

Supplementary Materials: Synthesis, Antimicrobial, and Antioxidant Activities of Chalcogen-Containing Nitrones Derivatives from (*R*)-citronellal

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Experimental Section

General Information: The reactions were monitored by TLC carried out on Merck silica gel (60 F254) by using UV light as visualizant agent and 5% vanillin in 10% H₂SO₄ and heat as developing agents. Baker silica gel (particle size 0.040–0.063 mm) was used for flash chromatography. Proton nuclear magnetic resonance spectra (¹H NMR) were obtained at 300 MHz on a Varian Gemini NMR and at 400 MHz on Bruker DPX 400 spectrometer. Spectra were recorded in CDCl₃ solutions. Chemical shifts are reported in ppm, referenced to tetramethylsilane (TMS) as the external reference. Coupling constants (J) are reported in Hertz. Abbreviations to denote the multiplicity of a particular signal are s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). Carbon-13 nuclear magnetic resonance spectra (¹³C NMR) were obtained at 75 MHz on a Varian Gemini NMR and at 100 MHz on Bruker DPX 400 spectrometers. Chemical shifts are reported in ppm, referenced to the solvent peak of CDCl₃. Low-resolution mass spectra were obtained with a Shimadzu GC-MS-QP2010 mass spectrometer.

1. Synthesis of compounds, biological activities, spectral and analytical data

1.1. Synthesis of α -phenylselenanyl citronellal **3a**, α -phenylthio citronellal **3b** and β -phenylthio citronellal **8**

The synthesis of α -phenylchalcogen citronellal **3a–b** was performed according to the methodology developed by Nazari and Movassagh [1], with modifications. In a 25 mL vial was added (*R*)-citronellal (**1**, 0.308 g, 2 mmol), diphenyl disulfide (**2a**, 1.5 mmol) or diphenyl diselenide (**2b**, 2 mmol) and PEG-400 (4.0 mL) under N₂ atmosphere. Then, Al₂O₃/KF 40% (0.324 g, 1.5 mmol) was added and the temperature was slowly increased to 60°C. The progress of the reaction was monitored using thin layer chromatography (TLC) and after 22 h, compounds **1a–b** were isolated and identified. The synthesis of β -phenylchalcogen citronellal **8** was performed according to previously described by our group [2]. In a test tube was added citral (**6**, 0.304 g, 2 mmol), benzenethiol (**7**, 0.352 g, 2.4 mmol) and Al₂O₃/KF 40% (0.140 g, 0.65 mmol) under magnetic stirring at room temperature. The progress of the reaction was monitored using thin layer chromatography (TLC) and after 24 h, compound **8** was isolated.

1.2. General procedure for the synthesis of nitrones **5a–d** derived from citronellal

Using a synthetic route adapted from Isager *et al.* [3] in a 25 mL vial was added the aldehyde **1**, **3a–b** or **8** (0.5 mmol), N-methyl-hydroxylamine hydrochloride (**4**, 0.084 g, 1 mmol) and water (2 mL) as the solvent and the mixture was stirred at room temperature for 30 min. Then, a 1M solution of Na₂CO₃ (1.0 mL) was added and the stirring was continued for additional 24 h. Compound **5a** was purified by preparative chromatographic plate (silicagel) and compounds **5b–d** were isolated by column chromatography using neutral alumina as a stationary phase and a solution of hexanes/ethyl acetate as the eluent (90:10). The NMR spectra of nitrones **5a** (Figure S1 and Figure S2), **5b** (Figure S3 and Figure S4), **5c** (Figure S5 and Figure S6) and **5d** (Figure S7 and Figure S8) are in accordance with those expected for the compounds.

2. Synthesis of the selenium-containing oxime **10**

Using a synthetic route adapted from Isager *et al.* [3], in a 25 mL vial was added α -phenylseleno citronellal (**5b**, 0.156 g, 0.5 mmol), hydroxylamine hydrochloride (**9**, 0.069 g, 1 mmol) and water (2

mL) as the solvent. After stirring for 30 min at room temperature, it was added 0.5 mL of an aqueous solution of Na_2CO_3 (0.027 g, 0.26 mmol) and the stirring was continued for additional 22 h. After this time, the oxime **10** was isolated by column chromatography using silicagel as a stationary phase and a solution of hexanes/ethyl acetate (95:5) as the eluent. The NMR spectra of oxime **10** (Figure S9 and Figure S10) are in accordance with those expected for the compound.

