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Electronic Supplementary Information

Platinum(IV) Oxaliplatin peptide conjugate targeting memHsp70+ phenotype

in colorectal cancer cells

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Experimental details

General

All chemicals and solvents were used as received from commercial suppliers. *cis,cis,trans*-diamminodichloridodisuccinato-platinum(IV), [Pt(DACH)(ox)(suc)$_2$], was synthesized as previously reported.[1] Protected amino acids Fmoc-Lys(Boc)-OH, Fmoc-Asp(OiBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Phe-OH, Fmoc-Glu(O*tB*u)-OH, were purchased from Merck (*Merck Millipore, Cork, Ireland*). Fmoc-Thr(*t*Bu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(*t*Bu)-OH, Rink amide 4-methylbenzhydrylamine (MBHA) resin, *N,N'*-diisopropylcarbodiimide (DIC, Sigma), oxyma pure (Sigma), piperidine, *N*-methyl-2-pyrrolidone (NMP, Life Sciences), N,N'-Carbonyldiimidazole (CDI), piperidine, phenol, trifluoroacetic acid (TFA), succinic anhydride, anhydrous DMF and anhydrous DCM were purchased from Sigma Aldrich (*Sigma Aldrich Ireland Ltd., Wicklow*, *Ireland*). Syringe reactors were purchased from MultiSynTech GmbH (*MultiSynTech GmbH,Witten, Germany*). Peptide conjugates were purified on a Shimadzu Prominence LC system (*Shimadzu Corp. Kyoto, Kyoto Prefecture, Japan*) using reverse-phase semi-preparative HPLC with a C18 Gemini column (110 Å, 5 µm, 10 x 250 mm) (*Phenomenex,* Macclesfield, Cheshire, UK). The purity of the peptides conjugated was checked by analytical reverse-phase HPLC on a C18 Atlantis column (100 Å, 5 µm, 4.6 x 250 mm) (*Waters Chromatography Ireland LTD, Dublin 9, Ireland*).

Mass spectrometry

Mass spectrometry experiments were performed by Mr. Jimmy Muldoon and Mr. Kevin Conboy on a Quattro Micro™ liquid chromatography instrument at the School of Chemistry and Chemical Biology, University College Dublin. MALDI-TOF experiments were performed by Dr Martin Feeney on a MADLDI-Q-ToF Premier at the School of Chemistry, Trinity College Dublin.

Synthesis of TPP and ScP

Peptide sequences **TPP** (TKDNNLLGRFELSG) and **ScP** (LNLETRLGFGDNKS) were assembled by automated solid phase peptide synthesis on a CEM Liberty Blue™ Microwave Peptide Synthesizer (*CEM Microwave Technology, (Ireland) Ltd, Dublin*) using L-amino acids protected with Fmoc on a 0.5 mmol and 0.1 mmol scale respectively. The synthesis was carried out in accordance to the Fmoc/*t*Bu strategy from a Rink amide MBHA resin using DIC/oxyma pure coupling chemistry. Removal of Fmoc was carried out using 20% (v/v) piperidine in NMP. The resin was washed with DCM (3x), dried under vaccum and stored at 4 °C until required for conjugation to [Pt(DACH)(ox)(suc)$_2$].

Prior to testing the purity of **TPP** was confirmed by analytical HPLC (Figures S1). ESI-MS spectra of **TPP** are shown in Figures S6 and S7 in the ESI† and the following characteristic m/z values were found: 1561.71 (M+H$^+$), 782.05 (M+2H$^+$)$^{2+}$.

Synthesis of 1, 2, 3 and 4

Attachment of $[Pt(DACH)(ox)(suc)_2]$ to the Rink Amide MBHA resin-bound **TPP** and **ScP** was performed manually in a 5 ml syringe (0.1 mmol or 0.05 mmol scale, respectively) fitted with a Teflon frit and stopcock. The resin was agitated in DMF for 30 min to facilitate swelling after which the DMF was drained. Quantities of the reagents were calculated relative to the original substitution of the resin. To $[Pt(DACH)(ox)(suc)_2]$ (2eq) in anyhdrous DMF (1.5 mL) at 60 °C was added a solution of CDI (4.2 eq) in anhydrous DMF (1.5 mL) and the mixture stirred at 60 °C for a further 10 min. The reaction was cooled to rt and added to the pre-swollen resin. The vessel was subsequently agitated for 48 h after which the medium was drained. The resin was washed with DMF (3x) in order to remove any excess or unreacted activated Pt precursor $[Pt(DACH)(ox)(suc)_2]$ and then washed with DCM (3x) and dried under vacuum.

