Azacoumarin-based "turn-on" fluorescent probe for detection and imaging of hydrogen peroxide in living cells

Supporting Information

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1. Materials and methods.

All chemicals were purchased from Sigma-Aldrich, TCI Chemicals, SRL Chemicals, and Avra and used as received. Molychem silica gel (60-120 mesh) was used for column chromatography, and thin-layer chromatography was performed on Merck pre-coated silica gel 60-F254 plates. All other chemicals and solvents were obtained from commercial sources and purified using standard methods. The ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker-Advance 500 and 600 MHz spectrometers. Chemical shifts (δ) are reported in parts per million (ppm), using TMS (δ = 0) as an internal standard and $CDCl_3$ as NMR solvents. The mass spectrum of the compounds has been obtained by Waters Q-ToF Premier Mass Spectrometer. UV absorbance was measured on an Agilent Cary UV 60 UV-visible spectrophotometer. Fluorescence data were recorded on a Molecular Devices SpectraMax M5 multimode plate reader. Solvents used for absorption and emission spectra of azacoumarins: dimethylsulfoxide (DMSO), dichloromethane (DCM), methanol (MeOH) and phosphate-buffered saline (PBS, 1X) (SRL Chemicals). GraphPad Prism ver. 7.0a (GraphPad Software, Inc.) and Origin2017 softwares were used to analyze data and generate graphs. MCF-7 breast cancer cell lines and HEK-293 normal cell lines were secured from the National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in DMEM (Dulbecco's modified eagle's medium), supplemented with 10% FBS (Gibco, Thermo Scientific, Waltham, MA, USA) and 1% anti-biotic anti-mitotic solution (Himedia, Mumbai, India) (10,000 U Penicillin, 10 mg streptomycin, 25 µg Amphotericin B per ml) and grown at 37°C temperature in a humidified incubator (Heracell VIOS 160i, ThermoFisher. MA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Fetal bovine serum (FBS) were procured from Thermo Scientific, Waltham, MA, USA.

Photophysical Characterization of compounds 5, 5a, 6 and 7. Maximum absorption wavelengths (λ_{abs}) of all the compounds were recorded using Agilent Cary UV 60 UV–Visible Spectrophotometer. Molar absorption coefficients (ε) were quantified using solutions of compounds **5, 5a, 6** and **7** at concentrations ranging between 10^{-6} - 10^{-5} M in PBS (1X) by direct application of the Beer-Lambert's law. The compound stock solutions were prepared using DMSO (2 mL) and then diluted with PBS (upto 100 mL). Maximum emission wavelengths (λ_{em}) were recorded on a Horiba Scientific FluoroMax-4. λ_{abs} and λ_{em} were recorded in three solvents, namely, Dichloromethane (DCM), Methanol (MeOH) and phosphate buffered saline [PBS, pH = 7.4] (SRL Chemicals) (Supporting information, Table S1). All measurements were taken at ambient

temperature (22 ± 2 °C) using 1-cm path length, 4 mL quartz cuvettes. GraphPad Prism ver. 7.0a (GraphPad Software, Inc.) and Origin2017 softwares were used to analyze all the data and generate the corresponding graphs. The spectral data have been normalized for better understanding.

Quantum Yield (φ) Measurement. For quantum yield measurement, the compounds (**5, 5a, 6** and **7**) were dissolved in DMSO and PBS mixture (in the ratio of 2:8 v/v) to prepare their respective stock solutions. Next, 3 mL of each sample solution was taken in a 1 cm quartz cuvette and their absorbance values were measured and adjusted between 0.07 to 0.09. After that, five serial dilutions from this 3 mL volume were prepared, and their corresponding absorbance and emission wavelength values were measured. The area of the obtained fluorescence intensity was plotted against their corresponding absorbance intensities in a graph to generate the slope of individual linear equations. The quantum yield of each sample was then calculated using the formula mentioned below, with compound **2a** as the reference standard.

$$\mathbf{Q = Q(r) \times (m/m_r) \times (n^2/n_r)}$$

Q(r) = absolute quantum yield of **2a** in PBS

m and m_r = equation slopes derived from the plots of Absorbance vs Area of Fluorescence of the sample and reference **2a,**

n and n_r = refractive indices of the solvents dissolving the test compounds and reference compound, respectively.

pH stability study. For the pH stability study, the buffers having pH ranges 4-8 (4.80, 5.70, 6.64 and 7.48) were prepared using deionized water following standard protocol and freshly used. Compound **6** in a fixed concentration was placed in each buffer system and incubated for 30 mins at 37 ^{o}C before measuring the fluorescence intensities.

Photostability study. For photostability tests, compound **6,** reference compound **2a** and standard cell labeling dye, **coumarin 6,** were dissolved in DMSO:PBS mixture (in 2:8 v/v ratio) in separate vials, each containing an equimolar concentration of the compounds and placed under continuous irradiation with a 500W tungsten lamp emitting white light. Their emission intensities were then measured from 0 to 360 minutes of irradiation, and the obtained fluorescence intensities were plotted into a curve against time.

***In vitro* cytotoxicity testing of compound 6 using MTT assay.** Cytotoxicity of compound **6** against the MCF-7 cancer cell line was analyzed in a 96-well cell culture plate, which contained 1 × 10^4 cells/well. The cells were evenly distributed onto the entire plate, with each well containing 100 µl of the complete culture medium. The culture plate was then placed in a CO_2 incubator with 5% CO_2 at 37°C for 24 h to allow cell adherence. After 24 h of incubation, the media was aspirated, and fresh media was added comprising of compound **6** at different concentrations within a range of 10 - 100 µM and incubated for another 24h. After the completion of treatment, media was aspirated, and fresh media (100 µL) containing 500 µg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well and incubated for an additional 3h at 37°C. The MTT-containing media was then aspirated, followed by the addition of 100 µL of DMSO in each well and subsequent incubation for another 30 mins to dissolve the purple-colored formazan precipitate. The plates were incubated for the next 10 mins with gentle shaking. The optical density of each well was measured at 570 nm using a Bio-Tek microplate reader. A similar protocol was followed for non-cancer HEK-293 cells.

Intracellular uptake assay of compound 6. The MCF-7 cells were seeded in Petri dishes at a density of 5 x 10^5 cells/well and incubated for 24 h in a CO_2 incubator with 5% CO_2 at 37°C to allow cell adherence. After 24 h, the media was aspirated, and the cells were trypsinized and collected, followed by treatment with 500 µl of compound **6** (20 µM). It was then incubated for 30 mins. After incubation, the cells were harvested using 1 mM EDTA in PBS and processed further for experimentation through flow cytometry (CytoFlex LX, Beckman coulter, MA, USA) for the measurement of fluorescence intensities. The experiment was performed in triplicate.

UV-VIS and fluorescence measurements of probe PYCB.

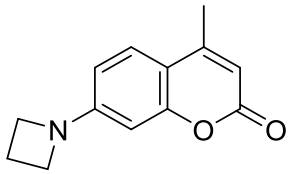
The stock solution of probe **PYCB** (100 µM) was made in 25 mM PBS mixed with DMSO in a ratio of 8:2 (pH 7.4). It was then diluted to 2 µM concentration using PBS and used freshly. The stock solution of H_2O_2 (10 mM) was prepared in PBS and then diluted to serial concentrations ranging between 5 – 200 µM and used freshly. The absorption and emission wavelengths of the resultant solution of the probe were measured in the absence and in the presence of H_2O_2 (5 µM solution in PBS) and plotted into graphs. For pH stability study, the buffers having pH ranges 2-8 (2.40, 3.33, 4.50, 5.40, 6.46, 7.48, and 8.5) were prepared using deionized water following standard

protocol and freshly used. Probe **PYCB** (10 μM) was placed in each buffer system and incubated for 30 mins at 37 ºC before measuring the fluorescence intensities. Stock solutions (100 mM) of Na^+, K^+, Fe^{3+}, Cl^-, HCO_3^-, Cys, and GSH were prepared respectively in deionized water and used freshly. Stock solutions (100 μM) of HOONO, HClO, H_2O_2, TBHP and *t*BuNO ($NO^{\cdot}$) were prepared immediately before use. Fluorescence spectra were obtained 90 mins after mixing at 37 ºC.

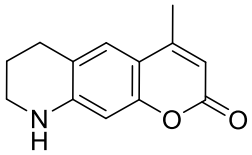
***In vitro* cytotoxicity testing of PYCB using MTT assay.** Cytotoxicity of probe **PYCB** against MCF-7 cell lines was analyzed in a 96-well cell culture plate, which contained 1×10^4 cells/well. The cells were distributed such that each well contained 100 μL of the complete culture medium. The culture plate was then placed in a CO_2 incubator with 5% CO_2 at 37°C for 24 h to allow cell adherence. After 24 h of incubation, the media was aspirated, and fresh media was added comprising of probe **PYCB** at different concentrations within a range of 2.5 - 100 μM and incubated for another 24h. After 24 h, media was aspirated, and fresh media (100 μL) containing 500 μg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well and incubated for an additional 3h at 37°C. The MTT-containing media was then aspirated, and 100 μL of DMSO was added to each well and subsequently incubated for another 30 mins to dissolve the formazan precipitate. The plate was incubated for the next 10 mins with gentle shaking. The optical density of each well was measured at 570 nm using a Bio-Tek microplate reader.

Fluorescence imaging of PYCB-treated MCF-7 cells. MCF-7 cells were seeded in a chambered well plate at 3 x 10^4 cells/well density and incubated overnight in a CO_2 incubator with 5% CO_2 at 37°C to obtain cell adherence. After 24 h of incubation, the media was aspirated, and fresh media was added containing 2 μM **PYCB** for 1 h, followed by incubation with H_2O_2 (10 μM and 50 μM) separately for another 2 h. Then, another group MCF-7 cells pre-treated with probe **PYCB** (2 μM), were incubated with H_2O_2 (50 μM) and *N*-Acetylcysteine (100 μM) for another 2 h. After completion of treatment, cells were washed with cold PBS three times, and images were captured using an inverted fluorescence microscope (Olympus BX53, Tokyo, Japan) in the blue channel (λ_{abs}: 357/44 nm and λ_{em}: 447/60 nm) at 400 X magnification.

