SUPPORTING INFORMATION

Platinum (II) complex-nuclear localization sequence peptide hybrid for overcoming platinum resistance in cancer therapy

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GENERAL INFORMATION

Materials.

Chemicals were purchased from Acros Organics: potassium carbonate 99%, sodium hydride 60% in mineral oil, diethyl methylmalonate 99%, silver nitrate, cis-dichlorodiamineplatinum (II) 99%, 5-hexynoic acid 97%, copper (I) iodine 99.995%; Alfa Aesar: propargyl bromide 97%, (80% in toluene), 6-chloro-1-hexyne 98%, 1,6-dibromohexane 97%, ninhydrin 99%; Fisher Scientific: uranine powder 40%, sodium azide, L-ascorbic acid, phenol, sodium hydroxide; Chem-Impex INT'L INC.: 6-bromohexanoic acid 99.2%,

Chemicals for solid phase peptide synthesis (SPPS) were purchased from Chem-Impex INT'L INC.: Fmoc-L-Pro 99.44%, Fmoc-L-Val 4-alkoxybenzyl alcohol resin (0.332 meq/g) and N^{α}-Fmoc-N^{ω}-Pbf-L-Arg 99.1%, triisopropylsilane (TIPS); Acros Organics: N^{α}-Fmoc-N^{ε}-Boc-L-Lys, ANASPEC INC.: 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU); Alfa Aesar: piperidine 99%, trifluoroacetic acid 99% (TFA) and Oakwood Chemical: N,N-diisopropylethylamine (DIPEA).

All solvents were bought and used without further purification. Fischer Scientific (ACS grade): chloroform, ethyl acetate, methylene chloride, anhydrous ethyl ether, 2-propanol (IPA), N,N-dimethylformamide (DMF), methanol, hexanes; Acros Organic: acetonitrile HPLC grade; Alfa Aesar: anhydrous tetrahydrofuran (THF) and Koptec: ethyl alcohol 190 proof.

NMR spectra of all the synthetic samples were recorded on Bruker 400 MHz Ultra Shield instrument.

LC/MS: Ultra High Performance Liquid Chromatography System Agilent Technologies 1200 series Accurate-Mass TOF LC/MS 6220.

IR: Nicolet iS10 FT-IR Spectrophotometer Thermo Scientific

AAS: Atomic Absorption Spectrophotometer AAnalyst 800 Perkin Elmer

HPLC: High Performance Liquid Chromatography Agilent Technologies 1260 Infinity

Methods.

Cell culture methods.

The human cancer derived cell line, A2780 and its isogenic clone CP70 and SKOV-3, OV-90, TOV-21G, ES-2 were purchased from ATCC. The cell lines were cultured in Dulbecco's Modified Eagle Medium (Sigma Aldrich) supplemented with 10% (A2780, CP70, SKOV-3, ES-2) or with 15% (TOV-21G, OV-90) fetal bovine serum (HyClone) with L-Glutamine (HyClone) and penicillin/streptomycin (HyClone). All cells were grown in a 5% CO_2, water saturated atmosphere at 37°C. For *in vitro* experiments, $3x10^5$ cells were seeded in each 96-well plate and pre-cultured overnight. All stock solutions of standards and drugs were prepared in water. Figures S19 to S24 describe the determination of IC_{50} carboplatin for each cell line. Figures S25 to S30 describe the determination of IC_{50} Pt-NLS for each cell line. Concentration of all substrates was adjusted to match molar concentration of carboplatin. The volume of substrates in the solution was adjusted to not exceed 10% of the media volume. After 72 hours of incubation, media was removed and the cell viability/cytotoxicity was evaluated using TACS MTT Cell Proliferation assay (Trevigen) according to the manufacturer instructions and analyzed by plate reader (SpectraMax M3 by Molecular devices).

Isolation of DNA.

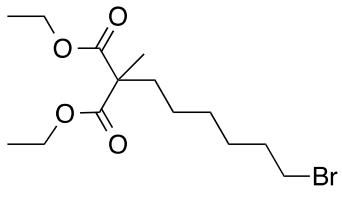
Cell lines type A2780 and CP70 were seeded onto petri dish at the density of $3x10^5$ cells/petri dish and incubated for 72h according to procedure described above. Media was removed and each dish was washed three times with PBS. Cells were treated with trypsin for approximately two minutes then media was added. After centrifugation at 1250 rpm for 10 min, cells were washed with PBS three times and centrifuged again. Whole cellular DNA was isolated using QIAamp ® DSP DNA Mini Kit (QIAGEN) according to the manufacturer instructions. The concentration of DNA was measured using NanoDrop 2000C Spectrophotometer (Thermo Scientific).

Confocal images.

Cell line type CP70 was seeded onto 35mm MatTek confocal dish at the density of $3x10^5$ cells per dish and pre-cultured overnight and incubated with FITC modified NLS peptide in media for 72h. After incubation, media was removed and the cells were washed three times with PBS (Dulbecco's phosphate buffered saline, Corning). Glutaraldehyde (Alfa Aesar) was added and after 2 hours' cells were washed three times with PBS again. DAPI was used to stain nucleus. Distribution of drug was visualized by confocal microscopy (Fluoview FV10i OLIMPUS).

EXPERIMENTAL SECTION

Synthesis of compound 1

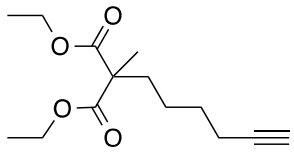


All glassware was flame dried and cooled under argon atmosphere. All steps were carried under argon atmosphere.

Diethyl methylmalonate 10.00g [58.5mmol] in 20ml of dry THF was added dropwise into sodium hydride 3.51g [87.8mmol] suspension in 20ml of dry THF at 0°C. Hydrogen gas evolution was observed. Reaction mixture was allowed to warm up to room temperature and stirred for 30min. Reaction mixture was transferred via cannula into a solution

of 1,6-dibromohexane 16.73ml [87.8mmol] in 30ml of dry THF. Color of reaction mixture changed to yellow/brown with stirring. Progress of the reaction was monitored by TLC [hexane/ethyl acetate 9:1]. Upon completion (about 17 hours), reaction was quenched with 5ml of saturated ammonium chloride solution, diluted with 20 ml of water and extracted with diethyl ether [3x50ml]. Organic layer was dried with anhydrous sodium sulfate. Crude product was purified by flash chromatography using solvent gradient starting with 1:0 to 9:1 hexane to ether ratio. Afforded 11g of pure product [56% yield]. ^{1}H NMR ($CDCl_3$) δ 4.166 (q, 4H), δ 3.391 (t, 2H) δ 1.860 (m, 4H), δ 1.458 (m, 2H), δ 1.419 (s, 3 H), δ 1.351 (m, 2H), δ 1.241 (t, m overlap, 8 H). ^{13}C NMR ($CDCl_3$) δ 172.42, 61.11, 53.61, 35.37, 33.77, 32.66, 28.98, 27.89, 24.07, 19.84, 14.06 (Fig S1). HRMS-ESI: m/z $[M + H]^+$ calc. for $C_{14}H_{25}BrO_4$: 337.1014; found: 337.0992.

Synthesis of compound 2

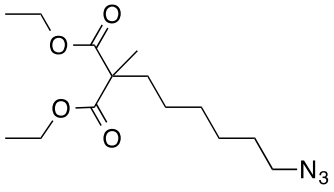


All glassware was flame dried and cooled under argon atmosphere. All steps were carried under argon atmosphere.

To a solution of sodium iodide 3.71g [24.7mmol] in 15ml of acetone, 6-chloro-1-hexyne was added 1.00ml [8.25mmol] and stirred under reflux overnight. Progress of the reaction was monitored by NMR. Upon completion acetone was removed under vacuum. Remaining solid was diluted with 20 ml of water and extracted with diethyl ether [3x30 ml]. Organic fraction was washed with brine [20 ml] then dried with anhydrous magnesium sulfate. Afforded 1.52g of product [88.9% yield]. ^{1}H NMR ($CDCl_3$) δ 3.209 (t, 2 H), δ 2.226 (dt, 2H, J = 2.4 Hz) δ 1.960 (m, 3H), δ 1.647 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 172.34, 84.11, 68.36, 61.13, 53.57, 34.91, 28.60, 23.33, 19.79, 18.13, 14.04.

Diethyl methylmalonate 1.00g [5.85mmol] in 20ml of dry THF was added dropwise into sodium hydride 0.35g [87.8mmol] suspension in 20ml of dry THF at 0°C. Hydrogen gas evolution was observed. Reaction mixture was allowed to warm up to room temperature and stirred for 30min. 6-iodo-1-hexyne 1.15g [7.28mmol] was added in one portion. Brown color of the solution was observed. Progress of the reaction was monitored by TLC [hexane:ethyl acetate 9:1]. Upon completion (about 17 hours), reaction was quenched with 3 ml of saturated ammonium chloride solution, diluted with 10 ml of water and extracted with diethyl ether [3x20ml]. Organic layer was dried with anhydrous sodium sulfate. Crude product was purified by flash chromatography using solvent gradient starting with 1:0 to 9:1 hexane to ether ratio. Afforded 1.48g of pure product [99.3% yield]. ^{1}H NMR ($CDCl_3$) δ 4.165 (q, 4 H), δ 2.195 (dt, 2H, J = 2.8 Hz) δ 1.917 (t, 1H), δ 1.887-1.835 (m, 2H), δ 1.539 (m, 2H), δ 1.401 (s, 3H), δ 1.351 (m, 2H), δ 1.241 (t, 6H).

^{13}C NMR ($CDCl_3$) δ 172.34, 84.11, 68.36, 61.13, 53.57, 34.91, 28.60, 23.33, 19.79, 18.13, 14.04 (Fig S2). HRMS-ESI: m/z $[M + H]^+$ calc. for $C_{14}H_{22}O_4$: 255.1596; found: 255.1560.

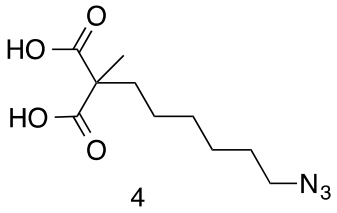


Synthesis of compound 3

To a solution of bromide 8.11g [24.1 mmol], **1** in 75ml of DMF, sodium azide 4.69g [72.1mmol] was added. Not all azide dissolved. Reaction mixture was allowed to stir over weekend. Progress of the reaction was monitored by NMR. Upon completion DMF was removed under vacuum at 45°C. Afforded colorless oil 7.20g, quantitative yield.

^{1}H NMR ($CDCl_3$) δ 4.173 (q, 4H), δ 3.239 (t, 2H), δ 1.831 (m, 2H), δ 1.574 (m, 2H), δ 1.406-1.245 (s,m,t, 15H). ^{13}C NMR ($CDCl_3$) δ 172.39, 61.07, 53.57,51.36, 35.33, 29.33, 28.70, 26.41, 24.07, 19.81, 14.02 (Fig S3). HRMS-ESI: m/z $[M + H]^+$ calc. for $C_{14}H_{25}N_3O_4$ 300.1926; found: 300.2019.

Synthesis of compounds 4, 5



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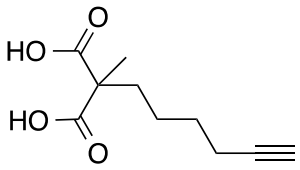
To a solution of azide **3** 3.78g [12.6mmol] in 15ml of methanol, sodium hydroxide 3.03g [75.8mmol] was added. Pale yellow solution was observed. Progress of the reaction was monitored by TLC [hexane:ethyl acetate 4:1]. Methanol was removed under vacuum. Water was added 20ml and aqueous fraction was extracted with ether [3x20ml]. Organic phase was discarded. Aqueous phase was acidified with 10% HCl and extracted with ethyl acetate [3x35ml] Organic phase was dried over anhydrous sodium sulfate. Afforded white solid 2.78g [90% yield]. ^{1}H NMR (DMSO) δ 12.566 (bs, 2H), δ 3.305 (t, 2 H), δ 1.700 (m, 2H), δ 1.512 (m, 2H), δ 1.301-1.159 (m,s,m, 9H). ^{13}C NMR (DMSO) δ 173.63, 52.69, 50.56, 35.04, 28.86, 28.14, 25.93, 23.77, 19.64 (Fig S4). HRMS-ESI: m/z $[M + Na]^+$ calc. for $C_{10}H_{17}N_3O_4$ 266.1117; found: 266.1136. IR spectrum (Fig S6).



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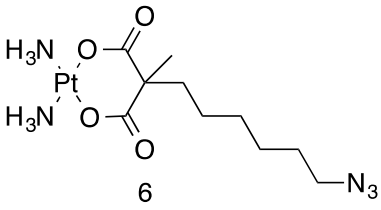
Product **5** was synthesized by the same procedure from substrate **2**. Afforded white solid with [94% yield]
^{1}H NMR (DMSO) δ 12.610 (bs, 2 H), δ 2.730 (s, 1 H), δ 2.154 (dt, 2H J=2.4Hz), δ 1.699 (m, 2H), 1.427 (m, 2H), 1.281-1.243 (m,s, 5H). ^{13}C NMR (DMSO) δ 173.63, 52.69, 50.56, 35.04, 28.86, 28.14, 25.93, 23.77, 19.64 (Fig S5). HRMS-ESI: m/z $[M + Na]^+$ calc. for $C_{10}H_{14}O_4$ 221.0790; found 221.0798. IR spectrum (Fig S7).

Synthesis of Pt-N_3 (6) and Pt-CCH (7) complexes



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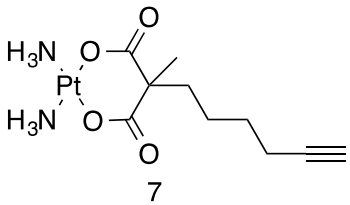
To a suspension of cisplatin 101.6mg [0.338mmol] in 190 proof ethanol 10ml, silver nitrate was added 112.2mg [0.660mmol]. White precipitate formed upon silver nitrate addition. Reaction was stirred in the dark at 40-50^{o}C untill test for silver +1 with 10% HCl was negative. Usually 1 to 2 hours. Most of the white precipitate was removed by centrifugation. The remaining solid was removed via syringe filter 0.2µm. Filtrate was tested for presence of platinum with tin (II) chloride.