The peptide was then cleaved from the resin and concomitantly deprotected. The cleavage cocktail (3 ml for 0.05 mmol, 5ml for 0.1 mmol of resin) consisted of TFA (90 %), phenol (5%) and water (5%). The mixture was gently stirred for 2 h. The resin was filtered and the peptide precipitated by addition of cold diethyl ether (0 °C, ~10 ml), isolated by centrifugation and washed with 5 ml of diethyl ether (x3). The dry pellet was dissolved in H_2O and the solution lyophilized. Peptides were purified by semi-preparative reverse-phase HPLC with a C18 Gemini column (110 Å, 5 µm, 10 x 250 mm). The purity of the peptides was checked by analytical reverse-phase HPLC on a C18 Atlantis column (100 Å, 5 µm, 4.6 x 250 mm). Crude peptides were dissolved in a water methanol (95:5) mixture containing 0.1% TFA. Mobile phase A: 0.1% TFA in water and mobile phase B: 0.1% TFA in MeOH were used with a gradient of 5 – 85 – 5% B over 65 min with a flow rate of 1 mL/min (analytical) or 3 mL/min (semi-preparative). Wavelength detection at 254 nm was used.

The monoconjugate **1** was eluted at 38 min, followed by the diconjugate **2** at 39 min. The fractions, containing the products, were collected and lyophilized, yielding 7.5 mg of the monoconjugate **1** and 11.3 mg of the diconjugate **2** (Yields: 3.5% and 3.1% with respect to the resin loading). Purity was confirmed by analytical HPLC (Figures S2 and S3). ESI-MS spectra of **1** and **2** are shown in Figures S8 and S9 in the ESI† and the following characteristic m/z values were found: monoconjugate **1**: 1088.88 $(M+2H^+)^{2+}$, 733.27 $(M+3H^+)^{3+}$; diconjugate **2**: 1240.54$(M+2H^+)^{2+}$, 931.12$(M+3H^+)^{3+}$. In MALDI-TOF MS measurements, only the TPP-succinate moiety common to both **1** and **2** was found: monoconjugate **1**: 1662.80; diconjugate **2**: 1662.85. calculated: $(+H)^+$ 1662.84 (Figure S10 and S11 in the ESI†).

The monoconjugate **3** was eluted at 37 min, followed by the diconjugate **4** at 38 min. The fractions, containing the products, were collected and lyophilized, yielding 4 mg of the monoconjugate **3** and 3.7 mg of the diconjugate **4** (Yields: 3.7% and 2% with respect to the resin loading). Purity was confirmed by analytical HPLC (Figures S4 and S5). ESI-MS spectra of **3** and **4** are shown in Figures S12 and S13 in the ESI† and the following characteristic m/z values were found: monoconjugate **3**: 1088.88 $(M+2H^+)^{2+}$, 733.27 $(M+3H^+)^{3+}$; diconjugate **4**: 1240.54$(M+2H^+)^{2+}$, 931.12$(M+3H^+)^{3+.}$

Cell lines

HT29, loVo, HCT116 wt, and HCT116 p53 -/- colorectal cancer cell lines were kindly gifted by Dr. Caoimhin Concannon at the Department of Physiology and Medical Physics, and the Centre for Systems Medicine at the Royal College of Surgeons in Ireland. Normal human dermal fibroblasts (NHDF) were sourced from American Type Culture Collection (PCS-201-010) (*ATCC®, LGC Standards, Middlesex, UK*). HT29 and LoVo cell lines were cultured in

Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin-streptomycin, 1% L-glutamine and 10% fetal bovine serum (FBS). HCT-116 wt and HCT-116 p53-/- cell lines were cultured in Roswell Park Memorial Institute Medium -1640 (RPMI) supplemented with 1% penicillin-streptomycin, 1% L-glutamine and 10% FBS. NHDF cells were cultured in Fibroblast Basal Medium (FBM) supplemented with Hfgf-B, insulin, 5% FBS and gentamicin/amphotericin-B (FGM-2 BulletKit Suppl. & Growth Factors) (*Lonza, Basal, Switzerland).* Cells were grown under standard conditions; 37 °C, 95% v/v humidity, 5% v/v CO_2, and passaged twice a week. Cells were used in exponential phase and their viability (> 95%) confirmed prior to use using trypan blue dye exclusion. All cells were cultured in a sterile mycoplasma free facility and were tested regularly for the presence of mycoplasma contamination with MycoAlert^{TM} Mycoplasma Detection Kit (*Lonza, Basal, Switzerland*).

***In vitro* cytotoxicity assays**

Stock solutions of cisplatin and oxaliplatin were made freshly in medium and diluted to required concentrations with further medium. Cell growth was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt, (MTS test, Promega, Southampton, UK), a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt to blue formazan. 3×10^4 cells were seeded per well onto 96-well plates in 100 µL of the appropriate culture medium. Twenty-four hours after seeding, the medium was removed and the cells were treated by adding 100 µL of the test compound solutions at appropriate concentrations.
After 72 h of treatment, 20 µL of the MTS reagent was added to each well and the plates incubated for 2 h at 37 °C. The absorbance was measured at 490 nm using a Wallac 1420 Victor 3 V plate reader (Perkin-Elmer Life Sciences, Boston, MA, USA). The percentage of surviving cells relative to untreated controls was then determined. The IC_{50} value, defined as the drug concentration required to inhibit cell growth by 50%, was estimated graphically from dose-response plots using GraphPad Prisim, a scientific 2D graphing and statistics software. Evaluation is based on averages from three independent experiments and each experiment comprised three replicates per concentration level.

Western blot analysis

For western blot analysis, whole cell pellets were lysed on ice using radioimmunoprecipitation assay (RIPA) buffer (NaCl 150 mM, sodium dodecyl sulfate 0.1%, sodium deoxycholate 0.5%, NP40 1%, Tris 50 mM pH 8, 1:100 protease inhibitor, 1:00 phosphatase inhibitor). Protein content was determined using a micro BCA protein assay (*Thermo Fisher Scientific, Waltham, Massachusetts, USA*) - 20 µg samples were boiled in gel-loading buffer and separated on 10-15% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes using the iBlot® gel transfer device (*Life Technologies, Ireland*). The membranes were incubated with a monoclonal antibody directed against HSP70 (IgG1; *Enzo Life Sciences Ltd., Exeter, UK)* overnight at 4 °C. Bound antibodies were visualised using horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG1; *Enzo Life Sciences Ltd., Exeter, UK),* ECL Chemillumisecent HRP developing kit *(Milipore, Carrigtwohill, Cork)* and a FujiFilm LAS-3000 image reader (*Fuji, Sheffield, UK*

Flow cytometric analysis of memHSP70

The expression of the membrane form of HSP70 on tumor cell lines was determined by flow cytometry using a memHSP70 specific murine monoclonal antibody cmHSP70.1-FITC (IgG1; *Multimmune GmbH, Germany*) which binds to the exposed sequence of memHSP70. After incubation of viable cells ($5x10^4$) with the antibody for 2 h at 4 °C and following three washing steps, cell were analysed with a BD LSR II flow cytometer (*BD Biosciences, Oxford, UK*). FITC was excited at 488 nm and fluorescence emission was collected in the FL1 channel through a 520 nm band-pass filter. BDFACSDiva^{TM} software (version 8.0.1) was used to determine the mean fluorescence per sample and to generate histograms based on the gated area of interest determined by unstained controls. Cells for analysis were identified on the basis of forward and side light scatter characteristics (FSC, SSC respectively) and confirmed as being single cells using the FSC-A(rea) and SSC-H(eight) parameters.

