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Supplementary information

Novel quinoline-based fluorescent bioimaging probe, KSNP117, for sentinel lymph node mapping

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Supplementary information

Table of Contents

Experimental Section

General information

All reagents and solvents used for chemical synthesis and biological evaluation were purchased from commercial suppliers and used with no further purification. Reaction were monitored by thin layer chromatography (TLC) on aluminum TLC plates, F_{254} fluorescent-coated silica gel 60 (Merk). Flash column chromatography was performed using silica gel 60 (220-240 mesh). Nuclear magnetic resonance (NMR) were recorded on a 400 AMX Bruker Advance (Germany) at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR in $CDCl_3$ solution at room temperature. The chemical shifts (δ) were scored in parts per million (ppm) on the scale by using tetramethylsilane as an internal standard. Coupling constants (*J*) were recorded in Hz, and the spin multiplicities were expressed by s (singlet), d (doublet), dd (doublet of doublet), t (triplet), and m (multiplet). The absorption spectra was detected on a Jasco V-730 (UV-Vis) spectrophotometer (Japan), using quartz cuvettes of 10 mm path length in the wavelength range of 300-600 nm. Fluorescence measurements were performed on a FlexStation 3 spectrofluorophotometer (Molecular Devices, USA), using black clear-bottom 96 well plate at room temperature in the wavelength range of 450-800 nm. High-resolution mass spectrum (HRMS) was obtained by Time-of-Flight (TOF) Mass spectrometer (Shimadzu, Japan). High-performance liquid chromatography (HPLC) was used to determine the purity of KSNP 117. HPLC was performed on CORTECS C18 1.6 µm (2.1 x 100 mm) column (USA) by using a gradient elution of acetonitrile in water with a flow rate of 0.3 mL/min.

Synthesis

6-Methoxy-2-methylquinoline (**1**). A mixture of 4-methoxy aniline (5 g, 40.5 mmol) and hydrochloric acid (6 M, 100 mL) was heated to 110°C. Crotonaldehyde (5.69 g, 81 mmol) was then added dropwise as a solution in toluene (25 mL) and the reaction mixture was heated for another 2 hours. After cooling, the aqueous layer was separated and neutralized by Na_2CO_3. The solution was then extracted with ethyl acetate (3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 5:1, v/v) to afford **1** as white solid (4.6 g, 66%). ^{1}H NMR (400 MHz) δ 7.97 (d, *J*= 8.8 Hz, 1H), 7.94 (d, *J*= 9.2 Hz, 1H), 7.36 (dd, *J*= 2.8 Hz, 9.2 Hz, 1H), 7.25 (s, 1H), 7.07 (d, *J*= 2.8 Hz, 1H), 3.95 (s, 3H), 2.74 (s, 3H). ^{13}C NMR (100 MHz) δ 157.0, 156.0, 143.7, 134.8, 129.8, 127.2, 122.0, 121.7, 105.0, 55.2, 24.8.
6-Methoxyquinoline-2-carbaldehyde (**2**). Compound **1** (500 mg, 2.9 mmol) was added to a stirred suspension of selenium (IV) dioxide (641 mg, 5.8 mmol) in 10 mL dioxane. The mixture was then heated to 80°C for 6 hours under N_2 atmosphere. The mixture was filtered through Celite and washed once with water. Solvents were removed in vacuo, and the residue was purified by column chromatography (*n*-hexane/ ethyl acetate 6:1, v/v) affording compound **2** as a white solid (438 mg, 81%), ^{1}H NMR (400 MHz) δ 10.21 (s, 1H), 8.21 (d, *J*= 8.8 Hz, 1H), 8.16 (d, *J*= 9.2 Hz, 1H), 8.03 (d, *J*= 8.4 Hz, 1H), 7.50 (dd, *J*= 2.8 Hz, 9.2 Hz, 1H), 7.17 (d, *J*= 2.8 Hz, 1H), 4.01 (s, 3H). ^{13}C NMR (100 MHz) δ 193.6, 160.0, 150.7, 144.1, 135.7, 131.9, 131.7, 123.7, 117.9, 105.0, 55.7.

N,6-dimethoxy-*N*-methylquinoline-2-carboxamide (**3**). (i) A solution of sodium dihydrogen phosphate (4.10 g, 34.2 mmol) and sodium chlorite (3.87 g, 42.7 mmol) in water (10 mL) was added carefully to a stirred solution of 6-methoxyquinoline-2-carbaldehyde (**2**) (0.8 g, 4.27 mmol) in *tert*-butanol (20 mL). The reaction mixture was stirred at room temperature for 2 h. The mixture was acidified to pH 4 and the product was extracted by ethyl acetate (3 × 20 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude residue (865 mg, 100%) was used without further purification for the next step. (ii) To a stirred DMF solution (1 mL) of the resulting product (0.5 g, 2.46 mmol) was added *N*,*O*-dimethylhydroxyamine hydrochloride (195 mg, 3.20 mmol), trimethylamine (747 mg, 7.38 mmol), and HATU (1.03 g, 2.71 mmol). The reaction mixture was stirred for 6 h at room temperature. The mixture was then poured into water, and the product was extracted by ethyl acetate (3 × 20 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by silica gel chromatography (*n*-hexane/ethyl acetate 1:1, v/v) affording **3** as a pale-yellow powder (390 mg, overall 64%), ^{1}H NMR (400 MHz) δ 8.13 (d, *J*= 8.8 Hz, 1H), 8.03 (d, *J*= 9.2 Hz, 1H), 7.72 (brs, 1H), 7.41 (dd, *J*= 2.8 Hz, 9.2 Hz, 1H), 7.10 (d, *J*= 2.4 Hz, 1H), 3.95 (s, 3H), 3.82 (s, 3H), 3.49 (s, 3H). ^{13}C NMR (100 MHz) δ 158.6, 151.1, 150.4, 142.8, 135.3, 131.2, 129.6, 122.9, 120.6, 104.9, 61.4, 55.6, 38.6.
1-(6-Methoxyquinolin-2-yl)ethan-1-one (**4**). Methyl magnesium bromide (1.848 mL, 1.848 mmol, 1M in THF), was added dropwise to a solution of **3** (350 mg, 1.42 mmol) in THF (10 mL) at 0° C. The mixture was stirred at 0° C for 1 h and at room temperature for 12 hr. The mixture was then poured into water and the product was extracted by ethyl acetate (5 × 20 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by silica gel chromatography (*n*-hexane/ethyl acetate 5:1, v/v) to afford **4** as a white powder (206 mg, 72%), ^{1}H NMR (400 MHz) δ 8.12-8.06 (m, 3H), 7.41 (dd, *J*= 2.8 Hz, 9.2 Hz, 1H), 7.09 (d, *J*= 2.8 Hz, 1H), 3.95 (s, 3H), 2.84 (s, 3H). ^{13}C NMR (100 MHz) δ 200.5, 159.5, 151.3, 143.2, 135.3, 132.1, 131.0, 123.1, 118.4, 104.9, 55.7, 25.5.
Tert-butyl (4-iodophenyl)(methyl)carbamate (**6**). Sodium hydride (180 mg, 7.5 mmol) and methyl iodide (0.8 g, 5.6 mmol) were added to a stirred solution of **5** (1.2 g, 3.8 mmol) in THF (10mL) at 0° C. The reaction mixture was stirred at room

temperature for 6 hr. The reaction mixture was poured into water, followed by extraction with methylene chloride (3 × 15 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (*n*-hexane/ ethyl acetate 10:1, v/v) to give **6** as a colorless oil (1.07 g, 85%), ^{1}H NMR (400 MHz) δ 7.62 (d, *J*= 8.4 Hz, 2H), 7.00 (d, *J*= 8.4 Hz, 2H), 3.23 (s, 3H), 1.45 (s, 9H). ^{13}C NMR (100 MHz) δ 154.4, 143.6, 137.6, 127.3, 89.5, 80.7, 37.1, 28.3.

4-Iodo-N-methylaniline (**7**). TFA (1.24 mL, 16.2 mmol) was cautiously added to a stirred solution of **6** (1.07 g, 3.2 mmol) in DCM (5 mL) at 0° C. The reaction mixture was allowed to stir for 2 h at room temperature. The mixture was neutralized carefully with saturated aqueous $NaHCO_3$ solution (pH 7-8). Then, the mixture was poured into water and extracted with DCM (3 x 15 mL). The organic extract was dried over anhydrous Na_2SO_4 and concentrated under vacuum. The residue was purified by 10% ethyl acetate in *n*-hexane to form **7** as a colorless oil (745 mg, 99%), ^{1}H NMR (400 MHz) δ 7.42 (d, *J*= 8.8 Hz, 2H), 6.38 (d, *J*= 8.8 Hz, 2H), 3.72 (brs, 1H), 2.79 (s, 3H). ^{13}C NMR (100 MHz) δ 148.9, 137.7, 114.6, 77.7, 30.6.

2-((4-Iodophenyl)(methyl)amino)ethan-1-ol (**8**). 2-iodoethanol (0.8 mL, 9.55 mmol) and potassium carbonate (880 mg, 6.37 mmol) were added to a 1 mL DMF solution of **7**. The reaction mixture was stirred at 70° C for 4 h. The mixture was poured into water and extracted with DCM (3 x 15 mL). The combined organic extracts were dried over anhydrous Na_2SO_4, and the solvents were removed under reduced pressure. Column chromatography (*n*-hexane/ ethyl acetate 3:1, v/v) was used to purify the product **8** as a pale-yellow solid (550 mg, 62%), ^{1}H NMR (400 MHz) δ 7.45 (d, *J*= 9.2 Hz, 2H), 6.54 (d, *J*= 9.2 Hz, 2H), 3.77 (t, *J*= 5.6 Hz, 2H), 3.43 (t, *J*= 5.6 Hz, 2H), 1.85 (brs, 1H). ^{13}C NMR (100 MHz) δ 149.5, 137.7, 115.1, 77.9, 60.0, 55.1, 38.8.