2. Synthetic Procedures and Spectral Data

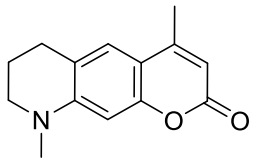


Procedure for the synthesis of 7-(azetidin-1-yl)-4-methyl-2H-chromen-2-one (2a): A round bottom flask was charged with 4-methyl-7-nonafluorobutylsulfonyloxy coumarin (1 mmol, 500 mg, 1equiv.), azetidine (2 mmol), tris(dibenzylideneacetone)palladium (5 mol %), xantphos (10 mol %), caesium carbonate (2 mmol), TBAF.3H_2O (1 mmol), and dioxane (4.0 mL) under argon. The resulting solution was heated at 100 ◦C with rapid stirring for 12 h. The reaction mixture was allowed to cool to room temperature and then diluted with water (20 mL) and extracted with ethyl acetate (25 mL × 3). After drying with anhydrous Na_2SO_4, the organic phase was evaporated to dryness and purified by column chromatography using 20% ethyl acetate: hexane. **2a** was obtained as a brownish-yellow solid with 88% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39 (d, *J* = 8.5 Hz, 1H), 6.32 (dd, *J* = 2.0 Hz, 2.5 Hz, 1H), 6.22 (d, *J* = 1.8 Hz, 1H), 5.98 (d, J = 1Hz, 1H), 4.00 (t, *J* = 7.5 Hz, 4H), 2.47–2.42 (m, 2H), 2.35 (d, *J* = 1 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 162.0, 155.6, 154.0, 153.0, 125.4, 110.3, 109.4, 107.7, 97.1, 51.8, 18.6, 16.5. HRMS (ESI) m/ z calculated for $C_{13}H_{13}O_2N$ $[M + H]^+$ calculated as 216.1019, found 216.1039. [1]

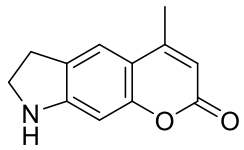


Procedure for the synthesis of 4-methyl-6,7,8,9-tetrahydro-2H-pyrano[3,2-g]quinolin-2-one (5): 1,2,3,4-tetrahydroquinolin-7-ol (**A**, 2.01 mmol, 300 mg, 1 equiv.) was dissolved in ethanol (2mL) in a round-bottomed flask of 25 mL capacity. Then zinc chloride (3.015 mmol, 1.5 equiv.) was added to the slurry and stirred for 5 mins. Ethyl acetoacetate (2.41 mmol, 1.2 equiv.) was then added to the vessel and the reaction mixture was refluxed at 85 – 90 ºC for 16 h to yield the condensation product.[2] After cooling the reaction mixture, the solvent was evaporated *in vacuo* and the crude mixture was then diluted with water (10 mL) and extracted with ethyl acetate (15 mL×3). After drying with anhydrous Na_2SO_4, the organic phase was evaporated to dryness and the compound was purified by column chromatography using 50% ethyl acetate:hexane. Compound

5 was obtained as bluish-green solid with 75 % yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.02 (s, 1H), 6.32 (s, 1H), 5.85 (s, 1H), 4.61 (s, 1H, NH), 3.30 (t, *J* = 5.5 Hz, 2H), 2.71 (t, *J* = 6.0 Hz, 2H), 2.24 (s, 3H) 1.87 (quint, *J* = 6.0 Hz, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 161.3, 153.1, 152.0, 147.3, 123.7, 117.0, 109.0, 107.7, 98.1, 40.5, 25.8, 20.4, 17.5. HRMS (ESI) m/z calculated for $C_{13}H_{23}NO_2$ $[M + H]^+$ calculated as 216.1019, found 216.1017.

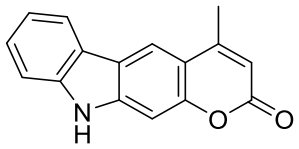


Procedure for the synthesis of 4,9-dimethyl-6,7,8,9-tetrahydro-2H-pyrano[3,2-g]quinolin-2-one (5a): 5 (0.96 mmol, 206 mg, 1 equiv.) and potassium carbonate (K_2CO_3) were dissolved in acetonitrile (ACN, 2 mL) in a round-bottomed flask of 25 mL capacity and stirred for 30 mins at room temperature. [2] Then, methyl iodide (1.5 equiv.) was dissolved in ACN (1 ml), added dropwise to the slurry and stirred at 65 ºC for 6 h to obtain the methylated derivative (**5a**). After cooling the reaction mixture, the solvent was evaporated *in vacuo,* and the crude mixture was then diluted with water (10 mL) and extracted with ethyl acetate (15 mL×3). After drying with anhydrous Na_2SO_4, the organic phase was evaporated to dryness, and the compound was purified by column chromatography using 35% ethyl acetate:hexane. Compound **5a** was obtained as a yellowish-green solid with a 50 % yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.00 (s, 1H), 6.30 (s, 1H), 5.85 (s, 1H), 3.28 (t, *J* = 6.6 Hz, 2H), 2.88 (s, 3H), 2.70 (t, *J* = 6.0 Hz, 2H), 2.24 (s, 3H) 1.87 (quint, *J* = 6.0 Hz, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 162.4, 154.7, 152.9, 149.3, 123.6, 119.4, 109.0, 108.6, 96.6, 50.7, 38.8, 27.5, 21.9, 18.5. HRMS (ESI) m/z calculated for $C_{14}H_{15}NO_2$ $[M + H]^+$ calculated as 230.1176, found 230.1169.

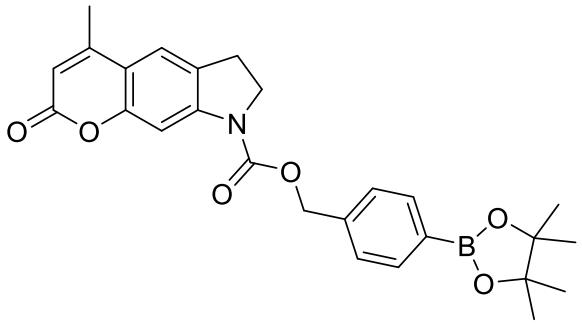


Procedure for the synthesis of 4-methyl-7,8-dihydropyrano[3,2-f]indol-2(6*H*)-one (6): Indole-6-ol (**B**) was converted to indoline-6-ol using literature reference. [3,4] Indolin-6-ol (**C**, 2.22 mmol, 300 mg, 1 equiv.) was dissolved in ethanol (2mL) along with zinc chloride (3.33 mmol, 1.5 equiv.) in a round-bottomed flask of 25 mL capacity and stirred for 5 mins. Ethyl acetoacetate (2.67 mmol,

1.2 equiv.) was then added to the vessel, and the reaction mixture was refluxed at 85 – 90 °C for 6 h to yield the condensation product. After cooling the reaction mixture, the solvent was evaporated *in vacuo* and the crude mixture was then diluted with water (10 mL) and extracted with ethyl acetate (15 mL×3). After drying with anhydrous Na_2SO_4, the organic phase was evaporated to dryness and the compound was purified by column chromatography using 50 % ethyl acetate:hexane. Compound **6** was obtained as brown solid with 75 % yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.14 (s, 1H), 6.36 (s, 1H), 5.86 (s, 1H), 3.61 (t, J = 8.4 Hz, 2H), 3.00 ((t, J = 8.4 Hz, 2H), 2.24 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 161.3, 154.5, 154.4, 152.3, 125.3, 118.7, 110.0, 107.7, 94.3, 46.4, 27.4, 17.8. HRMS (ESI) m/z calculated for $C_{12}H_{11}NO_2$ $[M + H]^+$ calculated as 202.0863, found 202.0865.



Procedure for the synthesis of 4-methylpyrano[2,3-b]carbazol-2(10*H*)-one (7): 9*H*-carbazol-2-ol (**D**, 1.63 mmol, 300 mg, 1 equiv.) was dissolved in ethanol (2mL) in a round-bottomed flask of 25 mL capacity. Ethyl acetoacetate (1.96 mmol, 1.2 equiv.) was then added to the vessel, and the reaction mixture was kept for stirring. 200 µL of conc. H_2SO_4 was slowly glided through the wall of the flask, and after the complete addition of the acid, the slurry refluxed at 85 – 90 °C for 16 h.[5] After ensuring the product formation via TLC, the reaction mixture was cooled, and the solvent was evaporated *in vacuo.* The crude mixture was then diluted with water (10 mL) and extracted with ethyl acetate (15 mL×3). After drying with anhydrous Na_2SO_4, the organic phase was evaporated to dryness and the compound was purified by column chromatography using 35% ethyl acetate:hexane. Compound **7** was obtained as a white solid with 55 % yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.87 (s, 1H), 8.21 (s, 1H), 8.07 (d, J = 7.0 Hz, 1H), 7.51 – 7.41 (m, 2H), 7.32 (s, 1H), 7.25 – 7.22 (m, 1H), 6.15 (s, 1H), 2.56 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.5, 158.5, 157.2, 147.2, 145.9, 131.0, 127.2, 125.6, 124.9, 124.4, 120.7 (d, J = 8.75 Hz), 117.5, 116.0, 115.7, 102.4 (d, J = 8.75 Hz), 23.9. HRMS (ESI) m/z calculated for $C_{16}H_{11}NO_2$ $[M + H]^+$ calculated as 250.0863, found 250.0859.



Procedure for the synthesis of 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl-4-methyl-2-oxo-6,7-dihydropyrano [3,2-f]indole-8(2H)-carboxylate (PYCB): A round-bottomed flask charged with (4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)methanol (**E**, 2.13 mmol, 500 mg, 1 equiv.) and dried Na_2CO_3 (2.9 g, 10 equiv.), dissolved in toluene (2 mL), was kept at 0 ^oC on an ice bath. In a glass beaker, crystals of triphosgene (1.2g, 2 equiv.) were dissolved in toluene (2 mL), and this solution was gradually added to the slurry in the round-bottomed flask.[6] After the addition of triphosgene, the reaction vessel was removed from the ice bath and gradually brought to rt. The reaction mixture was then stirred for 6 h at rt. After completion of the reaction, the slurry was diluted with ice-cold water (50 mL) and extracted with ethyl acetate (15 mL x 3). After drying with anhydrous Na_2SO_4, the organic phase was evaporated to dryness, and the compound F was obtained as a faint pink-colored liquid with about 90% yield. Next, the obtained compound **F** (0.67 equiv.) was reacted with compound **6** (1 mmol, 150 mg, 1 equiv.) in THF under reflux conditions overnight. After cooling to rt, the reaction mixture was diluted with water (20 mL) and extracted with ethyl acetate (10 mL x 3). After drying *in vacuo,* the probe **PYCB** was purified via column chromatography using 15% ethyl acetate:hexane and was isolated as a white, flaky solid in 72% yield. ^{1}H NMR (600 MHz, d^6-DMSO) δ 9.30 (s, 1H), 7.70 (d, J = 9 Hz, 2H), 7.43 (d, J = 7.2 Hz, 2H), 6.97 (d, J = 7.8 Hz, 1H), 6.36 (dd, J = 7.8 Hz, 1.8 Hz, 1H), 5.24 (s, 2H), 3.99 (s, 2H), 3.32 (s, 3H), 2.97 (t, J = 7.8 Hz, 2H), 1.29 (s, 12H). ^{13}C NMR (150 MHz, d^6-DMSO) δ 162.05, 157.3, 148.4, 145.2, 139.8, 139.5, 132.0, 130.9, 130.2, 126.2, 114.4, 107.4, 88.9, 71.1, 53.1, 31.3, 29.8. HRMS (ESI) m/z calculated for $C_{26}H_{28}BNO_6$ $[M + H]^+$ calculated as 462.2082, found 462.2075.

3. Photophysical Characterization of Compounds 5, 5a, 6 and 7.

Table S1: Photophysical properties of compounds 5, 5a, 6 and 7 in different solvents.

Compound	λ_{abs} (nm)[a]			λ_{em} (nm)[b]			ε ($M^{-1}cm^{-1}$)	ϕ	Brightness ($M^{-1}cm^{-1}$)
	DCM	MeOH	PBS	DCM	MeOH	PBS			
5	363	375	378	420	452	459	6458	0.901	5818
5a	375	379	383	437	455	465	14920	0.779	11622
6	360	371	359	420	451	456	22370	0.760	17001
7	362	363	362	448	460	464	5217	0.095	495
2a	360	368	355	430	451	478	12100	0.935	11320

λ_{abs} = Absorbance maximum; λ_{em} = emission maximum; ε = Molar absorptivity in PBS; ϕ = Quantum yield in PBS.

