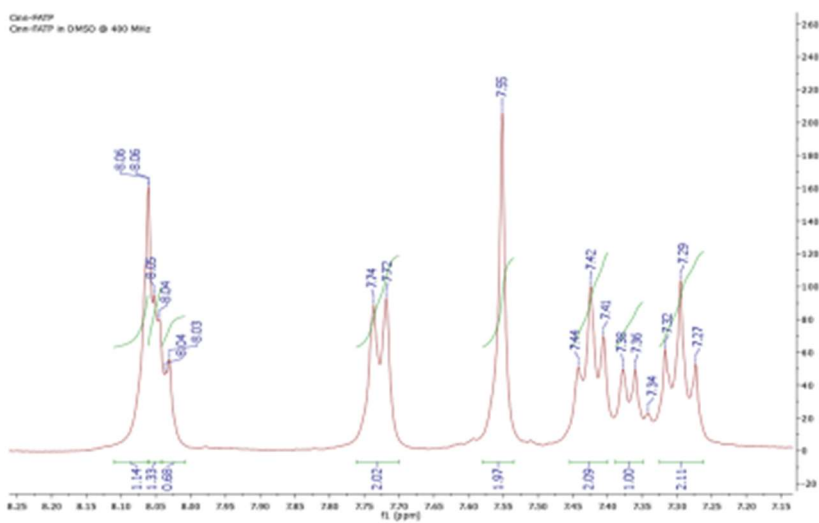
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Orb-847P in DMSO @ 400 MHz



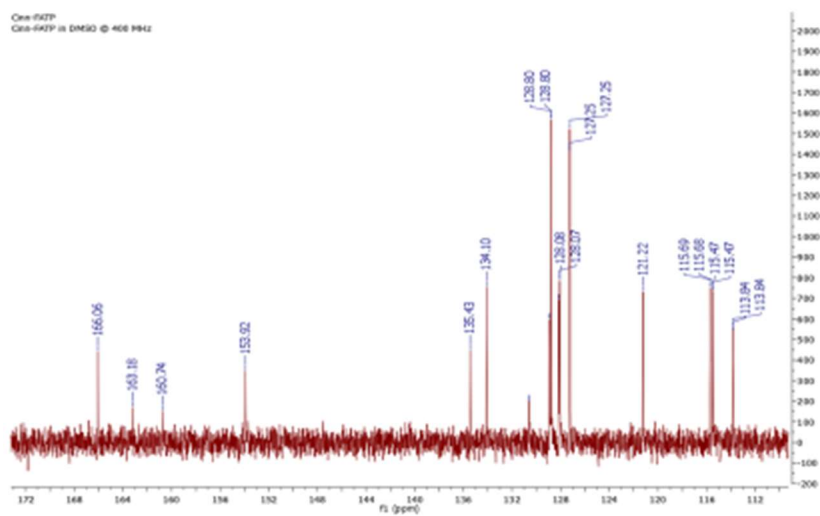
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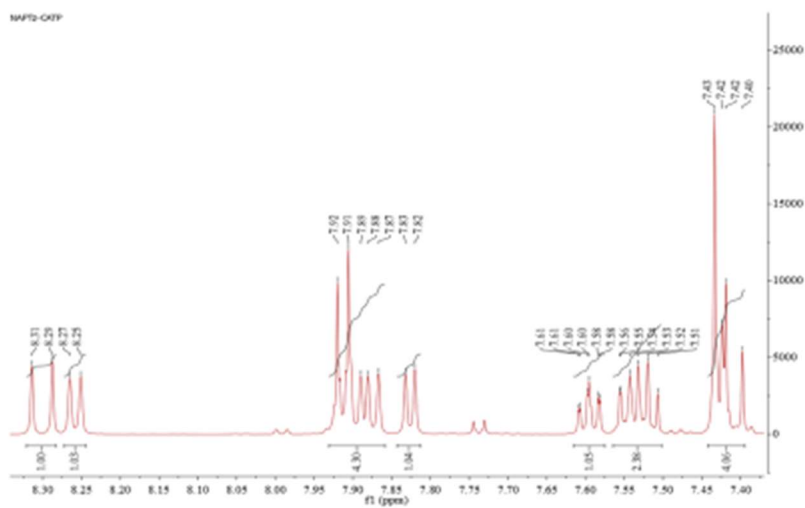
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 Chem 1027P in DMF-D₂O © 406 9942