2.1. Antimicrobial activity assay using the disk diffusion test

The disk diffusion test followed the methodology recommended by the Clinical Laboratory Standards Institute CLSI [4]. The inoculum was standardized by standard McFarland to the concentration of 10^8 CFU.mL⁻¹ and spread on the surface of a Petri dish containing Mueller-Hinton (MH) agar. Then, the paper disc (6 mm) was put on a plate impregnated with 20 μL of the testing compound previously diluted in DMSO (1:1) and incubated at 37 °C for 24 hours. Zone of inhibition ≥ 20 mm were considered as strong inhibition, <20–12 mm as moderate inhibition, and <12 mm no inhibition. A positive control with streptomycin antibiotic (10 μg) and a negative one, with paper discs impregnated with distilled water, were used

2.2. Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC was determined using the macro dilution tube method, in accordance with Rota *et al.* [5], with modifications. The tested concentrations were 20, 18, 15, 12, 10, 7 and 5 $\mu\text{L.mL}^{-1}$ of each compound. In a tube with 1 mL of MH broth, was inoculated 10^5 UFC.mL⁻¹, the compound in a predetermined concentration and, to facilitate the solubility, 2 drops of TWEEN 80. The mixture was incubated at 37 °C for 24 h under stirring (150 rpm). As the positive control, MH broth and inoculum concentration in 10^5 UFC.mL⁻¹ and as the negative control MH, the inoculum concentration in 10^5 UFC.mL⁻¹ and streptomycin (10 μg) were used. MIC was defined as the lowest concentration of compound which showed no visible growth in broth. After 24 h, tubes with no visible growth were seeded in a 100 mL TSA-YE tube and incubated at 37 °C for 24 h. The MBC is the lowest concentration of compound where 99.9% of the initially inoculated cells were killed.

2.3. Antioxidant activity assays

The antioxidant properties of the synthesized compounds were evaluated by three different methods *in vitro*: DPPH and ABTS⁺ radical scavenging activity and ferric ion reducing antioxidant power (FRAP). All drugs were dissolved in dimethyl sulfoxide (DMSO). The experimental results were given as the means \pm standard deviation (SD) to show the variations among the groups. The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test when appropriate. The differences were considered statistically significant at a probability of less than 5% ($p < 0.05$). All tests were performed at least three times in duplicate. The IC₅₀ values (the concentration of sample required to scavenge 50% of the free radicals) were calculated from the graph of the scavenging effect percentage versus the compound concentration.

2.4. Radical Scavenging Activity

To determine if compounds **5a–d** and **10** present *in vitro* antioxidant activity against free radicals, DPPH and ABTS⁺ scavenging capability were evaluated at different concentrations.

The DPPH scavenging activity of compounds **5a–d** and **10** (10–500 μM) was determined in accordance with the method of Sharma and Bhat [6] and the decrease in the absorbance at 517 nm was recorded.

The ABTS radical scavenging activity was determined according to the method described by Erel [7]. Different concentrations of compounds **5a–d** and **10** (10–500 μM) were mixed with the ABTS⁺ solution, and the decrease in the absorbance at 734 nm was recorded.

The values are expressed as the percentages of radical inhibition (I %) in relation to the control values, as calculated by the following equation:

$$I\% = [(A_c - A_s) / A_c] \times 100 \quad (1)$$

Where A_c is the absorbance of the control excluding the test compounds, and A_s is the absorbance of the tested compounds.

3. Ferric ion reducing antioxidant power (FRAP)

The ferric ions (Fe^{3+}) reducing antioxidant power (FRAP) method was used to measure the reducing capacity of compounds **5a–d** and **10**. The assay was performed as described by Stratil *et al.* [8] with slight modifications. Different concentrations of **5a–d** and **10** (1–500 μM) and FRAP reagent were added to each sample, and the mixture was incubated at 37 °C for 40 min in the dark. The absorbance of the resulting solution was measured at 593 nm with a spectrophotometer.

In vitro toxicity

The activity of δ -ALA-D in the presence of compounds **5a–d** and **10** at different concentrations (10–500 μM) was determined according to the method described by Sassa [9]. Tissues were removed from the mice, liver and kidneys (1:10 *w/v*) and brain (1:5 *w/v*). They were homogenized in a 0.1 M Tris-HCl buffer, pH 7.4 and centrifuged at 2500 RPM for 10 min. The supernatants were used for determination of the δ -ALAD activity.

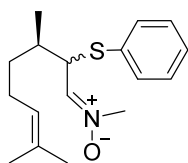
Firstly, 200 μL of the tissue supernatant was incubated for 10 min at 37 °C with 50 μL of potassium phosphate buffer (TFK), 150 μL of distilled water, and 10 μL of the test compound. Then, 100 μL of substrate (12 mM ALA aminolevulinic acid) was added and the incubation was carried out at 37 °C for 1 h for liver and kidney and 3 h for the brain; the reaction was stopped by adding 250 μL of trichloroacetic acid (TCA) solution with 10% 10 mM HgCl_2 followed by centrifugation (2500 RPM for 10 min). Then, 500 μL of the supernatant was collected and added to 1000 μL of Ehrlich's reagent and 500 μL of distilled water. The resulting mixture was incubated for 10 min at room temperature and the absorbance was read at 555 nm.