Flow cytometric analysis of apoptosis

Cells were grown in T75 flasks for 48 h, at which time they were harvested using trypsin and counted using trypan blue dye exclusion. Viable cells were transferred and cultivated in 24-well culture plates ($5x10^4$ cells/well). The medium was removed 24 hours after seeding and cells were treated with a particular compound solution at various working concentrations. After 72 h of treatment the test compound solutions were removed, the cells were harvested using trypsin, transferred to eppendorfs and pelleted (500 rcf, 5 min). The cell pellets were re-suspended in a solution of binding buffer (HEPES 10 mM NaCl 135 mM, $CaCl_2$ 5 mM) containing annexin-V FITC conjugate and propidium iodide (PI) (*annexin-v-fitc apoptosis kit*) (*BioVision Inc., Milpitas, California, USA*). The cell suspension were directly transferred to a 96-well plate and analysed with a BD LSR II flow cytometer (*BD Biosciences, Oxford, UK*). Excitation of Annexin V-FITC was done with a 488 nm laser and fluorescence emission was collected in the FL1 channel through a 520 nm band pass filter. PI was excited at 561 nm and fluorescence emission was collected through a 605/40 nm band-pass filter and a 570 nm long pass filter. BDFACSDiva^{TM} software (version 8.0.1) was used to determine the mean fluorescence per sample and to generate histograms based on the gated area of interest. Cells for analysis were confirmed as being single cells using the FSC-A(rea) and SSC-H(eight) parameters.

Statistical Analysis

The Student's T-test was used to analyse differences between two test compounds at a single concentration, with the significance level set to $p<0.05$.

Figures

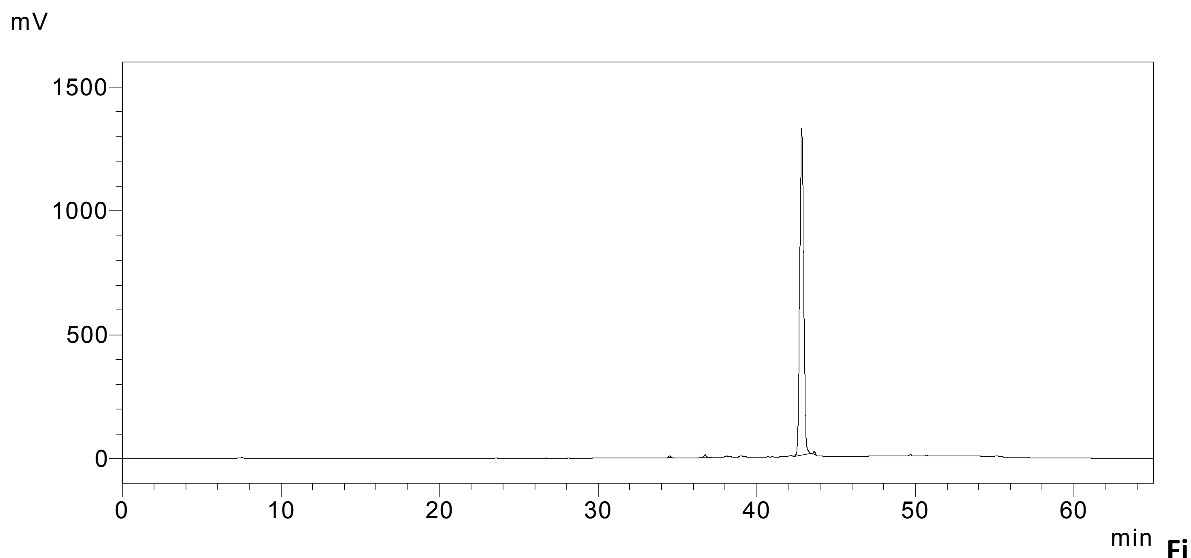

Figure S1. Analytical trace of **TPP** under HPLC conditions; A: 0.1% TFA in water and B: 0.1% TFA in MeOH with a gradient of 5 – 85 – 5% B over 65 min with 0.1% TFA; 1 ml/min; detector λ 254nm.