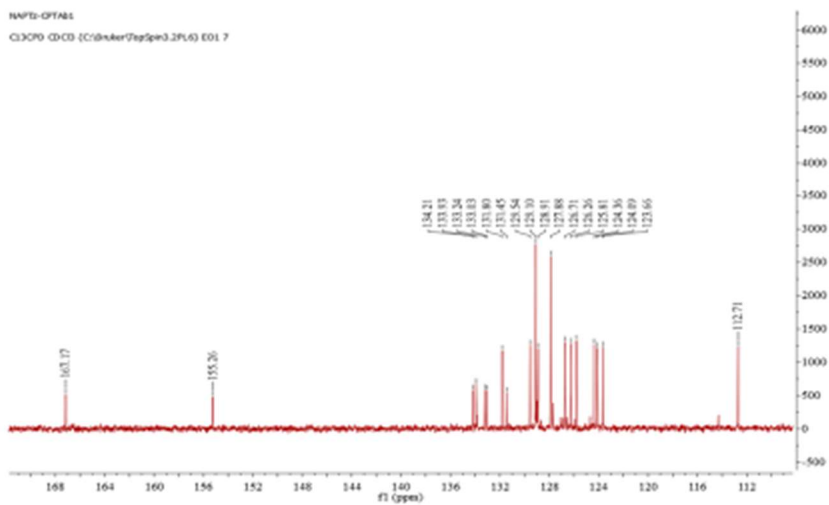
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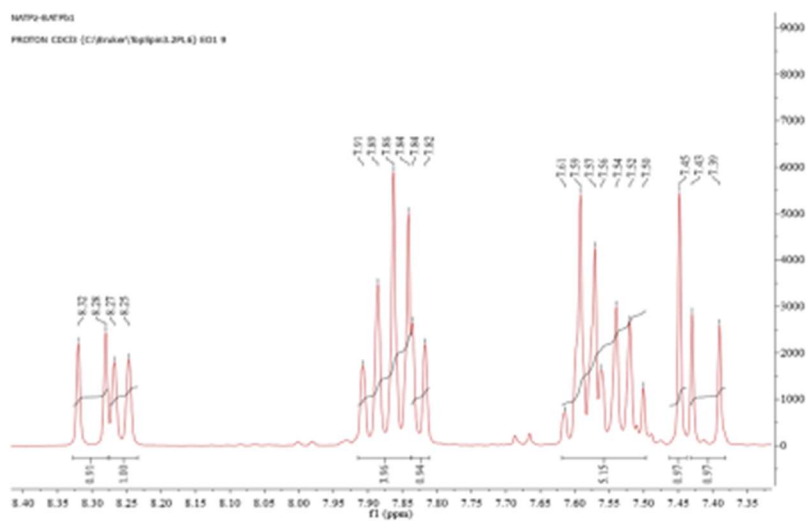
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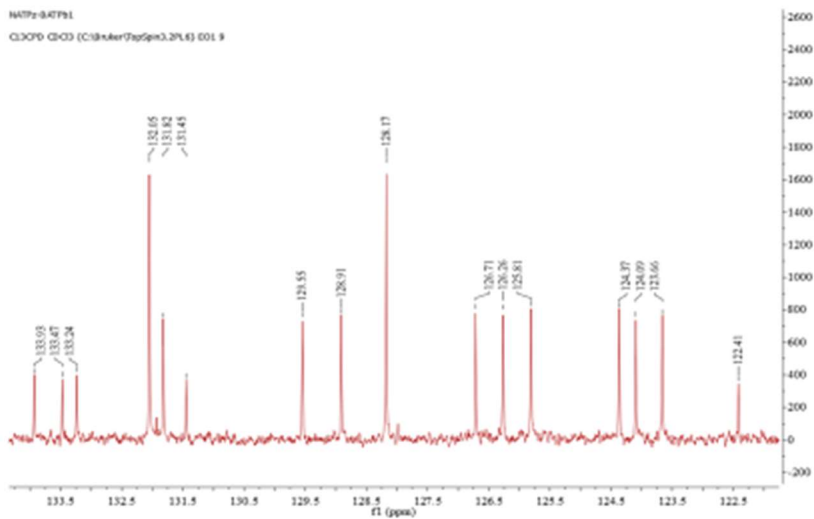


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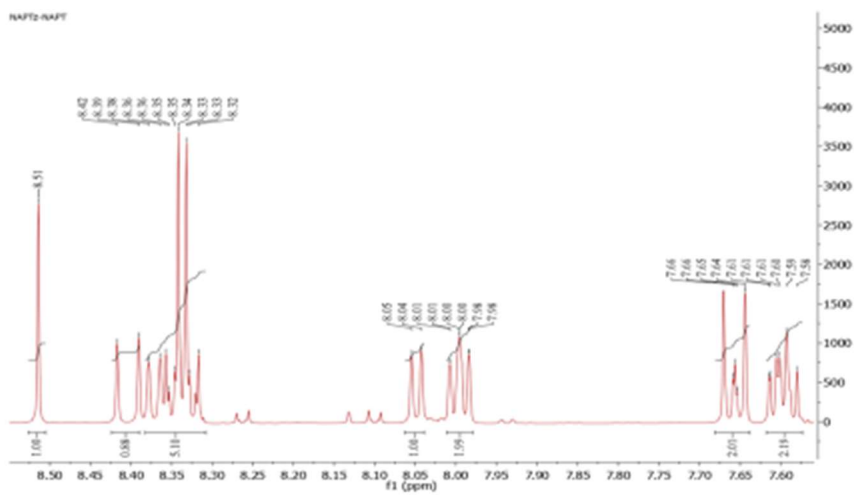
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