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Synthesis, characterization and multi-modal intracellular mapping of cisplatin nano-conjugates

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Contents

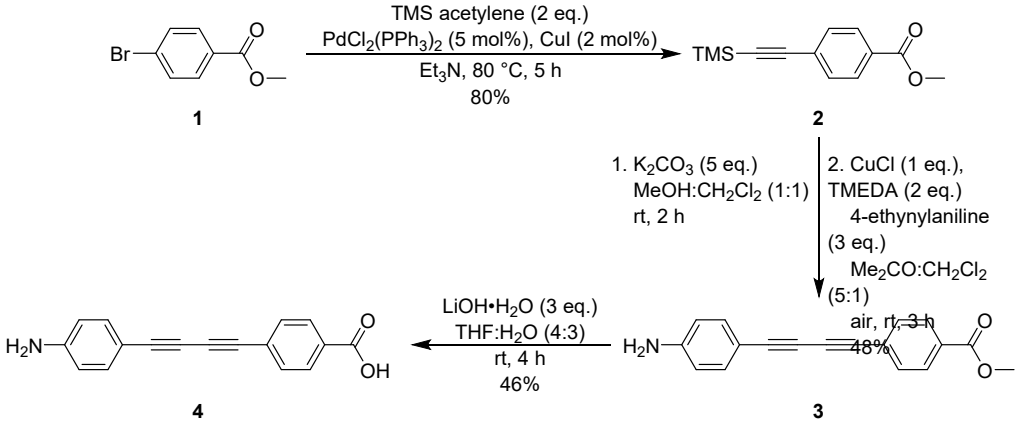
Materials and Methods

Materials

Cisplatin ($Pt(NH_3)_2Cl_2$) and N,N-Dimethylformamide were purchased from Sigma Aldrich (Gillingham, UK). For the cellular accumulation studies, MCF-7 human breast cancer cells (ATCC® HTB-22™) were acquired from American Type Culture Collection (ATCC) (Teddington, UK). All glassware was decontaminated with aqua regia (3 HCl: 1 $HN0_3$) prior to use.

Methods

1. Synthesis of alkyne linker



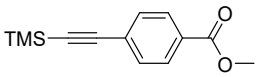
1.1. General Information

All reagents were obtained from Sigma-Aldrich, Alfa Aesar or Fluorochem and used without purification. Solvents were acquired from commercial sources and used without further purification unless otherwise stated. Flash column chromatography was carried out using Fischer Scientific chromatography grade silica 60 Å particle size 35–70 micron. Analytical thin layer chromatography was carried out using aluminium-backed plates coated with Machery-Nagel pre-coated TLC sheets, coated in 0.20 mm silica gel 60 with UV_{254} fluorescent indicator. Sheets were visualized under UV light (at 254 nm) or stained using potassium permanganate solution. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III spectrometer operating at 400 MHz (^1H) and 101 MHz (^{13}C). Chemical shifts were reported in parts per million (ppm) in the scale relative to $CDCl_3$, 7.26 ppm for ^1H NMR and 77.16 for ^{13}C NMR; $(CD_3)_2SO$ (dimethylsulfoxide), 2.50 ppm for ^1H NMR and 39.52 for ^{13}C NMR. Multiplicities are abbreviated as: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; ddd, doublet of doublets of doublets; td, triplet of doublets; app.t, apparent triplet; app.td, apparent triplet of doublets; app.p, apparent pentet; hept, heptet; dhept, doublet of heptets; m, multiplet; br, broad. Coupling constants are measured in Hertz (Hz). Low-resolution mass spectra (LRMS) were recorded on an Agilent 6130 single quadrupole with

APCI/ESI dual source, on a ThermoQuest Finnigan LCQ DUO electrospray, or on an Agilent 7890A GC system equipped with a 30 m DB5MS column connected to a 5975C inert XL CI MSD with TripleAxis Detector and were determined using atmospheric pressure chemical ionization (APCI) unless otherwise stated. ESI refers to electrospray ionization, CI refers to chemical ionization (methane) and EI refers to electron ionization. Melting points were obtained on a Gallenkamp Griffin MPA350.BM2.5 device. Infrared spectra were recorded on an Agilent Technologies 5500 series FTIR. *In vacuo* refers to evaporation under reduced pressure using a rotary evaporator connected to a diaphragm pump, followed by the removal of trace volatiles using a high vacuum (oil) pump.

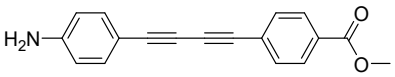
1.2 Experimental Procedures

Methyl 4-((trimethylsilyl)ethynyl)benzoate 2.[1]



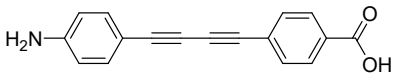
A flame dried microwave vial was charged with aryl bromide **1** (250 mg, 1.16 mmol), $PdCl_2(PPh_3)_2$ (41 mg, 0.06 mmol) and CuI (4 mg, 0.02 mmol) before being sealed and purged with N_2 gas. Triethylamine (Et_3N, 2.50 mL) was added and the mixture was degassed with 4 freeze-pump-thaw cycles. The mixture was then warmed to ambient temperature prior to the addition of TMS acetylene (321 µL, 2.33 mmol), after which the reaction was stirred at 80 °C for 5 h. The mixture was allowed to cool to ambient temperature, diluted with Et_2O (20 mL) and filtered through Celite®. The filtrate was concentrated *in vacuo* and purification by flash chromatography on silica gel (5% EtOAc/petroleum ether 60–80) afforded the *title compound* as an off white solid (215 mg, 0.92 mmol, 80%). **M.P.:** 52–55 °C (lit.[1] 54–55 °C); **FTIR (ATR, cm^{-1})**: 2972, 2917, 2172, 1728, 1614; **^{1}H NMR (400 MHz, $CDCl_3$)**: δ 7.97 (d, *J* = 8.7 Hz, 2H), 7.51 (d, *J* = 8.7 Hz, 2H), 3.91 (s, 3H), 0.26 (s, 9H); **^{13}C NMR (101 MHz, $CDCl_3$)**: δ 166.7, 132.0, 129.9, 129.5, 127.9, 104.2, 97.9, 52.4, 0.0; **LRMS (ES + APCI)**: *m/z* calc. 232.1 found 233.1 $[M+H]^+$.