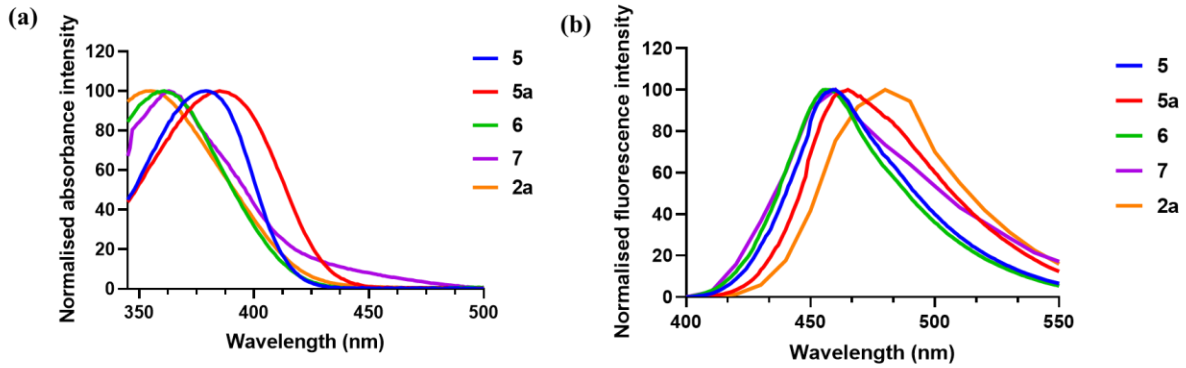


Figure S1. **(a)** Normalised absorbance intensities and **(b)** normalised fluorescence emission intensities of compounds **5-7** in PBS.

4. pH Stability and Photostability Studies on Compound 6.

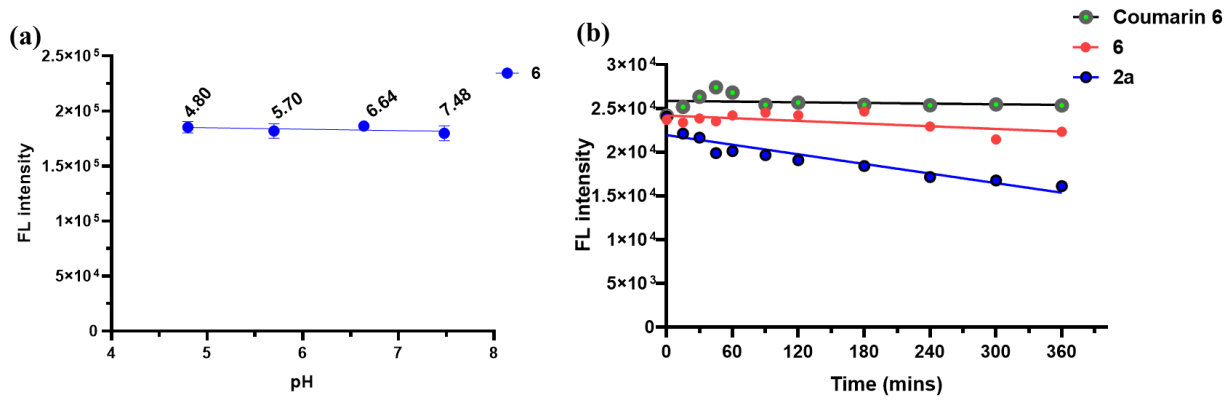


Figure S2: (a) pH stability study of compound **6** in a pH range of 4-8, and **(b)** Photostability study of compound **6** measured in terms of change of fluorescence intensity in comparison to **2a** and **coumarin 6** with time (0 – 360 minutes).

5. Cytotoxicity Assay of Compound 6 in Cancer and Normal Cell Lines

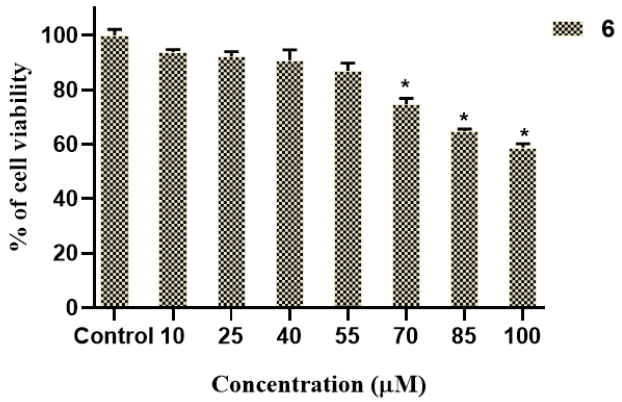


Figure S3: Measurement of percent viability of MCF-7 cells incubated with compound **6** at various concentrations ranging from 10 μM to 100 μM. Statistical analysis has been done by One-way ANOVA followed by Tukey's test where * denotes a significant difference ($p<0.05$).

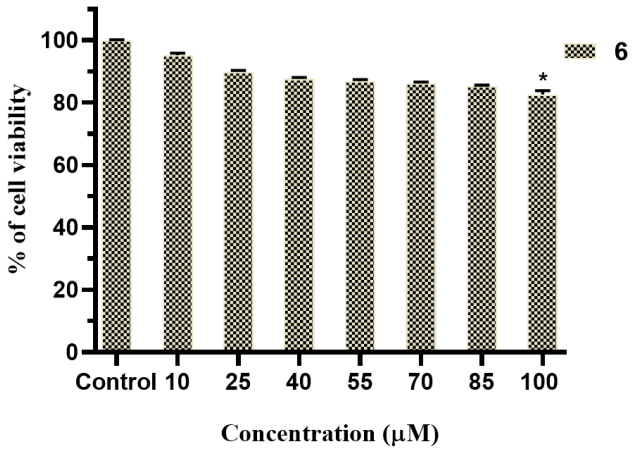


Figure S4: Measurement of percent viability of HEK-293 cells incubated with compound **6** at various concentrations ranging from 10 µM to 100 µM. Statistical analysis has been done by One-way ANOVA followed by Tukey's test where * denotes a significant difference ($p<0.05$).

6. Cellular uptake of compound 6 in MCF-7 breast cancer cells

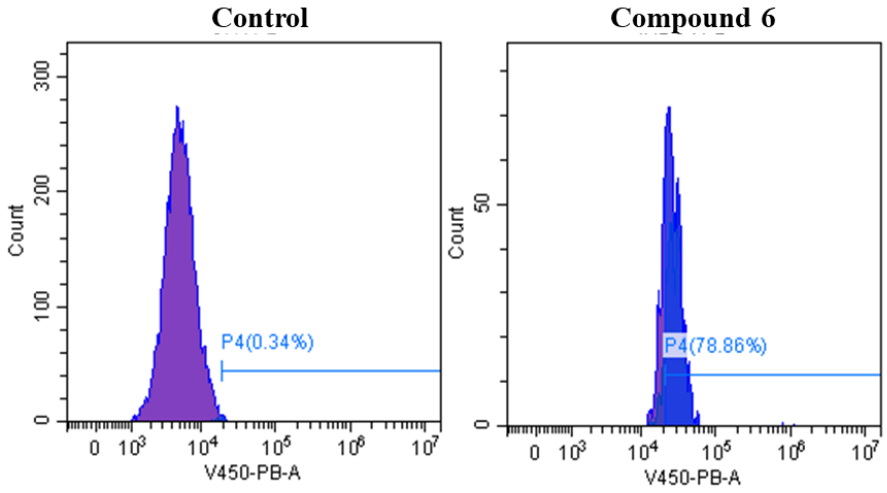


Figure S5. Average fluorescence intensity of compound **6** with respect to control determined through flow cytometry in MCF-7 cell lines after 30 minutes of incubation at 20 µM concentration.

7. Time-based response study of PYCB at variable concentrations of H_2O_2

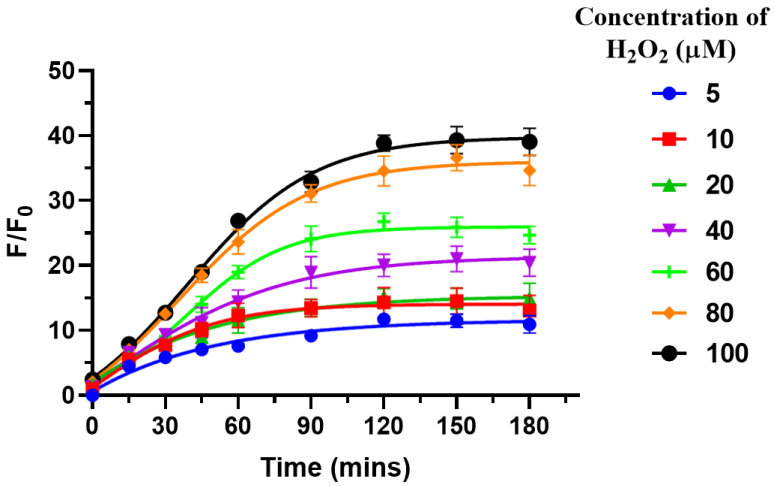


Figure S6. Time-based response of **PYCB** (2 μM) to increasing concentrations of H_2O_2 (5-100 μM) at 460 nm. (λ_{ex}: 350 nm) over a total time period of 3 h where the probe reaches maximum equilibrium at about 2 h in the presence of H_2O_2 at all possible concentrations.

8. References

1 S. Das, P. Goswami, V. K. Verma, H. K. Indurthi, M. Kumar, B. Koch and D. K. Sharma, *Dyes Pigm.*, 2023, **217**, 111407.

2 R. L. Atkins and D. E. Bliss, *J. Org. Chem.*, 1978, **43**, 1975–1980.

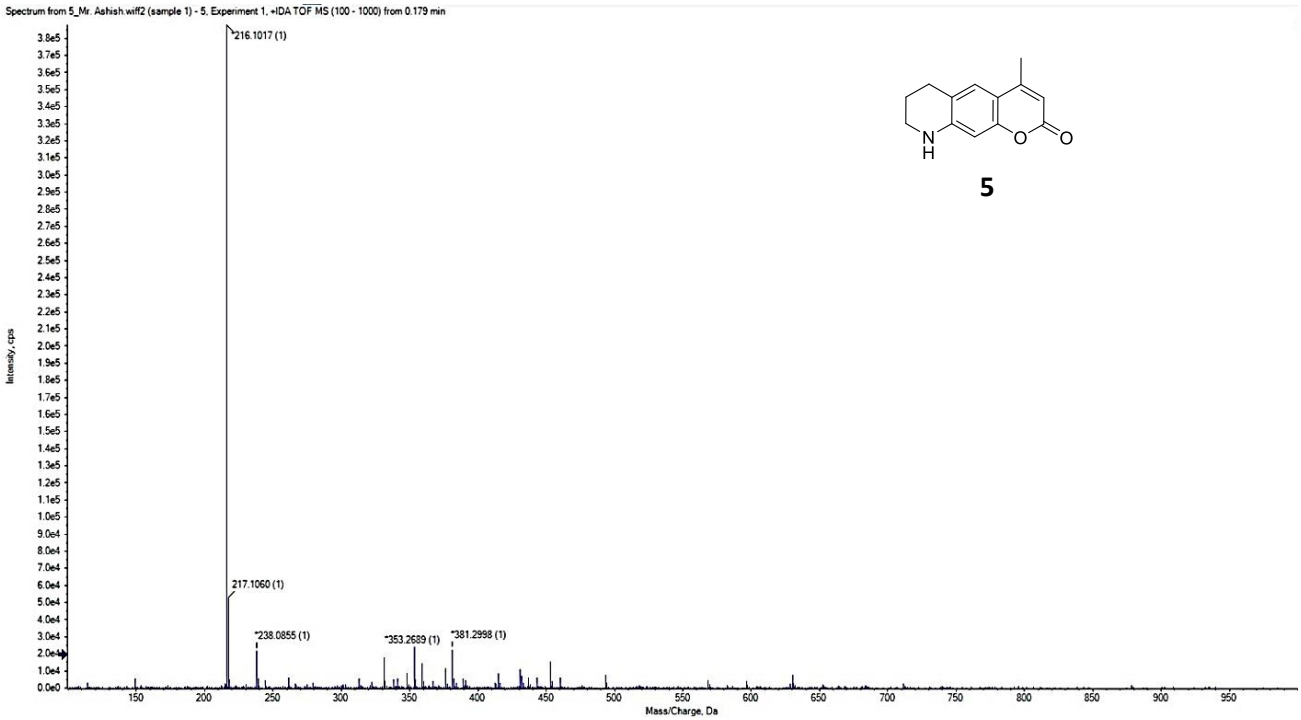
3 D. K. Sharma, B. Rah, M. R. Lambu, A. Hussain, S. K. Yousuf, A. K. Tripathi, B. Singh, G. Jamwal, Z. Ahmed, N. Chanauria, A. Nargotra, A. Goswami and D. Mukherjee, *Med. Chem. Commun.*, 2012, **3**, 1082–1091.