(*E*)-3-(4-((2-Hydroxyethyl)(methyl)amino)phenyl)acrylaldehyde (**9**). Acrolein diethyl acetal (0.89 mL, 5.85 mmol), potassium carbonate (404 mg, 2.92 mmol), potassium chloride (145 mg, 1.95 mmol), tetrabutyl ammonium acetate (1175 mg, 3.90 mmol), and palladium acetate (13.1 mg, 0.06 mmol) were added to a stirred solution of **8** in 2 mL DMF. The reaction mixture was stirred for 3 h at 90° C. After cooling, 2N HCl was slowly added, and the mixture was further stirred at room temperature for 30 min. The product was then extracted with DCM (3 x 20mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by column chromatography (*n*-hexane/ ethyl acetate 2:1 to 1:1, v/v) as an orange solid (135 mg, 34%). ^{1}H NMR (400 MHz) δ 9.54 (d, *J*= 8.0 Hz, 1H), 7.43 (d, *J*= 9.2 Hz, 2H), 7.35 (d, *J*= 15.6 Hz, 1H), 6.75 (d, *J*= 8.8 Hz, 2H), 6.49 (dd, *J*= 8.0, 15.6 Hz, 1H), 3.86 (t, *J*= 5.6 Hz, 2H), 3.60 (t, *J*= 5.6 Hz, 1H), 3.10 (s, 3H), 2.33 (brs, 1H). ^{13}C NMR (100 MHz) δ 193.9, 154.0, 151.9, 130.7, 123.7, 122.0, 111.9, 60.1, 54.5, 39.0.

(2*E*,4*E*)-5-(4-((2-Hydroxyethyl)(methyl)amino)phenyl)-1-(6-methoxyquinolin-2-yl)penta-2,4-dien-1-one (**KSNP 117**). The methyl ketone **4** (55 mg, 0.27 mmol) and the conjugated aldehyde **9** (56.1 mg, 0.27 mmol) were dissolved in methanol (3 mL). Sodium hydroxide (11 mg, 0.27 mmol) was added, and the mixture was allowed to stir at room temperature for 12 hr. The solid was filtered and washed with cold methanol. Further purification was performed by column chromatography (*n*-hexane/ ethyl acetate 2:1 to 1:1, v/v) to obtain the final probe as a red solid (89 mg, 84%). ^{1}H NMR (400 MHz) δ 8.62 (d, *J*= 8.8 Hz, 1H), 8.29 (d, *J*= 8.8 Hz, 1H), 8.19 (d, *J*= 9.2 Hz, 1H), 7.91 (d, *J*= 15.2 Hz, 1H), 7.81-7.75 (m, 1H), 7.59 (d, *J*= 9.6 Hz, 1H), 7.41 (d, *J*= 8.8 Hz, 2H), 7.33-7.29 (m, 1H), 7.01 (d, *J*= 15.2 Hz, 1H), 6.96 (d, *J*= 15.6 Hz, 1H), 6.74 (d, *J*= 8.8 Hz, 2H), 4.10 (s,3H, OCH_3), 3.86 (t, *J*= 5.4 Hz, 2H), 3.55 (t, *J*= 5.6 Hz, 2H), 3.05 (s, 3H, NCH_3), 1.80 (t, *J*= 5.4 Hz, 1H, OH). ^{13}C NMR (100 MHz) δ 189.1, 154.2, 152.9, 150.5, 146.3, 143.2, 142.8, 132.8, 130.7, 129.1, 128.5, 124.9, 123.4, 121.8, 120.2, 116.8, 116.0, 112.3, 60.3, 57.0, 54.7, 38.9. HRMS *m / z*: calcd for $C_{24}H_{24}N_2O_3$, 389.1787; found, 389.1865, M + H^+.

Stability test

Changes in FLI signal intensity of 10 μM KSNP117 were examined using a IVIS Lumina III (Perkin Elmer). KSNP117 (10 μL) was incubated in solutions of DMSO, FBS, and 10% BSA (90 μL) at 37°C for 5 min and 60 min. Changes in signal intensity for these different solutions were monitored by use of fluorescent imaging (FLI).

Animals

Specific pathogen-free six-week-old, female Balb/c nude mice were obtained from SLC, Inc. (Shizuoka, Japan). All animal experimental procedures were conducted in strict accordance with the appropriate institutional guidelines for animal research. The study protocol was approved by the Committee on the Ethics of Animal Experiments of the Laboratory Animal Center of Daegu-Gyeongbuk Medical Innovation Foundation (approval no. DGMIF-20032403-00).

Cells

Chinese hamster ovary (CHO) cells were grown in RPMI 1640 medium (Hyclone, Logen UT, USA) supplemented with 10% fetal bovine serum (FBS: Hyclone) and 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA). Murine macrophage cells (Raw264.7) were grown in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 1.0 M β-mercaptoethanol (Sigma).

Cell proliferation assay

Cell proliferation assay was performed using a Cell Counting Kit (CCK-8, Dojindo Laboratories, Tokyo, Japan). CHO and Raw 264.7 cells were seeded at 1×10^4 cells per well in 96-well plates, followed by incubation with various concentration of KSNP117 for 24 h. Ten microliters of CCK-8 solution were added to each well at 24 h after incubation with various concentration of KSNP117, and then the plate was incubated at 37°C for 1 h. Absorbance at 450 nm was measured using a microplate reader (BMG Labtech, Offenburg, Germany).

ROS assay

ROS assay was performed using a ROS-Glo H_2O_2 assay (Promega, USA). CHO and Raw 264.7 cells were seeded at 1×10^4 cells per well in 96-well plates, followed by incubation with various concentration of KSNP117 for 24 h. Twenty microliters of H_2O_2 substrate solution were added to each well for 6 h, and then ROS-Glo detection solution was incubated at 37°C for 20 min. Luminescent signals was measured using a microplate reader (BMG Labtech, Offenburg, Germany).

Animal studies

Study 1.

Mice (n=5) received 3 mg/kg either KSNP117 or ICG via tail vein injection, and fluorescence images were acquired at 1- and 30 min post-injection. In vivo fluorescence images was acquired using IVIS Spectrum CT (PerkinElmer) at indicated times post-injection. After imaging acquisition, mice were sacrificed and all organs excised, followed by measurement of fluorescent signal in the excised organs.

Study 2.

KSNP-117 at concentrations of 1.5, 3, and 30 mg/kg and ICG at a concentration of 3 mg/kg, were separately, subcutaneously injected into the footpad of mice (N=5), and fluorescence images were acquired at indicated times post-injection. Upon imaging acquisition, mice were sacrificed and all organs, including lymph nodes, excised and imaged using an IVIS Spectrum CT.

***In vivo* fluorescence imaging**

In vivo fluorescence images were acquired using an IVIS Spectrum CT (PerkinElmer). The scan times ranged from 1 s to 5 min depending on the intensity of the emitted fluorescence signal. All in vivo fluorescence images were obtained with the following settings: Ex/Em, 465 nm/720 nm; exposure time, 1-2 s; f/stop, 2; binning, 8; and field of view, 21.8. The image was thresholder to maximize visualization of the region of interest and minimize background fluorescence. Grayscale and fluorescence color images were superimposed using LIVINGIMAGE v 2.12 (Perkin Elmer) and IGOR Image Analysis FX (Wave Metrics, Lake Oswego, OR, USA) software. Signal intensity is expressed in units of Total Radiant Efficiency [p/s] / [μW/cm²].

Fluorescence Microscopy

After killing, sentinel lymph nodes and control lymph nodes were removed and frozen at -80°C. Frozen section (30 μm) of all the lymph nodes were prepared after embedding in Tissue Tek ® O.C.T. compound (Sakura Finetek, Torrance, CA, USA). Fluorescence was observed under an upright epifluorescence microscope (IX-71 Provis, Olympus, Rungis, France) equipped with a 100-W mercury vapor lamp and a Peltier cooled CCD camera (DP71, Olympus). The filter set used consisted of a 400- to 440-nm band-pass excitation filter, a 570-nm dichromic mirror, and a 590-nm long-pass filter. Fluorescence images were recorded at a magnification of ×40.

Acute toxicity study

To determine the acute toxicity of KSNP117 *in vivo*, ICR mice were intravenously injected with either vehicle (n =7) or 3 mg/kg KSNP117 (n =8) solution. The body weight and physical activities were observed within 7 days. All mice were sacrificed, and the collected blood samples were examined for serum biochemistry analysis according to the manufacturer's instructions (TBA 120-FR; Toshiba, JP). All tissues were fixed in 10% neutral buffered formalin (BBC Biochemicals, Mount Vernon, WA, USA) for histopathological evaluation. A tissue processor (Thermo Fisher Scientific, Inc., Runcorn, UK) was used to prepare tissue samples from the formalin-fixed samples for analysis by fixing, staining, and dehydrating. The paraffin-embedded tissue blocks were cut at a 4-μm thickness and mounted onto glass slides. Staining was performed with hematoxylin and eosin by using an autostainer (Dako Coverstainner; Agilent, Santa Clara, CA, USA). Histopathological blind evaluation of the samples was performed.