Methyl 4-((4-aminophenyl)buta-1,3-diyn-1-yl)benzoate 3.[2]



A microwave vial was charged with TMS protected alkyne **2** (100 mg, 0.43 mmol), K_2CO_3 (297 mg, 2.16 mmol) and a mixture of MeOH/CH_2Cl_2 (1:1, 4.0 mL). The mixture was stirred at rt for 2 h before being diluted with water (5 mL) and extracted with CH_2Cl_2 (3 × 5 mL). The combined organics were washed with brine (5 mL) and dried over $MgSO_4$ before being concentrated *in vacuo*. The crude terminal alkyne was used in the next step without further purification. A suspension of CuCl (43 mg, 0.43 mmol) and *N,N,N'*,*N'*-tetramethylethylenediamine (TMEDA, 128 μL, 0.86 mmol) in acetone (4.3 mL) was prepared and purged with air for 15 min. A solution of the terminal alkyne and 4-ethynylaniline (151 mg, 1.29 mmol) in CH_2Cl_2 (0.86 mL) was then prepared and added to the CuCl/TMEDA suspension. The reaction was stirred at rt for 3 h whilst open to air before being partitioned with sat. NH_4Cl solution (5 mL). The aqueous phase was extracted with EtOAc (3 × 5 mL) and the combined organics were washed with brine (5 mL) and dried over $MgSO_4$ before being concentrated *in vacuo*. Purification by flash chromatography on silica gel (20–40% EtOAc/petroleum ether 60–80) afforded the *title compound* as a yellow solid (57 mg, 0.21 mmol, 48%). **M.P.:** 170–173 °C; **FTIR (ATR, cm^{-1})**: 3495, 3397, 2975, 2218, 2175, 1725, 1608; **^{1}H NMR (400 MHz, $CDCl_3$)**: δ 7.99 (d, *J* = 8.3 Hz, 2H), 7.55 (d, *J* = 8.3 Hz, 2H), 7.34 (d, *J* = 8.6 Hz, 2H), 6.61 (d, *J* = 8.6 Hz, 2H), 3.97–3.87 (m, 5H); **^{13}C NMR (101 MHz, $CDCl_3$)**: δ 166.5, 148.0, 134.4, 132.4, 130.0, 129.6, 127.2, 114.8, 110.4, 84.6, 80.0, 77.6, 71.9, 52.4; **LRMS (ES + APCI)**: *m/z* calc. 275.1 found 276.0 $[M+H]^+$.

4-((4-Aminophenyl)buta-1,3-diyn-1-yl)benzoic acid 4.[2]



Methyl ester **3** (30 mg, 0.11 mmol) was dissolved in a mixture of THF/H_2O (1.4:1, 1.2 mL) before LiOH·H_2O (14 mg, 0.33 mmol) was added in one portion. The reaction was stirred at rt for 4 h before being acidified by the addition of 1M HCl. The resultant suspension was filtered and washed with 1M HCl to afford the *title compound* as a black solid (13 mg, 0.05 mmol, 46%). **M.P.:** decomposition > 230 °C; **FTIR (ATR, cm^{-1})**: 2840, 2563, 2221, 1691; **^{1}H NMR (400 MHz, DMSO-d_6)** δ 7.94 (d, *J* = 8.5 Hz, 2H), 7.66 (d, *J* = 8.5 Hz, 2H), 7.29 (d, *J* = 8.6 Hz, 2H), 6.59 (d, *J* = 8.6 Hz, 2H), 3 H missing; **^{13}C NMR (101 MHz, $CDCl_3$)**: δ 166.5, 143.5, 134.0, 132.4, 131.3, 129.6, 125.1, 118.1, 111.5, 84.3, 80.6, 76.5, 72.3; **LRMS (ES + APCI)**: *m/z* calc. 261.1 found 262.1 $[M+H]^+$. **HRMS (ESI)**: $[M+H]^+$ calc for $C_{17}H_{12}O_2N$ 262.08626 found 262.0854.

2. Synthesis of cisplatin conjugates

Gold nanoparticles (AuNPs) were synthesized using a citrate reduction method previously reported by Turkevich et al. More specifically, sodium tetrachloroaurate dihydrate solution (10 mL, 15 mM) was dissolved in 490 mL of double distilled deionized water (dddH2O) and heated until boiling under continuous stirring. Sodium citrate tribasic dihydrate (7.5 mL, 26 mM) was then added and the mixture was boiled under continuous stirring for approximately 1 hour. The resulting colloidal solution was then left to cool, maintaining stirring throughout.

The attachment of the active component of cisplatin to the AuNPs was facilitated through the alkyne linker **4**. For the functionalisation, a target concentration of 10 μM was used, as it resulted in a strong SERS signal without inducing aggregation at this stage of the synthesis on the colloidal AuNP dispersion. Briefly, 100 μL of 100 μM stock was added to 900 μL of AuNPs and 100 μL of borate buffer (pH 8.5) and incubated on a shaker plate for 30 minutes. The final stage in the assembly of the drug-nanoparticle conjugates was the coupling of cisplatin. To facilitate this, cisplatin was dissolved in DMF and incubated with the alkyne-nanoparticles overnight. Following this, the samples were centrifuged at 4000 rpm for 20 minutes and resuspended in dH_2O.

3. Characterization of cisplatin conjugates

Extinction Spectroscopy: Extinction spectra were measured using an Agilent Cary 60 UV-Visible spectrophotometer with Win UV scan V.2.00 software. Initially the system was left to equilibrate at RT, followed by the insertion of quartz micro cuvette (Micro Cell 115-QS, 10 mm Light Path) with 400

µL of sample in order to scan wavelengths from 200 – 800 nm. A baseline was obtained using double distilled dH2O in place of the sample.

Particle Tracking Analysis (PTA): PTA measurements were performed with an NS300 instrument, manufactured by Malvern Panalytical and equipped with a 405 nm diode laser source, sCMOS camera, syringe pump and NTA3.4 software. The PTA instrument was switched on at least 30 minutes before the measurements. All measurements were performed at room temperature. Videos were recorded with a camera level set to 13, over 60 seconds' duration with 10 seconds equilibration time prior to each measurement. Two independent preparations of each sample were measured five times under repeatability conditions (n=10). Measurements were performed in a flow mode, with a syringe pump set to injection speed of 40. The software assumed viscosity of water for the samples. For the analysis of the recorded videos, detection threshold was set to 5. The obtained data were further processed with Excel using modal diameter values generated by the software.

Microwave digestion and determination of total Pt concentration via Inductively coupled plasma mass spectrometry (ICPMS): Prior to analysis, the samples were digested using a Milestone Ethos UP system equipped with PFA micro-volume vessels (Milestone SRL, Sorisole, Italy). For each sample 300mg was weighed directly into a PFA micro-volume vessel and then 3 mL of 10% HCl+ 3% HNO_3 (UpA, Romil, Cambridge, UK) solution was added. The micro-volume vessels were placed inside the outer vessel which was loaded with 10 mL H_2O and 1 mL H_2O_2 (UpA, Romil, Cambridge, UK). Digestion of the samples was achieved by heating to 180 ^oC for 10 mins followed by a hold at 180 ^oC for a further 10 mins. Once cooled the samples were further diluted up to 10mL in ultra-pure H_2O (18.2 MΩ cm). Where necessary samples were further diluted 10-fold in 10% HCl+ 3% HNO_3 solution.