4 E. Quanten, P. Adriaens, F. C. De Schryver, R. Roelandts and H. Degreef, *Photochem. Photobiol.*, 1986, **43**, 485–492.

5 J. Mihara and K. Fujimoto, *Org. Biomol. Chem.*, 2021, **19**, 9860–9866.

6 D. Kim, G. Kim, S.-J. Nam, J. Yin and J. Yoon, *Sci. Rep.*, 2015, **5**, 8488.

9. HRMS, ^{1}H and ^{13}C NMR Spectra



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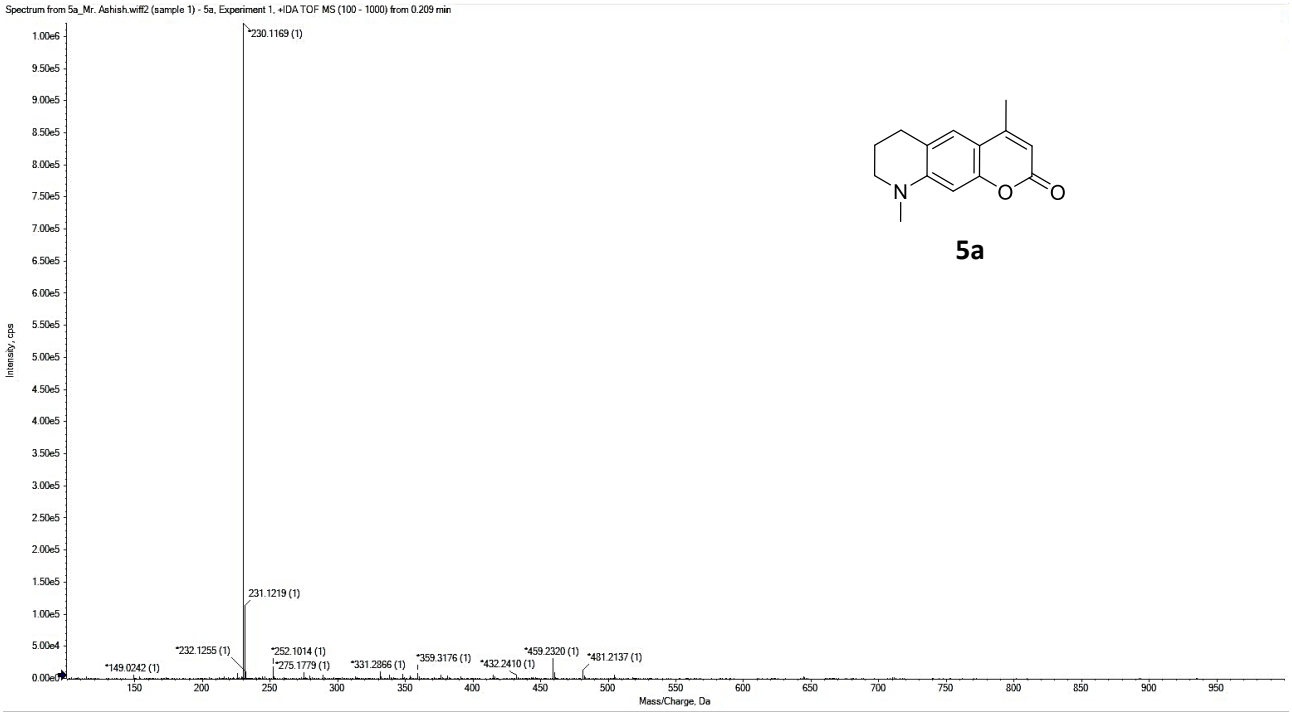
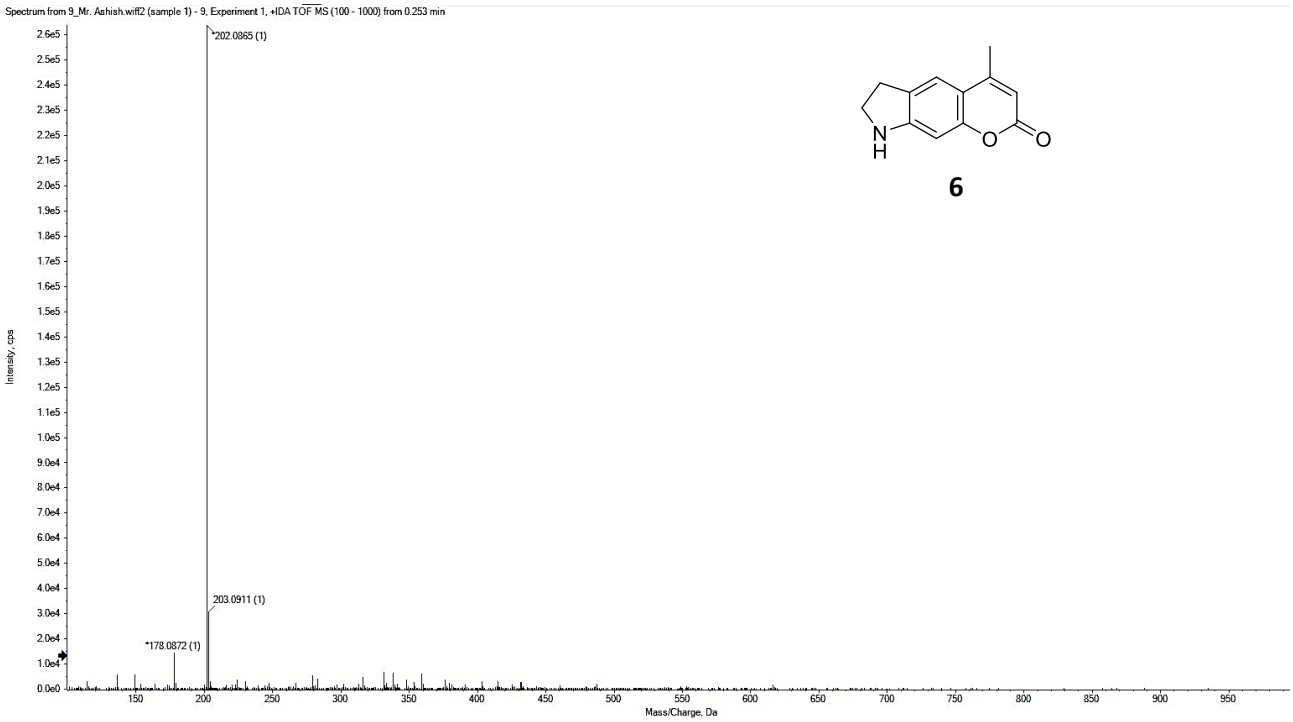
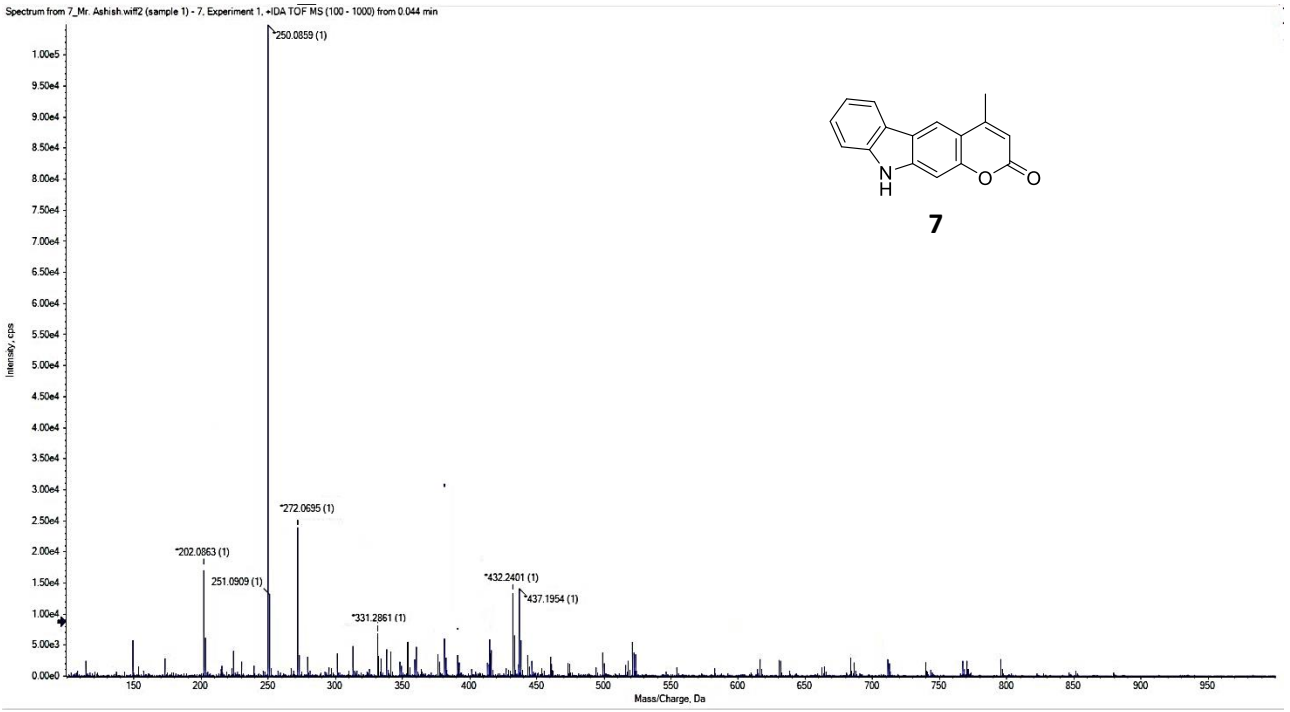