The results were expressed in $\text{nmol PBG} \cdot \text{mg} \cdot \text{tn}^{-1} \cdot \text{h}^{-1}$, according to the equation below:

$$\text{PBG} = [(2 \text{ nmol} / 0.061) (1/T) \times (1/\text{ptn}) \times (1/\text{tissue}) \times \text{Abs}] \quad (2)$$

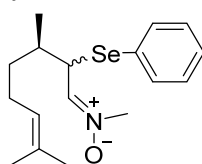
Where T is the incubation time, ptn is protein and tissue refers to the added amount, for example 0.2 to 200 μL of tissue and Abs refers to the absorbance of the tested compounds.

(3*R,E*)-*N*-3,7-trimethyl-2-(phenylthio)oct-6-en-1-imine oxide (**5a**)



Yield: 61%; yellow oil. ^1H NMR (CDCl_3 , 300 MHz): δ = 7.40–7.37 (m, 2H), 7.30–7.25 (m, 2H), 7.21–7.16 (m, 1H), 6.72–6.67 (m, 1H), 5.10–5.05 (m, 1H), 4.76–4.67 (m, 1H), 3.61 (s, 3H), 2.09–1.94 (m, 3H), 1.68 (s, 3H), 1.59 (s, 3H), 1.38–1.25 (m, 2H), 1.09 (t, J = 6.56 Hz, 3H). ^{13}C NMR (CDCl_3 , 75 MHz): δ = 140.0, 139.2, 134.5, 131.9, 130.1, 130.1, 128.9, 126.5, 123.8, 52.6, 52.6, 48.4, 47.6, 35.9, 35.6, 34.5, 34.2, 25.7, 25.6, 25.4, 17.7, 17.6, 17.2, 16.6. MS, m/z (rel. int.): 291[M]⁺(12), 182(34), 135(21), 123(18), 84(27), 69(66), 41(100).

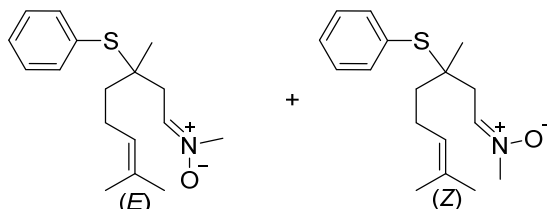
(3*R,E*)-*N*-3,7-trimethyl-2-(phenylselanyl)oct-6-en-1-imine oxide (**5b**)



Yield: 65%; yellow oil. ^1H NMR (CDCl_3 , 300 MHz): δ = 7.65–7.60 (m, 2H), 7.30–7.24 (m, 3H), 6.74

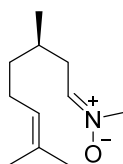
(dd, $J = 2.57$ Hz and 9.14 Hz, 1H), 5.07–5.00 (m, 1H), 4.70–4.62 (m, 1H), 3.56 (d, $J = 4.50$ Hz, 3H), 2.07–1.79 (m, 3H), 1.66 (s, 3H), 1.58 (d, $J = 6.40$ Hz, 3H), 1.35–1.23 (m, 2H), 1.07 (dd, $J = 6.72$ Hz and 14.83 Hz, 3H). ^{13}C NMR (CDCl_3 , 75 MHz): $\delta = 139.6, 139.0, 134.8, 134.7, 131.7, 128.8, 128.7, 128.1, 128.0, 127.6, 123.7, 52.3, 52.3, 45.3, 44.3, 35.5, 35.5, 35.1, 34.8, 25.6, 25.4, 25.1, 17.9, 17.5, 17.5, 17.2$. MS, m/z (rel. int.): 339[M]⁺(4), 182(99), 156(18), 135(14), 109(27), 84(31), 77(32), 69(61), 41(100).

(*E*)- and (*Z*)-*N*-3,7-trimethyl-3-(phenylthio)oct-6-en-1-imine oxide (**5c**)