A 7700 ICP-QQQ-MS instrument (Agilent Technologies California, USA) was used for the determination of total Pt concentration in the samples by monitoring the ^{195}Pt isotope in the 'no gas' mode. The instrument was calibrated using gravimetrically prepared standards of 0.1 – 5 µg/kg Pt from a Romil single element standard solution (Cambridge, UK). ^{209}Bi was used as an internal standard and added inline at a concentration of 1 µg/kg. A quality control sample was prepared by diluting the multielemental standard SM68-3 (VHG, LGC Standards) in the acidic solution, and was analysed before and after each batch.

4. Intracellular tracking of cisplatin-conjugates

Cell culture and treatment: MCF-7 human breast cancer cells were cultured in Rosewell Park Memorial Institute medium (RPMI 1640) containing 1% penicillin/streptomycin (10000 units per mL), 1% fungizone, and 10% heat-inactivated fetal bovine serum (FBS). The cells were incubated at 37 °C and 5% CO2 in a humidified incubator. For fixed cell microscopy the cells were resuspended in media at a final concentration of 0.25 10^6 cells per ml, seeded on 22 mm glass coverslips and incubated for 24 hours at 37 °C and 5% CO2 in a humidified incubator. For the treatment, 400 µL of the cisplatin-

conjugates were mixed with the media and incubated with the cells for 48 hours. For the control samples bare AuNPs were used. The coverslips were washed with PBS and fixed in 4% paraformaldehyde (PFA) for 15 minutes. The fixed cells were further washed with PBS and dH_2O and left to airdry prior to mounting on a standard microscope slide for imaging.

SERS imaging: A Renishaw InVia Raman confocal microscope equipped with a Leica 50x/NA 0.55 N PLAN EPI objective and a HeNe 633 nm laser excitation source was used. A grating of 1800 l mm-1 in high confocality mode and a laser power of 1.2 mW (10% power) with a 1 s acquisition time per point was used to map the cell areas. More specifically, 3D maps were acquired to investigate the depth profiles and establish the focal plane of the fixed cells in correlation with the white light images. The 3D maps were collected in edge Streamline HR high confocality mode, with a spatial resolution of 1 μm in the X and Y directions and 5 μm between Z stacks. For each map, a total of 5 Z planes were collected. After acquisition, all spectra were processed using Python 3. For each SERS spectrum, a 3rd order polynomial baseline was determined and subtracted from the original spectrum, yielding a baseline corrected spectrum. To generate the SERS maps, the total peak area between 2155 – 2280 cm^{-1} was determined for each pixel. Each map was subsequently reconstructed using Matlab.

LA-ICP-ToF-MS imaging: For this study, an ImageBio 266 nm laser ablation system (ESL, Montana, USA) equipped with a TwoVolume3 ablation cell and dual concentric injector was used. The laser ablation system was coupled to an inductively coupled plasma mass spectrometer with a Time-of-Flight mass analyzer ICP-ToF-MS (2R) system (TOFWERK, Thun, Switzerland). The isotopes ^{197}Au, ^{195}Pt and ^{31}P were measured in no gas mode. For each sample, multiple parallel line scans (80 lines per replicate) were ablated to create 2D distributions of the selected isotopes, with a 4 J cm^{-2} fluence, 50 Hz repetition rate and 10 μm laser spot size (n=3 replicates per condition). The data were exported to ImageJ where false color images were generated representing the intensity of each isotope per laser pulse.

Supplementary Tables

Table S1: Determination of total ^{195}Pt concentration by ICPMS, validating the cisplatin binding in the conjugate samples.

Sample	**^{195}Pt concentration, mean ± stdev, n=3 (mg kg^{-1}*) / rsd**
Bare AuNPs	< 0.07

Conjugates	2477 ± 156 / 6%

* ^{195}Pt LOQ: 0.07

Supplementary Figures

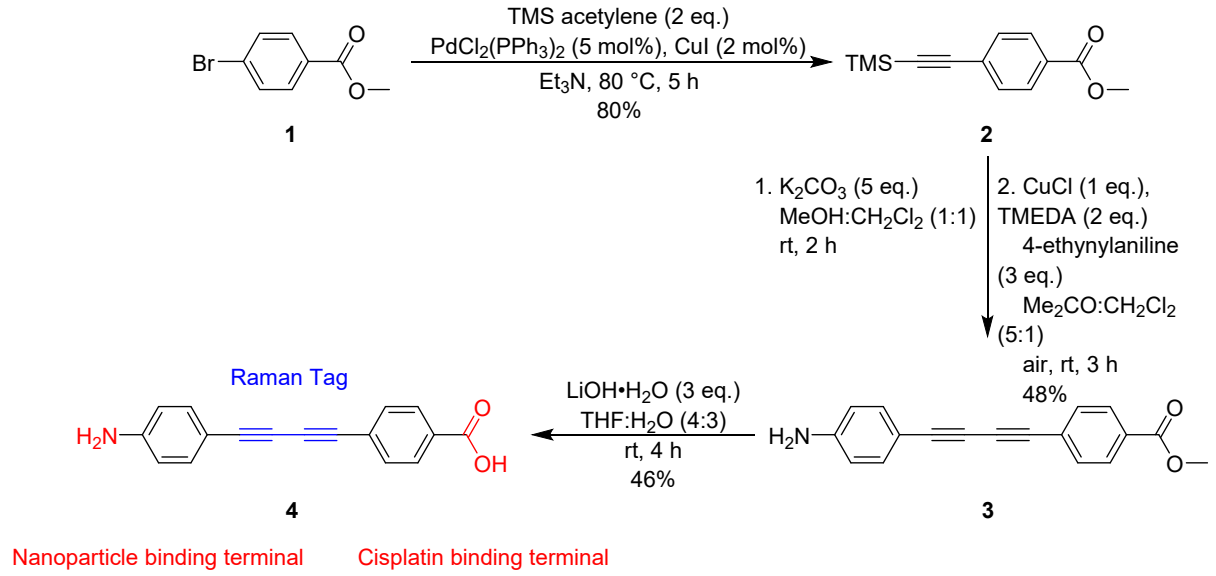


Figure S1: Synthesis of the organic linker **4** incorporating a *bis*alkyne moiety as the Raman tag and two terminals for facilitating the binding with cisplatin and with the nanoparticles.