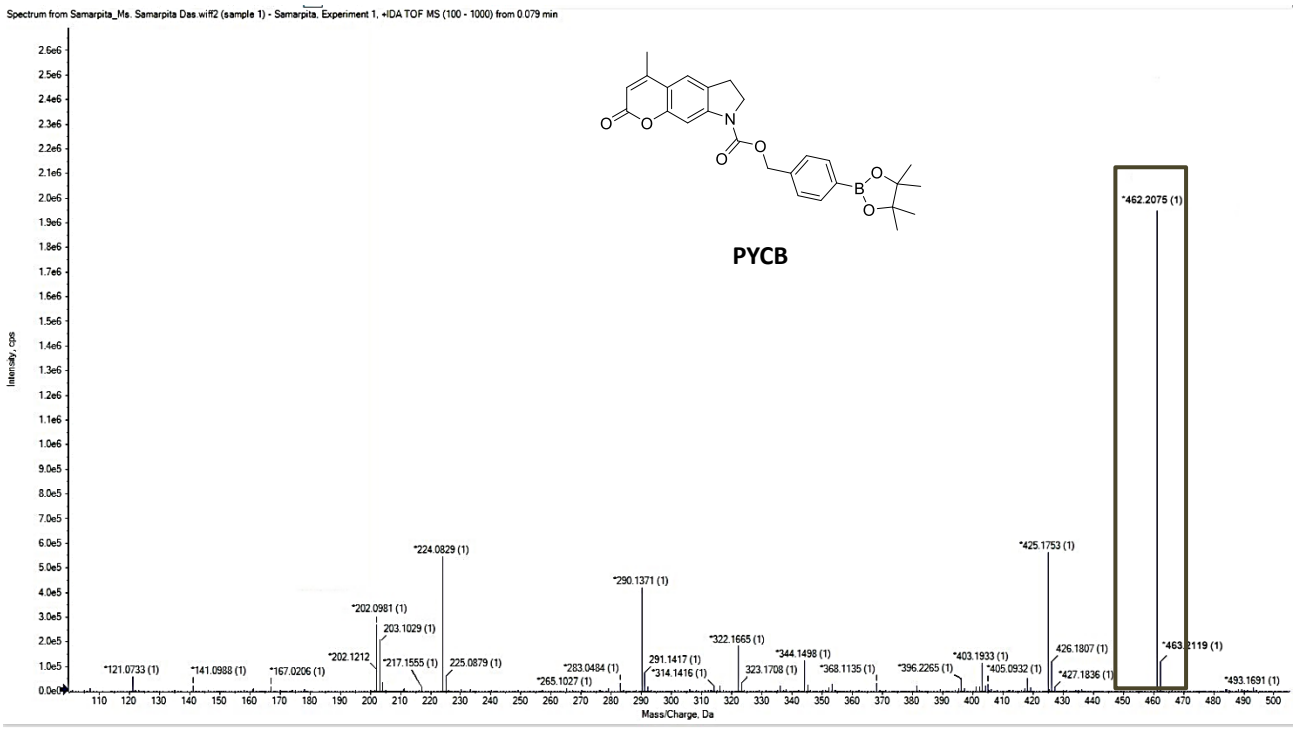
5a


Spectrum from 9_Mr. Ashish.wiff2 (sample 1) - 9, Experiment 1, +IDA TOF MS (100 - 1000) from 0.253 min
202.0865 (1)
203.0911 (1)
178.0872 (1)
Intensity, cps
Mass/Charge, Da

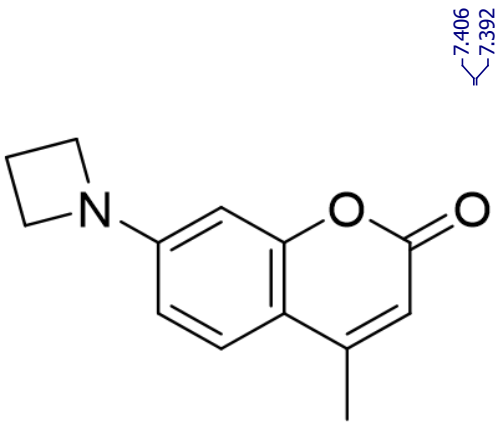
6


Spectrum from 7_Mr. Ashish.wiff2 (sample 1) - 7, Experiment 1, +IDA TOF MS (100 - 1000) from 0.044 min
250.0859 (1)
272.0695 (1)
202.0863 (1)
251.0909 (1)
432.2401 (1)
437.1954 (1)
331.2861 (1)
Intensity, cps
Mass/Charge, Da

7

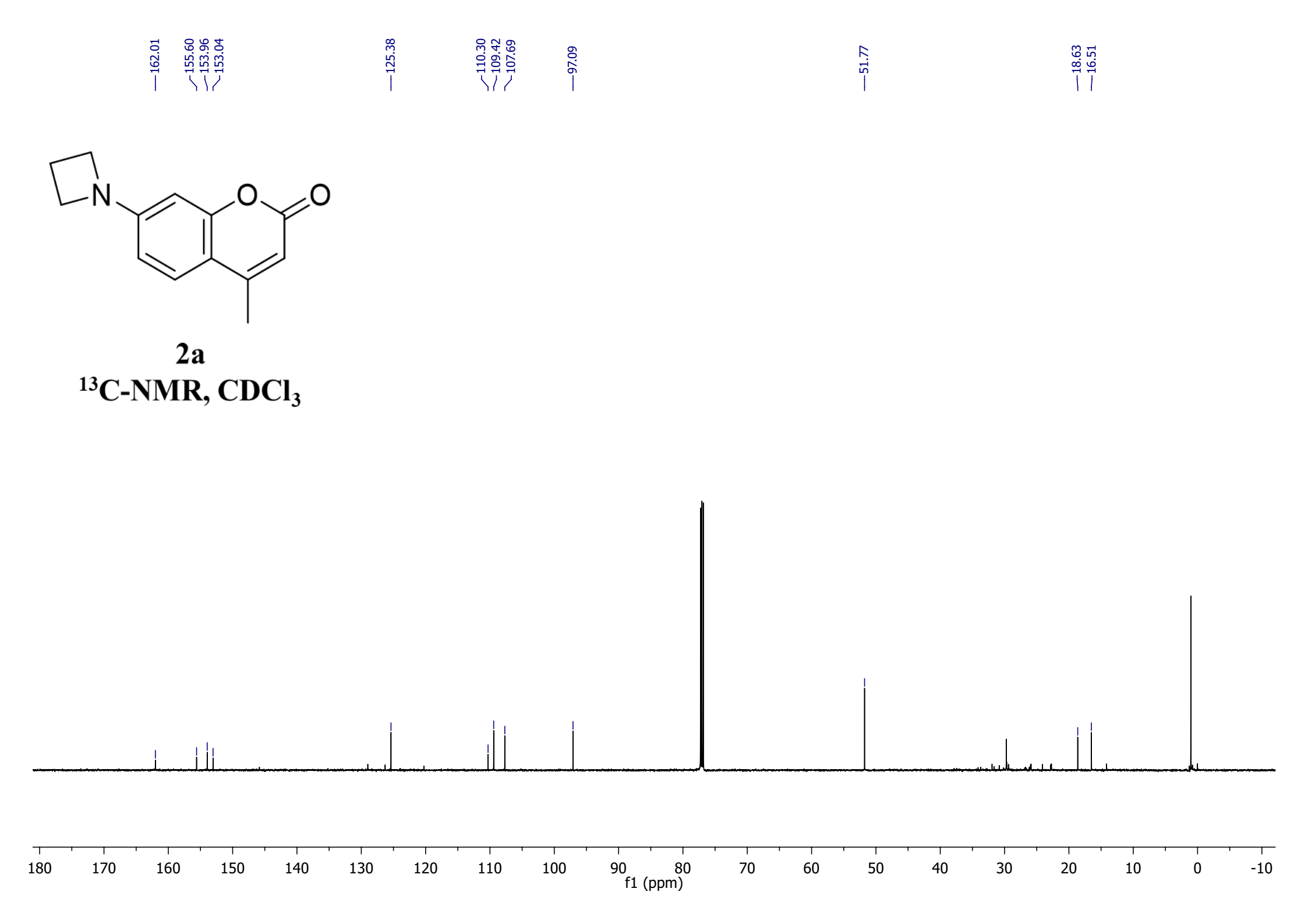

Spectrum from Samarpita_Ms. Samarpita Das.wiff2 (sample 1) - Samarpita, Experiment 1, +IDA TOF MS (100 - 1000) from 0.079 min
Intensity, cps
Mass/Charge, Da
*462.2075 (1)
*425.1753 (1)
*224.0829 (1)
*290.1371 (1)
*202.0981 (1)
203.1029 (1)
*322.1665 (1)
*344.1498 (1)
426.1807 (1)
*463.2119 (1)
*202.1212
*217.1555 (1)
225.0879 (1)
291.1417 (1)
*314.1416 (1)
323.1708 (1)
*368.1135 (1)
*403.1933 (1)
*405.0932 (1)
*427.1836 (1)
*121.0733 (1)
*141.0988 (1)
*167.0206 (1)
*283.0484 (1)
*265.1027 (1)
*396.2265 (1)
*493.1691 (1)