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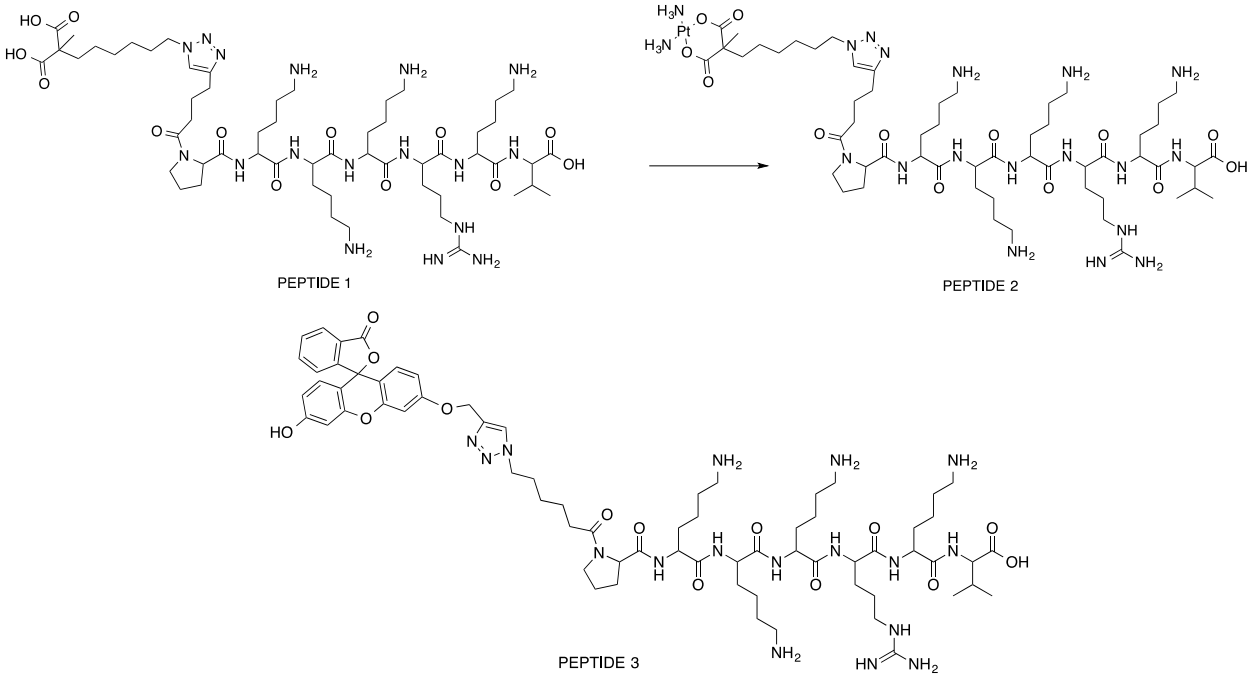
To dicarboxylic acid **4** 80.3mg [0.330mmol] solution in 2ml of ethanol, sodium hydroxide 26.4mg [0.660mmol] in 2 ml of ethanol was added. After 5 minutes of stirring, filtrate containig activated platinum from first step was added dropwise. Some white precipitation was observed. Stirred at room temperature over 2 hours. Progress of the reaction was monitored by HRMS due to poor solubility of substarte in DMSO and methanol. Solid was removed by centrifugation and washed twice with methanol 5ml and once with ether 20 ml Afforded white powder 66.7mg [43%yield].

Product **Pt-N_3 complexes:** No NMR due poor solubility. HRMS-ESI: m/z $[M + H]^+$ calc. for $C_{10}H_{21}N_5O_4Pt$ 471.1320; found 471.1313 (Fig S12). IR spectrum (Fig S8).
Product **Pt-CCH complexes**: Afforded off white powder 32.0mg [39.4%yield]. No NMR due poor solubility. HRMS-ESI: m/z $[M + H]^+$ calc. for $C_{10}H_{18}N_2O_4Pt$ 426.0993; found 426.0973 (Fig S13). IR spectrum (Fig S9).

General procedure for synthesis of precursor for Pt-NLS hybrid (peptide 1), Pt-NLS hybrid (peptide 2) and NLS-FITC (peptide 3).

Peptide sequence PKKKRKV



Precursor for Pt-NLS hybrid (peptide 1)

Standard SPPS method has been employed to synthesize PKKKRKV peptide. Briefly, 5g of Fmoc-L-Val 4-alkoxybenzyl alcohol resin (0.332 meq/g) were soaked in DMF 25ml for 1 hour prior use. Deprotection of Fmoc protecting amine group was carried with 20% piperidine/DMF solution 5 min followed by 20 min cycle. Upon completion resin was washed for 1min with each solvent as follows: DMF, IPA, DMF, IPA, DMF, IPA, DMF, DMF. Kaiser test was performed and if positive, coupling was performed overnight. Standard coupling conditions: Wang resin 1.65mmol, Fmoc amino acid 3.3mmol, TBTU 3.3mmol, DIPEA 6.6mmol. DMF 10ml. Amino acid was dissolved together with TBTU in DMF then DIPEA was added. Resulting solution was transferred to reaction vessel and shaken overnight. Next day resin was washed for 1 min with DMF, IPA, DMF and IPA, then Kaiser test was performed again. If negative, another deprotection cycle started followed by coupling of subsequent amino acid. If Kaiser test was positive coupling procedure was repeated.
N-terminal amino acid was derivatized with either 6-azido-hexanoic acid[1] or 5-hexynoic acid by the same coupling procedure. Peptide 1 was synthesized via click reaction. Briefly, to 0.315mmol of derivatized peptide 0.631mmol of solid copper iodide was added, followed by

0.631mmol of **4** and ascorbic acid 0.631mmol in 5 ml of deoxygenated 20% piperidine/DMF solution. Brown solution formed while shaking. Any exposure to air caused color change to green, therefore all solvents were degassed by passing argon gas through the solvent for at least 30 min. Speed of reaction when exposed to air decreases drastically probably due to oxidation of Cu^{1+} to Cu^{2+}. Progress of the reaction was monitored by HRMS. Upon completion resin was washed for 1 min with 5ml of DMF, IPA, DMF, Methanol, Dichloromethane, Methanol and diethyl ether. Cleavage of peptide 1 from resin was achieved with a solution of TFA/TIPS/H_2O ratio 95/2.5/2.5 over 3 hours. Crude peptide was precipitated out in cold diethyl ether, washed 3 times with cold ether then dried under vacuum. Crude product was lyophilized then purified by HPLC. For analytical purposes Agilent XDB-C18 5µm 4.6-150mm column was used. For purification purposes Agilent Zorbax RX-C8 5µm, 4.6-250mm was used. 1-35% solvent gradient over 25min of water/acetonitrile/TFA (95/5/0.01%) and acetonitrile/water/TFA (95/5/0.01%). Fractions containing pure product were collected and used in synthesis of Peptide 2. HRMS-ESI: m/z $[M + H]^+$ calc. for $C_{56}H_{102}N_{17}O_{13}$ 1220.7843; found 1220.7867 (Fig S15). IR spectrum Fig S10 and HPLC analysis (Fig S17).

Pt-NLS hybrid (peptide 2)

Cisplatin 12.3mg (0.041mmol) was suspended in 0.5ml of water, 13.2mg (0.079mmol) of silver nitrate in 0.5ml of water as added. Reactions mixture was stirred in the dark at 40-50^{o}C untill test for silver +1 with 10% HCl was negative. Usually 1 to 2 hours. Most of the white precipitate was centrifuged off and the remaining solid was removed via syringe filter 0.2µm. Aqueous filtrate was tested for presence of platinum with tin (II) chloride.

Peptide 1 23.8mg (0.0194mmol) was dissolved in 1 ml of water. Aqueous sodium hydroxide was added 1.56mg in 0.5 ml of water. Filtrate containing activated platinum was added dropwise to sodium salt of peptide 1. Reaction was allowed to stir at room temperature over 2 days. Progress of the reaction was monitored by HRMS. Crude product was purified by HPLC following the same protocol as Peptide 1. Pure product was tested *in vitro*. HRMS-ESI: m/z $[M + H]^+$ calc. for $C_{56}H_{108}N_{19}O_{13}Pt$ 1448.7943; found 1448.7873 (Fig S16). IR spectrum (Fig S11) and HPLC analysis (Fig S18).

NLS-FITC (peptide 3)

6-azido-hexanoic acid derivative of the NLS peptide obtained from synthesis of peptide 1 was coupled via click reaction with propargyl fluorescein[2]. Briefly, to 0.043mmol of resin with modified NLS, 0.017mmol of solid copper (I) iodide was added, followed by 0.172mmol of propargyl fluorescein and ascorbic acid 0.0.017mmol in 3 ml of deoxygenated 20% piperidine/DMF solution. Brown solution formed with shaking. Any exposure to oxygen caused color change to green. Progress of the reaction was monitored by HRMS. Upon completion resin was washed for 1 min with 5ml of DMF, IPA, DMF, Methanol, Dichloromethane, Methanol and diethyl ether. Cleavage of NLS-FITC from resin was achieved with a solution of TFA/TIPS/H_2O ratio 95/2.5/2.5 over 3 hours. Crude peptide was precipitated out in cold diethyl ether, washed 3 times with cold ether then dried under vacuum. After desalting and lyophilization product was used directly for confocal imaging study. Peptide 3. HRMS-ESI: m/z $[M + H]^+$ calc. for $C_{69}H_{102}N_{17}O_{14}$ 1372.7792; found 1372.7780 (Fig S14).

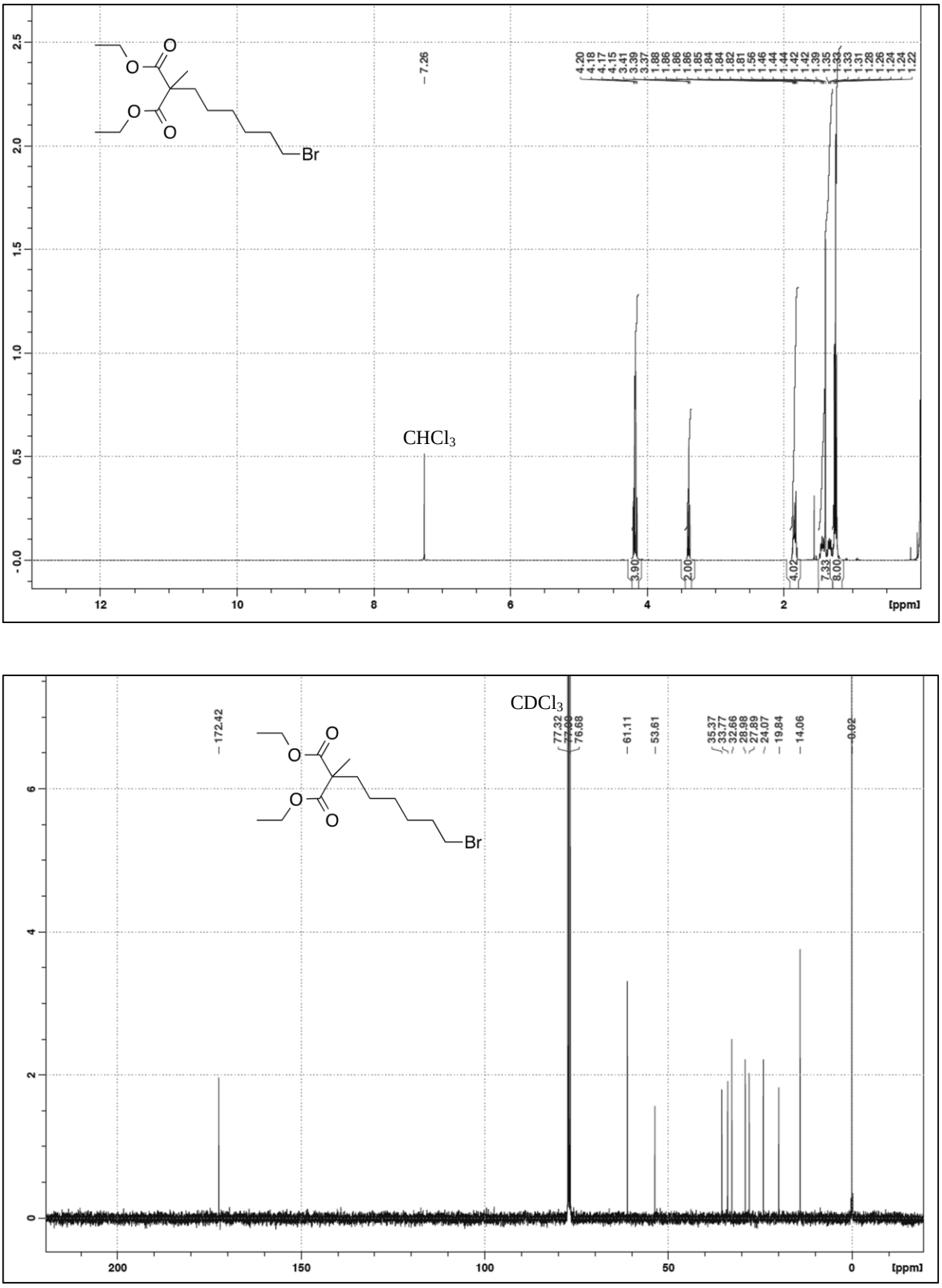


Figure S1. ^{1}H and ^{13}C NMR spectra of compound 1.