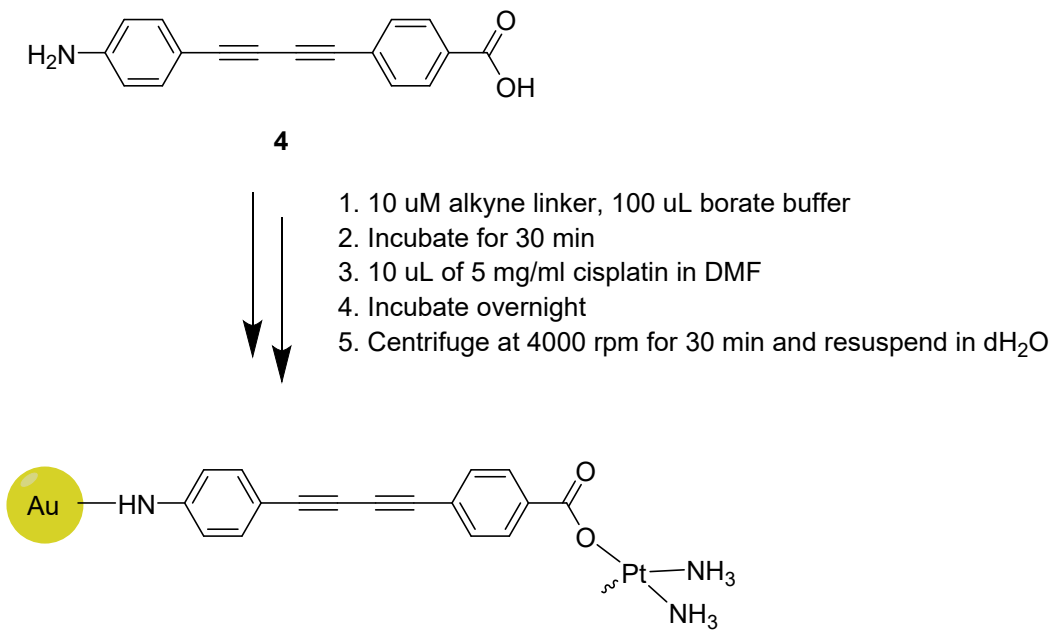


Figure S2: Synthesis of the cisplatin conjugates.

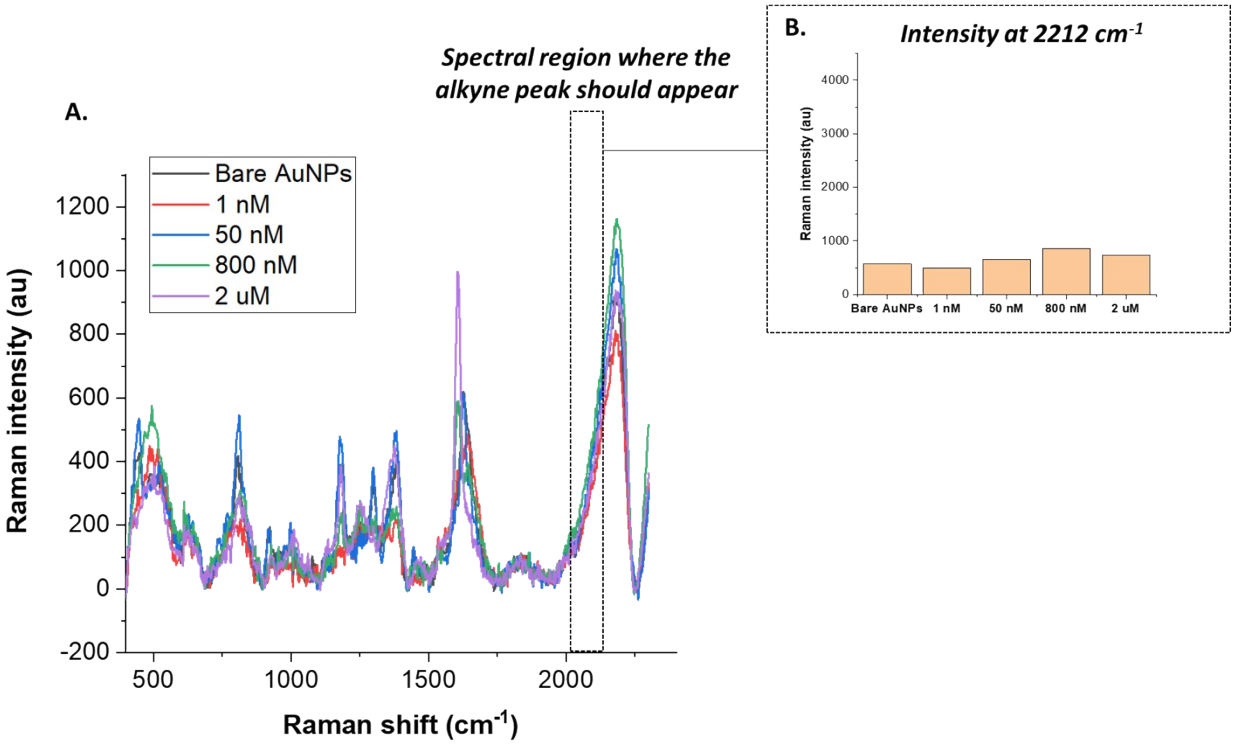


Figure S3: Optimization of the experimental conditions to facilitate the binding of the alkyne linker to the AuNPs. At the slightly acidic pH of AuNPs as prepared, no binding was observed. A. SERS spectra showing no SERS peaks originating from the alkyne linker for the investigated concentrations (1 nM – 2 µM). A Snowy Range CBEx 2.0 handheld Raman spectrometer (Snowy Range Instruments, Laramie WY USA) equipped with a 638 nm laser excitation with a maximum laser power of 40 mV and 1 s accumulation time was used to

acquire the spectra (n=3), which were then processed for baseline correction in Matlab. The spectra shown are an average of the collected spectra. B. No increase of the absolute Raman intensity at the spectral region of 2212 cm^{-1} (corresponding to the alkyne peak) with increasing concentration.