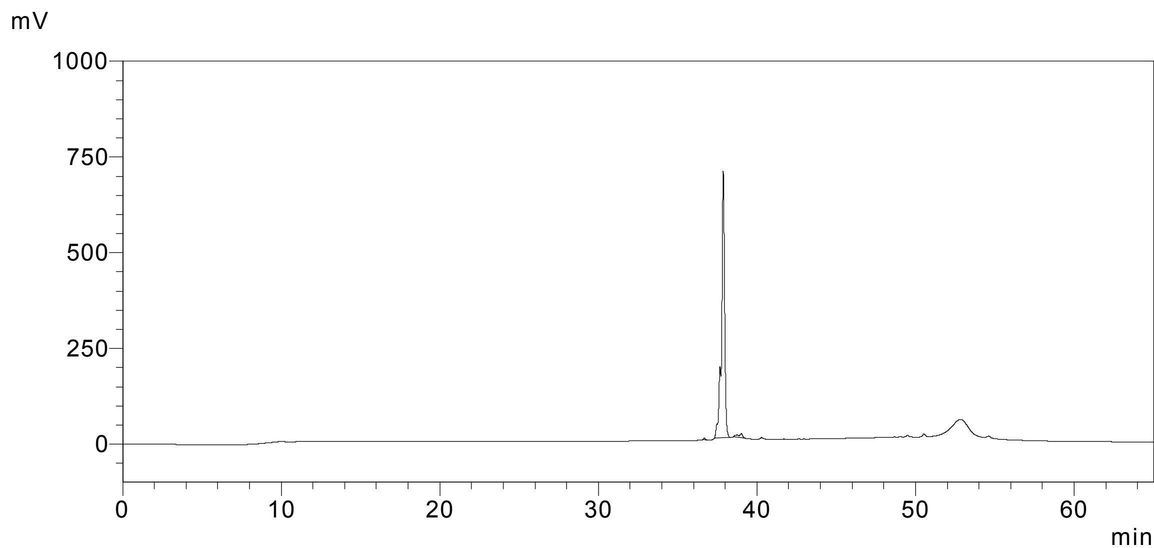

Figure S2. Analytical trace of **1** under HPLC conditions; A: 0.1% TFA in water and B: 0.1% TFA in MeOH with a gradient of 5 – 85 – 5% B over 65 min with 0.1% TFA; 1 ml/min; detector λ 254nm.

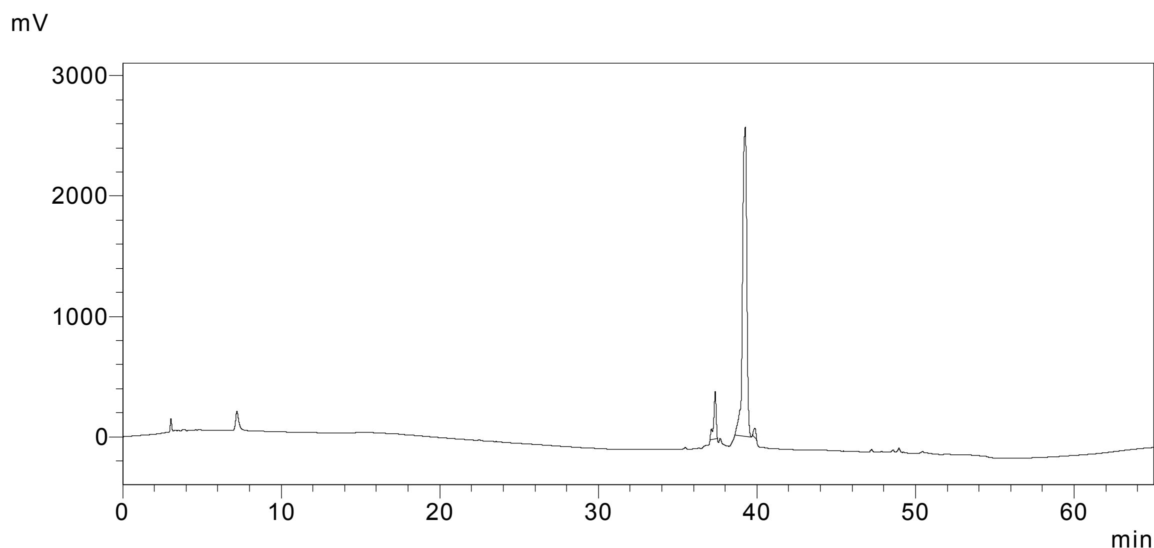

Figure S3. Analytical trace of **2** under HPLC conditions; A: 0.1% TFA in water and B: 0.1% TFA in MeOH with a gradient
of 5 – 85 – 5% B over 65 min with 0.1% TFA; 1 ml/min; detector λ 254nm.