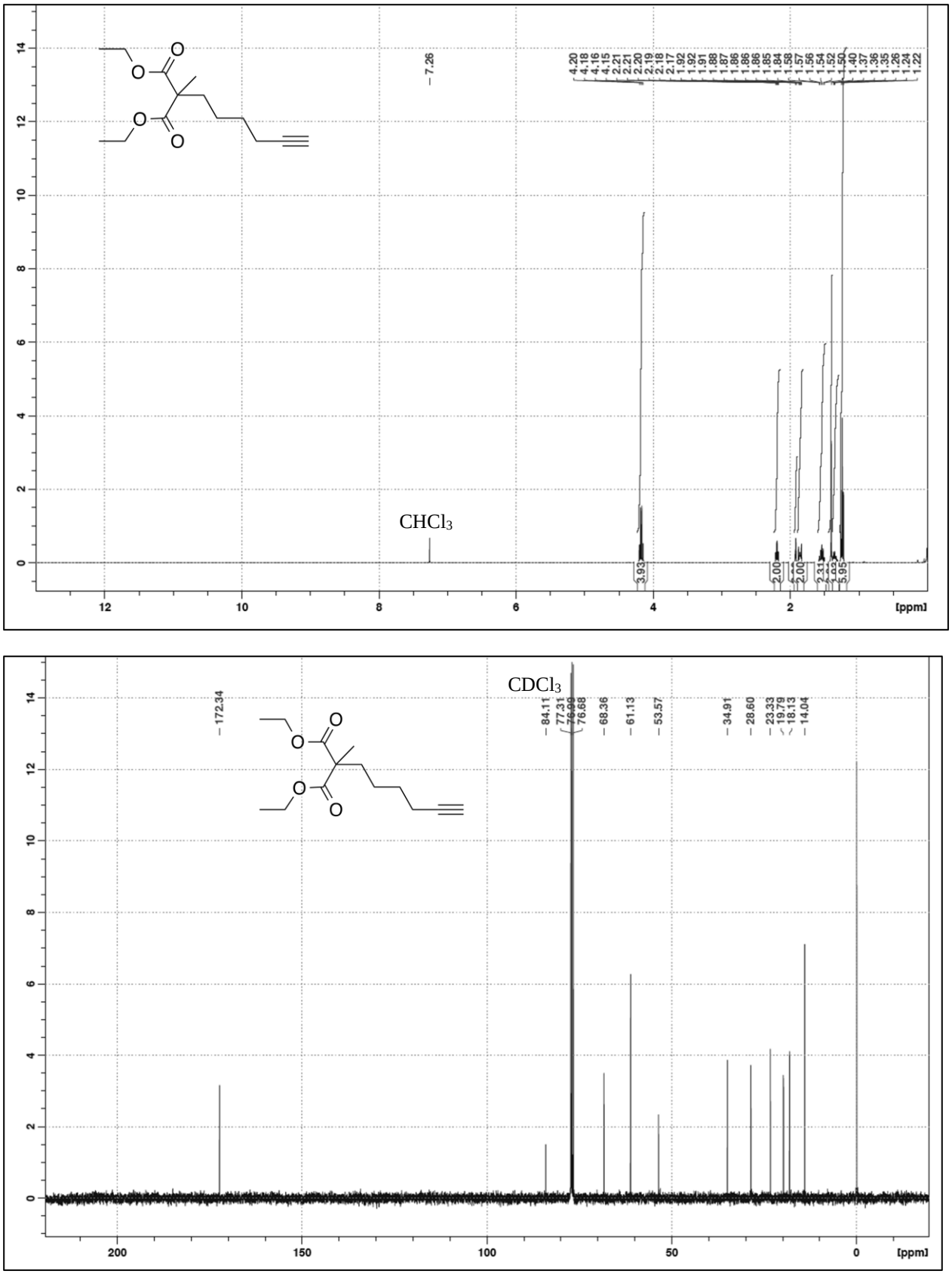
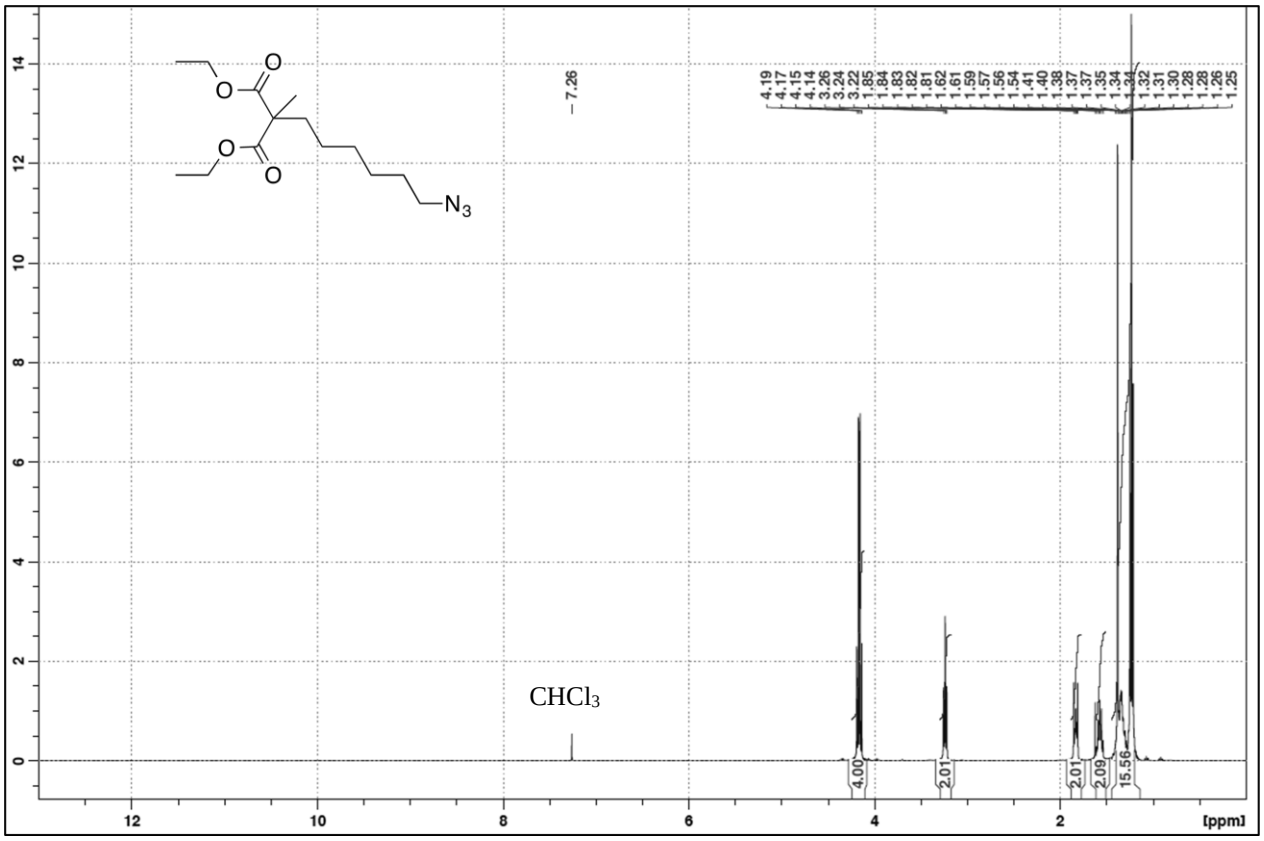


Figure S2. ^{1}H and ^{13}C NMR spectra of compound 2.



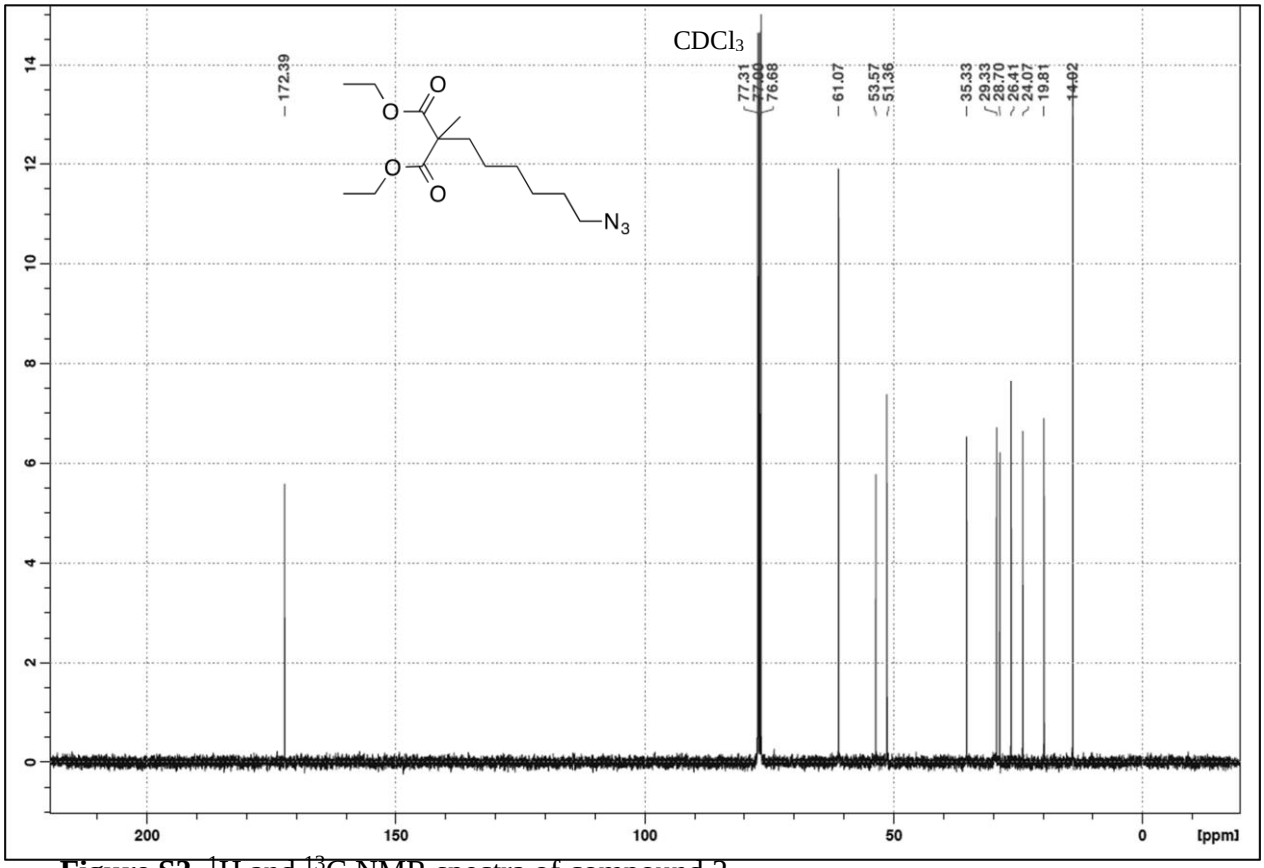


Figure S3. ^{1}H and ^{13}C NMR spectra of compound 3

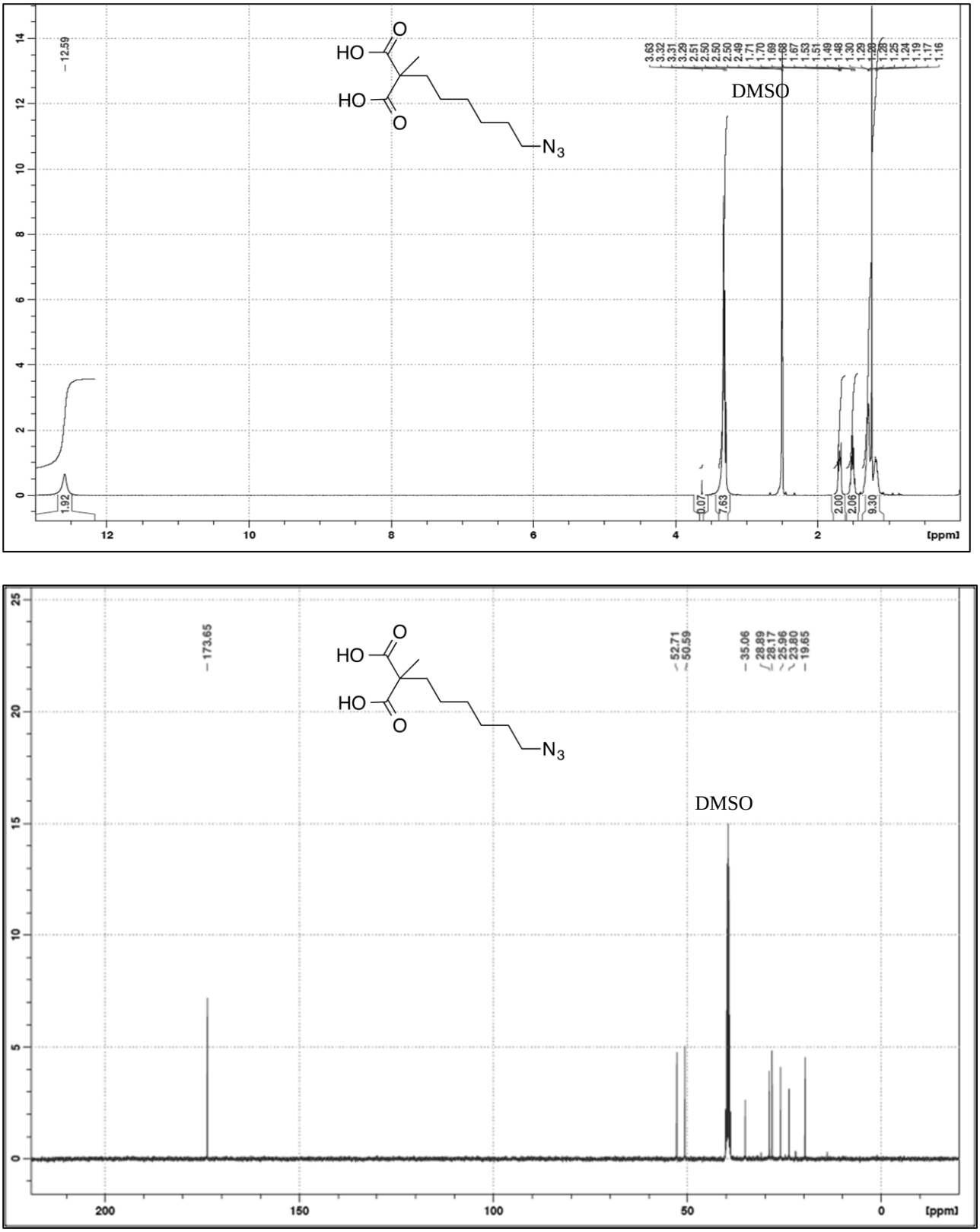
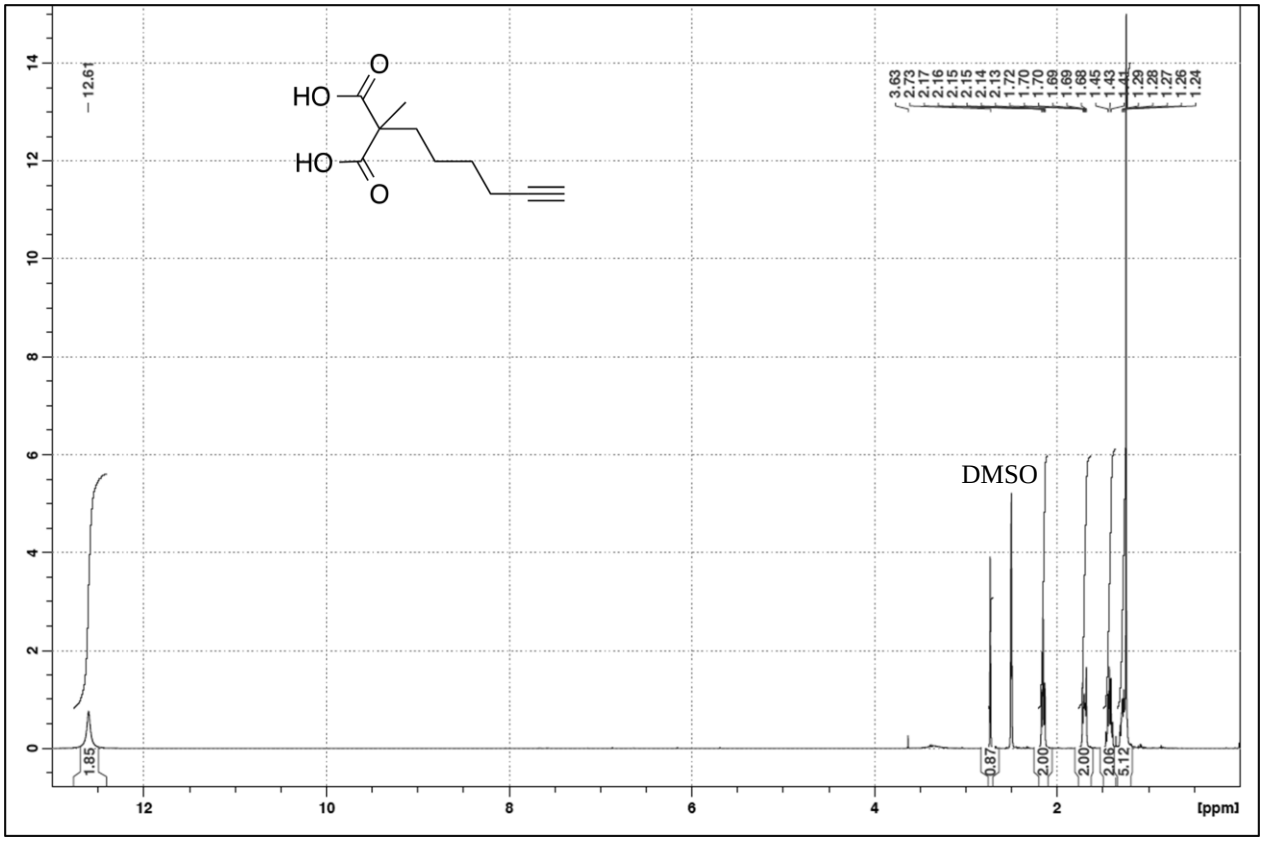


Figure S4. ^{1}H and ^{13}C NMR spectra of compound 4.