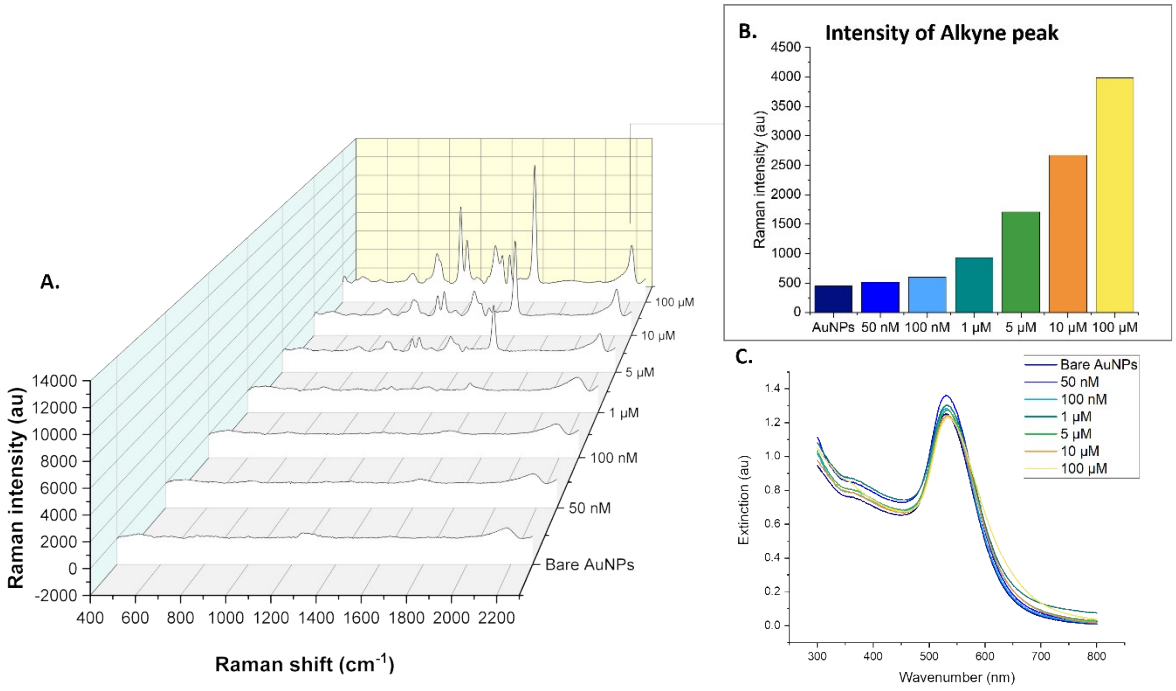
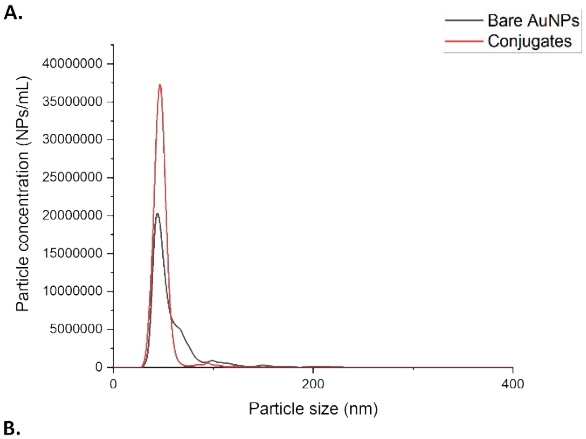
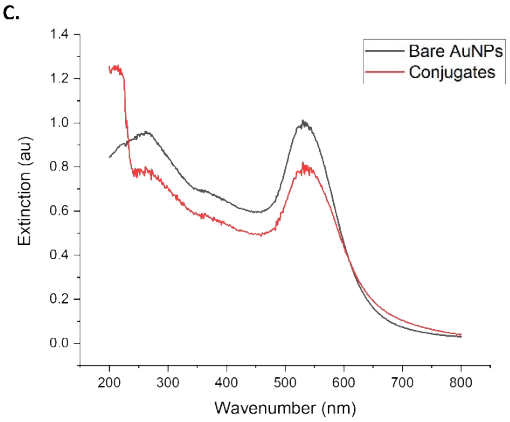


Figure S4: Optimization of the alkyne linker concentration (50 nM – 100 µM) for signal intensity. A. SERS spectra showing an increase in the intensity of SERS peaks with increasing concentration. A Snowy Range CBEx 2.0 handheld Raman spectrometer (Snowy Range Instruments, Laramie WY USA) equipped with a 638 nm laser excitation with a maximum laser power of 40 mV and 1 s accumulation time was used to acquire the spectra (n=3), which were then processed for baseline correction in Matlab. The spectra shown are an average of the collected spectra. B. The increase of the absolute Raman intensity of the alkyne peak at 2212 cm^{-1} with increasing concentration. C. Extinction spectra showing the colloidal stability of 50 nM – 10 µM concentrations and the aggregation starting to appear as peak shouldering in the higher concentration (100 µM).

A.



C.



B.

Sample	Particle number concentration, mean ± stdev, n=10 (kg^{-1}*) / rsd	Modal particle diameter mean ± stdev, n=10 (nm) / rsd
Bare	(4.4 ± 0.4) E13 / 8.6%	44.1 ± 1.8 / 4.0%
Conjugates	(5.6 ± 0.2) E13 / 4.1%	46.5 ± 1.7 / 3.8%

**assuming density of water 1.0 g/cm^3*

Figure S5: Characterization of cisplatin conjugates against control bare AuNPs. A. Average PTA size distribution graph, showing a monomodal character for the conjugates with minimal aggregates present (n=10 replicates) and a better colloidal stability compared to bare AuNPs. B. Summary table of the particle number concentration and modal particle diameter, as characterized by PTA. C. Extinction spectra showing the overall moderate colloidal stability of conjugates with respect to bare AuNPs. The increase around 200 nm suggests the presence of cisplatin in the conjugate samples. Details on the protocol used to acquire the data can be found in the methods section.

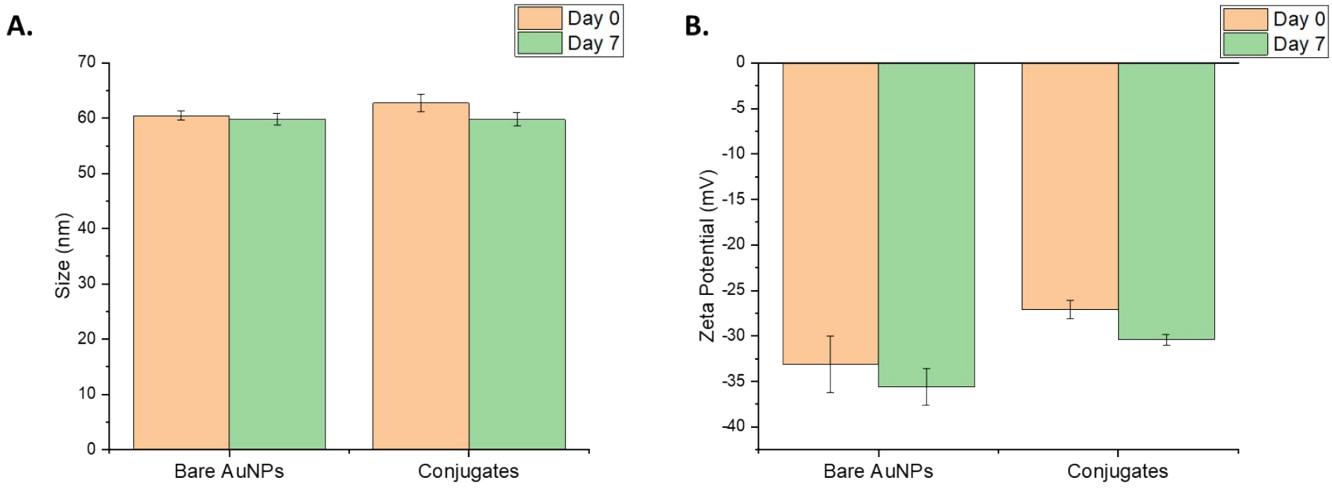


Figure S6: Dynamic light scattering (A) and zeta potential measurements (B) demonstrating the stability of conjugates in double distilled dH_20 for the duration of a week, when stored at 4 ºC. Error bars represent ± SD (n=3 replicates).