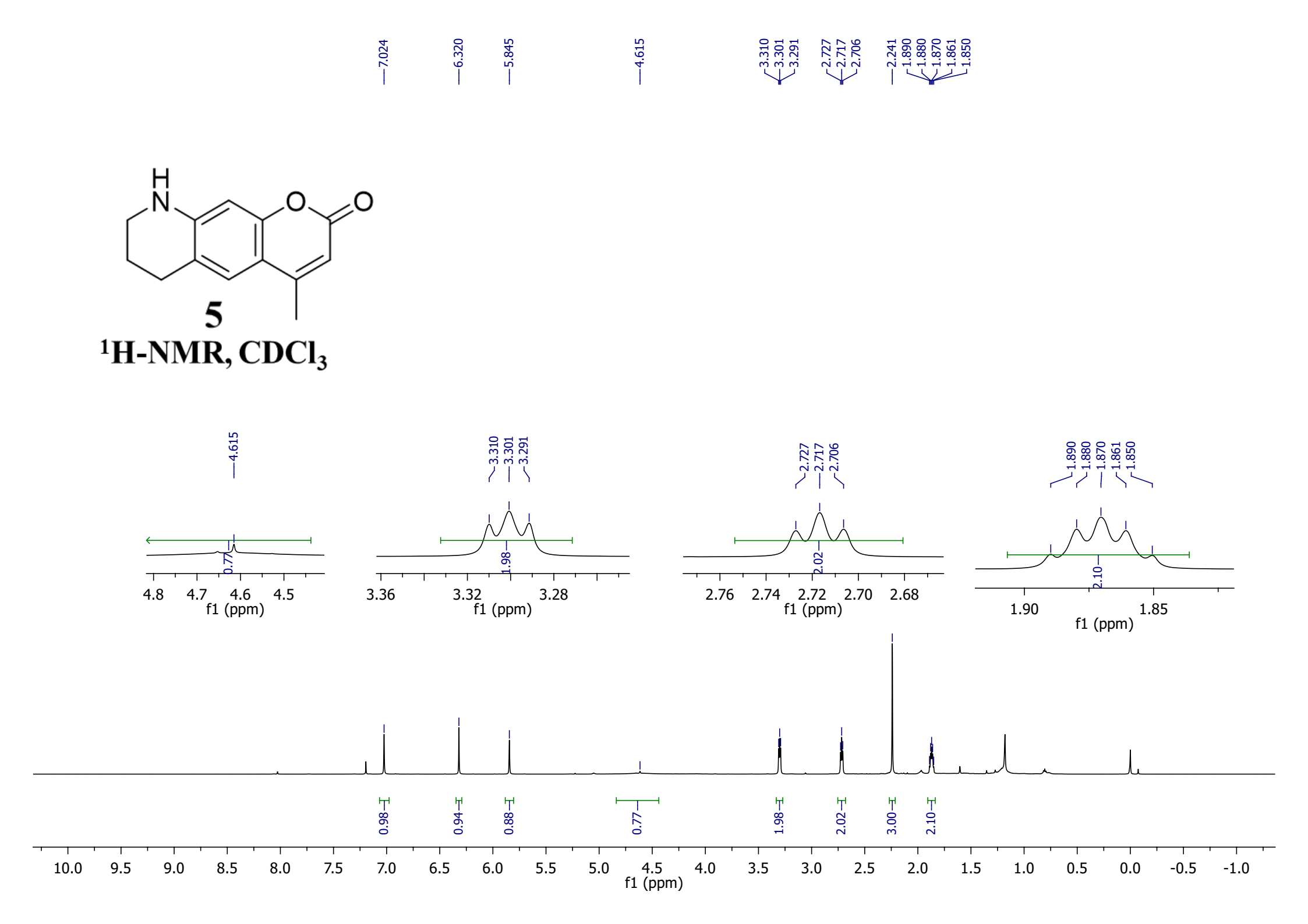
PYCB

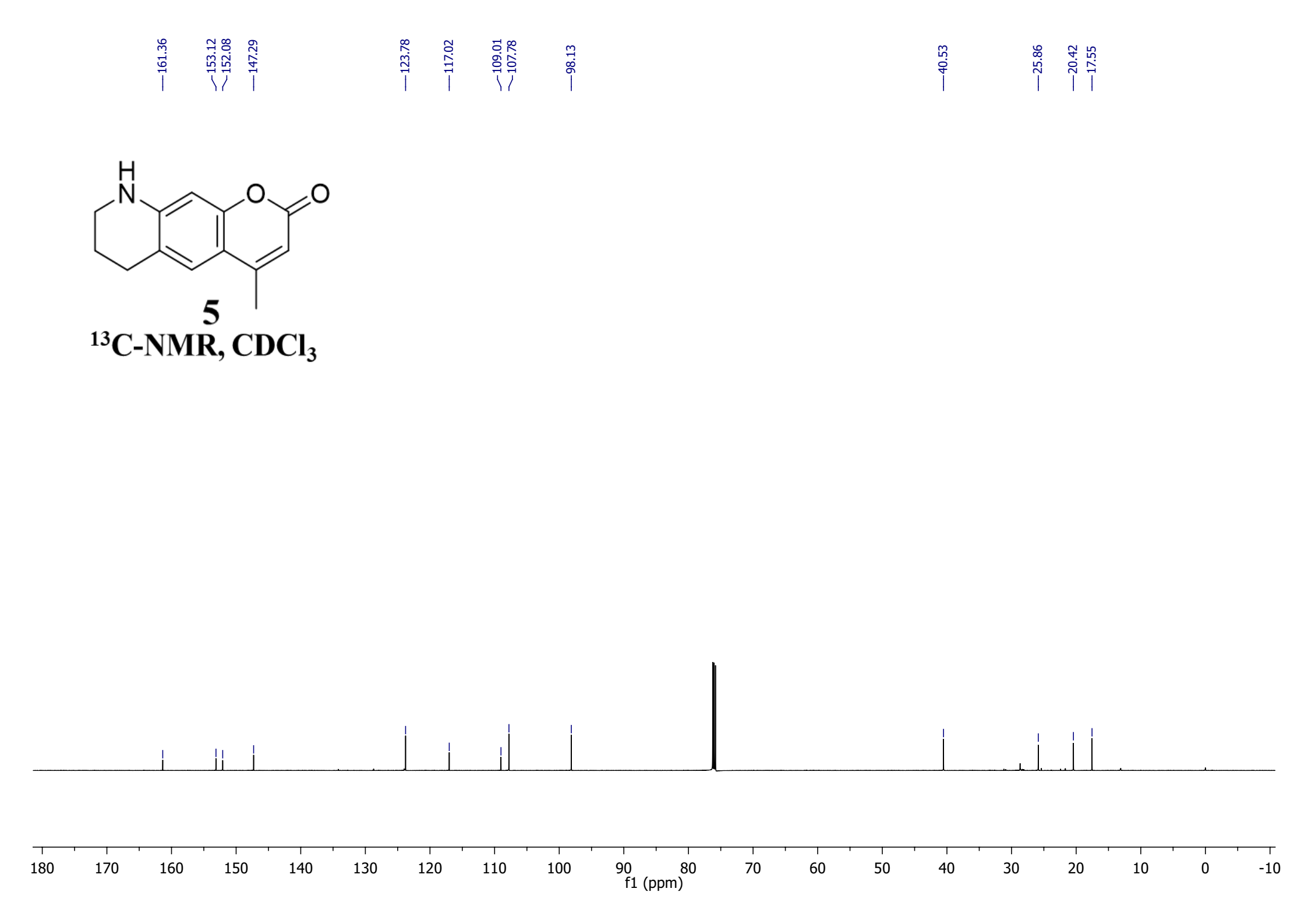


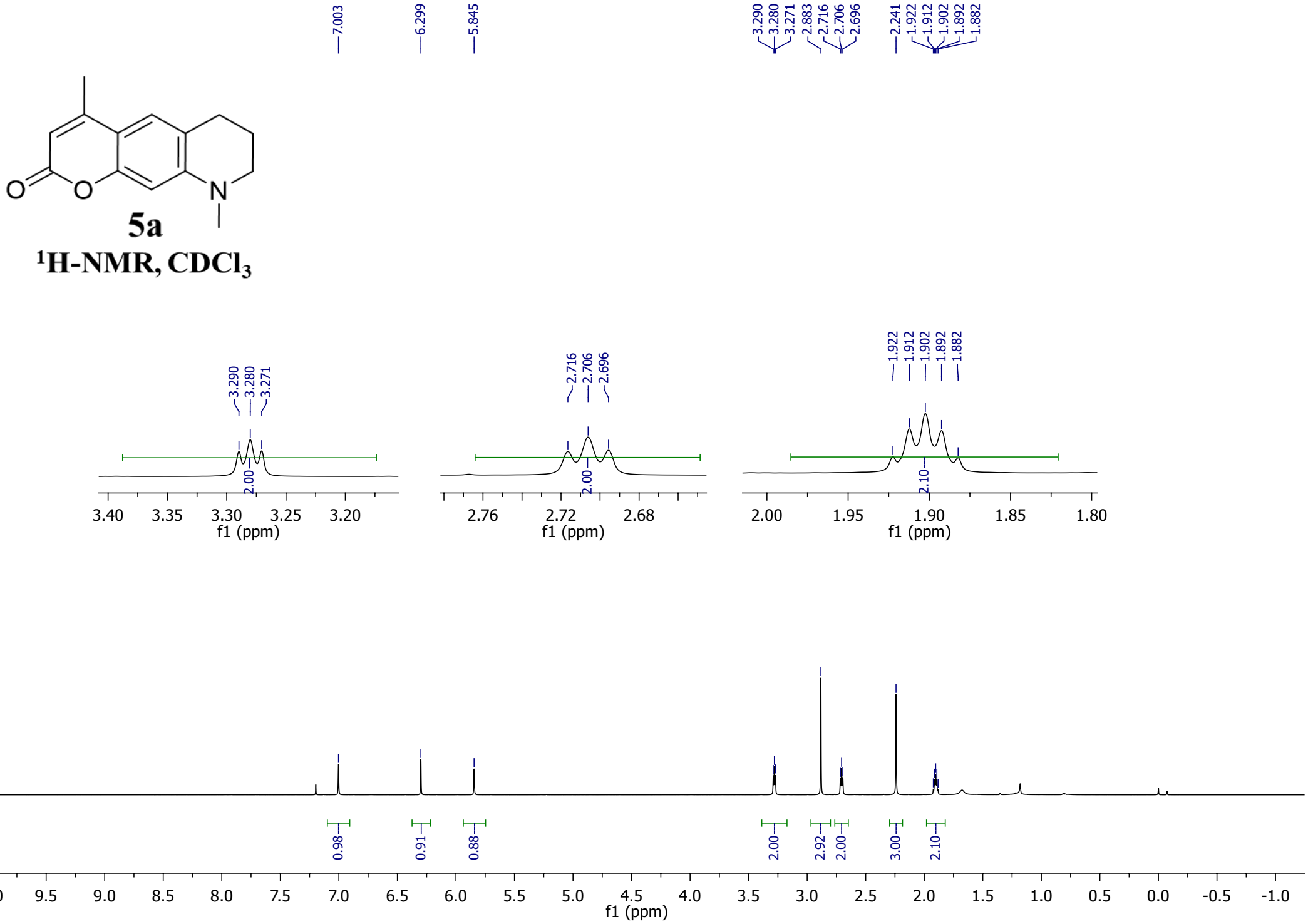
2a

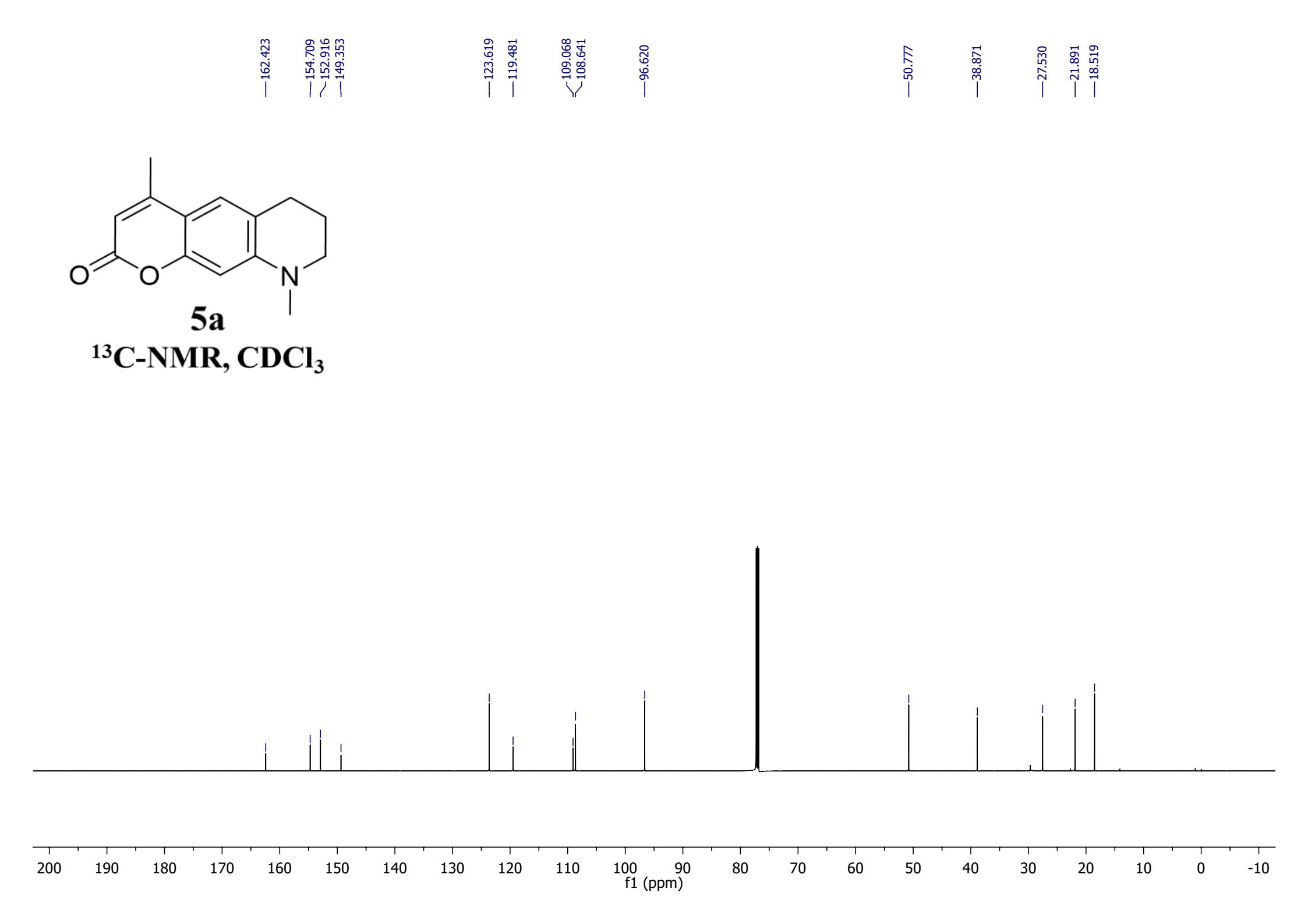
^{1}H-NMR, $CDCl_3$

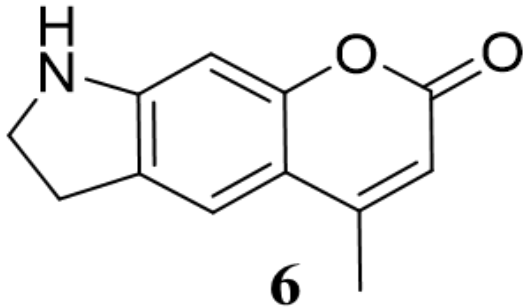

162.01
155.60
153.96
153.04
125.38
110.30
109.42
107.69
97.09
51.77
18.63
16.51
N
O
O
2a
13C-NMR, CDCl3
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)