Statistical analysis

All data are expressed are mean± standard deviation from at least three repeated experiments. Statistical significance was determined using unpaired Student's t-test with Graph Pad Prism version 8 statistical software (GraphPad software Inc.) Differences between analyzed groups with p-values lower than 0.05 were considered statistically significant.

Results

Table S1. Full optical characterization of KSNP117

CHARACTERISTIC	VALUE
MOLECULAR WEIGHT	388.47 g/mol
EXTINCTION COEFFICIENT	30,784$M^{-1}Cm^{-1}$
ABSORBANCE MAXIMUM	475 nm
EMISSION MAXIMUM	714 nm
STOKE SHIFT	239 nm
RELATIVE QUANTUM YIELD	8.90%
cLOGP[A]	4.42

[A] calculated using Chemdraw Ultra 12.0.

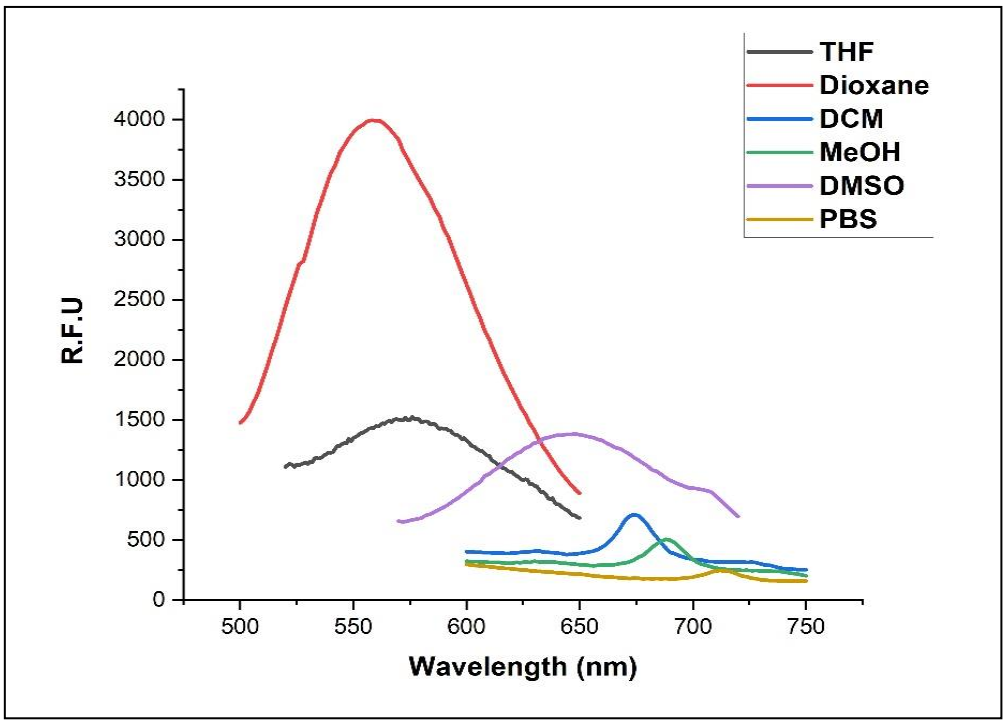


Figure S1. Fluorescence spectra of KSNP117 (10 µM) in different solvents in relation to the change in solvent polarity: Tetrahydrofuran (THF), dioxane, dichloromethane (DCM), methyl alcohol (MeOH), dimethyl sulfoxide (DMSO), and phosphate-buffered saline (PBS).

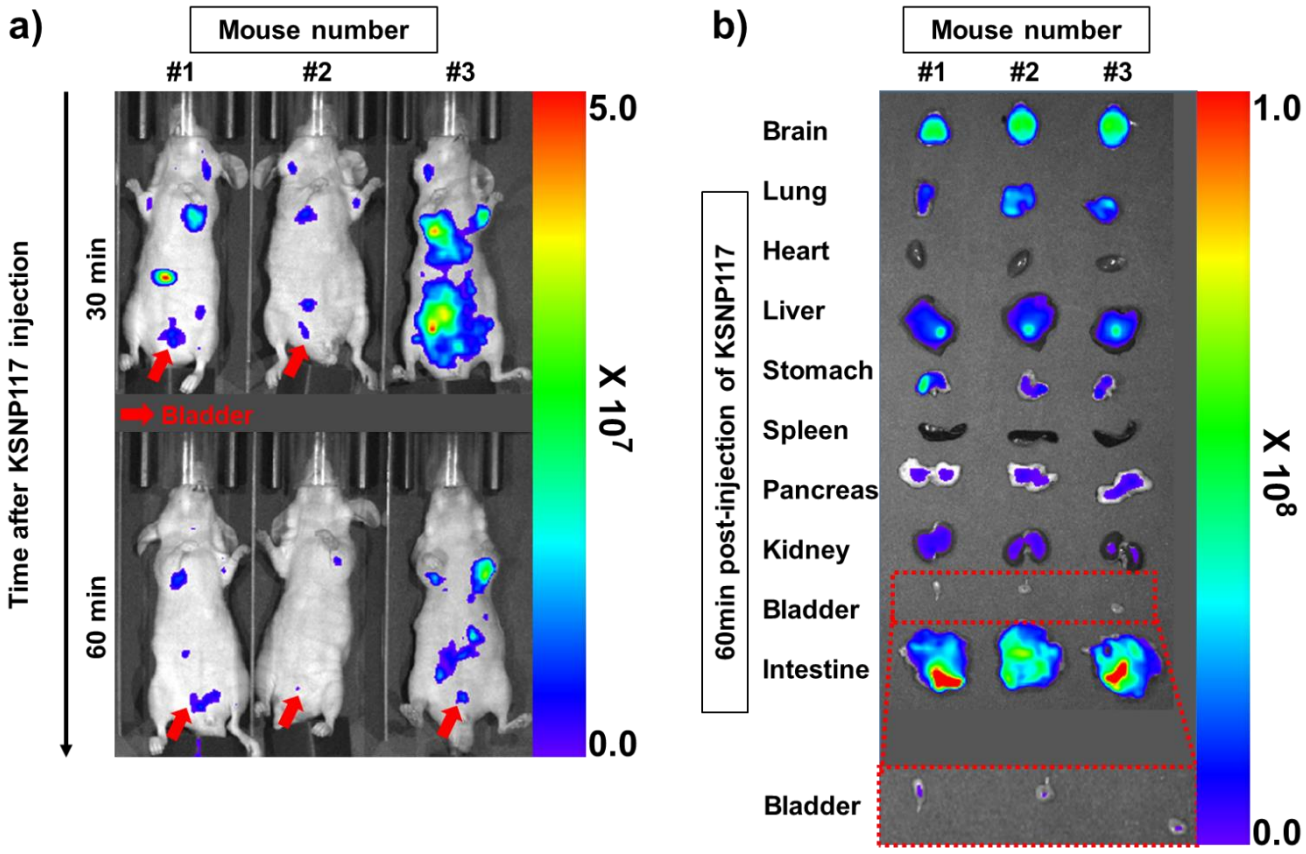


Figure S2. *In vivo* imaging of KSNP117 wash-out kinetics in kidney and bladder. (a) Representative imaging of mice after KSNP117 intravenous injection. (b) *Ex vivo* imaging of excised organs at 60 min post-injection. Inset: *ex vivo* imaging of excised bladder. The mice received 3 mg/kg KSNP117 via intravenous injection, and imaging was acquired at 30 and 60 min. The organs were excised 1 h post-injection, followed by *ex vivo* imaging.

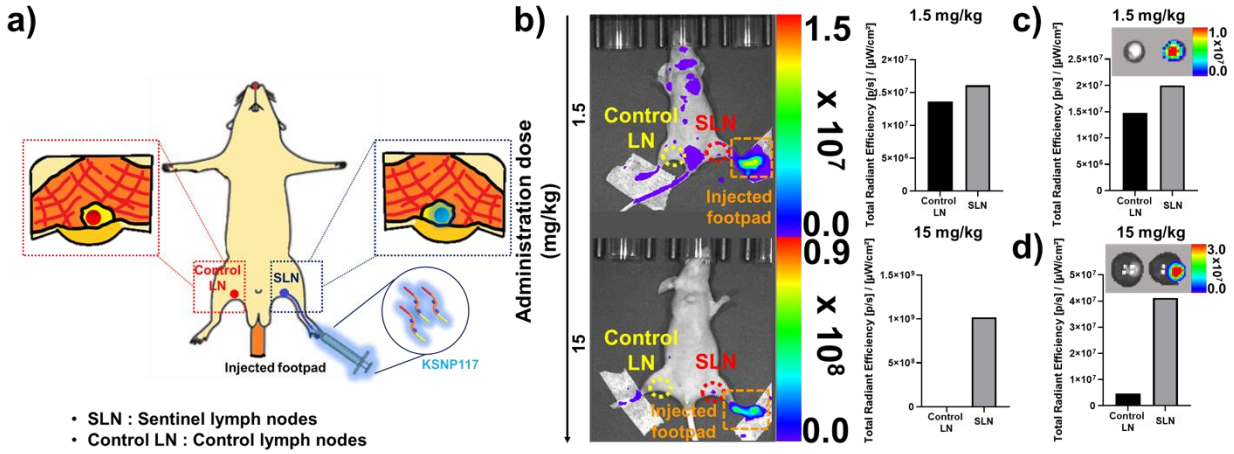


Figure S3. *In vivo* imaging of the sentinel lymph nodes (SLN) after injection with various concentrations of KSNP117. **(a)** Representative image showing the dose-dependent increase of the fluorescent imaging (FLI) signal in the SLN (left) and quantification of the FLI signal in the SLN (right). *Ex vivo* imaging of excised lymph nodes and quantification of the FLI signal in mice injected with **(b)** 1.5 mg/kg KSNP117 and **(c)** 15 mg/kg KSNP117. **(d)** Correlation between administration doses and FLI signal in the SLN. Various concentrations of KSNP117 were subcutaneously injected in the footpad of nude mice, followed by *in vivo* FLI at the indicated times. After image acquisition, the organs were excised and *ex vivo* imaging was performed. Data are presented as the mean ± standard deviation.

