Tumor Targeted Alpha Particle Therapy with an Actinium-225 Labelled Antibody for Carbonic Anhydrase IX

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Supporting Information

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1.1 Materials and Reagents

All solvents and reagents were purchased from standard commercial suppliers and were used as received.

1.2 Instrumentation

^{1}H, ^{13}C, COSY, HSQC, HMBC were all recorded using a Varian FT-NMR 400 or FT NMR 500 spectrometer with a cryoprobe (Bruker). All ^{1}H NMR spectra were acquired at 400 MHz or 500 MHz and ^{13}C spectra were acquired at 101 MHz or 126 MHz. The reported peaks were all referenced to solvent peaks in the order of parts per million at 25°C. Microwave reactions were performed on a Biotage Initiator. Non-radioactive analytical HPLC were performed on Agilent 1200 series HPLC system (System A) or a 1290 LC System (System B) fitted with an Alltech Hypersil BDS-C18 (4.6 x 150 nm, 5um, column A) or a Phenomonex Luna C18(2) column (4.6 mm x 150 mm, 5 µm, column B) and Phenomenex SecurityGuard™ C18 guard cartridge (4 mm x 30 mm) with a 1 mL/min flow rate; gradient elution of Buffer A = 0.1% TFA in H_2O and Buffer B = 0.1% TFA in acetonitrile (method A: 5 to 100% B in A over 25 min, method B: 0 to 100% B in A over 25 min) and UV detection at 214, 254, 280 nm and 350 nm. Semipreparative RP- HPLC (1260 Infinity II Preparative LC System with a mass detector on a Lunar C18 column, 100Å, 21.2 x 250 mm, 5 µm) with a 15 mL/min flow rate. Gradient elution of Buffer B = 0.1% FA in H_2O and Buffer B= 0.1% FA in acetonitrile Method C (5 to 95% B in A at 23 min, 95 % B in A at 25 min, 5 % B at 26 min) and detection at 280 nm and 254 nm or an Agilent 1200 LC System on a Lunar C18 column, 100Å, 21.2 x 250 mm, 5 µm) with an 8 mL/min flow rate. Gradient elution of Buffer B = 0.1% TFA in H_2O and Buffer B = 0.1% TFA in acetonitrile. Method D: 5 to 60 % B in A at 20 min, 60 to 95% B at 26 min, 95 to 5 % B in A at 30 min). ESI-QTOF MS was collected on an Exactive Plus Orbitrap Infusion mass spectrometer (Exactive Series, 2.8 Build 268801, ThermoFisher Scientific). Analysis was performed using

Xcalibur 4.0.27.10 (ThermoFisher Scientific). Protein samples were analysed on Agilent 6220 ESI-TOF LC/MS Mass Spectrometer coupled to an Agilent 1200 LC system (Agilent, Palo Alto, CA). All data were acquired, and reference mass corrected *via* a dual-spray electrospray ionisation (ESI) source. Acquisition was performed using the Agilent Mass Hunter Acquisition software version B.02.01 (B2116.30). Ionisation mode: Electrospray Ionisation; Drying gas flow: 7 L/min; Nebuliser: 35 psi; Drying gas temperature: 325°C; Capillary Voltage (Vcap): 4000 V; Fragmentor: 300 V; Skimmer: 65 V; OCT RFV: 250 V; Scan range acquired: 300–3200 m/z Internal Reference ions: Positive Ion Mode = m/z = 121.050873 & 922.009798. Protein desalting and chromatographic separation was performed using an Agilent Poroshell C18 2.1 x 75 mm, 5µm column using 5% (v/v) acetonitrile ported to waste (0 – 5min). Upon desalting of the sample, the flow was ported back into the ESI source for subsequent gradient elution with (5% (v/v) to 100% (v/v)) acetonitrile / 0.1% formic acid over 8 min at 0.25 mL/min. Analysis was performed using Mass Hunter version B.06.00 with BioConfirm software using the maximum entropy protein deconvolution algorithm; mass step 1 Da; Baseline factor 3.00; peak width set to uncertainty. ELISA assay measurements were performed on a FLUOstar Omega microplate reader (BMG Labtech) and analyzed with MARS data analysis software. Radio-TLC was analyzed using a Raytest Rita-Star TLC scanner. Non-radioactive SE-HPLC was performed on an Agilent 1260 Infinity II HPLC system fitted with a Phenomenex BioSep LC column (1 mL/min, 5 µm, 300 x 7.8 mm, 290Å, column C), or an Agilent 1200 series HPLC system fitted with a Phenomenex Yarra column (0.35 mL/ min, 3 µm, 300 x 4.6 mm, 290Å, column D) in 0.2M phosphate buffer, pH 6.8 as mobile phase. For analysis of radioactive SE-HPLC, samples were collected in 15-second intervals, allowed to reach secular equilibrium with daughter isotopes (>8 hours) and fractions were read on a Perkin Elmer 2470 Automatic Gamma Counter Wizard^2 (30 seconds of counting time).

1.3 Supporting Information Figures

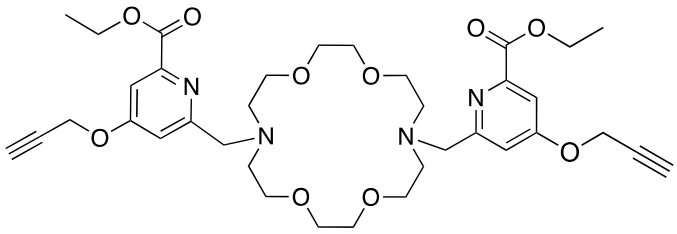


Figure S1: Chemical Structure of compound ***5_2***.

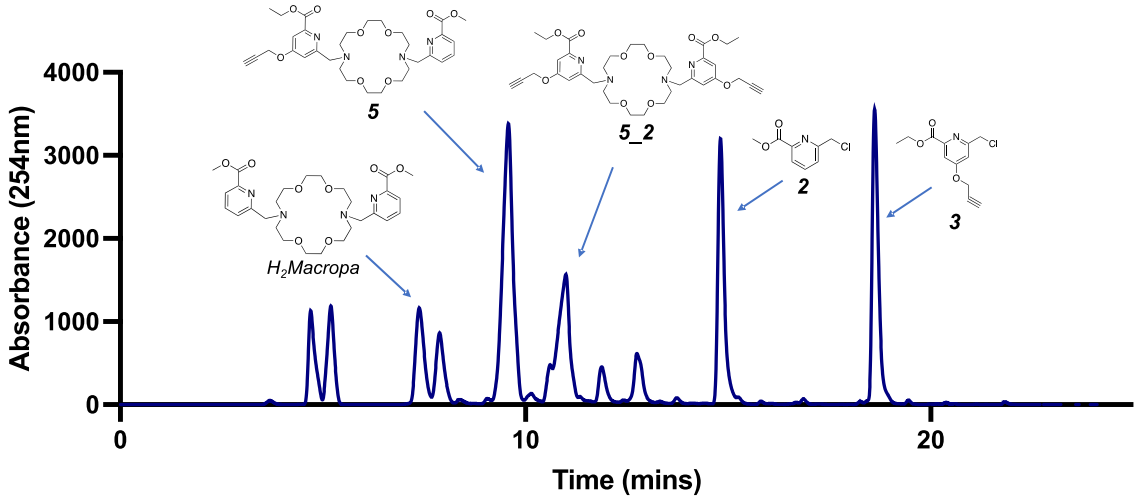


Figure S2: The preparative RP-HPLC/MS trace of the one-pot reaction of compound ***5***. Highlighted peaks were collected and used in future reactions. (Method C: 5 – 95 % CH_3CN in MilliQ with 0.1% formic acid at 15 mL/min.

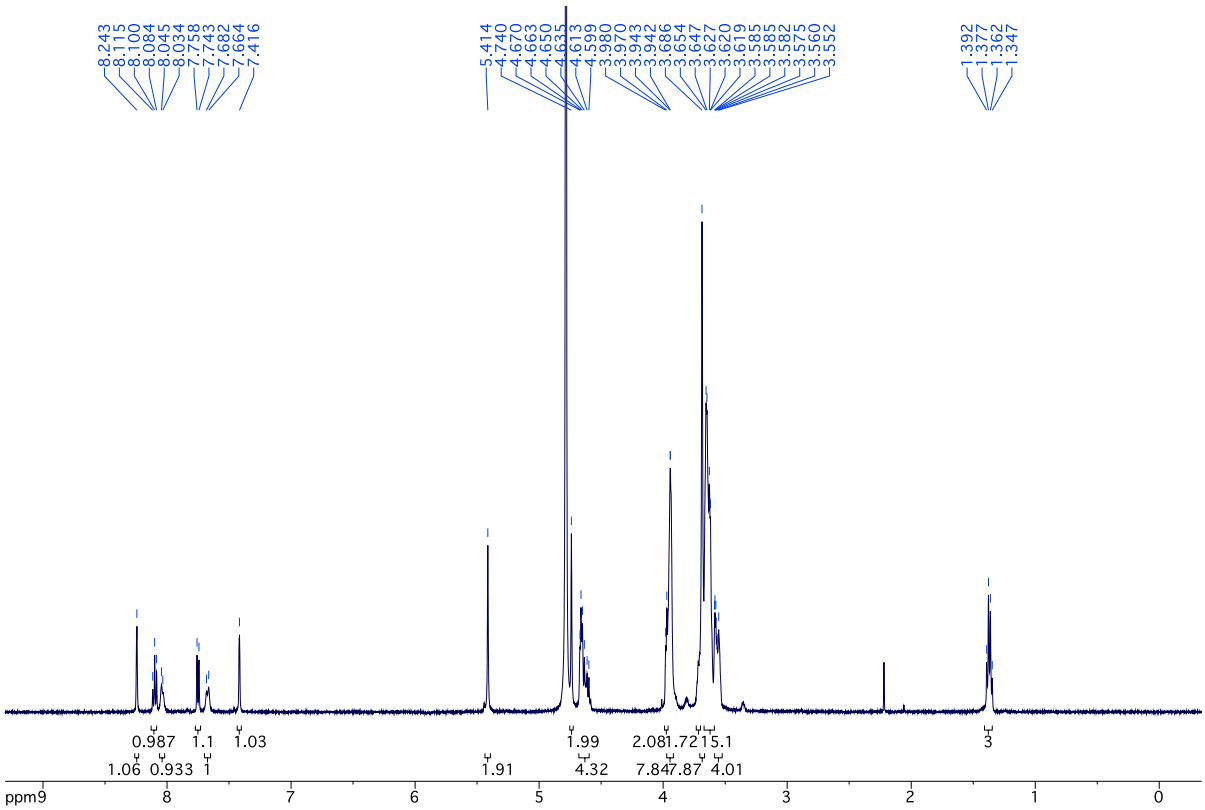


Figure S3: ^{1}H NMR (500 MHz; D_2O) of H_2MacropaSqOEt. Internal reference to Acetone (δ = 2.20 ppm).

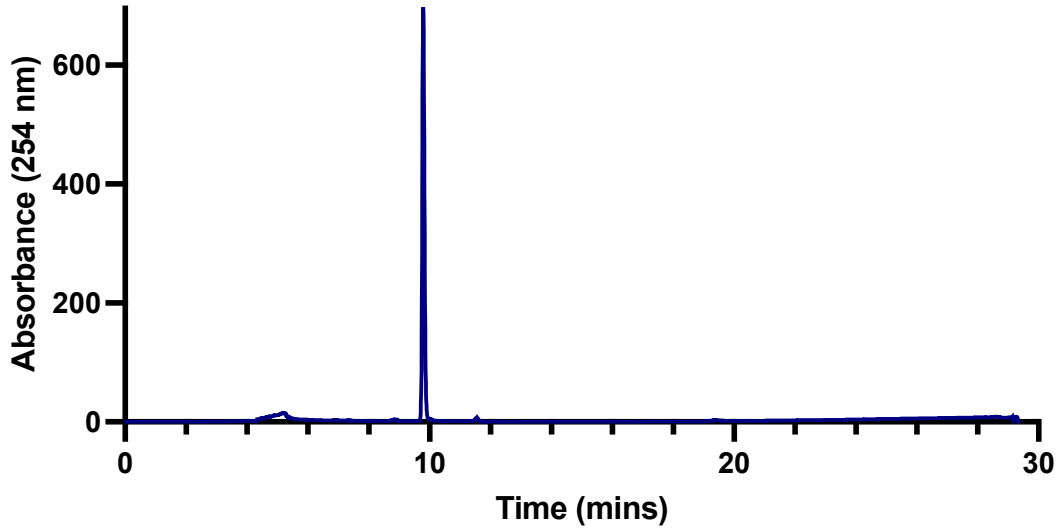


Figure S4: RP-HPLC of H_2Macropa-SqOEt stored at 4°C for >18 months. RP-HPC shows >95% purity.

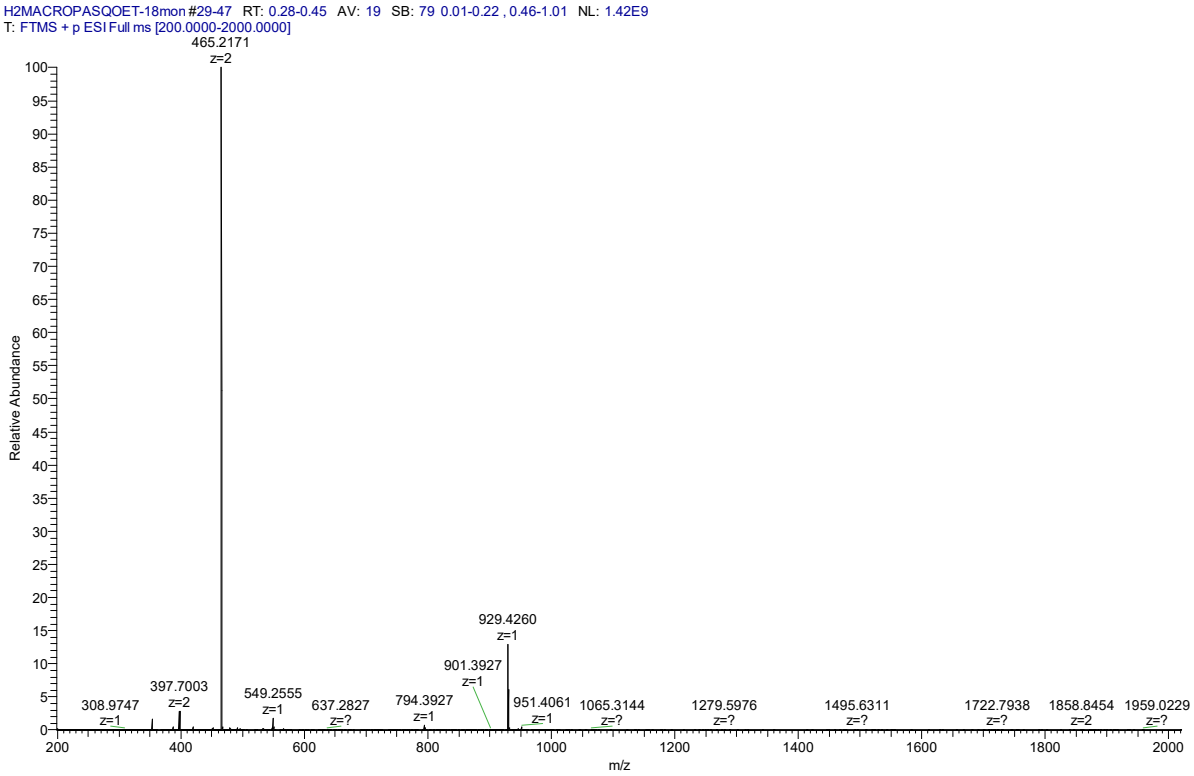


Figure S5: HR-MS of H_2Macropa-SqOEt stored at 4°C for >18 months. ESI-MS is comparable to freshly prepared H_2Macropa-SqOEt.

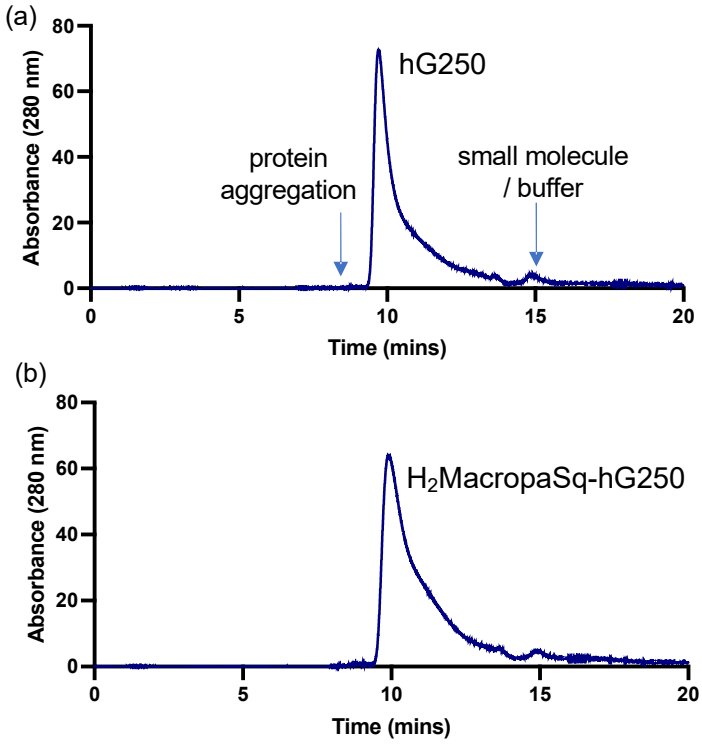


Figure S6: SE-HPLC of (a) hG250 and (b) H_2MacropaSq-hG250 (column D).