7.024
6.320
5.845
4.615
3.310
3.301
3.291
2.727
2.717
2.706
2.241
1.890
1.880
1.870
1.861
1.850
H
N
O
O
5
1H-NMR, CDCl3
0.98
0.94
0.88
0.77
1.98
2.02
3.00
2.10
f1 (ppm)


161.36
153.12
152.08
147.29
123.78
117.02
109.01
107.78
98.13
40.53
25.86
20.42
17.55
H
N
O
O
5
13C-NMR, CDCl3
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

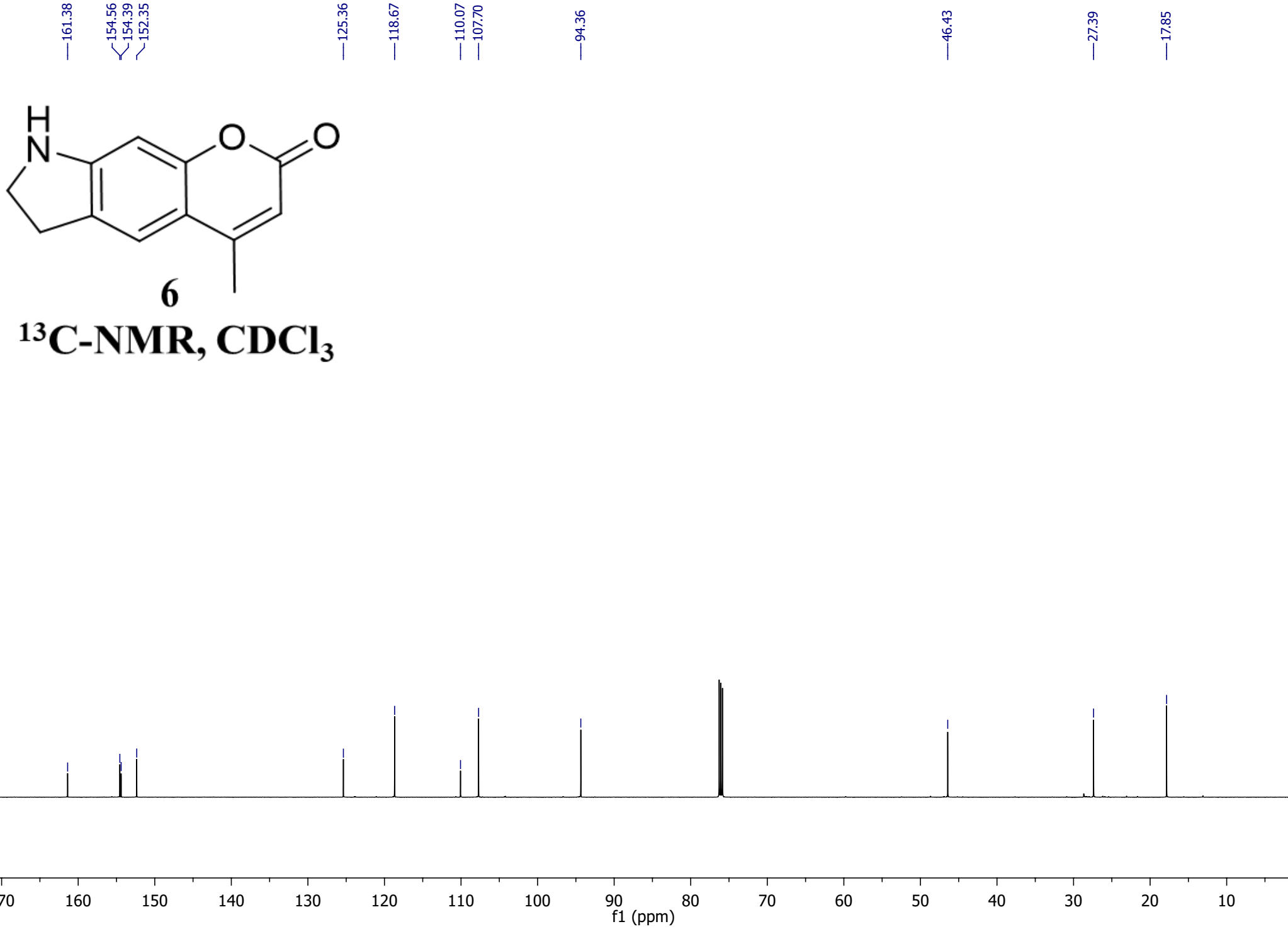

7.003
6.299
5.845
3.290
3.280
3.271
2.883
2.716
2.706
2.696
2.241
1.922
1.912
1.902
1.892
1.882
O
O
N
5a
1H-NMR, CDCl3
2.00
2.00
2.10
f1 (ppm)
0.98
0.91
0.88
2.00
2.92
2.00
3.00
2.10
f1 (ppm)

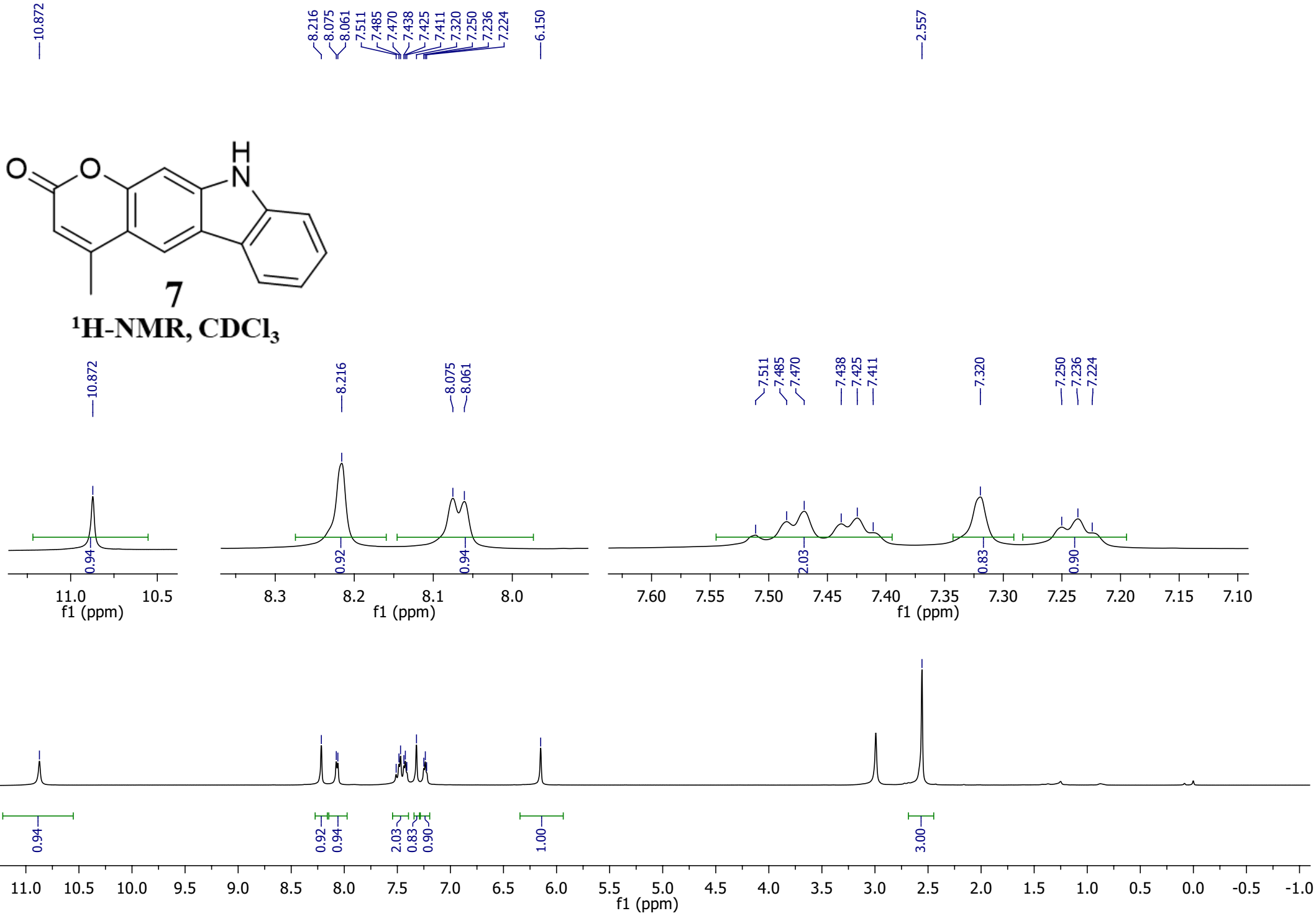

162.423
154.709
152.916
149.353
123.619
119.481
109.068
108.641
96.620
50.777
38.871
27.530
21.891
18.519
O
O
N
5a
13C-NMR, CDCl3
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