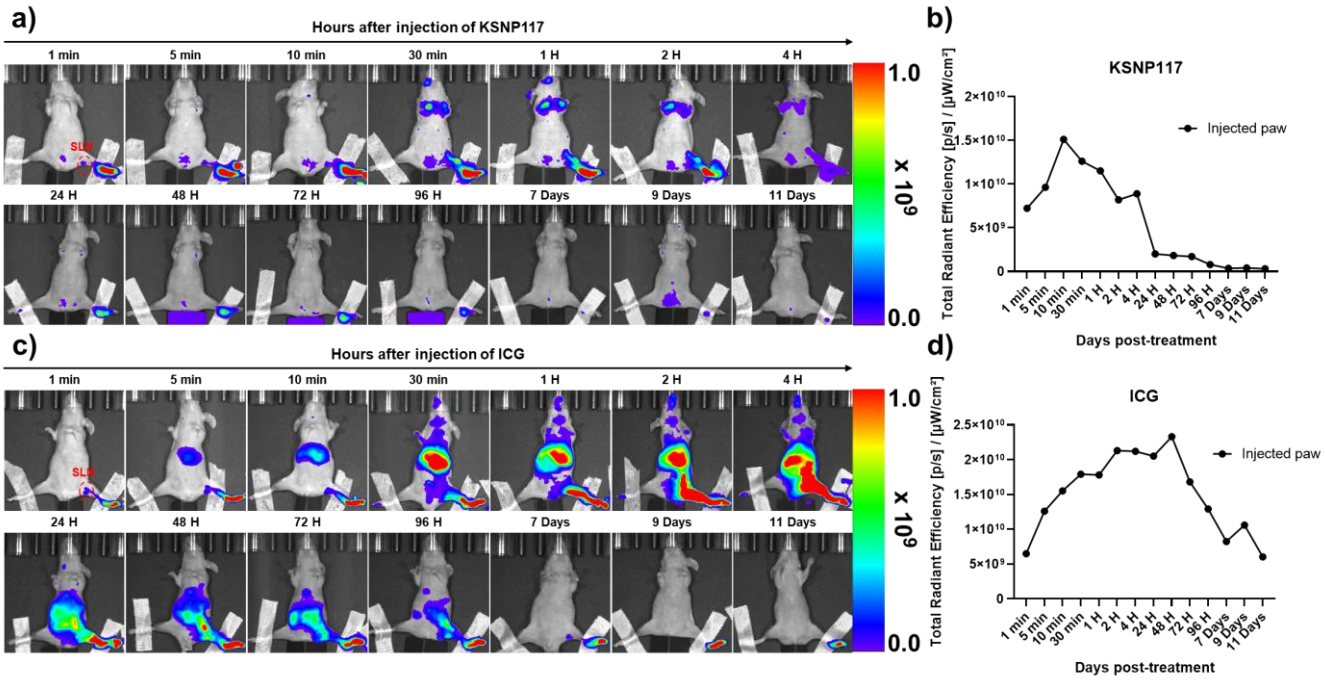
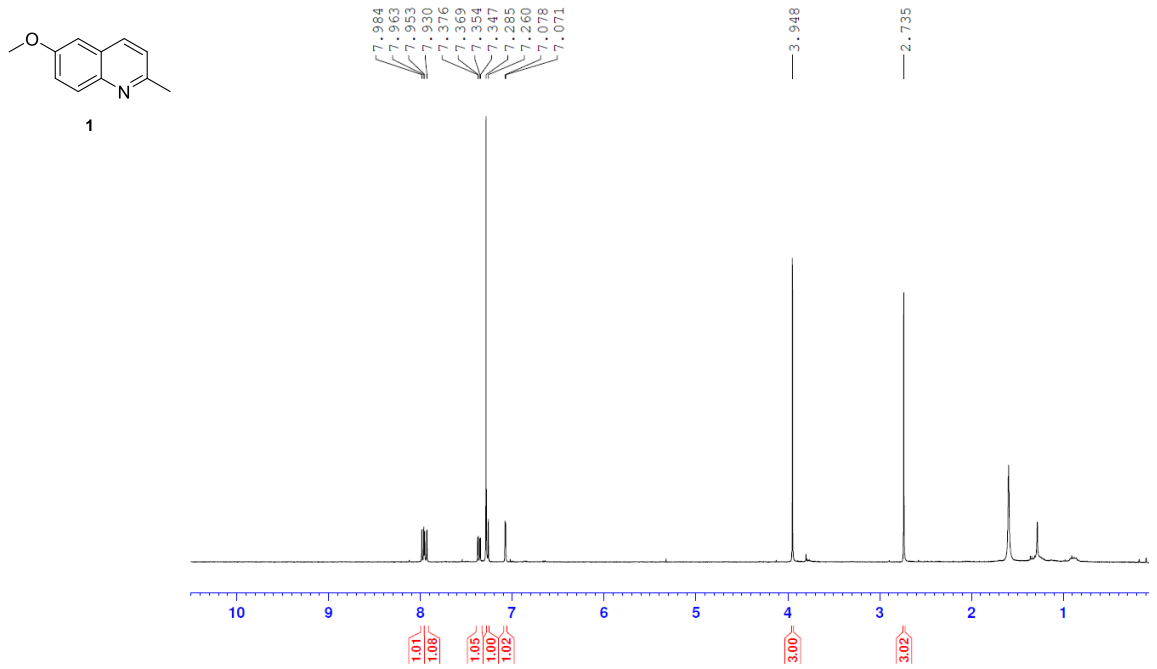


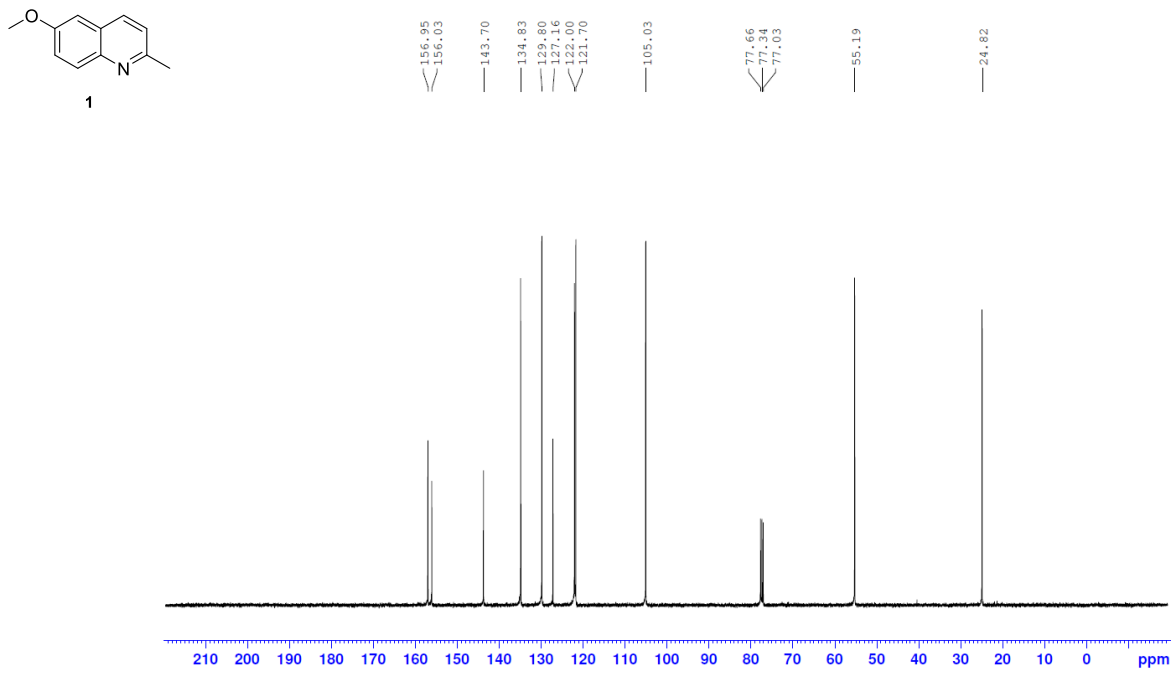
Figure S4. *In vivo* imaging of sentinel lymph nodes (SLN) after footpad injection with KSNP117 or ICG. (a) KSNP117 (3 mg/kg) or (c) ICG (3 mg/kg) were subcutaneously injected into the footpad of nude mice, and *in vivo* FLI was performed at the indicated times. (b and d) Quantification of FLI signals in the injected footpad.