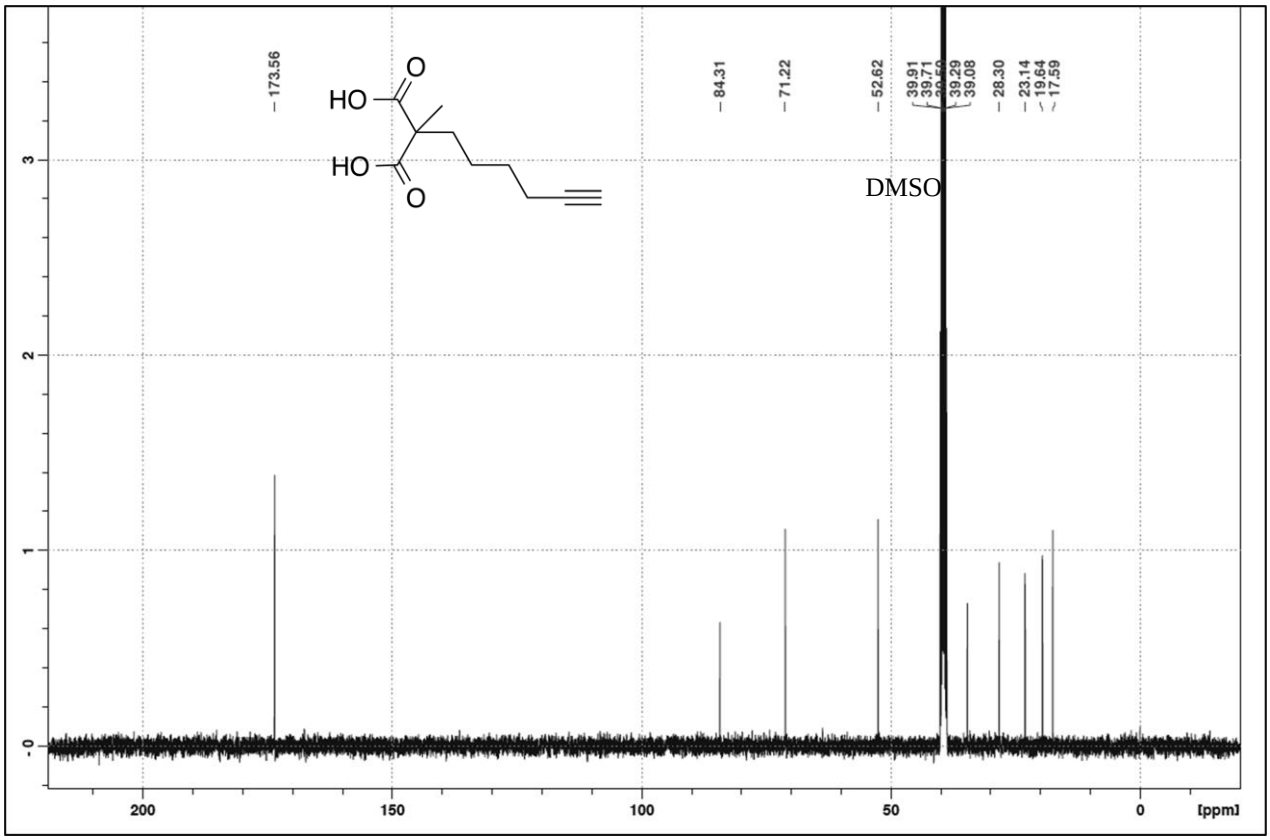


Figure S5. ^{1}H and ^{13}C NMR spectra of compound 5.

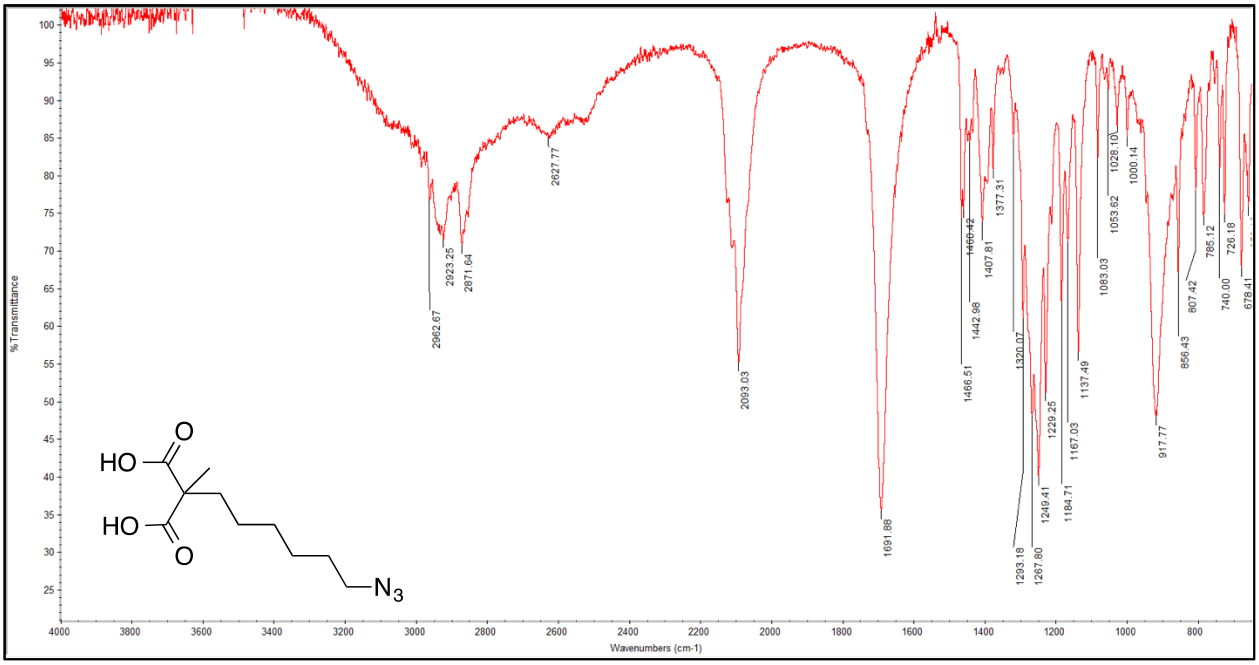


Figure S6. IR spectra of compound 4.

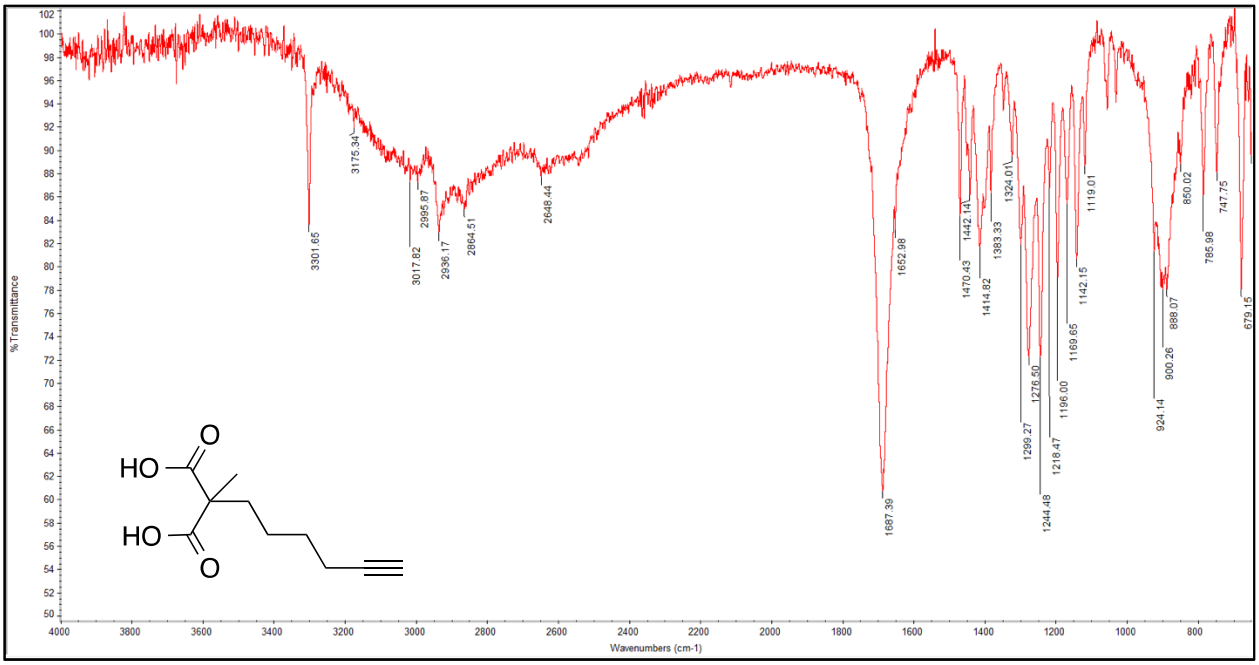


Figure S7. IR spectra of compound 5.

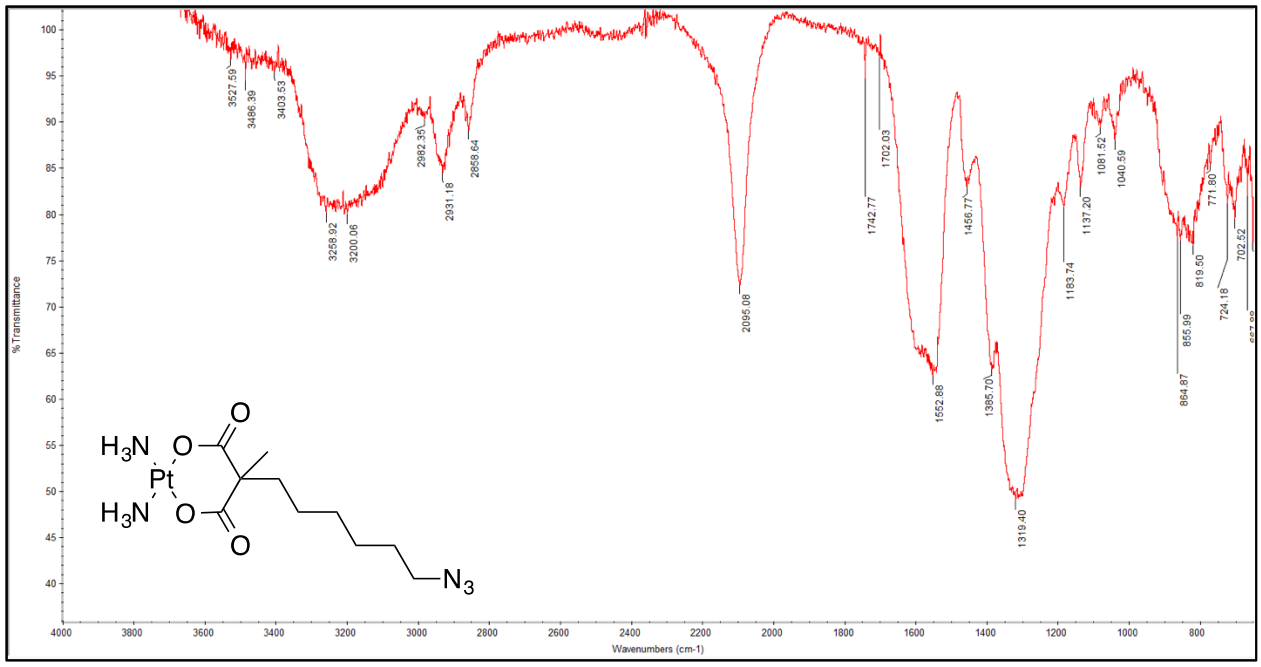


Figure S8. IR spectra of Pt-N_3 complex (6).

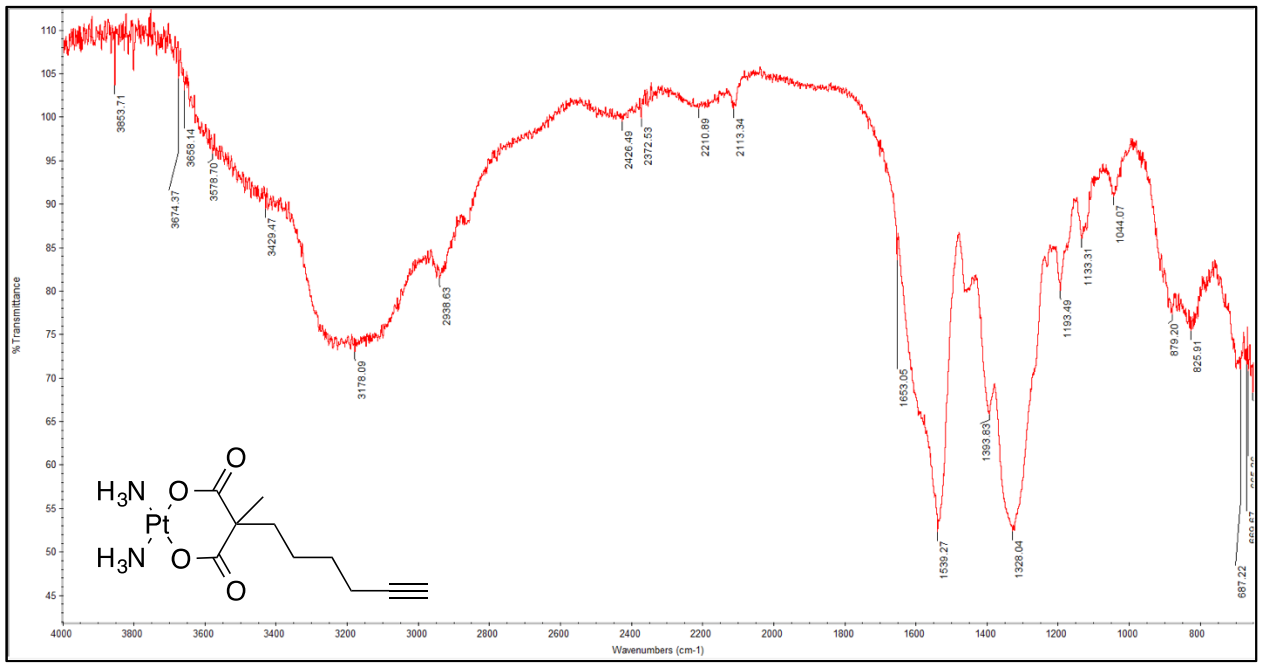


Figure S9. IR spectra of Pt-CCH complex (7).