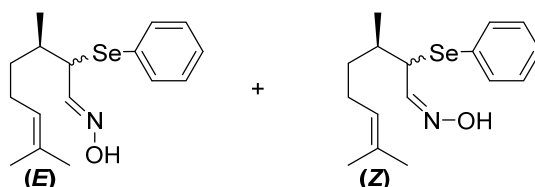
Yield: 67%; yellow oil. ^1H NMR (CDCl_3 , 300 MHz): $\delta = 7.51$ – 7.19 (m, 5H), 7.05–7.02 (m, 0.6H), 6.60–6.57 (m, 0.4H), 5.10–5.02 (m, 1H), 3.75 (s, 1.8H), 3.71 (s, 1.2H), 2.65 (d, $J = 5.42$ Hz, 1H), 2.23–2.12 (m, 3H), 1.83 (s, 1H), 1.68 (s, 3H), 1.62 (s, 1.8H), 1.61 (s, 1.2H), 1.55–1.49 (m, 1H), 1.26 (s, 3H). ^{13}C NMR (CDCl_3 , 75 MHz): $\delta = 137.7, 137.3, 132.3, 132.1, 130.6, 129.1, 129.0, 128.8, 123.4, 123.1, 122.9, 116.6, 115.8, 52.6, 52.2, 52.2, 50.6, 40.5, 40.3, 37.2, 33.5, 26.5, 26.2, 25.9, 25.6, 24.4, 23.0, 17.9, 17.7$. MS, m/z (rel. int.): 291[M]⁺(1), 181(10), 135(17), 110(20), 98(100), 81(13), 69(64), 55(14), 41(75).

(*R,E*)-*N*-3,7-trimethyloct-6-en-1-imine oxide (**5d**)



Yield: 61%; yellow oil. ^1H NMR (CDCl_3 , 300 MHz): $\delta = 6.73$ – 6.69 (m, 1H), 5.10–5.04 (m, 1H), 3.70 (s, 3H), 2.75–2.67 (m, 1H), 2.56–2.31 (m, 2H), 2.05–1.72 (m, 3H), 1.68 (s, 3H), 1.60 (s, 3H), 1.43–1.19 (m, 2H), 0.96 (d, $J = 6.68$ Hz, 3H). ^{13}C NMR (CDCl_3 , 75 MHz): $\delta = 139.9, 131.5, 124.0, 52.3, 36.8, 33.9, 30.1, 25.6, 25.3, 19.8, 17.5$. MS, m/z (rel. int.): 183[M]⁺(19), 137(20), 109(31), 100(70), 81(37), 69(69), 55(36), 41(100).

(3*R,E*)- and (3*R,Z*)-3,7-dimethyl-2-(phenylselanyl)oct-6-enal oxime (**10**)



Yield: 90%; yellow oil. ^1H NMR (CDCl_3 , 400 MHz): $\delta = 8.98$ (brs, 0.5H), 8.65 (brs, 0.5H), 7.57–7.48 (m, 2H), 7.44–7.36 (m, 0.5H), 7.25–7.17 (m, 3H), 6.79–6.77 (m, 0.5H), 5.11–5.04 (m, 1H), 4.66–4.57 (m, 0.5H), 3.80–3.73 (m, 0.5H), 2.38–1.76 (m, 3H), 1.67 (s, 3H), 1.59 (s, 3H), 1.42–1.16 (m, 2H), 1.11–0.91 (m, 3H). ^{13}C NMR (CDCl_3 , 100 MHz): $\delta = 151.6, 151.3, 151.2, 150.6, 150.5, 135.4, 135.3, 135.0, 134.9, 131.8, 131.7, 131.6, 131.3, 128.9, 128.8, 128.3, 128.2, 127.8, 127.6, 124.3, 124.3, 124.0, 123.9, 123.8, 50.2, 49.0, 42.9, 41.7, 36.8, 36.6, 36.3, 35.7, 35.4, 35.3, 35.2, 34.7, 34.6, 31.9, 30.9, 30.5, 25.6, 25.5, 25.4, 25.3, 25.2, 19.7, 19.4, 17.9, 17.6, 17.5, 17.1, 17.0$. MS, m/z (rel. int.): 325[M]⁺(1), 308(4), 168(63), 157(23), 135(9), 123(22), 109(21), 77(39), 69(97), 41(100).