NMR spectra of compounds

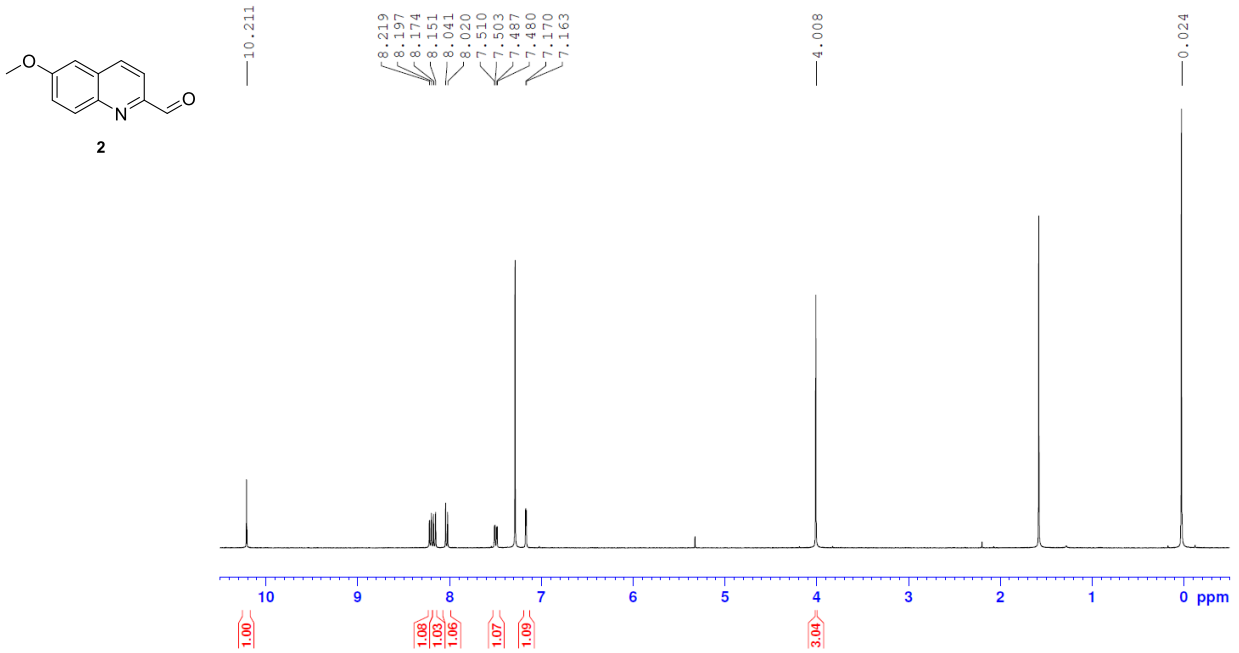
^{1}H-NMR ($CDCl_3$) spectrum of compound **1**



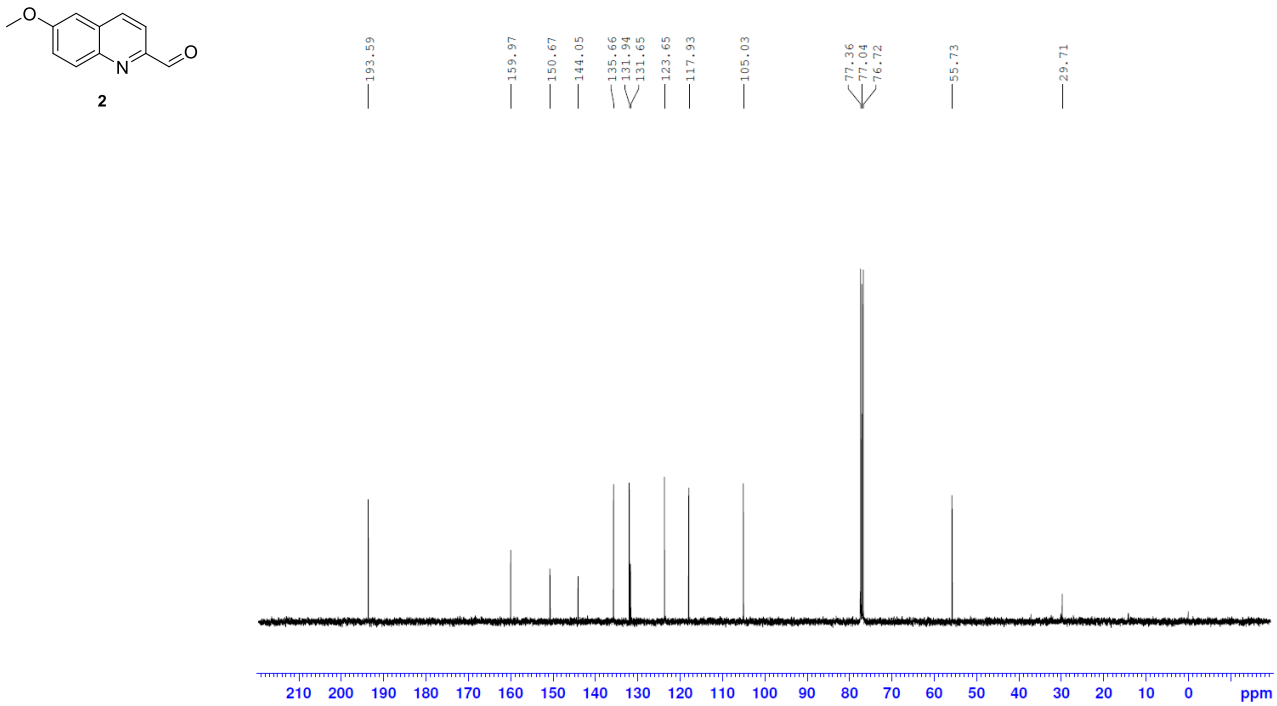
^{13}C-NMR ($CDCl_3$) spectrum of compound **1**



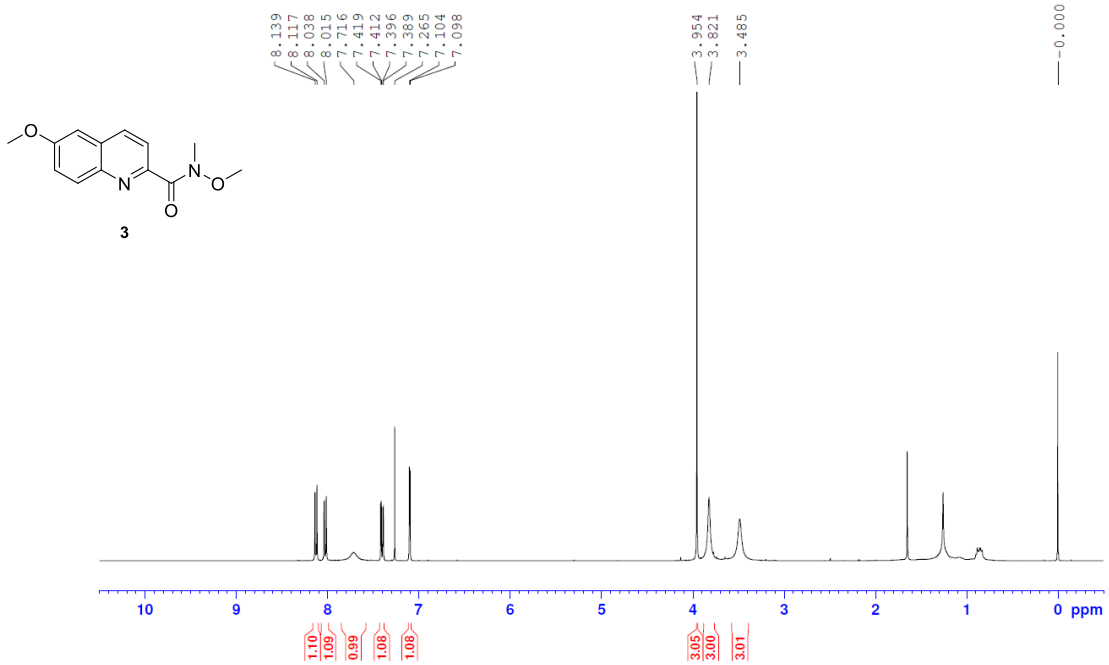
^{1}H-NMR ($CDCl_3$) spectrum of compound **2**



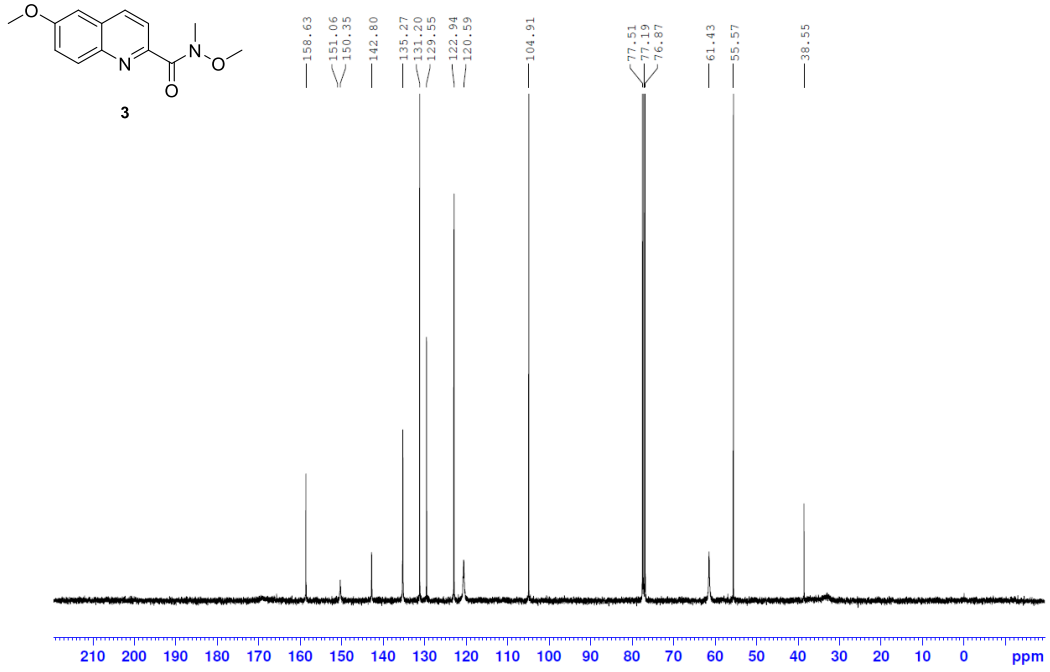
^{13}C-NMR ($CDCl_3$) spectrum of compound **2**



