Supporting information

Negative allosteric modulators of NMDA receptors with GluN2B subunit: synthesis of β-aminoalcohols by epoxide opening and subsequent rearrangement

Marvin Korff,[#] [a,c] Judith Lüken,[#] [a] Judith Schmidt,[a] Dirk Schepmann,[a] Gunnar Goerges,[b] Nadine Ritter,[b,c] Paul Disse,[b,c] Julian A. Schreiber,[a,b] Guiscard Seebohm,[b,c] Bernhard Wünsch[a,c]*

[#] both authors contributed equally

[a] Universität Münster, Institut für Pharmazeutische und Medizinische Chemie, Corrensstr. 48, D-48149 Münster, Germany
Tel.: +49-251-8333311; Fax: +49-251-8332144; E-mail: wuensch@uni-muenster.de

[b] University Hospital Münster, Cellular Electrophysiology and Molecular Biology, Institute for Genetics of Heart Diseases (IfGH), Department of Cardiovascular Medicine, Robert-Koch-Str. 45, D-48149 Münster, Germany

[c] Universität Münster, GRK 2515, Chemical biology of ion channels (Chembion), Corrensstraße 48, D-48149 Münster, Germany

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1. Receptor binding studies

1.1. Materials

Guinea pig brains, rat brains and rat livers were commercially available (Harlan-Winkelmann, Borchen, Germany). The recombinant L(tk-) cells stably expressing the GluN2B receptor were obtained from Prof. Dr. Dieter Steinhilber (Frankfurt, Germany). Homogenizers: Elvehjem Potter (B. Braun Biotech International, Melsungen, Germany) and Soniprep® 150 (MSE, London, UK). Centrifuges: Cooling centrifuge model Eppendorf 5427R (Eppendorf, Hamburg, Germany) and High-speed cooling centrifuge model Sorvall® RC-5C plus (Thermo Fisher Scientific, Langenselbold, Germany). Multiplates: standard 96 well multiplates (Diagonal, Muenster, Germany). Shaker: self-made device with adjustable temperature and tumbling speed (scientific workshop of the institute). Harvester: MicroBeta® FilterMate 96 Harvester. Filter: Printed Filtermat Typ A and B. Scintillator: Meltilex® (Typ A or B) solid state scintillator. Scintillation analyzer: MicroBeta® Trilux (all Perkin Elmer LAS, Rodgau-Jügesheim, Germany).

1.2. Cell culture and preparation of membrane homogenates from GluN2B cells

Mouse L(tk-) cells stably transfected with the dexamethasone-inducible eukaryotic expression vectors pMSG GluN1a, pMSG GluN2B (1:5 ratio) were grown in Modified Earl's Medium (MEM) containing 10 % of standardized FBS Superior (Biochrom AG, Berlin, Germany). The expression of the NMDA receptor at the cell surface was induced after the cell density of the adherent growing cells had reached approximately 90 % of confluency. For the induction, the original growth medium was replaced by growth medium containing 4 µM dexamethasone and 4 µM ketamine (final concentration). After 24 h, the cells were rinsed with phosphate buffered saline solution (PBS, Biochrom AG, Berlin, Germany), harvested by mechanical detachment and pelleted (10 min, 1,200 x g).

For the binding assay, the cell pellet was resuspended in PBS solution and the number of cells was determined using a Scepter® cell counter (MERCK Millipore, Darmstadt, Germany). Subsequently, the cells were lysed by sonication (4 °C, 6 x 10 s cycles with breaks of 10 s). The resulting cell fragments were centrifuged with a high performance cool centrifuge (23,500 x g, 4 °C). The supernatant was discarded and the pellet was

resuspended in a defined volume of PBS yielding cell fragments of approximately 500,000 cells/mL. The suspension of membrane homogenates was sonicated again (4 °C, 2 x 10 s cycles with a break of 10 s) and stored at -80 °C.

1.3. Preparation of membrane homogenates from guinea pig brain

5 guinea pig brains were homogenized with the potter (500-800 rpm, 10 up and down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1,200 x g for 10 min at 4 °C. The supernatant was separated and centrifuged at 23,500 x g for 20 min at 4 °C. The pellet was resuspended in 5-6 volumes of buffer (50 mM TRIS, pH 7.4) and centrifuged again at 23,500 x g (20 min, 4 °C). This procedure was repeated twice. The final pellet was resuspended in 5-6 volumes of buffer and frozen (-80 °C) in 1.5 mL portions containing about 1.5 mg protein/mL.

1.4. Preparation of membrane homogenates from rat liver

Two rat livers were cut into small pieces and homogenized with the potter (500-800 rpm, 10 up and down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1,200 x g for 10 min at 4 °C. The supernatant was separated and centrifuged at 31,000 x g for 20 min at 4 °C. The pellet was resuspended in 5-6 volumes of buffer (50 mM TRIS, pH 8.0) and incubated at rt for 30 min. After the incubation, the suspension was centrifuged again at 31,000 x g for 20 min at 4 °C. The final pellet was resuspended in 5-6 volumes of buffer and stored at -80 °C in 1.5 mL portions containing about 2 mg protein/mL.

1.5. Protein determination

The protein concentration was determined by the method of Bradford,[1] modified by Stoscheck.[2] The Bradford solution was prepared by dissolving 5 mg of Coomassie Brilliant Blue G 250 in 2.5 mL of EtOH (95 %, v/v). 10 mL deionized H_2O and 5 mL phosphoric acid (85 %, m/v) were added to this solution, the mixture was stirred and filled to a total volume of 50 mL with deionized water. The calibration was carried out using bovine serum albumin as a standard in 9 concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 and 4.0 mg /mL). In a 96 well standard multiplate, 10 µL of the calibration solution or 10 µL of the membrane receptor preparation were mixed with 190 µL of the Bradford solution, respectively. After 5 min, the UV absorption of the protein-dye

complex at λ = 595 nm was measured with a plate reader (Tecan Genios®, Tecan, Crailsheim, Germany).

1.6. General procedures for the binding assays

The test compound solutions were prepared by dissolving approximately 10 µmol (usually 2-4 mg) of test compound in DMSO so that a 10 mM stock solution was obtained. To obtain the required test solutions for the assay, the DMSO stock solution was diluted with the respective assay buffer. The filtermats were presoaked in 0.5 % aqueous polyethylenimine solution for 2 h at rt before use. All binding experiments were carried out in duplicates in the 96 well multiplates. The concentrations given are the final concentration in the assay. Generally, the assays were performed by addition of 50 µL of the respective assay buffer, 50 µL of test compound solution in various concentrations (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol/L), 50 µL of the corresponding radioligand solution and 50 µL of the respective receptor preparation into each well of the multiplate (total volume 200 µL). The receptor preparation was always added last. During the incubation, the multiplates were shaken at a speed of 500-600 rpm at the specified temperature. Unless otherwise noted, the assays were terminated after 120 min by rapid filtration using the harvester. During the filtration, each well was washed five times with 300 µL of water. Subsequently, the filtermats were dried at 95 °C. The solid scintillator was melted on the dried filtermats at a temperature of 95 °C for 5 min. After solidifying of the scintillator at rt, the trapped radioactivity in the filtermats was measured with the scintillation analyzer. Each position on the filtermat corresponding to one well of the multiplate was measured for 5 min with the [^{3}H]-counting protocol. The overall counting efficiency was 20 %. The IC_{50} values were calculated with the program GraphPad Prism® 3.0 (GraphPad Software, San Diego, CA, USA) by non-linear regression analysis. Subsequently, the IC_{50} values were transformed into K_i values using the equation of Cheng and Prusoff.[3] The K_i values are given as mean value ± SEM from three independent experiments.

Performance of the binding assays

1.7. Ifenprodil binding site of GluN2B subunit containing NMDA receptors

The competitive binding assay was performed with the radioligand [^{3}H]ifenprodil (60 Ci/mmol; BIOTREND, Cologne, Germany). The thawed cell membrane preparation from the transfected L(tk-) cells (about 20 µg protein) was incubated with various

concentrations of test compounds, 5 nM [^{3}H]ifenprodil, and TRIS/EDTA-buffer (5 mM TRIS/1 mM EDTA, pH 7.5) at 37 °C. The non-specific binding was determined with 10 µM unlabeled ifenprodil. The K_d value of ifenprodil is 7.6 nM.[4]

1.8. σ_1 Receptor assay

The assay was performed with the radioligand [^{3}H]-(+)-pentazocine (22.0 Ci/mmol; Perkin Elmer). The thawed membrane preparation of guinea pig brain (about 100 µg of the protein) was incubated with various concentrations of test compounds, 2 nM [^{3}H]-(+)-pentazocine, and TRIS buffer (50 mM, pH 7.4) at 37 °C. The non-specific binding was determined with 10 µM unlabeled (+)-pentazocine. The K_d value of (+)-pentazocine is 2.9 nM.[5]

1.9. σ_2 Receptor assay

The assays were performed with the radioligand [^{3}H]di-*o*-tolylguanidine (specific activity 50 Ci/mmol; ARC, St. Louis, MO, USA). The thawed rat liver membrane preparation (about 100 µg protein) was incubated with various concentrations of the test compound, 3 nM [^{3}H]di-*o*-tolylguanidine and buffer containing (+)-pentazocine (500 nM (+)-pentazocine in TRIS buffer (50 mM TRIS, pH 8.0)) at rt. The non-specific binding was determined with 10 µM non-labeled di-*o*-tolylguanidine. The K_d value of di-*o*-tolylguanidine is 17.9 nM.[6]

2. Two-electrode voltage clamp experiments

2.1. Molecular biology and TEVC measurements

cRNAs generation, oocyte preparation and TEVC recordings were mentioned before.[7; 8] In detail, cDNAs encoding GluN1a and GluN2B were linearized and transcribed into cRNA (mMESSAGE mMACHINE T7 Transcription Kit, Invitrogen, Carlsbad, USA). Defolliculated oocytes stage V (EcoCyte Bioscience, Dortmund, Germany) were injected with 0.8 ng GluN1a and 0.8 ng GluN2B cRNA. After cRNA injection, the oocytes were incubated for 5 days in Barth's solution (88 NaCl mM, 1 mM KCl, 0.4 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.6 mM $MgSO_4$, 5 mM TRIS-HCl, 2.4 mM $NaHCO_3$, supplemented with 80 mg/L theophylline, 63 mg/L benzylpenicillin, 40 mg/L streptomycin, and 100 mg/L gentamycin). TEVC recordings were performed at a holding potential of -70 mV with recording pipettes filled with 3M KCl (resistance 0.5-1.5 MΩ) in Ba^{2+}-Ringer solution (10 mM HEPES, 90 mM NaCl, 1 mM KCl, 1.5 mM $BaCl_2$, adjusted to pH 7.4 with 1 M NaOH), the agonist solution was composed of 10 µM (*S*)-glutamate and 10 µM glycine in Ba^{2+}-Ringer solution. Compound activity was evaluated at concentration levels of 1 µM and 10 µM compound diluted with the agonist solution. For dose response curves, the compounds were tested by applying ascending concentrations, ranging from 1 nM up to 10 µM, in at least five independent oocytes expressing GluN1-1a/GluN2B. Exact numbers of oocytes are given for each condition in Table 1 and 2. Ifenprodil was purchased from Sigma Aldrich, Germany.

2.2. Data analysis and statistics

The inhibition was calculated with following equation:

$$\text{Inhibition}\ (\%) = 1 - \frac{\mathrm{I_c} - \mathrm{I_h}}{\mathrm{I_a} - \mathrm{I_h}}$$

where I_h represents the current without agonists, I_c the current with agonist and I_a the current with agonist and test compound. Significance of mean differences was analyzed by one-way-ANOVA with post hoc mean comparison Tukey test indicated by ns for $p > 0.05$, * for $p < 0.05$ ** for $p < 0.01$ and *** for $p < 0.001$.

Dose response curves were fitted to the following logistic equation:

$$y = \frac{A_1 - A_2}{1 + \left(\frac{x}{x_0}\right)^p} + A_2$$

A_1 describes the minimal inhibition of a compound and was set to 0. A_2 represents the maximal inhibition of a compound; p is the slope of the curve; x_0 is defined as the concentration at half-maximal inhibition and x is the tested concentration.

3. Determination of log$D_{7.4}$ value [9;10]

Instruments and parameters for LC-MS standard analysis (in general, if not stated otherwise)

UPLC-UV/MS (Agilent Technologies): degasser: 1260 HiP (G4225A); pump: 1260 Bin Pump (G1212B); autosampler: 1260 HiP ALS (G1367E); column oven: 1290 TCC (G1316C), 30 °C; UV/Vis detector: 1260 VWD (G1314F); MS-detector: 6120 Quadrupole LC/MS (G1978B). MS source: multimode source (G1978B); ESI mode; SIM mode (*m*/*z* given for each compound). Data acquisition and settings were performed with OpenLab CDS (ChemStation Edition, Agilent). Guard column: Zorbax Eclipse Plus-C18 (Agilent, Waldbronn, Germany) (2.1 mm x 12.5 mm, 5.0 µm particle size). Main column: Zorbax SB-C18 (Agilent, Waldbronn, Germany) (2.1 mm x 50 mm, 1.8 µm particle size). Spray chamber: vaporizer temperature: 200 °C; drying gas: 12 L/min; nebulizer pressure: 40 psi; capillary voltage: 3000 V; corona current: 4 µA; charging voltage: 2000 V; fragmentor voltage: 100 V; drying gas temperature: 250 °C. 2 mL safe lock tubes (Eppendorf), 2 mL LC-MS vials (Agilent).

LC-MS standard method (LC parameters in general, if not stated otherwise)

Eluents: solvent A: H_2O/CH_3CN 95:5 + 0.1 % formic acid; solvent B: H_2O/CH_3CN 5:95 + 0.1 % formic acid; gradient elution (A %): 0 – 2.5 min: gradient from 100 % to 0 %, 2.5 – 3.5 min: 0 %, 3.5 – 4.0 min: gradient from 0 % to 100 %, 4.0 – 8.0 min: 100 %. Change valve position: after 1.0 min the valve was switched from ”waste“ to ”MS source“. Flow rate: 0.4 mL/min. Injection volume: 1.0 µL to 100 µL (given for each compound, 1.0 µL if not stated otherwise).

Chemicals, solvents and stock solutions

3-Morpholinopropanesulfonic acid (MOPS) (Fisher Chemical, 372.5 mg, 8.9 mM) and MOPS sodium salt (Sigma-Aldrich, 513.4 mg, 11.1 mM) were dissolved in dist. H_2O (200 mL) to prepare a 20 mM buffer solution with pH 7.4. A mixture of *n*-octanol (Sigma Aldrich) and MOPS buffer (20 mM, pH 7.4) in the ratio 1:1 was stirred overnight at room temperature (500 rpm) to saturate both liquids with each other. Afterwards, the aqueous and organic layers were separated.

10 mM stock solutions of the test compounds in DMSO (MERCK-Schuchardt, Hohenbrunn, Germany) were prepared by dissolving an exactly weighted amount of

the test compound and adding the calculated amount of DMSO. Depending on the lipophilicity, either the 10 mM stock solution was used directly or the stock solution was diluted 1:100 with MOPS buffer to a concentration of 100 µM.

General procedure

In order to determine the log$D_{7.4}$ value, the micro shake flask method was used. [9; 10] To create physiological conditions a buffer with pH 7.4 was used to analyze the lipophilicity (log$D_{7.4}$). The log$D_{7.4}$ value was determined by using three different volume ratios of buffer and *n*-octanol (1:1, 2:1, 1:2).

Method LA (standard procedure): The 10 mM DMSO stock solution of the test compound (7.5 µL) was added to three different volumes of MOPS buffer (750 µL, 1000 µL, 500 µL) in 2 mL Eppendorf tubes. Afterwards, the tubes were filled up to 1500 µL with *n*-octanol (750 µL, 500 µL, 1000 µL). Each ratio was produced as a triplicate. The tubes were vortexed at rt and centrifuged at 4 °C with 16,000 rpm for 2 min.

Method LB (for very hydrophilic compounds): The 100 µM MOPS solution of the test compound (75 µL) was added to three different volumes of MOPS buffer (675 µL, 925 µL, 425 µL) in 2 mL Eppendorf tubes. *n*-Octanol was added to fill up the tubes to a total volume of 1500 µL (750 µL, 500 µL, 1000 µL). Each ratio was produced as a triplicate. Afterwards, the tubes were vortexed at rt and centrifuged at 4 °C with 16,000 rpm for 2 min.

An aliquot of the aqueous layer was analyzed by LC-MS standard method. For matrix-matched calibration to calculate log$D_{7.4}$ value, the samples were diluted with MOPS buffer within a range of 1.56 nM to 1.0 µM or 39 nM to 10 µM. All samples were measured once.

4. Structural analysis of regioisomers 7a–e and 9a,9e

To unambiguously prove the constitution of β-aminoalcohols **7a-e** obtained by opening of epoxides **6a-e** with 4-benzylpiperidine, the isolated reaction product **7a** was analyzed by GC-EI-MS. (Figure S1) If epoxide **6a** was opened at the desired position, **9a** would be formed, which would give fragments 1’ and 2’ with a mass to charge ratios of 137.06 and 202.16, respectively, during the fragmentation, induced by the electron impact ionization. If **7a** was formed, it is expected to give fragments 1 and 2 as the characteristic fragmentation pattern. As can be seen in figure S1 A, the main component elutes at 7.36 min from the GC column. At this time point, the mass spectrum shows the main ion with a mass to charge ratio of 294.2, indicating the formation of regioisomer **7a** during the epoxide opening of **6a**, instead of **9a**.

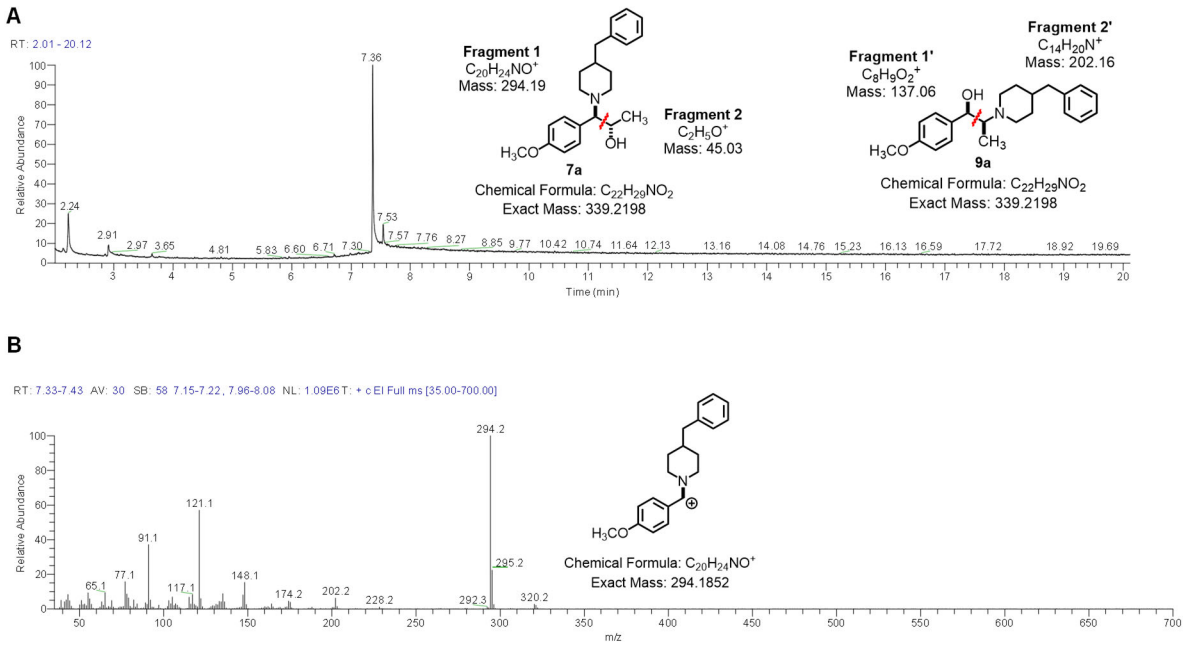

Figure S1: GC-EI-MS analysis of the reaction product of opening of epoxide **6a** to determine, which regioisomer had been formed. **A**: Chromatogram with total ion count from retention time range from 2–20 min. **B**: Mass spectrum at time range 7.33–7.43 min.

Being regioisomers, and therefore sharing similar physicochemical properties, this is also true for the NMR analysis. However, by recording gHSQC NMR spectra patterns were identified to distinguish the regioisomers **7a–e** and **9a**,**9e**. Figure S2 shows exemplary the spectra of **7a** and **9a**, but the discussed observations applies for **7b–e** and **9e** as well. For the benzylamine **7a**, the signal for the benzylic proton was found in the high field at 3.02 ppm (Figure S2, top, signal **A**), whereas the signal for the proton of the C*H*OH moiety was detected in the lower field at 4.35 ppm (Figure S2, top, signal

B). The corresponding C-atoms display the signals in a reversed order: The C-atom in benzylic position resonates at 74.9 ppm (Figure S2, top, signal **A**), whereas the signal for the *C*HOH group appears at higher field at 65.3 ppm (Figure S2, top, signal **B**).

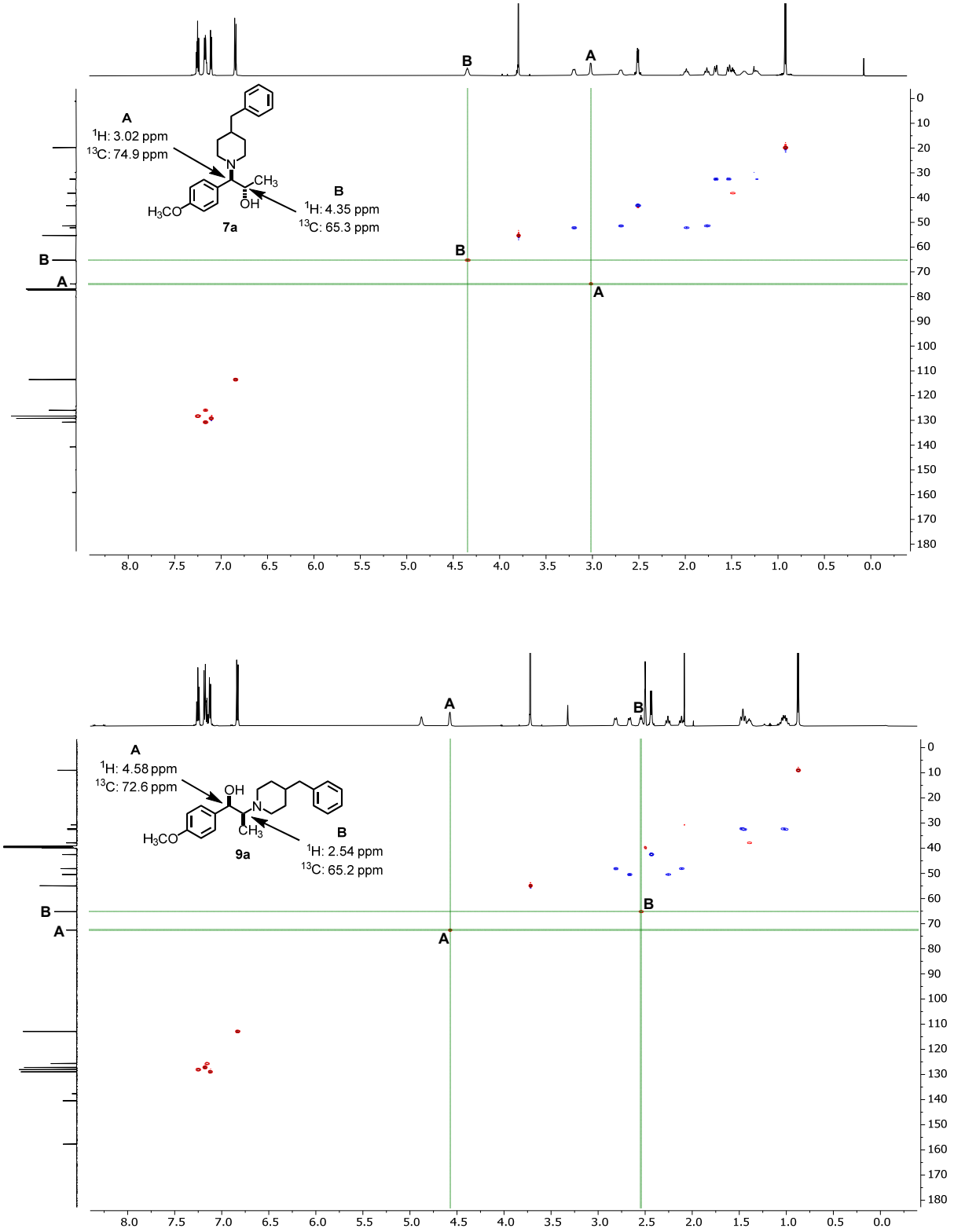

Figure S2: gHSQC NMR spectra of **7a** (top) and **9a** (bottom) to allocate unambiguously the structure of the regioisomers **7a** and **9a**.

In the case of benzylic alcohol **9a**, both the signals for the proton and C-atom in benzylic position were found in the low field at 4.58 ppm and 72.6 ppm (Figure S2, bottom, signals **A'**). In contrast, the signals for the $CHNR_2$ moiety appear at higher field at 2.54 ppm and 65.2 ppm (Figure S2, bottom, signals **B'**).

5. References

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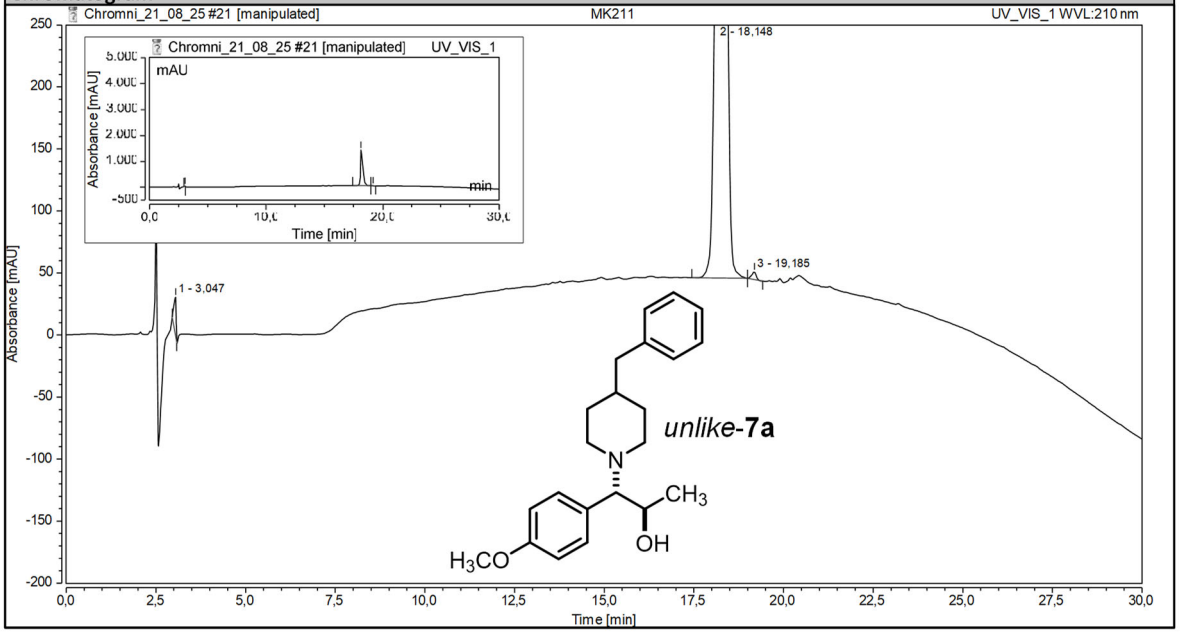
6. HPLC chromatograms (purity)

Chromatogram and Results

Injection Details

Injection Name:	**MK211**	*Run Time (min):*	**30,00**
Vial Number:	**GC1**	*Injection Volume:*	**5,00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**210,0**
Instrument Method:	**Chromni**	*Bandwidth:*	**n.a.**
Processing Method:	**Chromni Processing Method**	*Dilution Factor:*	**1,0000**
Injection Date/Time:	**26.Aug.21 00:36**	*Sample Weight:*	**1,0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3,047	2,210	30,890	0,67	2,19	n.a.
2		18,148	328,275	1376,075	99,07	97,38	n.a.
3		19,185	0,860	6,201	0,26	0,44	n.a.
Total:			**331,345**	**1413,166**	**100,00**	**100,00**	

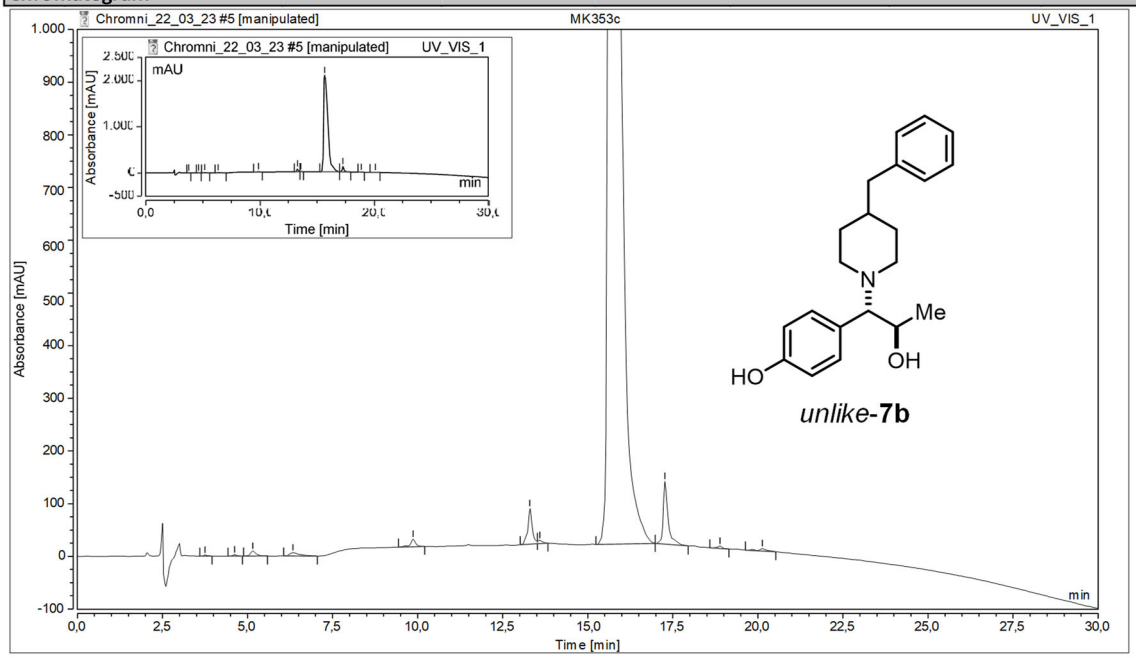
HPLC chromatogram of aminoalcohol *unlike*-**7a** showing the purity of **7a** (t_R = 18.15 min, HPLC method 1).

Chromatogram and Results

Injection Details

Injection Name:	**MK353c**	*Run Time (min):*	**30,00**
Vial Number:	**GA3**	*Injection Volume:*	**5,00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**210,0**
Instrument Method:	**Chromni**	*Bandwidth:*	**n.a.**
Processing Method:	**Chromni Processing Method**	*Dilution Factor:*	**1,0000**
Injection Date/Time:	**23.Mrz.22 17:22**	*Sample Weight:*	**1,0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3,755	0,280	2,325	0,03	0,10	n.a.
2		4,620	0,399	2,598	0,04	0,11	n.a.
3		5,157	1,832	9,575	0,20	0,41	n.a.
4		6,333	2,181	6,240	0,24	0,27	n.a.
5		9,867	2,563	14,612	0,28	0,63	n.a.
6		13,298	9,607	68,073	1,06	2,93	n.a.
7		13,593	0,958	6,220	0,11	0,27	n.a.
8		15,672	864,551	2088,340	95,63	89,75	n.a.
9		17,270	19,609	119,319	2,17	5,13	n.a.
10		18,882	0,819	4,696	0,09	0,20	n.a.
11		20,127	1,306	4,759	0,14	0,20	n.a.
Total:			**904,104**	**2326,756**	**100,00**	**100,00**	

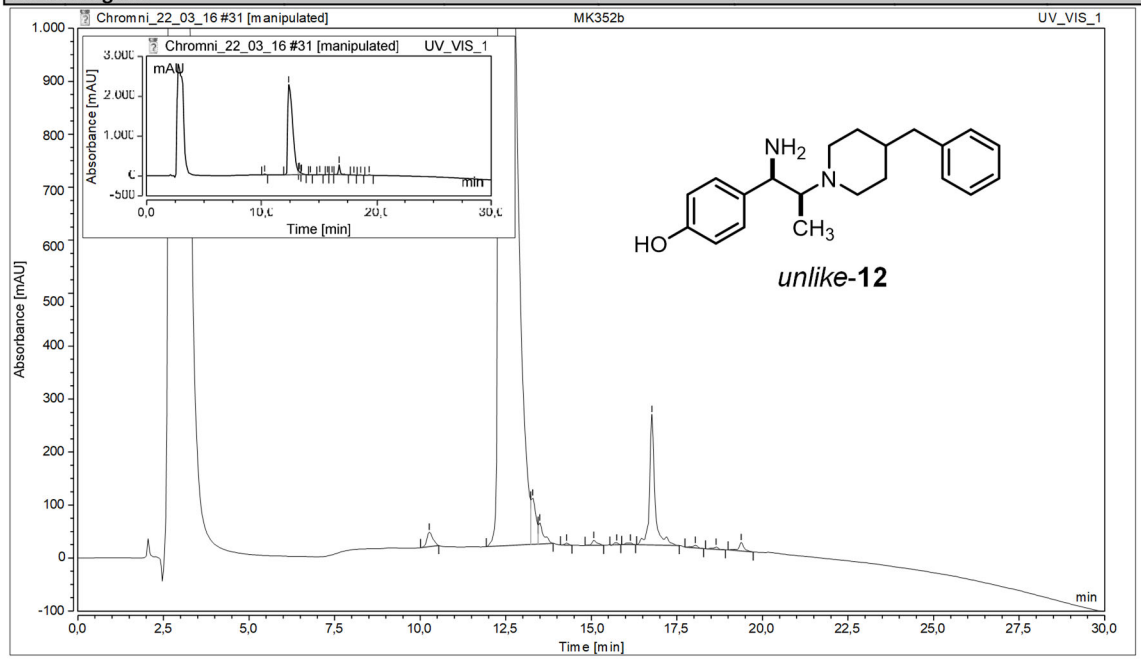
HPLC chromatogram of aminoalcohol *unlike*-**7b** showing the purity of **7b** (t_R = 15.67 min, HPLC method 1).