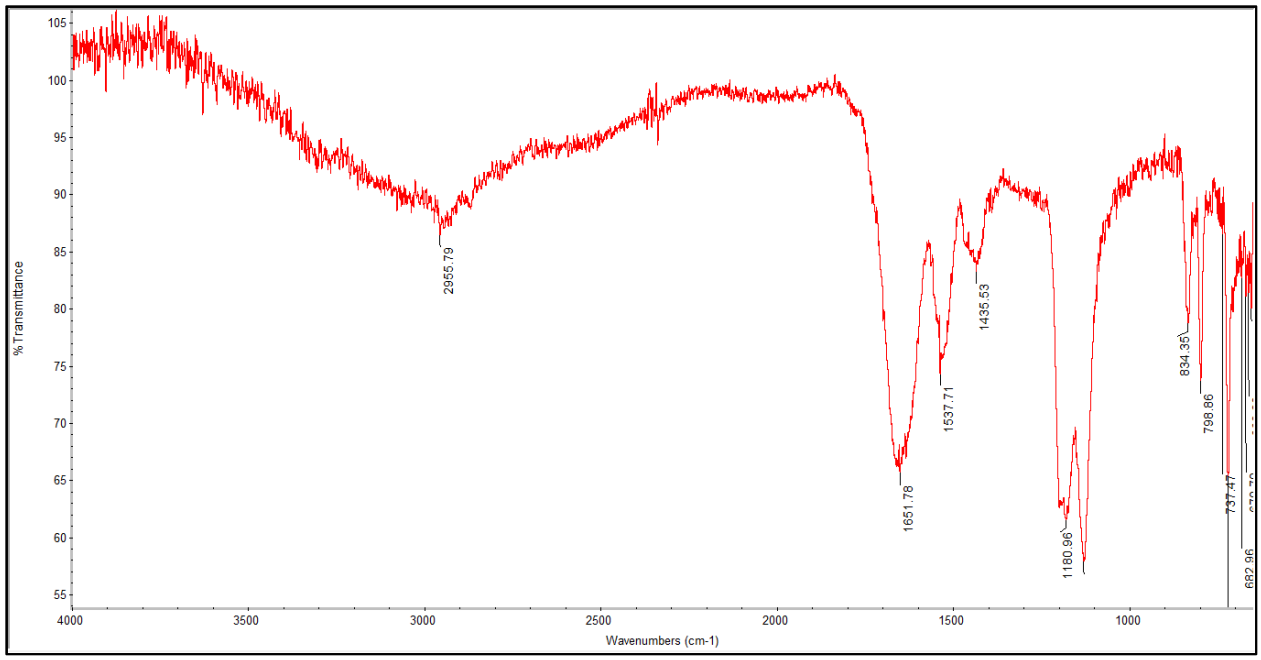


Figure S10. IR spectra of precursor for Pt-NLS hybrid (peptide 1).

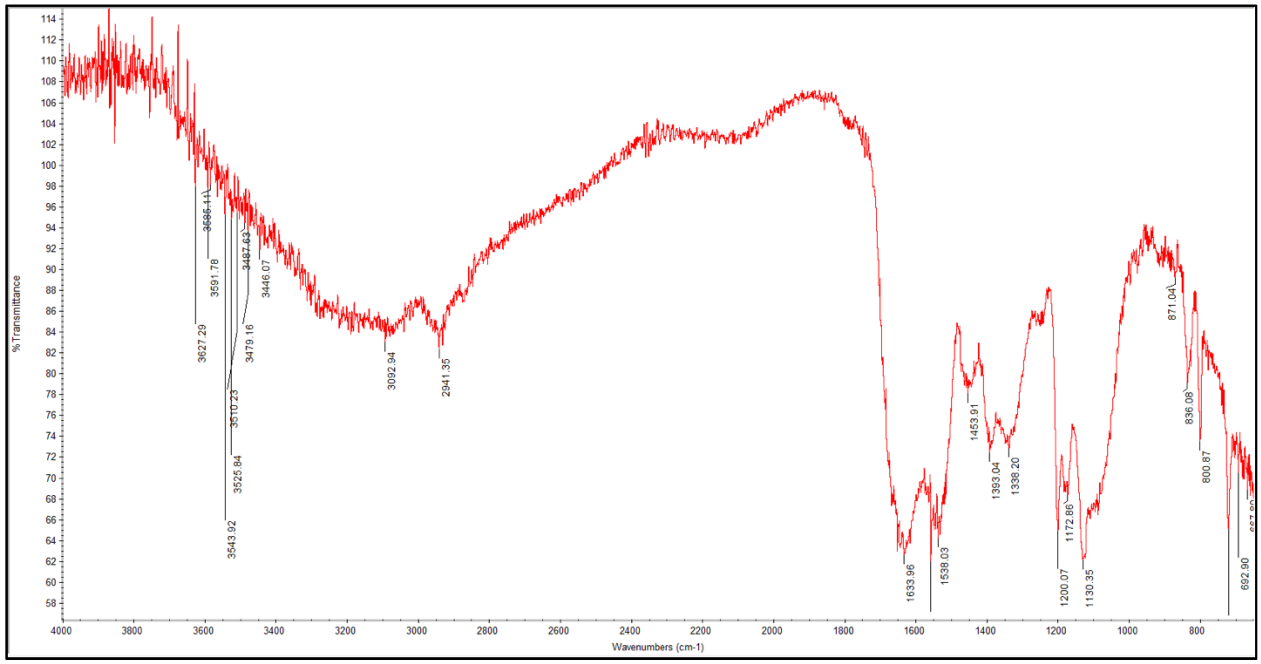


Figure S11. IR spectra of Pt-NLS hybrid (peptide 2).

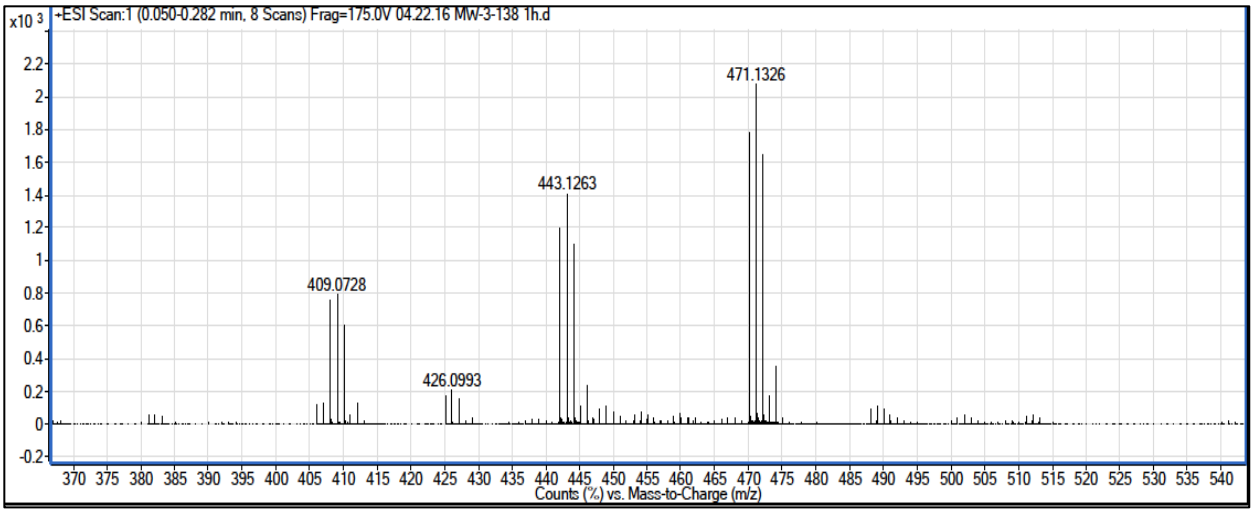


Figure S12. HRMS of Pt-N_3 complex (6).

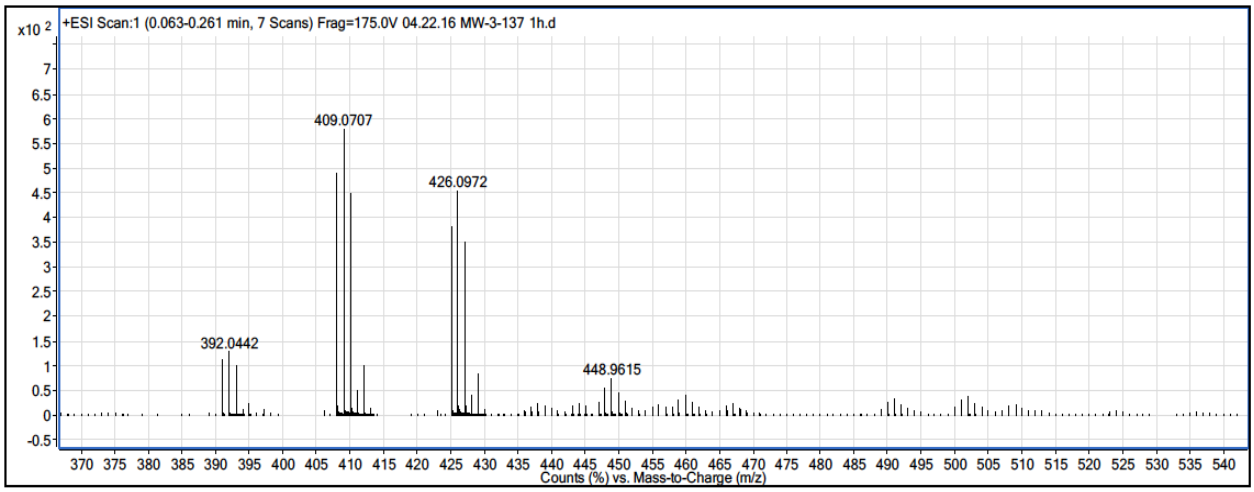


Figure S13. HRMS of Pt-CCH complex (7).

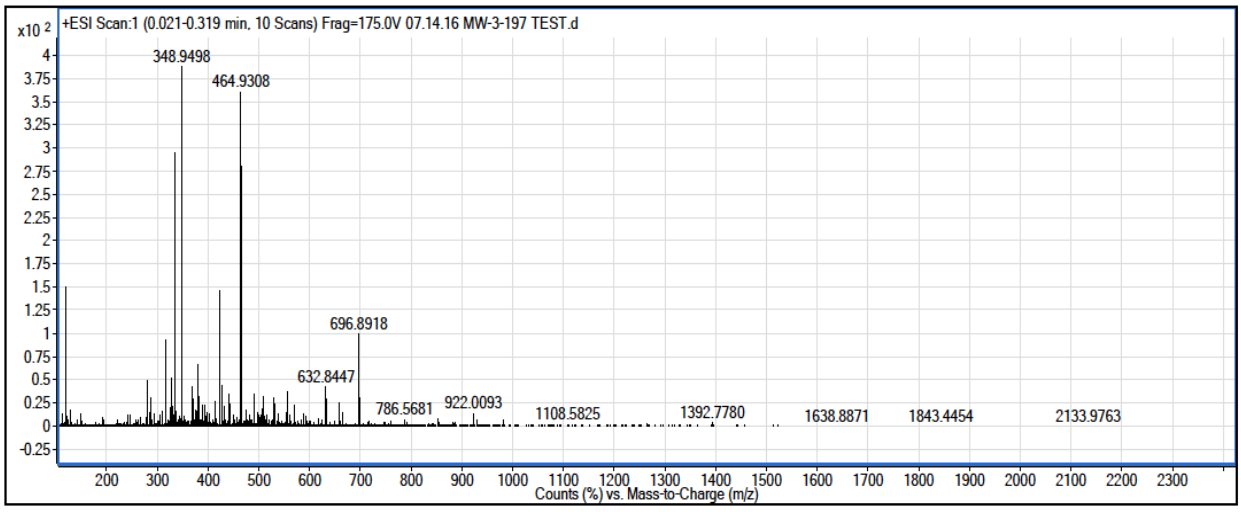


Figure S14. HRMS of NLS-FITC (Peptide 3).

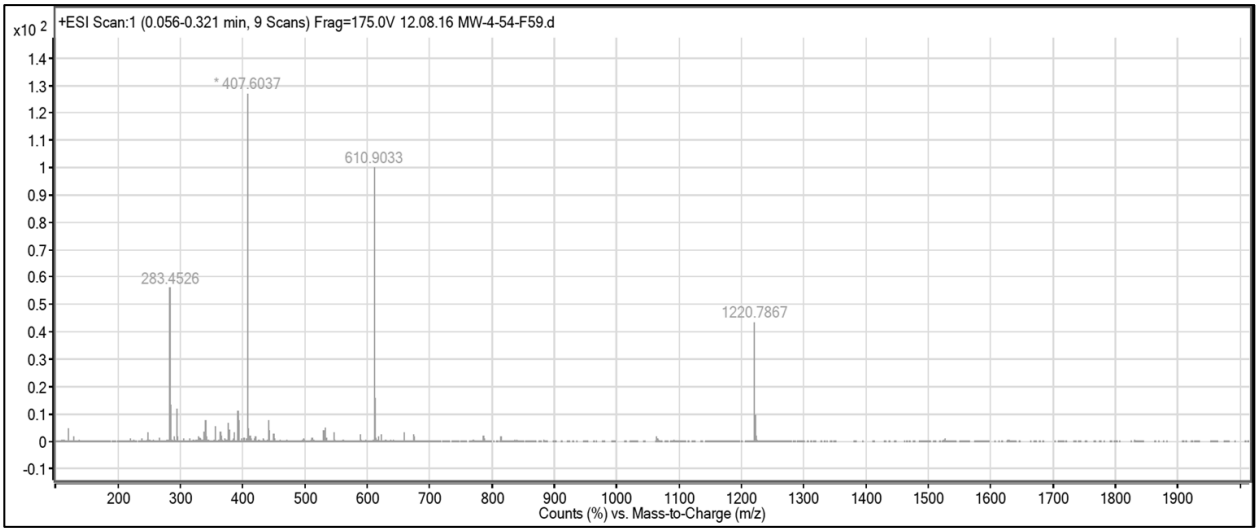


Figure S15. HRMS of precursor for Pt-NLS hybrid (peptide 1).