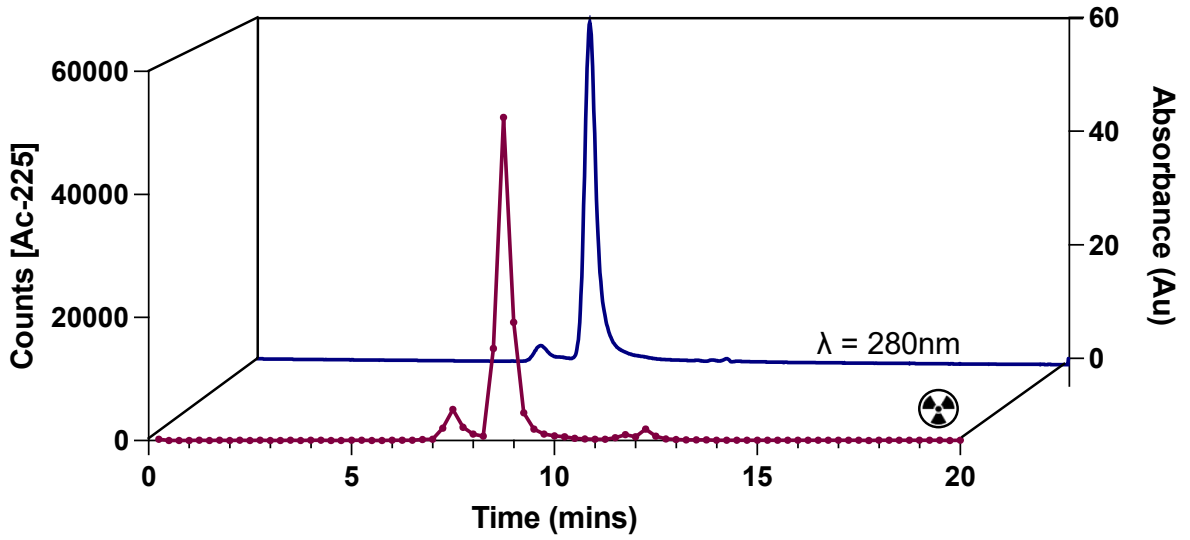


Figure S7: SE-HPLC chromatogram of [^{225}Ac]Ac(MacropaSq-hIgG1). Radioactive fractions in red, compared to the UV trace of H_2MacropaSq-hG250 in blue (λ = 280 nm), column C.

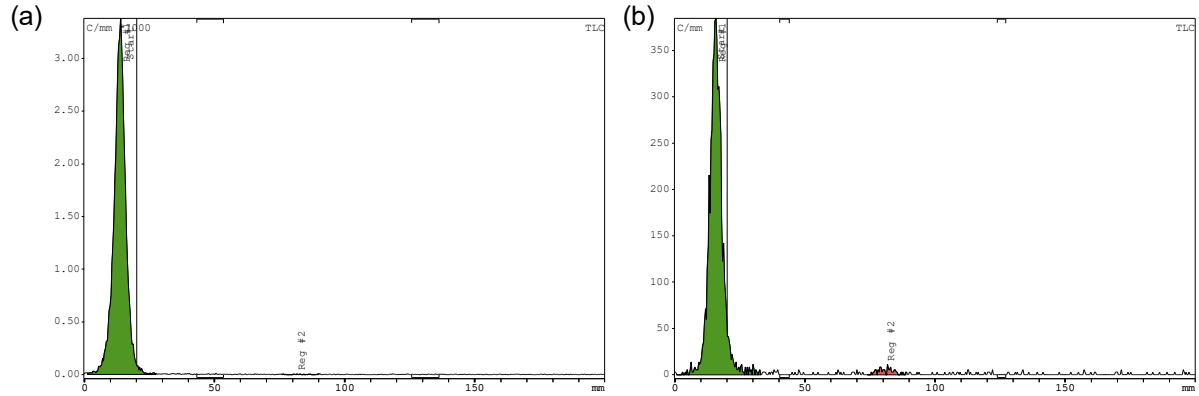


Figure S8: RadioTLC showing radiochemical yield of [^{225}Ac]Ac(MacropaSq-hG250), when incubated in human serum at 37°C on (a) day zero (RCP 100%) and (b) day seven (RCP 97.8%). [^{225}Ac]Ac(MacropaSq-hG250 is retained at the baseline (green) and [^{225}Ac]Ac^{III} travels to solvent front (red).

Table S1: Competition experiment of [^{225}Ac]Ac(MacropaSq-hG250) over seven days.

Day 0 (RCP = 100 %)	Hour 1	Day 1	Day 2	Day 7
PBS	99.9	99.9	99.7	99.7
PBS + La^{III} (5x)	99.8	99.9	100	99.4
PBS + La^{III} (50x)	99.9	100	99.9	99.4
PBS + La^{III} (500x)	99.9	99.9	99.8	99.2
PBS + H_4EDTA (5x)	98.4	99.2	99.5	99.6
PBS + H_4EDTA (50x)	99.9	100	99.8	99.1

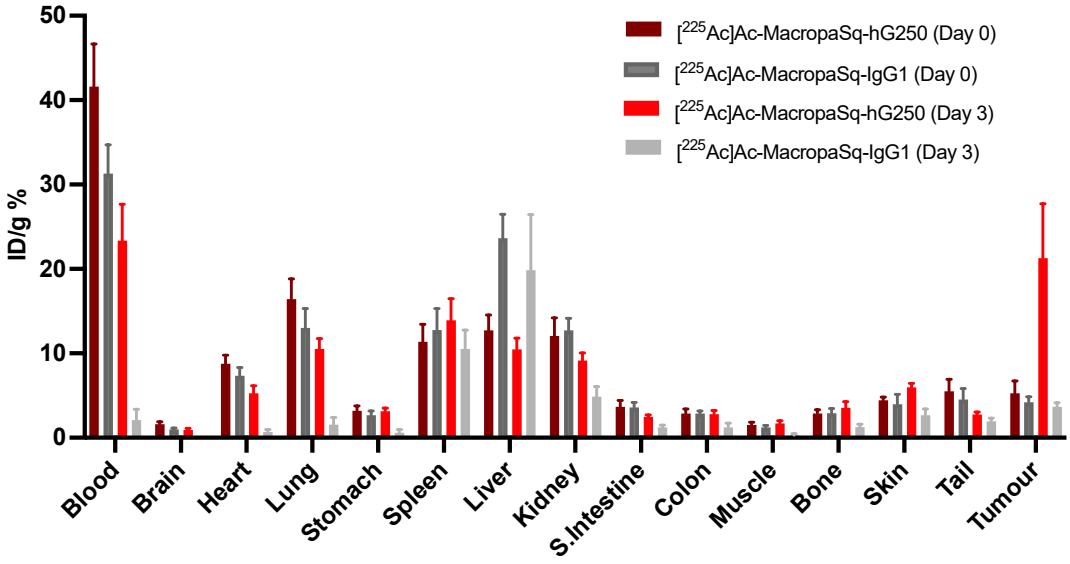


Figure S9: Biodistribution in Balb/c nude mice bearing SK-RC-52 tumors (n = 5). Mice were injected with 14.8 kBq (0.4 µCi) of [^{225}Ac]Ac(MacropaSq-IgG1) control and sacrificed at Days 0 and 3.

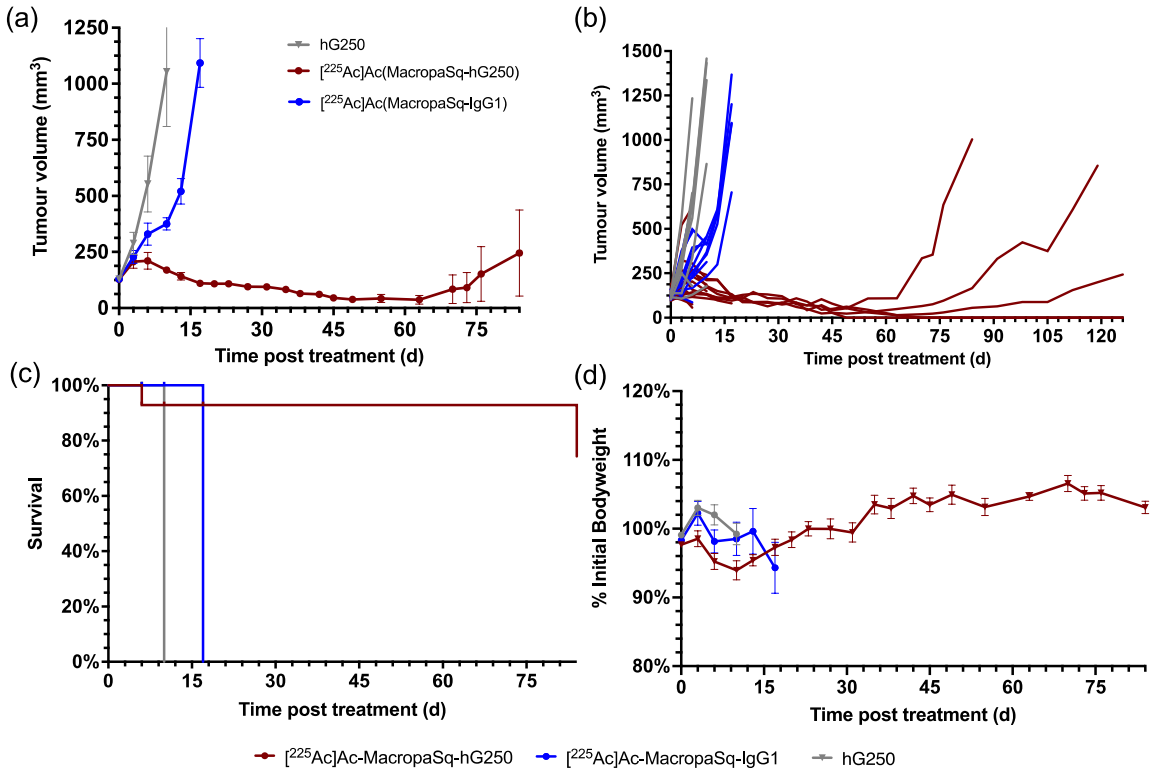


Figure S10: (a) Mean tumor volume (mm^3) as a function of time post-treatment with [^{225}Ac]Ac(MacropaSq-hG250) (14.8 kBq, 30 µg), [^{225}Ac]Ac(MacropaSq-IgG1) (14.8 kBq, 30 µg) or hG250 (30 µg) in mice with SK-RC-52 tumor xenografts, (b) individual tumor volume (mm^3) as a function of time post-treatment with [^{225}Ac]Ac(MacropaSq-hG250) (14.8 kBq, 30 µg), (c) Kaplan Meier plot shows the percentage survival of the cohort as a function of time (days); (d) Percentage body weight change after treatment with [^{225}Ac]Ac(MacropaSq-hG250) (14.8 kBq), [^{225}Ac]AcMacropaSq-hIgG1(14.8 kBq) or hG250(30 µg) over 84 days.

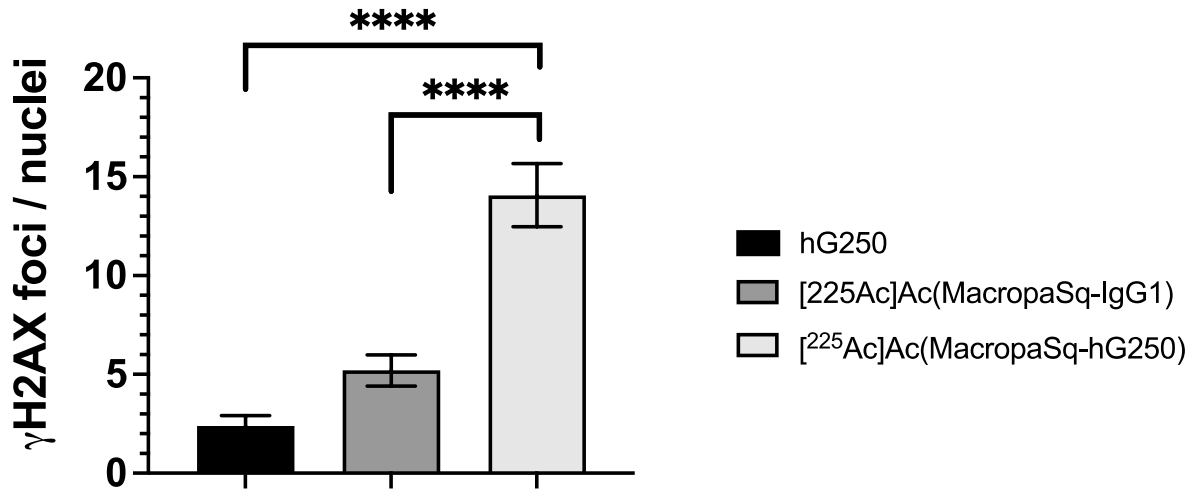


Figure S11: [^{225}Ac]Ac(MacropaSq-hG250) induces double-strand DNA breaks in an SK-RC-52 tumor model. Average foci per nuclei from each treatment group (mean ± SEM). Nuclei quantitated for [^{225}Ac]Ac(MacropaSq-hG250) = ≥55. Nuclei quantitated for [^{225}Ac]Ac-MacropaSq-IgG1 and hG250 = ≥36 ****p= ≤0.0001.

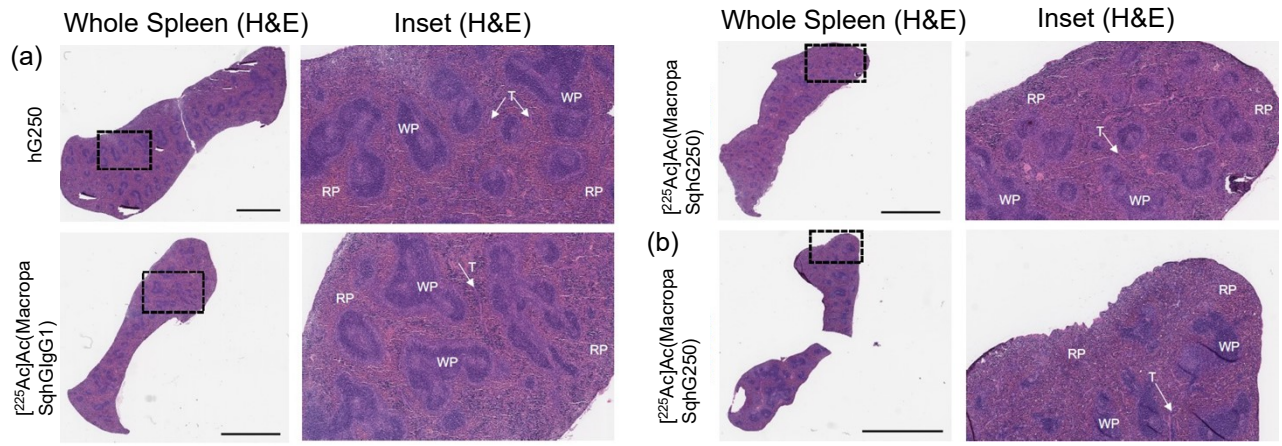


Figure S12: Spleen morphology and ultrastructure (A) Day 7 H&E staining of Spleens from treatment groups as indicated (B) Day 125 H&E staining of Spleens from [^{225}Ac]Ac(MacropaSq-hG250) group. Scale bar = 1 mm. For all insets T; trabecula, WP; white pulp, RP; red pulp.

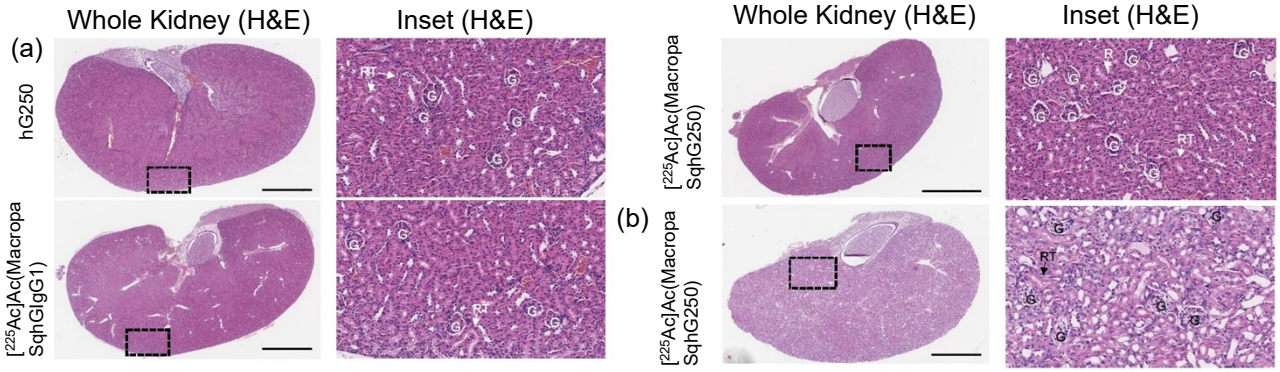
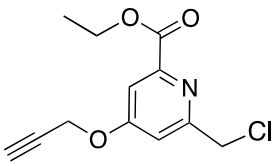


Figure S13: Kidney morphology and ultrastructure (A) Day 7 H&E staining of Kidneys from treatment groups as indicated (B) Day 125 H&E staining of Kidneys from [^{225}Ac]Ac(MacropaSq-hG250) group. Scale bar = 800 µm. For all insets G; glomerulus, RT; Renal tubes.