Chromatogram and Results

Injection Details

Injection Name:	**MK352b**	*Run Time (min):*	**30,00**
Vial Number:	**GD2**	*Injection Volume:*	**5,00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**210,0**
Instrument Method:	**Chromni**	*Bandwidth:*	**n.a.**
Processing Method:	**Chromni Processing Method**	*Dilution Factor:*	**1,0000**
Injection Date/Time:	**17.Mrz.22 11:27**	*Sample Weight:*	**1,0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		10,268	5,423	27,586	0,45	1,02	n.a.
2		12,378	1130,101	2264,268	93,42	83,47	n.a.
3		13,290	14,872	87,763	1,23	3,24	n.a.
4		13,497	6,982	39,888	0,58	1,47	n.a.
5		14,280	0,465	3,823	0,04	0,14	n.a.
6		15,077	1,650	9,652	0,14	0,36	n.a.
7		15,745	0,552	3,634	0,05	0,13	n.a.
8		16,143	0,702	3,139	0,06	0,12	n.a.
9		16,768	44,277	246,825	3,66	9,10	n.a.
10		18,040	0,944	5,235	0,08	0,19	n.a.
11		18,647	0,830	4,399	0,07	0,16	n.a.
12		19,382	2,859	16,517	0,24	0,61	n.a.
Total:			**1209,658**	**2712,727**	**100,00**	**100,00**	

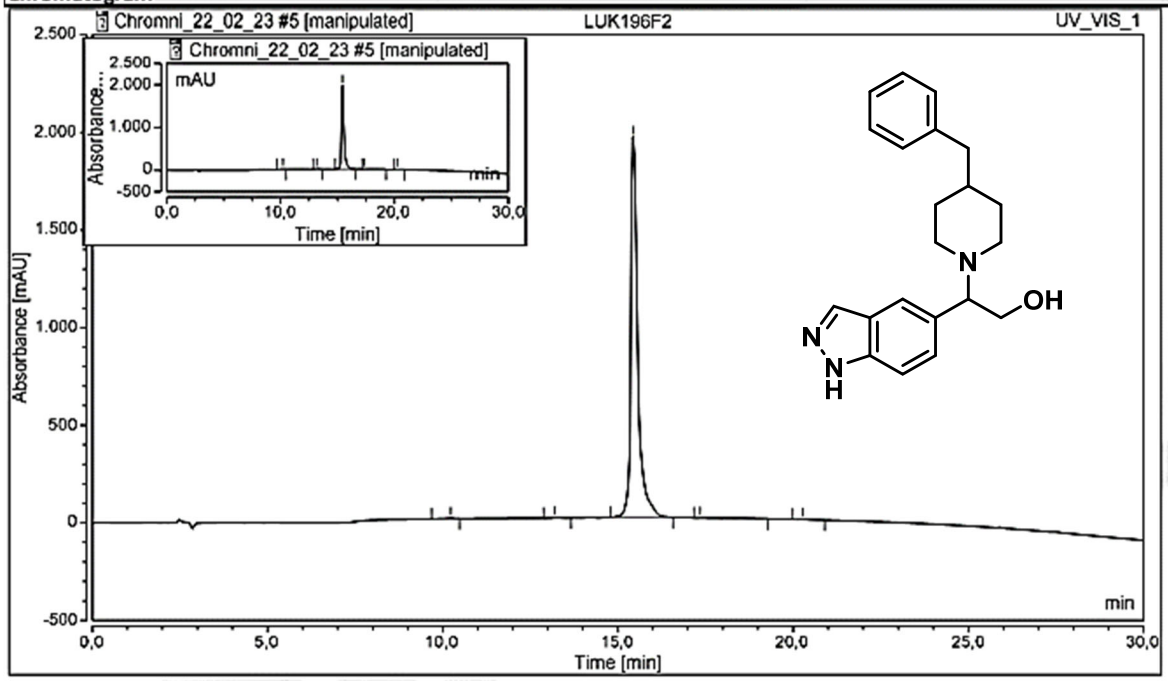
HPLC chromatogram of diamine *unlike*-**12** showing the purity of **12** (t_R = 12.38 min, HPLC method 1).

Chromatogram and Results

Injection Details

Injection Name:	**LUK196F2**	*Run Time (min):*	**30,00**
Vial Number:	**GA3**	*Injection Volume:*	**5,00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**210,0**
Instrument Method:	**Chromni**	*Bandwidth:*	**n.a.**
Processing Method:	**Chromni Processing Method**	*Dilution Factor:*	**1,0000**
Injection Date/Time:	**23.Feb.22 18:16**	*Sample Weight:*	**1,0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		10,227	1,201	4,659	0,26	0,24	n.a.
2		13,198	0,958	3,225	0,21	0,16	n.a.
3		15,433	457,699	1948,915	98,73	99,38	n.a.
4		17,352	2,609	1,365	0,56	0,07	n.a.
5		20,277	1,135	2,867	0,24	0,15	n.a.
Total:			**463,602**	**1961,030**	**100,00**	**100,00**	

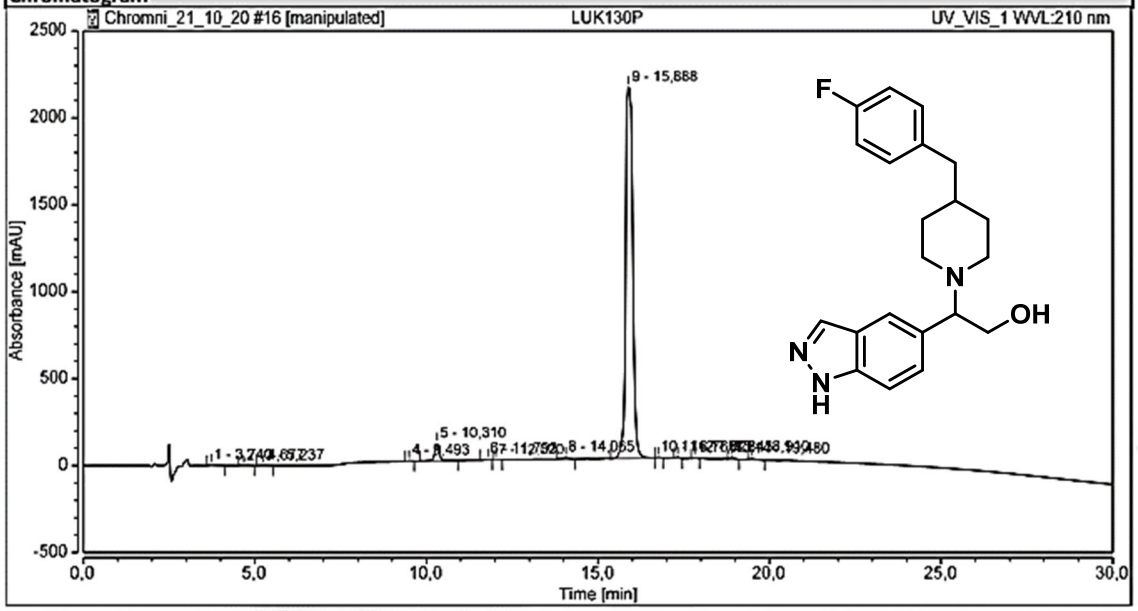
HPLC chromatogram of indazole **16a** showing the purity of **16a** (t_R = 15.4 min, HPLC method 1).

Chromatogram and Results

Injection Details

Injection Name:	**LUK130P**	*Run Time (min):*	**30,00**
Vial Number:	**GB5**	*Injection Volume:*	**5,00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**210,0**
Instrument Method:	**Chromni**	*Bandwidth:*	**n.a.**
Processing Method:	**Chromni Processing Method**	*Dilution Factor:*	**1,0000**
Injection Date/Time:	**21.Okt.21 00:09**	*Sample Weight:*	**1,0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		3,740	1,057	5,347	0,20	0,23	n.a.
2		4,677	0,326	1,725	0,06	0,08	n.a.
3		5,237	0,223	1,404	0,04	0,06	n.a.
4		9,493	0,209	1,581	0,04	0,07	n.a.
5		10,310	15,272	101,526	2,88	4,42	n.a.
6		11,793	0,460	3,264	0,09	0,14	n.a.
7		12,020	0,155	1,257	0,03	0,05	n.a.
8		14,065	1,697	10,658	0,32	0,46	n.a.
9		15,888	505,789	2136,003	95,50	92,99	n.a.
10		16,768	0,564	5,015	0,11	0,22	n.a.
11		17,322	0,933	8,185	0,18	0,36	n.a.
12		17,843	0,535	4,489	0,10	0,20	n.a.
13		18,910	1,540	11,047	0,29	0,48	n.a.
14		19,480	0,876	5,412	0,17	0,24	n.a.
Total:			**529,633**	**2296,913**	**100,00**	**100,00**	

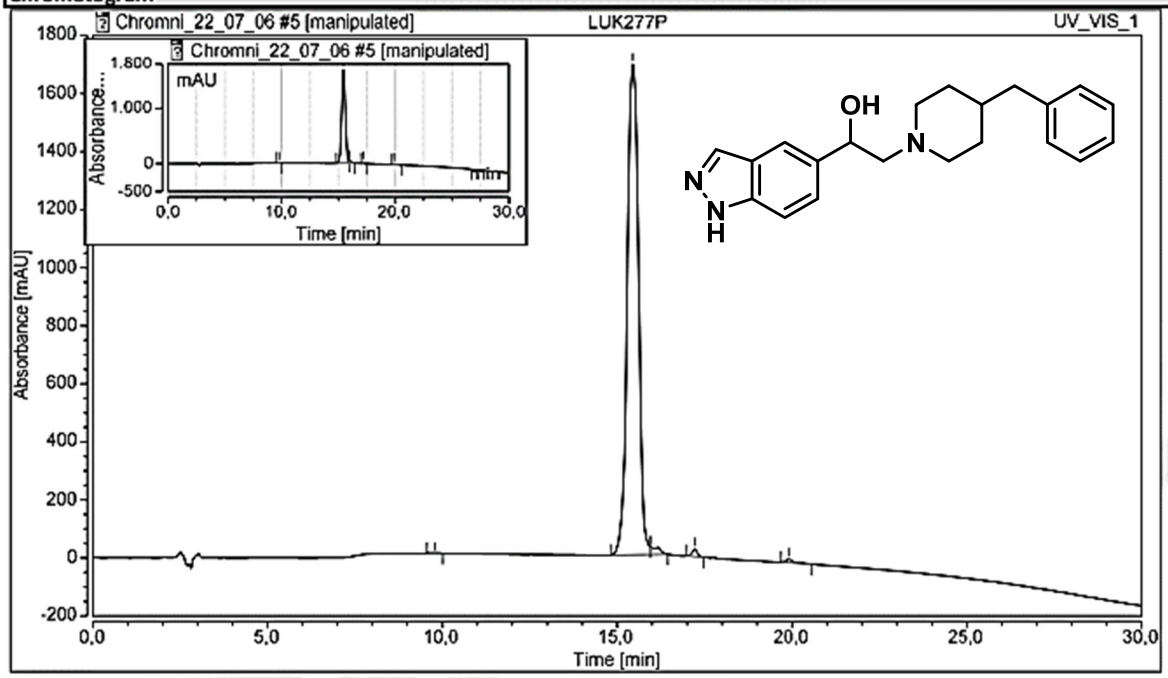
HPLC chromatogram of indazole **16b** showing the purity of **16b** (t_R = 15.9 min, HPLC method 1).

Chromatogram and Results

Injection Details

Injection Name:	**LUK277P**	*Run Time (min):*	**30,00**
Vial Number:	**GA2**	*Injection Volume:*	**5,00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**210,0**
Instrument Method:	**Chromni**	*Bandwidth:*	**n.a.**
Processing Method:	**Chromni Processing Method**	*Dilution Factor:*	**1,0000**
Injection Date/Time:	**06.Jul.22 18:16**	*Sample Weight:*	**1,0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		9,790	0,310	1,147	0,05	0,07	n.a.
2		15,447	632,359	1692,413	97,73	96,23	n.a.
3		15,952	7,130	26,301	1,10	1,50	n.a.
4		17,223	4,210	26,350	0,65	1,50	n.a.
5		19,910	3,046	12,426	0,47	0,71	n.a.
Total:			**647,055**	**1758,637**	**100,00**	**100,00**	

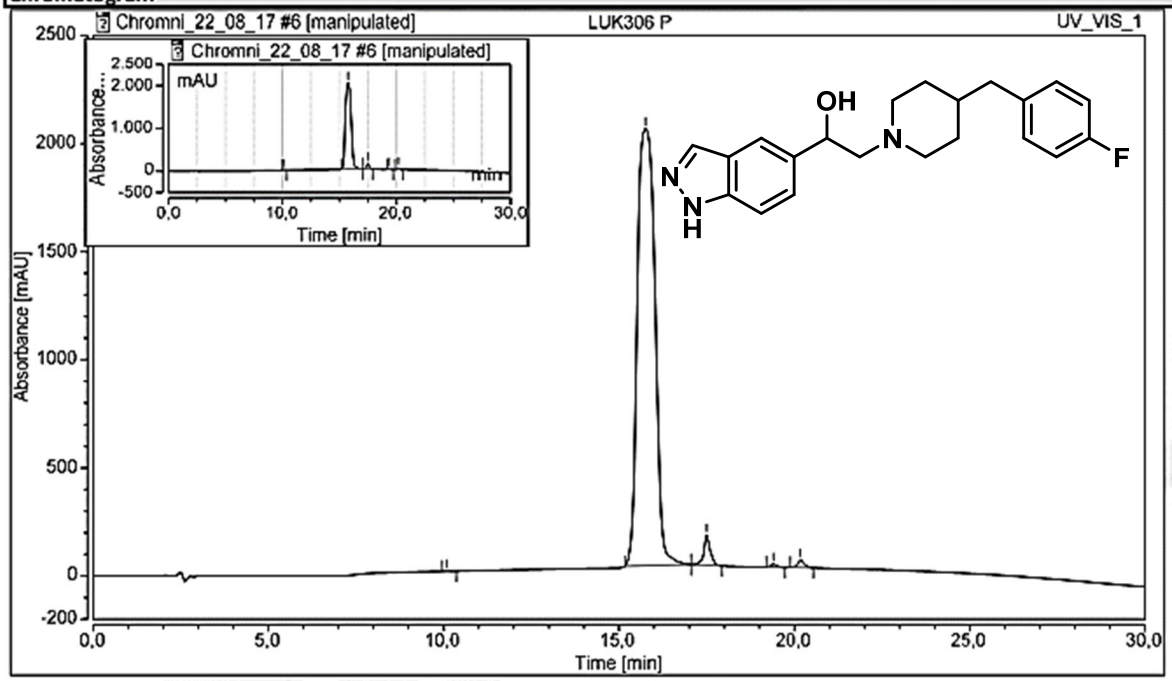
HPLC chromatogram of indazole **18a** showing the purity of **18a** (t_R = 15.4 min, HPLC method 1).

Chromatogram and Results

Injection Details

Injection Name:	**LUK306 P**	*Run Time (min):*	**30,00**
Vial Number:	**GA4**	*Injection Volume:*	**5,00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**210,0**
Instrument Method:	**Chromni**	*Bandwidth:*	**n.a.**
Processing Method:	**Chromni Processing Method**	*Dilution Factor:*	**1,0000**
Injection Date/Time:	**17.Aug.22 19:11**	*Sample Weight:*	**1,0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		10,093	0,215	1,357	0,02	0,06	n.a.
2		15,755	1177,358	2024,399	96,79	91,25	n.a.
3		17,498	29,172	140,691	2,40	6,34	n.a.
4		19,403	2,588	15,552	0,21	0,70	n.a.
5		20,173	7,068	36,458	0,58	1,64	n.a.
Total:			**1216,400**	**2218,458**	**100,00**	**100,00**	

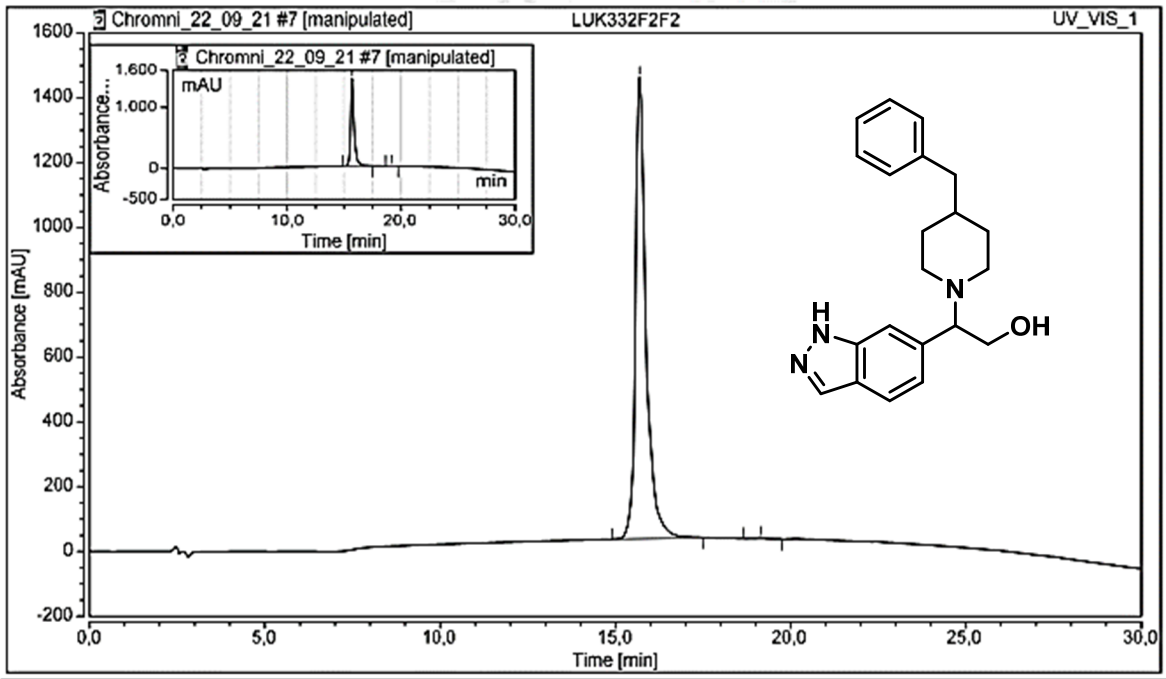
HPLC chromatogram of indazole **18b** showing the purity of **18b** (t_R = 15.8 min, HPLC method 1).

Chromatogram and Results

Injection Details

Injection Name:	**LUK332F2F2**	*Run Time (min):*	**30,00**
Vial Number:	**GA3**	*Injection Volume:*	**5,00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**210,0**
Instrument Method:	**Chromni**	*Bandwidth:*	**n.a.**
Processing Method:	**Chromni Processing Method**	*Dilution Factor:*	**1,0000**
Injection Date/Time:	**21.Sep.22 19:42**	*Sample Weight:*	**1,0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		15,690	500,209	1423,938	99,72	99,66	n.a.
2		19,152	1,425	4,899	0,28	0,34	n.a.
Total:			**501,635**	**1428,838**	**100,00**	**100,00**	

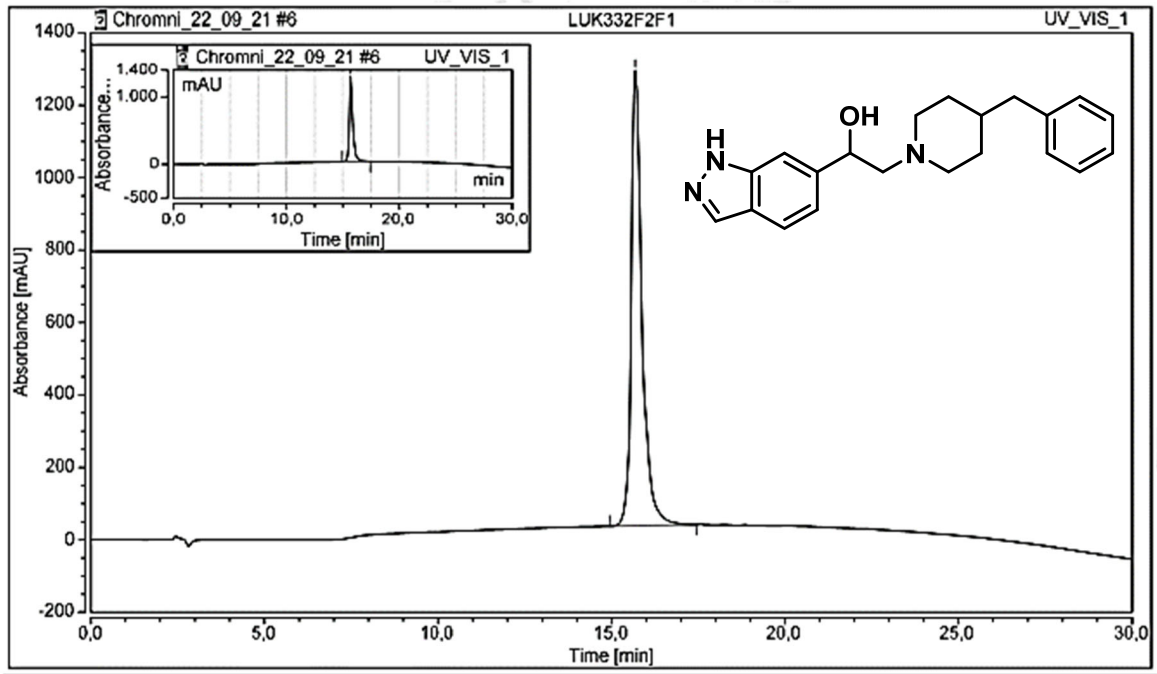
HPLC chromatogram of indazole **22a** showing the purity of **22a** (t_R = 15.7 min, HPLC method 1).

Chromatogram and Results

Injection Details

Injection Name:	**LUK332F2F1**	*Run Time (min):*	**30,00**
Vial Number:	**GA2**	*Injection Volume:*	**5,00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**210,0**
Instrument Method:	**Chromni**	*Bandwidth:*	**n.a.**
Processing Method:	**Chromni Processing Method**	*Dilution Factor:*	**1,0000**
Injection Date/Time:	**21.Sep.22 19:02**	*Sample Weight:*	**1,0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		15,688	455,970	1257,998	100,00	100,00	n.a.
Total:			**455,970**	**1257,998**	**100,00**	**100,00**	

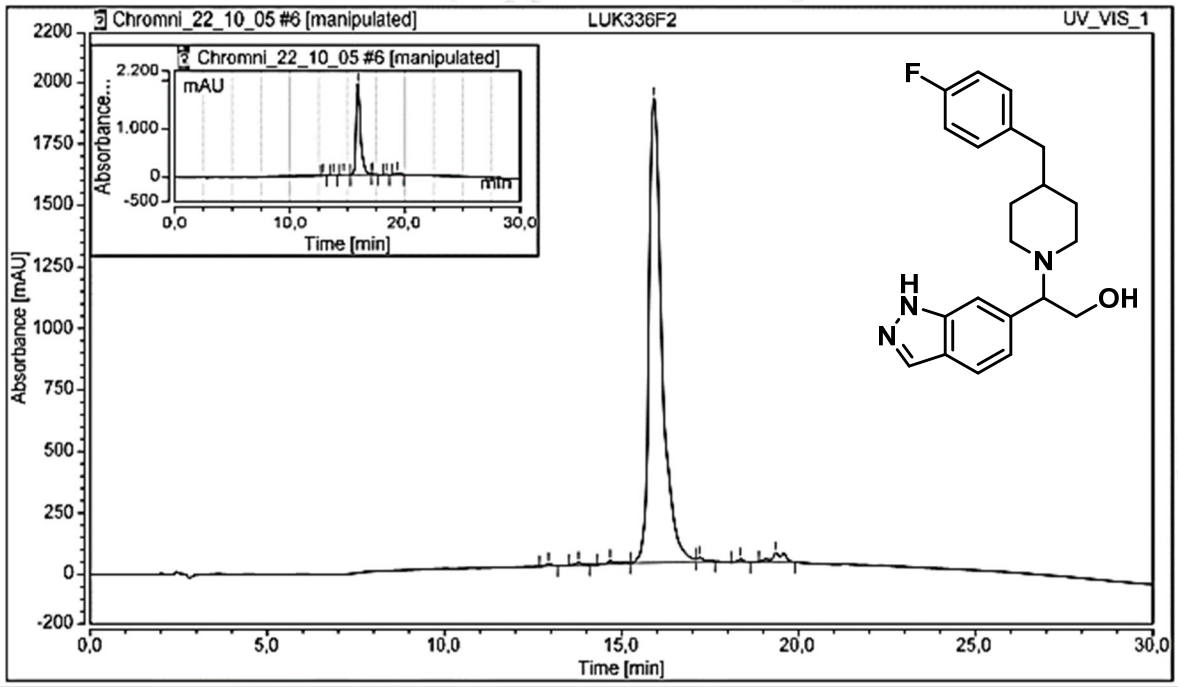
HPLC chromatogram of indazole **23a** showing the purity of **23a** (t_R = 15.7 min, HPLC method 1).

Chromatogram and Results

Injection Details

Injection Name:	**LUK336F2**	*Run Time (min):*	**30,00**
Vial Number:	**GA4**	*Injection Volume:*	**5,00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**210,0**
Instrument Method:	**Chromni**	*Bandwidth:*	**n.a.**
Processing Method:	**Chromni Processing Method**	*Dilution Factor:*	**1,0000**
Injection Date/Time:	**05.Okt.22 18:01**	*Sample Weight:*	**1,0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		12,940	1,632	9,429	0,19	0,47	n.a.
2		13,787	2,188	10,419	0,26	0,52	n.a.
3		14,680	3,731	13,911	0,44	0,70	n.a.
4		15,917	813,000	1887,516	96,43	94,78	n.a.
5		17,202	4,426	18,150	0,53	0,91	n.a.
6		18,363	2,652	14,028	0,31	0,70	n.a.
7		19,360	15,442	38,070	1,83	1,91	n.a.
Total:			**843,071**	**1991,522**	**100,00**	**100,00**	

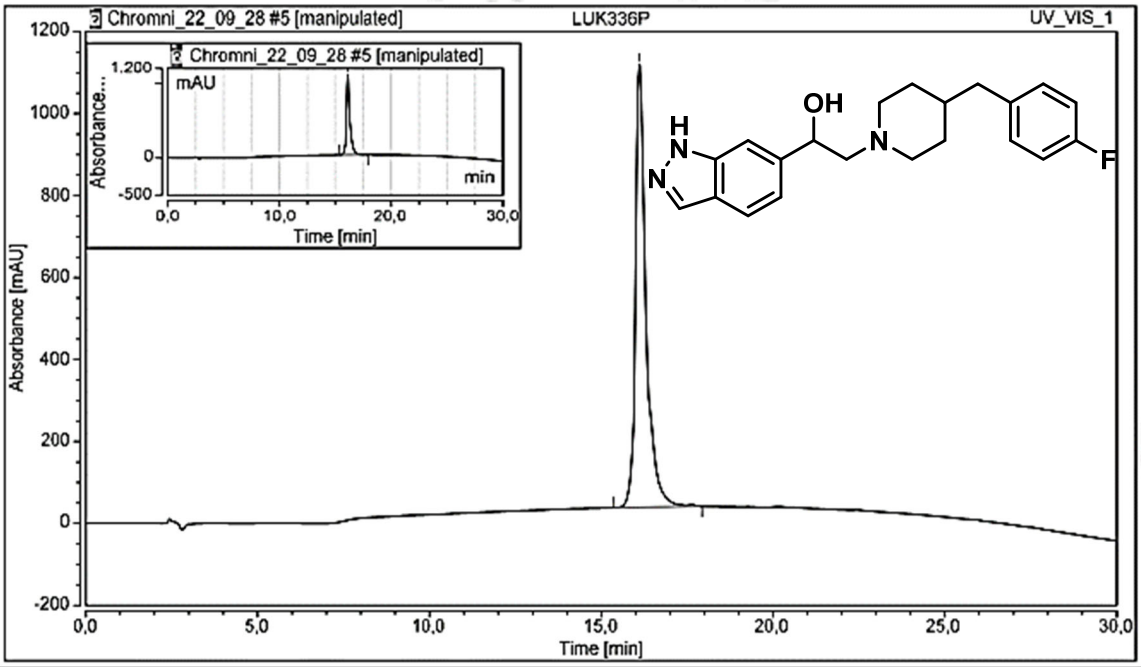
HPLC chromatogram of indazole **22b** showing the purity of **22b** (t_R = 15.9 min, HPLC method 1).

