Synthesis and Biological Evaluation of 2-Chloro-3-[(thiazol-2-yl)amino]-1,4-naphthoquinones

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Supplementary Material

NMR and HRMS spectra Pp.2-12

Bioassay Protocols Pp. 13-20

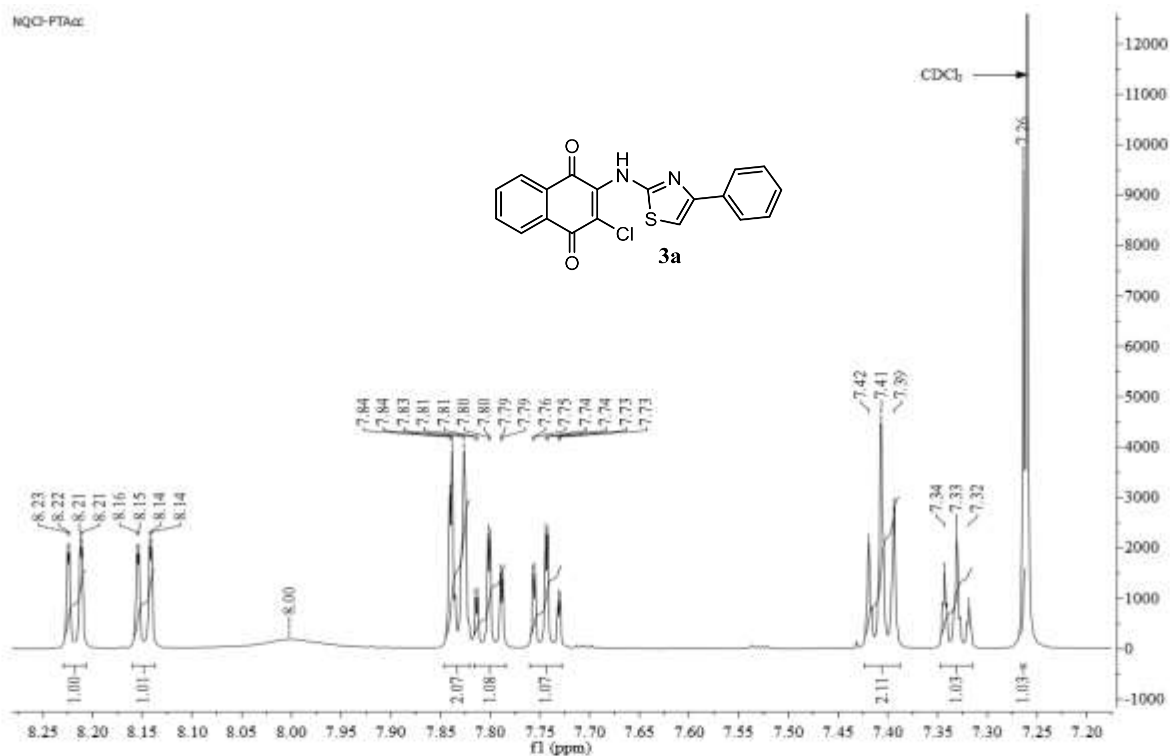


Figure 1: 400 MHz ^{1}H NMR spectrum of 2-chloro-3-[(4-phenylthiazol-2-yl)amino]-1,4-naphthoquinone **3a** in $CDCl_3$.

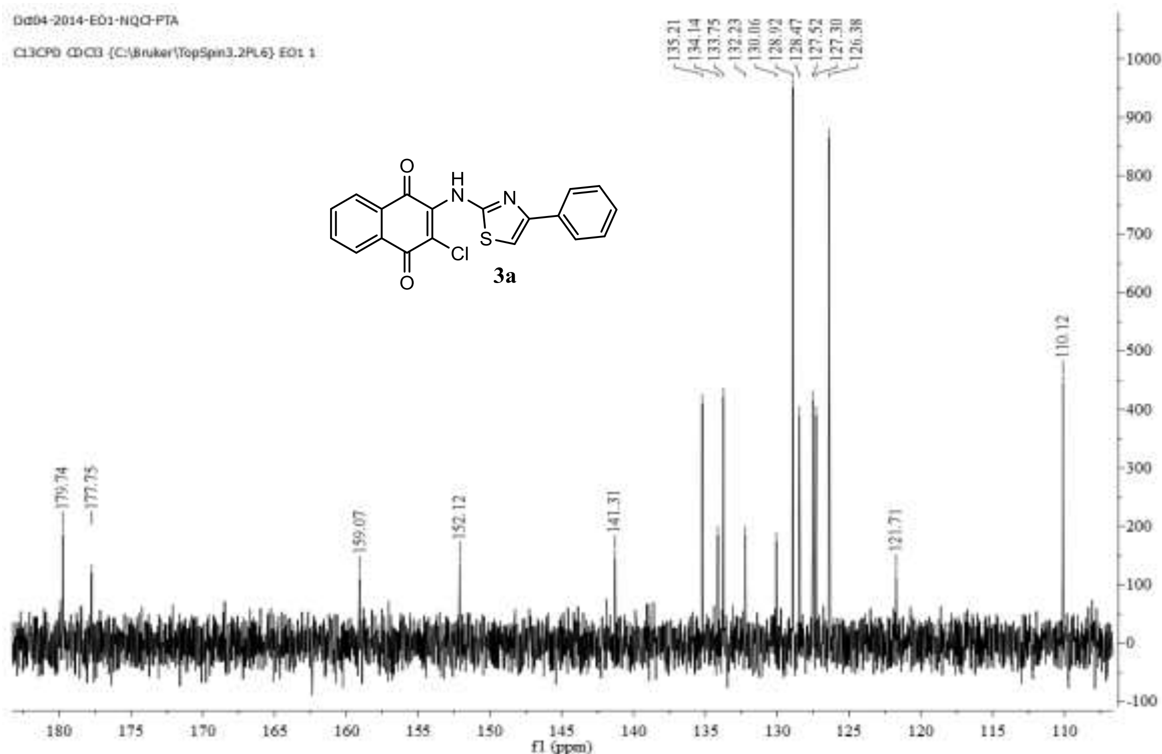


Figure 2: 100 MHz ^{13}C NMR spectrum of *2-chloro-3*-[(4-phenylthiazol-2-yl)amino]-1,4-naphthoquinone **3a** in $CDCl_3$.

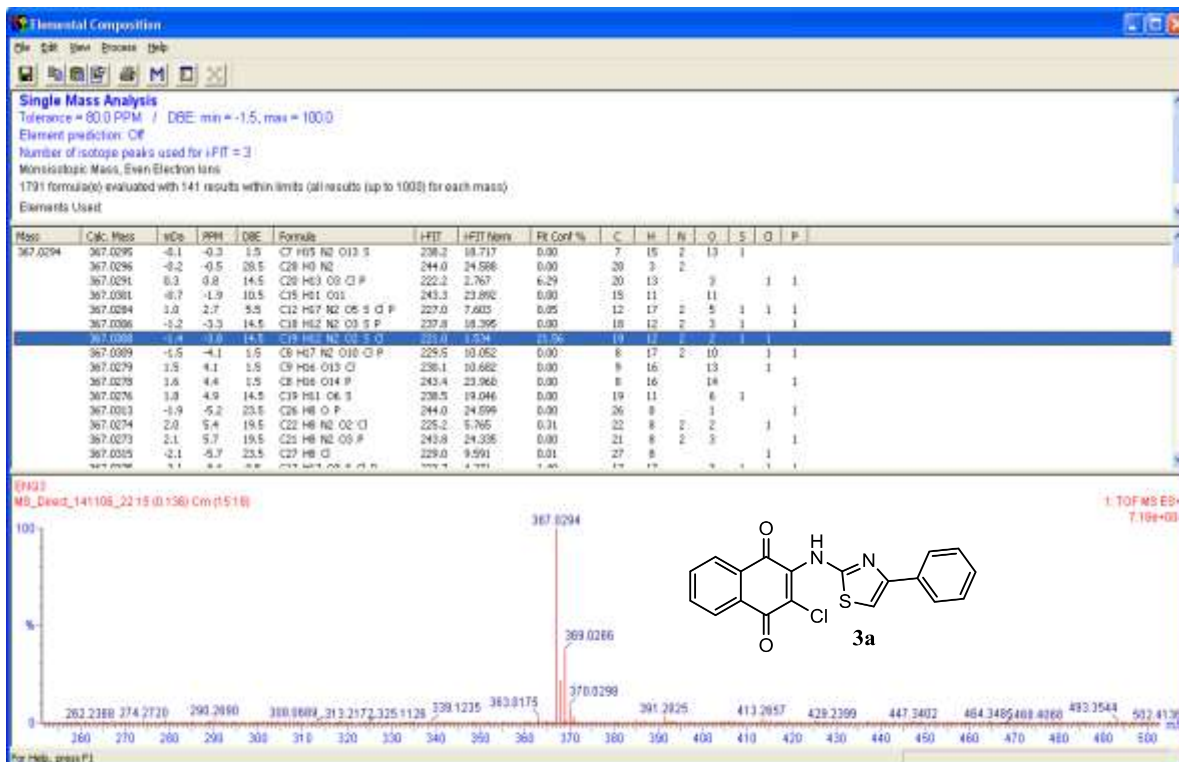


Figure 3: HRMS data of 2-chloro-3-[(4-phenylthiazol-2-yl)amino]-1,4-naphthoquinone **3a.**