1.4 Synthesis and Characterisation of H_2MacropaSqOEt

1.4.1 2-Chloromethyl 4-(prop-2-yn-1-yloxy)pyridine-6-ethylcarboxylate (3)



Thionyl Chloride (6 mL) was slowly added to 2-Hydroxymethyl 4-(prop-2-yn-1-yloxy)pyridine-6-ethylcarboxylate (0.4 g, 1.7 mmol) at 0 °C under an atmosphere of N_2 and stirred for 3 hours. After 3 hours, the thionyl chloride was removed in vacuo to yield a pale-yellow residue, which was dissolved in ethyl acetate (20 mL) and washed with saturated $NaHCO_3$ (30 mL) and water (30 mL). The organic fractions were dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure to yield a pale-yellow powder (0.33g, 77%). R_t = 13.7 mins (system B, method B, column B). ESI-MS $[M+H]^+$: 254.0580 calculated for $(C_{12}H_{13}ClNO_3)^+$: 254.0578. ^{1}H NMR (500 MHz; DMSO-d6): δ 7.60 (d, *J* = 2.4 Hz, 1H, CH_{Ar}), 7.41 (d, *J* = 2.4 Hz, 1H, CH_{Ar}), 5.03 (d, *J* = 2.4 Hz, 2H, $CH_2C\equiv$), 4.79 (s, 2H, CH_2Cl), 4.36 (q, *J* = 7.1 Hz, 2H, CH_2), 3.72 (t, *J* = 2.4 Hz, 1H, CH≡), 1.33 (t, *J* = 7.1 Hz, 3H, CH_3). ^{13}C NMR (126 MHz; DMSO-d6): δ 165.0 (COO), 164.3 (C_{Ar}), 158.45 (C_{Ar}), 149.3 (C_{Ar}), 113.3 (CH_{Ar}), 111.0 (CH_{Ar}), 79.6 ($CH_2\equiv$), 77.8 (CH≡), 61.5(CH_2), 56.2 ($CH_2C\equiv$), 46.3 (CH_2Cl), 14.1 (CH_3).

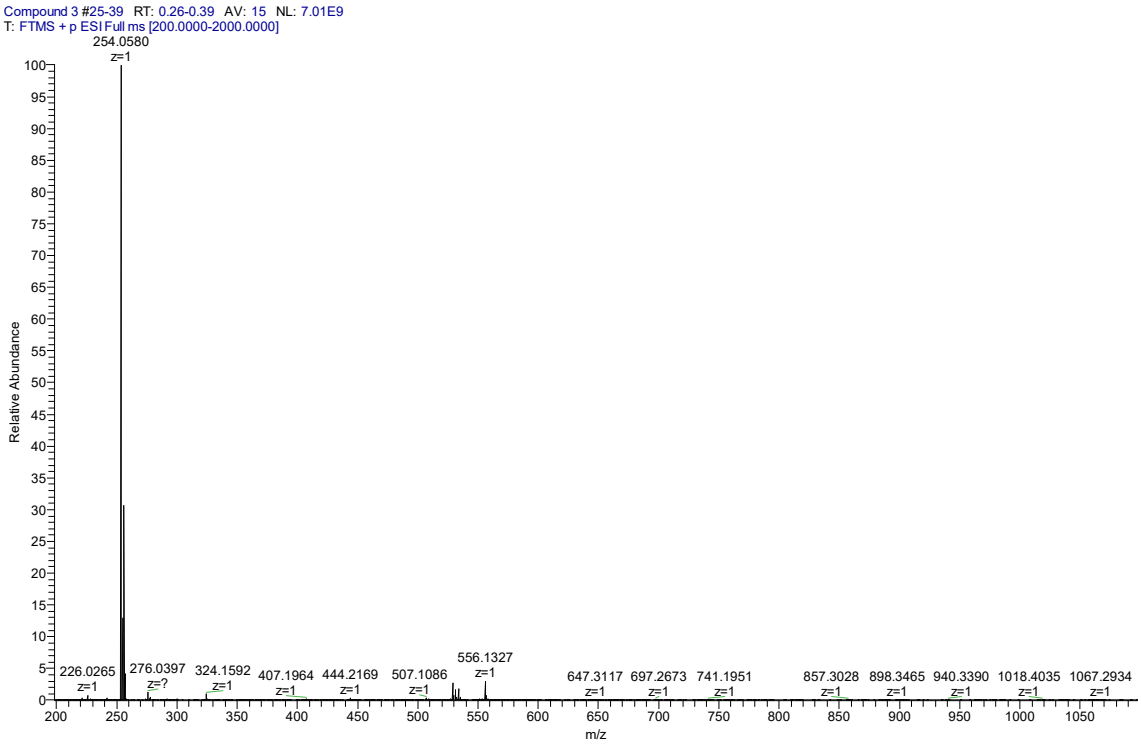


Figure S14: HR-MS of compound **3**, $[M+H]^+$ = 254.0580.

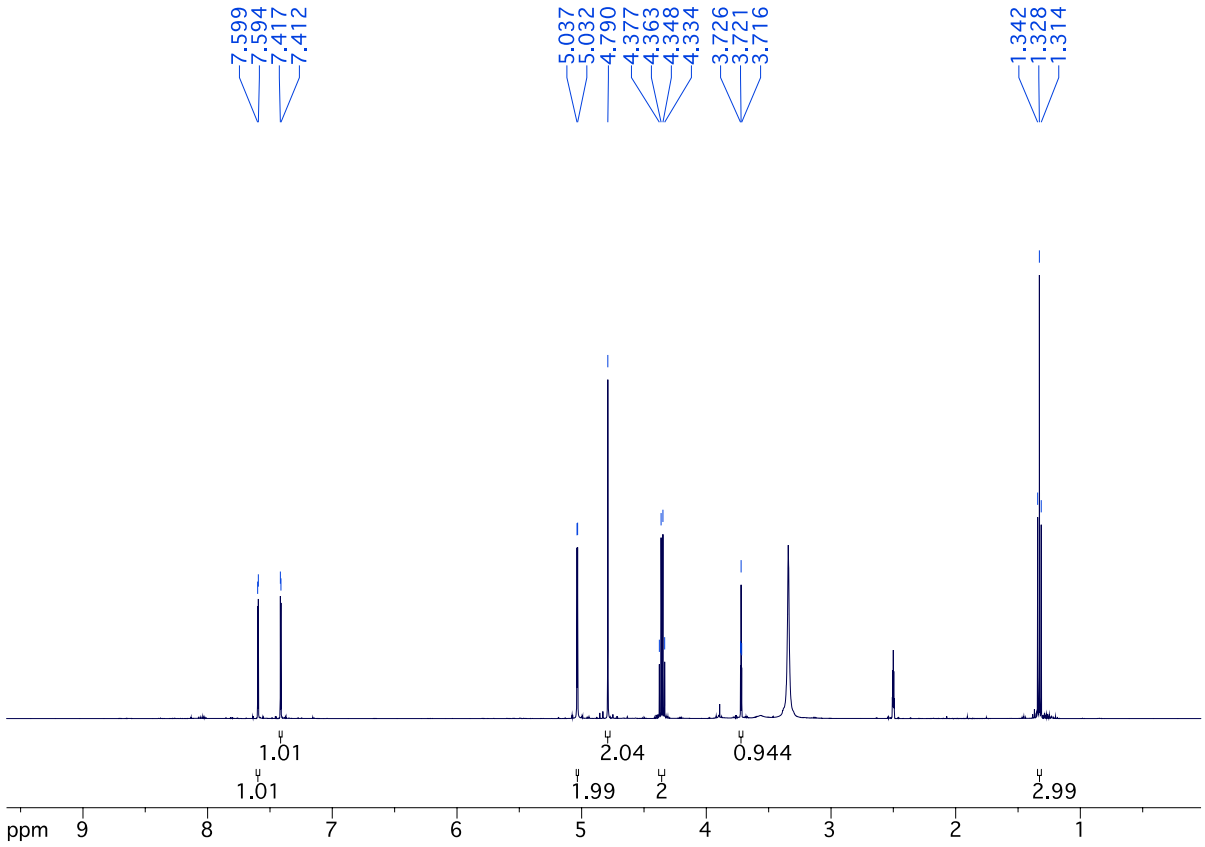


Figure S15: ^{1}H NMR (500 MHz; DMSO-d_6) of compound **3**.

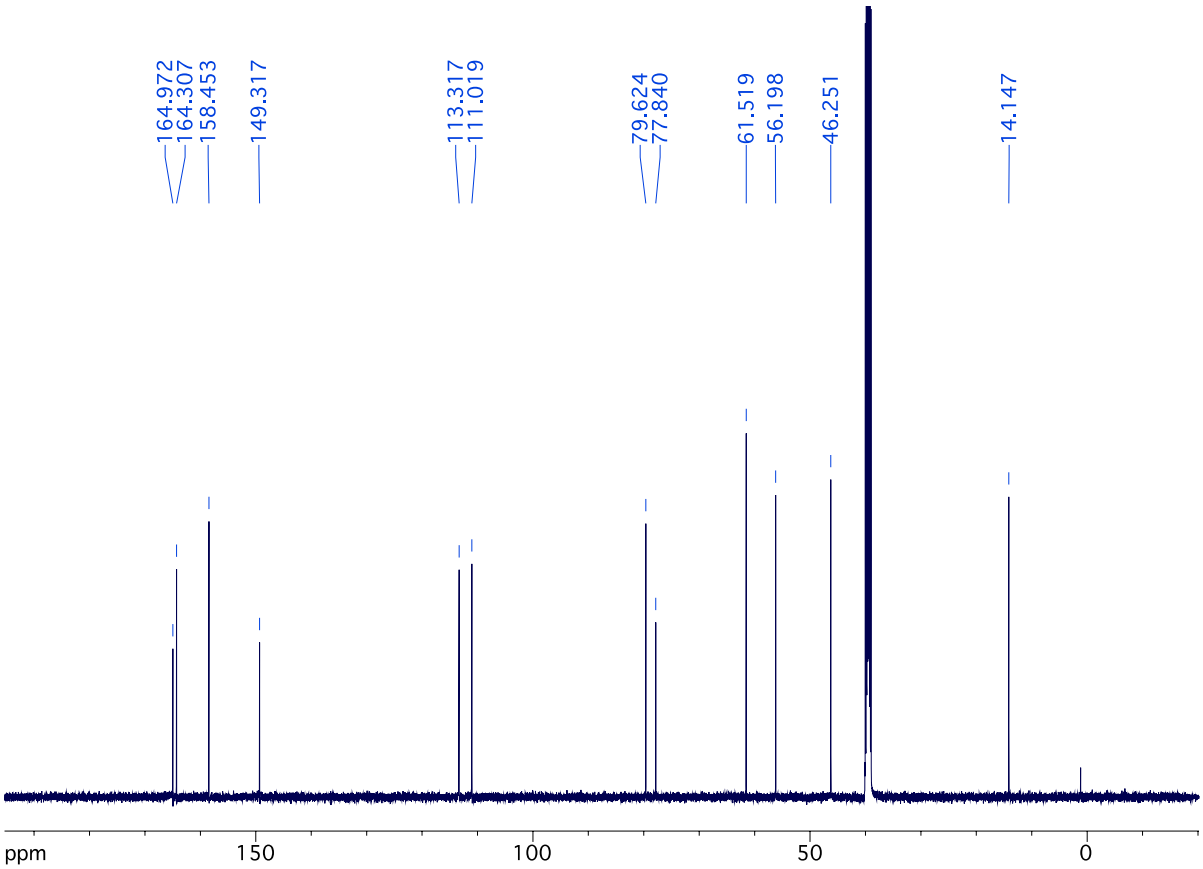


Figure S16: ^{13}C NMR (126 MHz; DMSO-d_6) of compound **3**.

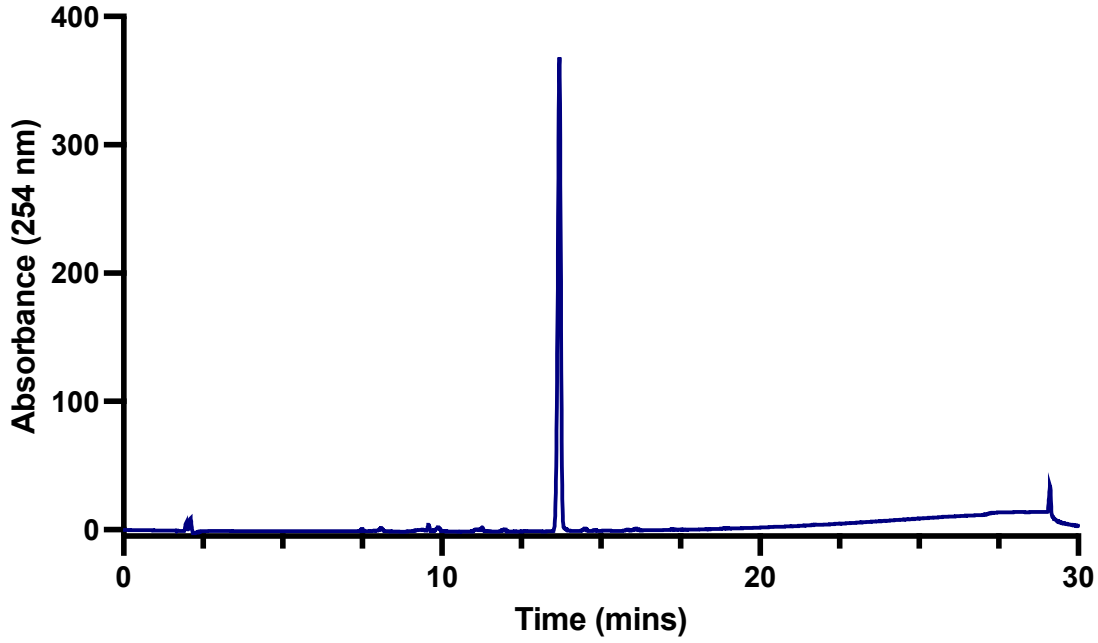
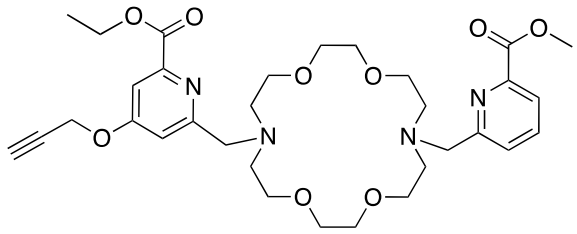


Figure S17: RP-HPLC of Compound *3* (method B).

1.4.2 Compound 5



Two-step synthesis: Methyl 6-((1,4,10,13-tetraoxa-7,16-diazacyclooctadecan-7-yl)methyl)picolinate (0.149 g, 0.4 mmol), 2-chloromethyl 4-(prop-2-yn-1-yloxy)pyridine-6-ethylcarboxylate (0.138 g, 0.54 mmol) and DIPEA (0.14 mL, 0.8 mmol) in CH_3CN (2 mL) were combined and the reaction was heated with microwave irradiation at 85°C for 1 hour. The resulting solution was taken to dryness under reduced pressure to yield a pale-yellow oil, which was purified *via* semi-preparative HPLC (method D), and appropriate fractions were combined and lyophilized to give a colorless oil (131 mg, 52%). *One-pot synthesis:* Diaza-18-crown-6 (50 mg, 0.19 mmol), compound **3** (46 mg, 0.24 mmol), compound **2** (76.2 mg, 0.31 mmol), DIPEA (70 µL 0.4 mmol) were dissolved in CH_3CN (2 mL) and heated with microwave irradiation at 90 °C for 1 hour. The resulting solution was diluted to a final ratio of MilliQ: CH_3CN of 2:1 and lyophilized. The resulting crude product was purified *via* semipreparative HPLC (method C). Pure fractions of compounds ***5***, ***5_2,*** and ***H_2Macropa*** were separated and lyophilized, giving compound ***5*** (57 mg, 48%). R_t = 10.38 min (system A, method A, column B). ESI-MS $[M+2H]^{2+}$: 315.1630 calculated for $(C_{32}H_{46}N_4O_9)^{2+}$: 315.1627, $[M+H]^+$: 629.3186 calculated for $(C_{32}H_{44}N_4O_9)^+$: 629.3181. ^{1}H NMR (500 MHz; $CDCl_3$): δ 8.04 (d, *J* = 7.5 Hz, 2H, CH_{Ar}), 7.89 (t, *J* = 7.8 Hz, 1H, CH_{Ar}), 7.73 (d, *J* = 2.3 Hz, 1H, CH_{Ar}), 7.62 (d, *J* = 2.5 Hz, 1H, CH_{Ar}), 4.88 (d, *J* = 2.4 Hz, 2H, $CH_2C\equiv$), 4.44 (q, *J* = 7.1 Hz, 2H, N-CH_2-), 4.31 (s, 2H, N-CH_2-), 4.26 (s, 2H, N-CH_2), 3.98 (s, 3H, CH_3), 3.80 (td, *J* = 5.1, 2.0 Hz, 8H, -OCH_2), 3.61 (s, 8H, CH_2-O), 3.21 (q, *J* = 5.1 Hz, 8H, -NCH_2), 2.63 (t, *J* = 2.4 Hz, 1H, CH≡), 1.41 (t, *J* = 7.1 Hz, 3H, CH_3). ^{13}C NMR (126 MHz;

CDCl3): δ 165.55 (FA), 165.52 (COO), 164.8 (C_{Ar}-O), 164.3 (C_{Ar}), 149.4 (C_{Ar}), 147.5 (C_{Ar}), 138.3 (CH_{Ar}), 128.0 (CH_{Ar}), 124.5 (CH_{Ar}), 113.4 (CH_{Ar}), 112.2 (CH_{Ar}), 77.09 (C≡), 76.91 (CH≡), 70.51 (CH_2N), 70.48 (CH_2N), 67.76 (OCH_2), 67.73 (OCH_2), 62.2 (CH_2CH_3), 59.57 (OCH_2) 59.44 (OCH_2), 54.74 (NCH_2), 54.62 (NCH_2), 56.6 (CH_2C≡), z), 53.1 (CH_3), 14.4 (CH_3CH_2).