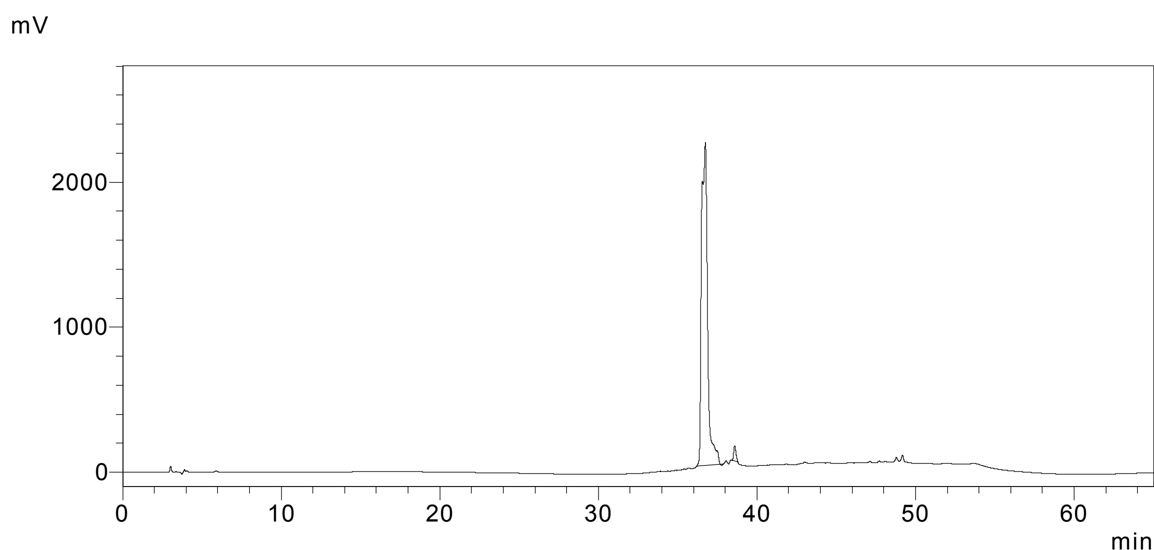

Figure S4. Analytical trace of **3** under HPLC conditions; A: 0.1% TFA in water and B: 0.1% TFA in MeOH with a gradient of 5 – 85 – 5% B over 65 min with 0.1% TFA; 1 ml/min; detector λ 254nm