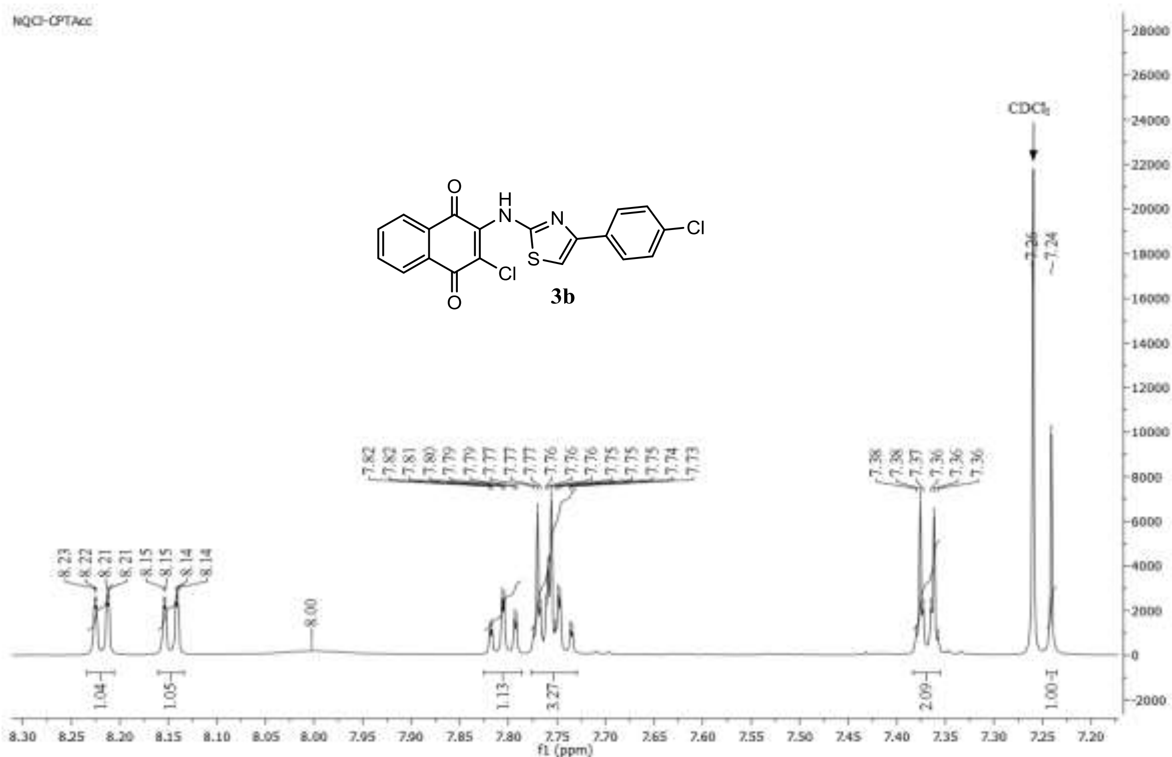


Figure 4: 400 MHz ^{1}H NMR spectrum of 2-chloro-3-{[4-(4-chlorophenyl)thiazol-2-yl]amino}-1,4-naphthoquinone **3b** in $CDCl_3$

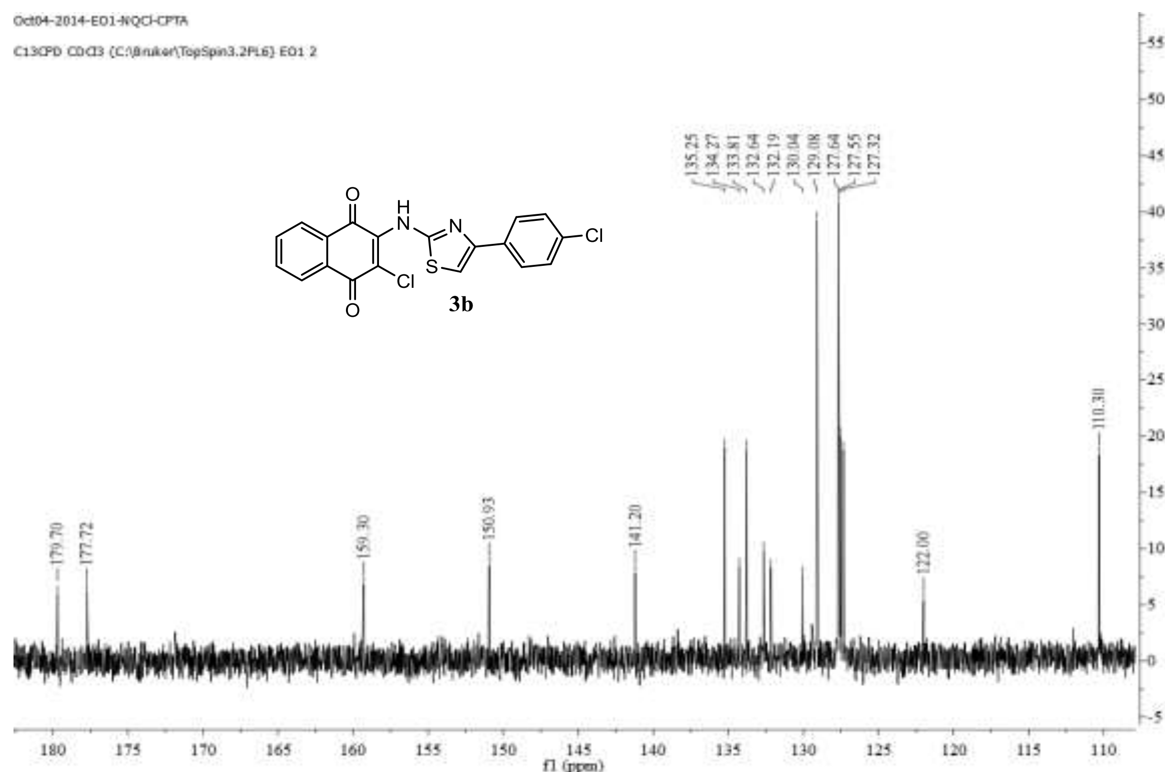


Figure 5: 100 MHz ^{13}C NMR spectrum of 2-chloro-3-{[4-(4-chlorophenyl)thiazol-2-yl]amino}-1,4-naphthoquinone **3a** in $CDCl_3$.

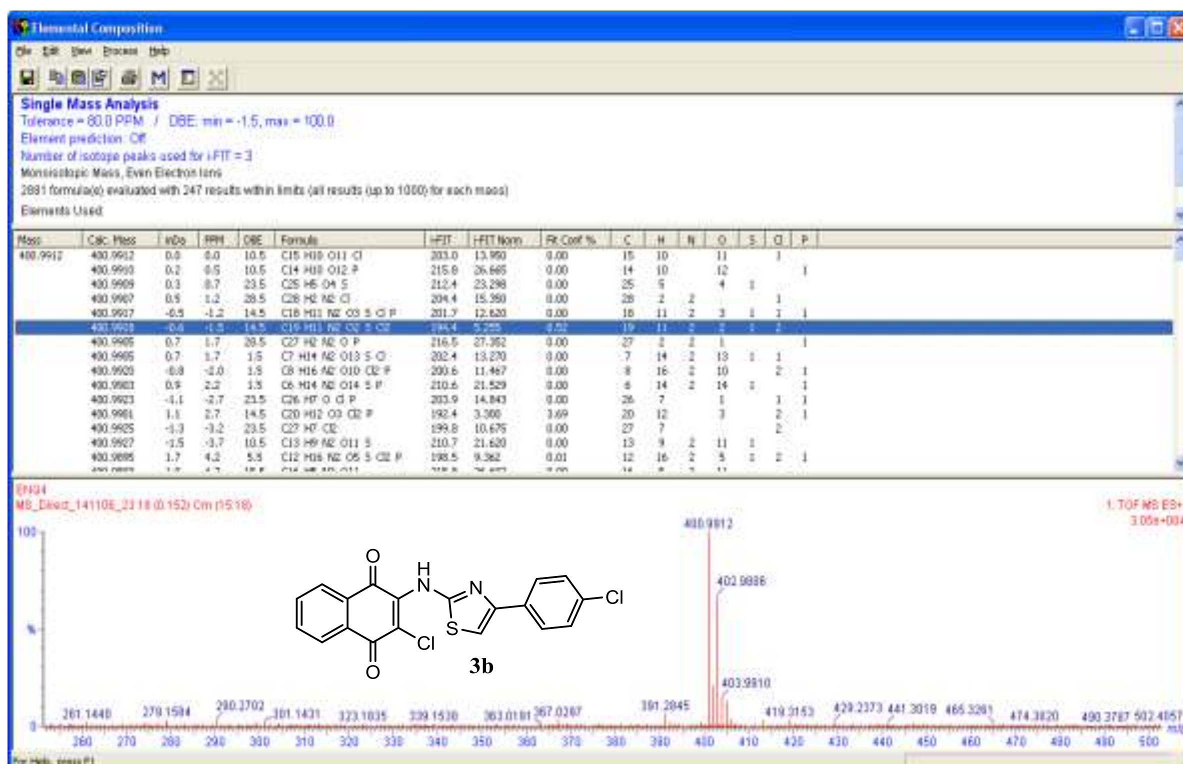


Figure 6: HRMS data 2-chloro-2-chloro-3-{[4-(4-chlorophenyl)thiazol-2-yl]amino}-1,4-naphthoquinone **3b.**

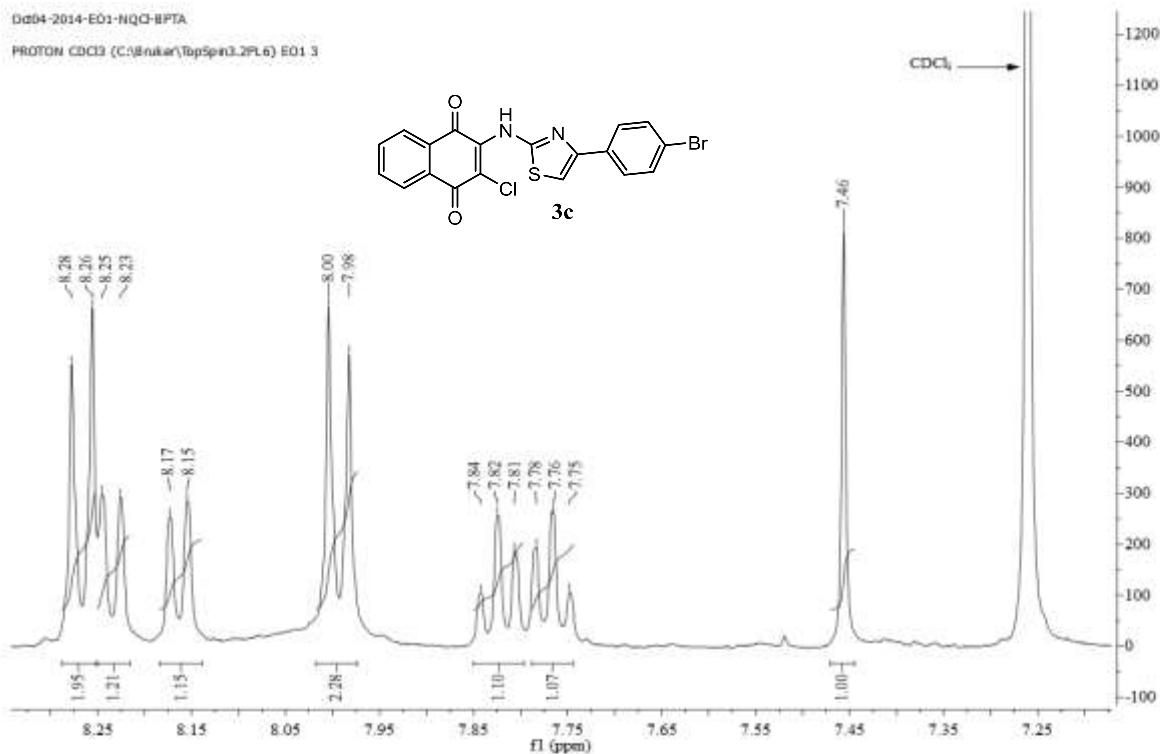


Figure 7: 400 MHz ^{1}H NMR spectrum of 2-chloro-3-{[4-(4-bromophenyl)thiazol-2-yl]amino}-1,4-naphthoquinone **3c** in $CDCl_3$