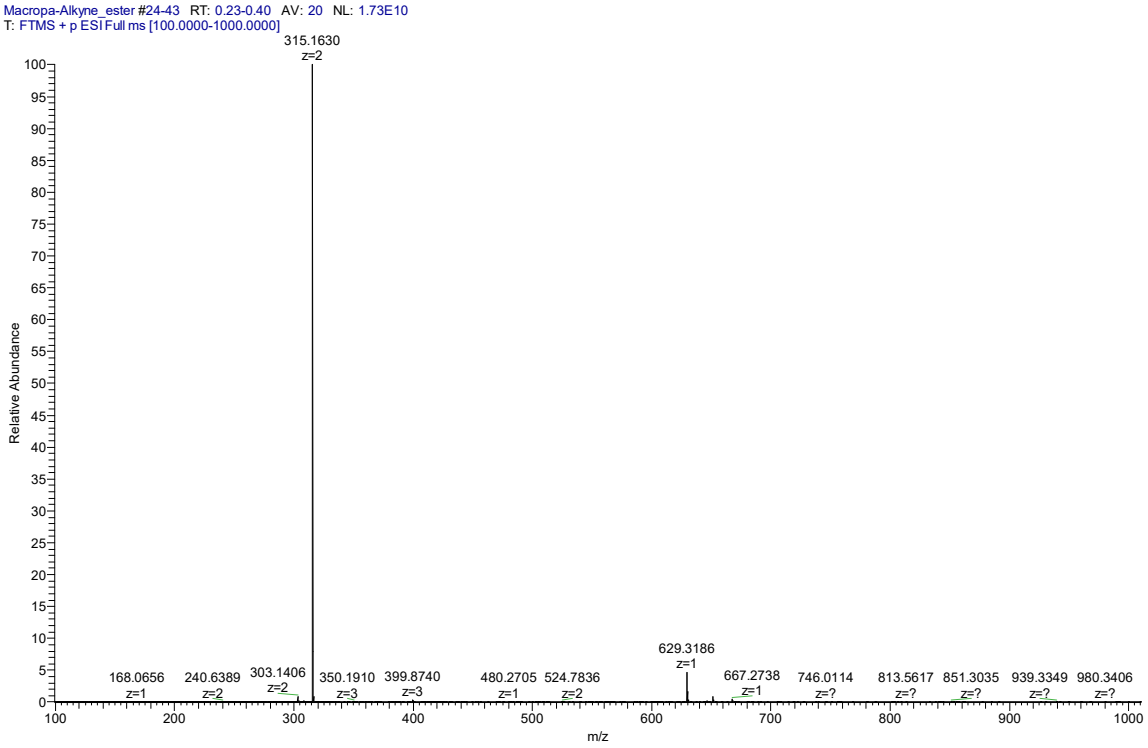


Figure S18: HR-MS of compound ***5***, $[M+2H]^{2+}$ 315.1632.

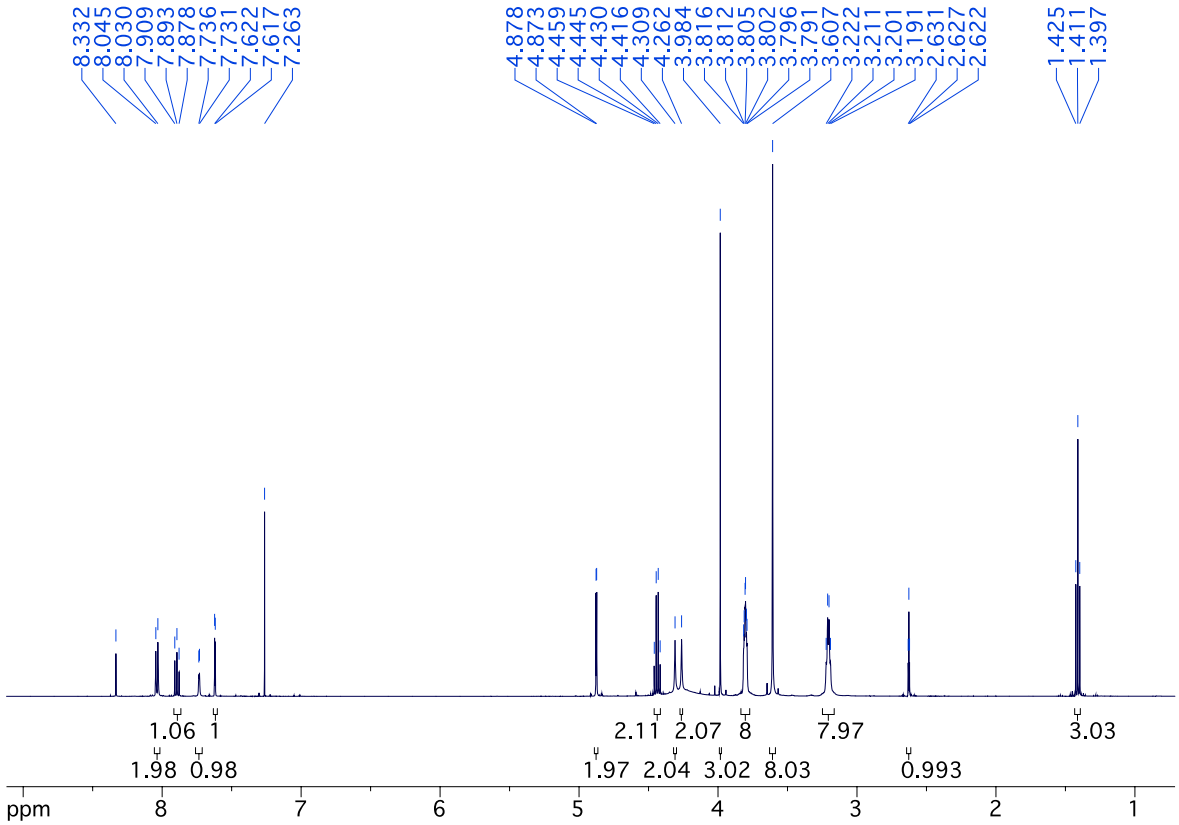


Figure S19: ^{1}H NMR (500 MHz; $CDCl_3$) of compound ***5***. Formic acid (δ = 8.33 ppm).

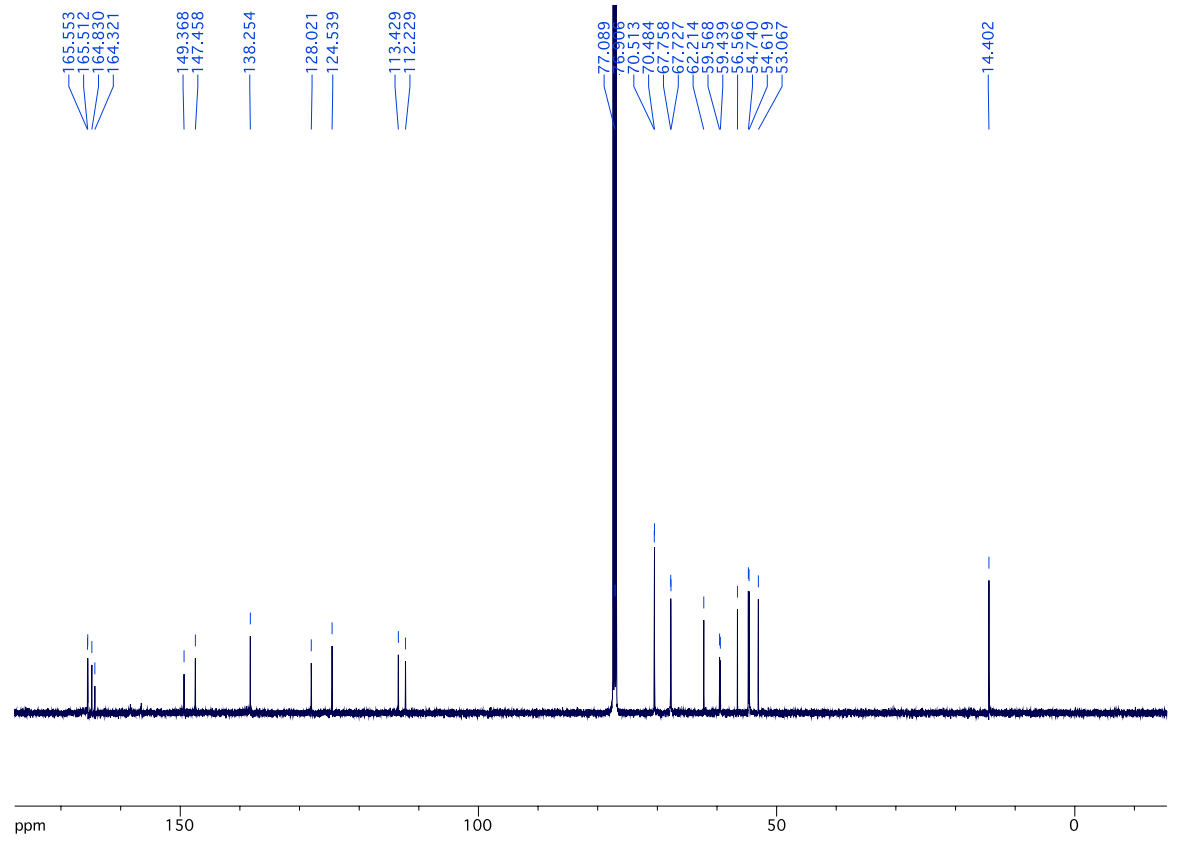


Figure S20: ^{13}C NMR (126 MHz; $CDCl_3$) of Compound ***5***. Formic acid (δ = 165.5 ppm).

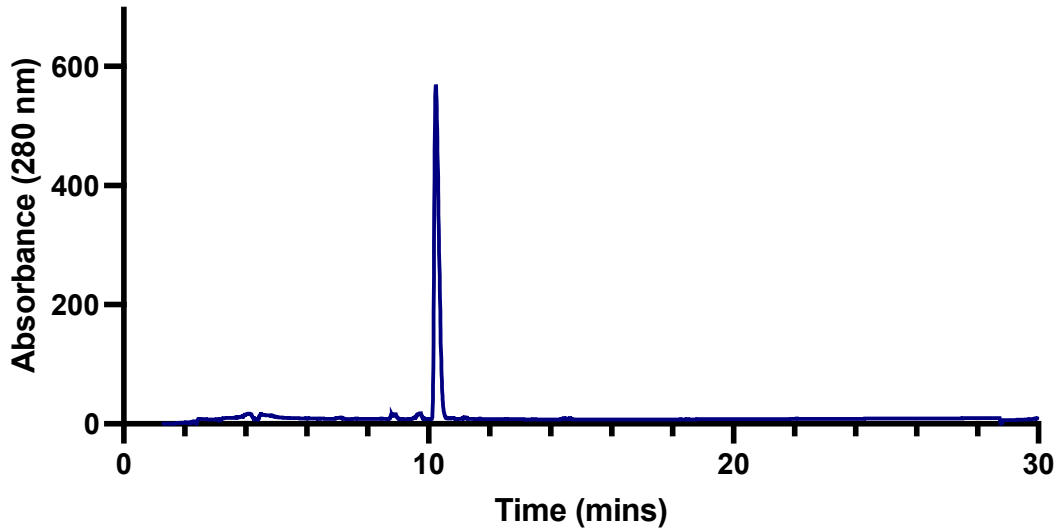
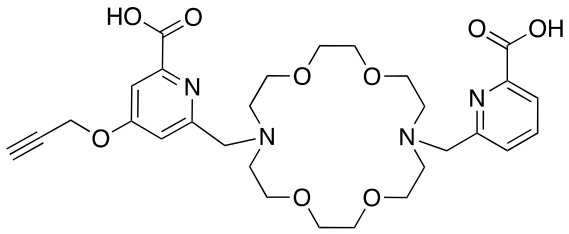


Figure S21: RP-HPLC of compound ***5*** (R_t = 10.3 min).

1.4.3 Compound 6



4-(prop-2-yn-1-yloxy)-6-((16-((6-carboxypyridin-2-yl)methyl)-1,4,10,13-tetraoxa-7,16-diazacyclooctadecan-7-yl)methyl)picolinic acid was synthesized using an adapted literature protocol.[1] To a solution of **5** (18.5 mg, 0.03 mmol) in DCM (450 mL), was added a suspension of NaOH in methanol (50 mL, 3 M), so the final concentration of NaOH was 0.3 M, and the final DCM: MeOH ratio was 9:1. The reaction mixture was stirred at room temperature for 1 hour, the reaction mixture was neutralized and the solvent was removed under reduced pressure. R_t = 8.54 mins (System A, method A, column B). ESI-MS $[M+2H]^{2+}$: 294.1396 calculated for $(C_{29}H_{40}N_4O_9)^{2+}$: 294.1393. $[M+H]^+$: 587.2715 calculated for $(C_{29}H_{39}N_4O_9)^+$: 587.2712.^{1}H NMR (500 MHz; $CDCl_3$): δ 8.10 (d, *J* = 7.7 Hz, 1H, CH_{Ar}), 7.89 (t, *J* = 7.7 Hz, 1H, CH_{Ar}), 7.71 (d, *J* = 2.4 Hz, 1H, CH_{Ar}), 7.67 (d, *J* = 7.4 Hz, 1H, CH_{Ar}), 7.30 (d, *J* = 2.5 Hz, 1H, CH_{Ar}),

4.86 (d, *J* = 2.4 Hz, 2H, $CH_2C\equiv$), 4.37 (s, 2H, N-CH_2-), 4.17 (s, 2H, N-CH_2-), 3.78 (t, *J* = 5.0 Hz, 4H, -OCH_2), 3.70 (t, *J* = 5.0 Hz, 4H, -OCH_2), 3.57 (q, *J* = 5.3 Hz, 8H, CH_2-O), 3.21 (t, *J* = 4.7 Hz, 4H, -NCH_2), 3.07 (t, *J* = 4.9 Hz, 4H, -NCH_2), 2.64 (t, *J* = 2.4 Hz, 1H, CH≡). ^{13} C NMR (126 MHz; CDCl3): δ 166.8 (FA), 166.00 (COO), 165.89 (COO), 164.8 , 149.6, 147.6, 138.8 (CH_{Ar}), 126.9 (CH_{Ar}), 123.2 (CH_{Ar}), 113.5 (CH_{Ar}), 109.3 (CH_{Ar}), 77.49 (C≡), 76.46 (CH≡), 70.44 (CH_2N), 70.37 (CH_2N), 68.4 (CH_2O), 67.8 (CH_2O), 59.2 (OCH_2), 58.9 (OCH_2), 56.7 (NCH_2), 55.1 (NCH_2), 54.7 ($CH_2C\equiv$).

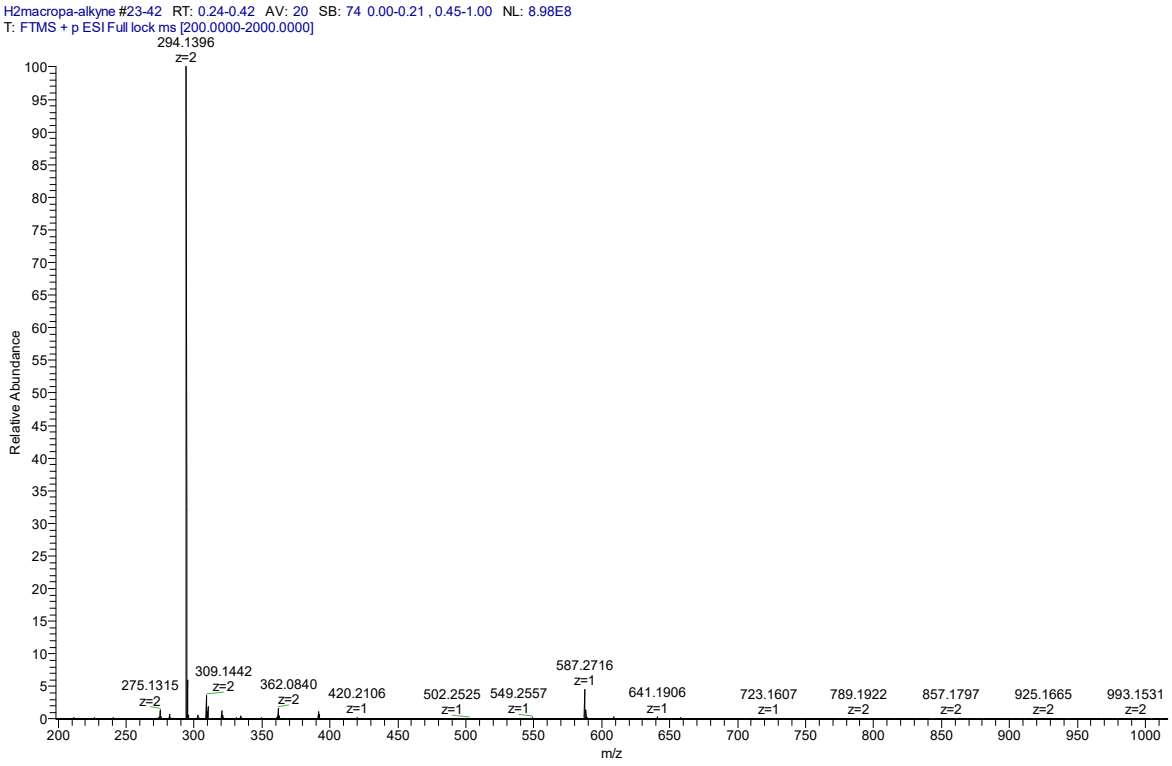


Figure S22: HR-MS of compound **6**, $[M+2H]^{2+}$ = 294.1396.