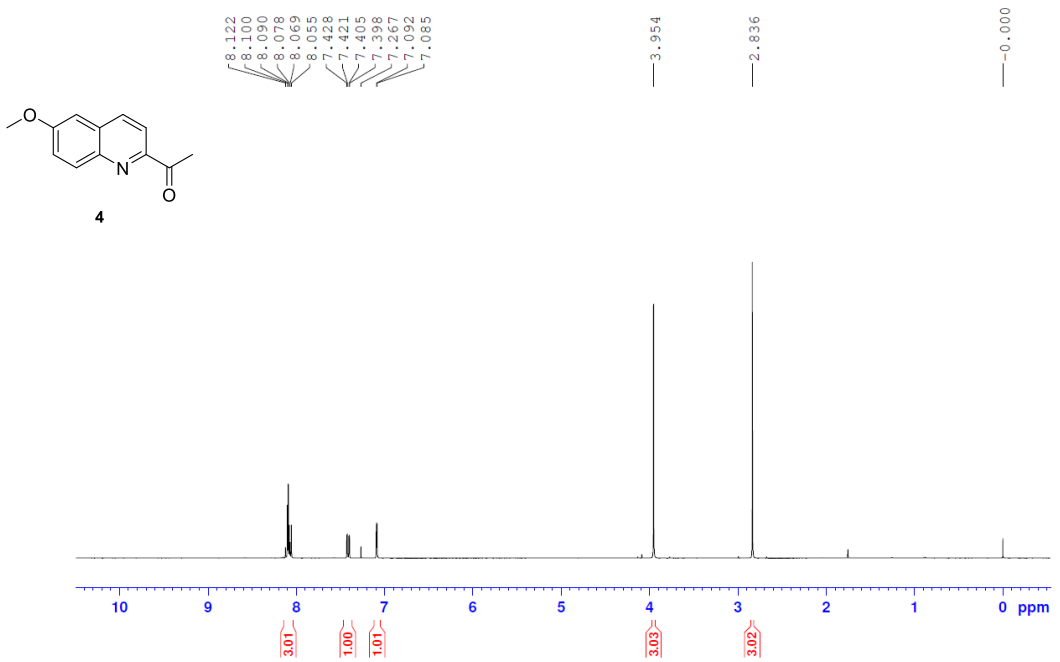
^{1}H-NMR ($CDCl_3$) spectrum of compound **3**



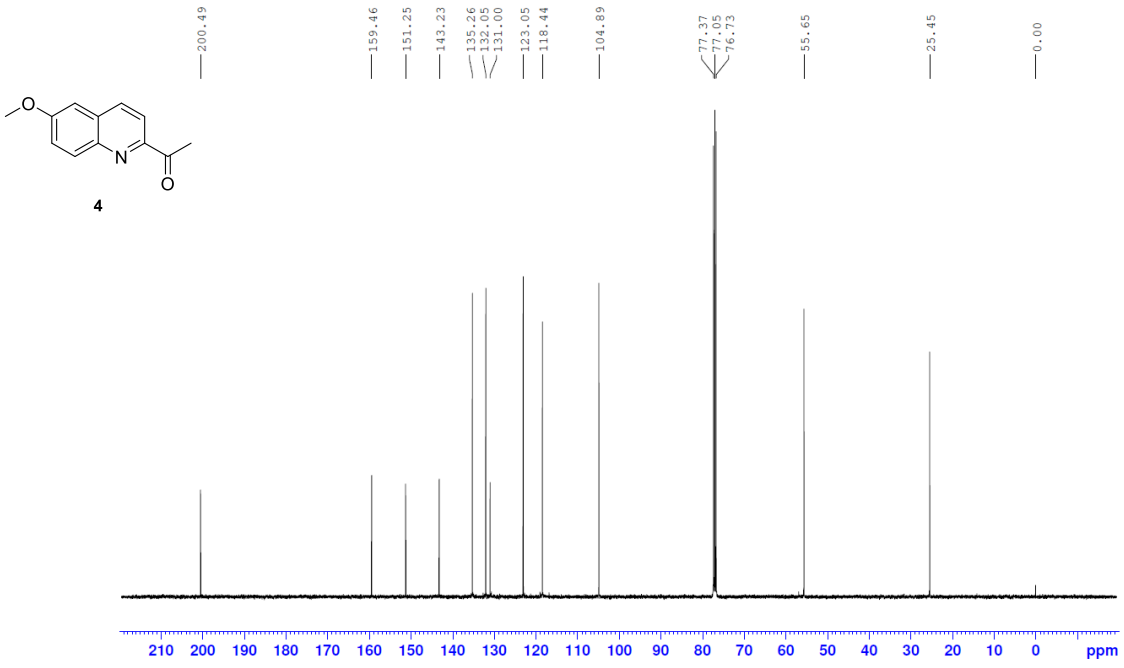
^{13}C-NMR ($CDCl_3$) spectrum of compound **3**



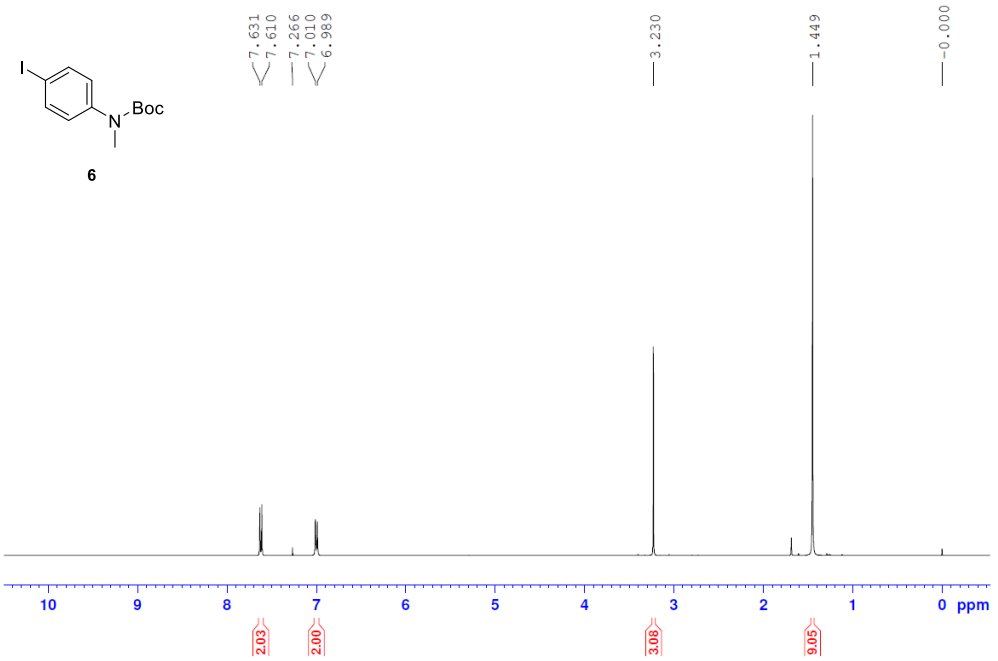
^{1}H-NMR ($CDCl_3$) spectrum of compound **4**



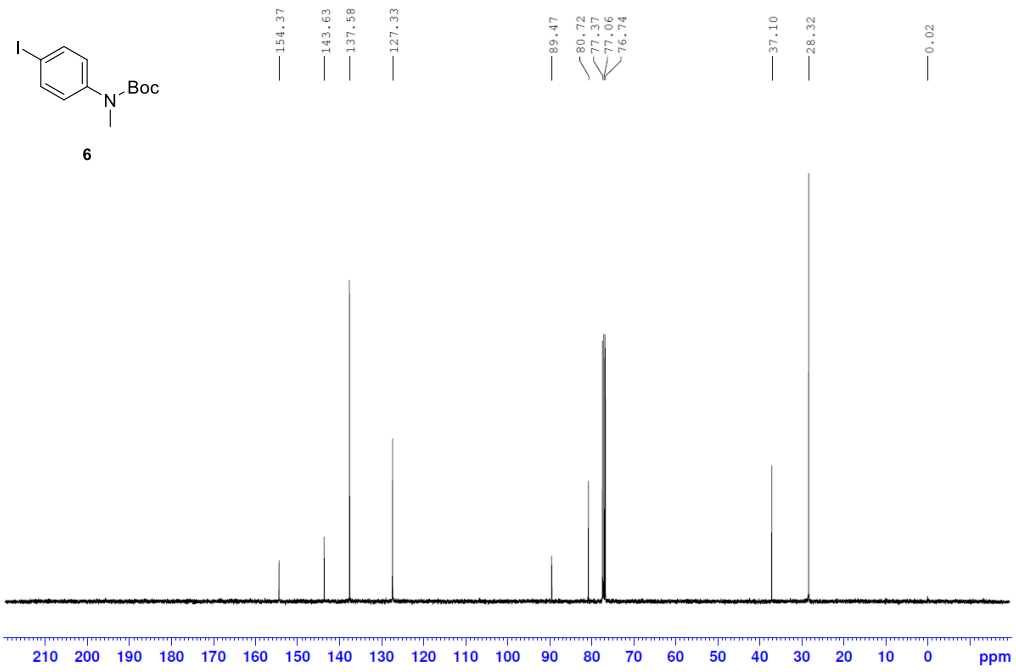
^{13}C-NMR ($CDCl_3$) spectrum of compound **4**