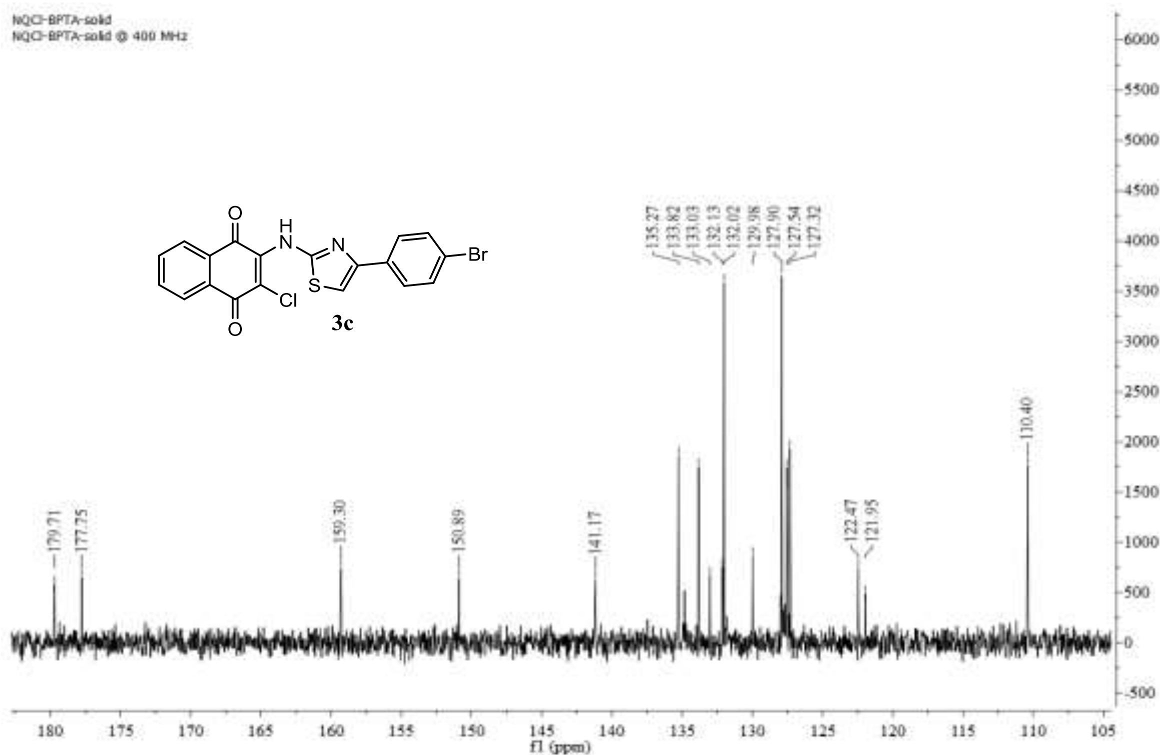


Figure 8: 100 MHz ^{13}C NMR spectrum of 2-chloro-3-{[4-(4-bromophenyl)thiazol-2-yl]amino}-1,4-naphthoquinone **3c** in $CDCl_3$.

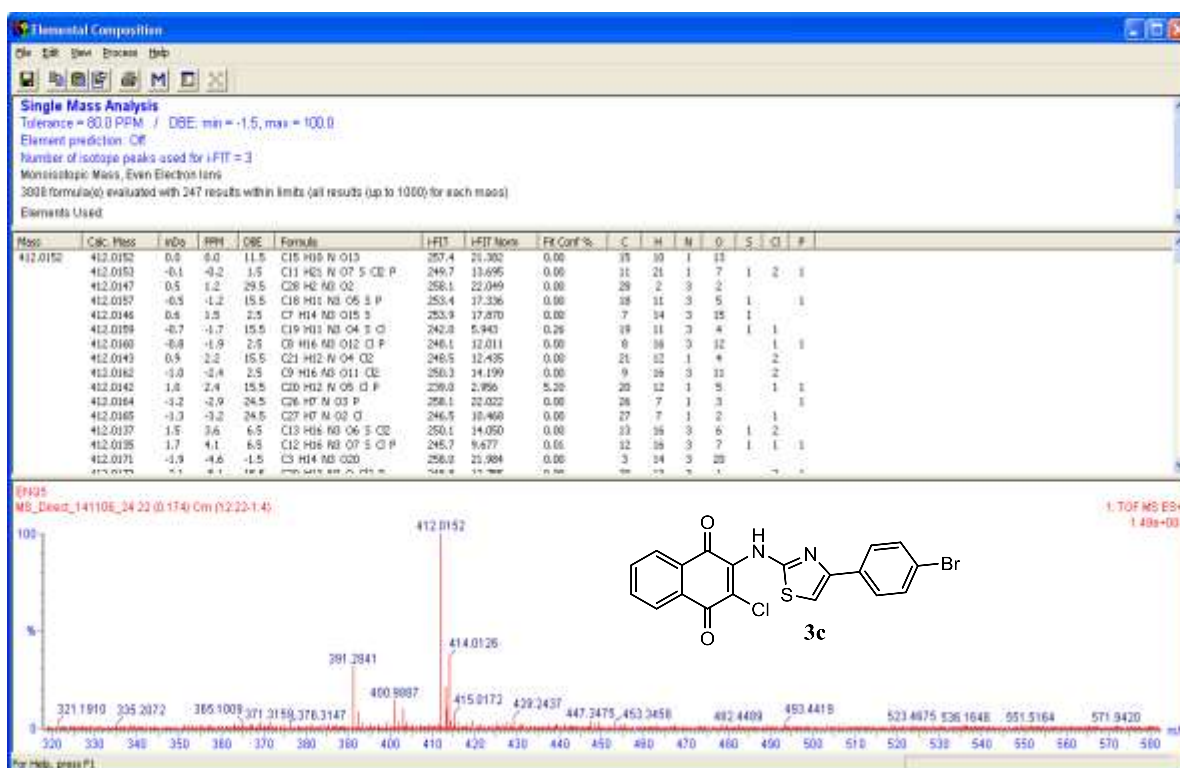


Figure 9: HRMS data of 2-chloro-3-{[4-(4-bromophenyl)thiazol-2-yl]amino}-1,4-naphthoquinone **3c.**

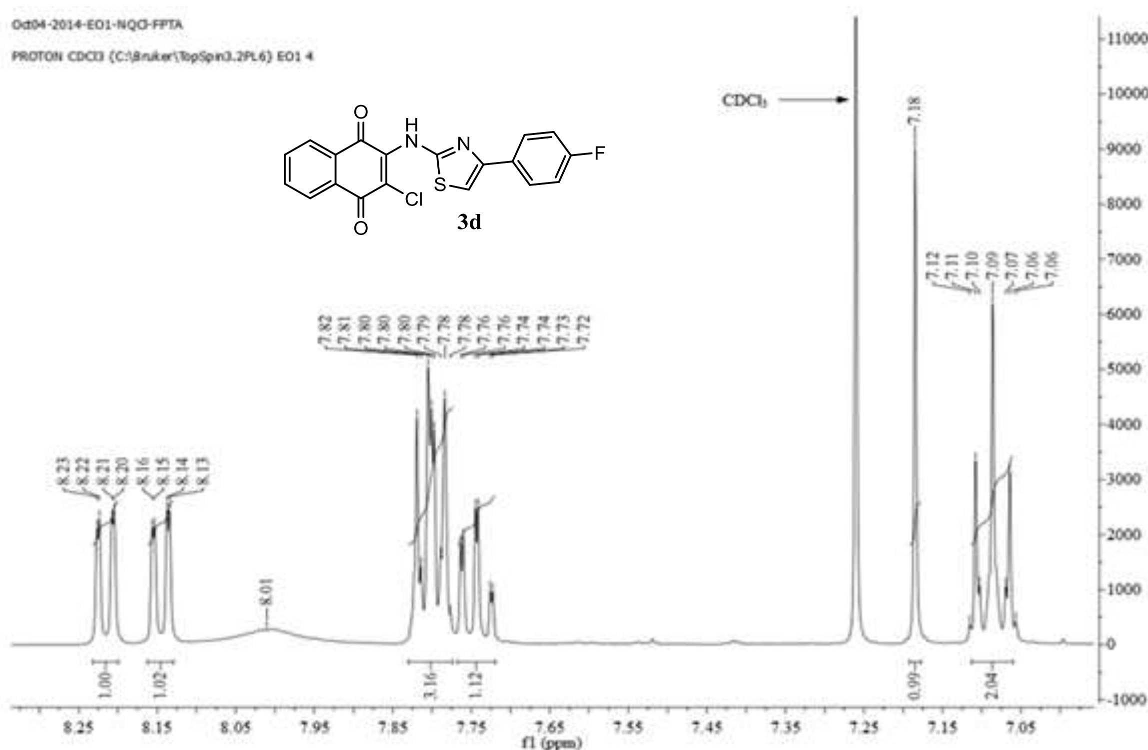


Figure 10: 400 MHz ^{1}H NMR spectrum of 2-chloro-3-{[4-(4-fluorophenyl)thiazol-2-yl]amino)-1,4-naphthoquinone **3d** in $CDCl_3$