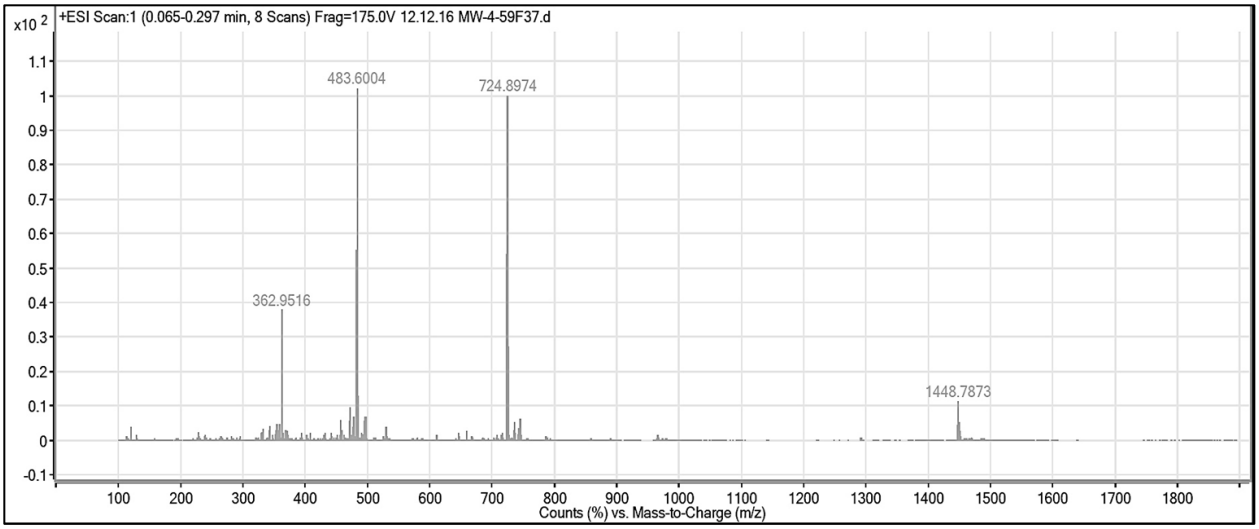


Figure S16. HRMS of Pt-NLS hybrid (peptide 2).

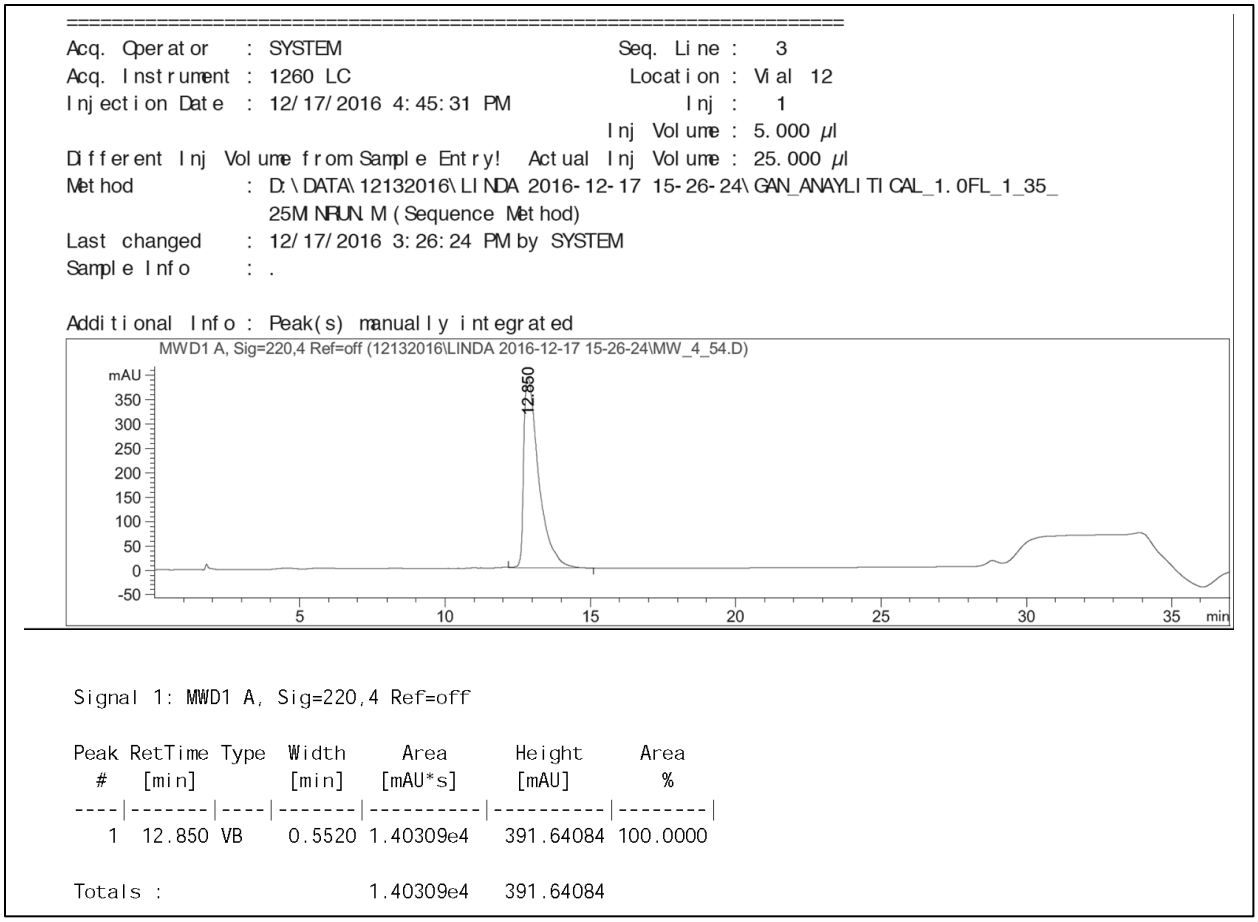


Figure S17. HPLC analysis of precursor for Pt-NLS hybrid (peptide 1).

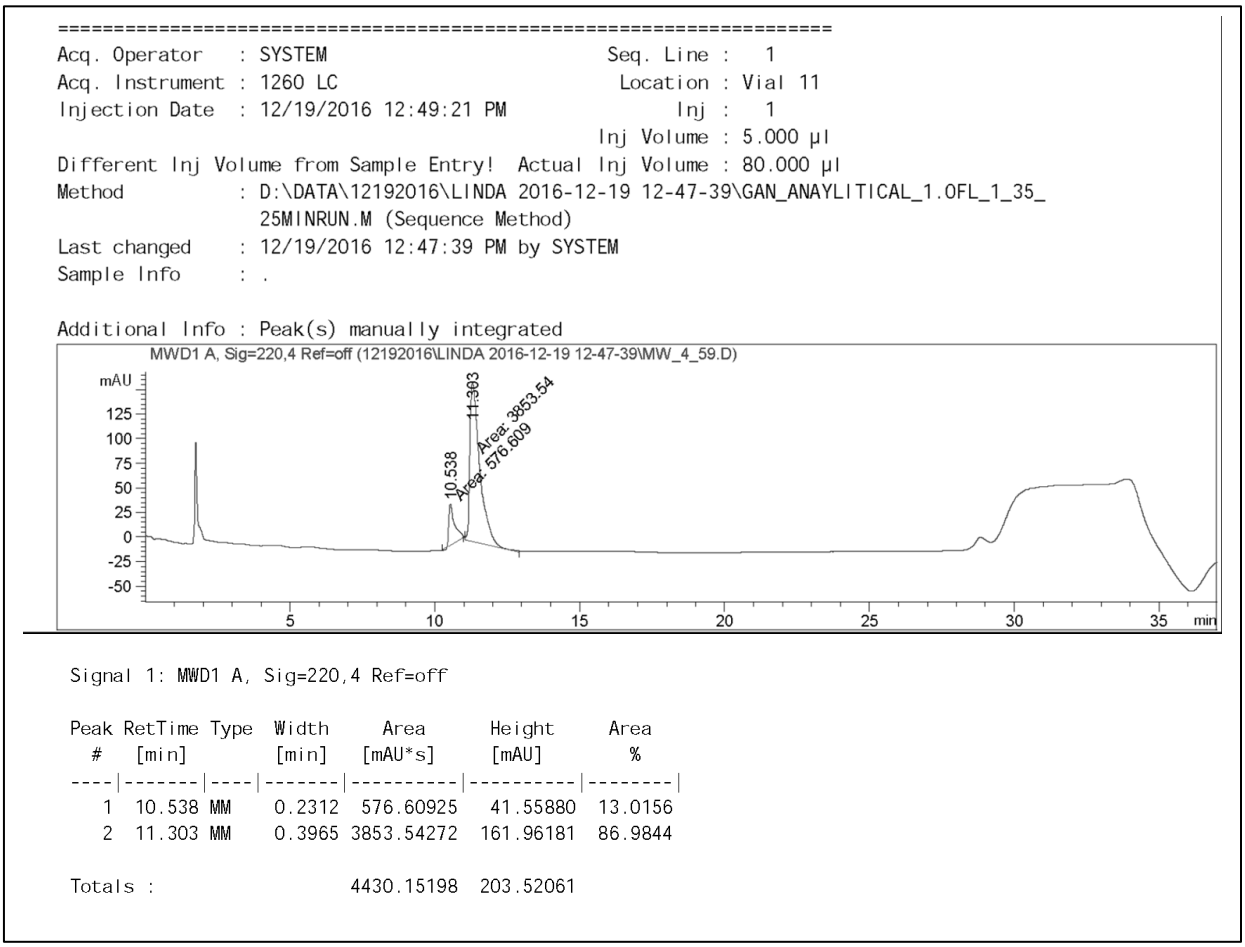


Figure S18. HPLC analysis of f Pt-NLS hybrid (peptide 2).

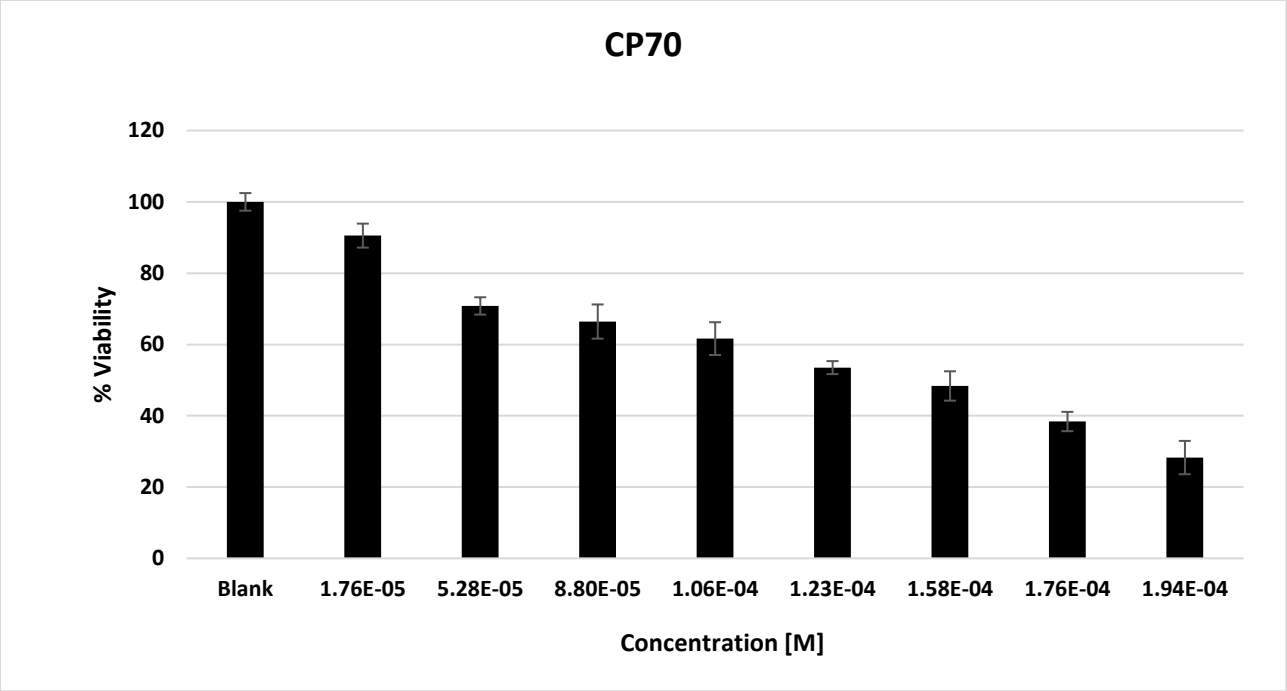


Figure S19. Determination of IC_{50} of carboplatin for CP70 cell line.

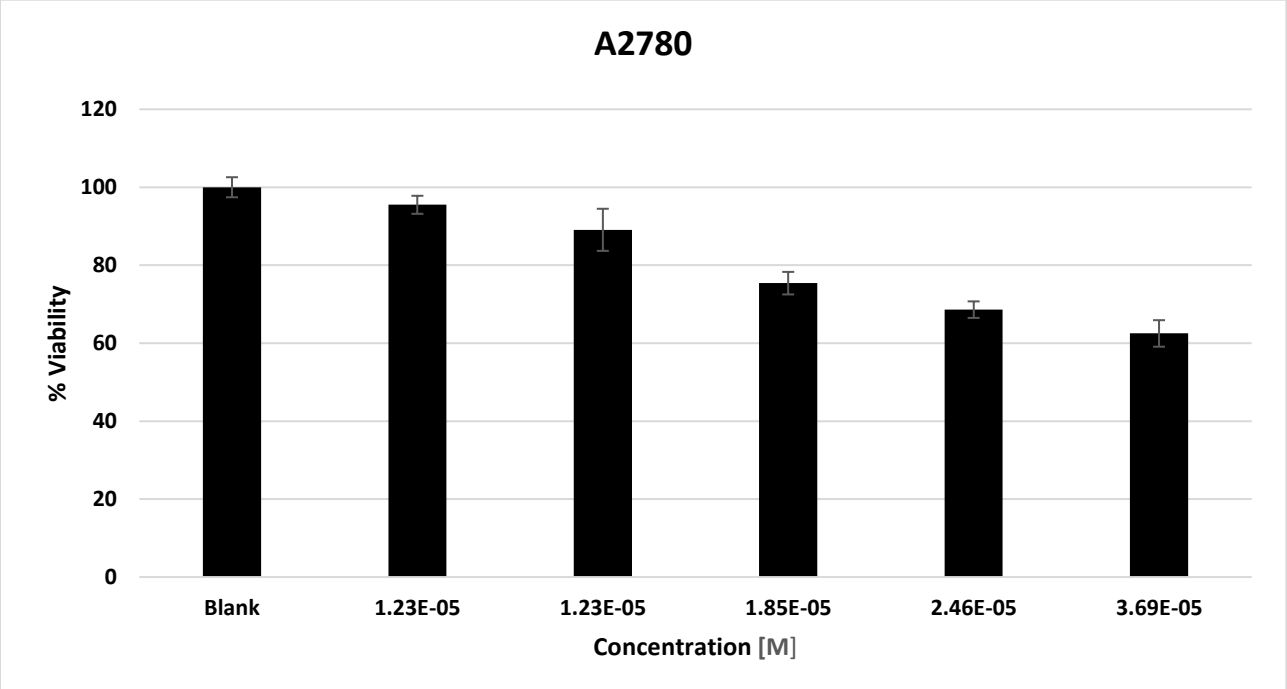


Figure S20. Determination of IC_{50} of carboplatin for A2780 cell line.