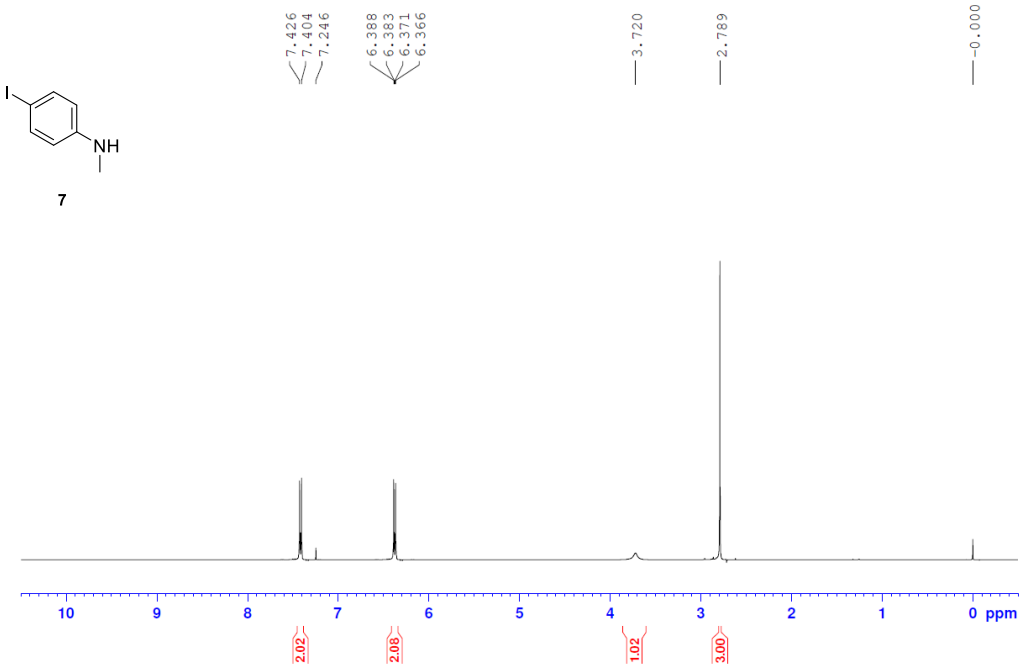
^{1}H-NMR ($CDCl_3$) spectrum of compound **6**



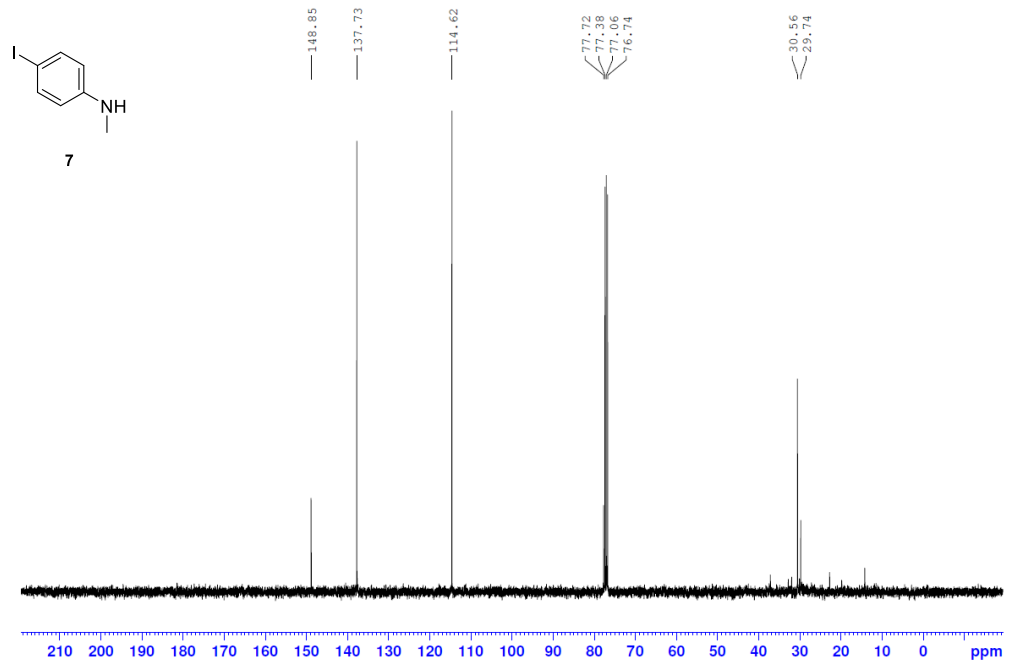
^{13}C-NMR ($CDCl_3$) spectrum of compound **6**



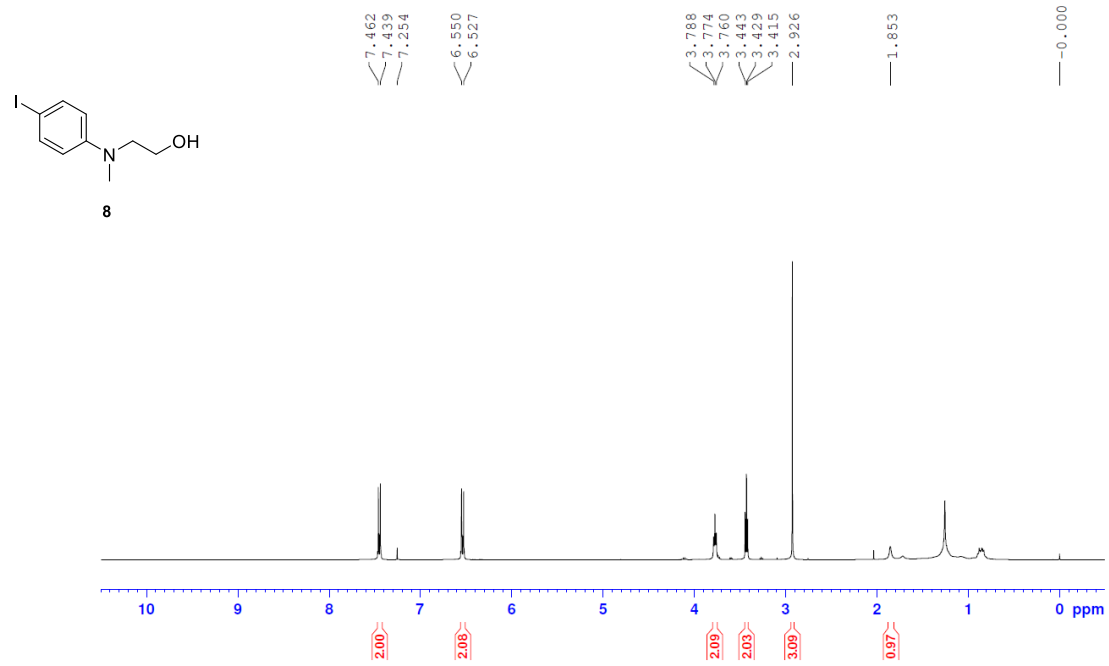
^{1}H-NMR (CDCl3) spectrum of compound **7**



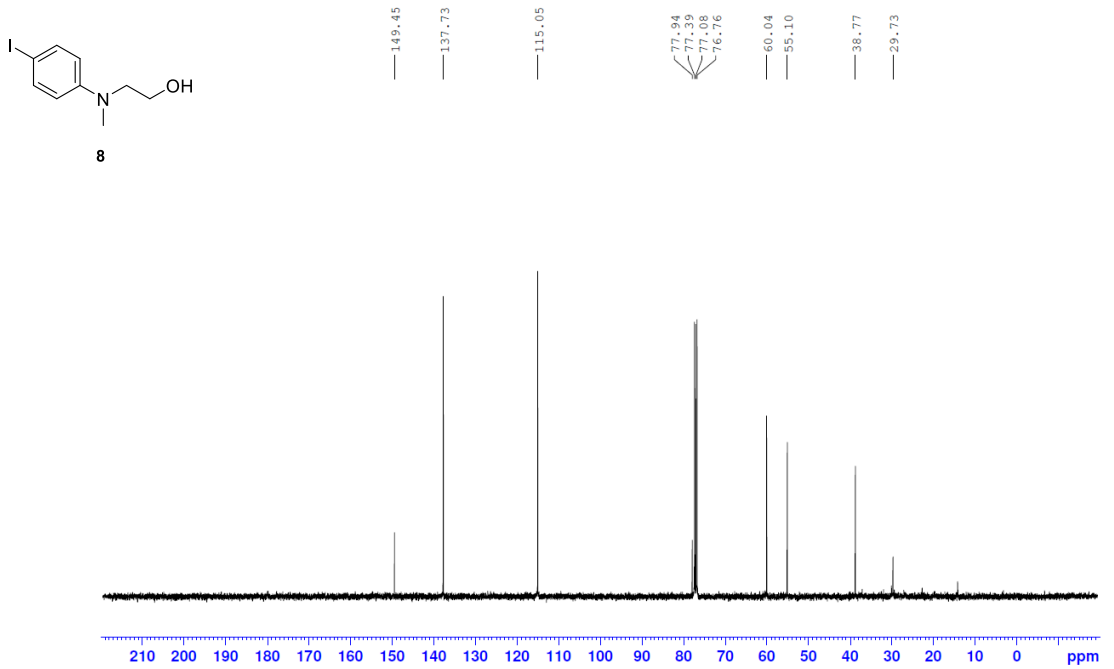
^{13}C-NMR ($CDCl_3$) spectrum of compound **7**