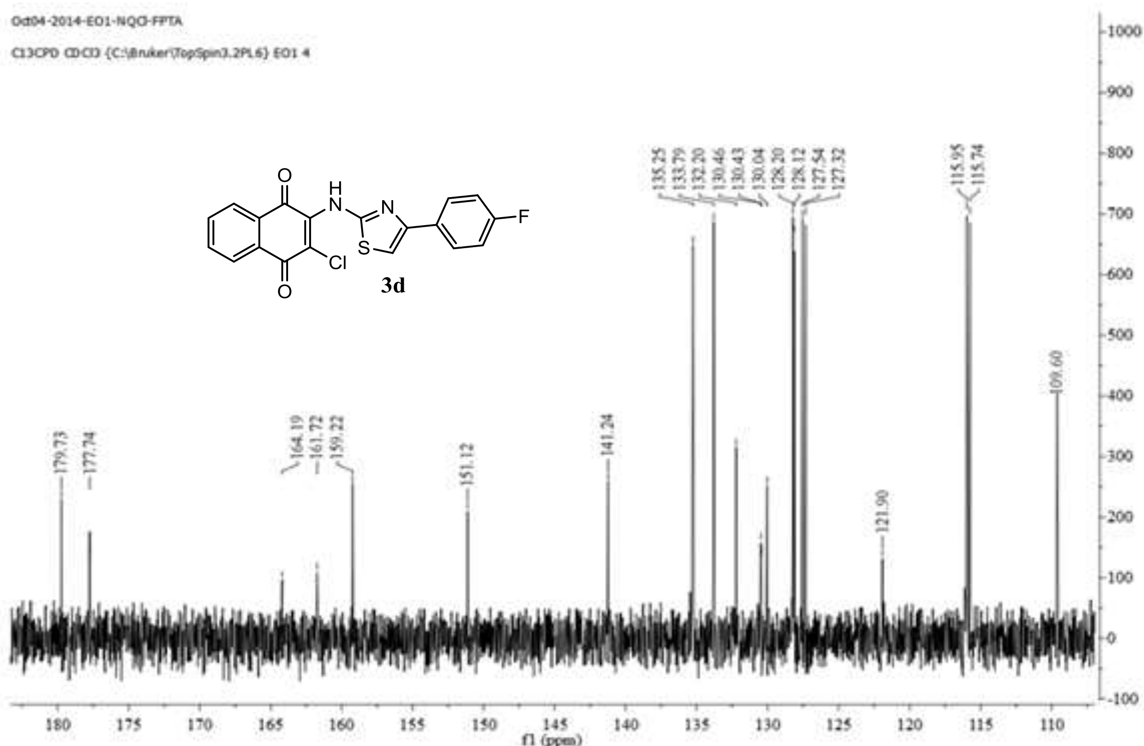


Figure 11: 100 MHz ^{13}C NMR spectrum of 2-chloro-3-{[4-(4-fluorophenyl)thiazol-2-yl]amino)-1,4-naphthoquinone **3d** in $CDCl_3$

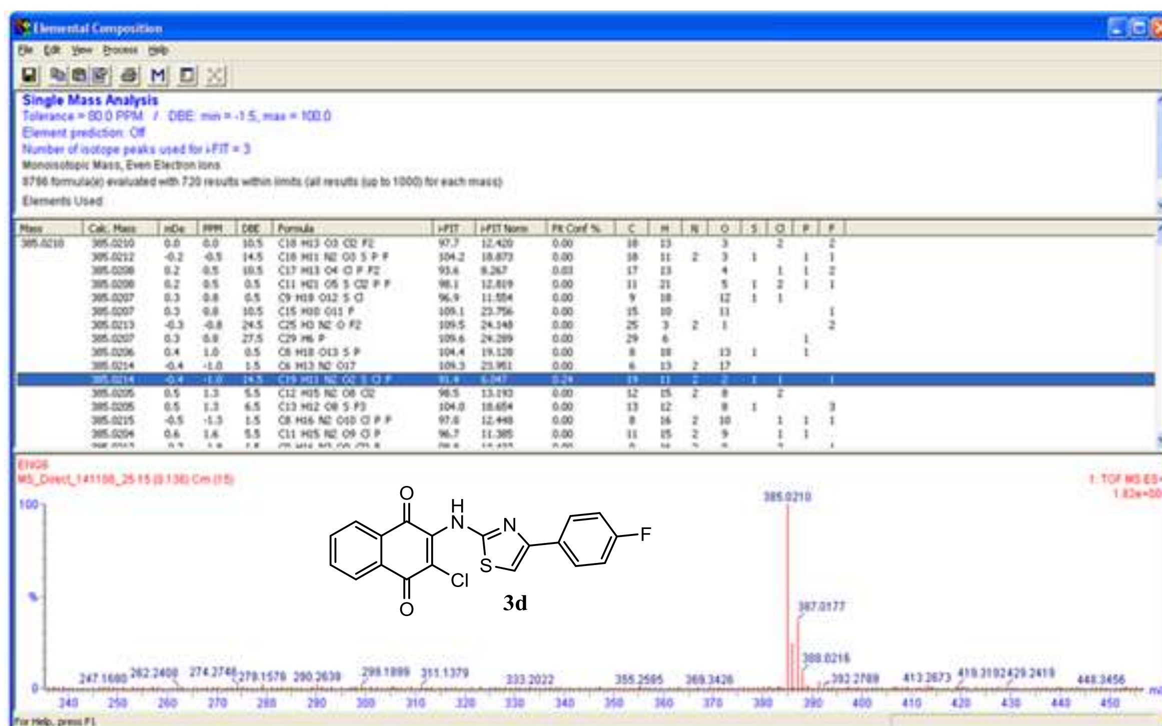


Figure 12: HRMS of 2-chloro-3-{[4-(4-fluorophenyl)thiazol-2-yl]amino)-1,4-naphthoquinone **3d** in $CDCl_3$

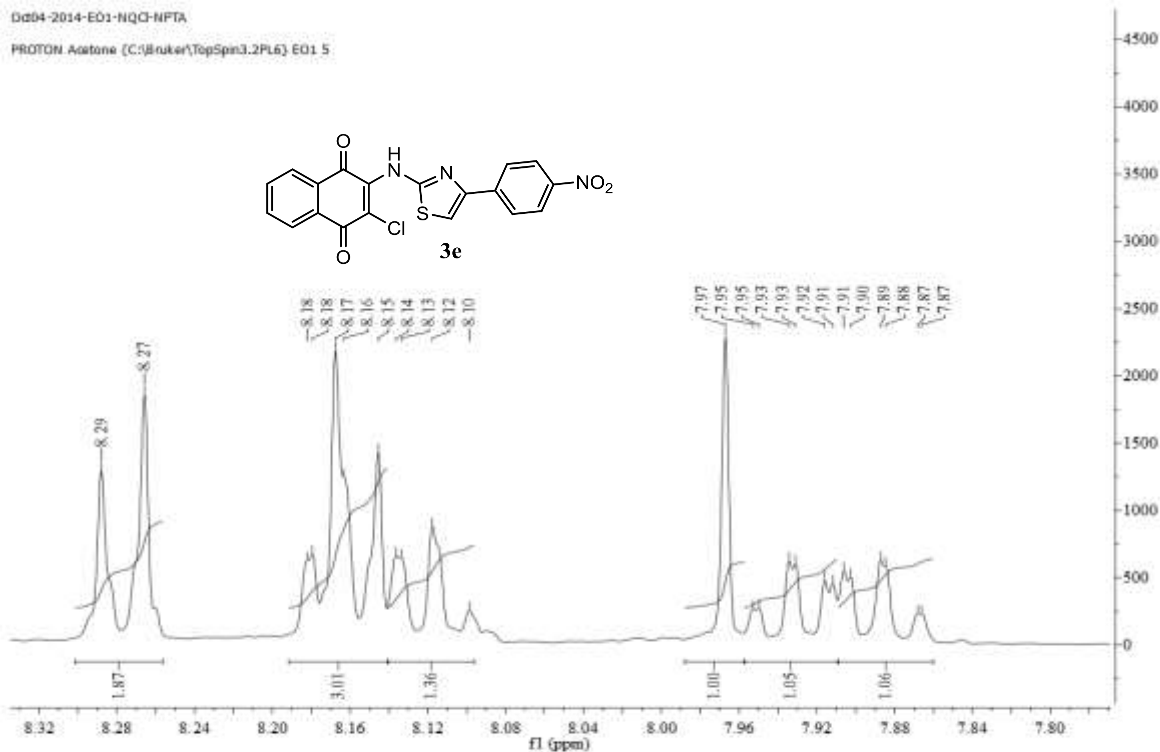


Figure 13: 400 MHz ^{1}H NMR spectrum of 2-chloro-3-{[4-(4-nitrophenyl)thiazol-2-yl]amino}-1,4-naphthoquinone **3e** in acetone-d_6

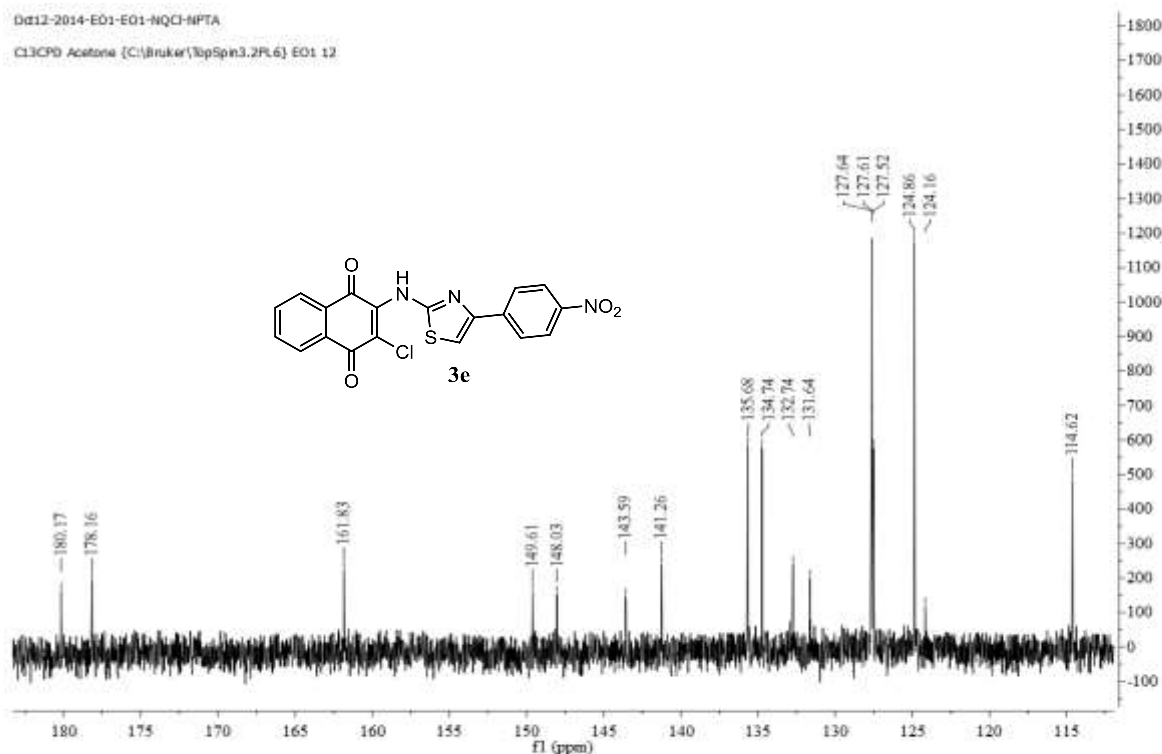


Figure 14: 100 MHz ^{13}C NMR spectrum of 2-chloro-3-{[4-(4-nitrophenyl)thiazol-2-yl]amino}-1,4-naphthoquinone **3e** in acetone-d_6