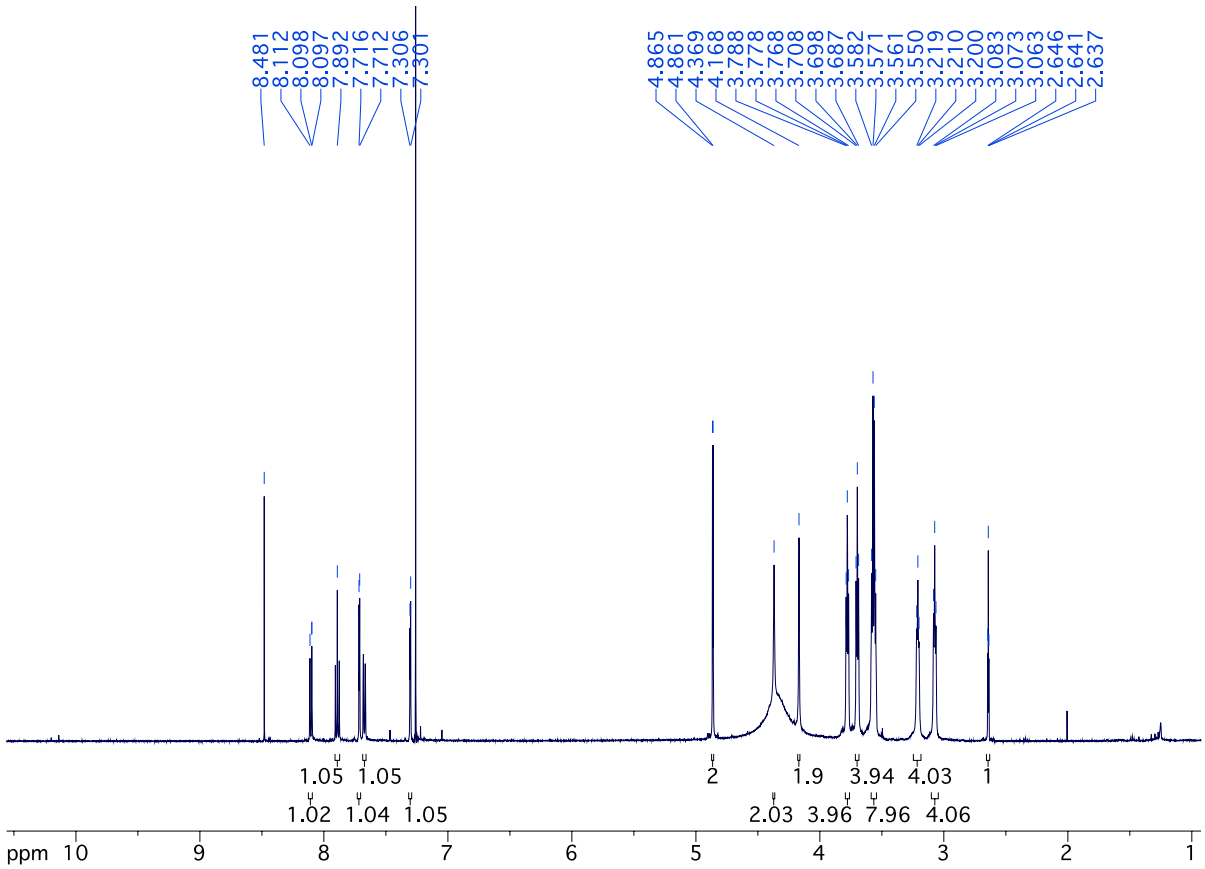


Figure S23: ^{1}H NMR (500 MHz; $CDCl_3$) of Compound ***6***. Formic acid (δ = 8.48 ppm).

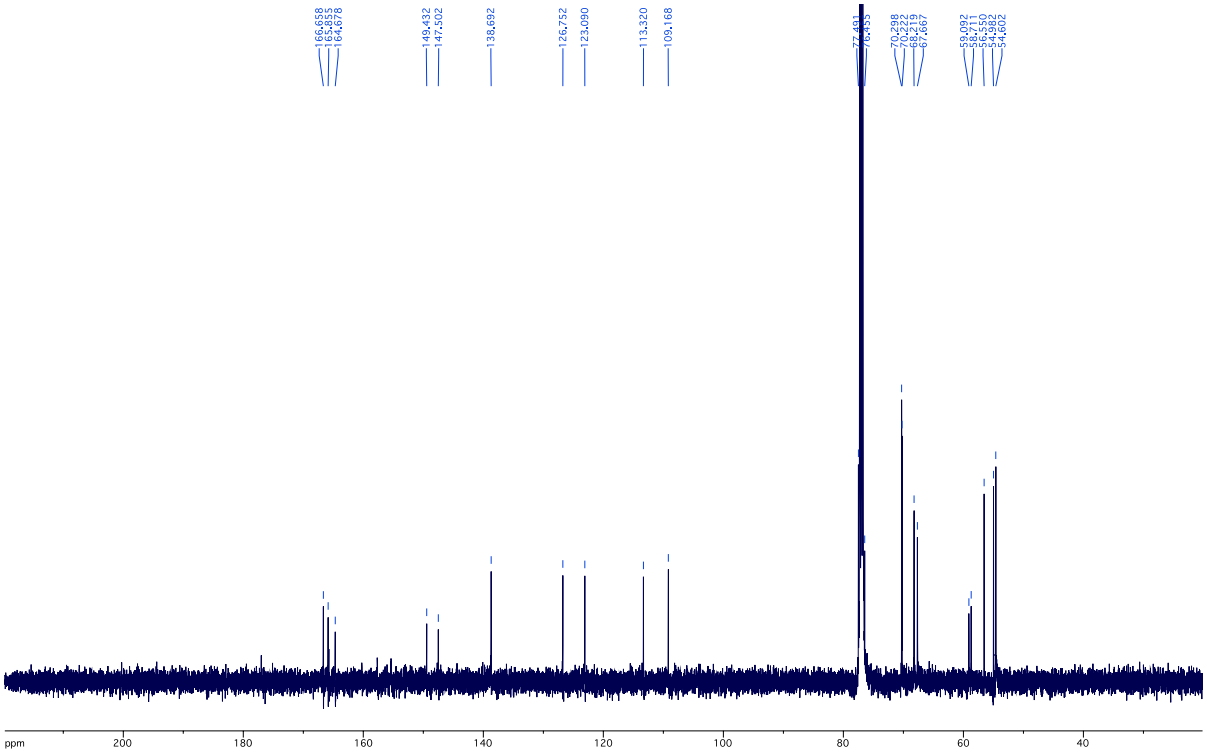


Figure S24: ^{13}C NMR (126 MHz; $CDCl_3$) of Compound ***6***.

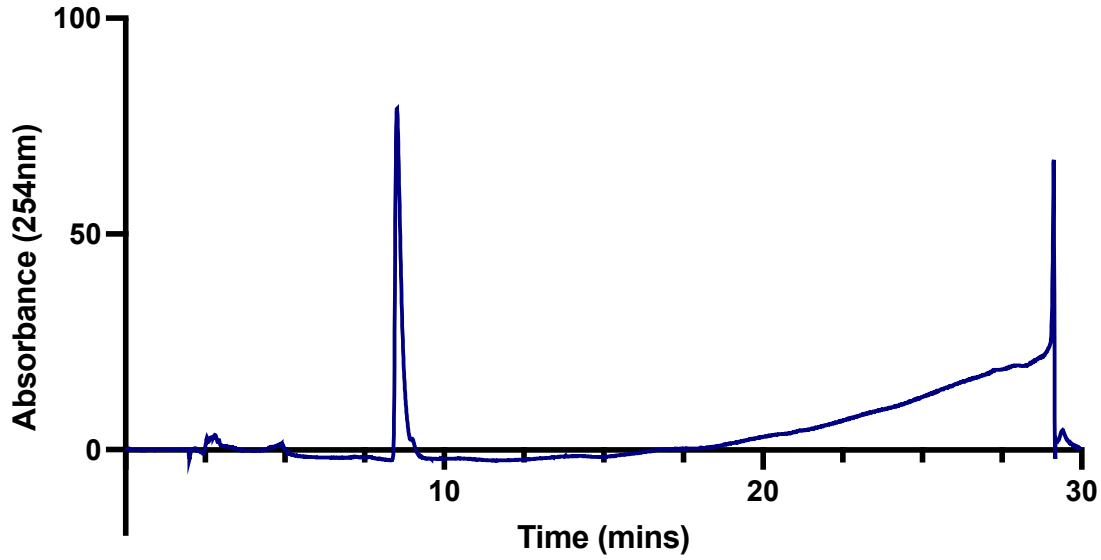
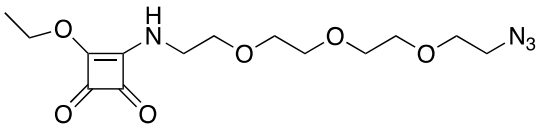


Figure S25: RP-HPLC of compound ***6*** (R_t = 8.54 min).

1.4.4 Compound 7



Freshly prepared diethyl squarate (0.23g, 1.32 mmol) was dissolved in ethanol (4 mL) before adding 1-Amino-11-azido-3,6,9-trioxaundecane (0.28g, 1.29 mmol) and DIPEA (1 eq) under nitrogen. The mixture was stirred at room temperature for 8 hours; then the colorless solution was taken to dryness under reduced pressure and purified *via* semi-preparative HPLC (method C). Fractions were combined and lyophilized to yield a colorless oil (209 mg, 49%). ESI-MS $[M+H]^+$: 343.1613 calculated for $(C_{14}H_{23}N_4O_6)^+$: 343.1612, $[M+2H]^+$: 685.3152 calculated for $(C_{28}H_{45}N_8O_{12})^+$: 685.3151. R_t = 7.33 mins (system A, method A, column B). ^{1}H-

NMR (500 MHz; DMSO-d_6): δ 8.78 (s, 0.5 H, NH_a), 8.61 (s, 0.5 H,NH_b), 4.64 (dd, *J* = 6.8, 4.6 Hz, 2H, CH_2), 3.59 (d, *J* = 5.4 Hz, 1H, CH_2NH_b), 3.57 (t, *J* = 4.8 Hz, 2H, CH_2O), 3.51 (t, *J* = 8.6 Hz, 10H, CH_2O), 3.42 (d, *J* = 5.4 Hz, 1H, CH_2NH_a), 3.36 (t, *J* = 4.9 Hz, 2H, CH_2-N_3), 1.35 (t, *J* = 7.1 Hz, 3H, CH_3). ^{13}C NMR (126 MHz; DMSO-d_6): δ 189.31 (CO-C-O), 189.16 (CO-C-O), 182.20 (CO-C-N), 182.04 (CO-C-N), 176.9 (C=C-O), 176.6 (C=C-O), 173.0 (HN-C=C), 172.5 (HN-C=C), 69.83 (CH_2-O), 69.78 (CH_2-O), 69.74 (CH_2-O), 69.69 (CH_2-O), 69.61 (CH_2-O), 69.25 (CH_2-O), 69.21 (CH_2-O), 68.79 (CH_2-O), 68.74 (CH_2CH_3), 50.0 (CH_2-N_3,) 43.7 (CH_2-NH), 15.6 (CH_3CH_2).

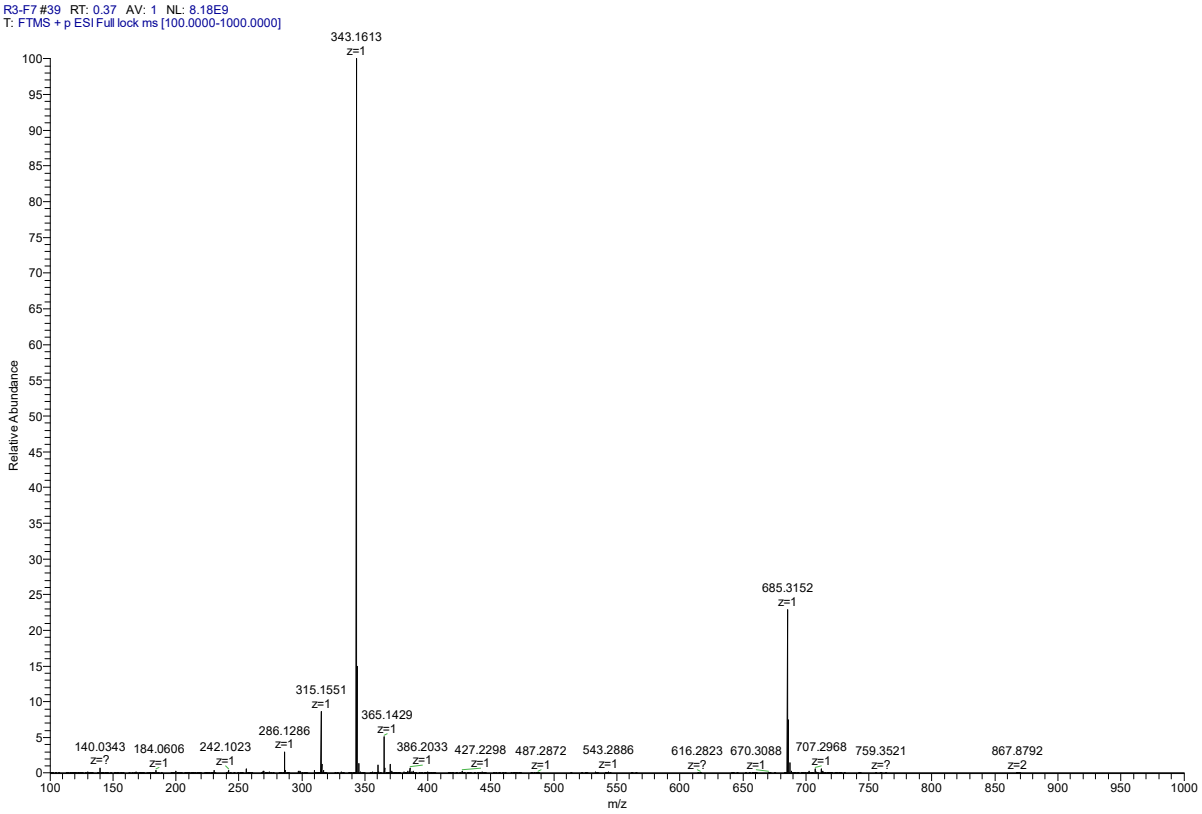


Figure S26: HR-MS of compound ***7***, $[M+H]^+$ = 343.1613, $[2M+H]^+$ = 685.3155.

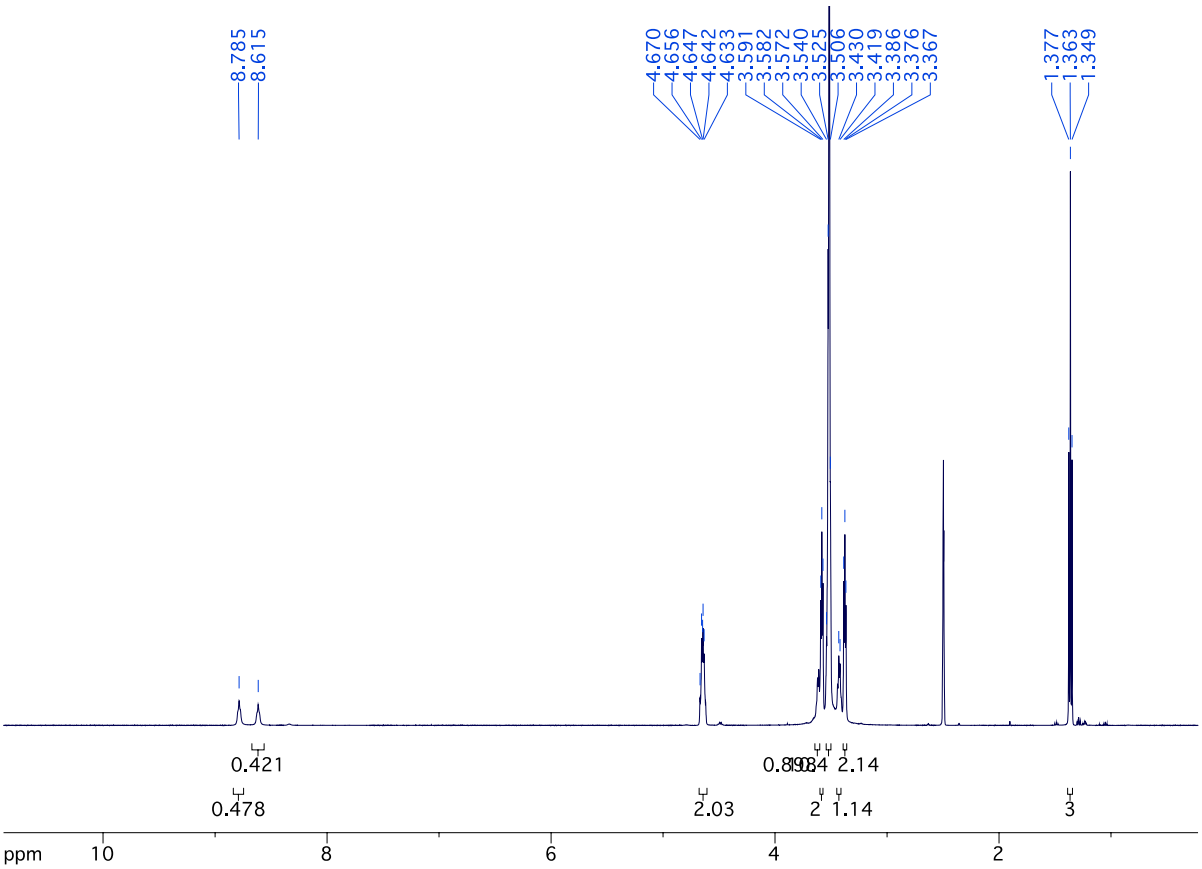


Figure S27: ^{1}H NMR (500 MHz, DMSO-d_6) of compound ***7***.