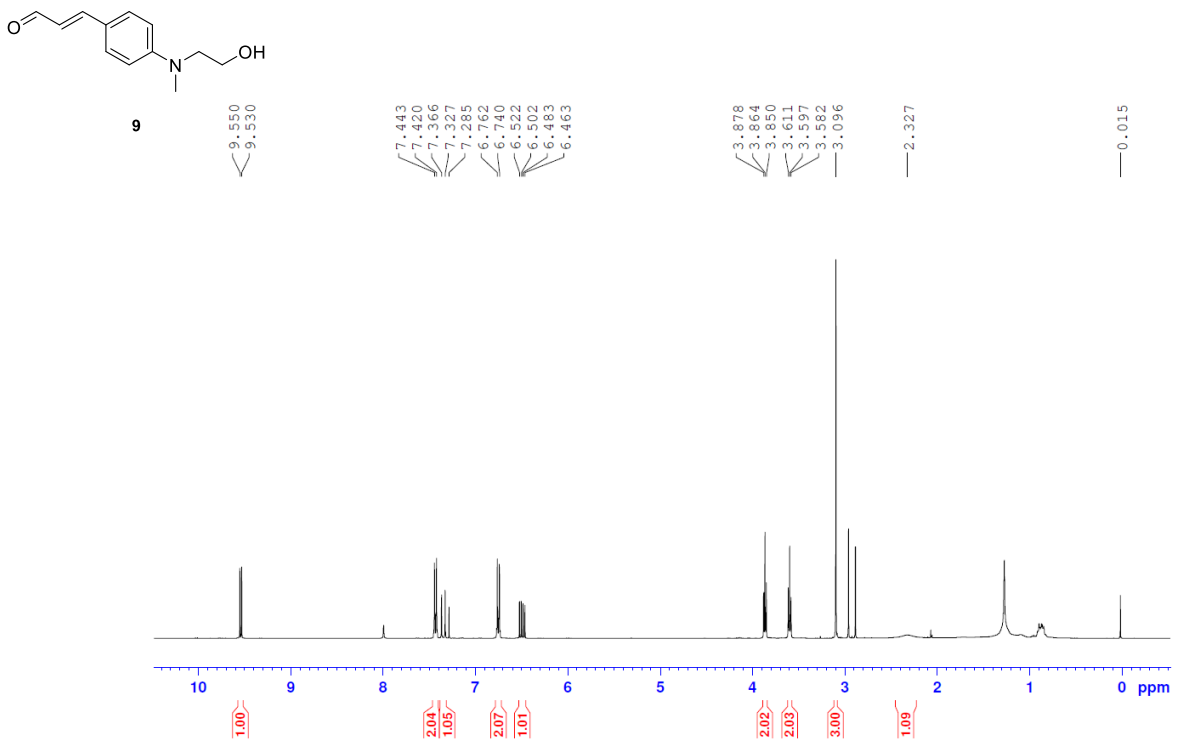
^{1}H-NMR ($CDCl_3$) spectrum of compound **8**



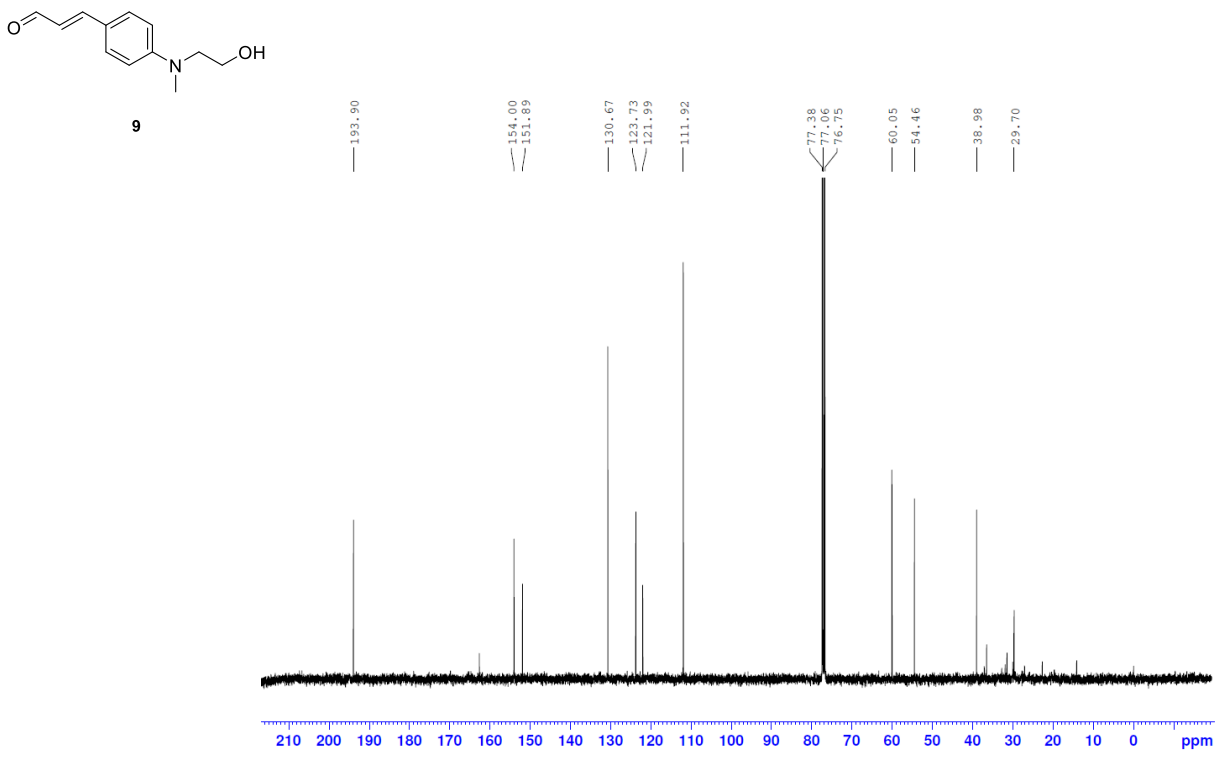
^{13}C-NMR ($CDCl_3$) spectrum of compound **8**



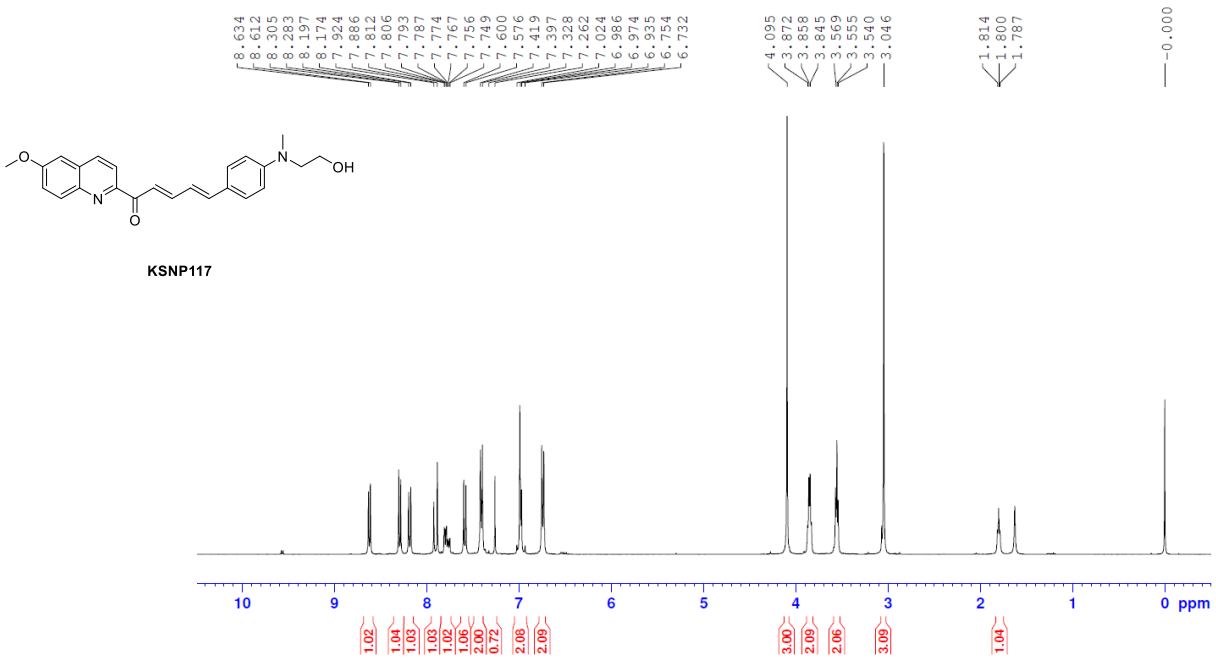
^{1}H-NMR ($CDCl_3$) spectrum of compound **9**



^{13}C-NMR ($CDCl_3$) spectrum of compound **9**



^{1}H-NMR ($CDCl_3$) spectrum of **KSNP117**



^{13}C-NMR ($CDCl_3$) spectrum of **KSNP117**