Chromatogram and Results

Injection Details

Injection Name:	**LUK336P**	*Run Time (min):*	**30,00**
Vial Number:	**GA2**	*Injection Volume:*	**5,00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**210,0**
Instrument Method:	**Chromni**	*Bandwidth:*	**n.a.**
Processing Method:	**Chromni Processing Method**	*Dilution Factor:*	**1,0000**
Injection Date/Time:	**28.Sep.22 17:24**	*Sample Weight:*	**1,0000**

Chromatogram

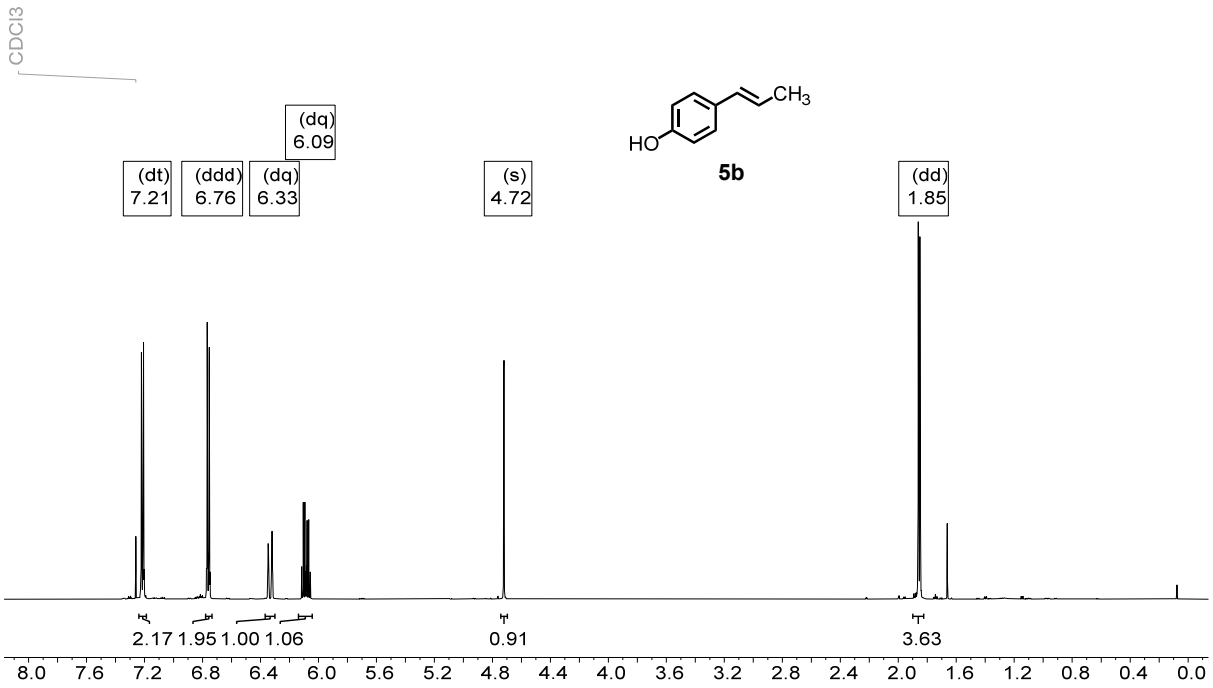


Integration Results

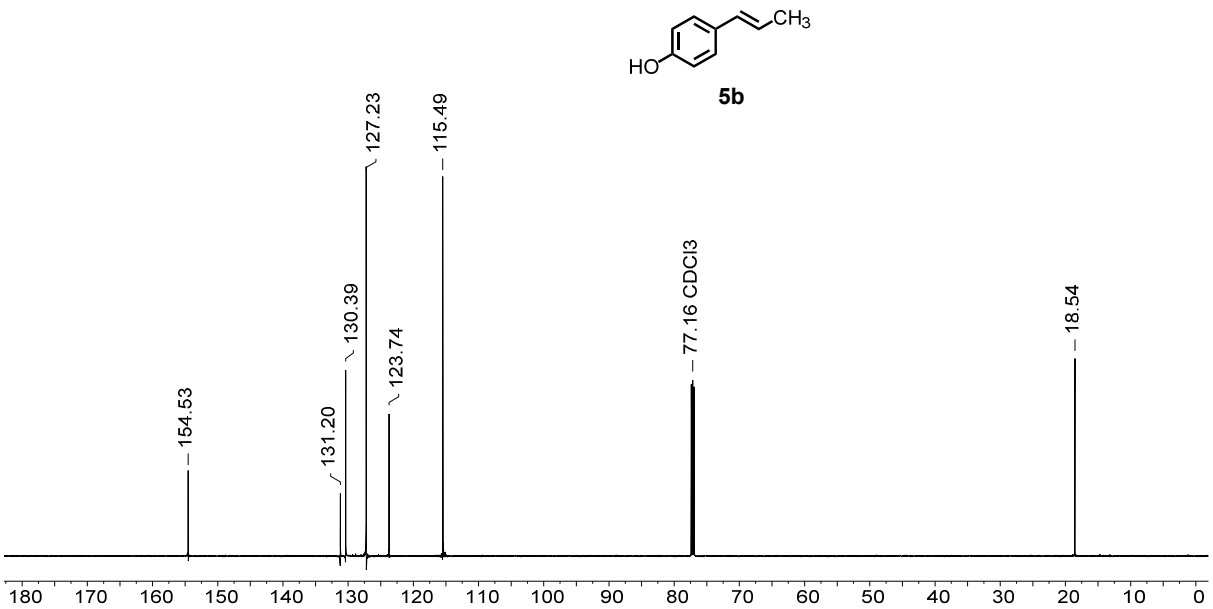
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		16,110	385,836	1082,208	100,00	100,00	n.a.
Total:			**385,836**	**1082,208**	**100,00**	**100,00**	

HPLC chromatogram of indazole **23b** showing the purity of **23b** (t_R = 16.1 min, HPLC method 1).

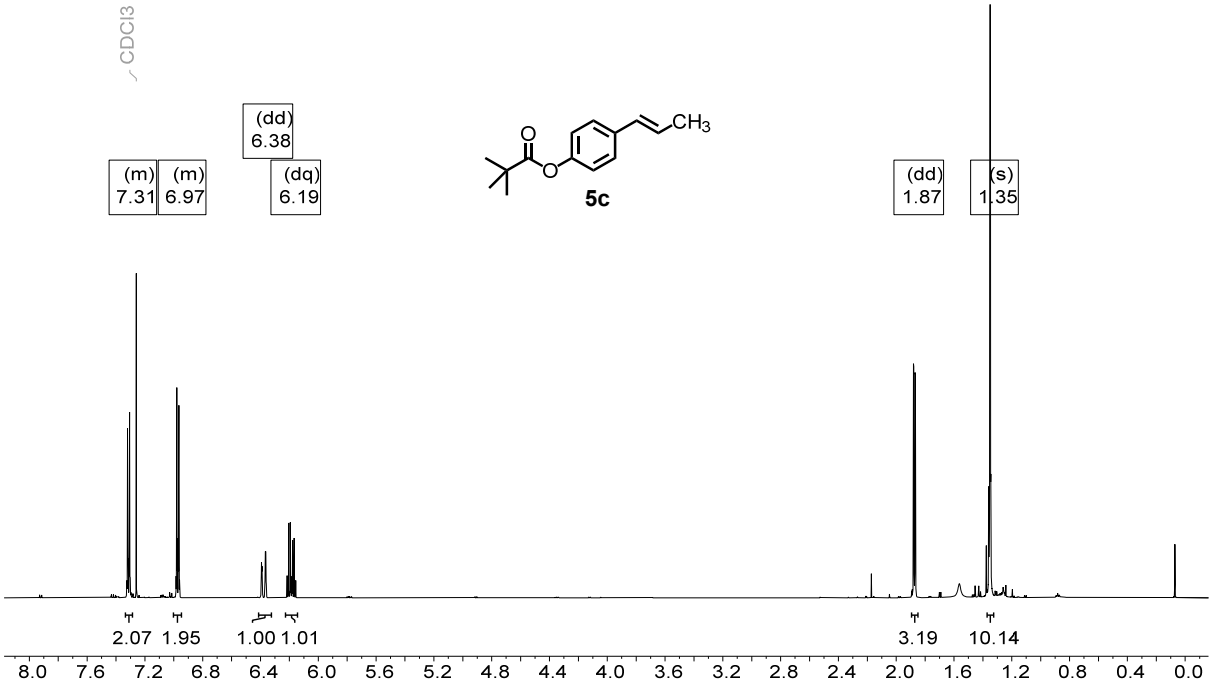
7. ^{1}H and ^{13}C NMR spectra



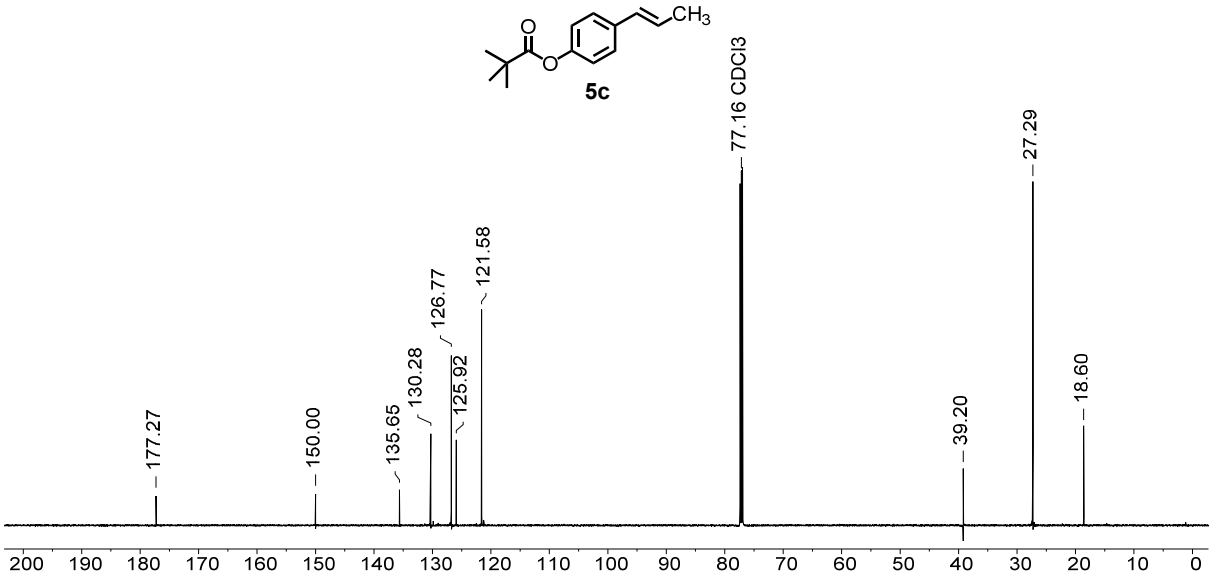
^{1}H NMR spectrum of **5b** in $CDCl_3$.



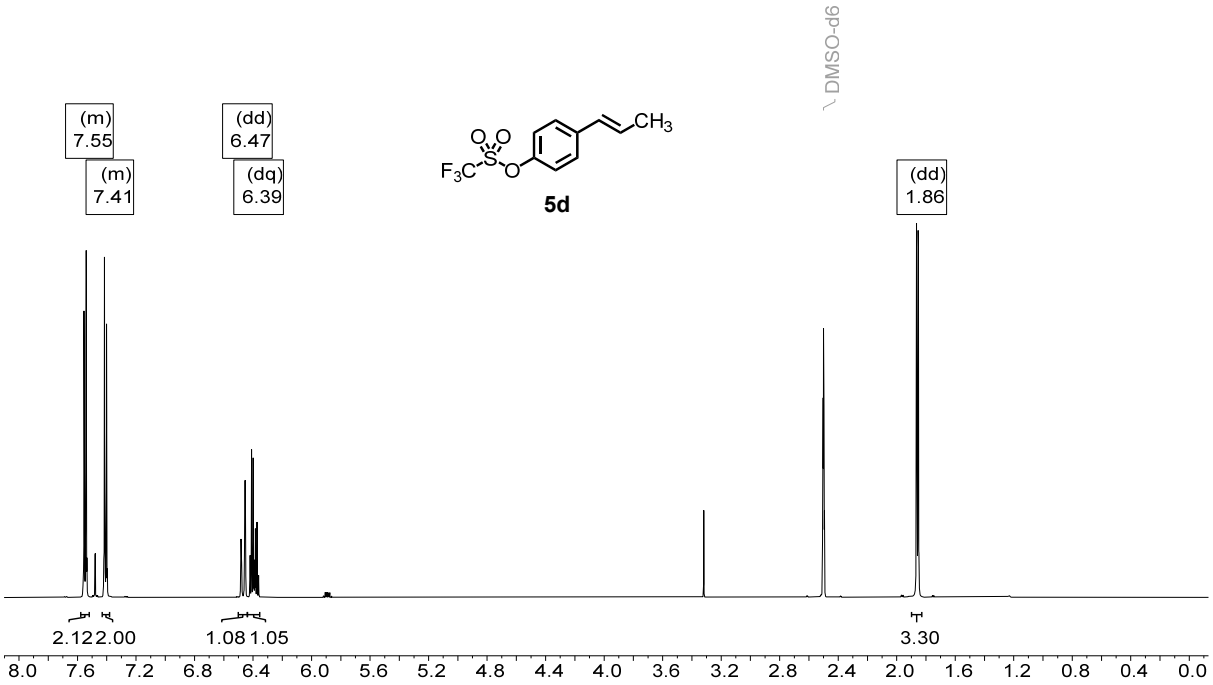
^{13}C NMR spectrum of **5b** in $CDCl_3$.



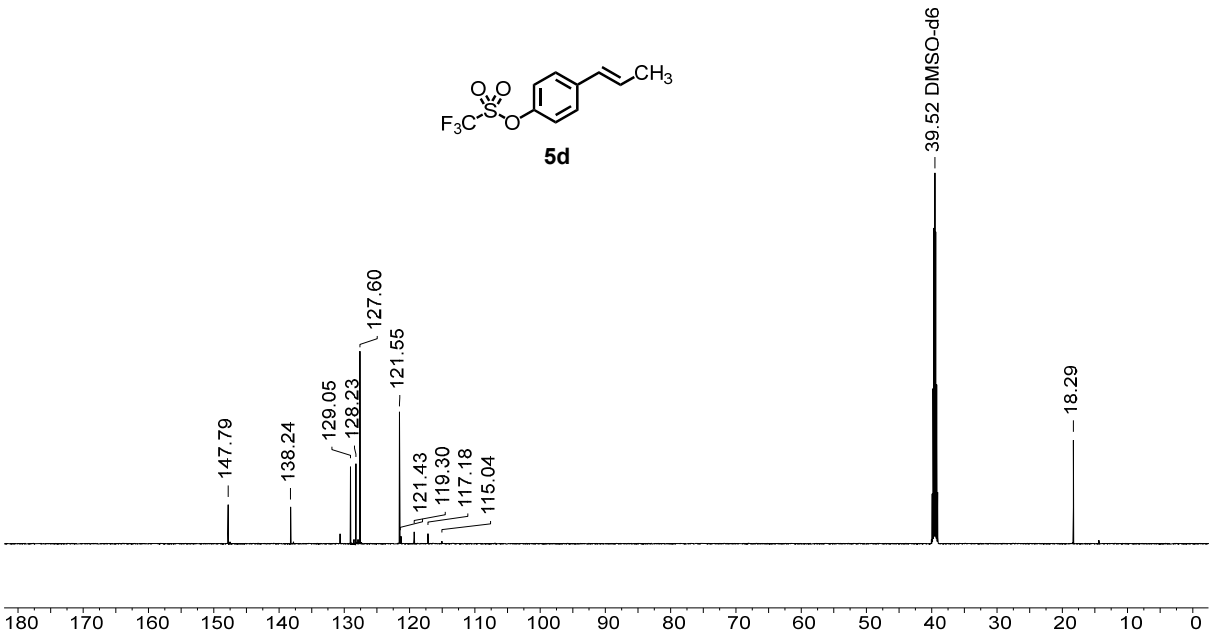
^{1}H NMR spectrum of **5c** in $CDCl_3$.



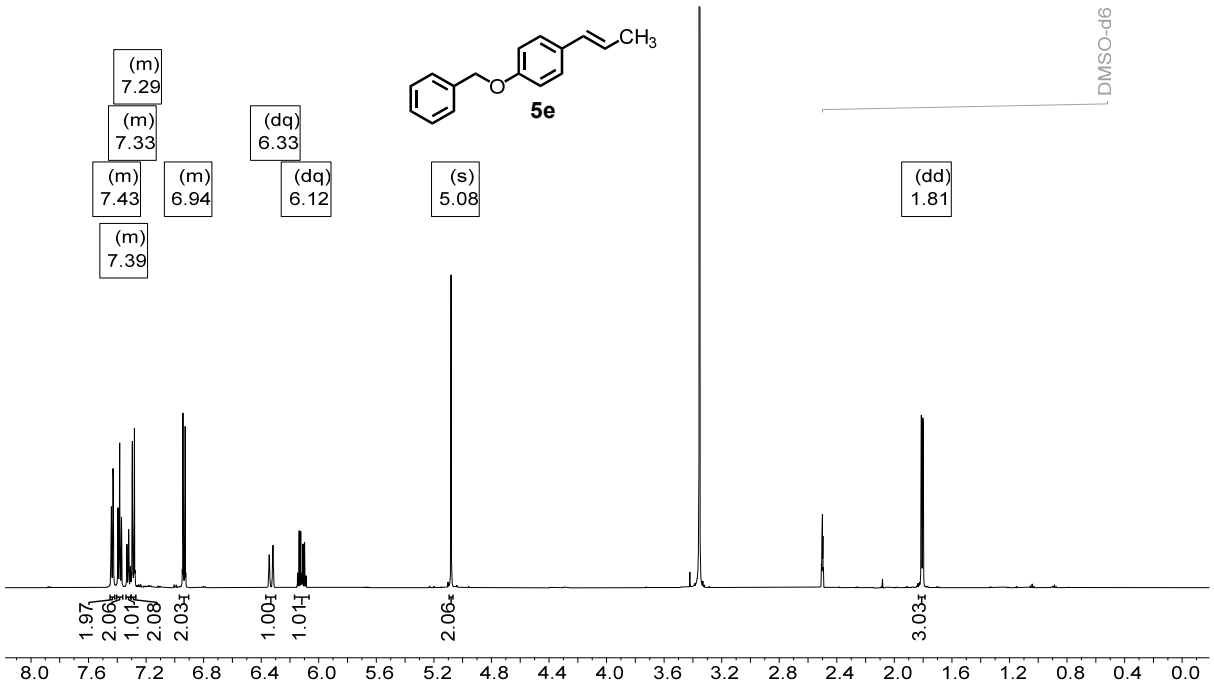
^{13}C NMR spectrum of **5b** in $CDCl_3$.



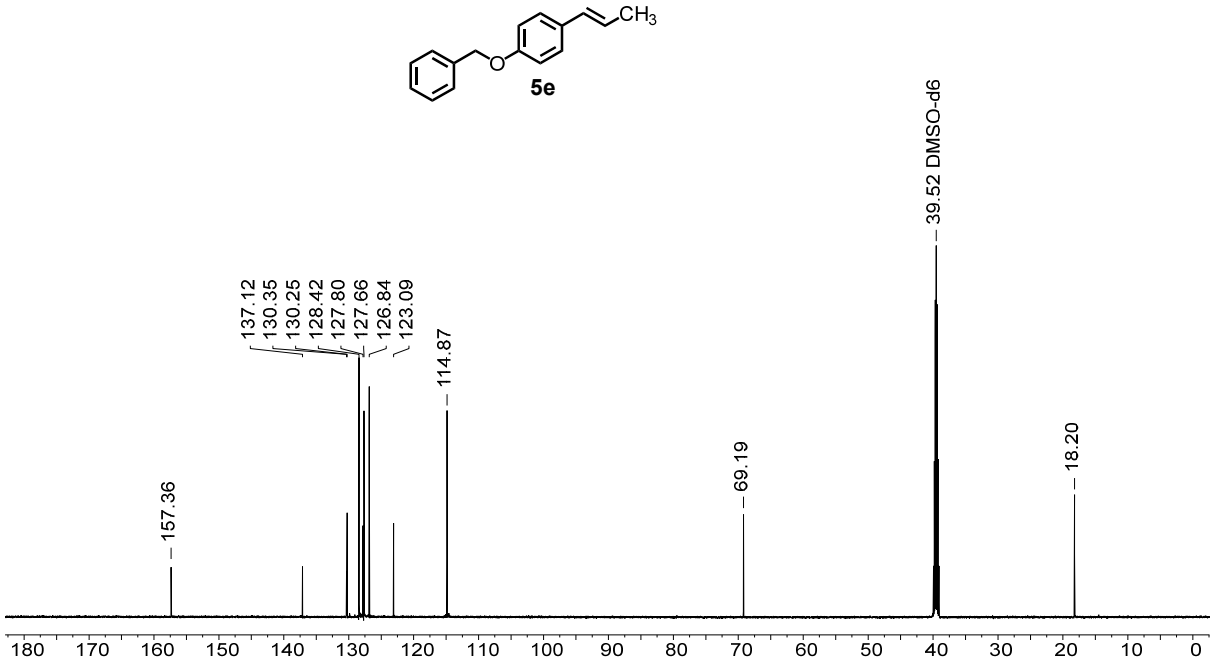
^{1}H NMR spectrum of **5d** in DMSO-d$_6$.



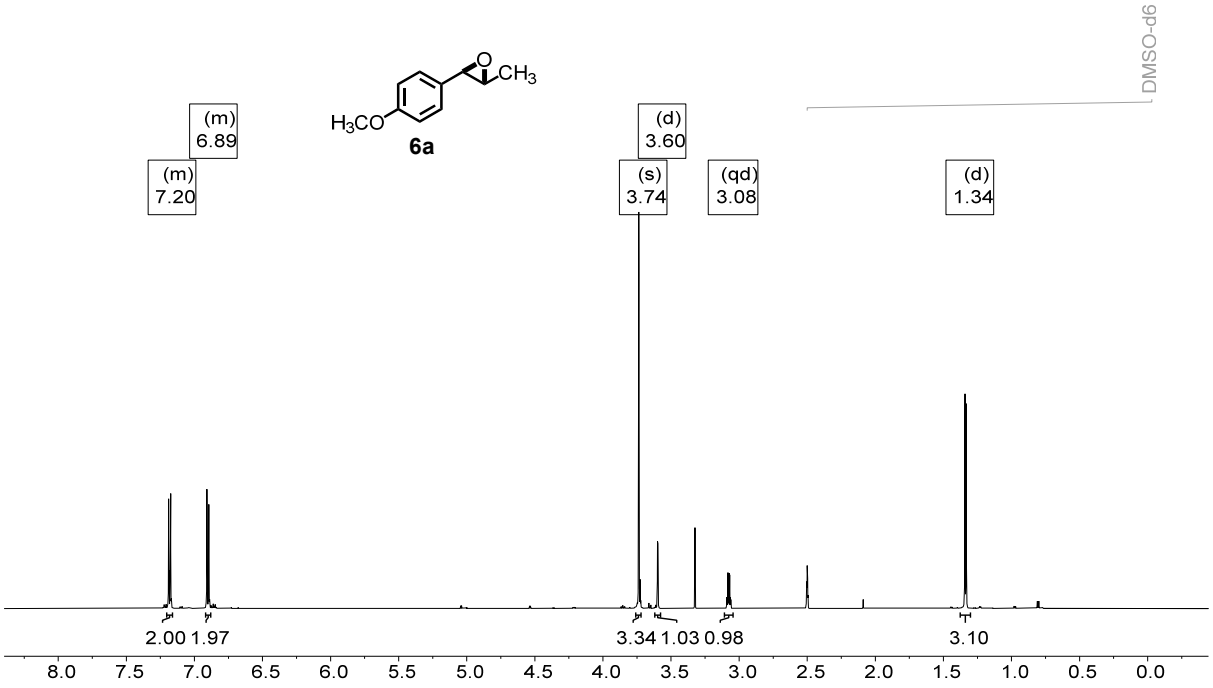
^{13}C NMR spectrum of **5d** in DMSO-d$_6$.



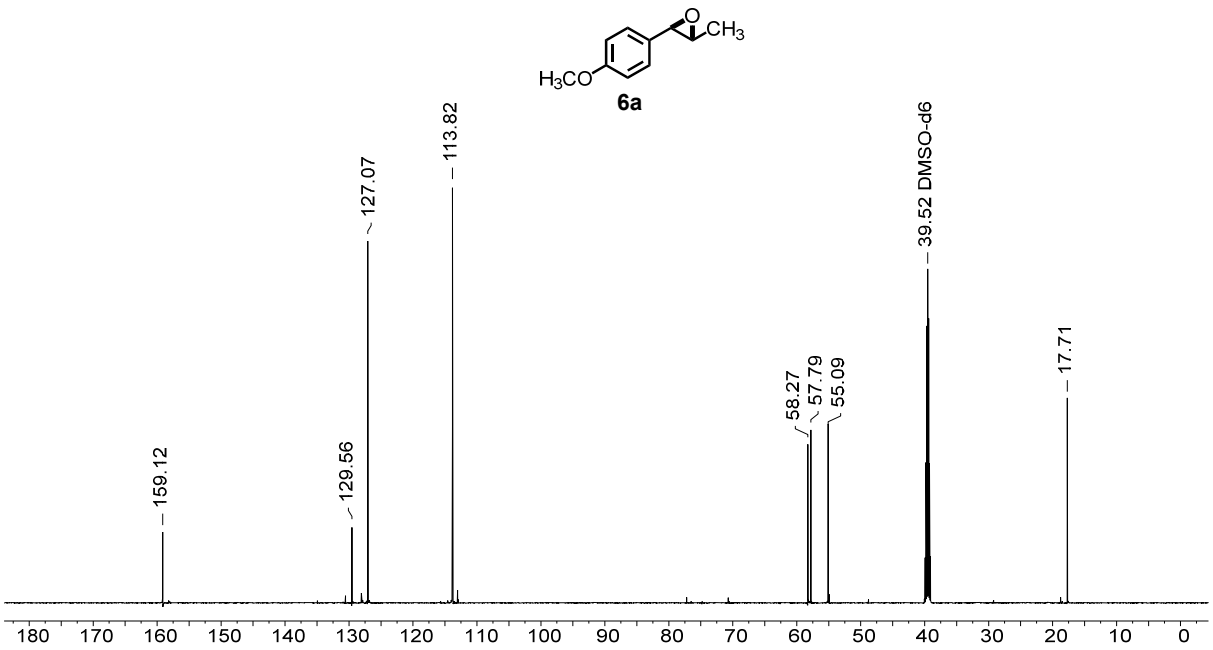
^{1}H NMR spectrum of **5e** in DMSO-d_6.



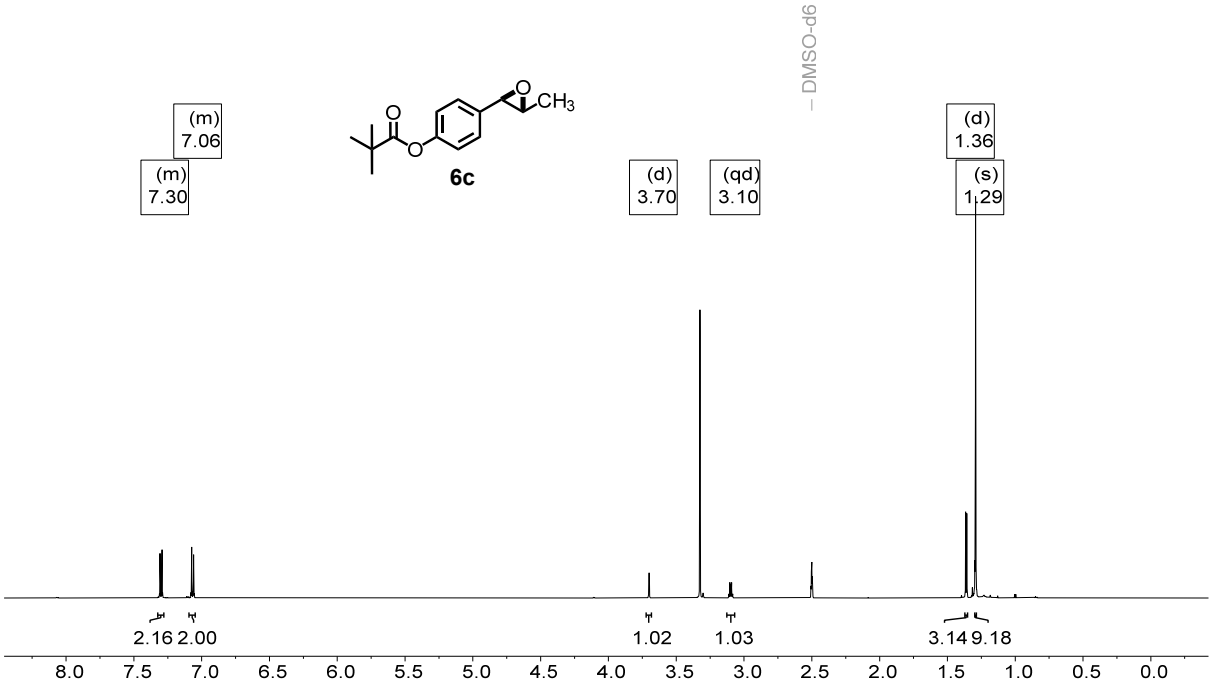
^{13}C NMR spectrum of **5e** in DMSO-d_6.



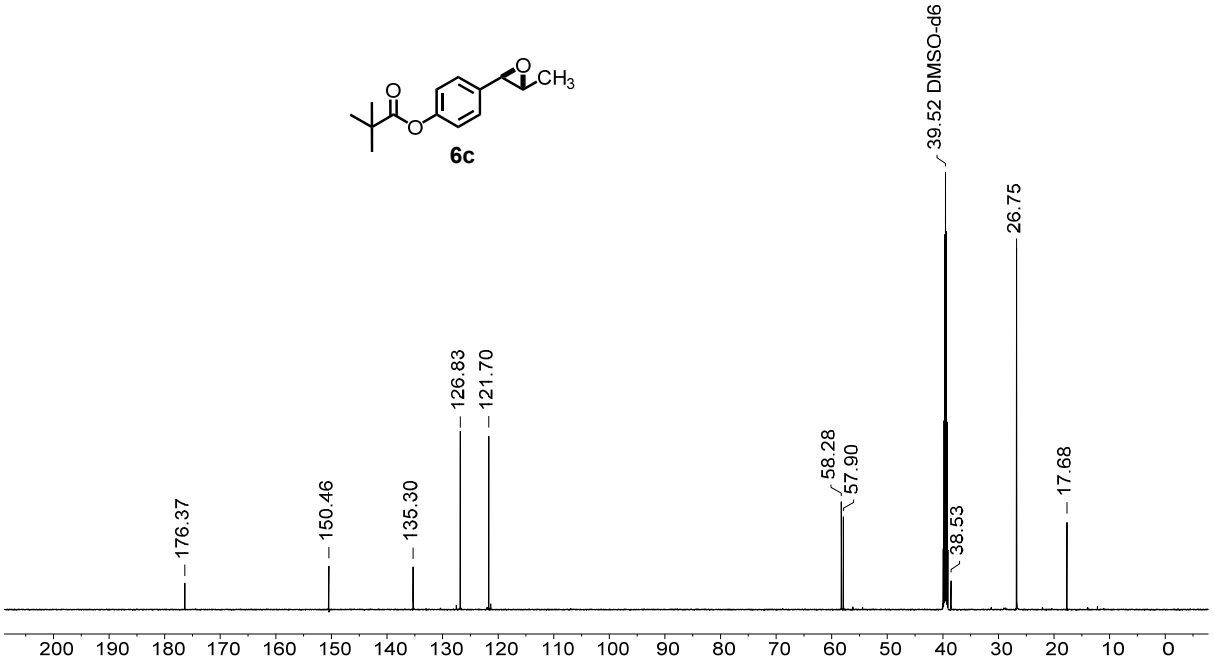
^{1}H NMR spectrum of **6a** in DMSO-d$_6$.



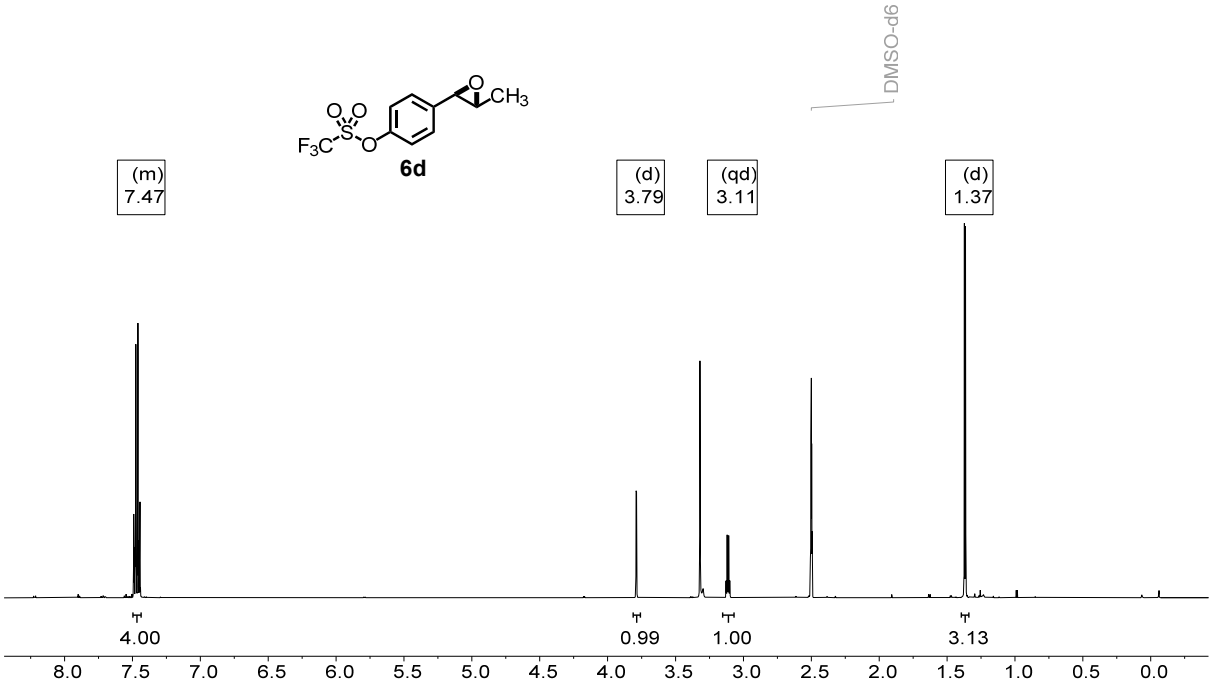
^{13}C NMR spectrum of **6a** in DMSO-d$_6$.