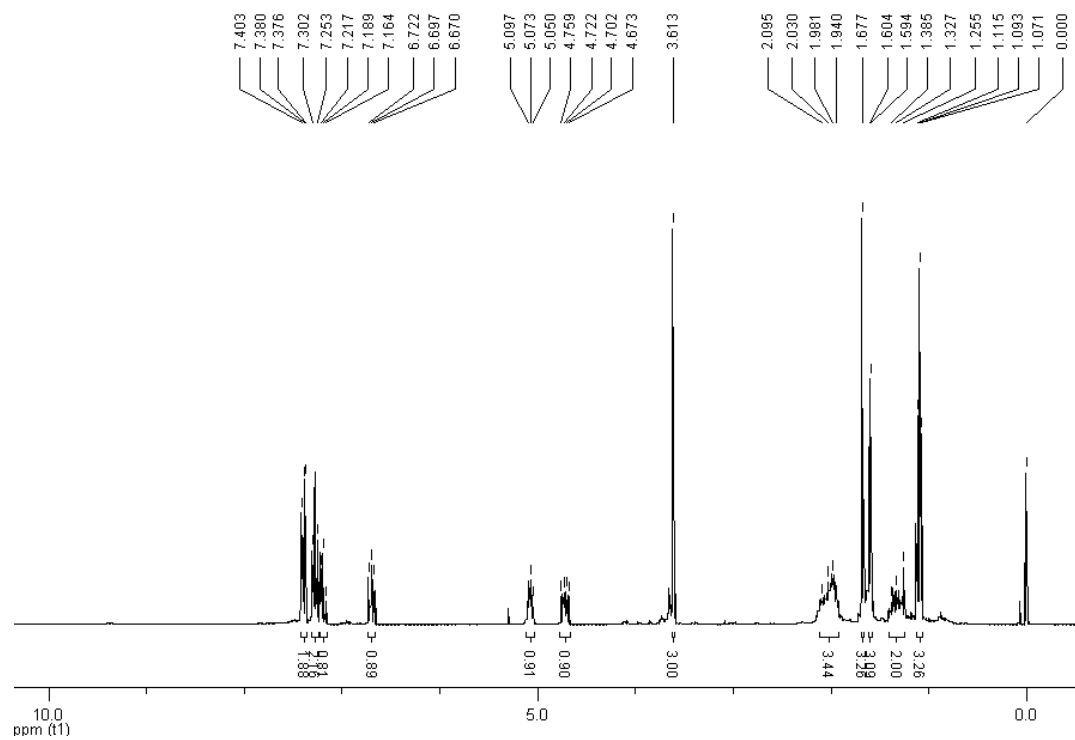


Figure S1. ^1H NMR of compound **5a** in CDCl_3 (300 MHz).

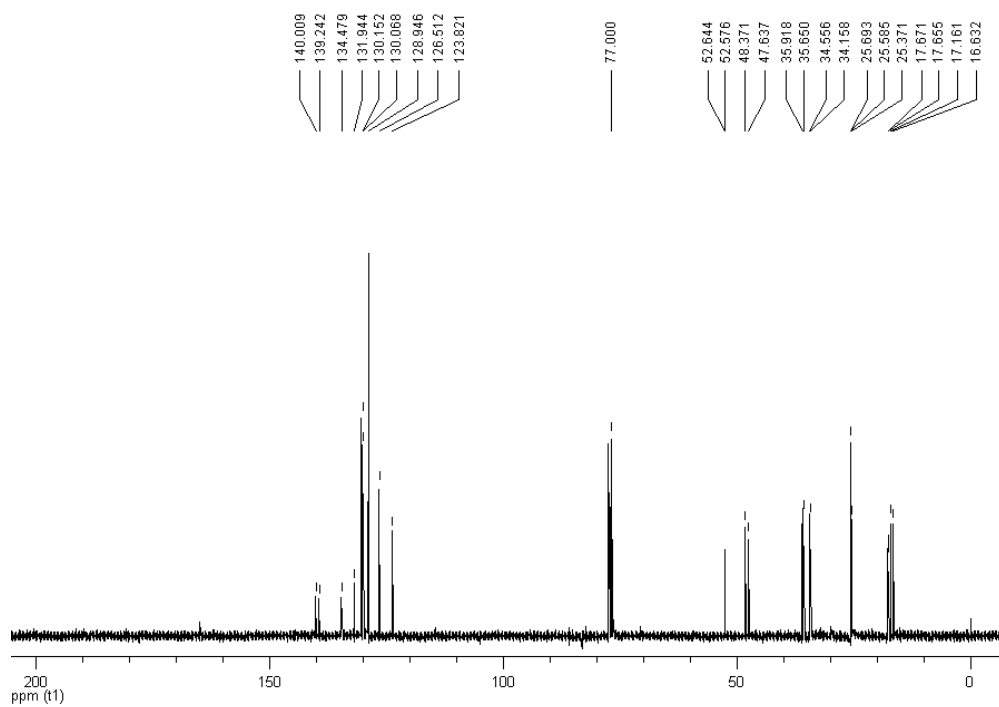


Figure S2. ^{13}C NMR of compound **5a** in CDCl_3 (75 MHz).