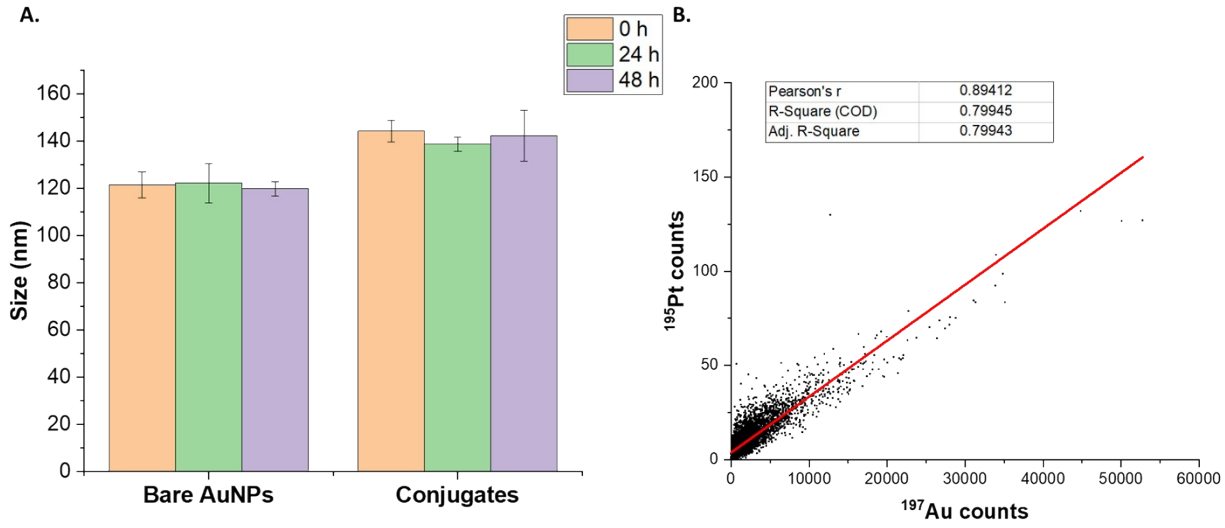


Figure S7: Investigation of the conjugate stability in biological environments. A. Dynamic light scattering measurements of the conjugates in presence of an RPMI media mixture demonstrated minor aggregation in presence of the biological components, however no further aggregation was observed for 48 hours. B. Multi-elemental pixel-by-pixel correlation of ^{197}Au and ^{195}Pt counts in conjugate treated MCF-7 cells, demonstrating that the observed minor aggregation did not inhibit the intracellular uptake of the conjugates. The correlation between the two supports the intracellular stability of the conjugates.

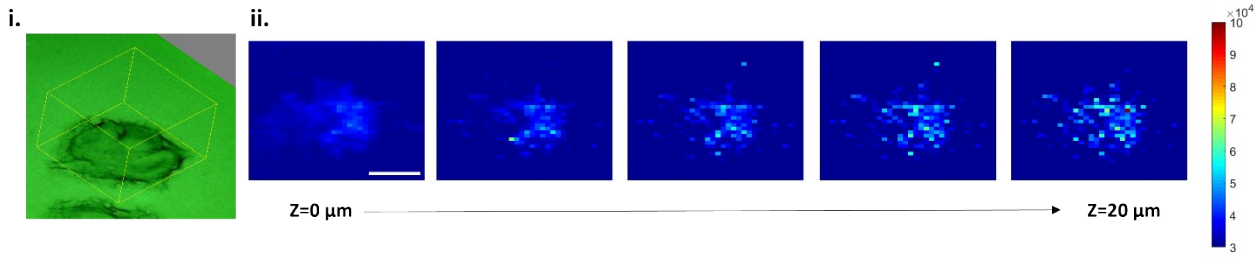


Figure S8: 3D SERS imaging of MCF-7 cells treated with bare AuNPs. i. White light image demonstrating the 3D area mapped. ii. False color images generated for each Z plane mapped, where each pixel corresponds to the total peak area between 2155–2280 cm^{-1}. The 3D maps were collected with a 633 nm laser in edge Streamline HR high confocality mode at 1 µm resolution in the X and Y directions and 5 µm between Z-stacks. A grating of 1800 l mm in high confocality mode and a laser power of 1.2 mW (10% power) with a 1 s acquisition time per point was used. The false color images were generated in Python and correspond to the presence or absence of the alkyne SERS peak. The scale bar is 10 µm.

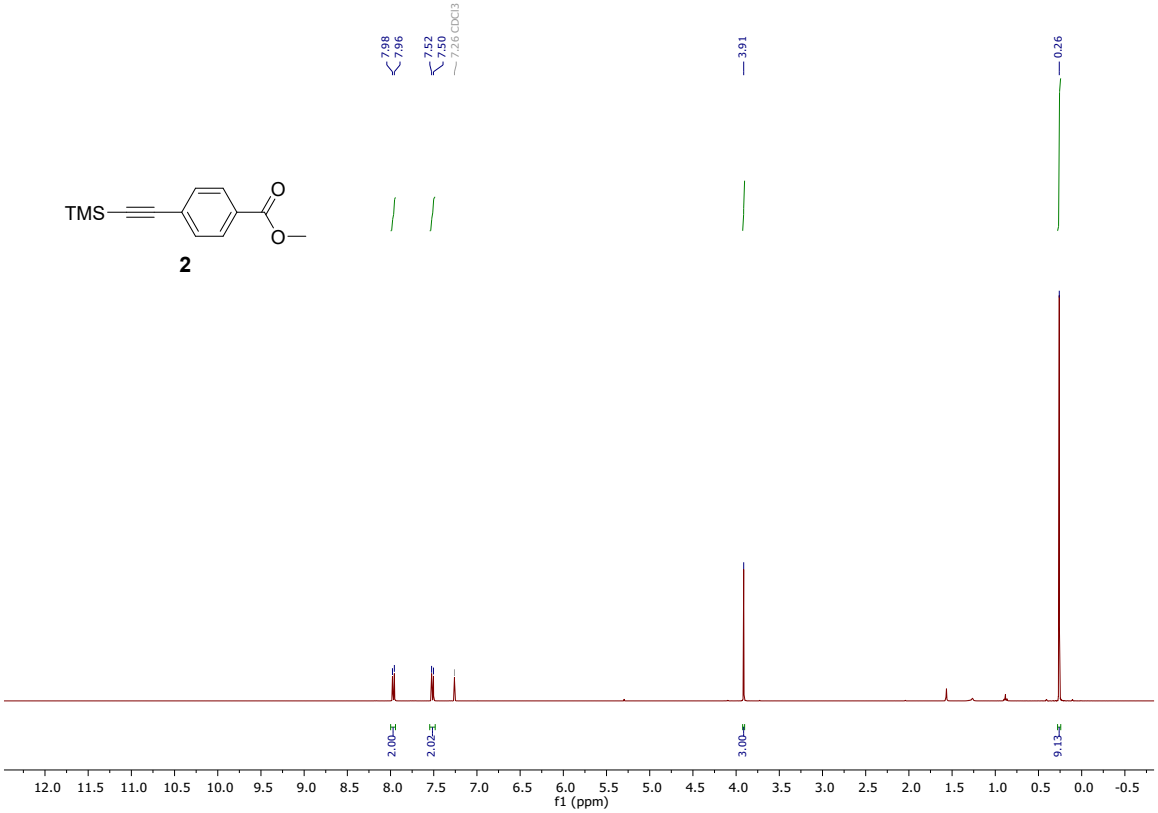
References

1 - S. R. Parsons, J. F. Hooper and M. C. Willis, *Org. Lett.*, 2011, **13**, 998–1000.