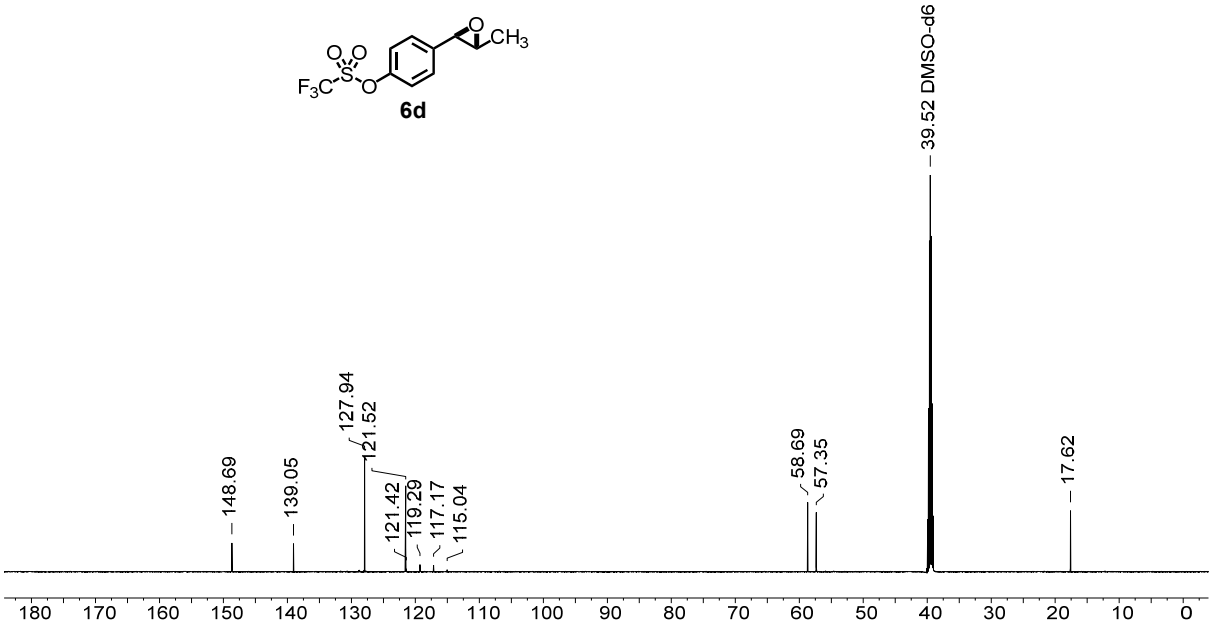
^{1}H NMR spectrum of **6c** in DMSO-d$_6$.



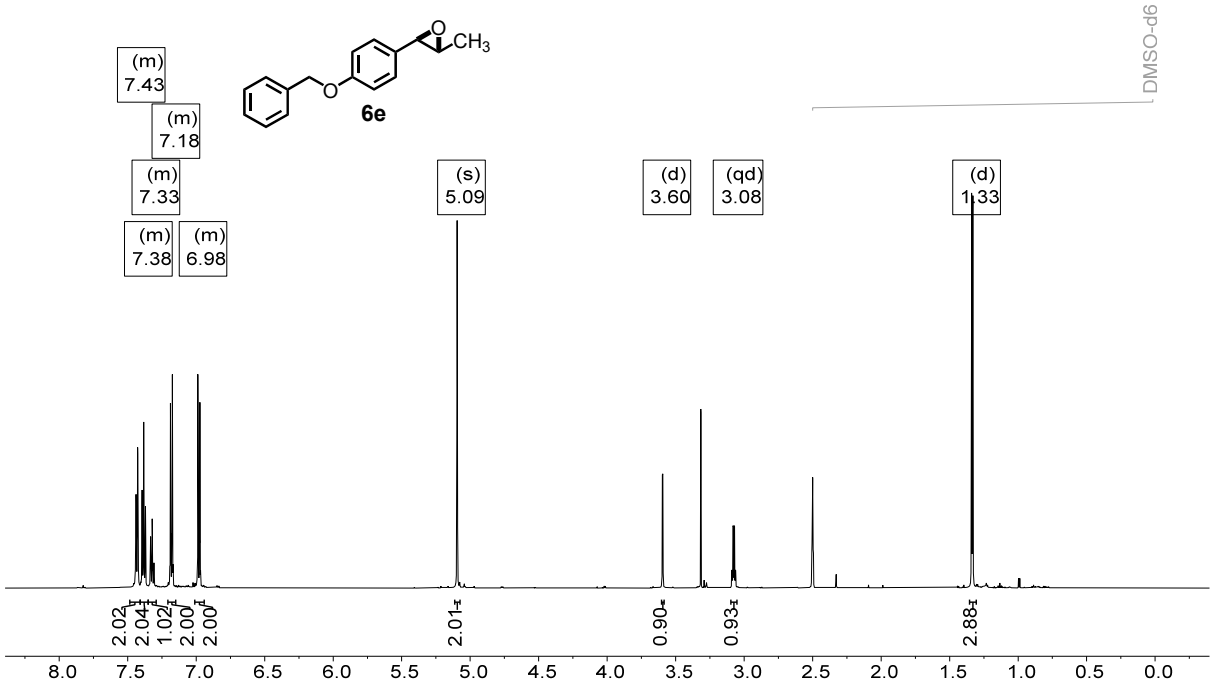
^{13}C NMR spectrum of **6c** in DMSO-d$_6$.



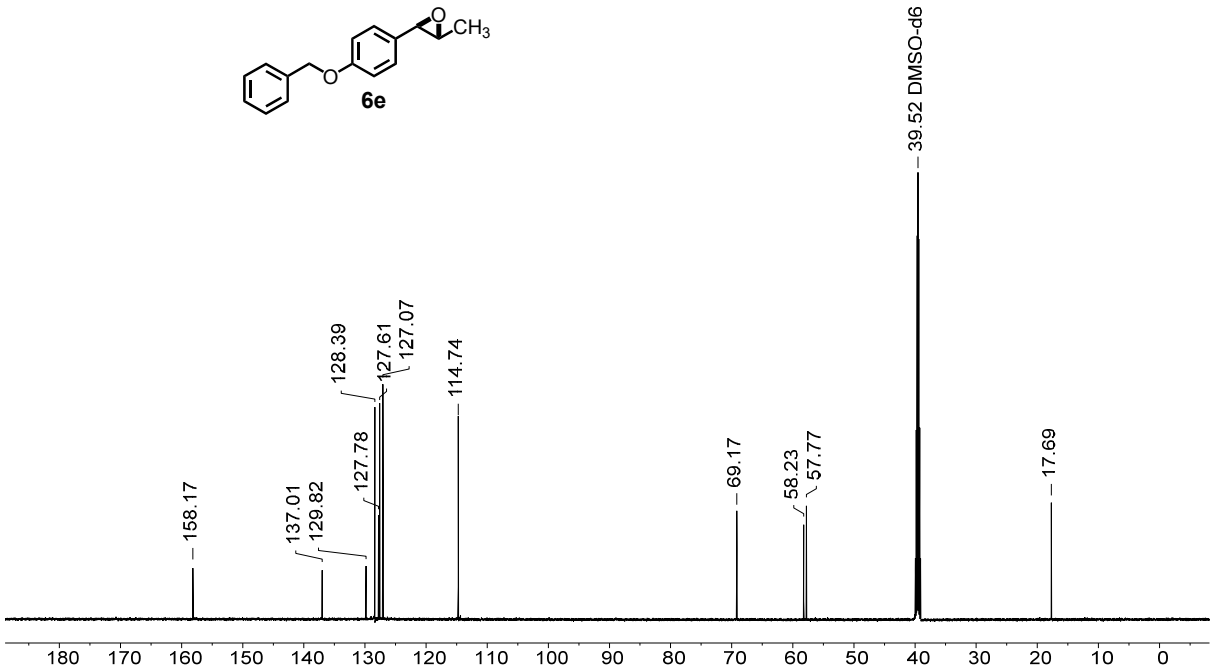
^{1}H NMR spectrum of **6d** in DMSO-d$_6$.



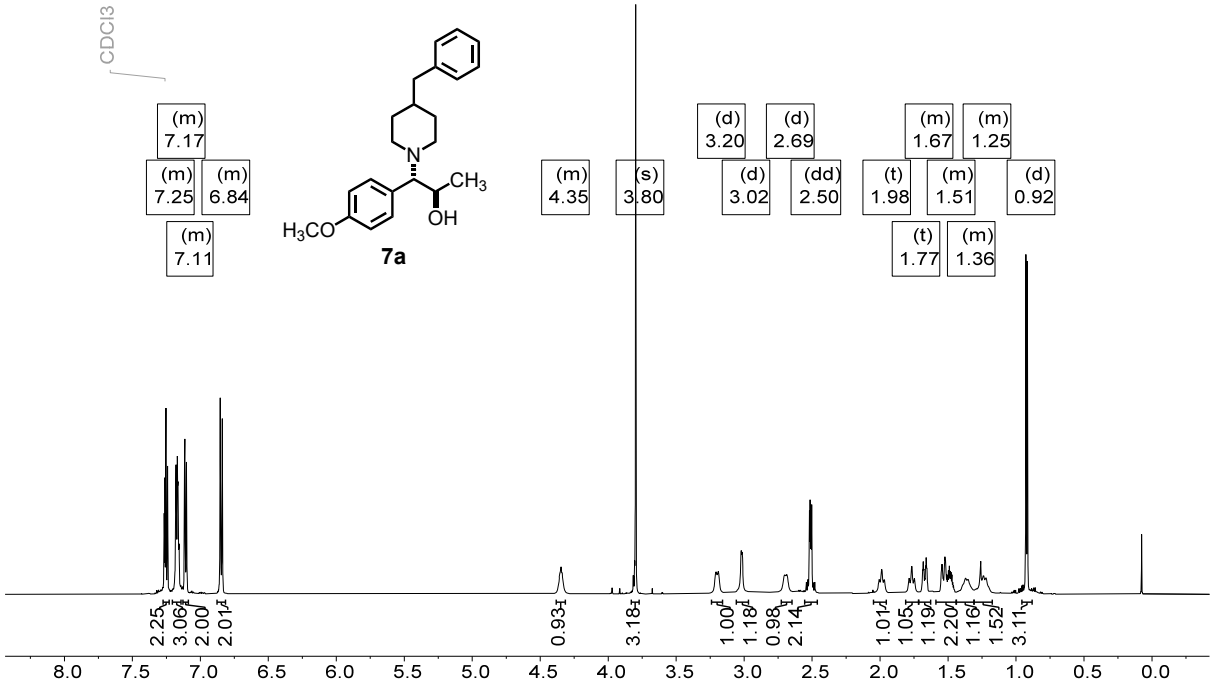
^{13}C NMR spectrum of **6d** in DMSO-d$_6$.



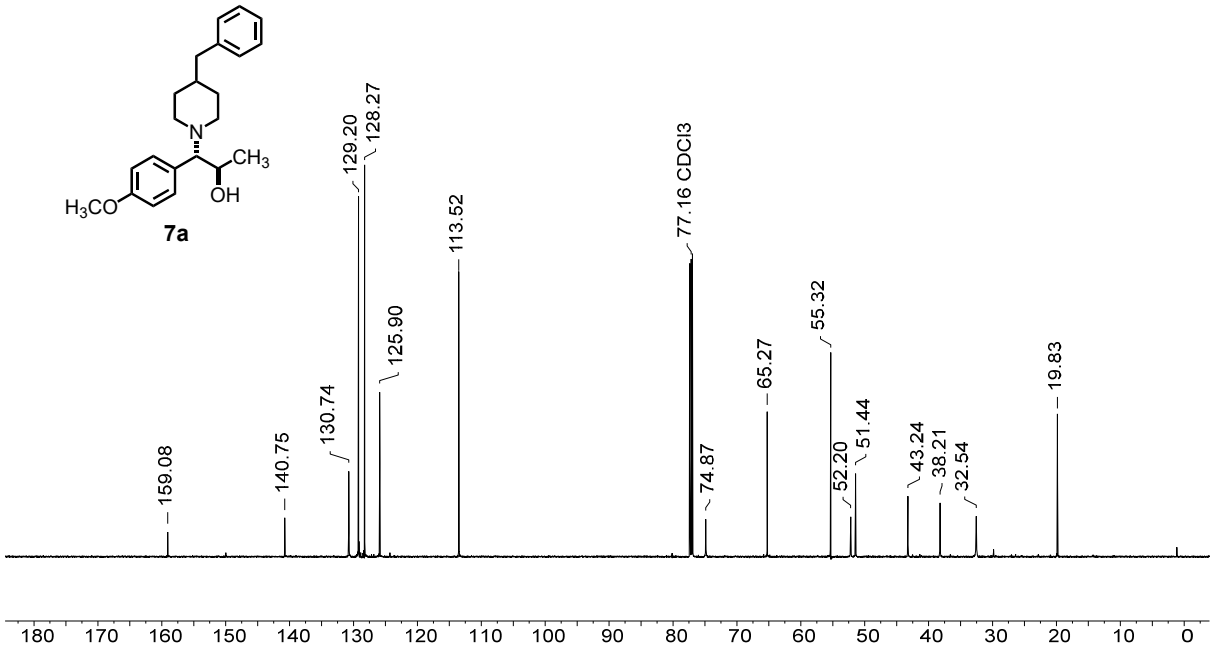
^{1}H NMR spectrum of **6e** in DMSO-d$_6$.



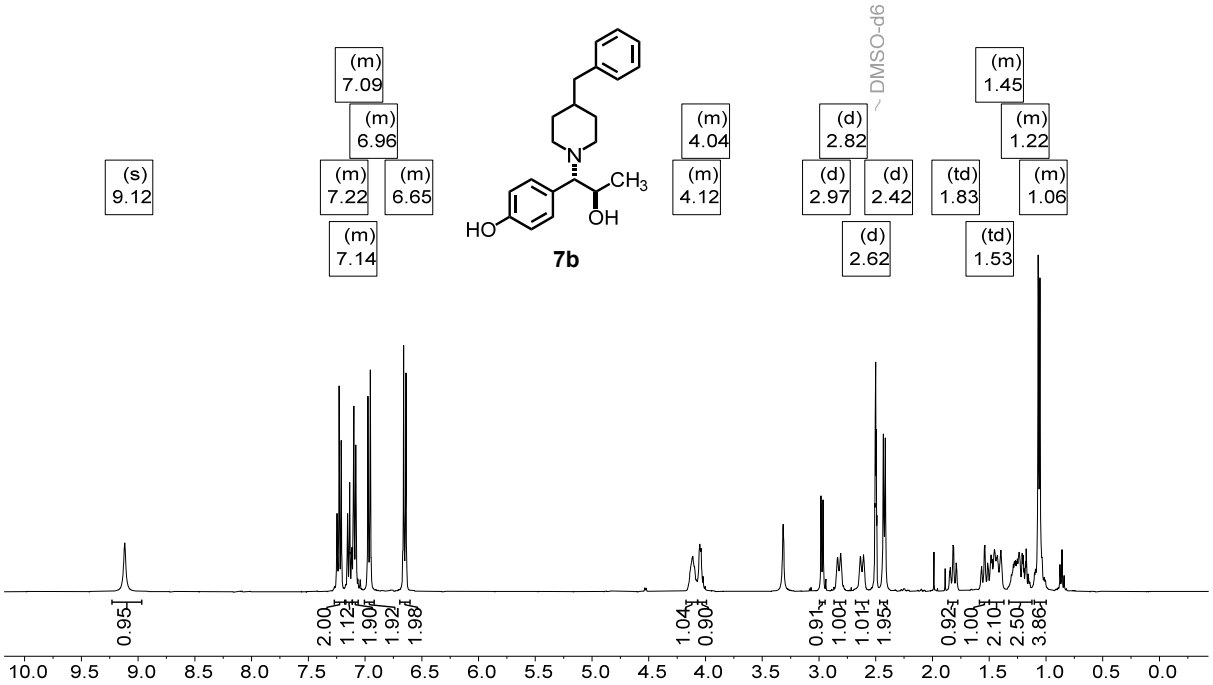
^{13}C NMR spectrum of **6e** in DMSO-d$_6$.



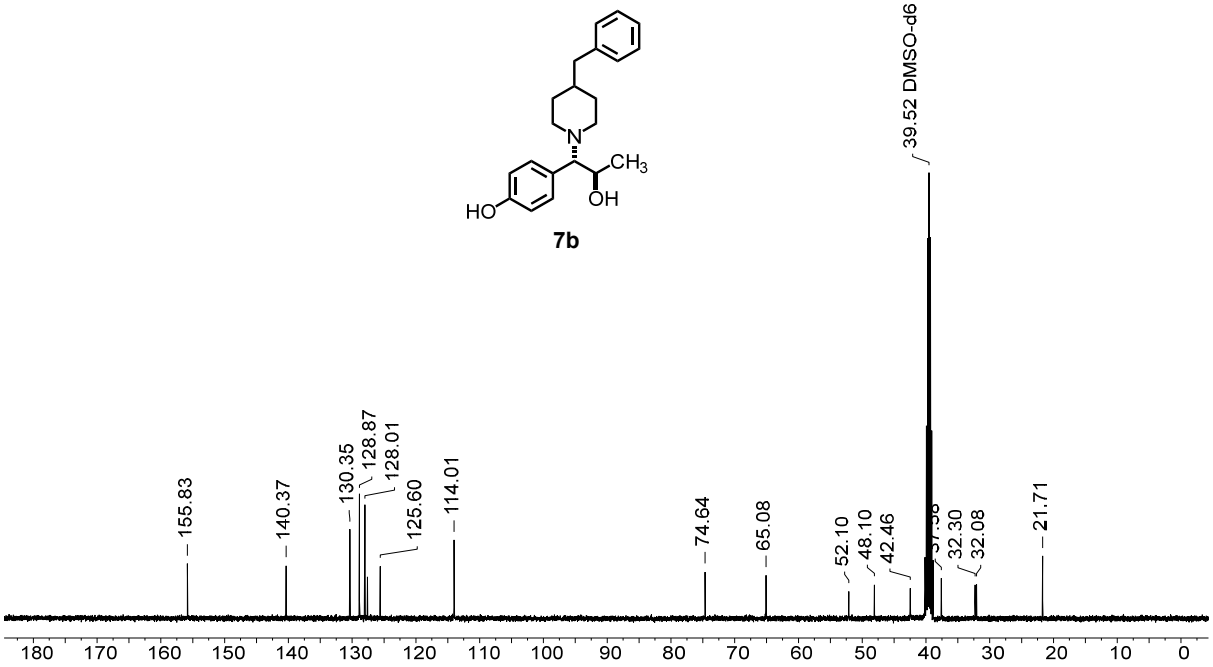
^{1}H NMR spectrum of **7a** in $CDCl_3$.



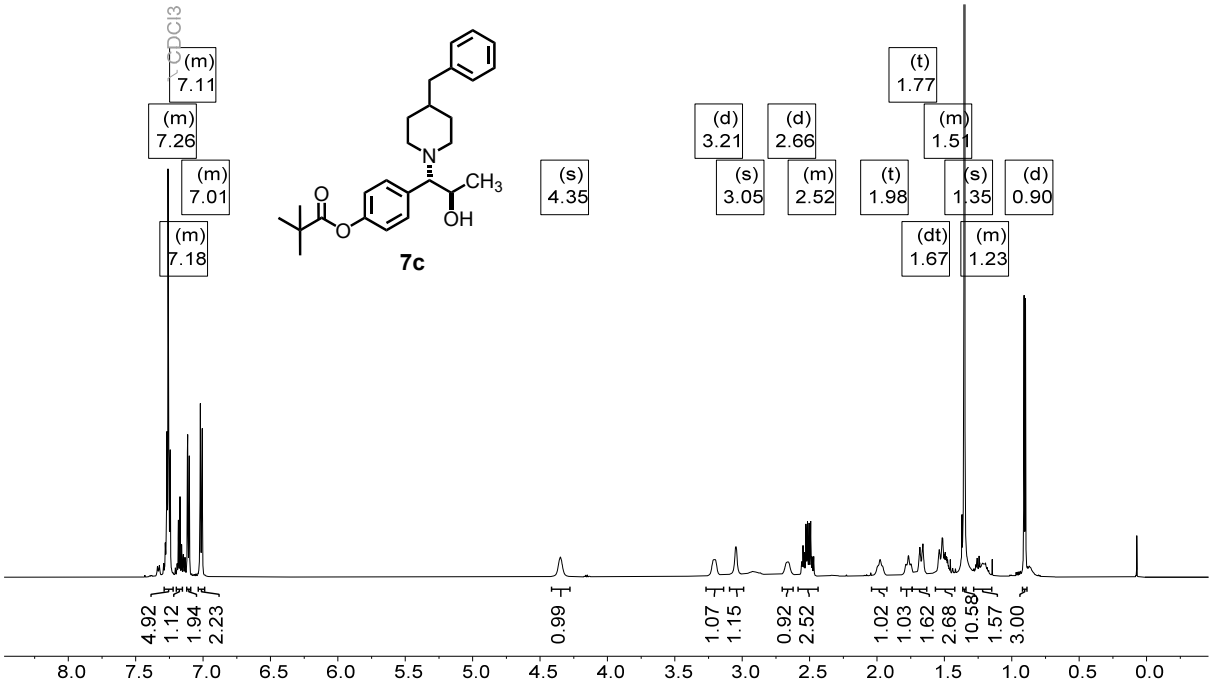
^{13}C NMR spectrum of **7a** in $CDCl_3$.



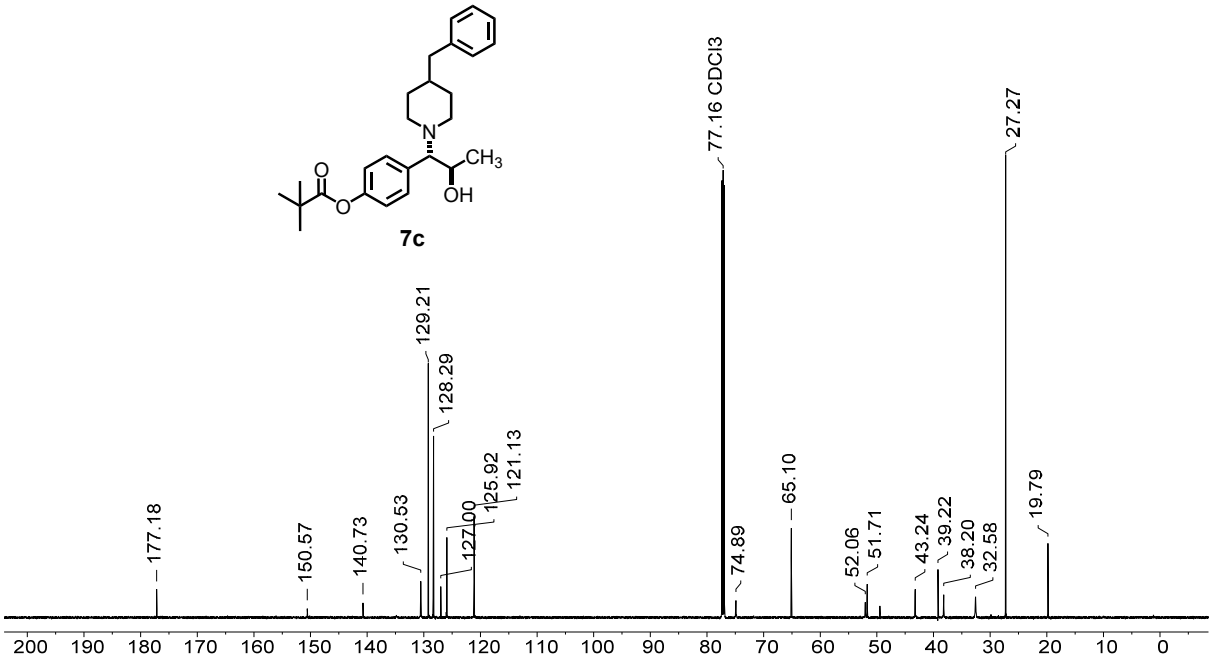
^{1}H NMR spectrum of **7b** in DMSO-d$_6$.



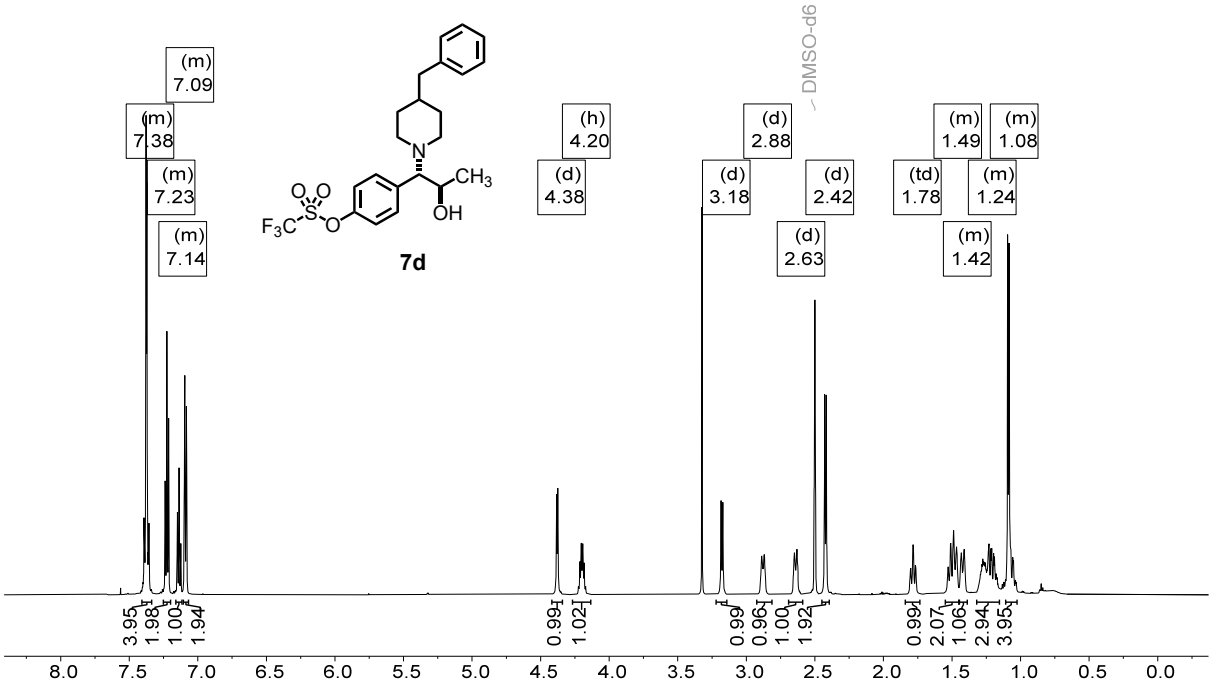
^{13}C NMR spectrum of **7b** in DMSO-d$_6$.



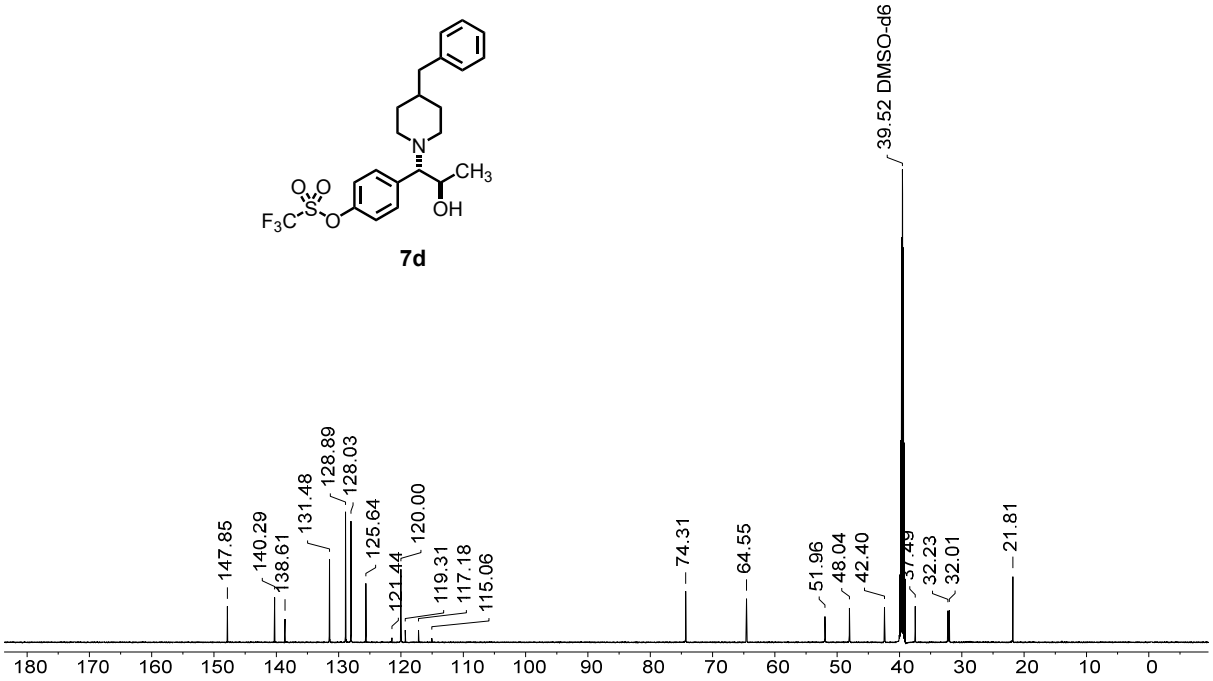
^{1}H NMR spectrum of **7c** in $CDCl_3$.



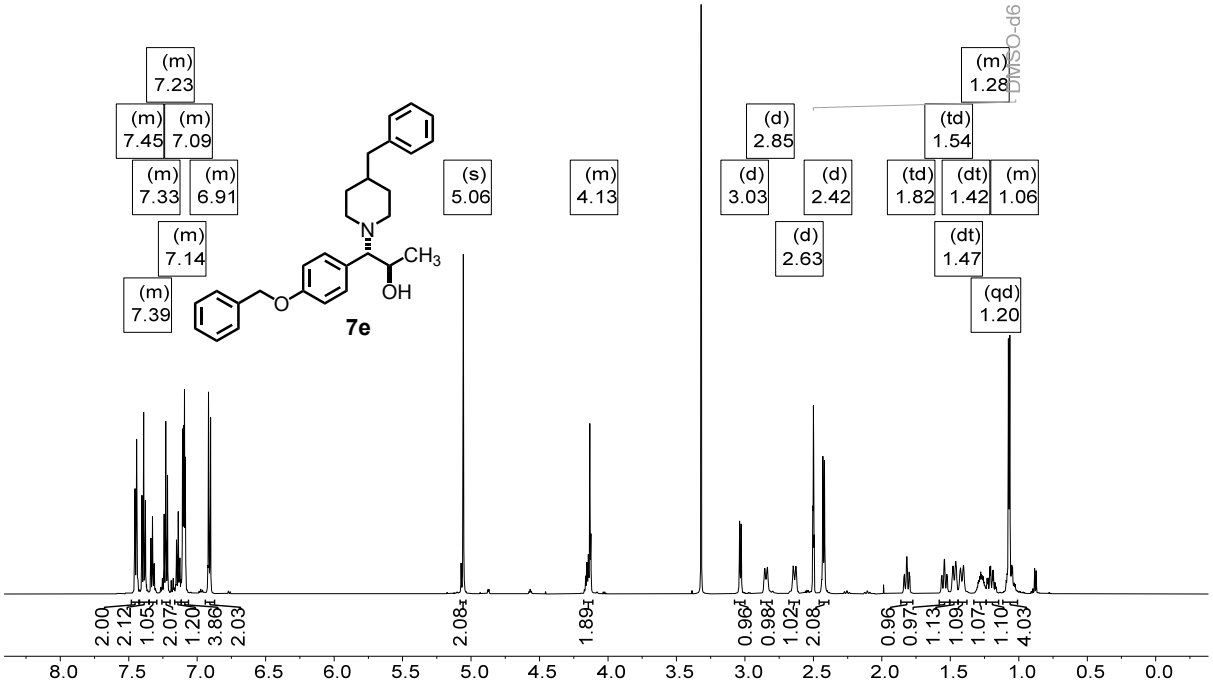
^{13}C NMR spectrum of **7c** in $CDCl_3$.



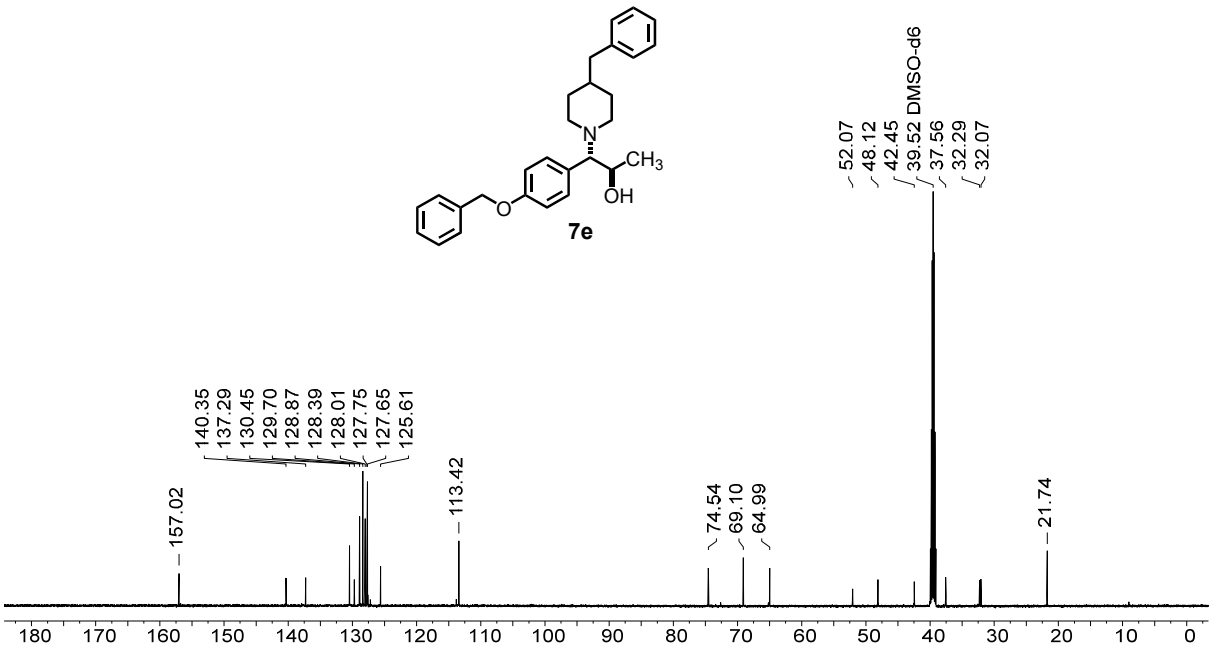
^{1}H NMR spectrum of **7d** in DMSO-d$_6$.