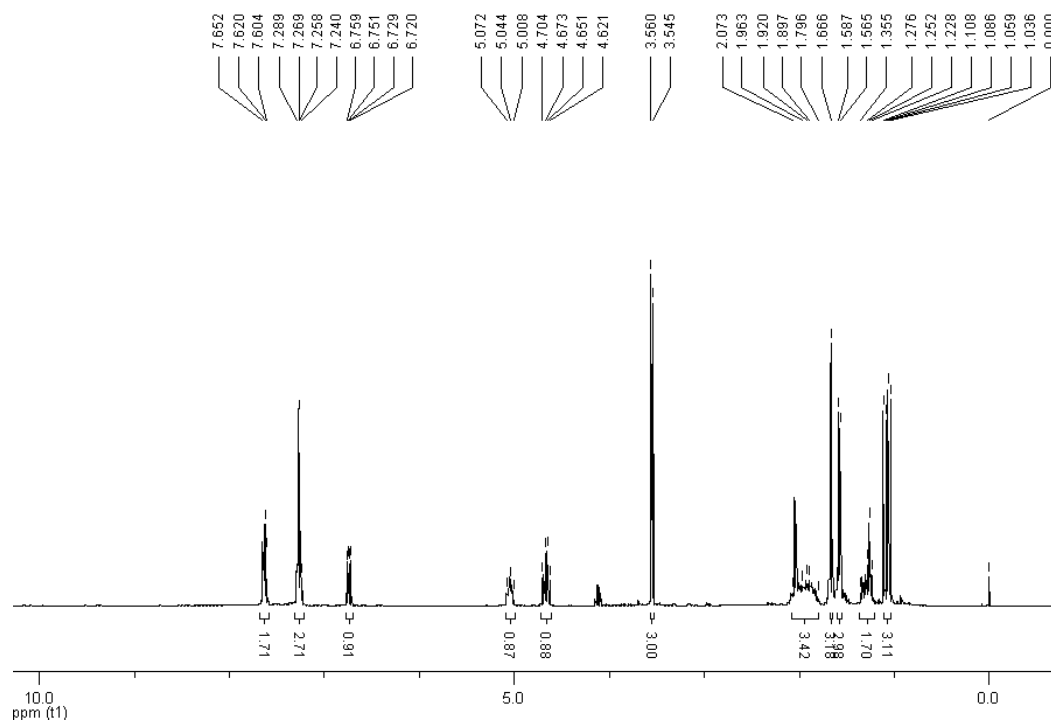


Figure S3. ^1H NMR of compound **5b** in CDCl_3 (300 MHz).

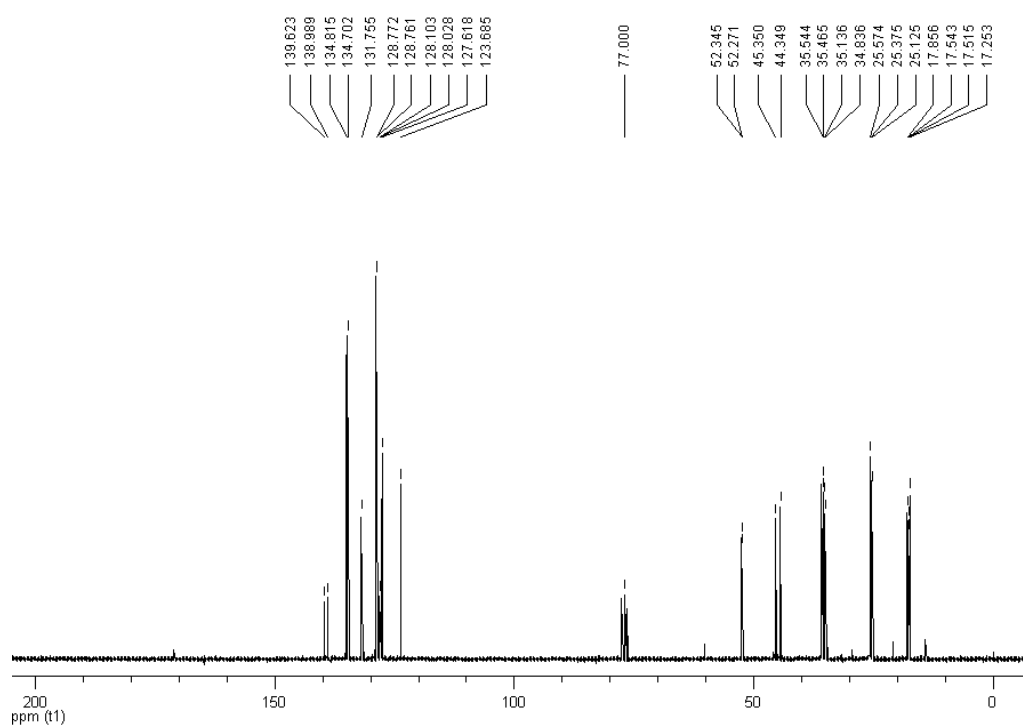


Figure S4. ^{13}C NMR of compound **5b** in CDCl_3 (75 MHz).