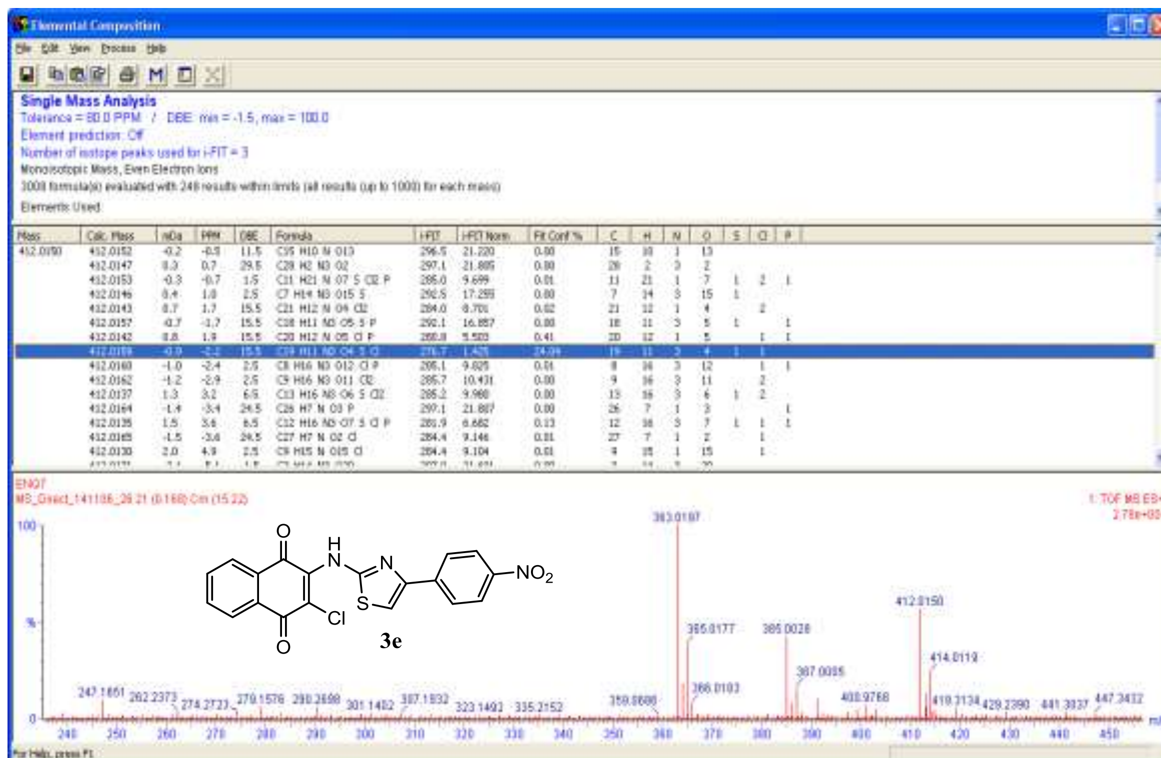


Figure 15: HRMS data of *2-chloro-3-{[4-(4-nitrophenyl)thiazol-2-yl]amino}-1,4-naphthoquinone* **3e.**

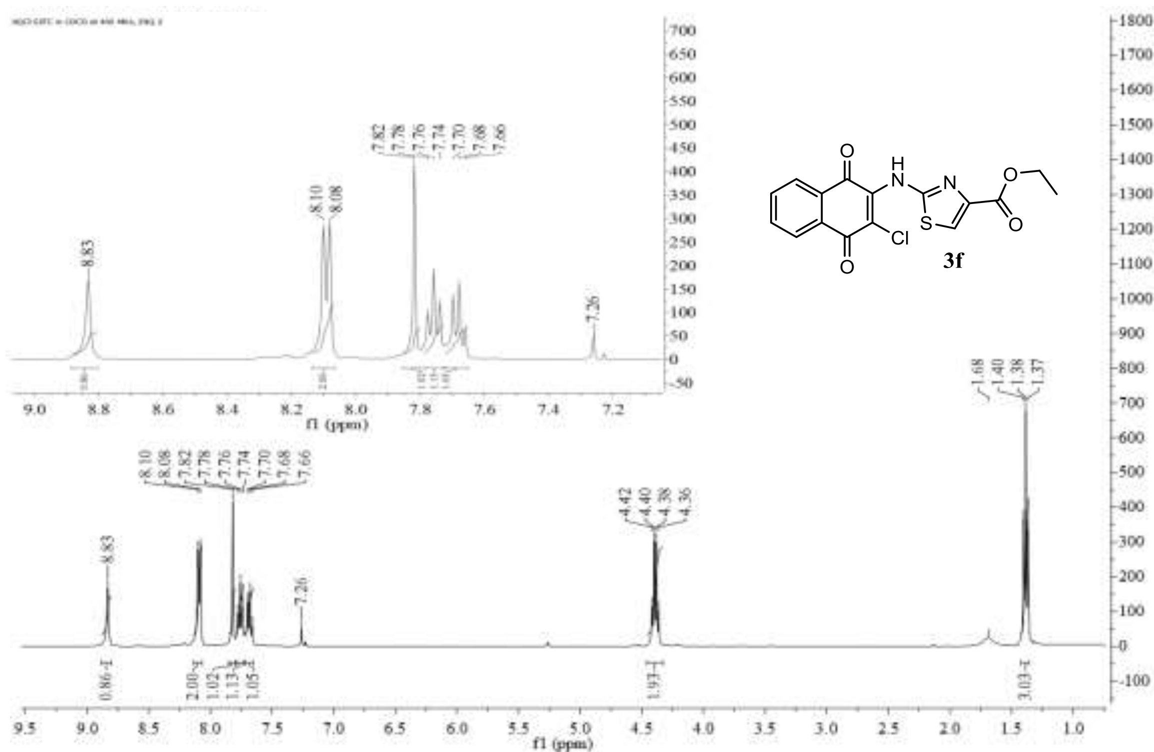


Figure 16: 400 MHz ^{1}H NMR spectrum of 3-[(4-carbethoxythiazol-2-yl)amino]-2-chloro-1,4-naphthoquinone **3f** in $CDCl_3$

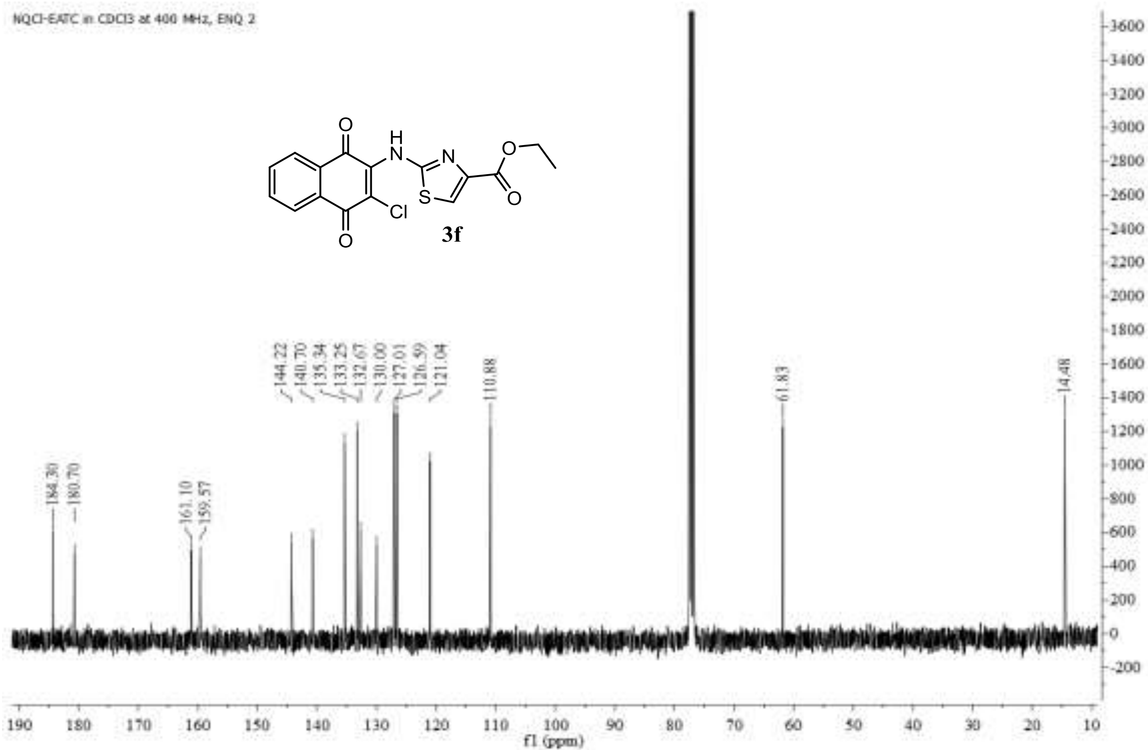


Figure 17: 100 MHz ^{13}C NMR spectrum of 3-[(4-carbethoxythiazol-2-yl)amino]-2-chloro-1,4-naphthoquinone **3f** in $CDCl_3$