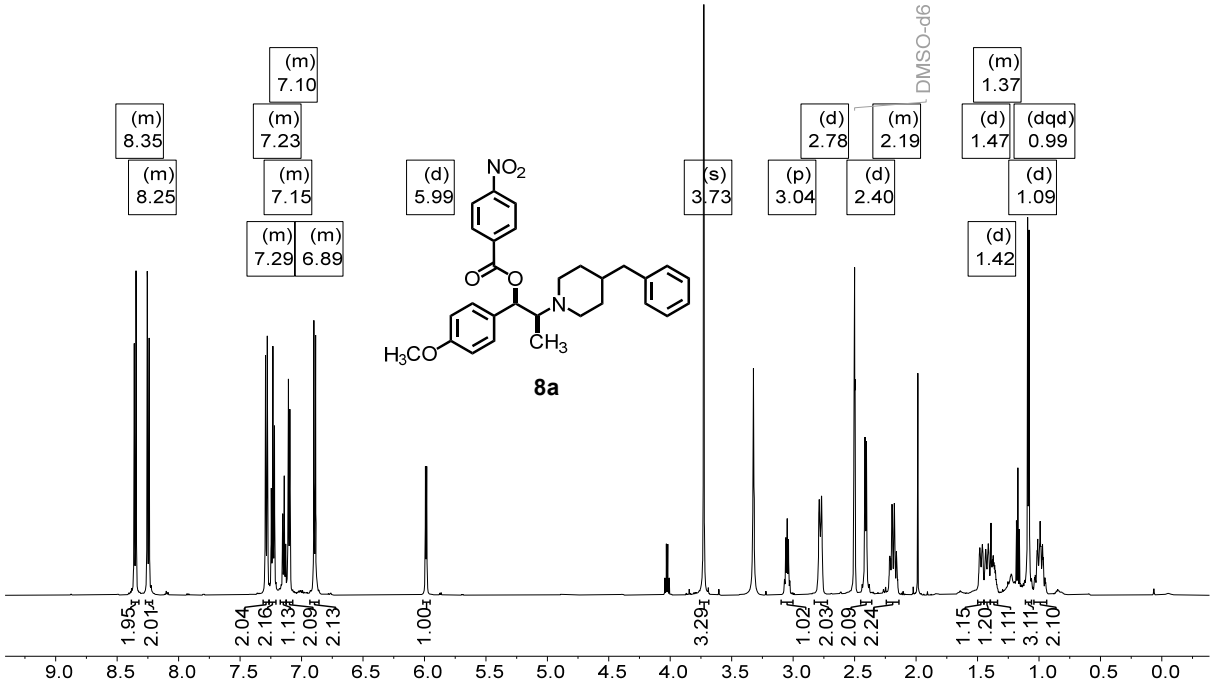
^{13}C NMR spectrum of **7d** in DMSO-d$_6$.



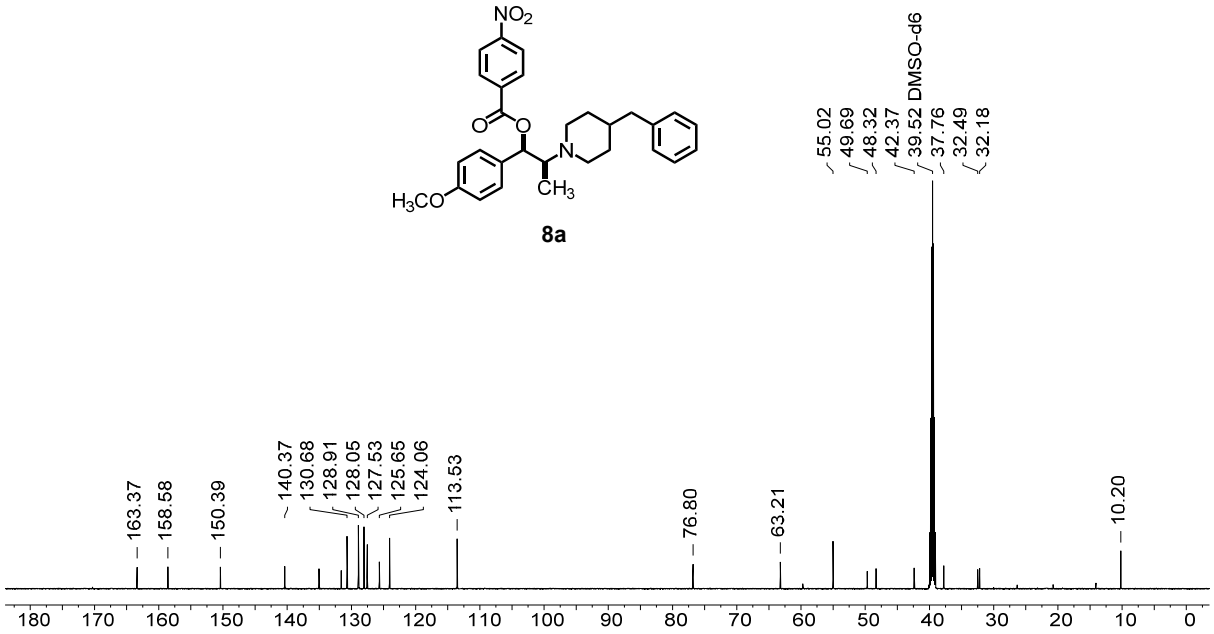
^{1}H NMR spectrum of **7e** in DMSO-d$_6$.



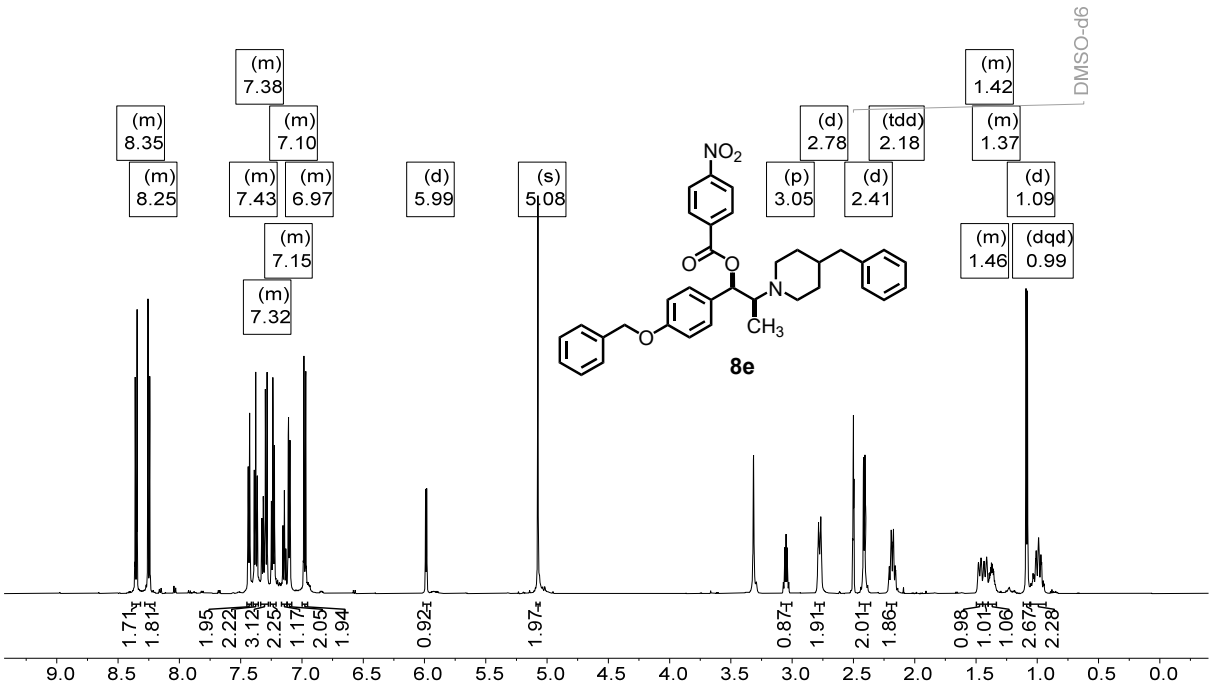
^{13}C NMR spectrum of **7e** in DMSO-d$_6$.



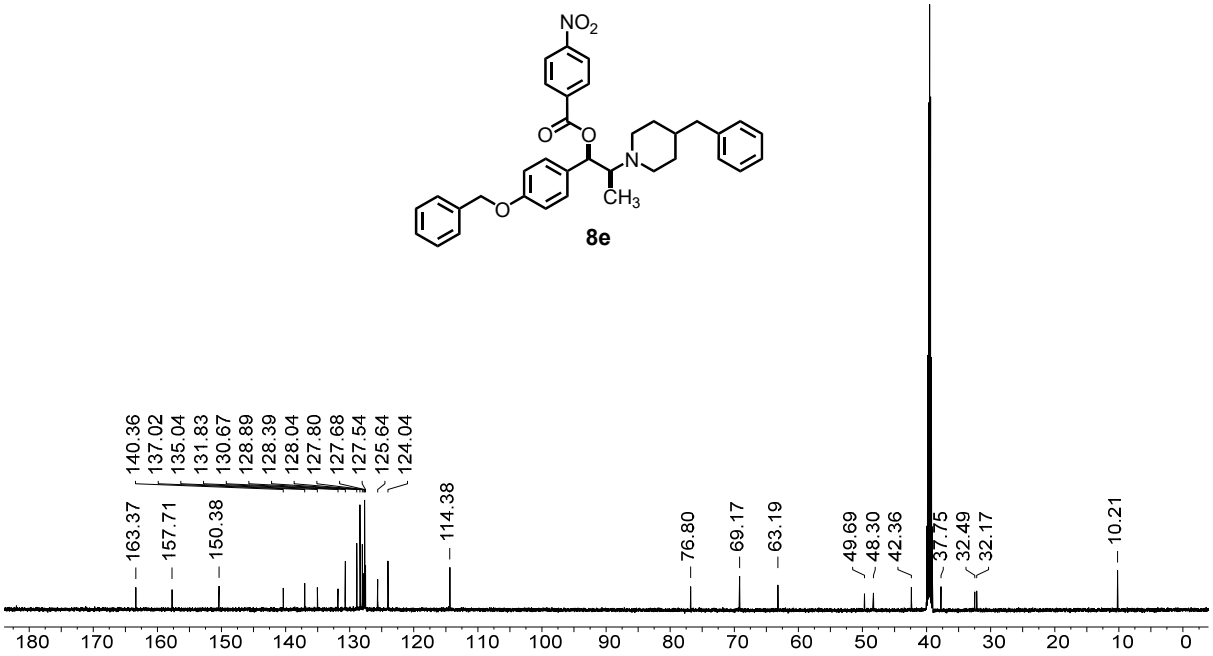
^{1}H NMR spectrum of **8a** in DMSO-d$_6$.



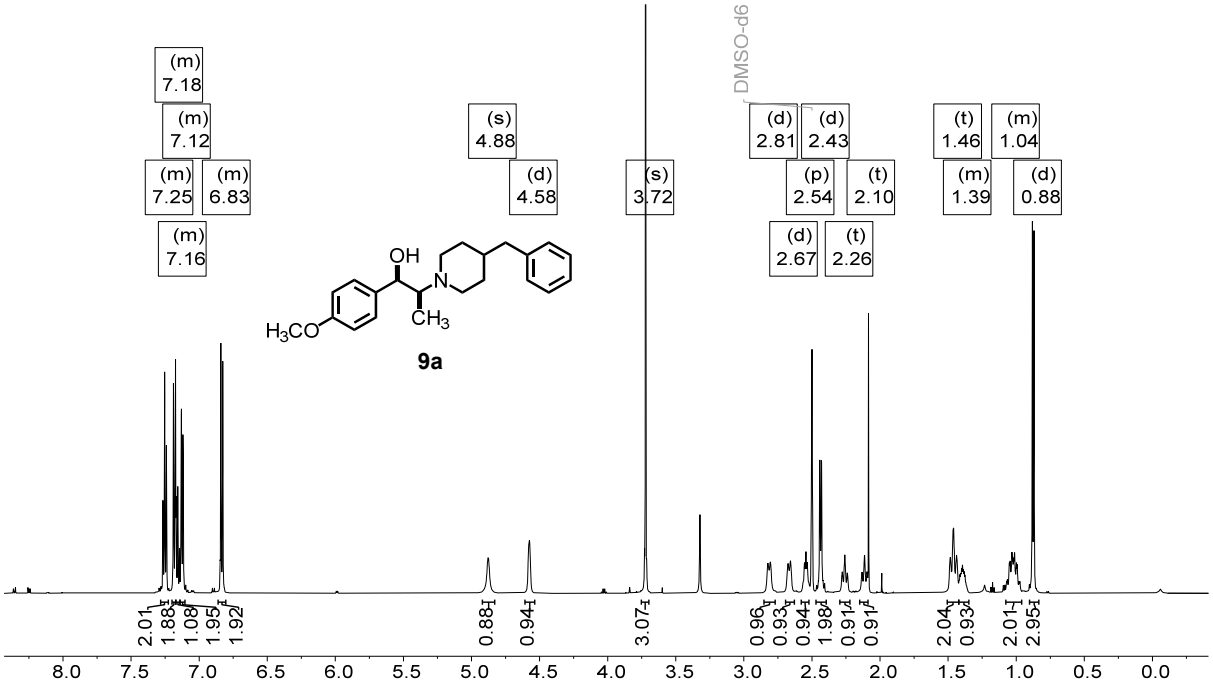
^{13}C NMR spectrum of **8a** in DMSO-d$_6$.



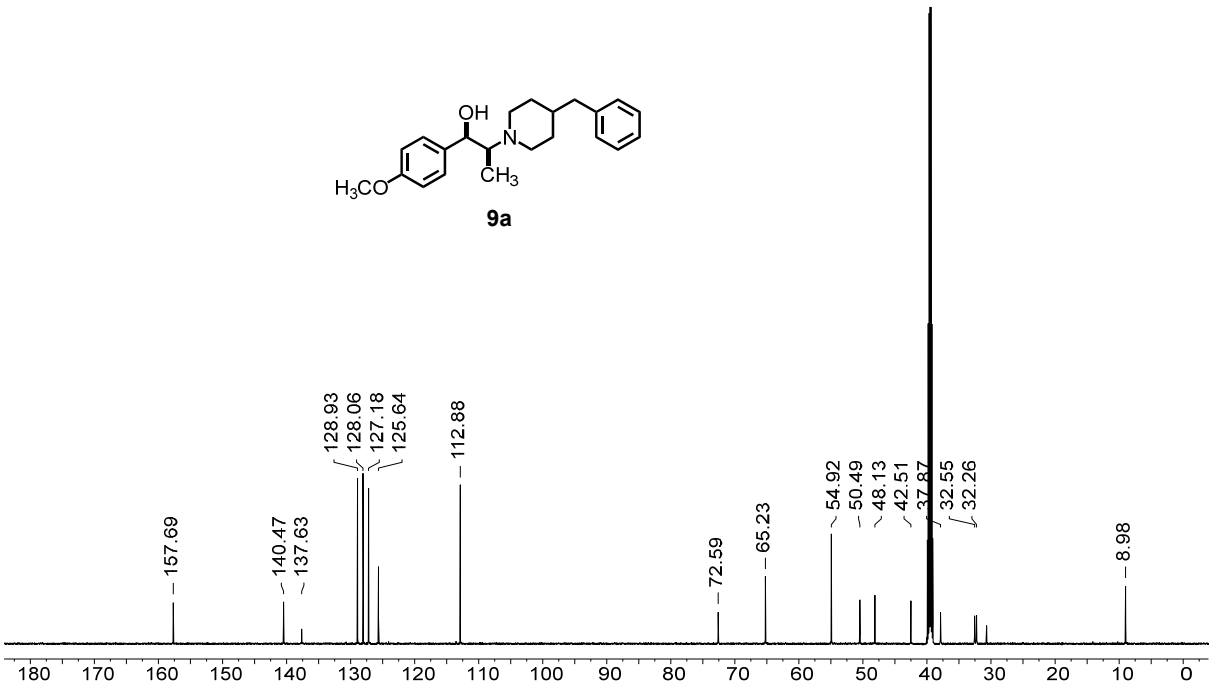
^{1}H NMR spectrum of **8e** in DMSO-d$_6$.



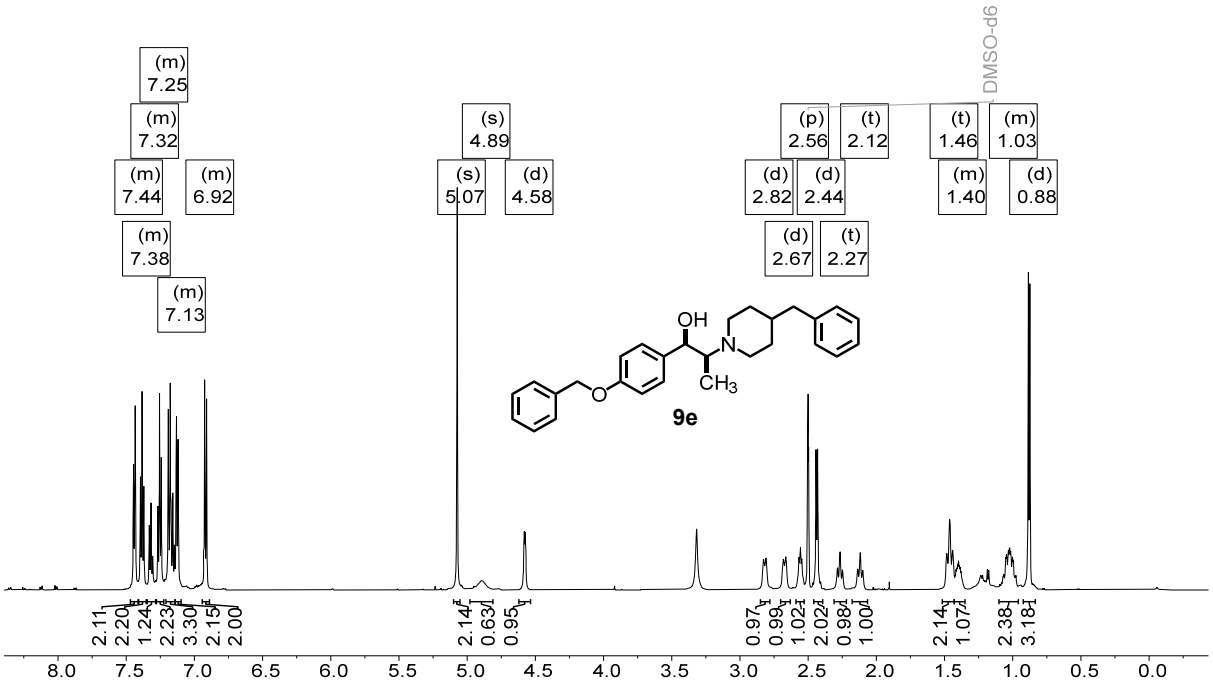
^{13}C NMR spectrum of **8e** in DMSO-d$_6$.



^{1}H NMR spectrum of **9a** in DMSO-d$_6$.

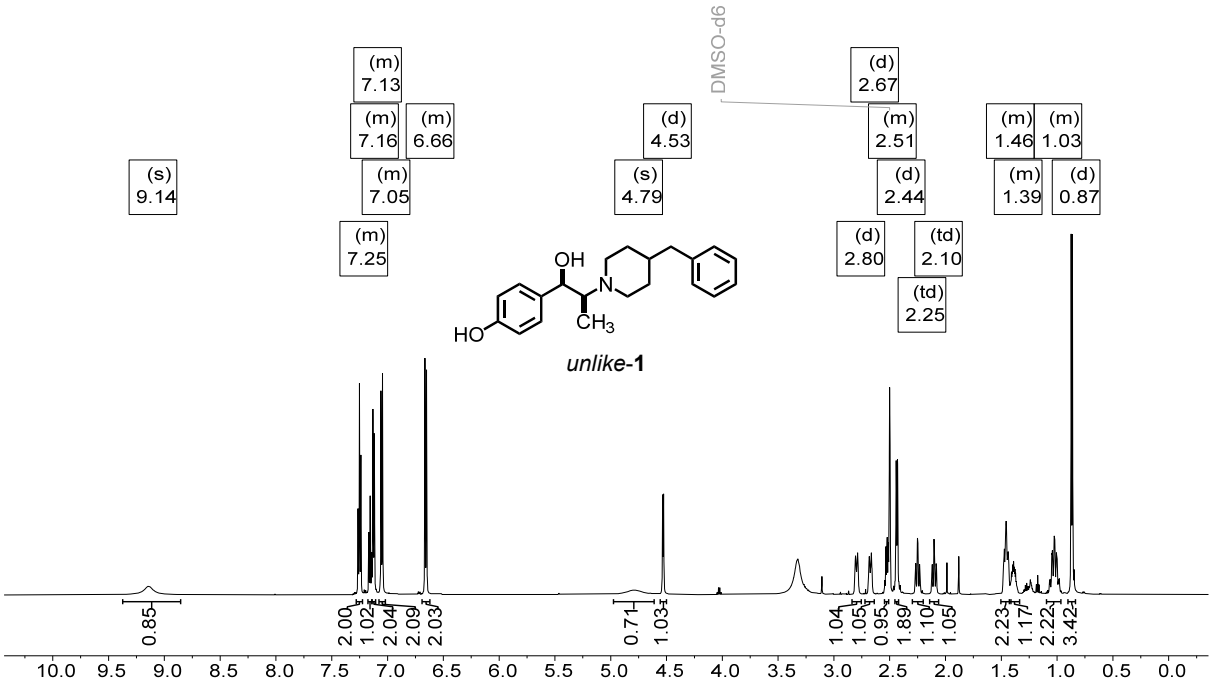


^{13}C NMR spectrum of **9a** in DMSO-d$_6$.

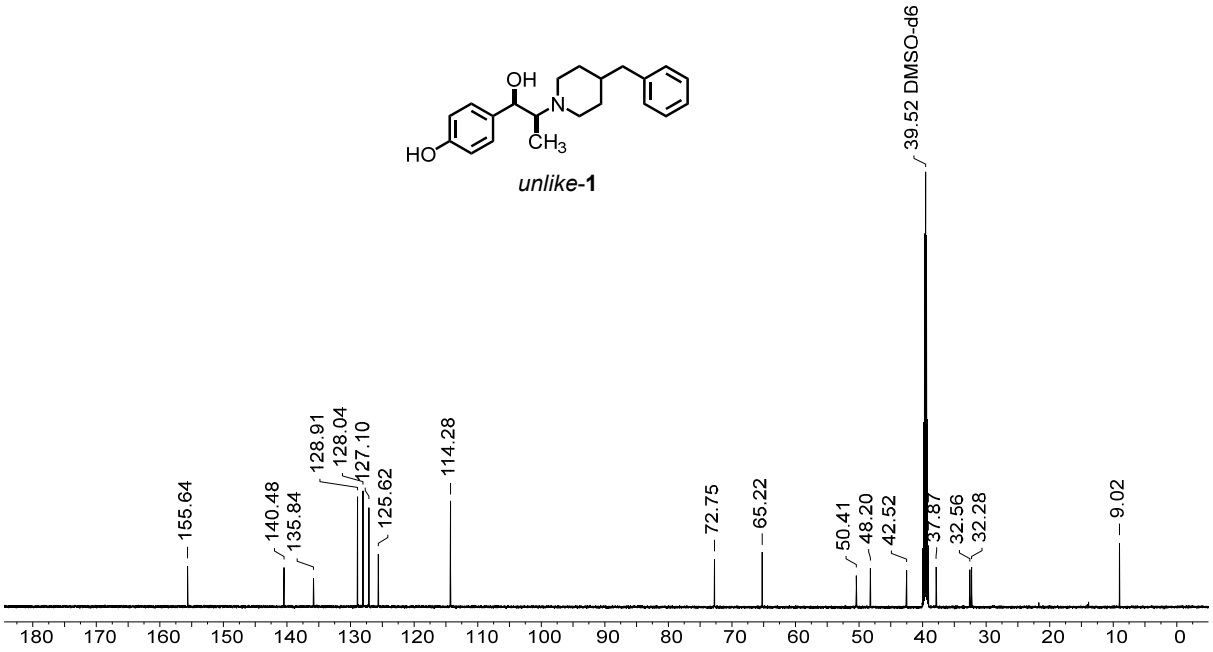


^{1}H NMR spectrum of **9e** in DMSO-d_6.

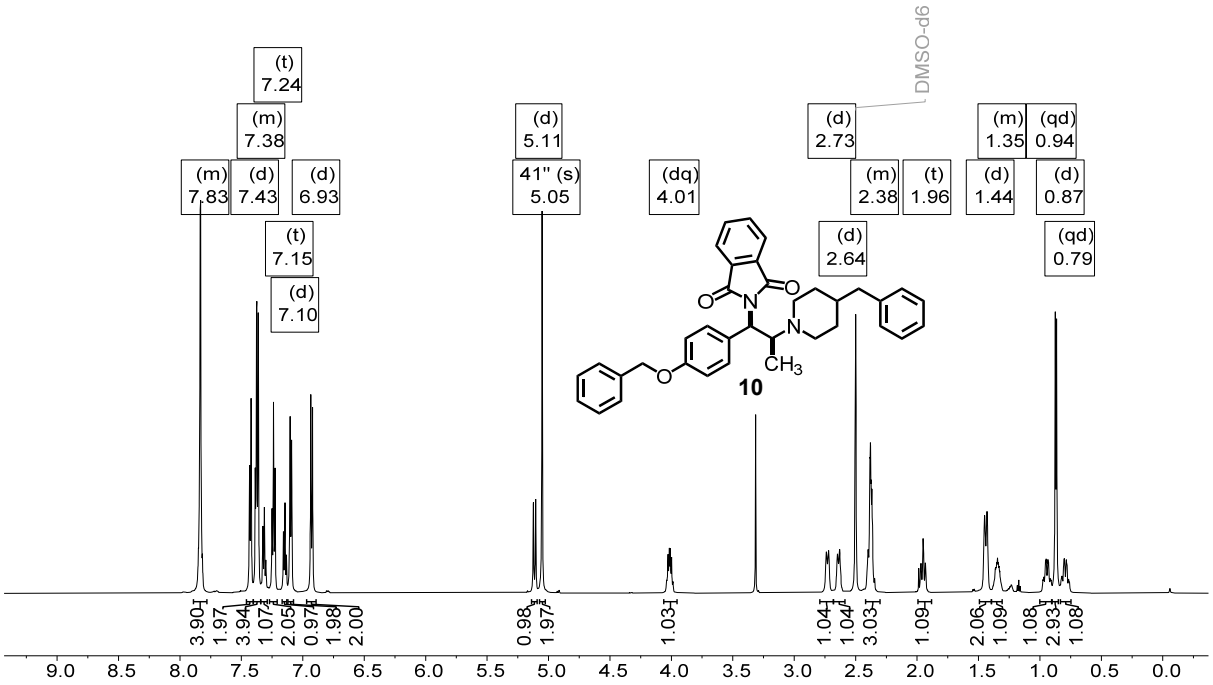
^{13}C NMR spectrum of **9e** in DMSO-d_6.



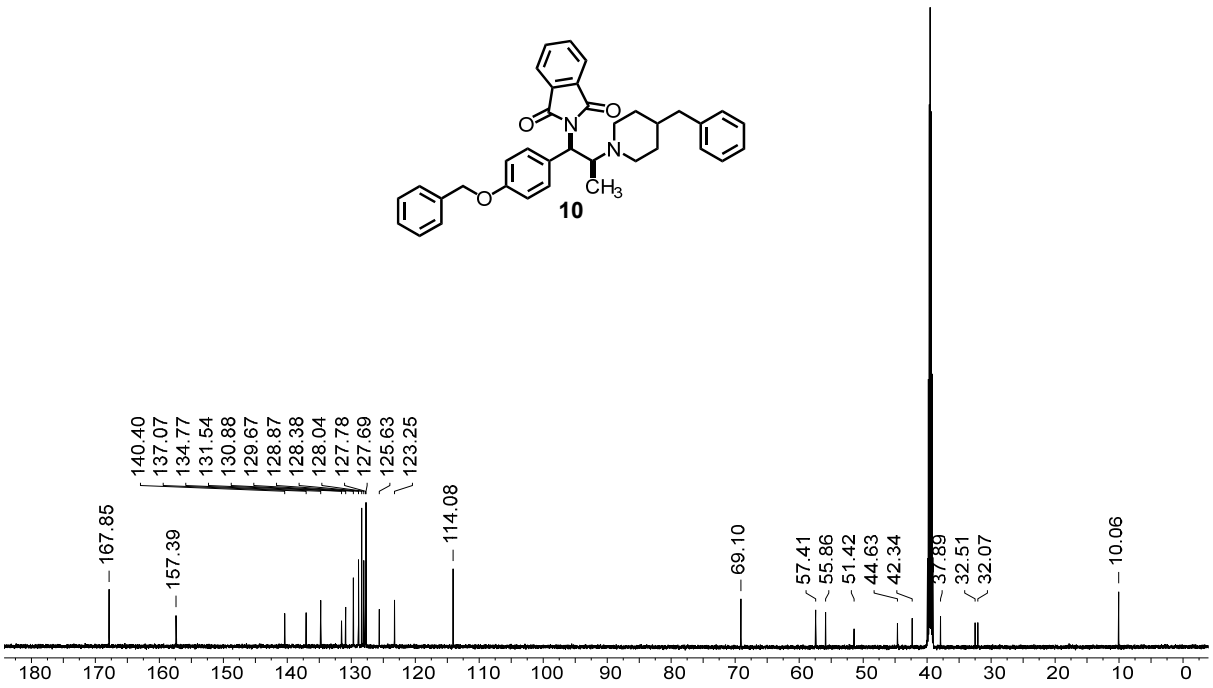
^{1}H NMR spectrum of *unlike*-**1** in DMSO-d$_6$.



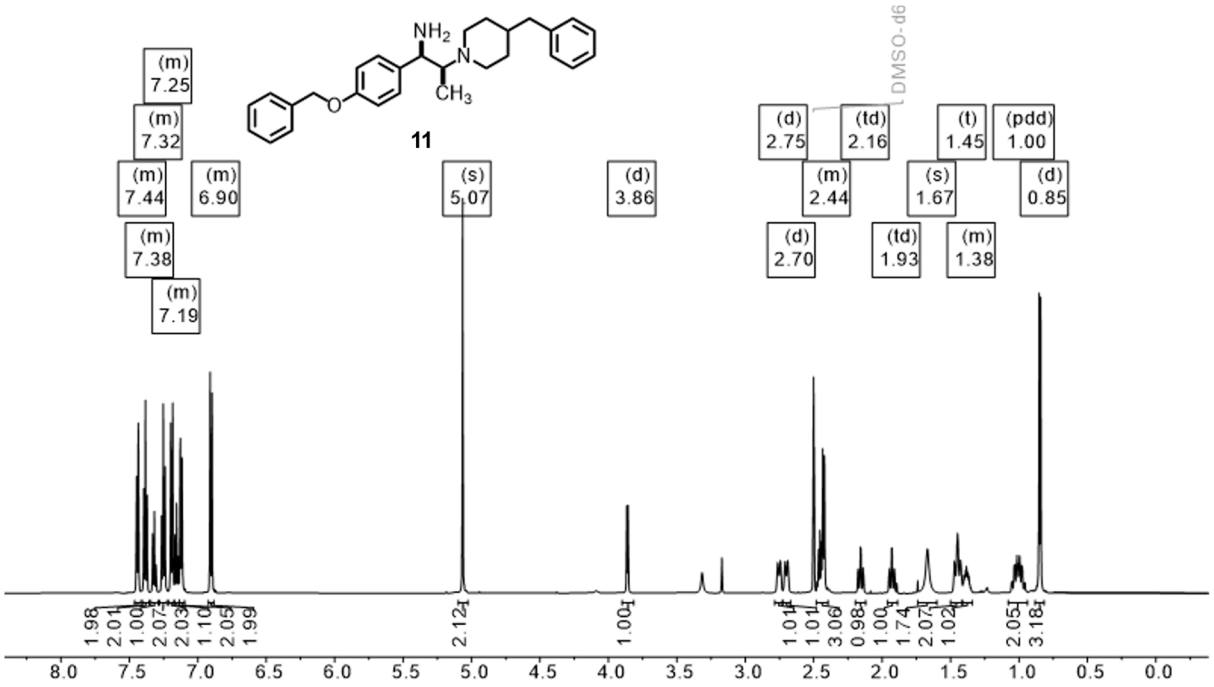
^{13}C NMR spectrum of *unlike*-**1** in DMSO-d$_6$.