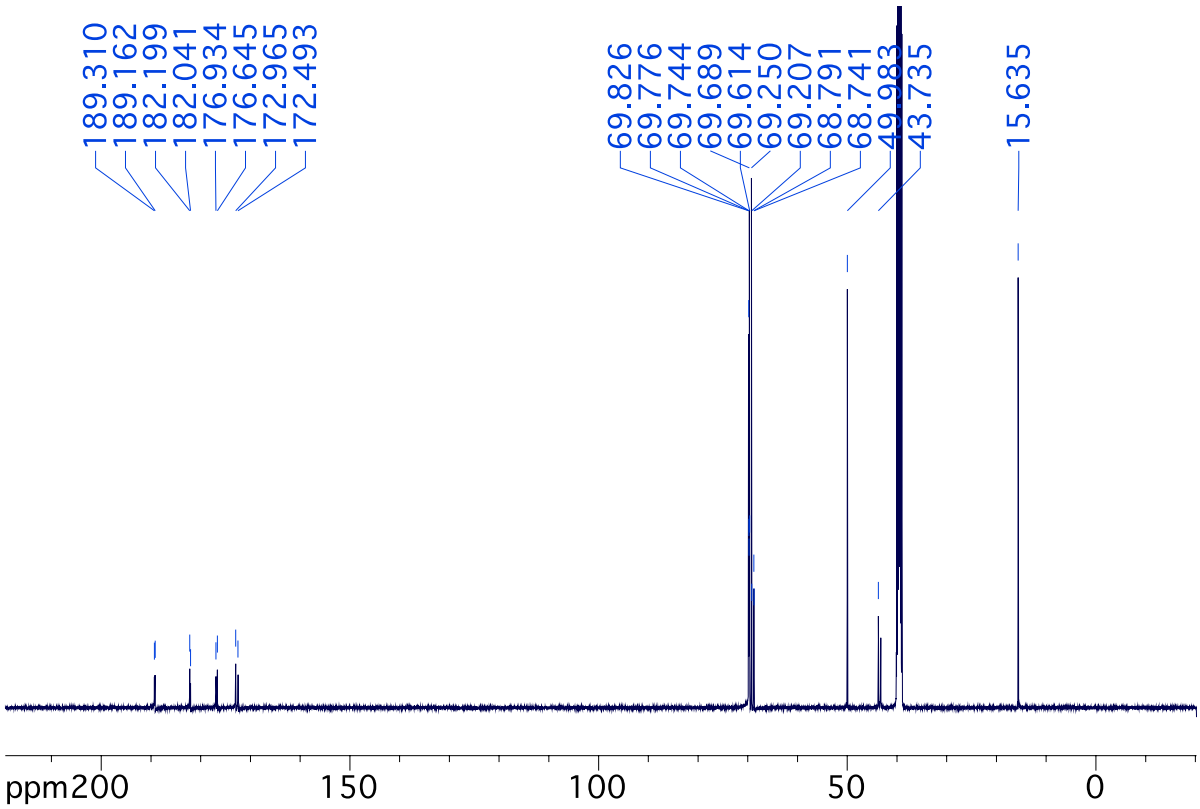


Figure S28: ^{13}C NMR (126 MHz, DMSO-d_6) of compound ***7***.

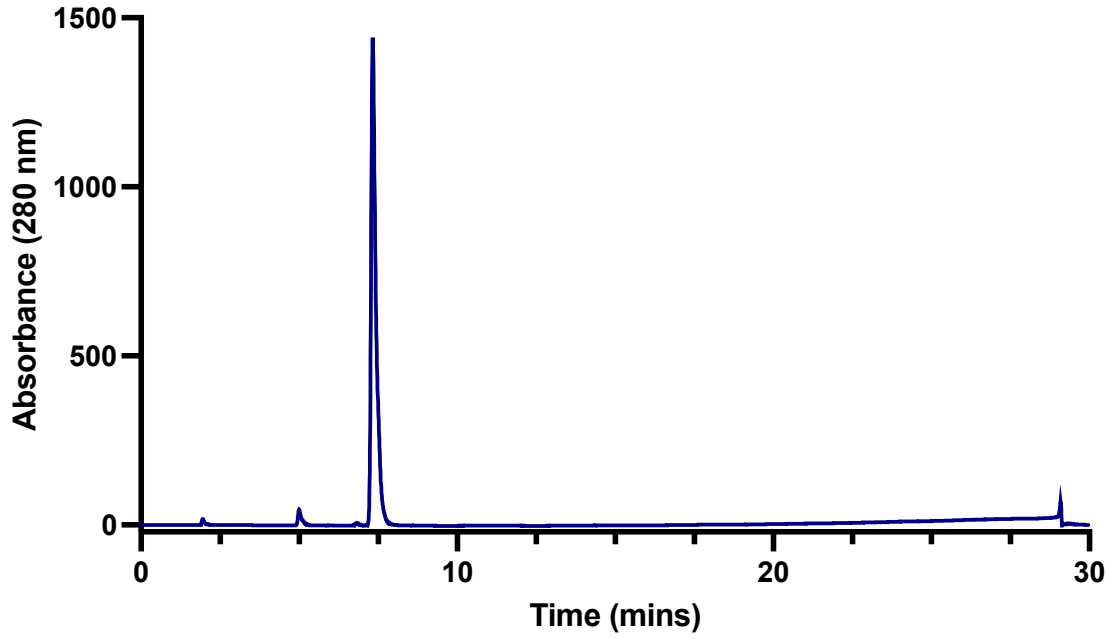
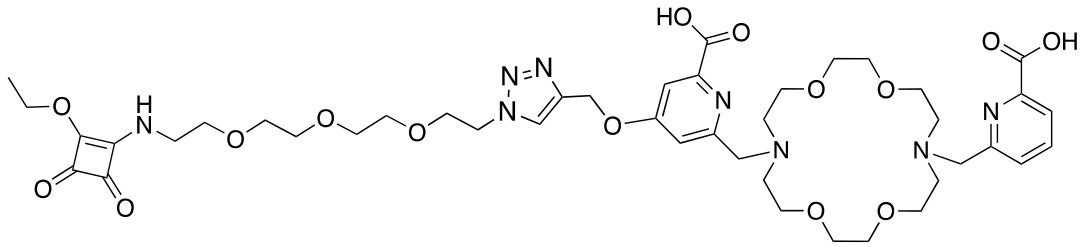


Figure S29: RP-HPLC of compound ***7*** (R_t = 7.33 min).

1.4.5 H_2MacropaSqOEt (8)



$CuSO_4$ (0.22 mg, 0.0014 mmol, 8% mmol) and Sodium Ascorbate (0.64 mg, 0.0032 mmol, 18% mmol) were combined to ensure complete reduction to copper (I) species. TBTA (2.8 mg, 0.0057 mmol, 32% mmol in DMF) was added as a stabilizing ligand with ***7*** (6.42 mg, 0.016 mmol) and reacted for 10 minutes. ***6*** (11 mg, 0.018 mmol) was added, and the reaction mixture was purged for 15 minutes with a stream of nitrogen. The reaction was monitored by RP-HPLC to ensure the complete removal of azide species (280 nm). After one hour, Na_2EDTA

was added to the reaction species to remove the catalytic copper. The pale green solution was taken to dryness under reduced pressure and dried *in vacuo.* The resulting precipitate was purified *via* semi-preparative HPLC (method D), and fractions were combined and lyophilized to yield an off-white powder (6.5 mg, 41 %). ESI-MS $[M+2H]^{2+}$: 465.2161 calculated for $(C_{43}H_{62}N_8O_{15})^{2+}$: 465.2162. Rt = 9.78 mins (system A, method B, column B). ^{1}H NMR (500 MHz, D_2O) δ 8.24 (d, *J* = 2.3 Hz, 1H, CH_{tz}), 8.10 (t, *J* = 7.8 Hz, 1H, CH_{Ar}), 8.04 (s, 1H, CH_{Ar}), 7.75 (d, *J* = 7.8 Hz, 1H, CH_{Ar}), 7.67 (d, *J* = 9.0 Hz, 1H, CH_{Ar}), 7.42 (d, *J* = 2.2 Hz, 1H, CH_{Ar}), 5.41 (s, 2H, CH_2C_{tz}), 4.74 (s, 2H, N-CH_2), 4.63 (m, 4H, N-CH_2 & CH_2CH_3), 3.98 (d, *J* = 4.9 Hz, 2H, CH_2O), 3.94 (t, *J* = 4.8 Hz, 8H, CH_2O), 3.72 (t, *J* = 5.0 Hz, 2H, CH_2N_{tz}), 3.69 (s, 8H, OCH_2), 3.64 (dq, *J* = 15.1, 5.0 Hz, 16H, CH_2O & NCH_2), 3.59 – 3.52 (m, 4H, $N_{sq}CH_2CH_2$), 1.37 (q, *J* = 7.4 Hz, 3H, CH_3).^{13}C NMR (126 MHz, D_2O) δ 191.20 (C_{sq}=O), 185.60 (C_{sq}=O), 179.69 (O-C_{sqa}), 179.47 (O-C_{sqb}), 176.04 (C_{sq}-NH_a), 175.99 (C_{sq}-NH_b) 169.48 (COO), 168.73 (COO), 165.81 – 164.96, (q, TFA), 153.90 (C_{Ar}), 153.85 (C_{Ar}), 152.35 (C_{Ar}), 150.78 (C_{Ar}), 148.92 (C_{Ar}), 144.34 (C_{tz}), 142.60 (CH_{Ar}), 130.59 (CH_A), 128.55 (CH_{tz}), 128.53 (29), 127.75 (CH_{Ar}), 116.77 (CH_{Ar}), 122.29 – 115.32 (q, TFA), 114.88 (CH_{Ar}), 114.80 (CH_{Ar}), 73.08 (CH_2CH_{3a}), 73.02 (CH_2CH_{3b}), 72.39 (OCH_2), 72.22 (OCH_2), 72.11 (OCH_2), 72.01 (OCH_2), 71.85 (OCH_2), 71.15 (OCH_2), 66.39 (OCH_2), 64.34 (CH_2C_{tz}), 59.82 (-CH_2N), 59.69, (CH_2N) 57.20 (NCH_2), 57.13 (NCH_2), 52.72 (CH_2N_{tz}), 46.47 ($N_{sq}CH_{2a}$), 46.33 ($N_{sq}CH_{2b}$), 17.51 (CH_{3a}), 17.46 (CH_{3b}).

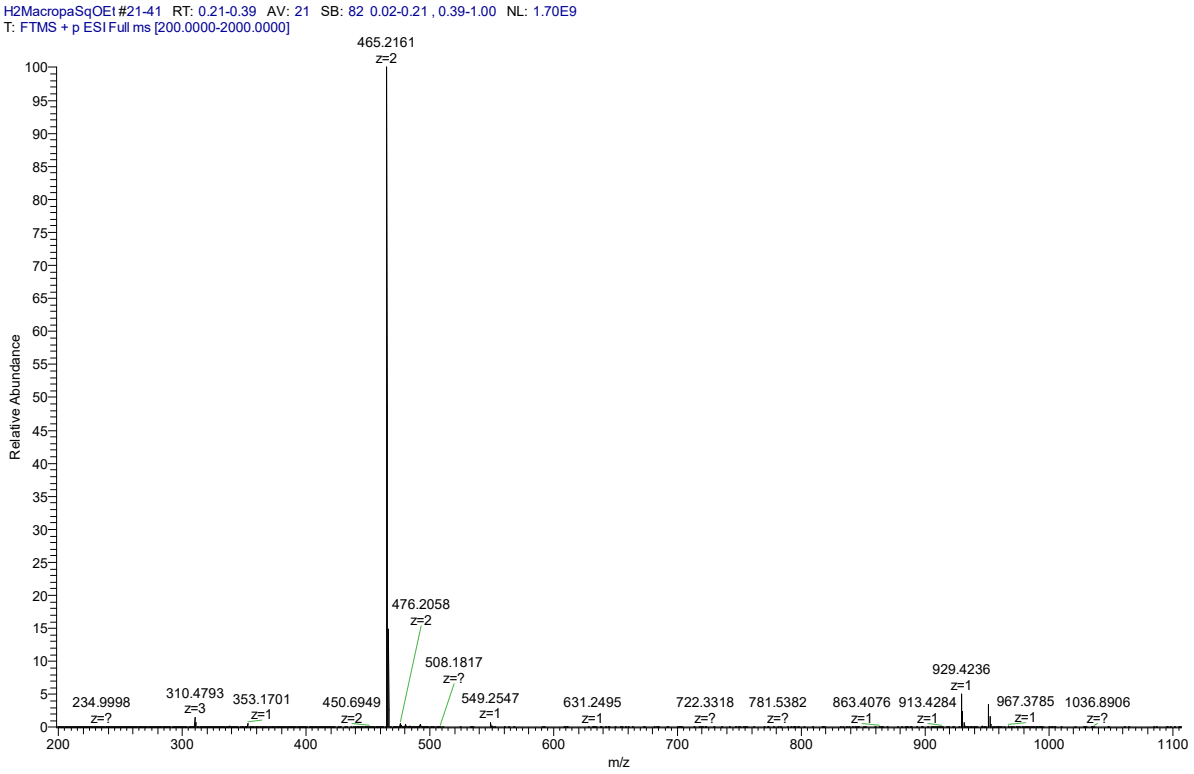


Figure S30: HR-MS of H_2MacropaSqOEt $[M+2H]^{2+}$ = 465.2161.

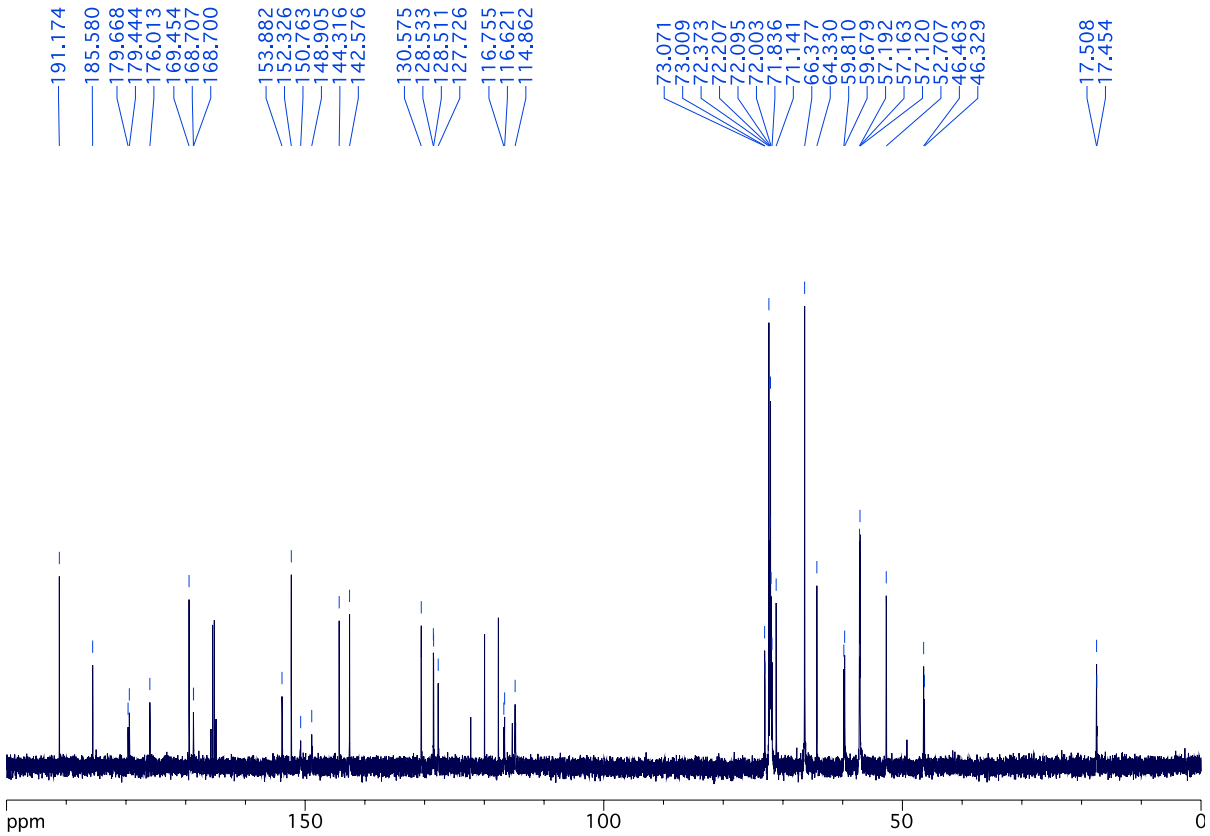


Figure S31: ^{13}C NMR (126 MHz; D_2O) of H_2MacropaSqOEt.