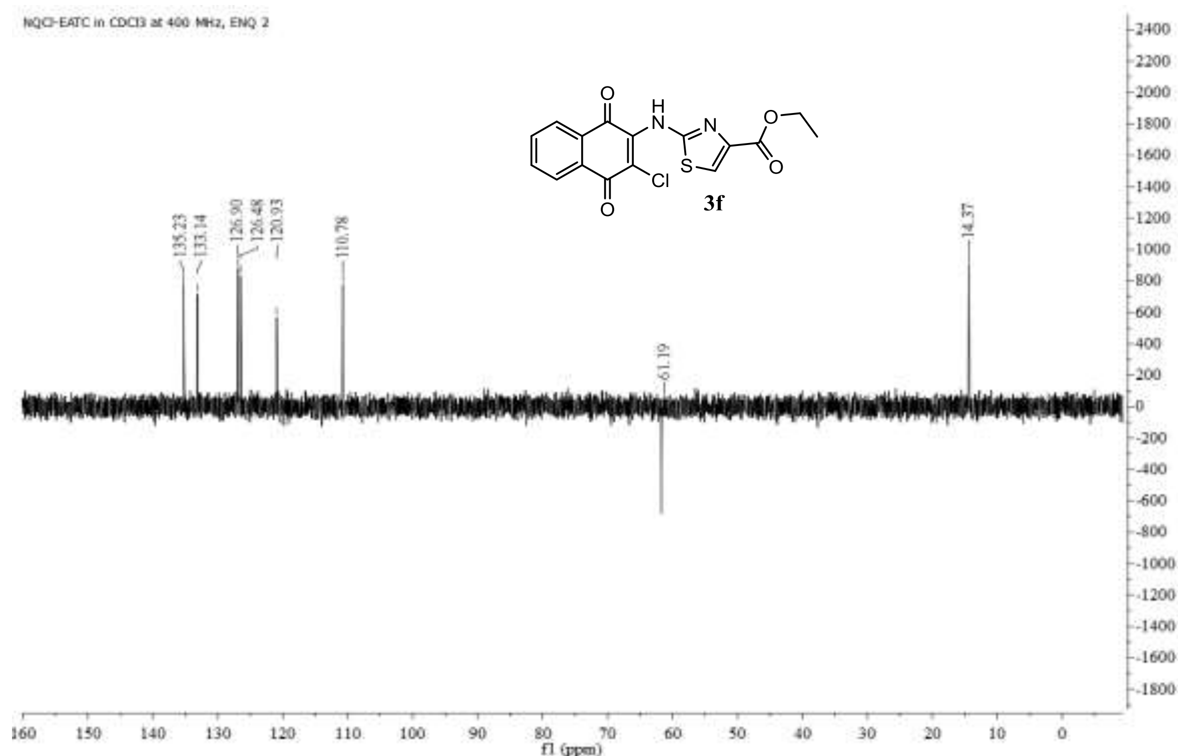


Figure 18: DEPT-135 spectrum of 3-[(4-carbethoxythiazol-2-yl)amino]-2-chloro-1,4-naphthoquinone **3f** in $CDCl_3$

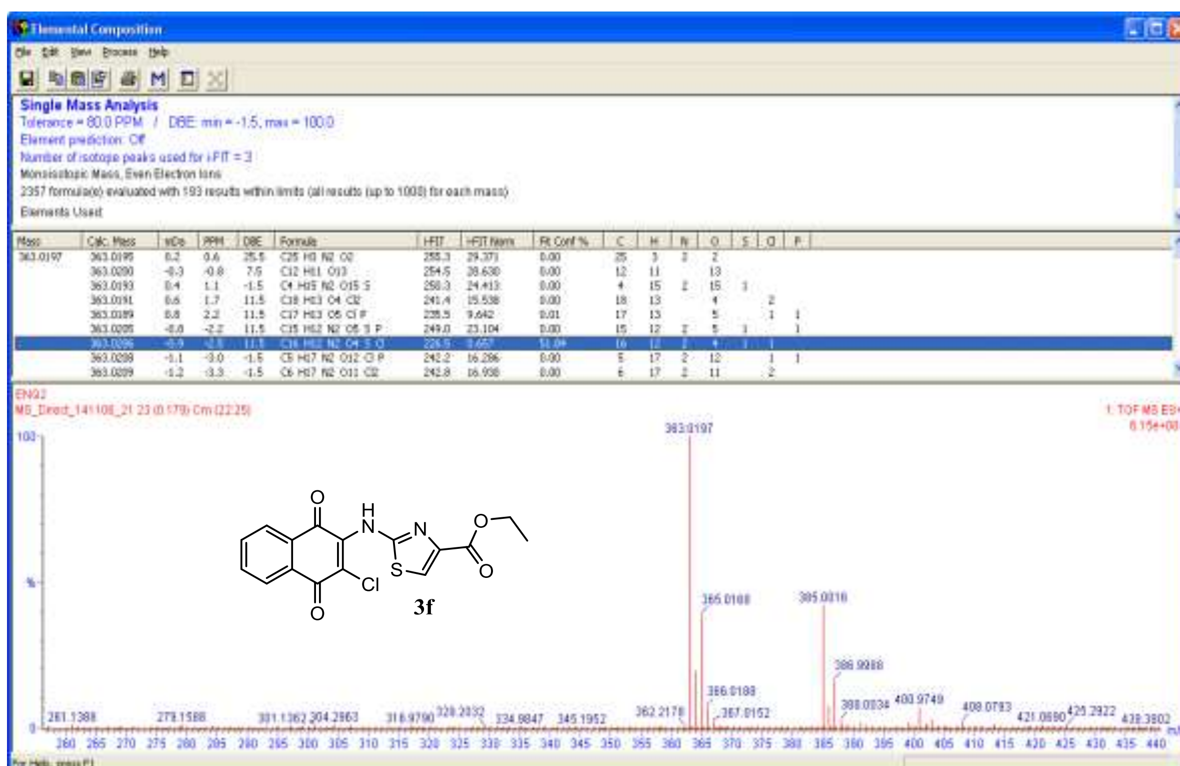


Figure 19: HRMS data of 3-[(4-carbethoxythiazol-2-yl)amino]-2-chloro-1,4- naphthoquinone **3f** in $CDCl_3$

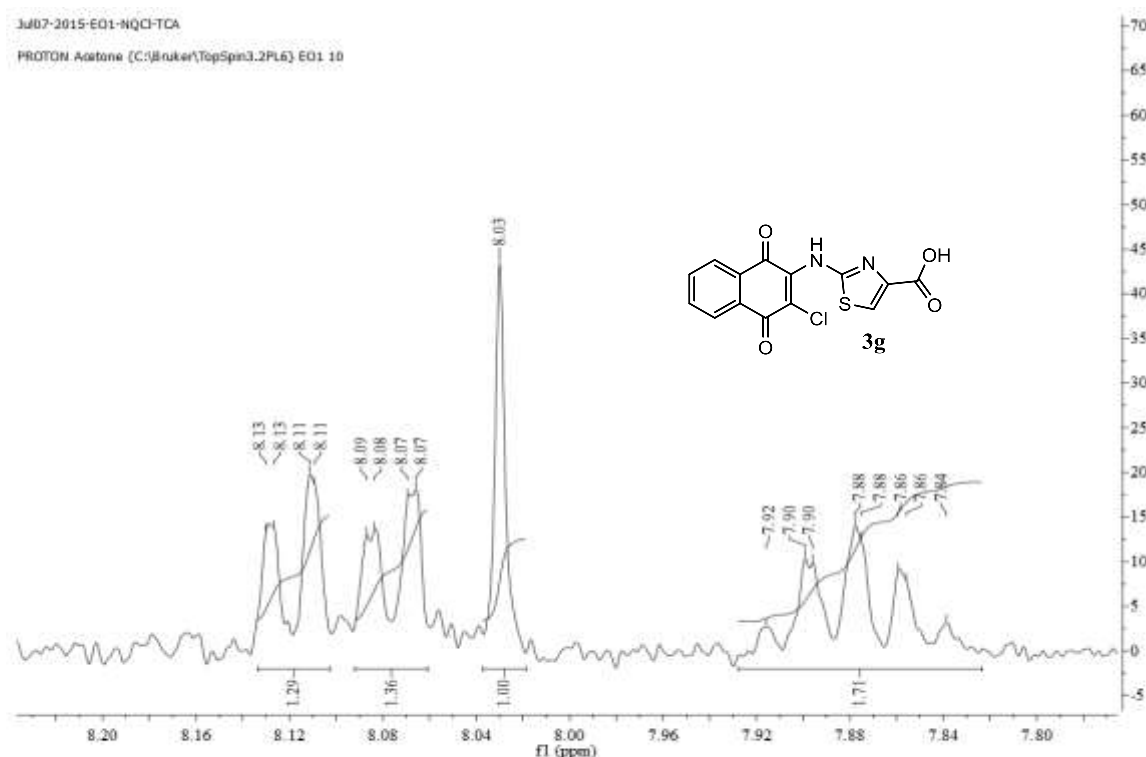


Figure 20: 400 MHz ¹H NMR spectrum of 3-[(4-carboxythiazol-2-yl)amino]-2-chloro-1,4-naphthoquinone **3g** in acetone-d_6