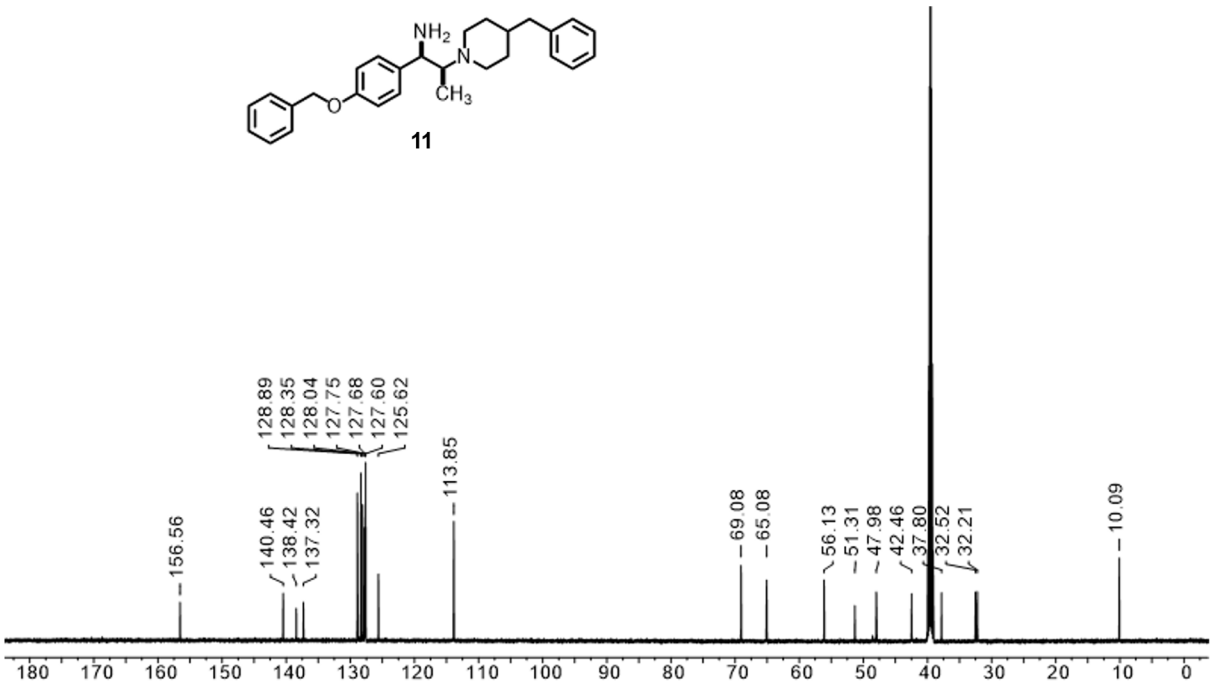
^{1}H NMR spectrum of **10** in DMSO-d_6.



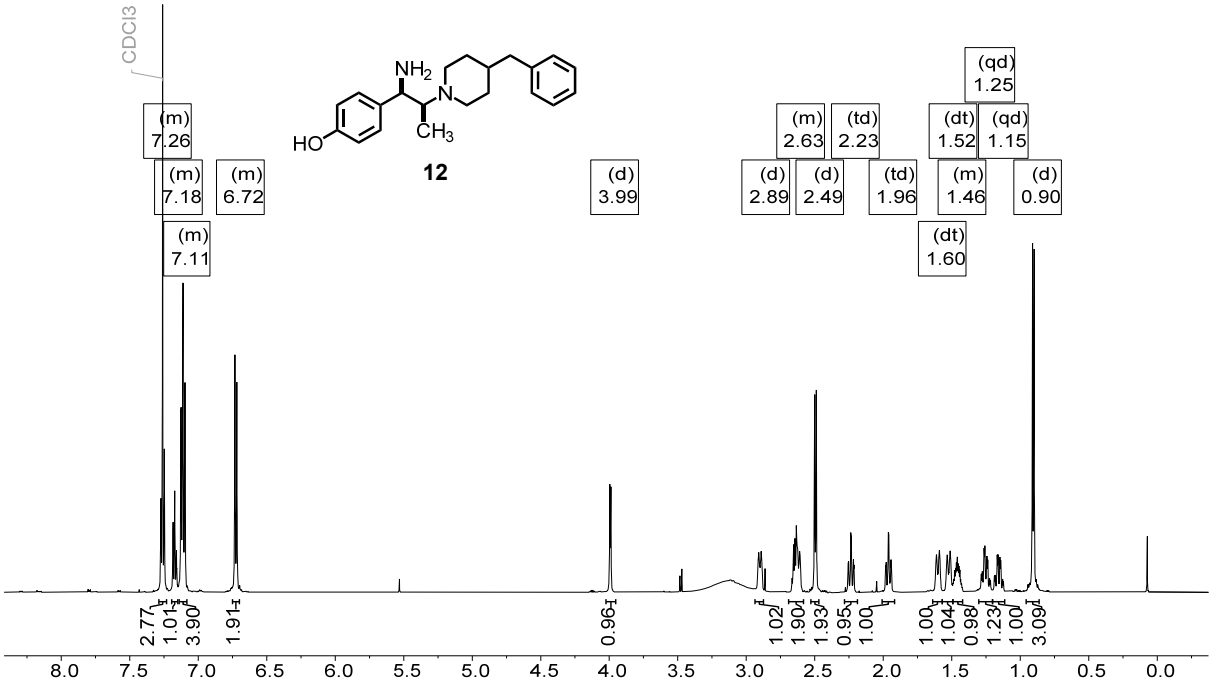
^{13}C NMR spectrum of **9e** in DMSO-d_6.



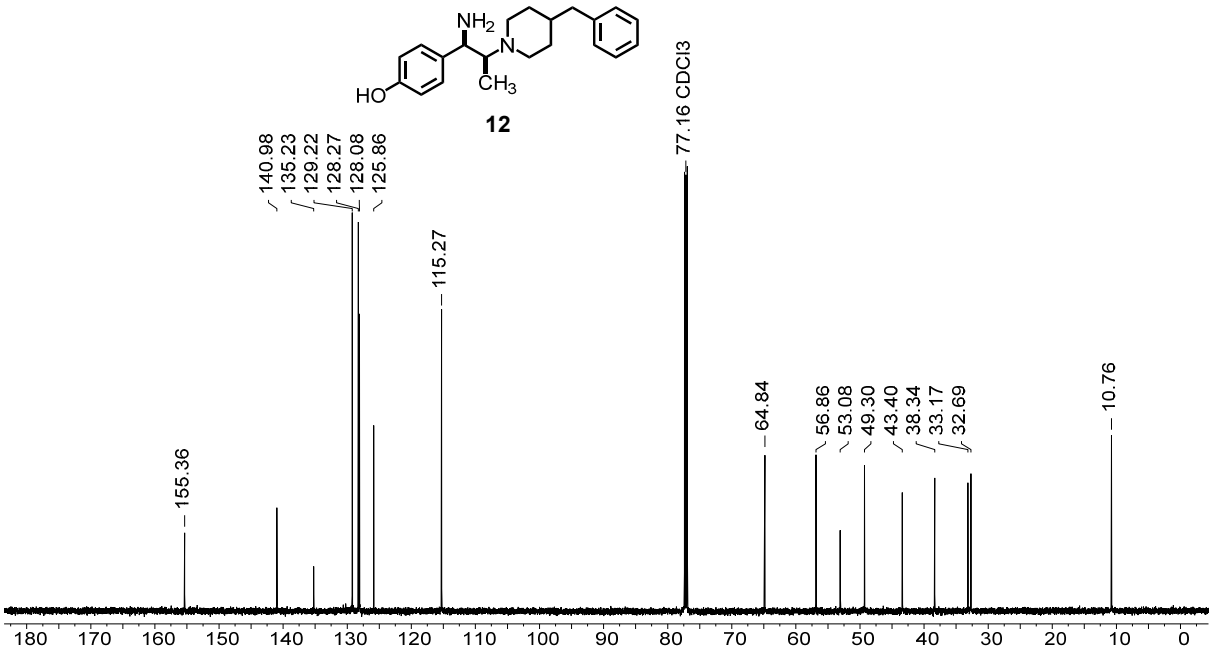
^{1}H NMR spectrum of **11** in DMSO-d_6.



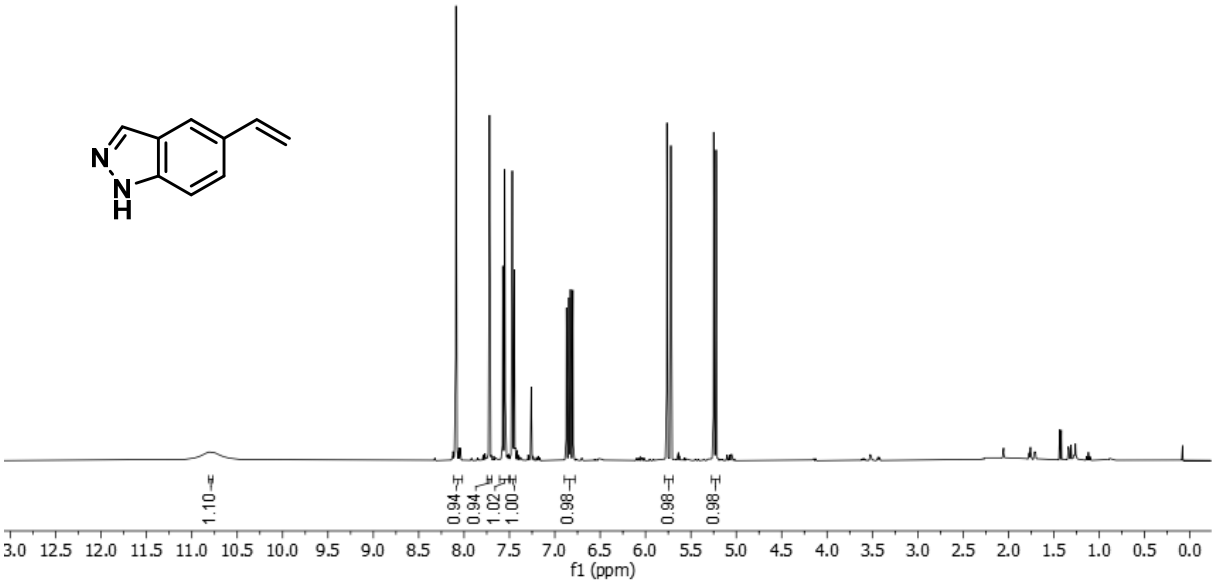
^{13}C NMR spectrum of **11** in DMSO-d_6.



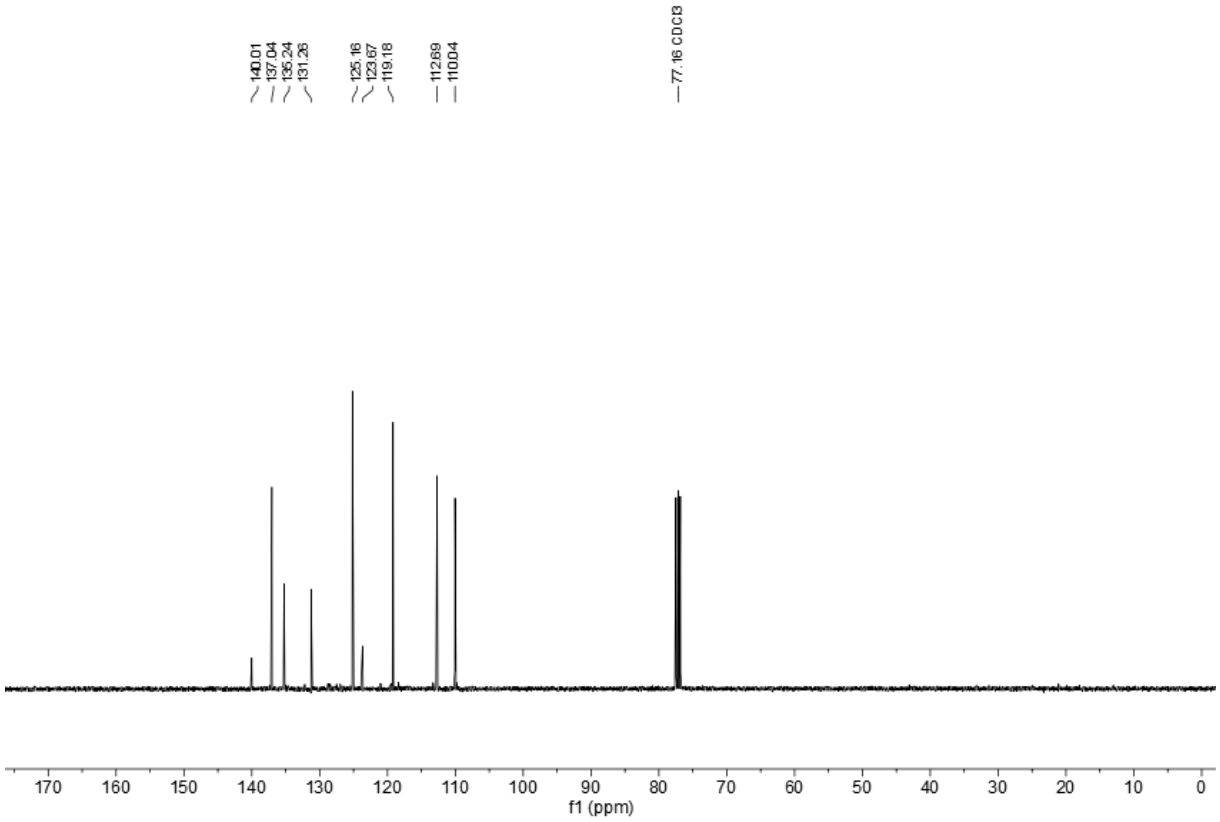
^{1}H NMR spectrum of **12** in $CDCl_3$.



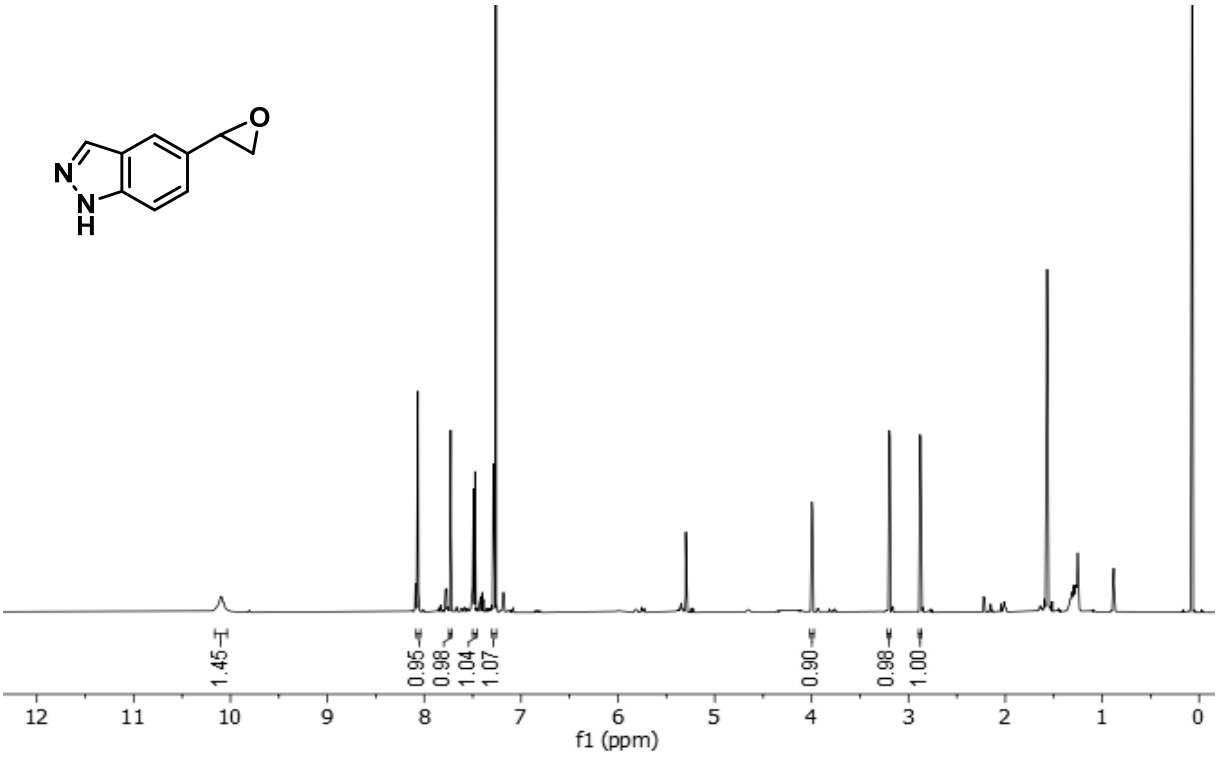
^{13}C NMR spectrum of **12** in $CDCl_3$.



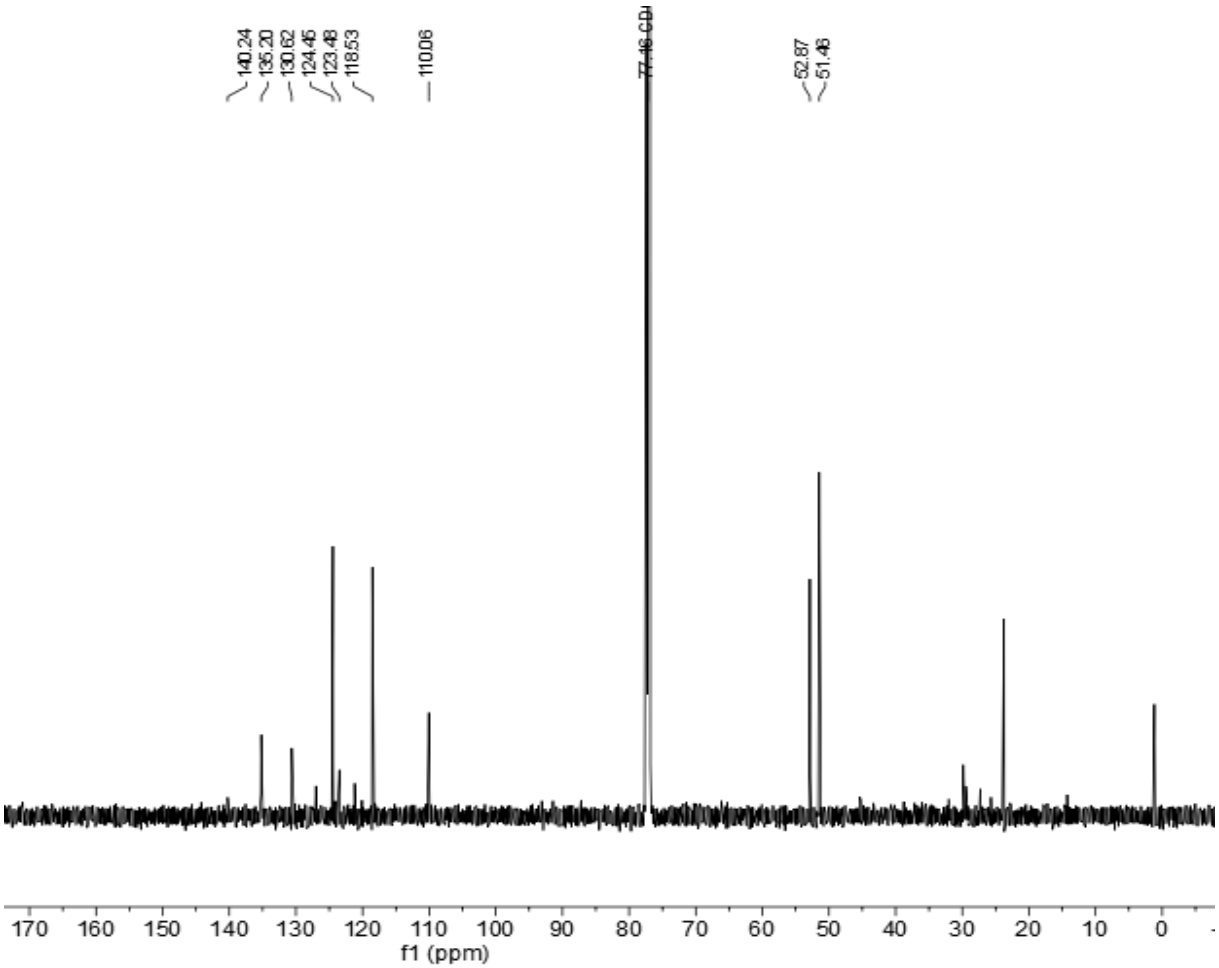
^{1}H NMR spectrum of **14** in $CDCl_3$.



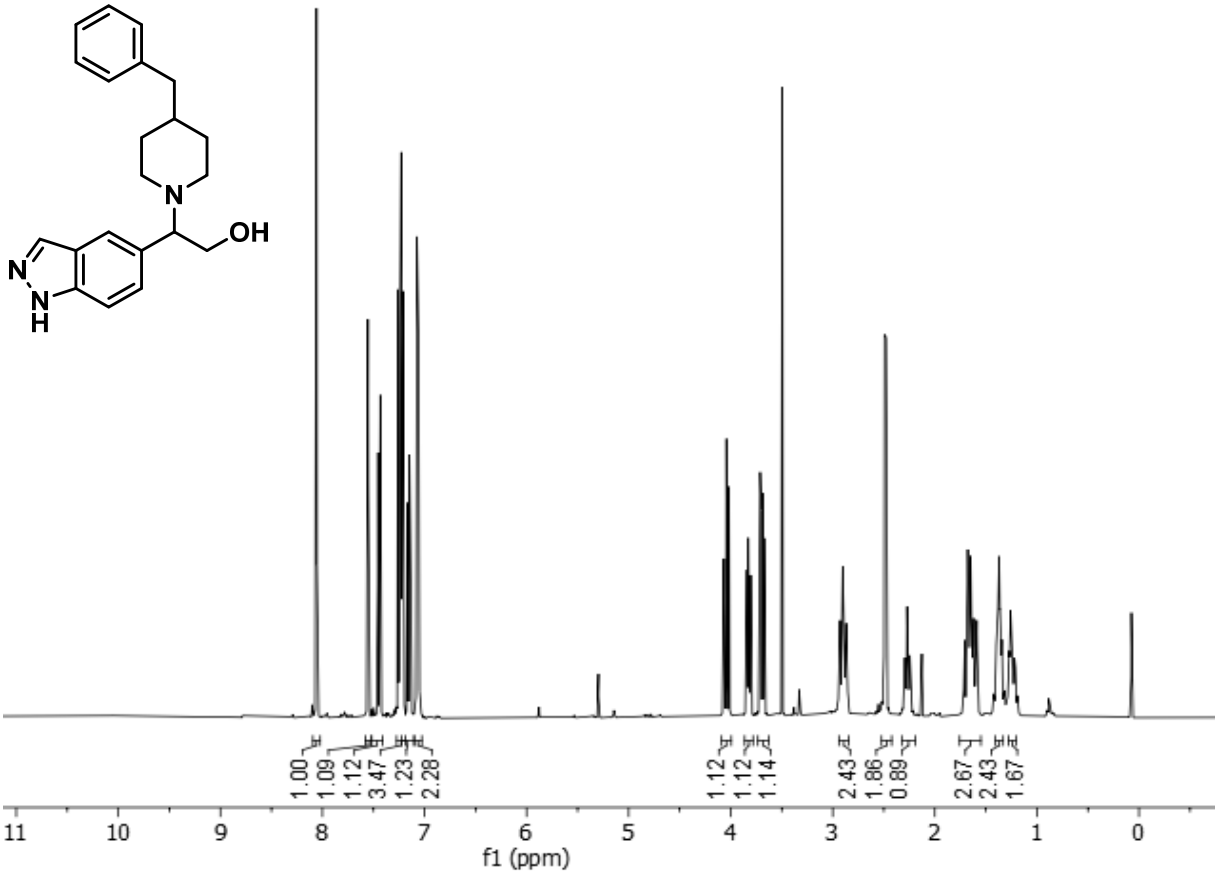
^{13}C NMR spectrum of **14** in $CDCl_3$.



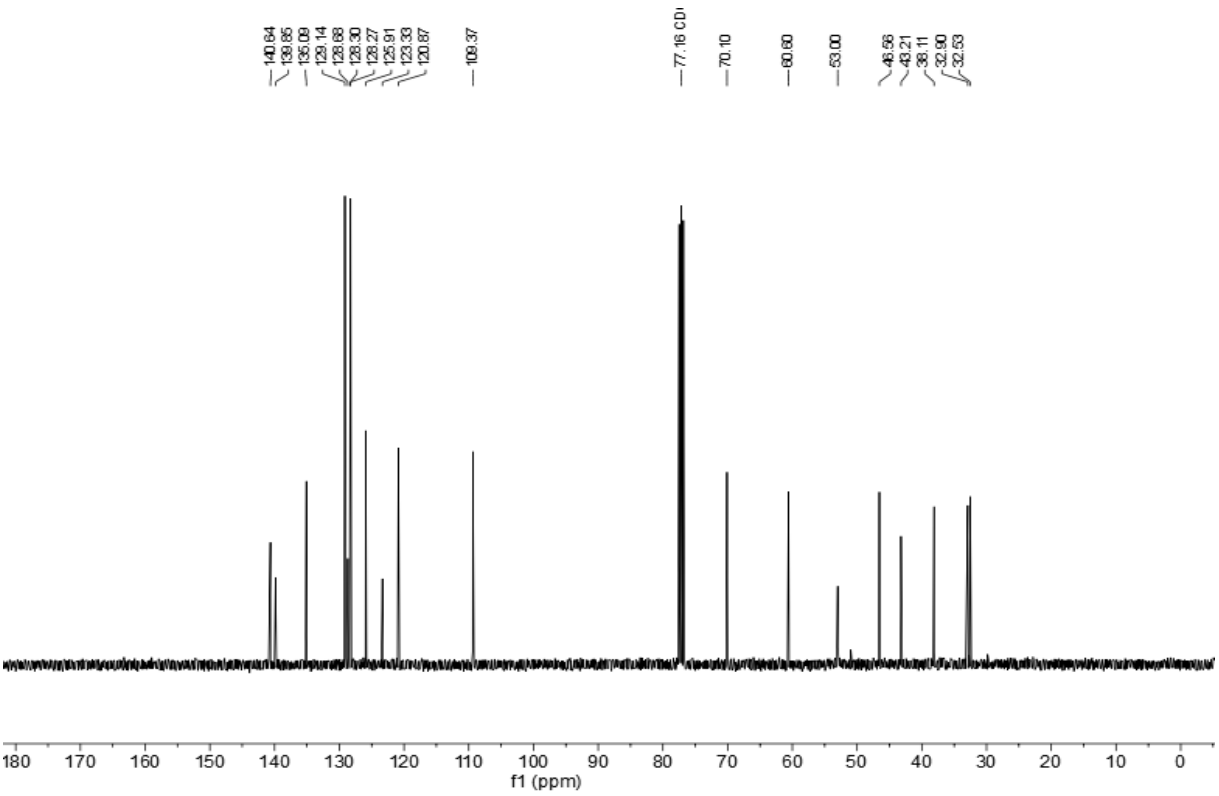
^{1}H NMR spectrum of **15** in $CDCl_3$.



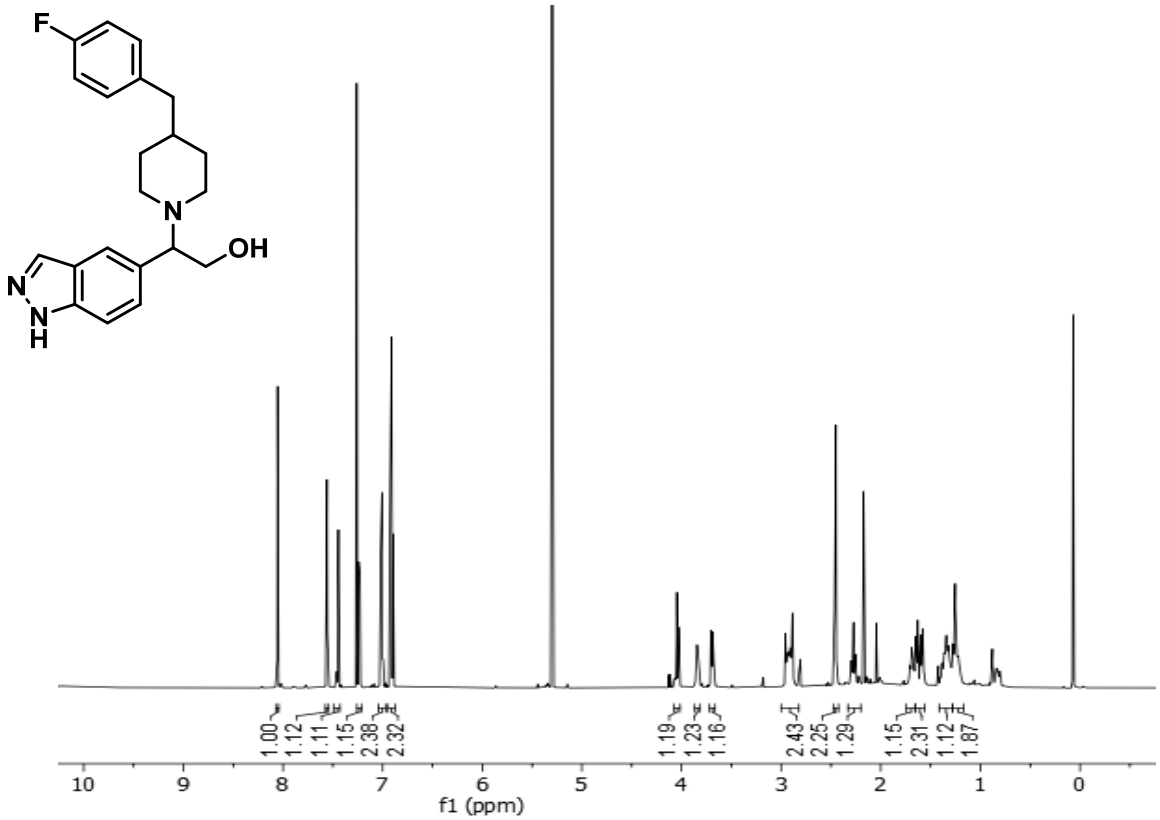
^{13}C NMR spectrum of **15** in $CDCl_3$.