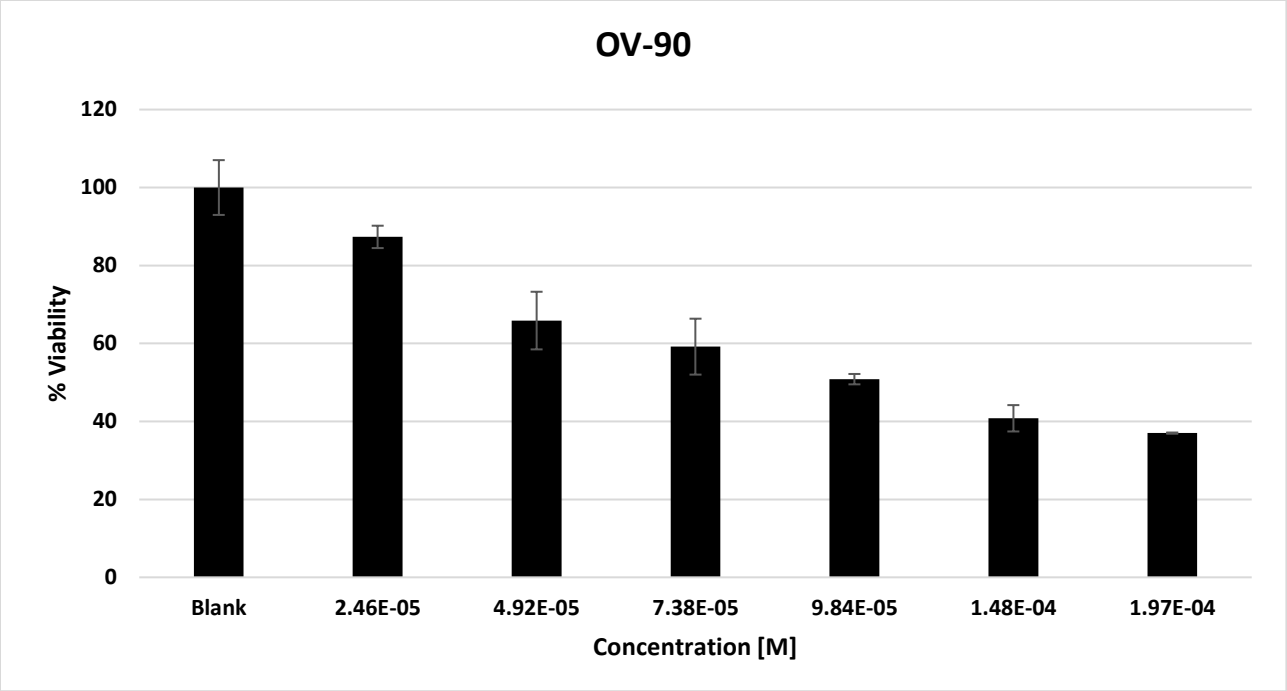


Figure S21. Determination of IC_{50} of carboplatin for OV-90 cell line.

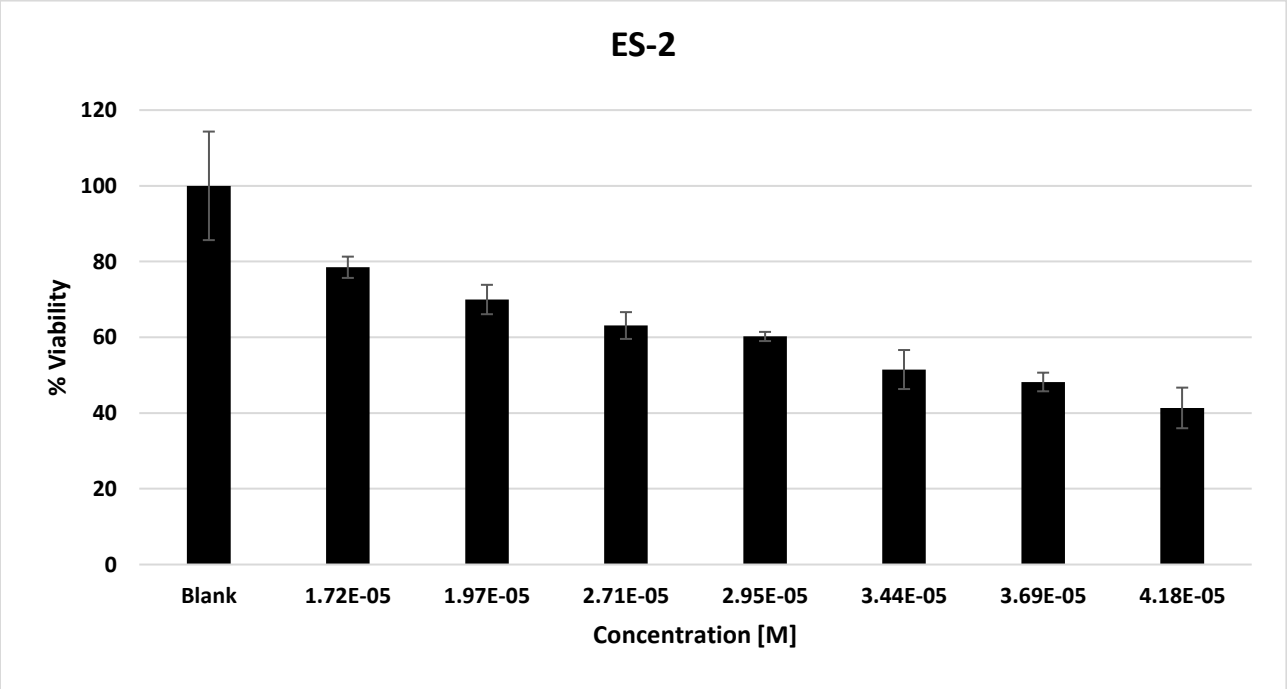


Figure S22. Determination of IC_{50} of carboplatin for ES-2 cell line.

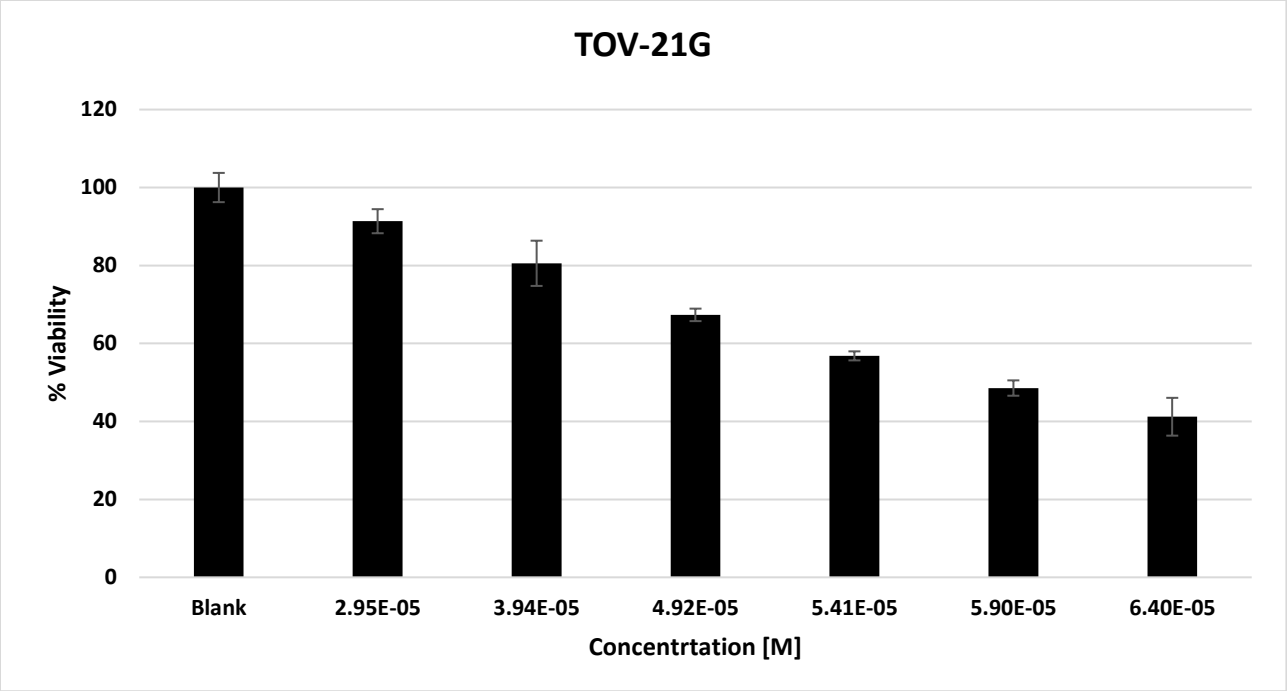


Figure S23. Determination of IC_{50} of carboplatin for TOV-21G cell line.

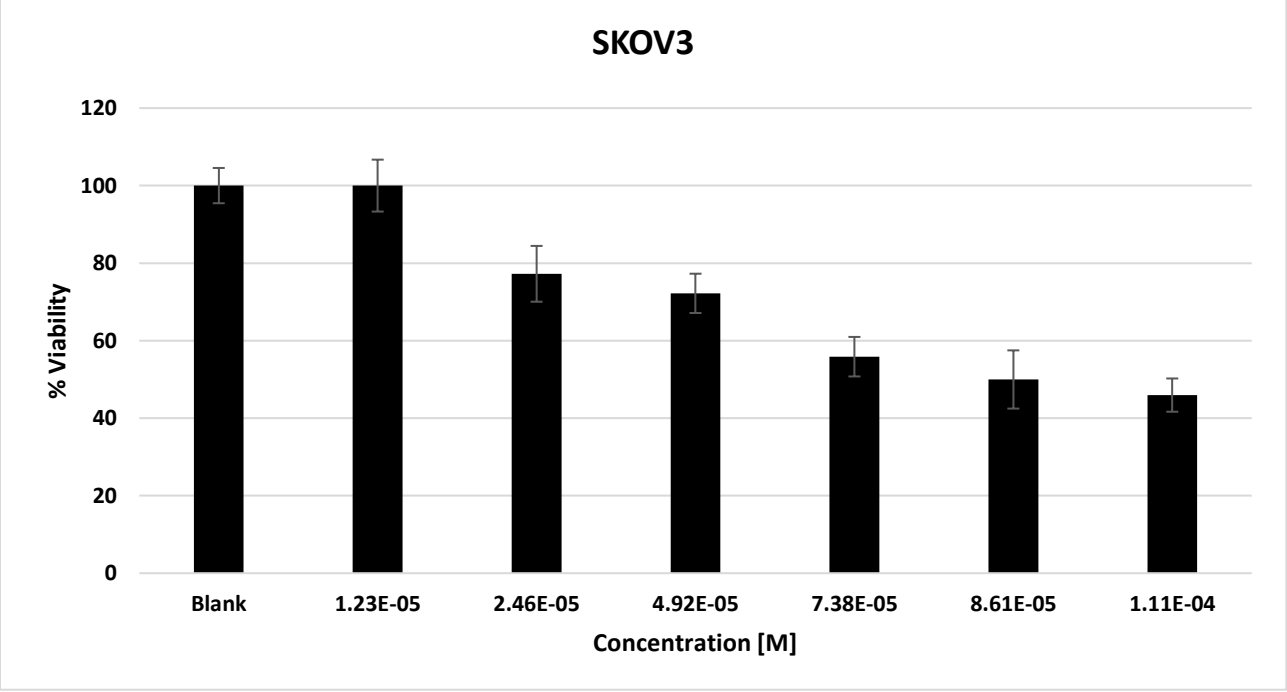


Figure S24. Determination of IC_{50} of carboplatin for SKOV-3 cell line.

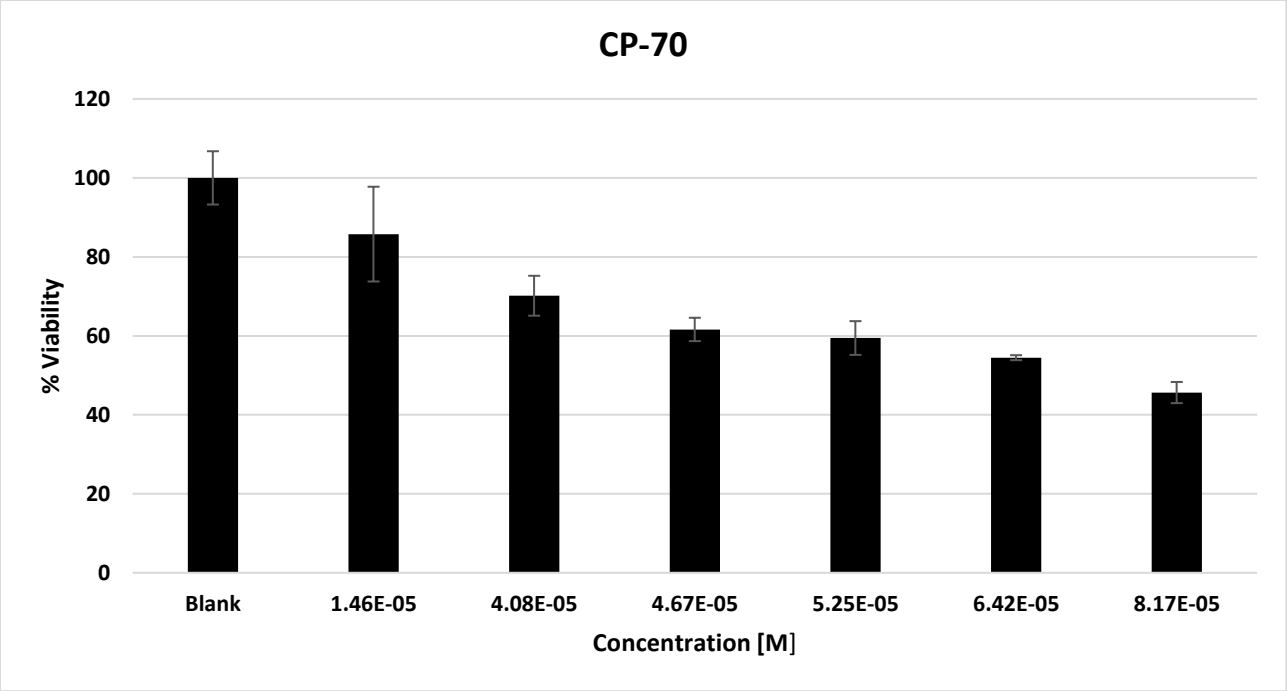


Figure S25. Determination of IC_{50} of Pt-NLS for CP-70 cell line.