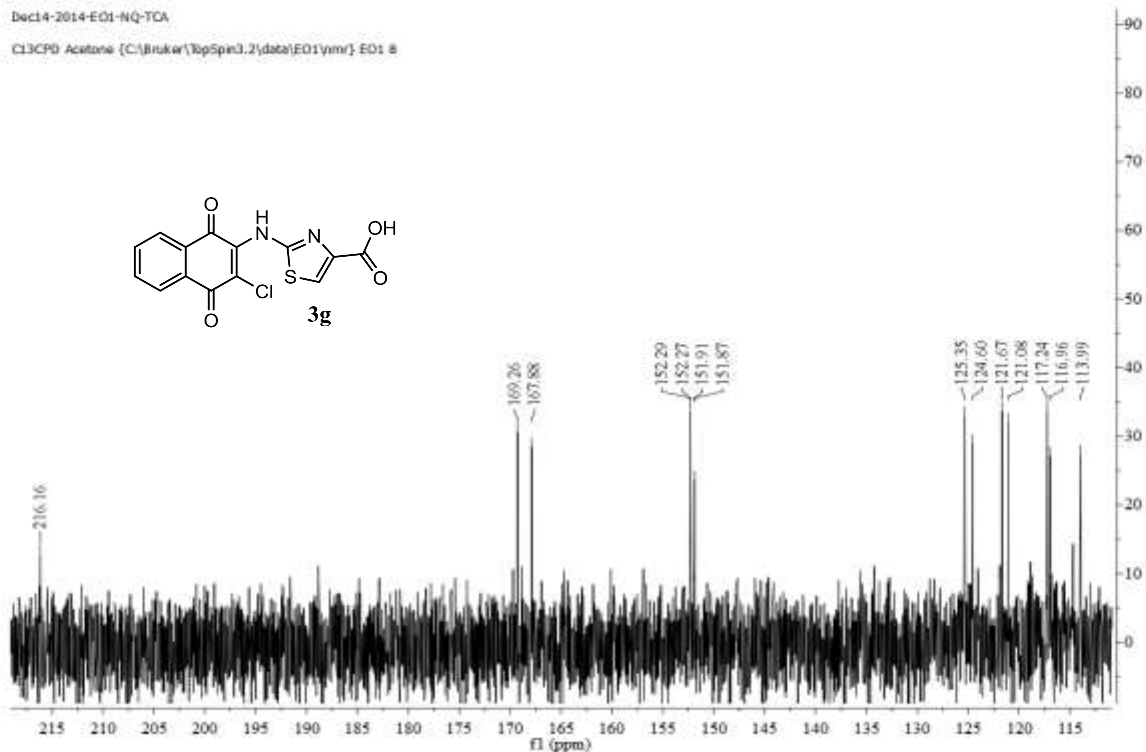
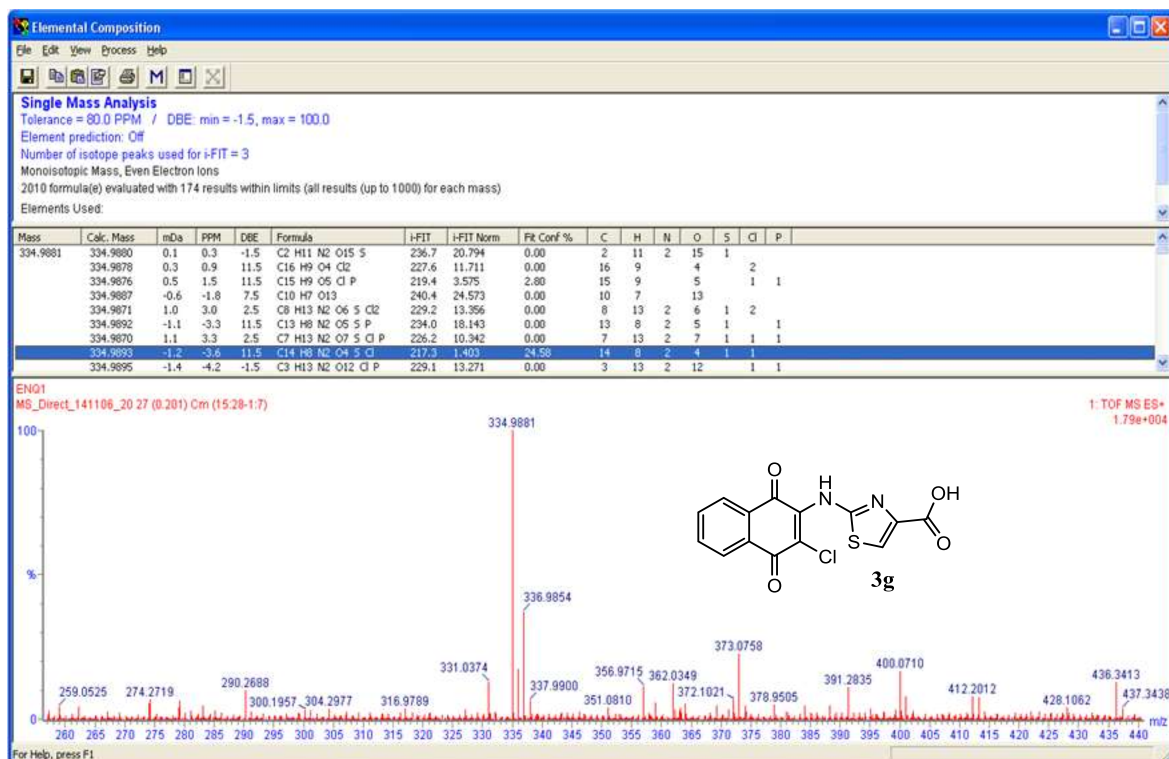


Figure 21: 100 MHz ^{13}C NMR spectrum of 3-[(4-carboxythiazol-2-yl)amino]-2-chloro-1,4-naphthoquinone **3g** in acetone-d_6



Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S	Cl	P
334.9881	334.9880	0.1	0.3	-1.5	C2 H11 N2 O15 S	236.7	20.794	0.00	2	11	2	15	1		
	334.9878	0.3	0.9	11.5	C16 H9 O4 Cl2	227.6	11.711	0.00	16	9		4		2	
	334.9876	0.5	1.5	11.5	C15 H9 O5 Cl P	219.4	3.575	2.80	15	9		5		1	1
	334.9887	-0.6	-1.8	7.5	C10 H7 O13	240.4	24.573	0.00	10	7		13			
	334.9871	1.0	3.0	2.5	C8 H13 N2 O6 S Cl2	229.2	13.356	0.00	8	13	2	6	1	2	
	334.9892	-1.1	-3.3	11.5	C13 H8 N2 O5 S P	234.0	18.143	0.00	13	8	2	5	1		1
	334.9870	1.1	3.3	2.5	C7 H13 N2 O7 S Cl P	226.2	10.342	0.00	7	13	2	7	1	1	1
	334.9893	-1.2	-3.6	11.5	C14 H8 N2 O4 S Cl	217.3	1.403	24.58	14	8	2	4	1	1	
	334.9895	-1.4	-4.2	-1.5	C3 H13 N2 O12 Cl P	229.1	13.271	0.00	3	13	2	12		1	1

Figure 22: HRMS data of 3-[(4-carboxythiazol-2-yl)amino]-2-chloro-1,4-naphthoquinone **3g.**

1. Anti-cancer bioassays

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

This was carried out as previously described.[1] Briefly, HeLa cells cultured in 96-well plates were incubated with individual compounds at a final concentration of 20 µM for 24 h, after which resazurin was added (13 µg/mL final concentration) and fluorescence (Exc_{560}/Em_{590}) read in a plate reader. Readings were converted to percentage cell viability relative to wells containing untreated cells.

2. Anti-cancer 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 (2mM), 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% CO_2.[2] The SH-SY5Y culture vessel, containing complete growth medium, was equally warmed to 37 °C in the incubator for 2 min 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 a G-force of 73 at 4 ˚C for 2 min. The cell pellets were re-suspended in DMEM in a T25 flask and incubated at 37 ˚C under 5% CO_2.[2] 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% CO_2 at 37 ˚C for 2 min until the cells were detached. Fresh medium (1 mL) was then added, aspirated and centrifuged at a G-Force of 202 at 4 ˚C for 2 min. The cell pellets were re-suspended in the medium, transferred into a new T75 flask, incubated under 5% CO_2 at 37 ºC and new medium was added after 4 days.[2]

2.2. Cytotoxicity assay using xCELLigence RTCA SP instrument

The optimal seeding concentration for the proliferation experiments of the SH-SY5Y was determined following the method reported by Malkoç and colleagues.[2] 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. Ten thousand (1

x 10^4) SH-SY5Y cells in 50 µL culture medium (DMEM Ham supplemented with 1% L-glutamine [2 mM], 10% FCS and 1% PBS were seeded into each well of the microelectronic censored E-Plate 96, and incubated at 37°C under 5% CO_2 in a humidified atmosphere.[2-4] The culture proliferation, attachment, and spreading of the cells were monitored every 30 min for 24 h 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% CO_2 at relative humidity of up to 98%, and scanned every 15 min for 24 h.[2] 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.[3-7] All cells were exposed for a total of 48 h.

The calculations were automatically computed by the RTCA-integrated software of the xCELLigence RTCA system. 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 (IC_{50} or log IC_{50}) at a given time point that produce 50% reduction of cell index (CI), relative to the SH-SY5Y control CI (100%). All data have been generated by the RTCA software, and are presented as mean (mmol/L) ± SEM (standard error of mean). 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.

3. Antimalarial bioassay

The activity of the compounds against erythrocytic stage *P. falciparum* (3D7 strain) parasites was assessed as previously described.[1] In summary, parasites cultured in 96-well plates were incubated with test compounds at a final concentration of 20 µM (or 3-fold serial dilutions of test compounds for dose-response assays) in 96-well plates for 48 h and residual parasite levels in individual wells determined by measuring parasite lactate dehydrogenase (pLDH) activity using a colorimetric assay.[8] Absorbance readings were converted to percentage parasite viability relative to untreated control wells and IC_{50} values determined by non-linear regression analysis of log[compound] vs. % viability plots.

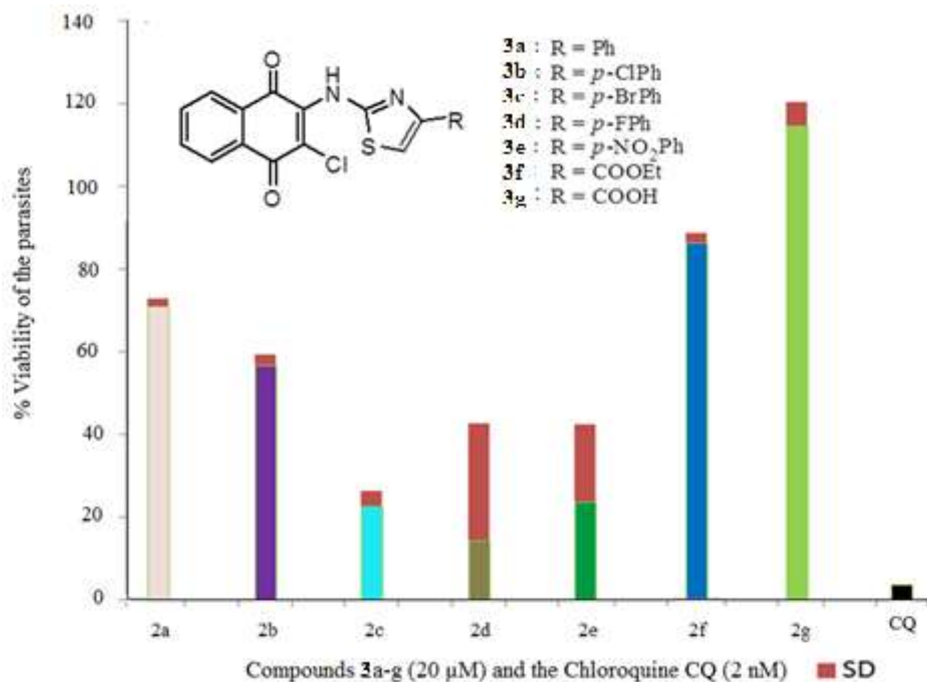


Figure 23. Antimalarial activities of 2-chloro-3-[(thiazol-2-yl)amino]-1,4-naphthoquinones **3a-g** against *Pf*LDH.