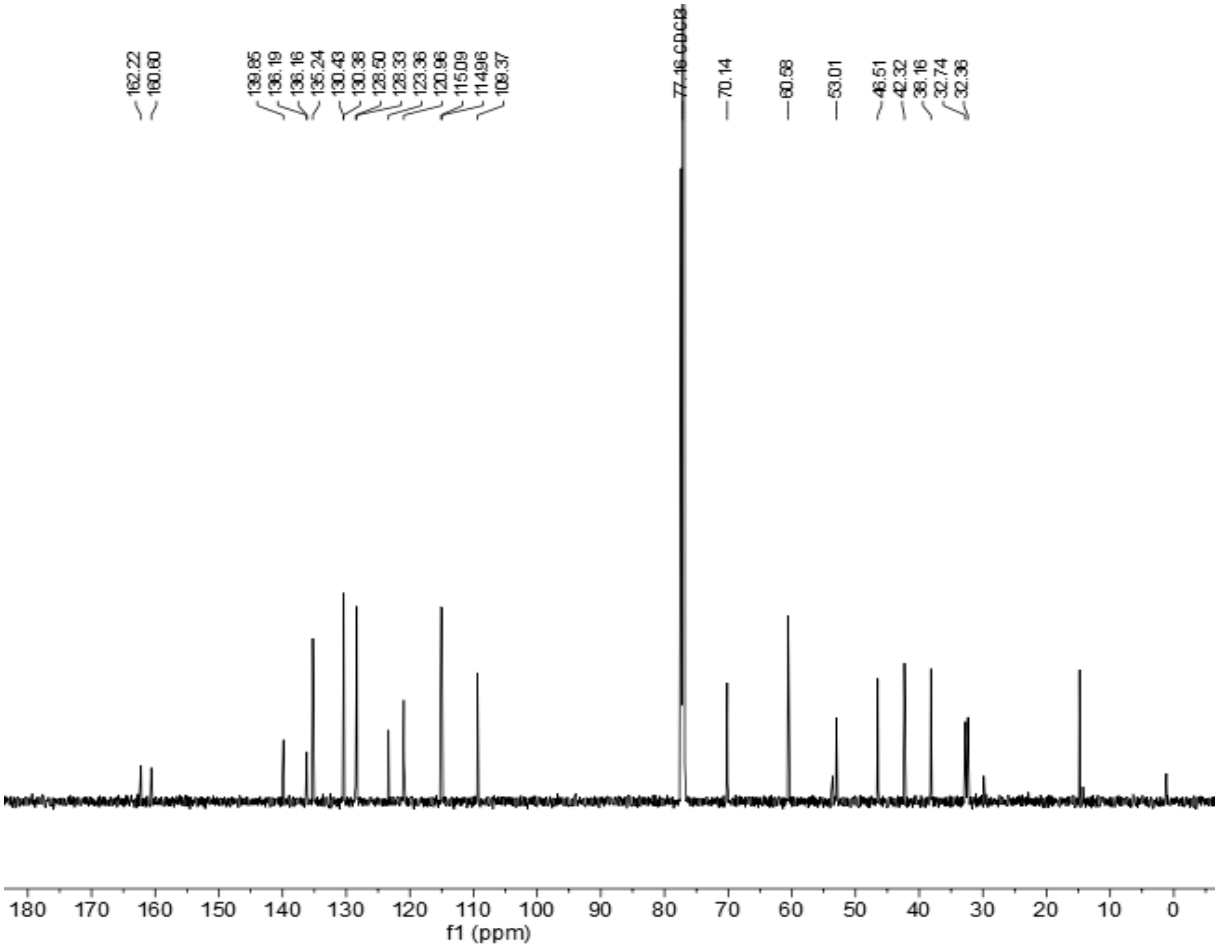
^{1}H NMR spectrum of **16a** in $CDCl_3$.



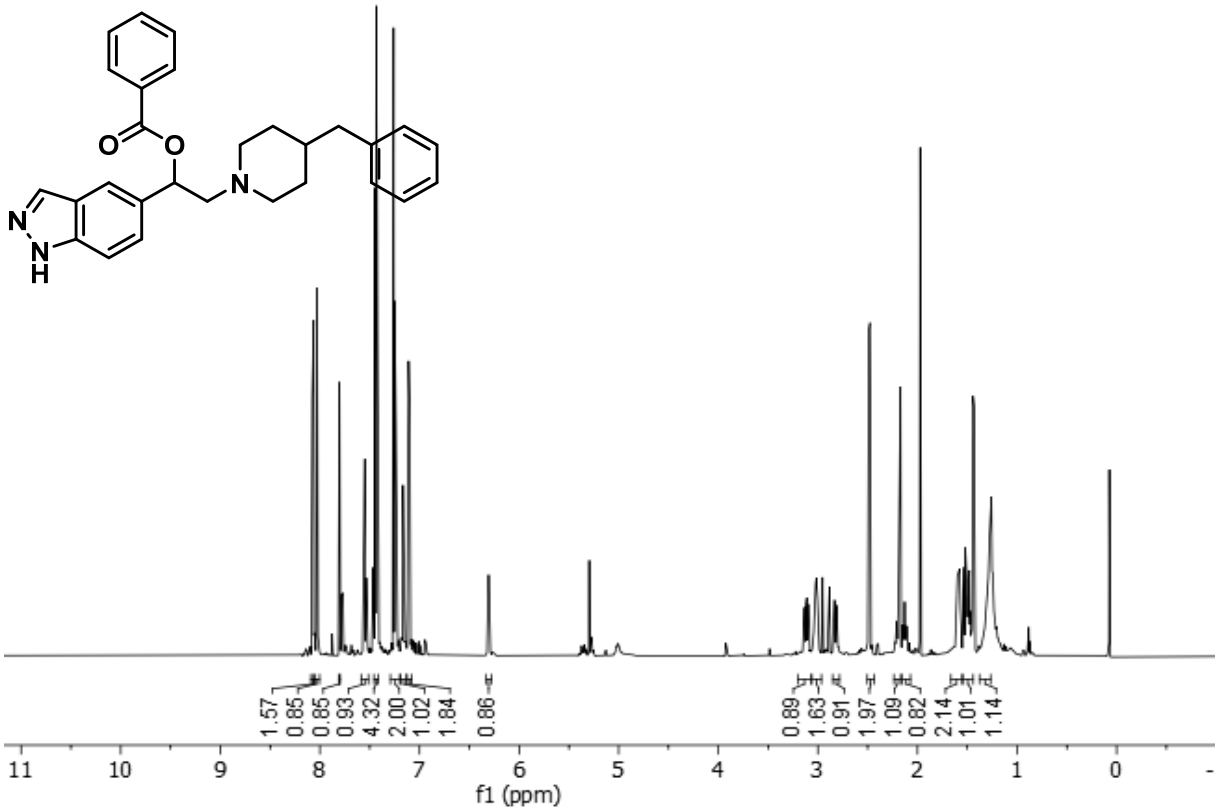
^{13}C NMR spectrum of **16a** in $CDCl_3$.



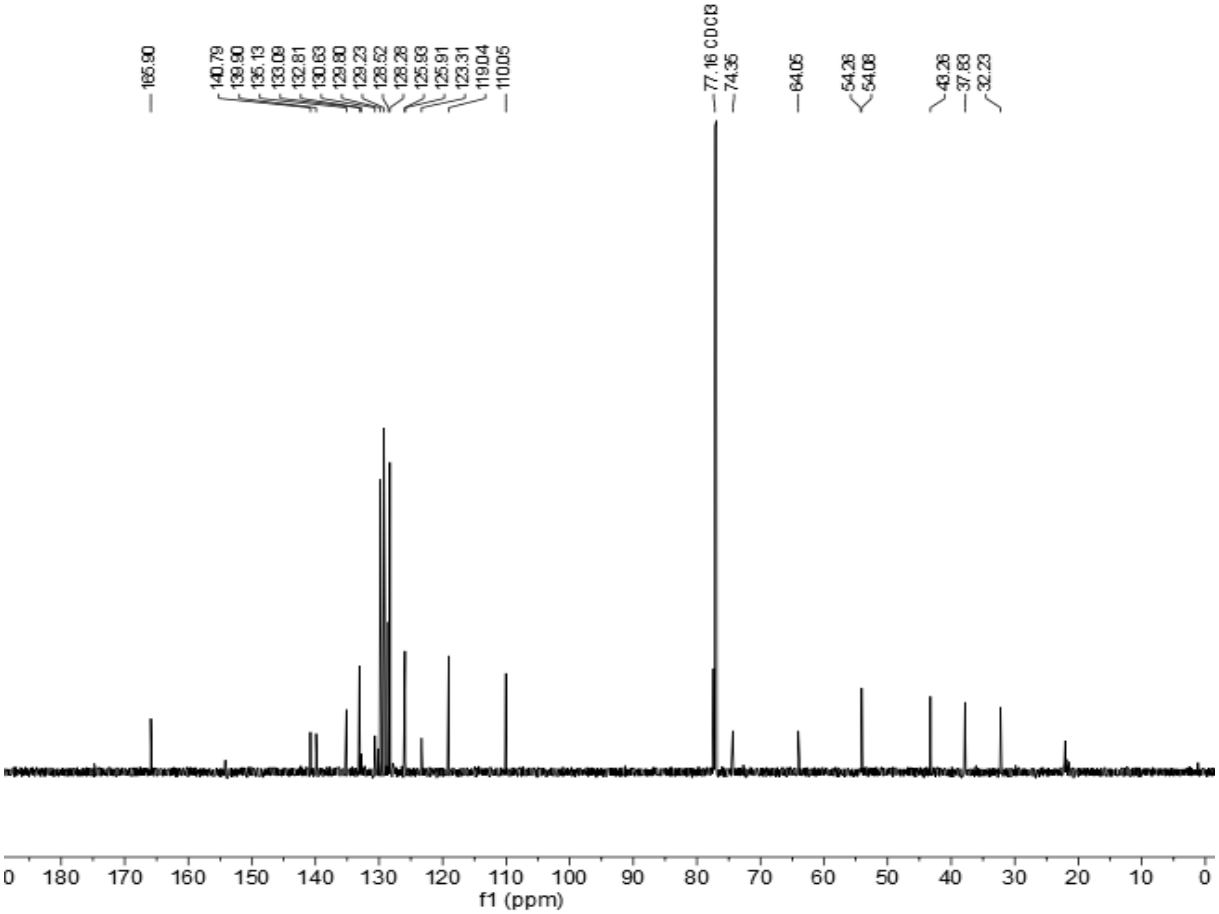
^{1}H NMR spectrum of **16b** in $CDCl_3$.



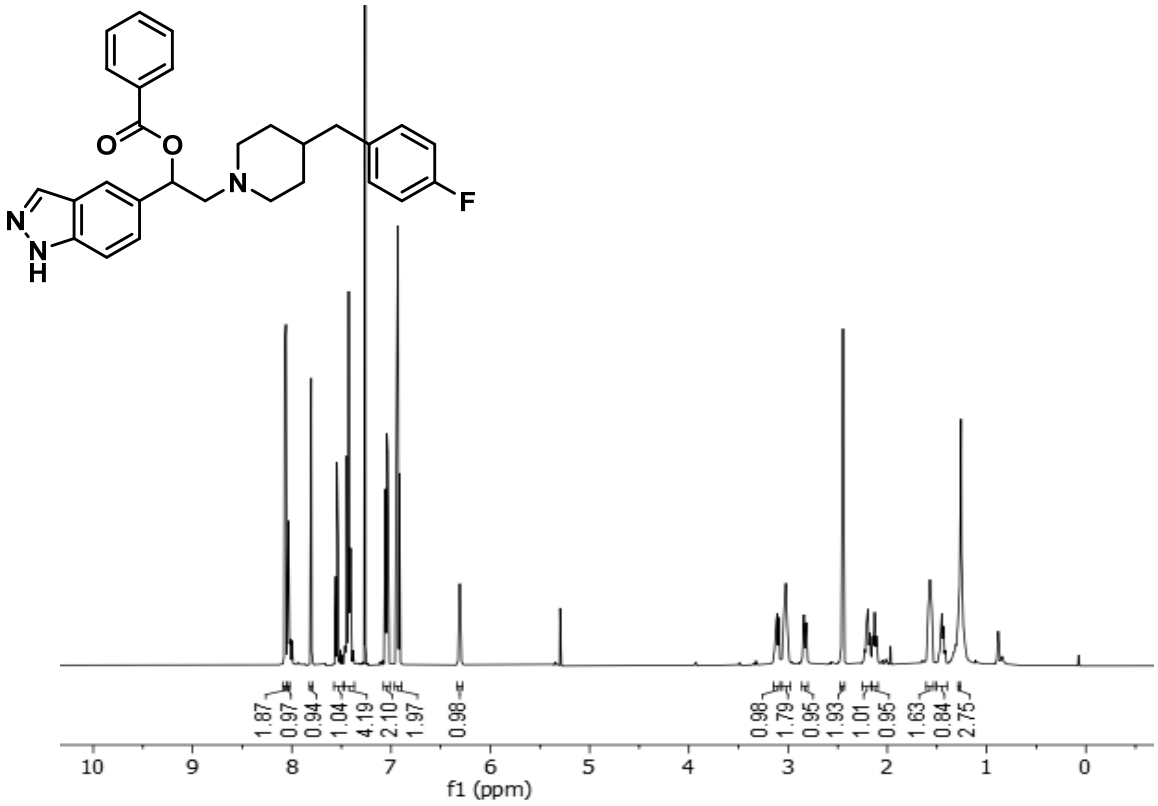
^{13}C NMR spectrum of **16b** in $CDCl_3$.



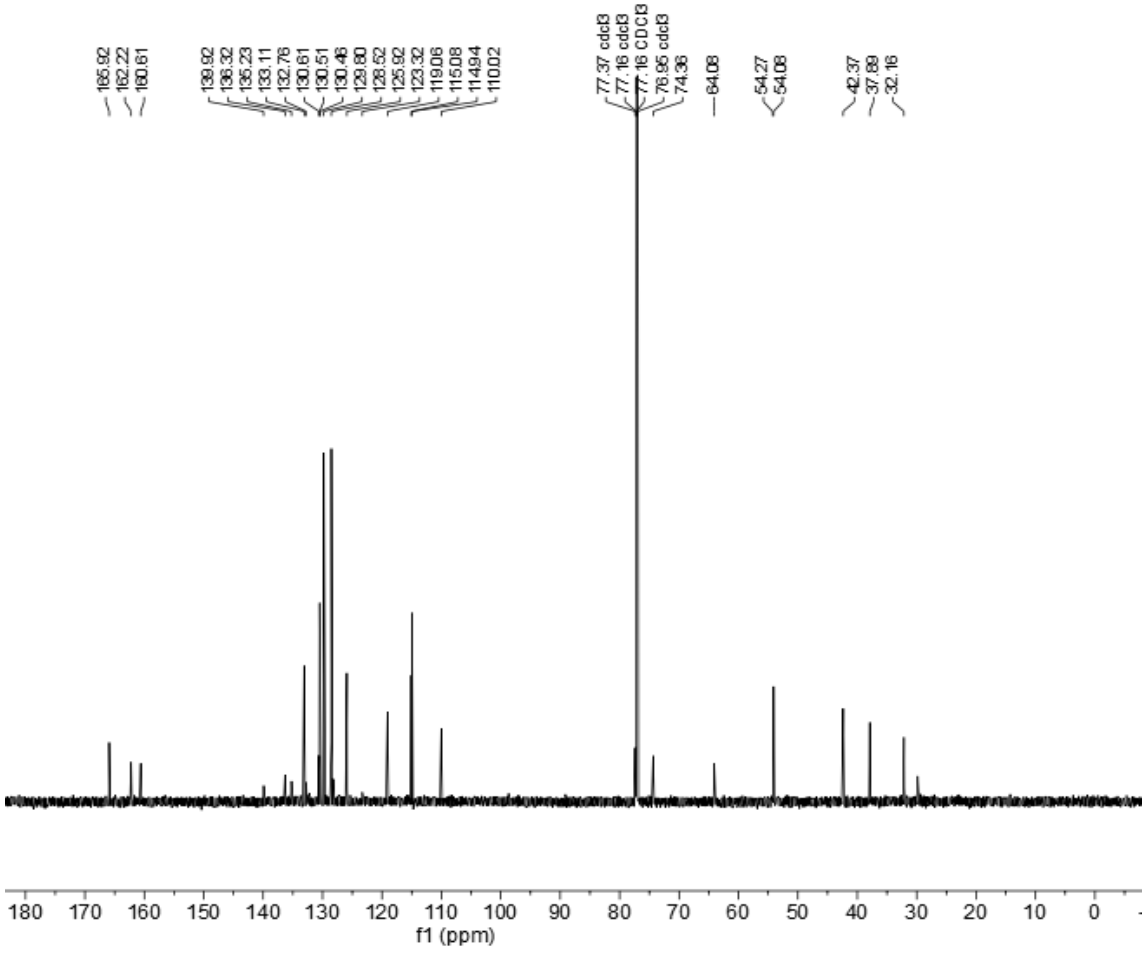
^{1}H NMR spectrum of **17a** in $CDCl_3$.



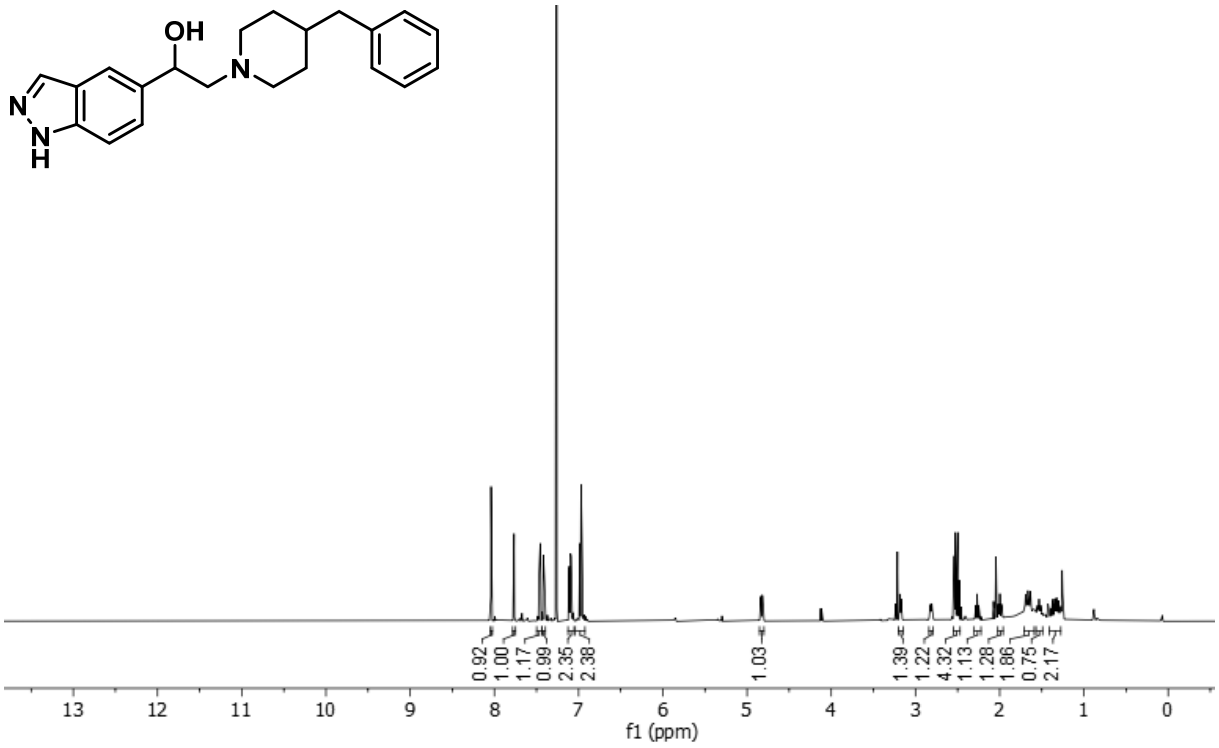
^{13}C NMR spectrum of **17a** in $CDCl_3$.



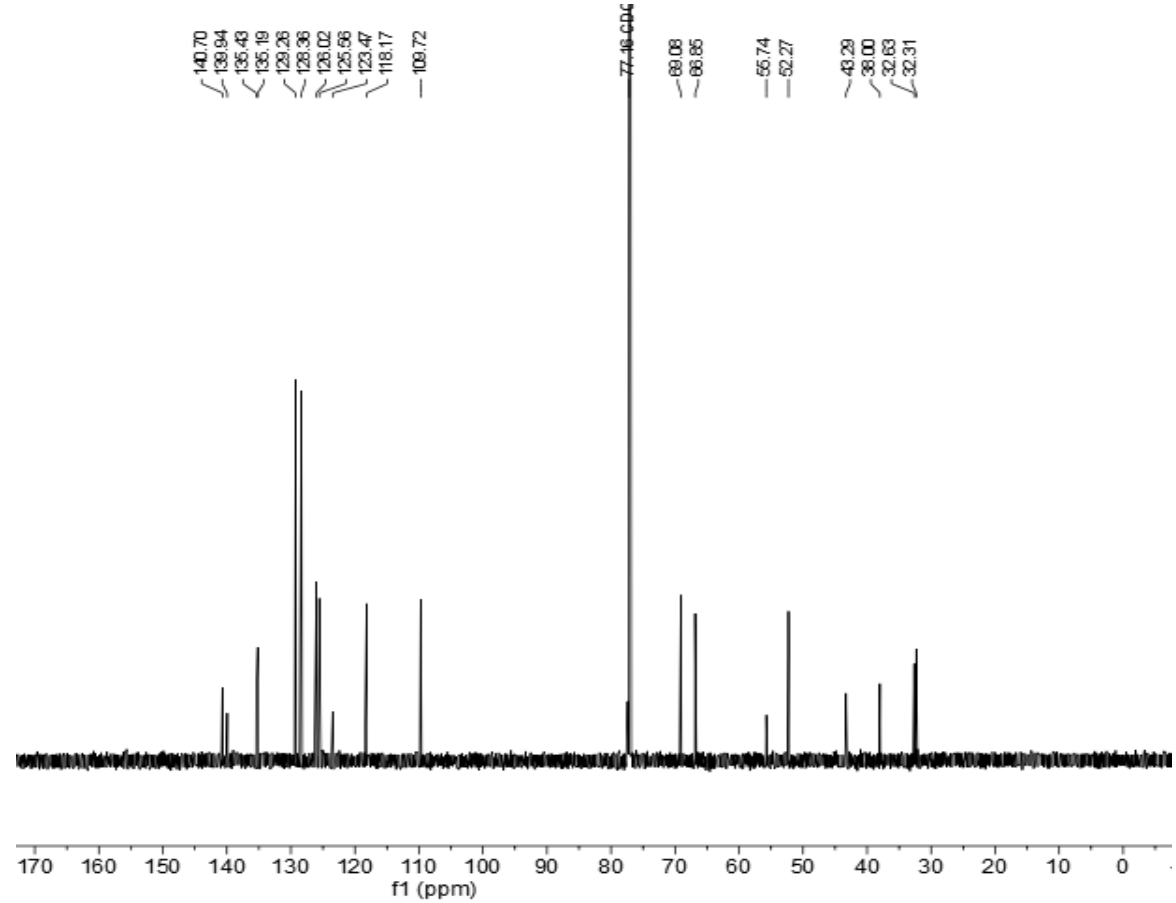
^{1}H NMR spectrum of **17b** in $CDCl_3$.



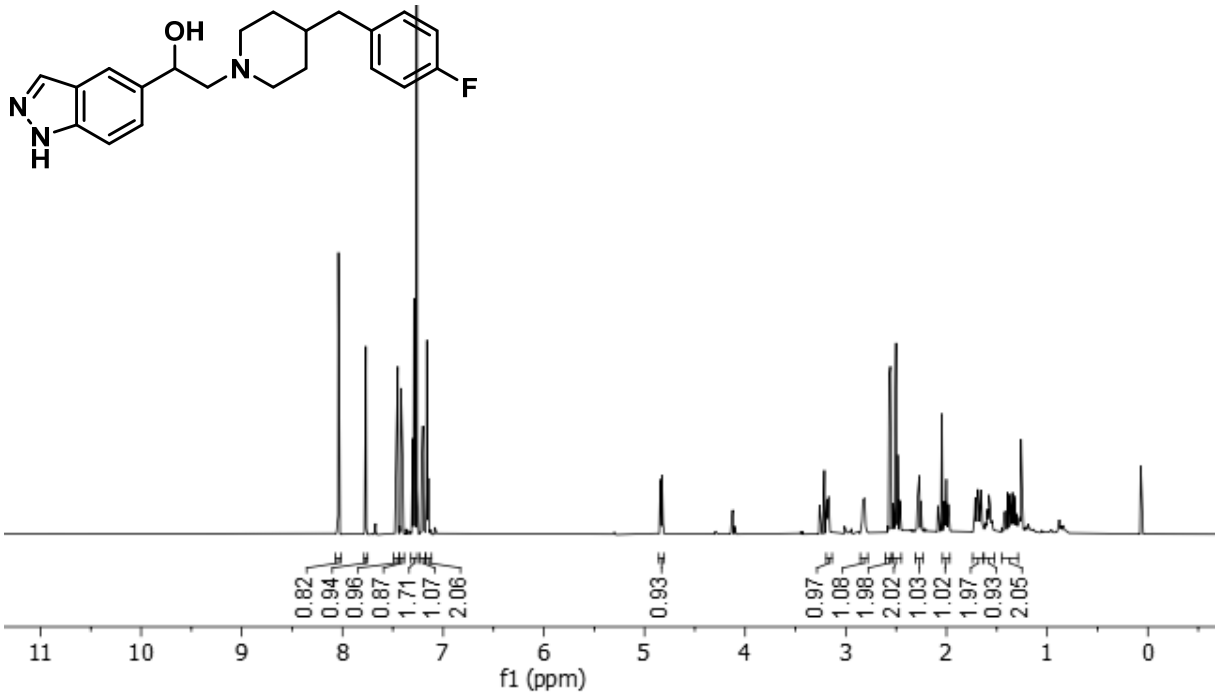
^{13}C NMR spectrum of **17b** in $CDCl_3$.



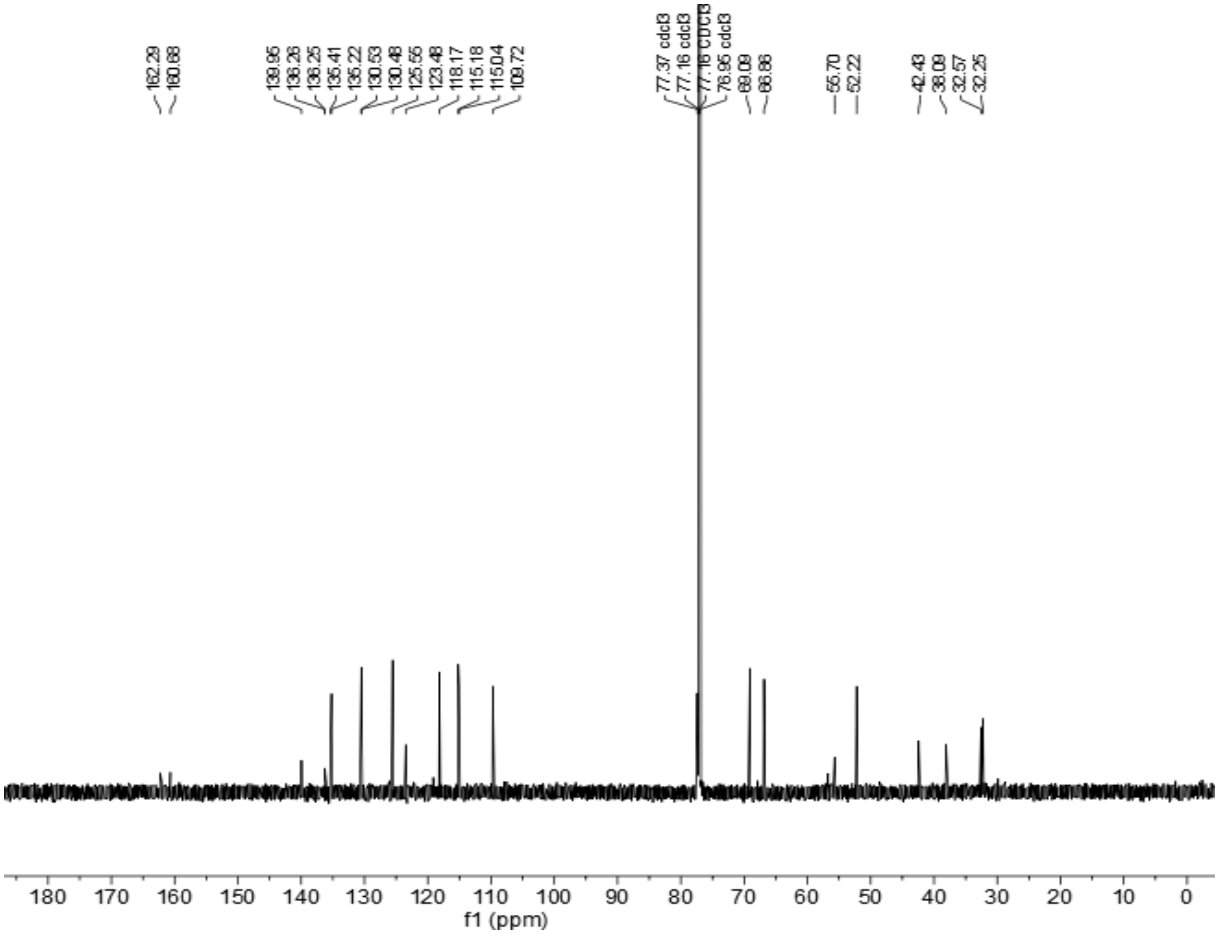
^{1}H NMR spectrum of **18a** in $CDCl_3$.



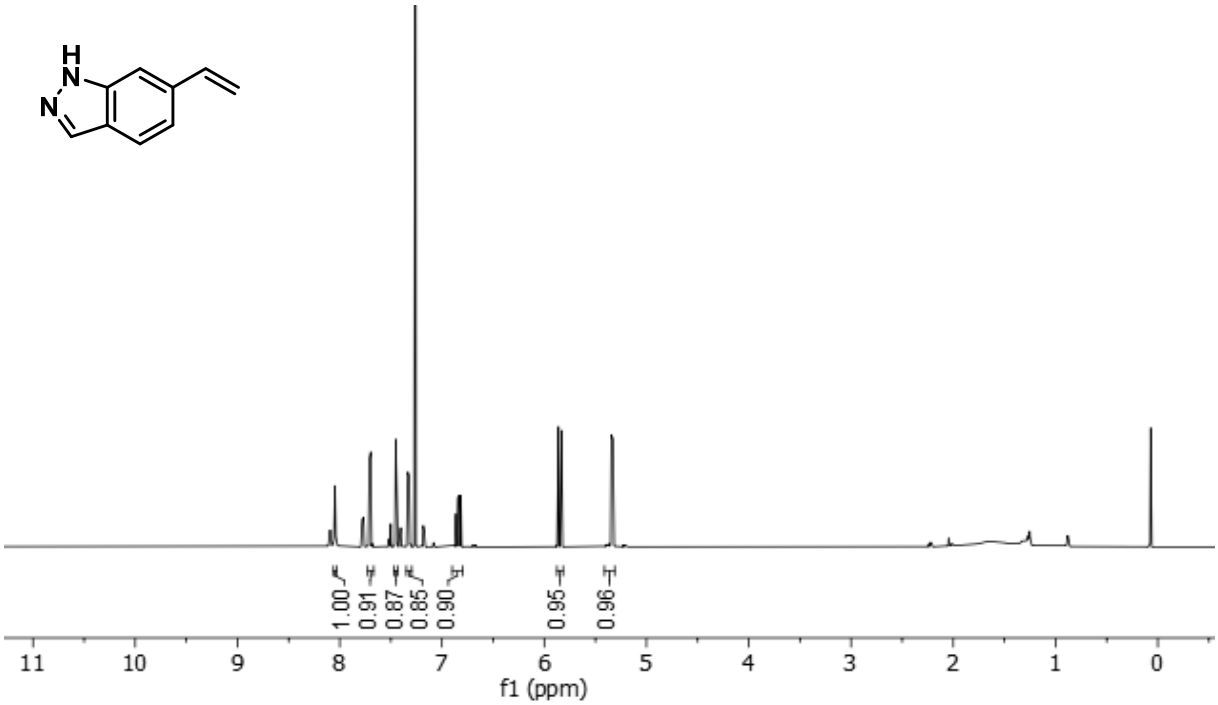
^{13}C NMR spectrum of **18a** in $CDCl_3$.



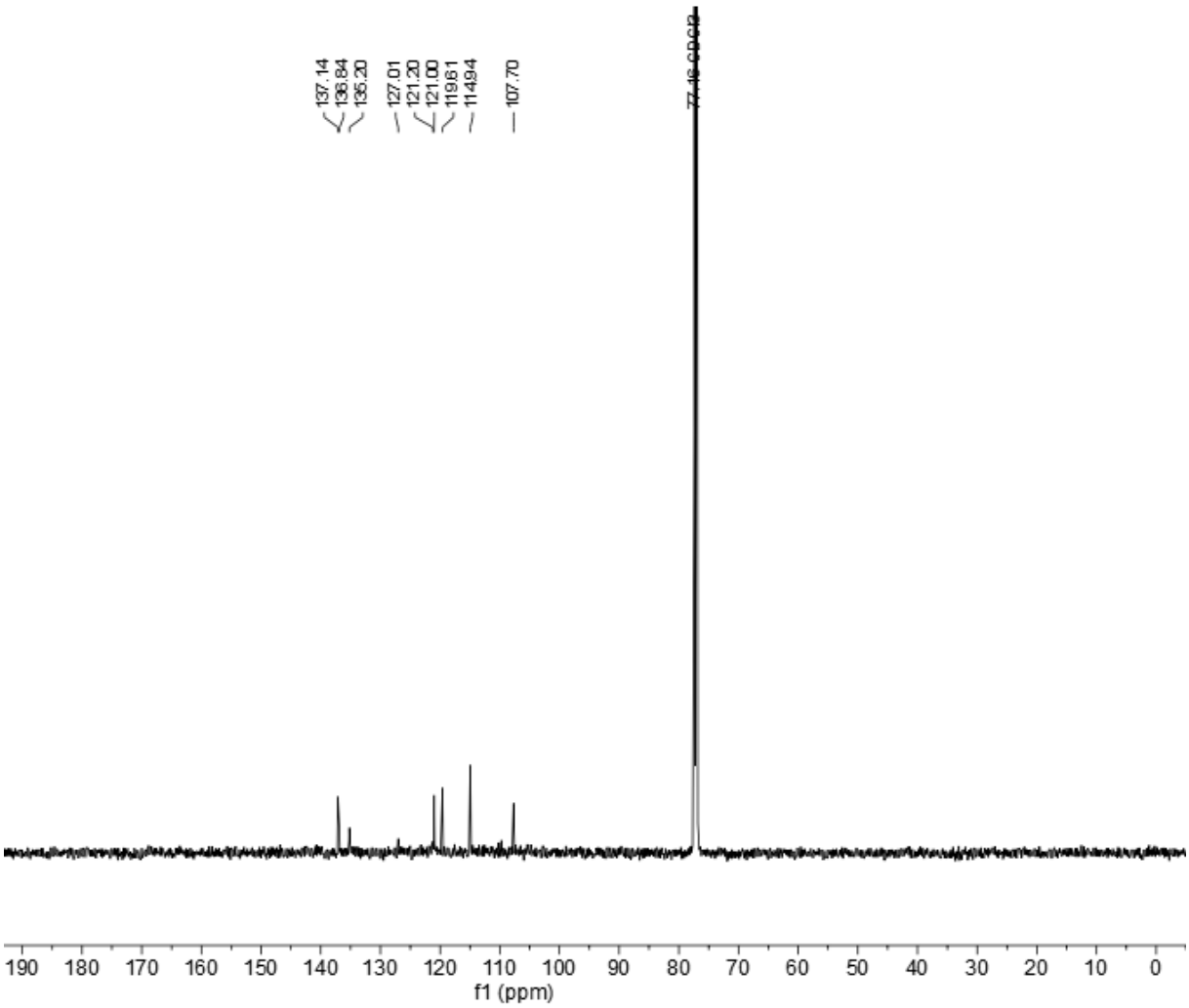
^{1}H NMR spectrum of **18b** in $CDCl_3$.