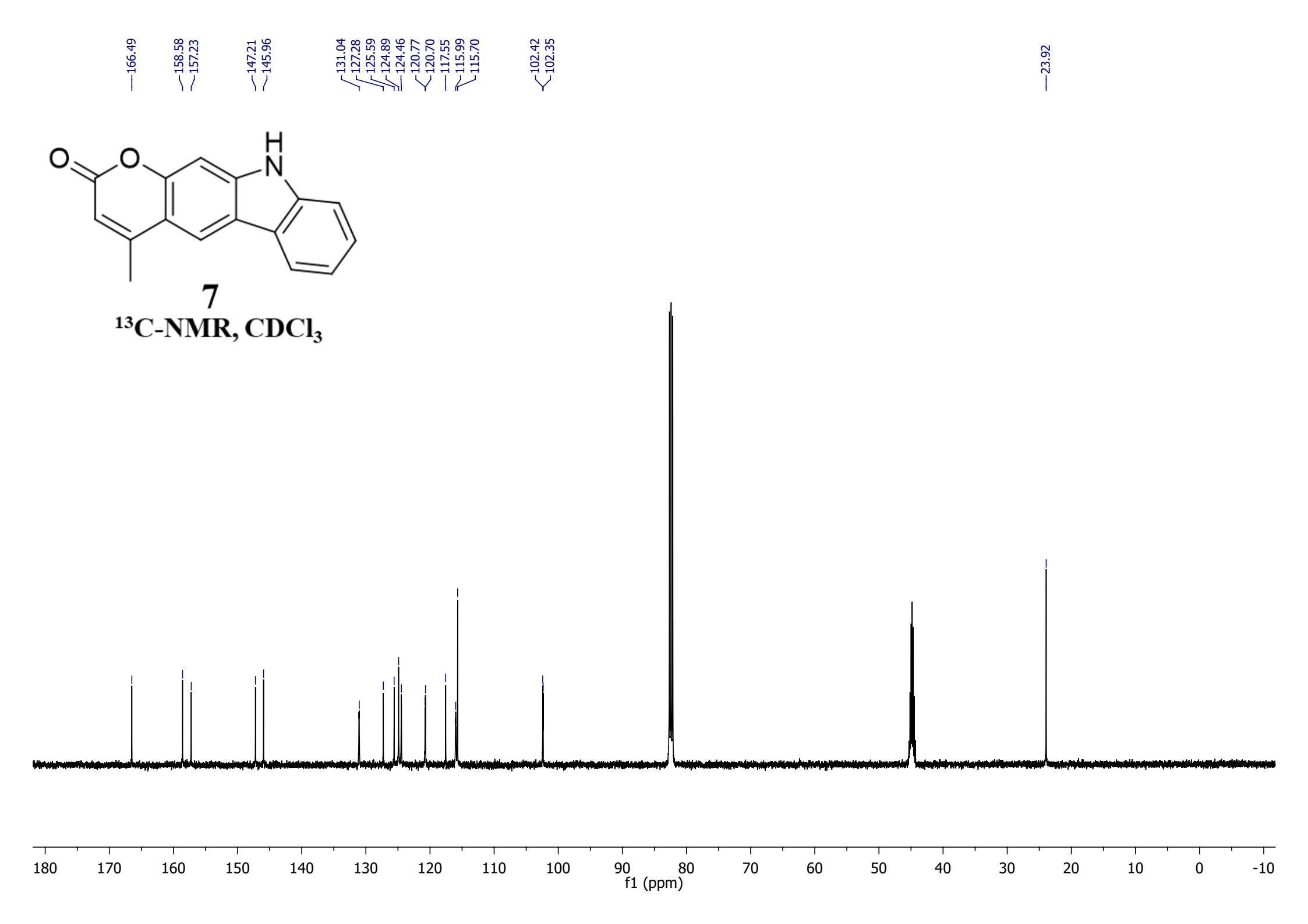


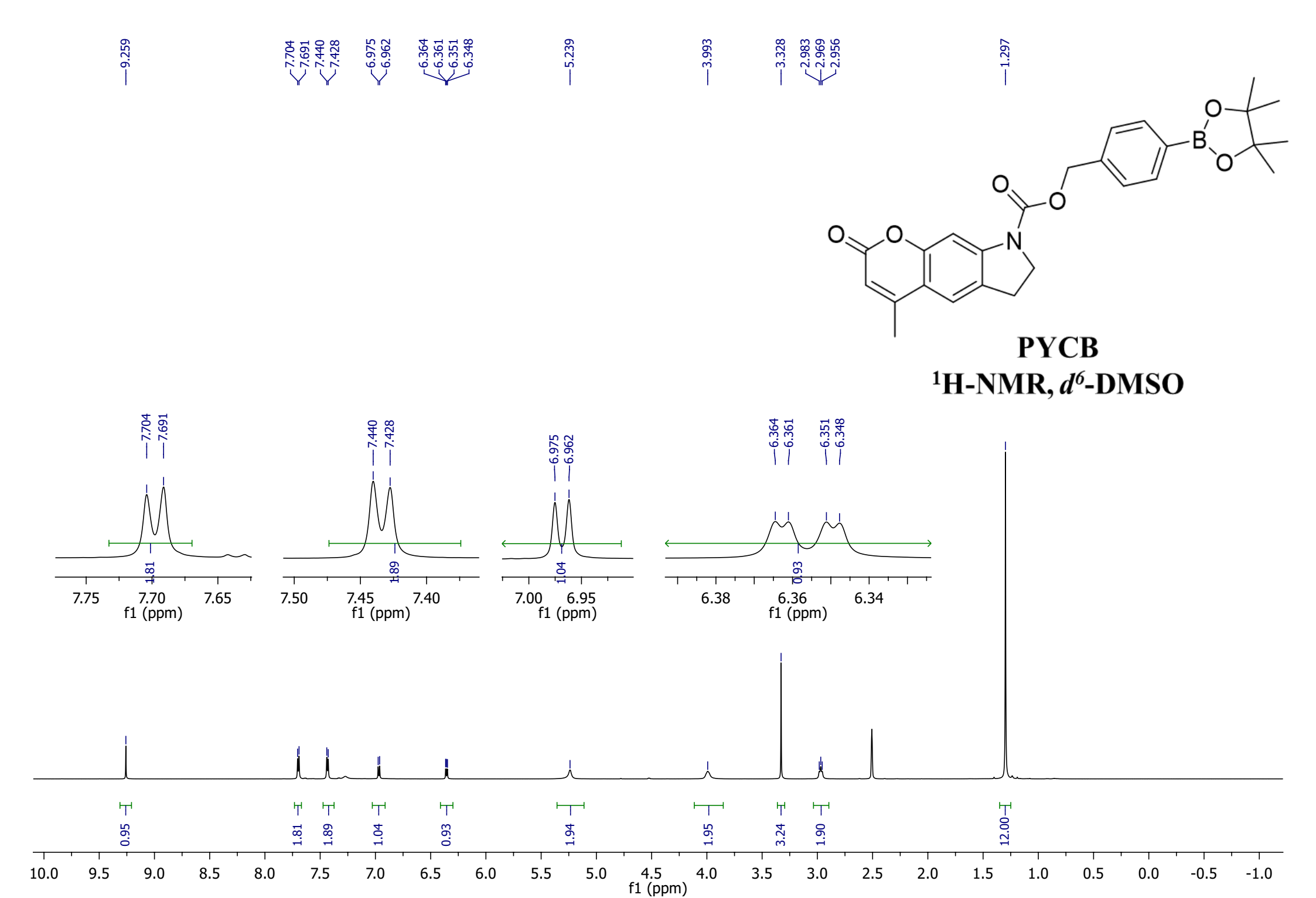
6

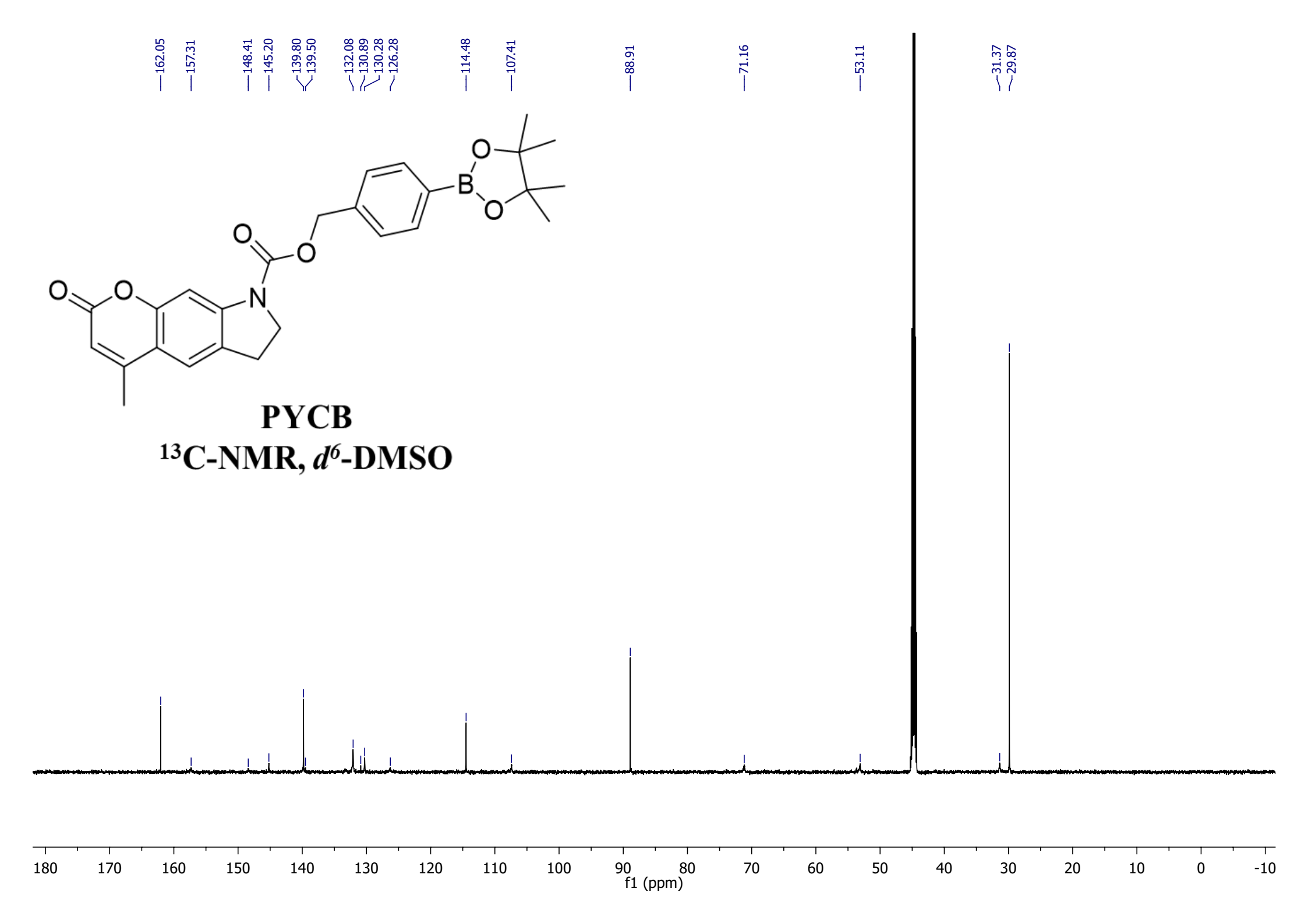
^{1}H-NMR, $CDCl_3$


161.38
154.56
154.39
152.35
125.36
118.67
110.07
107.70
94.36
46.43
27.39
17.85
H
N
O
O
6
13C-NMR, CDCl3
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


10.872
8.216
8.075
8.061
7.511
7.485
7.470
7.438
7.425
7.411
7.320
7.250
7.236
7.224
6.150
2.557
O
O
H
N
7
1H-NMR, CDCl3
0.94
0.92
0.94
2.03
0.83
0.90
1.00
3.00
f1 (ppm)


166.49
158.58
157.23
147.21
145.96
131.04
127.28
125.59
124.89
124.46
120.77
120.70
117.55
115.99
115.70
102.42
102.35
23.92
O
O
H
N
7
13C-NMR, CDCl3
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)


9.259
7.704
7.691
7.440
7.428
6.975
6.962
6.364
6.361
6.351
6.348
5.239
3.993
3.328
2.983
2.969
2.956
1.297
O
O
O
O
N
B
O
O
PYCB
1H-NMR, d6-DMSO
1.81
1.89
1.04
0.93
f1 (ppm)
0.95
1.81
1.89
1.04
0.93
1.94
1.95
3.24
1.90
12.00
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0


162.05
157.31
148.41
145.20
139.80
139.50
132.08
130.89
130.28
126.28
114.48
107.41
88.91
71.16
53.11
31.37
29.87
O
O
O
O
N
O
B
O
PYCB
13C-NMR, d6-DMSO
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)