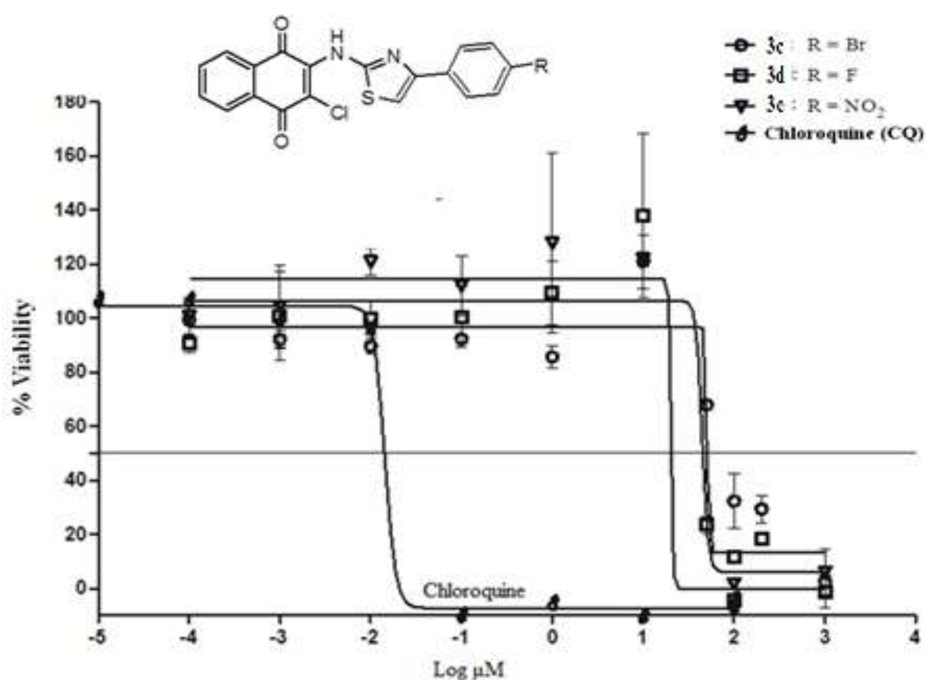


Figure 24. IC_{50} values of certain 2-chloro-3-[(thiazol-2-yl)amino]-1,4-naphthoquinones **3c-e** against *Pf*LDH.

4. *In vitro* antimycobacterial green fluorescent protein microplate assay (GFPMA)

MIC_{90} and MIC_{99}, which are the concentration of the thiazole-based compounds, which inhibit 90% and 99% of the growth of the virulent *M. tuberculosis*$H_{37}Rv$ (Mtb $H_{37}Rv$), respectively, were determined using the GFPMA in a microplate-based fluorometric assay.

A stock culture of the *Mtb::gfp* strain (H37RvMa::pMSP12GFP)[4] was grown to observe a density of 0.6-0.7, at 600 nm (i.e., OD_{600}), in 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 micro-well of a 96-well plate. In order to determine the MIC_{90} and MIC_{99}, the test compounds were dissolved in DMSO to make stock solutions of 12.8 mM, whereafter two-fold serial dilutions were prepared across a 96-well plate to provide ten dilutions of each compound in a final volume of 50 uL. The final concentration range was 160-0.078 µM. Rifampicin was used as positive control, while 5% DMSO and the Middlebrook 7H9-based media were used as negative controls. The plates were incubated for 14 days at 37 ^{o}C. Fluorescence (excitation 485 nM; emission 520 nM) was measured using a fluorometric plate reader on Day 7 and Day 14, post-inoculation. The lowest test and reference drug concentrations that inhibited the growth of 90% and 99% of the *Mtb::gfp* strain at day 7 and 14, were considered as the MIC_{90} and MIC_{99} values respectively (Figure 1).[9]

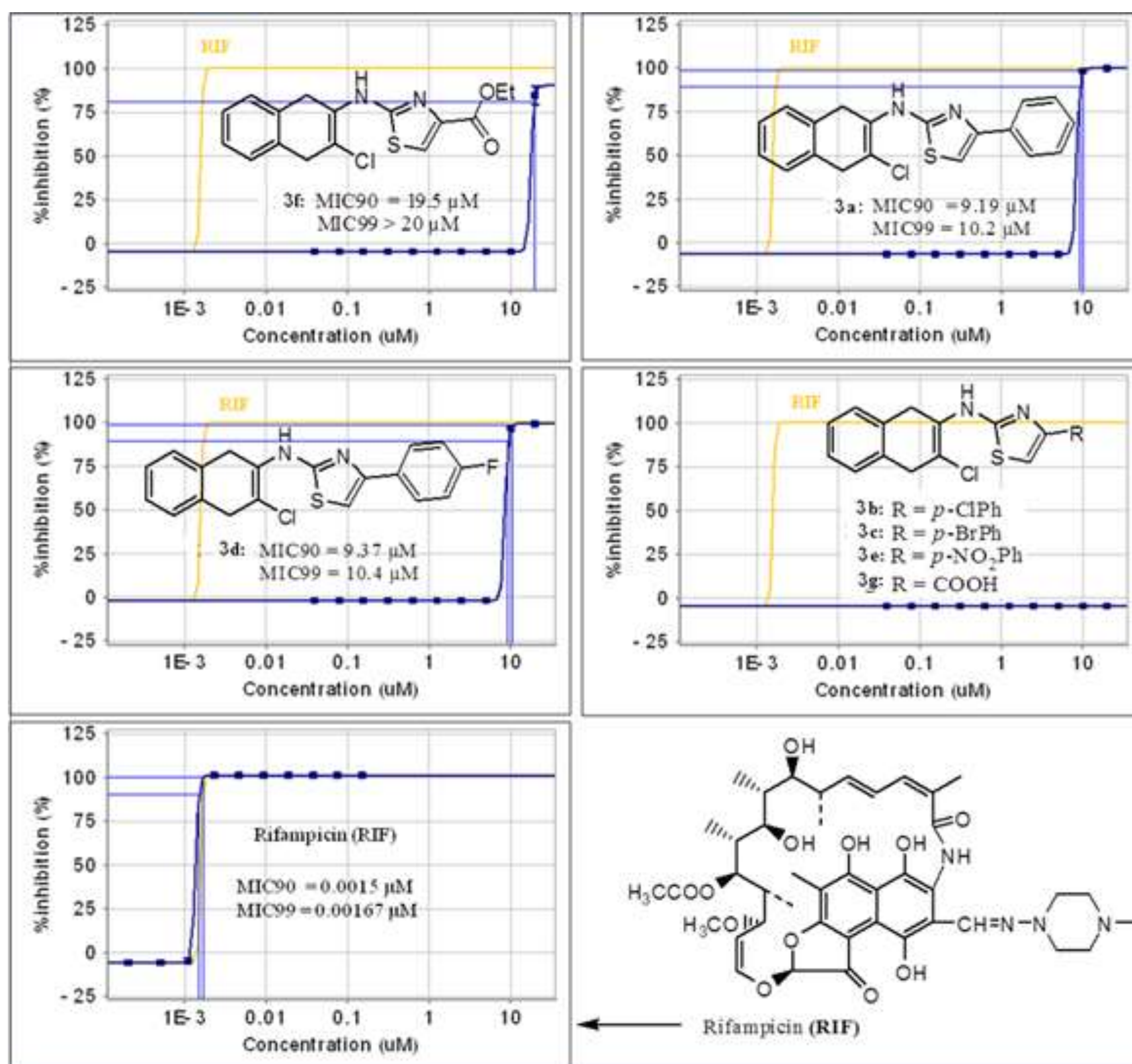


Figure 25. Dose-response plot of GFP-based assay for 2-chloro-3-[(thiazol-2-yl)amino]-1,4-naphthoquinones **3a-g** and rifampicin against *M. tuberculosis* $H_{37}Rv$.

5. Antibacterial disc susceptibility assay

5.1 Extraction of P. aeruginosa from soil samples

Soil samples, collected in Grahamstown, were weighed out in 5 g amounts, in triplicate, using a top loading balance [Zeiss West Germany (Pty) Ltd., Germany] and placed into sterile flasks. 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. Saline solution served as the negative control.[6] The samples were mixed for 2 min at 15 s intervals, after which serial dilutions (10^{-1}) of each of the samples were prepared, and 200 µL of both the undiluted and diluted solutions plated on cetrimide agar (90 mm in diameter). The plates were allowed to dry under a LabEAir laminar flow hood (Vivid Air, South Aftrica), for 10 s, incubated at 37 ^{o}C for 24 h and observed under a 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 37 ^{o}C for 24 h to obtain colonies with the same morphology, before the antibiotic assays were conducted.[7,8]

5.2. Disc diffusion antibacterial susceptibility test

5.2.1. 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.[9] Thereafter 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^{0} between streaking to obtain even inoculation; the lid was left ajar for about 5 min to allow for any surface moisture to be absorbed before applying the test compound-impregnated discs.[10]

5.2.2. 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.[11] Further serial dilutions of the 2000 µM aliquot solutions were made to obtain 1000 µM, 100 µM, 10 µM and 1 µM solutions.[7] 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 (Mast Diagnostics, Mast Group Ltd., Merseyside, UK) served as the positive controls (or reference drugs).[11,12] 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 h.

5.3.3. Data and interpretation

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 diameter (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.[11,12]

References

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