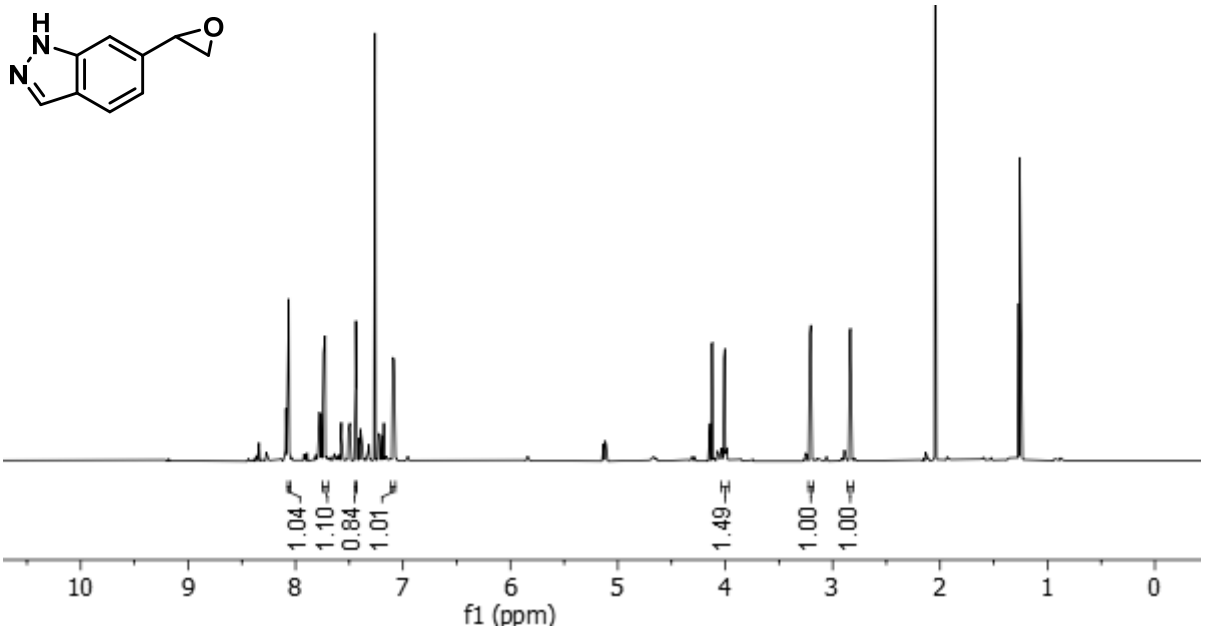
^{13}C NMR spectrum of **18b** in $CDCl_3$.



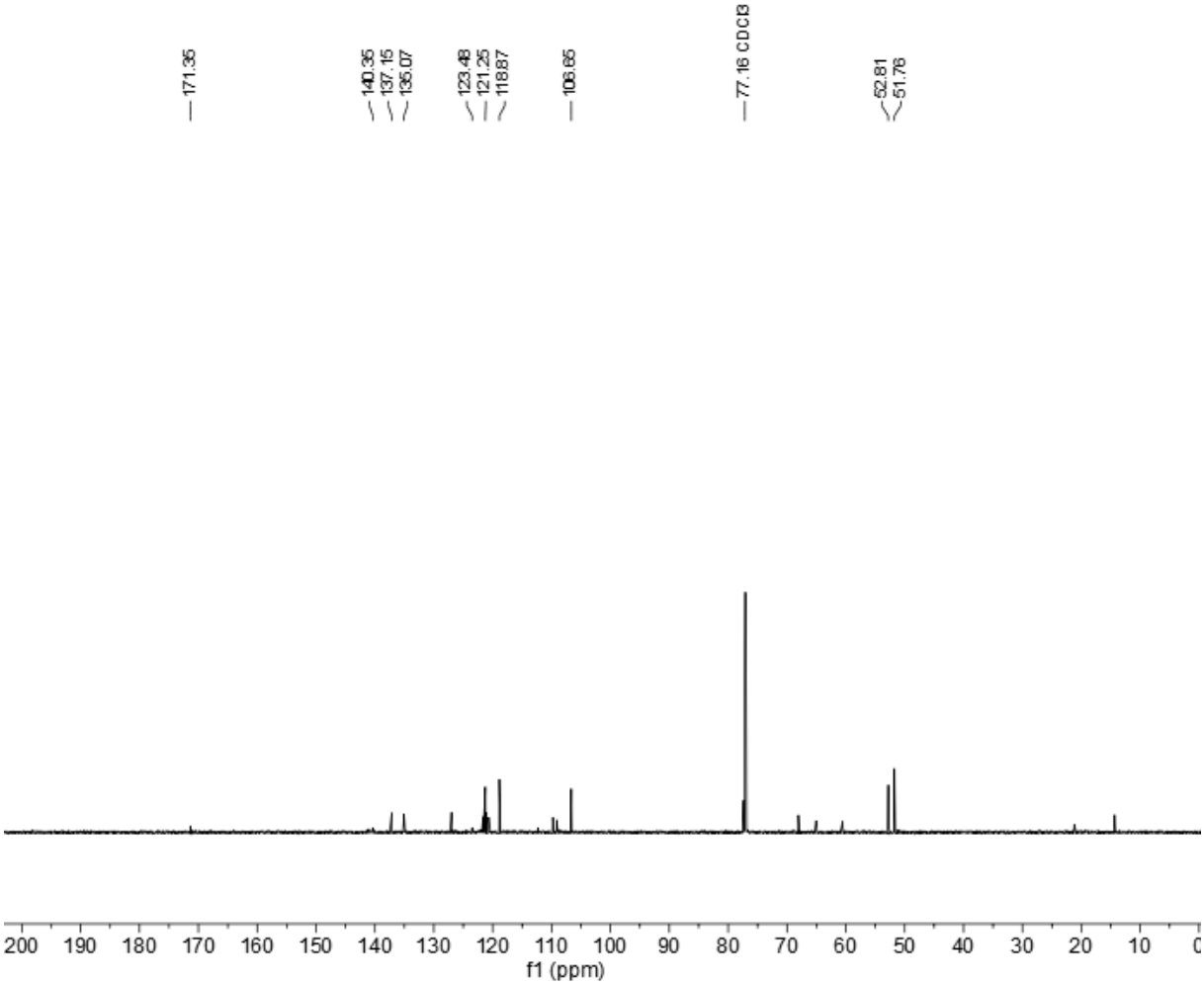
^{1}H NMR spectrum of **20** in $CDCl_3$.



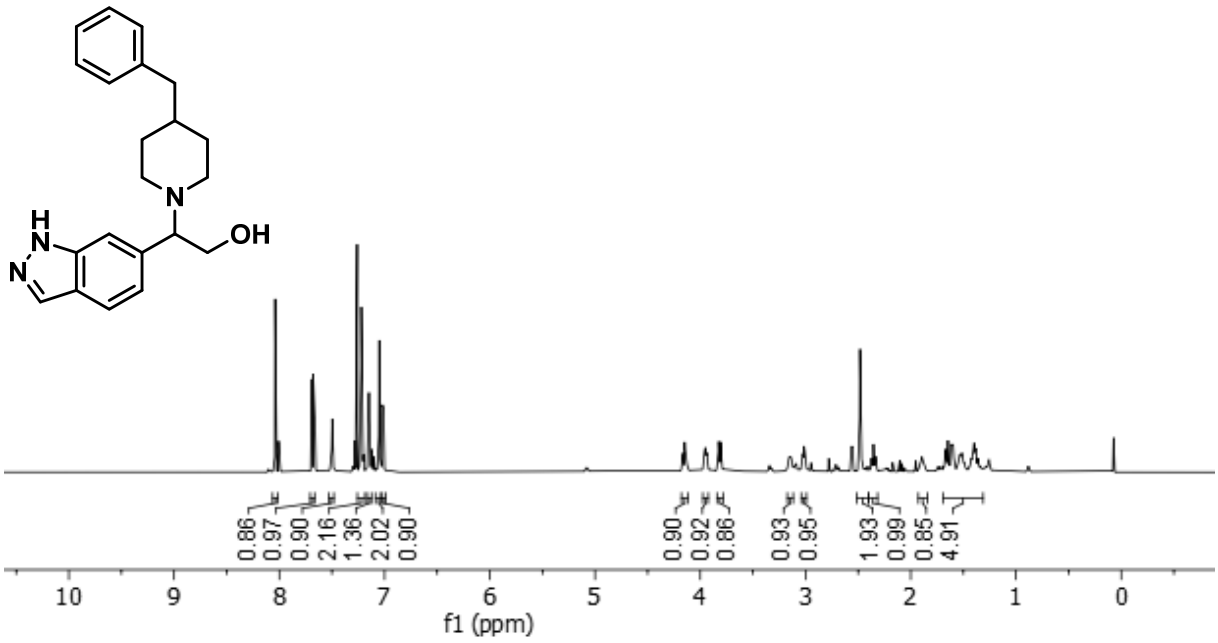
^{13}C NMR spectrum of **20** in $CDCl_3$.



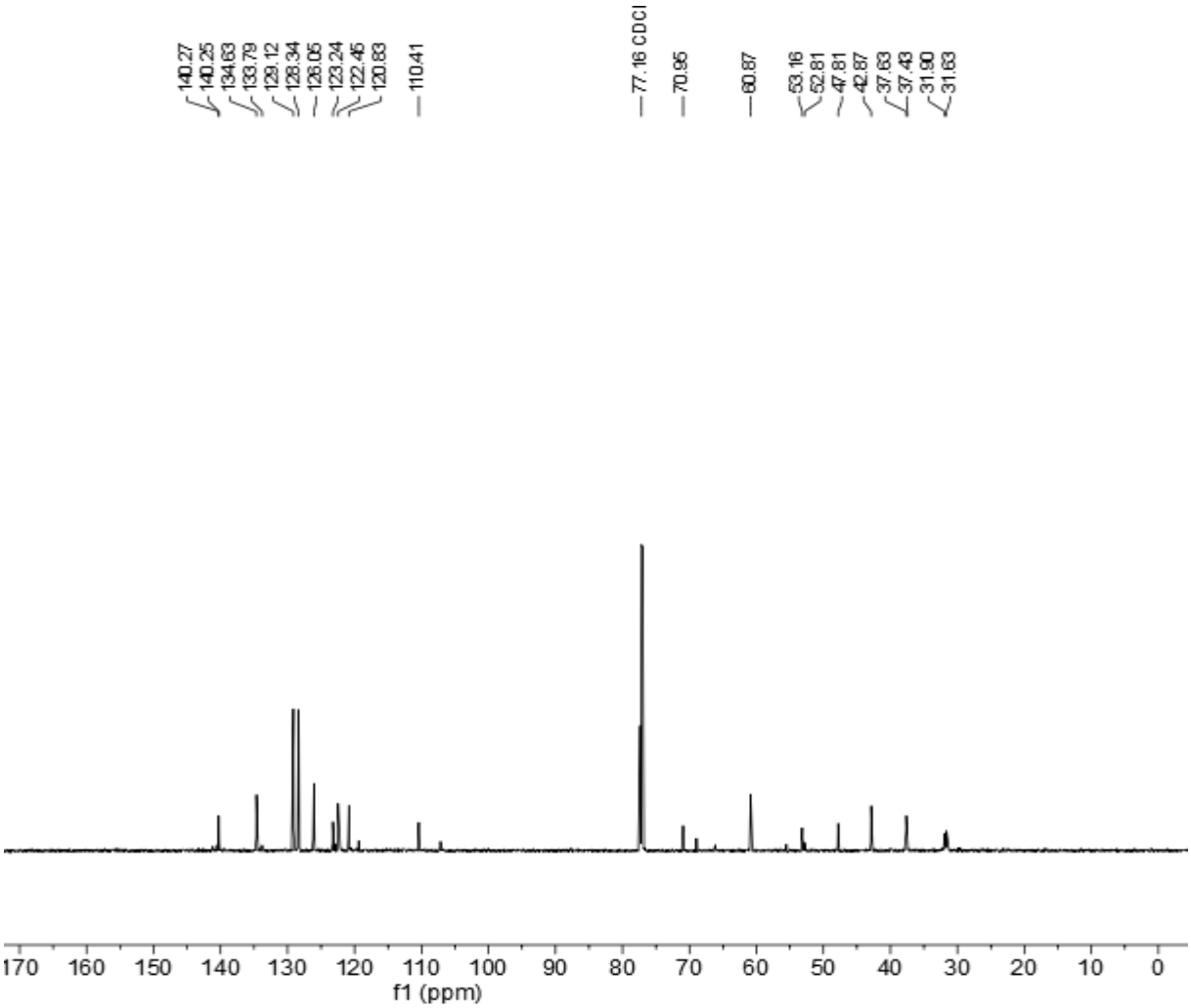
^{1}H NMR spectrum of **21** in $CDCl_3$.



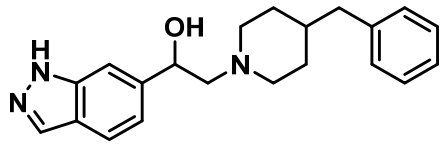
^{13}C NMR spectrum of **21**in $CDCl_3$.

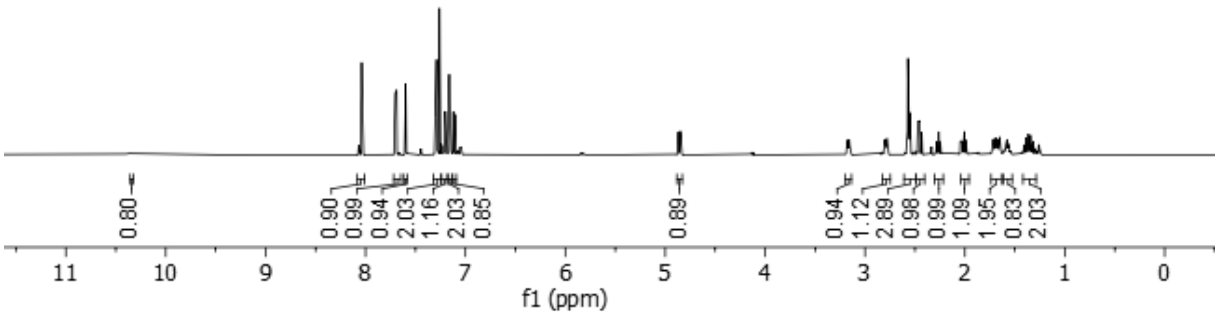


^{1}H NMR spectrum of **22a** in $CDCl_3$.

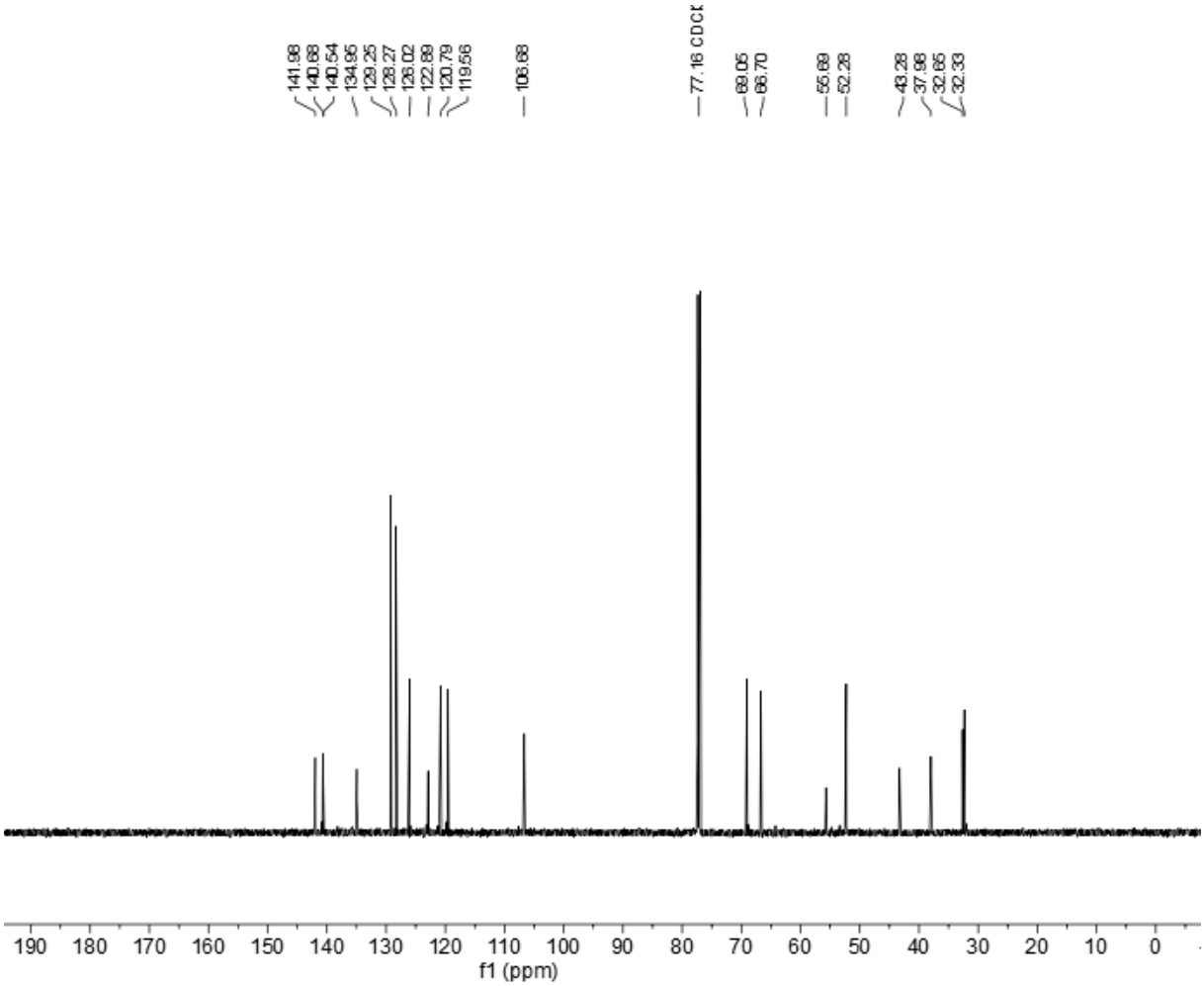


^{13}C NMR spectrum of **22a** in $CDCl_3$.

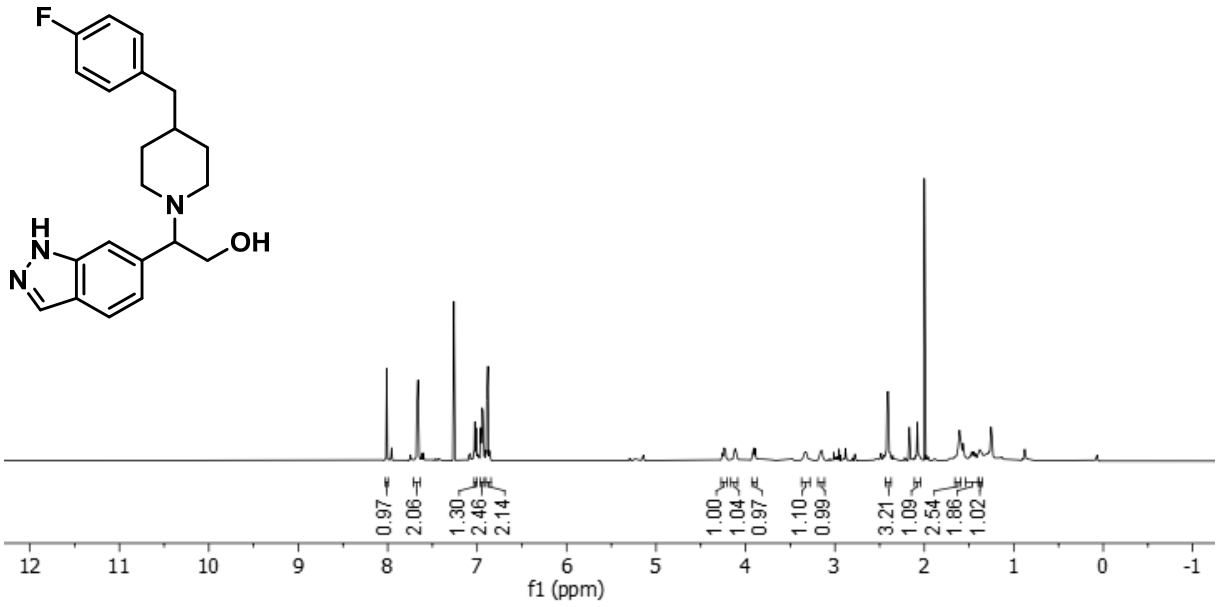




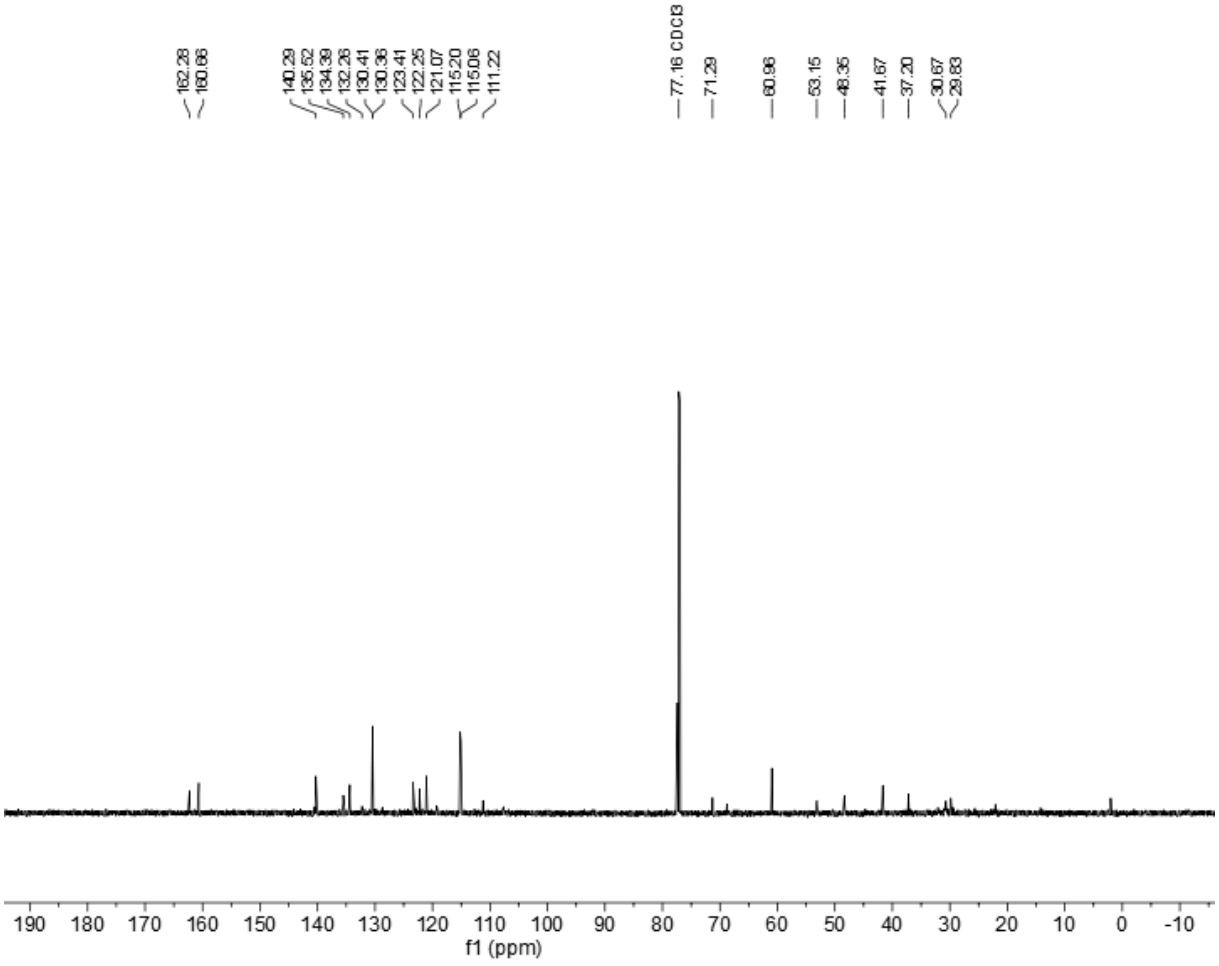
^{1}H NMR spectrum of **23a** in $CDCl_3$.



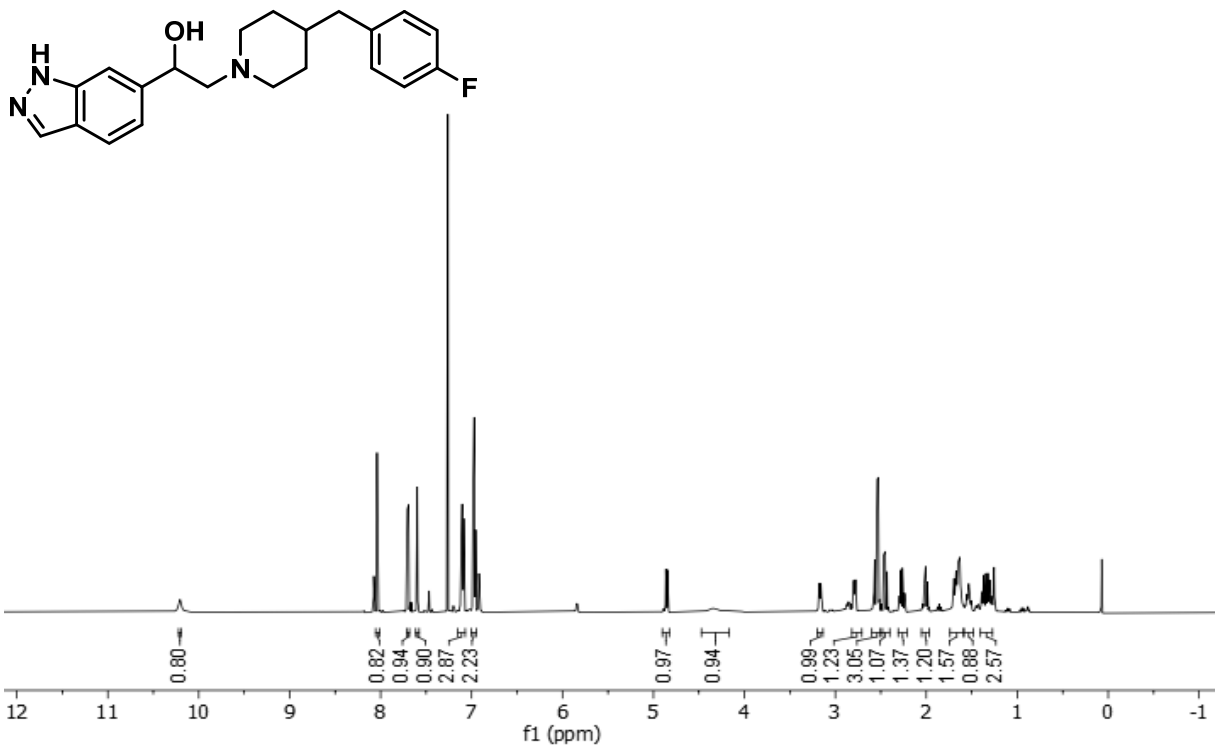
^{13}C NMR spectrum of **23a** in $CDCl_3$.



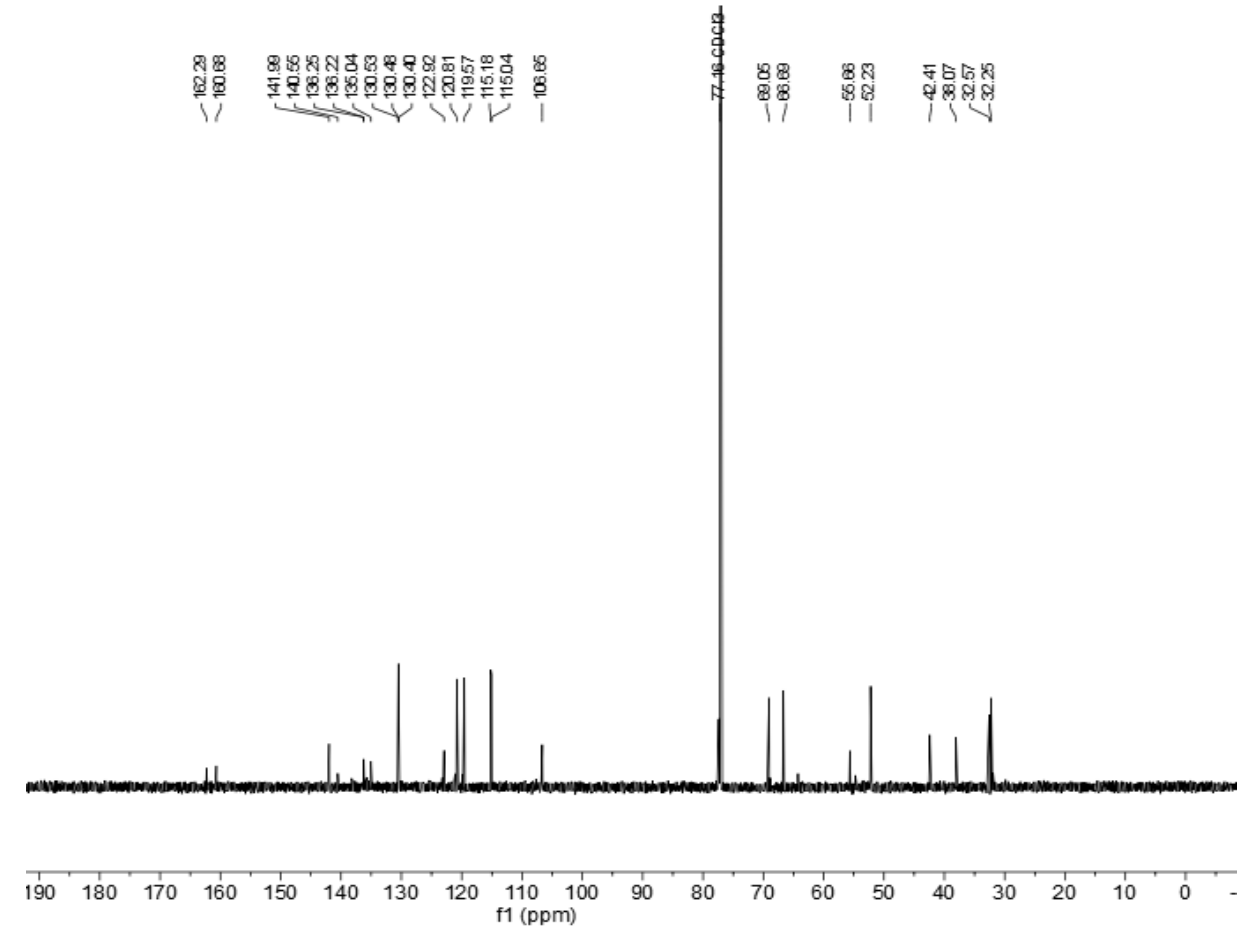
^{1}H NMR spectrum of **22b** in $CDCl_3$.



^{13}C NMR spectrum of **22b** in $CDCl_3$.



^{1}H NMR spectrum of **23b** in $CDCl_3$.



^{13}C NMR spectrum of **23b** in $CDCl_3$.