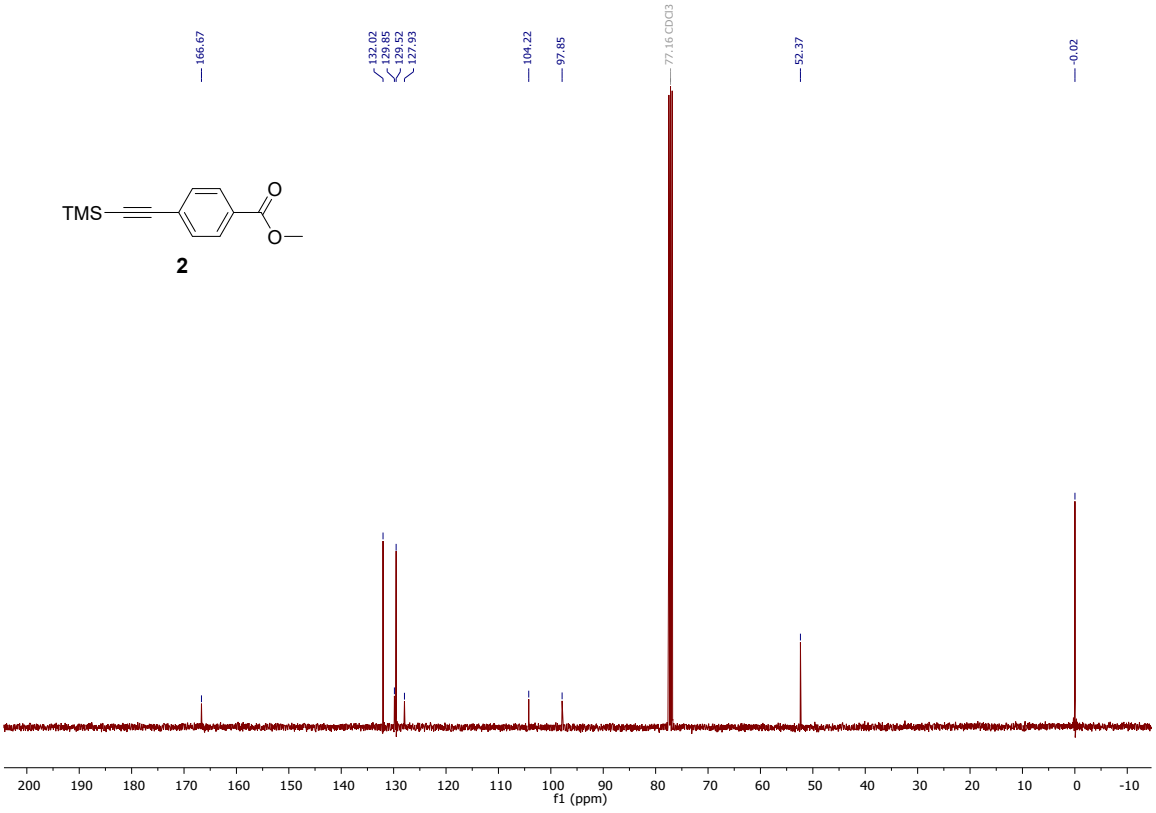
2 - X. Liang, C.-J. Lee, X. Chen, H. S. Chung, D. Zeng, C. R. H. Raetz, Y. Li, P. Zhou and E. J. Toone, *Bioorg. Med. Chem.*, 2011, **19**, 852–860.

Copies of NMR Spectra

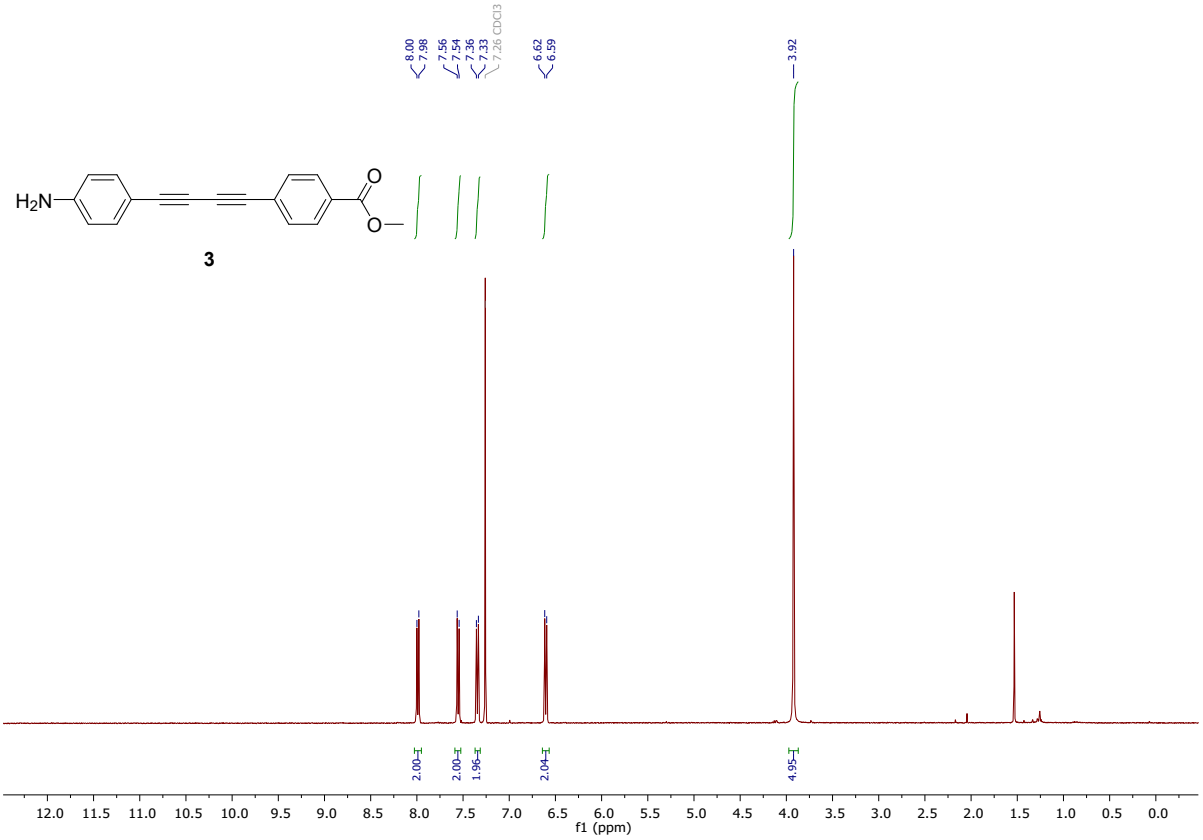
^{1}H NMR spectrum of **2** (400 MHz, $CDCl_3$)