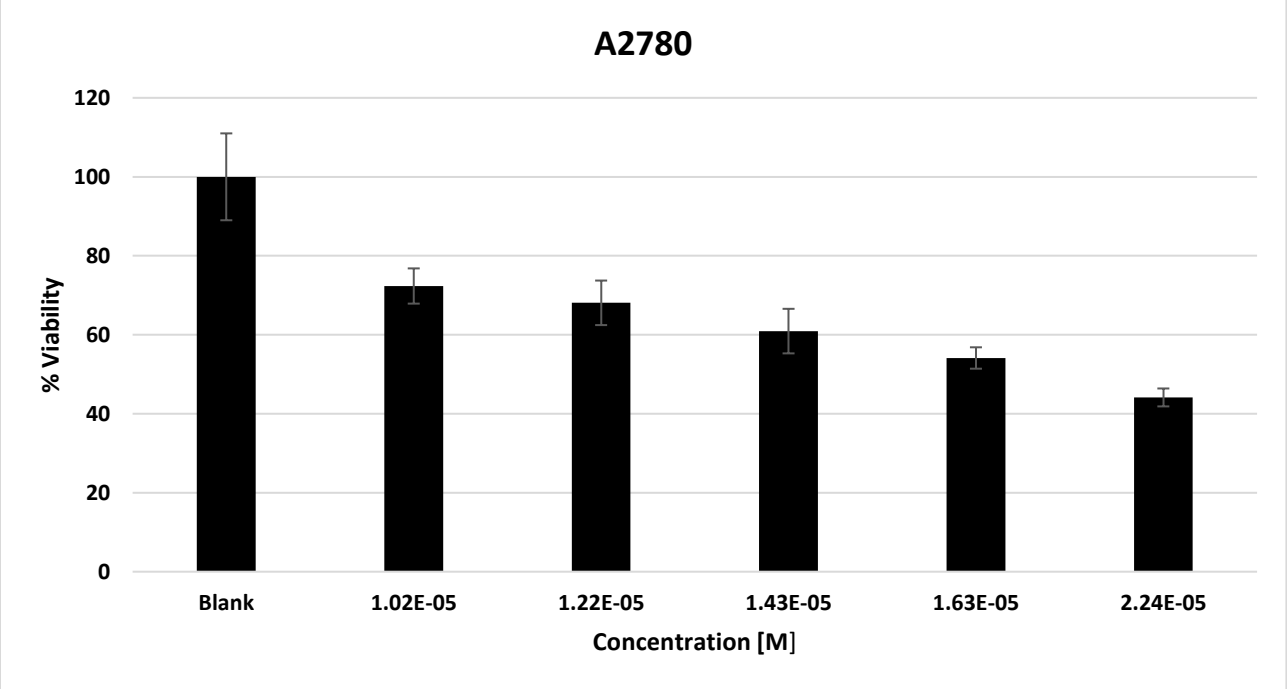


Figure S26. Determination of IC_{50} of Pt-NLS for A2780 cell line.

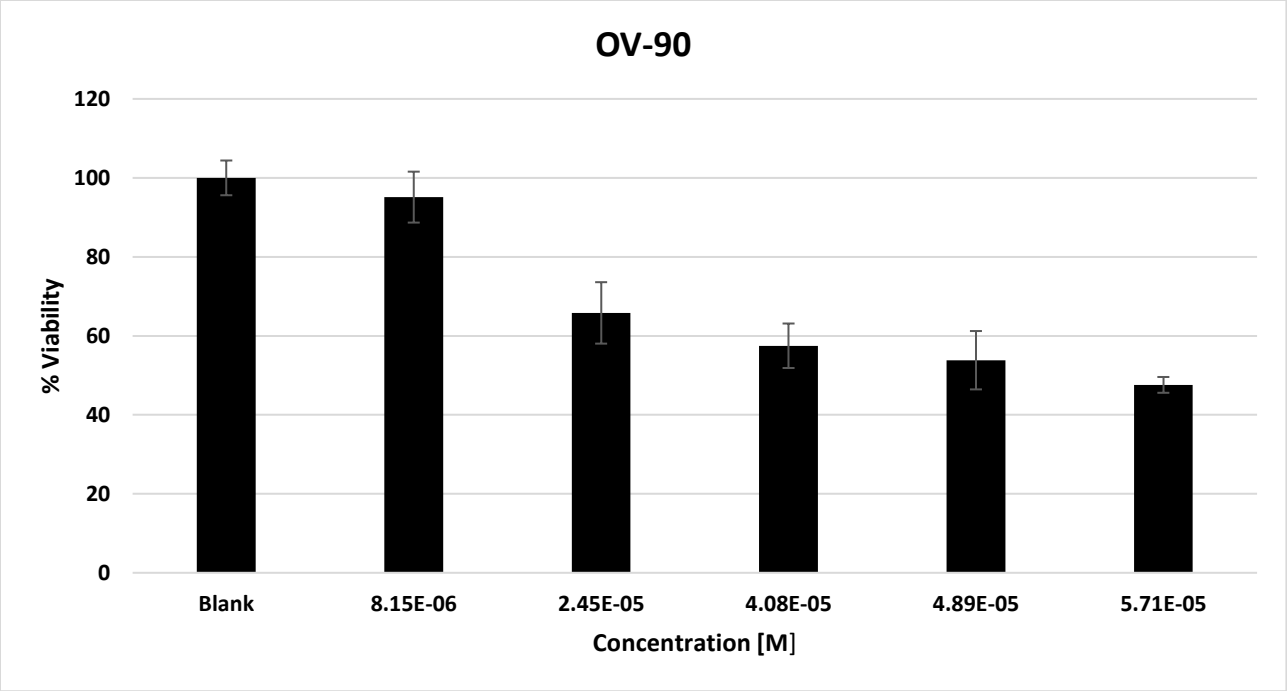


Figure S27. Determination of IC_{50} of Pt-NLS for OV-90 cell line.

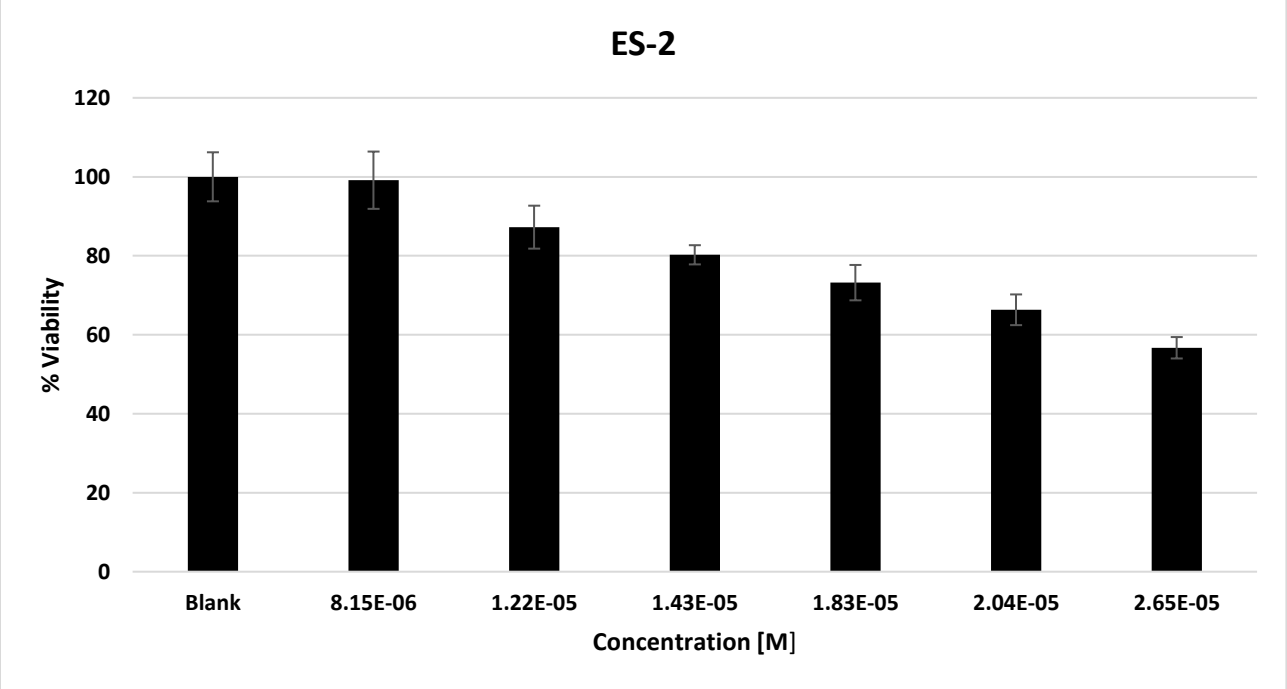


Figure S28. Determination of IC_{50} of Pt-NLS for ES-2 cell line.

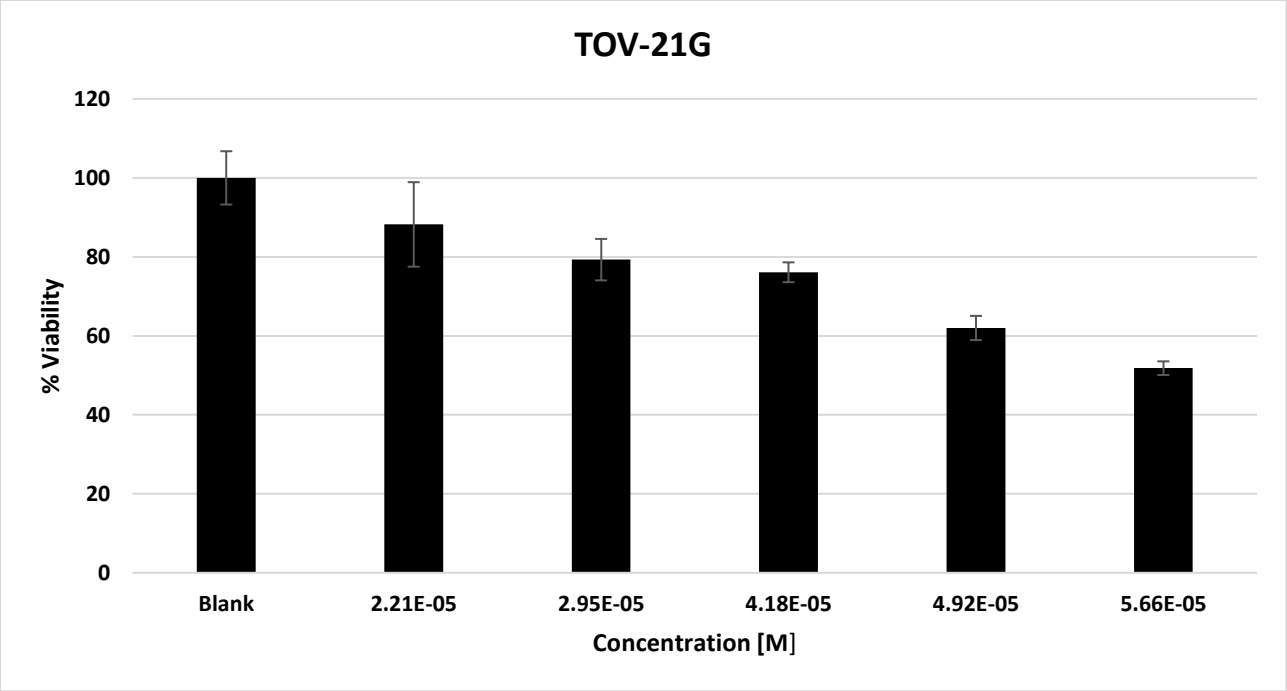


Figure S29. Determination of IC_{50} of Pt-NLS for TOV-21G cell line.

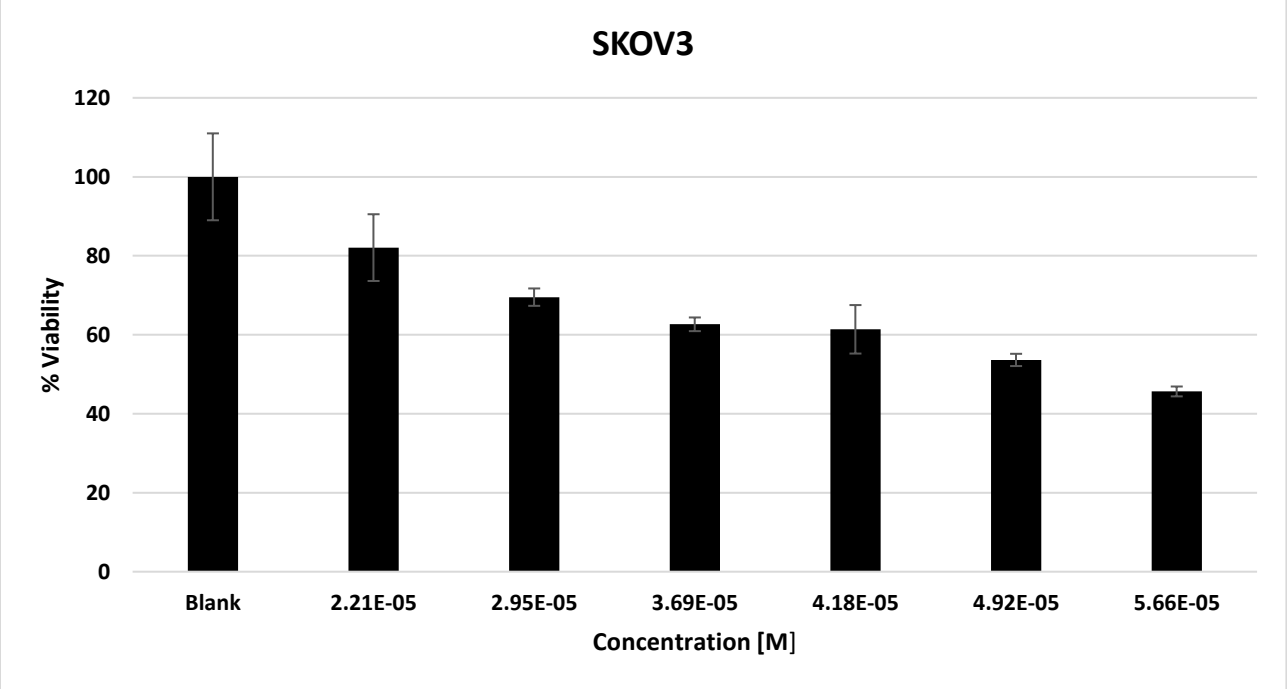


Figure S30. Determination of IC_{50} of Pt-NLS for SKOV3 cell line.

References

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2. J-G Boiteau Single step functionalization and cross-linking of hyaluronic acid. Patent WO 2015/0444455 A1, April 2, **2015**.