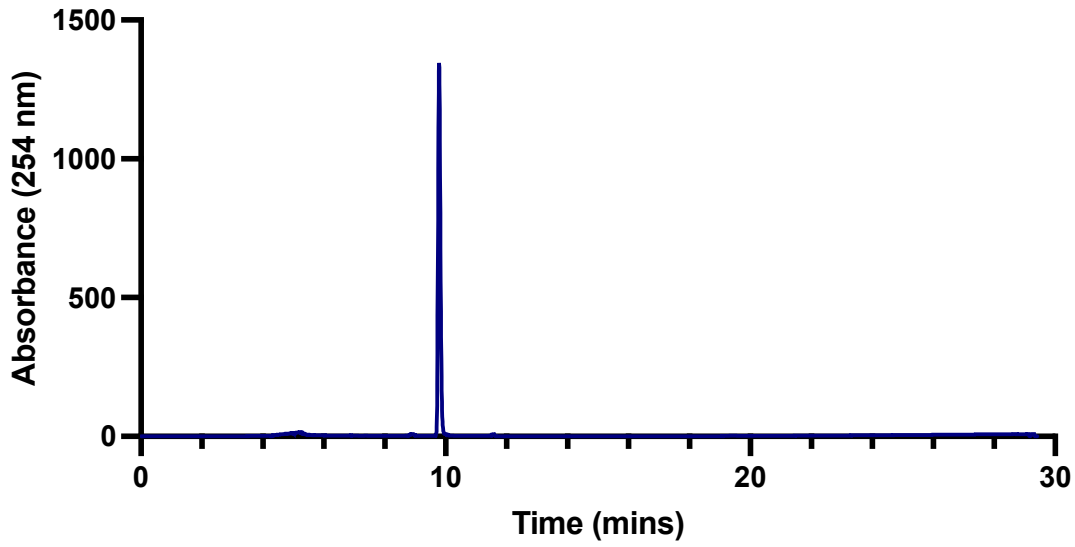


Figure S32: RP-HPLC of H_2MacropaSqOEt (R_t = 9.78 mins).

1.5 Antibody Conjugates

1.5.1 H_2MacropaSq-hG250

Humanized girentuximab, hG250 (5.03 mg/ mL, 200 µL), and IgG1 (3.0 mg/mL) in PBS were buffer exchanged into borate buffer (0.25 M, pH 9.0) and reconstituted to a final concentration of 5 mg/ mL. To the reaction mixture was added H_2MacropaSqOEt (15 mg / mL stock solution in DMSO – final DMSO concentration less than 4 %, 15 equivalents) and shaken at room temperature for four hours. Excess reagents were removed, and buffer was exchanged (sodium acetate, 0.15 M, pH 5.5) *via* spin filtration (50 KDa MW cut-off). H_2MacropaSq-IgG1 required further purification due to excess aggregation above suitable levels (> 4%). SE-HPLC was performed with the antibody conjugate collected at 15-second aliquots to separate aggregated protein.

1.5.2 ELISA

The ELISA protocol was adapted from Pantheon Biologics.[2] Human CAIX protein (50 µg) was reconstituted in MilliQ (stock concentration of 200 µg/mL), then diluted (3µg/ mL), coated on a 96-well plate (100 µL), sealed, and incubated overnight at 2-8 °C before washing with PBST wash solution (1x, 4 x 300 µL). Blocking buffer (1% BSA in PBS) was added, and the plate was sealed and incubated (60 mins), then washed with PBST wash solution. hG250, H_2MacropaSq-hG250, and the isotype control, IgG1 were diluted to 100 µg/mL in assay diluent. The dilutions were performed in a deep well dilution plate from a maximum concentration of 5000 ng/ mL to 2.28 ng/mL before 100 µL of the appropriate dilution was used for the assay. The assay plate was sealed and incubated (75 min) before washing with PBST wash solution. Secondary antibody-HRP conjugate was diluted to (15 ng/mL) and added to the assay plate (100 µL), then incubated in darkness (75 min) before washing with PBST wash solution. Ensure the TMB substrate has sufficient time to equilibrate to room temperature in the absence of light. After

at least 60 minutes, TMB substrate was added to the assay plate and incubated for 15 minutes in darkness before stop solution (2M H_2SO_4, 100 µL) was added to stop the reaction. The plate was checked to ensure no air bubbles, and then samples were run on a FLUOstar Omega microplate reader (BMG Labtech) and analyzed with MARS data analysis software.

1.5.3 Flow Cytometry of hG250 Antibodies and Conjugates

SK-RC-52 cells cultured in RPMI media were lifted with trypsin and pelleted *via* gentle centrifugation. Cells were resuspended in fresh media with a final concentration of 1 x 10^6 cells/ mL. Antibody samples (20 µg) were added and incubated at 4°C for 1 hour before excess antibody was removed *via* centrifugation and washing (x3 PBS). Populations of cells with no fluorescence will have a fluorescence intensity displayed on the histogram at approximately 0. The binding of hG250, as well as the conjugate H_2MacropaSq-hG250 to SK-RC-52 cells was confirmed by flow cytometry. There is no change in binding between the unmodified hG250 antibody and the H_2MacropaSq-hG250 conjugate.

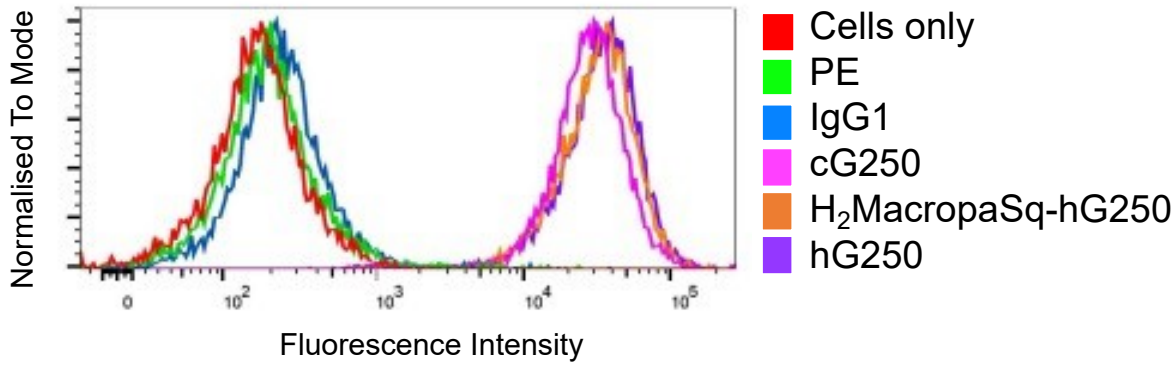


Figure S33: Flow Cytometry of hG250 antibodies, including H_2MacropaSq-hG250, against SK-RC-52 cells.

1.6 Radiochemistry

1.6.1 Cell culture and tumor models

SK-RC-52 was derived from a mediastinal metastasis of a primary RCC. SK-RC-52 cells were cultured in RPMI-1640 medium, supplemented with 20% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO_2. Before *in vitro* and *in vivo* experiments, cells were trypsinized and washed with RPMI-1640 supplemented with 20% FBS.

1.6.2 Radiolabelling of H_2MacropaSq Antibodies with Actinium-225

Actinium-225 was obtained from Oak Ridge National Laboratory as $[^{225}Ac]Ac(NO_3)_3$. $[^{225}Ac]Ac(NO_3)_3$ was reconstituted in HCl (0.2 M, 50 µL). $[^{225}Ac]AcCl_3$ (25µCi/ 925kBq, 5 µL) was neutralized with $NaCH_3CO_2$ buffer (0.15 M, 45 µL, pH 5.5, stock 1). To a solution of H_2MacropaSq-hG250 (1mg/ mL $NaCH_3CO_2$ buffer, pH 5.5, 10 µL) was added stock 1 (20 µL, 10 µCi/ 370 kBq) and left for 5 minutes. The radiolabelled constructs were used without further purification, RCP, <99% performed *via* radioTLC (Radio detector: raytest RITA, stationary phase glass microfiber chromatography paper impregnated with silica gel, mobile phase: 0.05 M citrate buffer, pH 5.0).

Table S2: RCP as a function of time at various concentrations of H_2MacropaSq-hG250.

Time (min)	1.5×10^{-7} M	1.5×10^{-6} M
1	94.6	99.3
5	99.8	99.9
15	99.9	99.9
30	99.9	99.9

Table S3: Immunoreactivity of $[^{225}Ac]Ac$(MacropaSq-hG250) incubated in human serum over seven days.

Immunoreactivity	Day 0 (%)	Day 2 (%)	Day 7 (%)
$[^{225}Ac]Ac$(MacropaSq-hG250)	99.9	86.0	86.9
Nonspecific binding	4.47	4.07	9.03

1.6.3 Lindmo-Scatchard Assay

Lindmo-Scatchard assays were performed as previously described.[3] Briefly, SK-RC-52 cells were suspended at concentrations of 5.0, 2.5, 1.25, 0.625, 0.31, 0.15, and 0.07 x 10^6 cells/mL in 500 µL PBS (pH 7.4). Aliquots of either [^{225}Ac]Ac(MacropaSq-hG250) (1µg of radiolabelled conjugate per 150µL of saline) were added to each tube, and the samples were gently shaken for 60 minutes at room temperature. The treated cells were then pelleted via centrifugation (3000 rpm for 5 minutes), re-suspended, and washed twice with cold PBS before removing the supernatant and counting the activity associated with the cell pellet. Self-blocking experiments with the radiolabelled antibody of extremely low specific activity (i.e., 1000-fold excess of unlabelled antibody) were performed as a negative control. The activity data were background-corrected and compared with the total number of counts in appropriate control samples. Immunoreactive fractions were determined by linear regression analysis of a plot of (total/bound) activity against (1/[normalized cell concentration]). In the radioactive Lindmo assay, [^{225}Ac]Ac(MacropaSq-hG250) was determined to have an immunoreactive fraction > 0.99. The Scatchard assay determined the number of antibody molecules bound per cell and extrapolated the association constant of the antibody-antigen binding interaction, calculated as K_a = 2.5 x 10^9 M^{-1}.

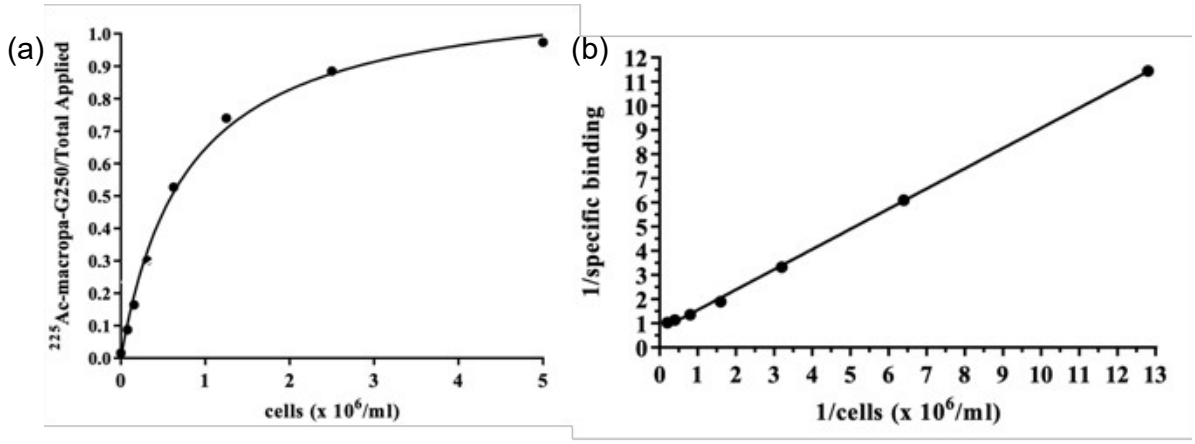


Figure S34: (a) Lindmo Assay showing the fraction of [^{225}Ac]Ac(MacropaSq-hG250) binding to SK-RC-52 cells as a function of cell number, (b) the inverse of specific binding plotted against the inverse of SK-RC-52 cell number

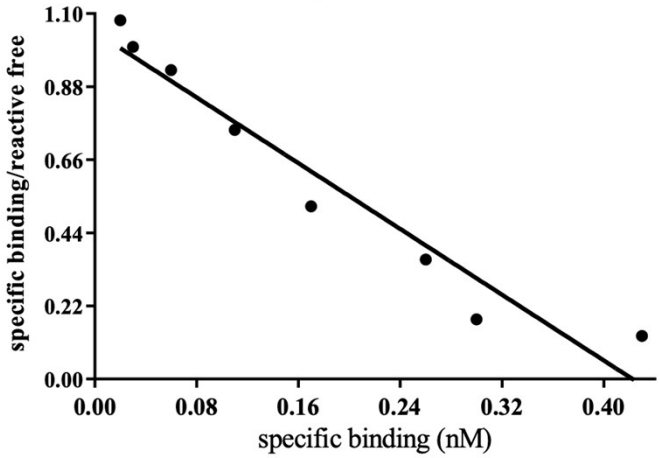


Figure S35: Scatchard Assay of [^{225}Ac]Ac(MacropaSq-hG250) binding to SK-RC-52 cells (K_a = 2.5 x 10^9 M^{-1}, with the maximum binding capacity to the antigen determined to be 5.1 x 10^5/cell).

1.6.4 Biodistribution Study in BALB/c Nude Mice bearing Established SK-RC-52 Xenografts

All animal studies were approved by the Austin Health Animal Ethics Committee. To characterise the biodistribution properties of [^{225}Ac]Ac(MacropaSq-hG250) in mice bearing SK-RC-52 renal cell carcinoma tumors BALB/c nude mice with established SK-RC-52 tumor xenografts (TV = 155.89 ± 21.47 mm^3) received intravenous injections of [^{225}Ac]Ac(MacropaSq-hG250) (30 µg, 0.4µCi/14.8 kBq, *n* =30) or isotype control [^{225}Ac]Ac(MacropaSq-IgG1) (30 µg, 0.4µCi/14.8 kBq; *n* = 10 mice). Following injection on day 0 of [^{225}Ac]Ac-MacropaSq-hG250, groups of mice (*n* = 5) were sacrificed by over-inhalation of isoflurane anesthesia and biodistribution was assessed on Day 0 (3 hrs p.i.) and day 1, 2, 3, 5, and 7 post-injection. Mice injected with isotype control were assessed at day 0 and 3 post-injection only. Mice were exsanguinated by cardiac puncture, and tumors and organs [liver, spleen, kidney, muscle, skin, bone (femur), lungs, heart, stomach, brain, small intestines, large intestines, tail, and colon] were collected immediately. All samples were counted in a dual-channel gamma scintillation counter (Wizard, PerkinElmer, Australia). Triplicate standards prepared from the injected material were counted at each time point with tissue and tumor samples enabling calculations to be corrected for the physical decay of the isotope. The tissue

distribution data were calculated as the mean ± SD percent injected activity per gram tissue (%IA g^{-1}) for each construct per time point.

SI Table 1: Biodistribution data of [^{225}Ac]Ac(MacropaSq-hG250).

	Day 1			***Day 2***			***Day 3***		
	IA g^{-1} %	*SD*	*n*	*IA g^{-1} %*	*SD*	*n*	*IA g^{-1} %*	*SD*	*n*
Blood	41.597	5.072	5	26.157	2.025	5	23.981	4.76	5
Brain	1.604	0.279	5	0.887	0.262	5	0.634	0.143	5
Heart	8.771	1.011	5	5.423	0.813	5	5.016	1.389	5
Lung	16.412	2.394	5	10.888	1.527	5	10.894	2.355	5
Stomach	3.192	0.583	5	2.986	0.397	5	2.834	0.862	5
Spleen	11.378	2.072	5	12.047	1.886	5	12.02	3.205	5
Liver	12.734	1.804	5	10.816	1.932	5	10.987	1.637	5
Kidney	12.07	2.129	5	9.122	0.97	5	8.705	1.749	5
S.Intestine	3.65	0.741	5	2.883	0.467	5	2.859	0.561	5
Colon	2.873	0.515	5	2.673	0.382	5	2.522	0.535	5
Muscle	1.528	0.312	5	2.33	0.542	5	1.91	0.414	5
Bone	2.869	0.437	5	3.274	0.498	5	3.356	1.016	5
Skin	4.441	0.369	5	7.162	1.101	5	5.827	0.654	5
Tail	5.516	1.39	5	3.335	0.356	5	2.895	0.235	5
Tumor	5.272	1.433	5	12.344	1.864	5	16.095	4.767	5

	Day 4			***Day 5***			***Day 7***		
	IA g^{-1} %	*SD*	*n*	*IA g^{-1} %*	*SD*	*n*	*IA g^{-1} %*	*SD*	*n*
Blood	23.362	4.282	5	17.32	4.109	5	14.793	3.334	5
Brain	0.918	0.19	5	0.625	0.251	5	0.351	0.137	5
Heart	5.28	0.856	5	3.32	1.196	5	2.624	1.251	5
Lung	10.54	1.2	5	7.976	2.191	5	6.573	1.681	5
Stomach	3.148	0.349	5	2.67	0.684	5	1.544	0.529	5
Spleen	13.899	2.567	5	10.221	3.027	5	8.288	1.788	5
Liver	10.464	1.345	5	8.629	2.176	5	9.149	2.04	5
Kidney	9.143	0.882	5	7.165	1.857	5	7.008	1.156	5
S.Intestine	2.462	0.225	5	2.092	0.33	5	1.777	0.476	5
Colon	2.78	0.432	5	1.924	0.406	5	1.297	0.446	5
Muscle	1.682	0.323	5	1.219	0.238	5	0.871	0.28	5

Bone	3.525	0.734	5	2.594	0.798	5	1.425	0.468	5
Skin	5.96	0.464	5	4.677	1.24	5	3.854	1.227	5
Tail	2.75	0.29	5	2.021	0.28	5	1.891	0.416	5
Tumor	21.26	6.45	5	19.119	7.064	5	21.416	10.134	5

SI Table 2: Biodistribution of [^{225}Ac]Ac(MacropaSq-IgG1).