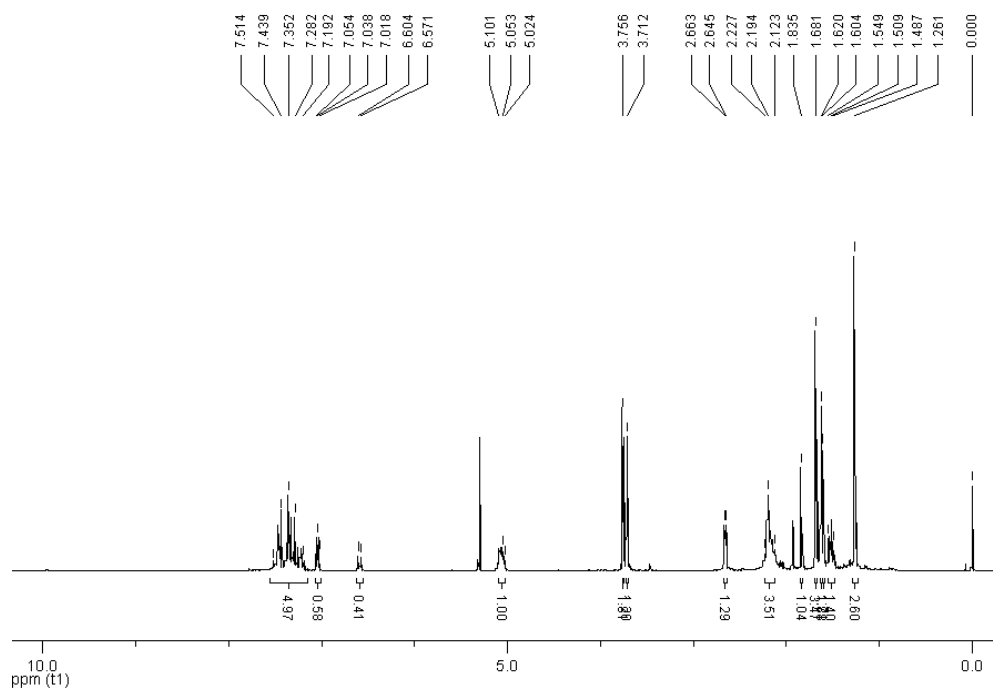


Figure S5. ¹H NMR of compound 5c in CDCl₃ (300 MHz).

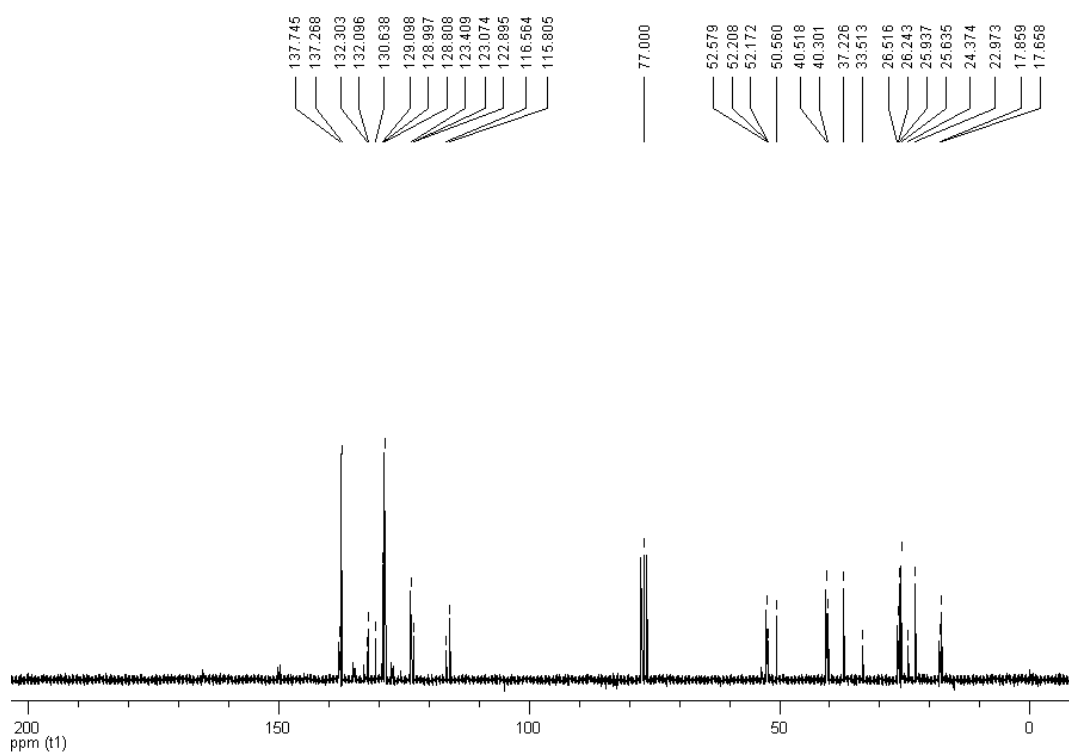


Figure S6. ¹³C NMR of compound 5c in CDCl₃ (75 MHz).