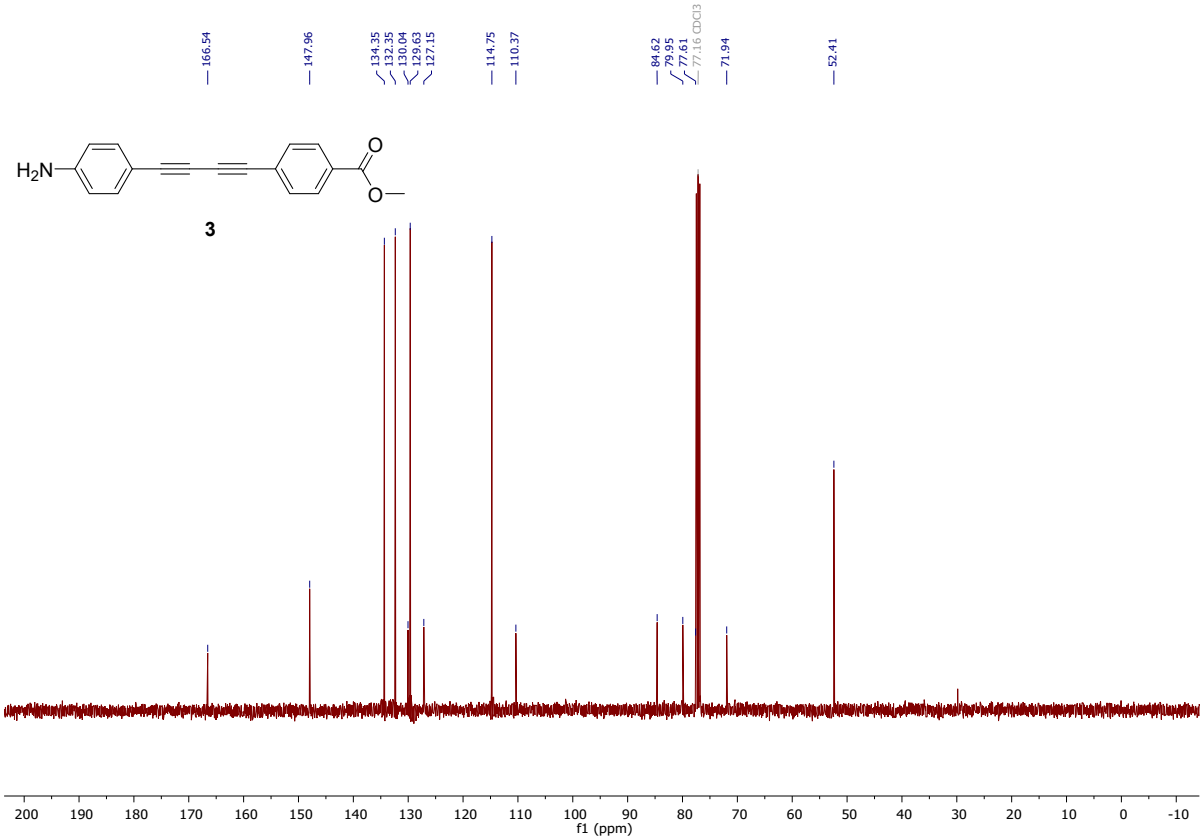
^{13}C NMR spectrum of **2** (101 MHz, $CDCl_3$)



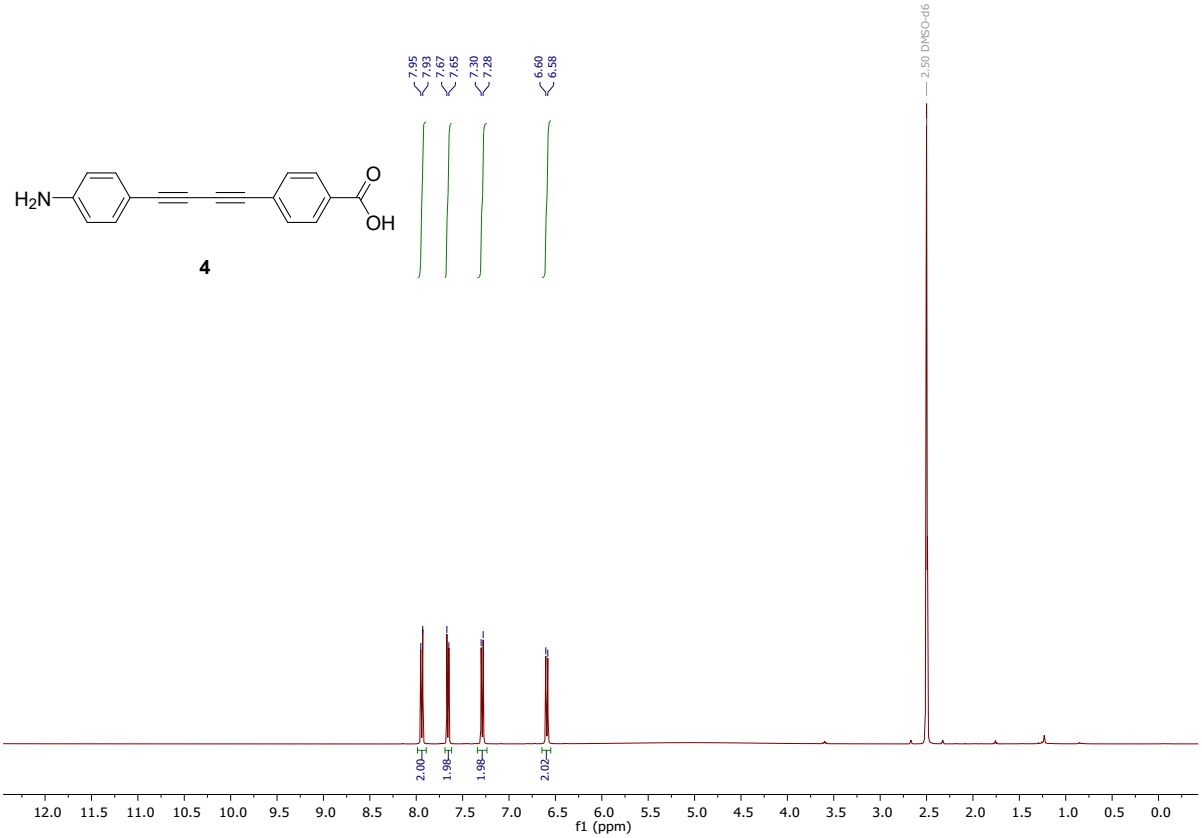
^{1}H NMR spectrum of **3** (400 MHz, $CDCl_3$)



^{13}C NMR spectrum of **3** (101 MHz, $CDCl_3$)



^{1}H NMR spectrum of **4** (400 MHz, DMSO-d_6)



^{13}C NMR spectrum of **4** (101 MHz, DMSO-d_6)