	Day 0			***Day 3***		
Organ	*IA g^{-1} %*	*SD*	*n*	*IA g^{-1} %*	*SD*	*n*
Blood	31.286	3.407	5	2.095	1.282	5
Brain	0.997	0.155	5	0	0	5
Heart	7.351	0.942	5	0.673	0.286	5
Lung	13.004	2.29	5	1.536	0.834	5
Stomach	2.673	0.486	5	0.585	0.386	5
Spleen	12.745	2.562	5	10.525	2.223	5
Liver	23.642	2.837	5	19.848	6.589	5
Kidney	12.721	1.408	5	4.876	1.168	5
S.Intestine	3.606	0.562	5	1.224	0.278	5
Colon	2.87	0.286	5	1.235	0.479	5
Muscle	1.238	0.231	5	0.297	0.181	5
Bone	2.899	0.545	5	1.267	0.328	5
Skin	3.97	1.145	5	2.66	0.745	5
Tail	4.534	1.299	5	1.945	0.386	5
Tumor	4.194	0.637	5	3.649	0.485	5

1.6.5 *In vivo* Longitudinal Studies with [^{225}Ac]Ac(MacropaSq-hG250)

All animal studies were approved by the Austin Health Animal Ethics Committee. For the longitudinal therapy study in mice bearing SK-RC-52 renal cell carcinoma tumors BALB/c nude mice with established SK-RC-52 tumor xenografts received intravenous injections of [^{225}Ac]Ac(MacropaSq-hG250) (30 µg, 0.4µCi/14.8 kBq, *n* =14, mean TV 127.6 ± 23.42 mm^3), isotype control [^{225}Ac]Ac(MacropaSq-IgG1) (30 µg, 0.4uCi/14.8 kBq; *n* = 11 mice, mean TV 129.7 ± 21.98 mm^3) or cold hG250 (30 µg, *n* = 8, mean TV 128.91 ± 13.94 mm^3). Following

injection on Day 0 of the three groups, the mice were sacrificed by over-inhalation of isoflurane anesthesia once they reached their ethical endpoint (tumor volume >1000m^3) or at the following collection time points: 3 mice per group at the time of growth curve separation (when anti-tumor effect becomes apparent), 3 mice per group at the time of hG250 cold control cull due to ethical endpoint + all tissues from hG250 cold control group (n = 5 remaining), 3 mice from [^{225}Ac]Ac(MacropaSq-hG250) group on the day of [^{225}Ac]Ac-MacropaSq-IgG1) cull due to ethical endpoint + all tissues from [^{225}Ac]Ac(MacropaSq-IgG1) group (n = 5 remaining), and all remaining tissues from [^{225}Ac]Ac(MacropaSq-hG250) at ethical endpoint (n = 5 remaining). The tumor volumes were measured every 3 – 4 days for the first 49 days, then every 3 – 7 days until day 126. A Logrank test of the Kaplan Meier survival determined the difference in survival between [^{225}Ac]Ac(MacropaSq-hG250) and the control groups to be statistically significant ($p \leq 0.0001$).

SI Table 3: Average tumor volumes of mice receiving [^{225}Ac]Ac(MacropaSq-hG250).

Time post treatment (d)	*Tumor Volume (mm^3)*	*SD*	*n*	*Time post treatment (d)*	*Tumor Volume (mm^3)*	*SD*	*n*
0	127.6	23.42	14	49	38.3	26.12	5
3	206.69	110.33	14	55	42.69	39.86	5
6	210.73	140.77	14	63	37.2	44.03	5
10	169.04	42.6	11	70	84.03	142.7	5
13	141.5	47.56	8	73	90.62	150.85	5
17	110.37	16.07	8	76	151.92	272.85	5
20	107.92	15.65	5	84	245.01	429.24	5
23	108.15	28.74	5	91	98.81	158.08	4
27	95.34	25.95	5	98	128.29	201.77	4
31	94.2	19.85	5	105	115.84	177.71	4
35	82.87	17.43	5	112	190.62	287.57	4

38	64.35	19.16	5	119	263.76	405.57	4
42	61.34	29.99	5	126	81.12	140.5	3
45	45.34	21.43	5				

SI Table 4: Average tumor volumes of mice receiving [^{225}Ac]Ac(MacropaSq-IgG1) and hG250.

	[^{225}Ac]Ac(MacropaSq-IgG1)			***hG250***		
Time post treatment	*Tumor Volume (mm^3)*	*SD*	*n*	*Tumor Volume (mm^3)*	*SD*	*n*
0	129.66	21.98	11	128.91	13.94	8
3	227.81	93.34	11	288.11	139.21	8
6	329.83	163.22	11	553.2	353.17	8
10	375.55	76.2	8	1054.13	546.29	5
13	520.24	127.17	5	-	-	-
17	1092.35	243.84	5	-	-	-

1.6.6 Immunofluorescence assays

Formalin-fixed, paraffin-embedded tumors were dewaxed by two washes in xylene, followed by rehydration in decreasing concentrations of alcohol (two washes in 100% ethanol for 5 minutes each, 95% ethanol, 5 minutes and 70% ethanol for 5 minutes). Slides were placed in water before antigen retrieval using citrate buffer, pH 6.0 (Sigma) at 90°C for 20 mins. Slides were left to cool for 1 hour at room temperature, washed with water, and blocked with 5% bovine serum albumin (BSA; Sigma) and 5% normal goat serum (Thermo Fisher Scientific) in Tris Buffered Saline with Tween 20 (TBST20; Sigma). Slides were incubated with anti-gH2AX

(1:200; Cell Signalling Technologies) and anti-F-actin (1:200; Abcam) primary antibodies in with 5% BSA (Sigma) and 5% normal goat serum (Thermo Fisher Scientific) in TBST20 (Sigma) overnight at 4°C. Primary antibodies were removed by three washes in TBST20, followed by rinsing in water. Primary antibodies were labeled using goat anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific) and goat anti-rat Alexa Fluor 568 (Abcam) for 1 hour at room temperature. DAPI (1 μg/mL) was used to stain nuclei. Fluoromount-G mounting media (Thermo Fisher Scientific) was used to set slides for microscopy and left overnight at 4°C prior to imaging. A one-way analysis of variance (ANOVA) determined the difference in both the hG250 and [^{225}Ac]Ac(MacropaSq-hG250) to be statistically significant ($p \leq 0.0001$). The same is true when comparing [^{225}Ac]Ac(MacropaSq-IgG1) to and [^{225}Ac]Ac(MacropaSq-hG250) ($p \leq 0.0001$).

1.6.7 Immunohistochemical assays

Hematoxylin/eosin staining was completed by Austin Pathology Services (Austin Health). Formalin-fixed, paraffin-embedded tumors were dewaxed by two washes in xylene, followed by rehydration in decreasing concentrations of alcohol (two washes in 100% ethanol for 5 minutes each, 95% ethanol, 5 minutes and 70% ethanol for 5 minutes). Slides were placed in water before antigen retrieval using citrate buffer, pH 6.0 (Sigma) at 90°C for 20 mins. Slides were left to cool for 1 hour at room temperature, washed with water and samples quenched in 3% hydrogen peroxide for 5 mins at room temperature. Slides blocked with 5% bovine serum albumin (BSA; Sigma) and 5% normal goat serum (Thermo Fisher Scientific) in Tris Buffered Saline with Tween 20 (TBST20; Sigma) for 1 hour. Anti Ki-67 antibody was applied overnight (1:200; 5% BSA 5% NGS in TBST20) at 4°C. Primary antibodies were removed by one wash in TBST20 followed by one wash in TBS. Slides were incubated with anti-rabbit Dako Envision+ Dual Link System HRP secondary antibodies for 1 hour at room temperature

(Aligent). DAB substrate (Abcam) was used to detect the presence of antibody staining at room temperature. Sections were thoroughly washed prior to counterstaining with Heamatoxylin followed by blueing in Scotts Tap water and dehydration in increasing ethanol percentages. Finally, slides were washed in xylene, and dehydrated in increasing concentrations of ethanol, followed by mounting in DPX mounting media (Sigma-Aldrich). Images of all H&E and Ki67 IHC sections were acquired on an Aperio AT2 Slide Scanner (Leica Biosystems). Images were processed using Aperio ImageScope software (Leica Biosystems). DAB Staining was quantitated using QuPath Software.[4] SK-RC-52 tumor sections that were treated with [^{225}Ac]Ac(MacropaSq-hG250) were compared to corresponding tumor sections treated with [^{225}Ac]Ac(MacropaSq-IgG1) and hG250 to evaluate tumor growth and inhibition. Hematoxylin and eosin (H&E), and Ki67 staining were used to assess tumor proliferation and apoptosis. Ki-67 staining indicated that tumors treated with [^{225}Ac]Ac(MacropaSq-hG250) exhibited reduced proliferation (20.2 ± 6.0%) compared with tumors treated with hG250 or [^{225}Ac]Ac(MacropaSq-IgG1) (84.3 ± 8.5% and 74.1 ± 6.4%, respectively) ANOVA determined the difference in both the hG250 and [^{225}Ac]Ac(MacropaSq-hG250) to be statistically significant ($p \leq 0.0001$). The same is true when comparing [^{225}Ac]Ac(MacropaSq-IgG1) and. and [^{225}Ac]Ac(MacropaSq-hG250) ($p \leq 0.0001$).

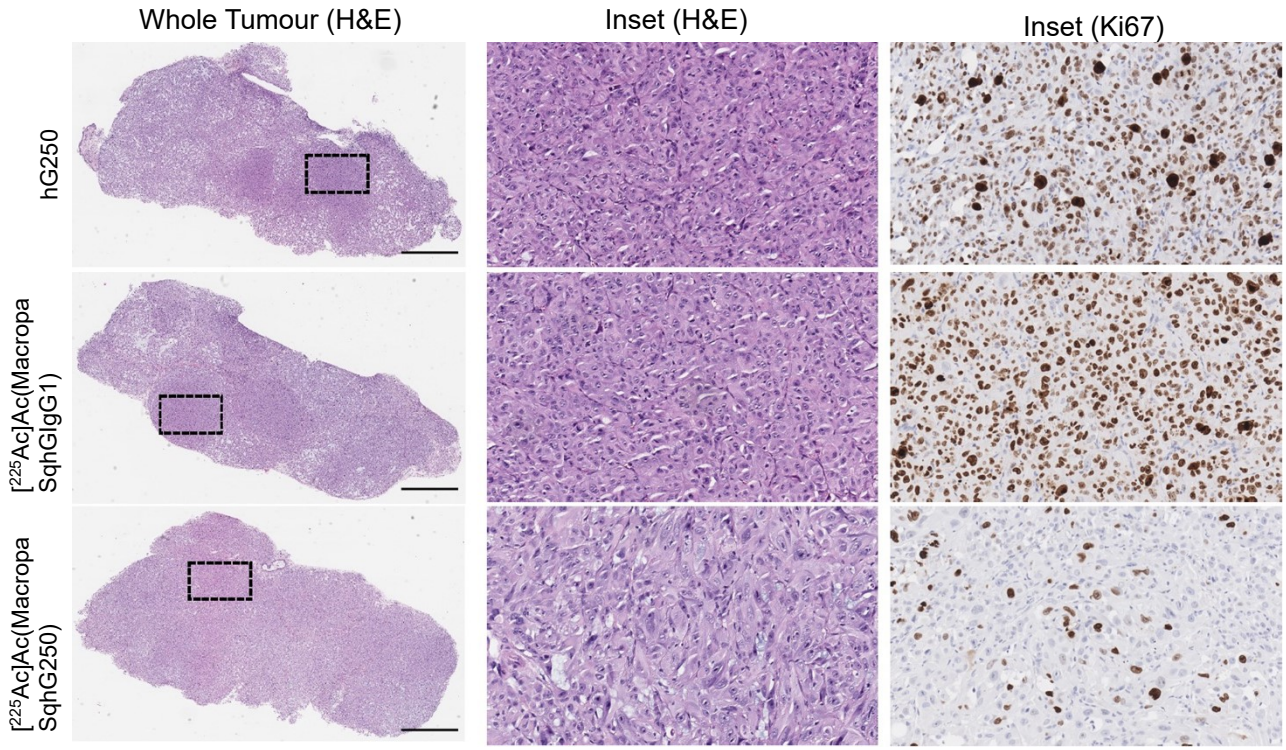


Figure S36: Treatment with [^{225}Ac]Ac(MacropaSq-hG250) inhibits cell proliferation in tumors compared to control groups. Tumor H&E and Ki67 staining. H&E and Ki67 Insets are area matched. Scale bar = 700 µm.

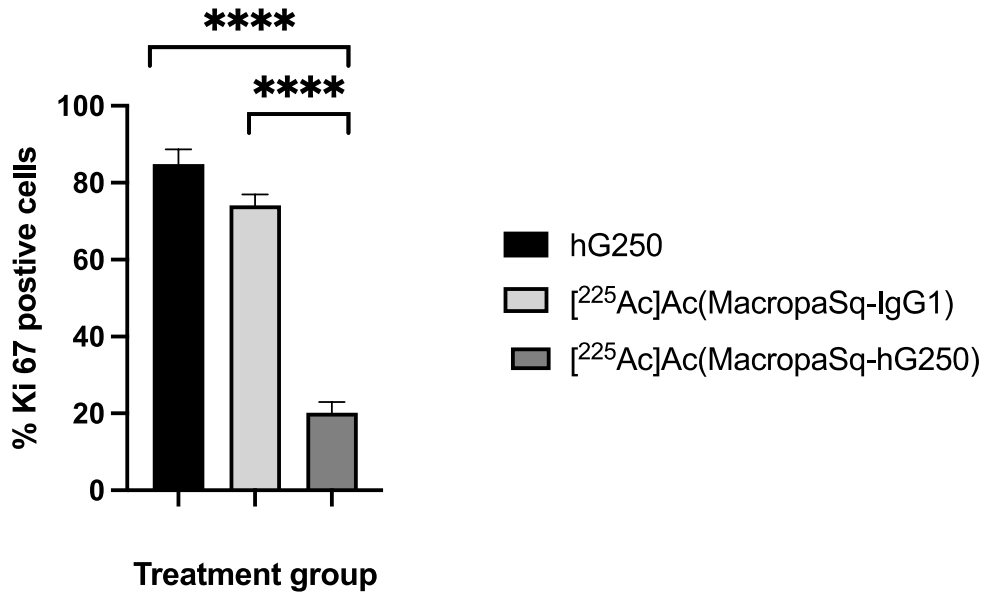


Figure S37: Treatment with 225Ac G250 induces dsDNA breaks and inhibits cell proliferation in tumor cells. Percentage of Ki67 cells of immunohistochemically stained 4 µm tumor sections, n= 6 representative 20 x images quantitated per treatment group ***p<0.0001.

1.6.8 Confocal Microscopy and gH2AX foci quantitation

Confocal microscopy was performed on a Zeiss LSM 980 Airyscan 2 microscope equipped with

2 x MA-PMT detectors, using 405 nm, 568, and 633 nm laser lines. Images were acquired on

either the 40 x or 63 x Axio oil objectives. Where required, Z-sectioning was performed using 1 µm sections. Images in all experimental groups were obtained with the same settings, except for detector gain adjustments that were performed to normalize saturation levels. All images were processed using either Zeiss ZEN software (Zeiss), MetaMorph software (Visitron Systems), and/or Image J software. Following image processing, average gH2AX foci per nuclei were determined by creating a region of interest around each tumor nuclei (mouse stroma were manually excluded) on deconvoluted, greyscale images. Each image was thresholded a maximum edge projection was performed and foci per nuclei counted.

1.6.9 Statistical Analysis

Visualization of gH2AX foci and Ki67 quantitation was performed using GraphPad Prism. A one-way ANOVA with Tukey's multiple comparisons test was used. All data is mean ± SEM with >36 nuclei analyzed per treatment group for H2AX quantitation and n=6 20 x images quantitated (>6000 cells/treatment group) for Ki67 staining.

1.7 References

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