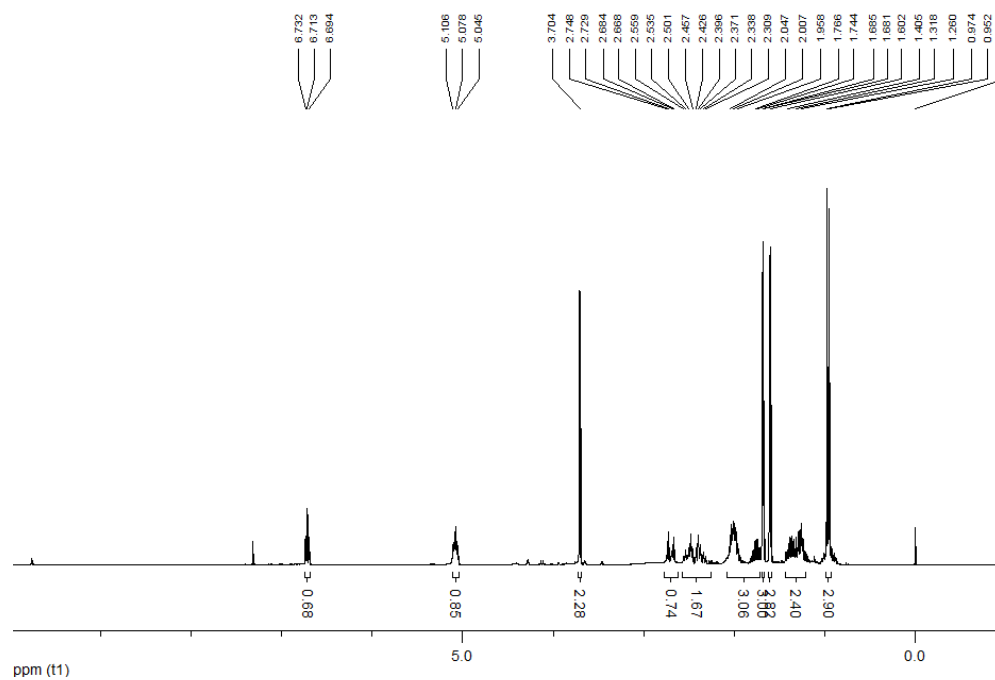


Figure S7. ¹H NMR of compound 5d in CDCl₃ (300 MHz).

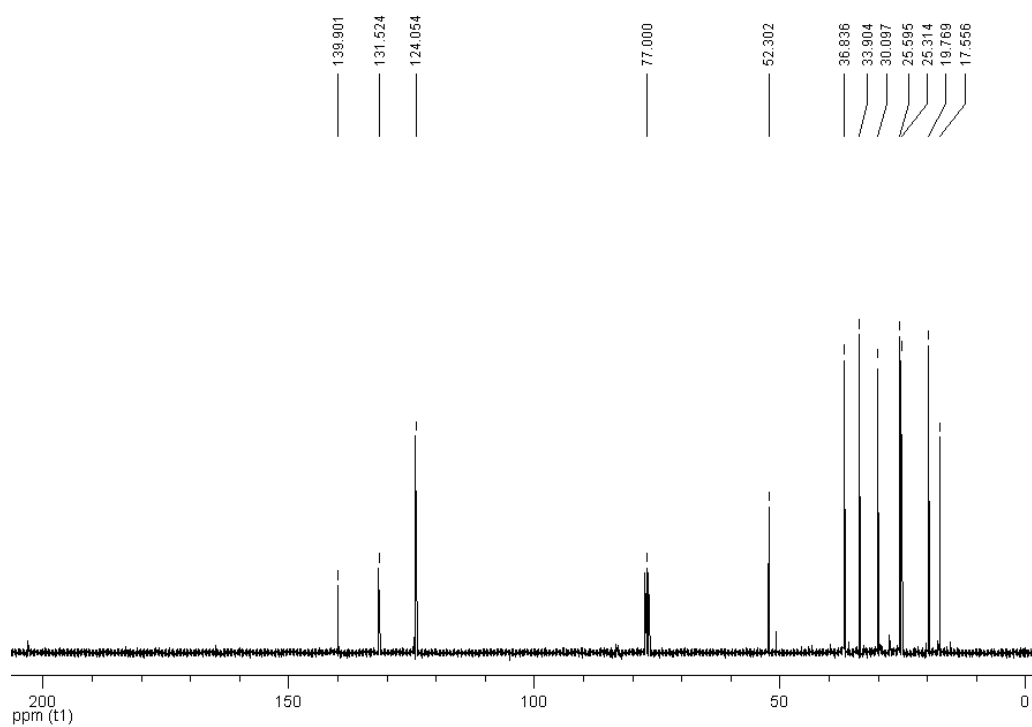


Figure S8. ¹³C NMR of compound 5d in CDCl₃ (75 MHz).

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