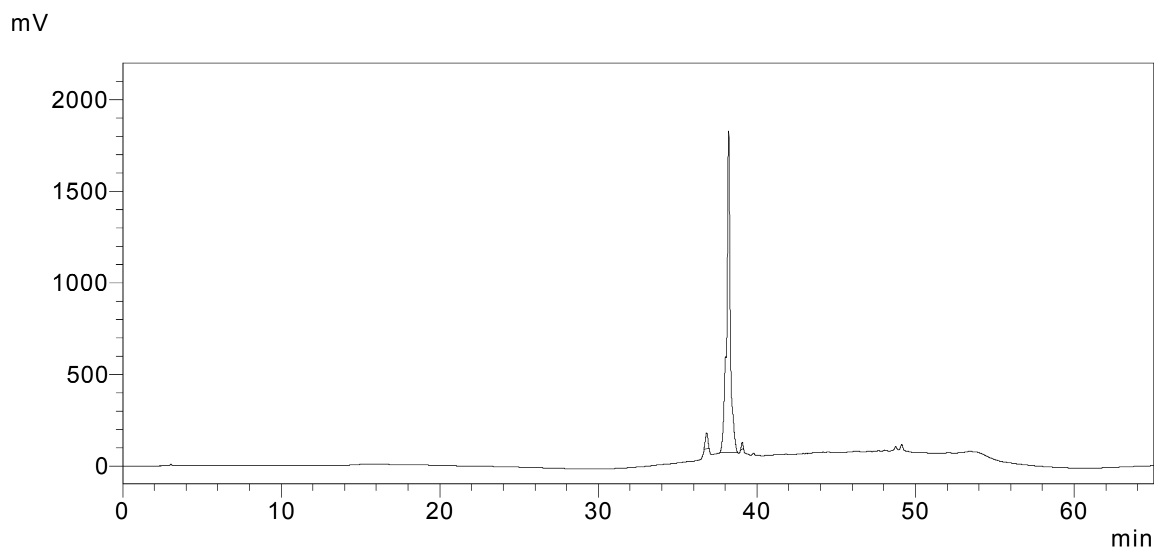

Figure S5. Analytical trace of **4** under HPLC conditions; A: 0.1% TFA in water and B: 0.1% TFA in MeOH with a gradient of 5 – 85 – 5% B over 65 min with 0.1% TFA; 1 ml/min; detector λ 254nm.

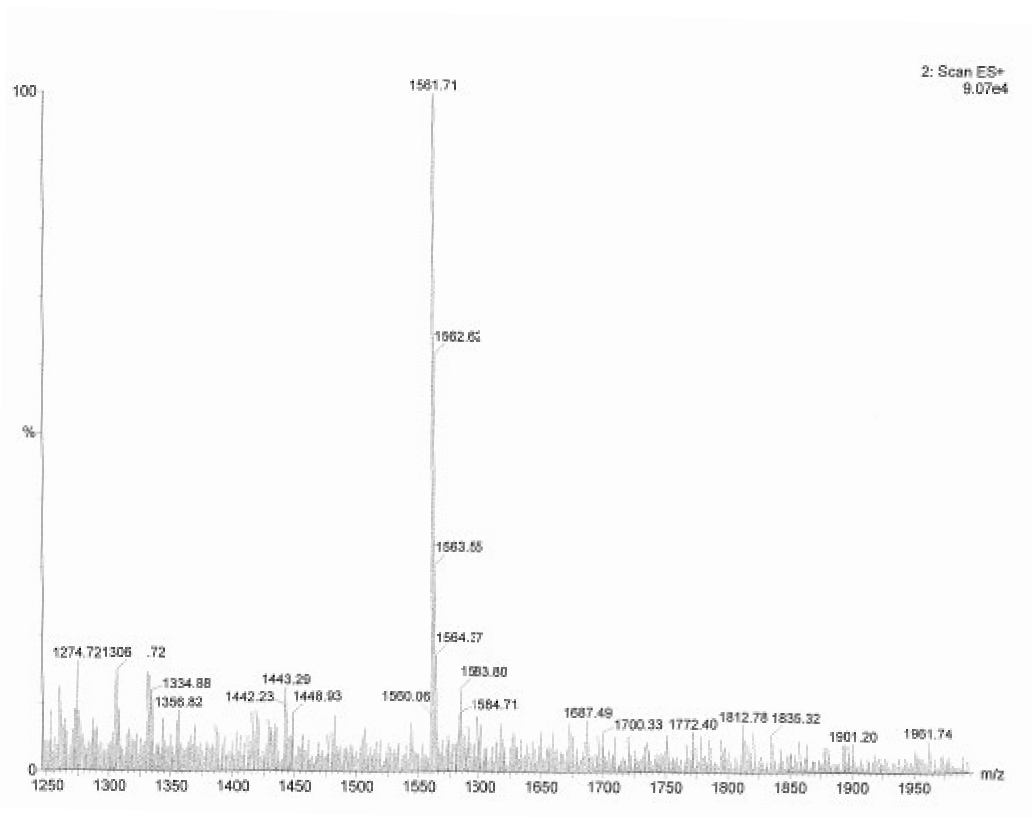

Figure S6. Expanded region between 1250-1950 of ESI-MS spectrum of **TPP** with characteristic m/z value: 1561.71 (M+H)+.

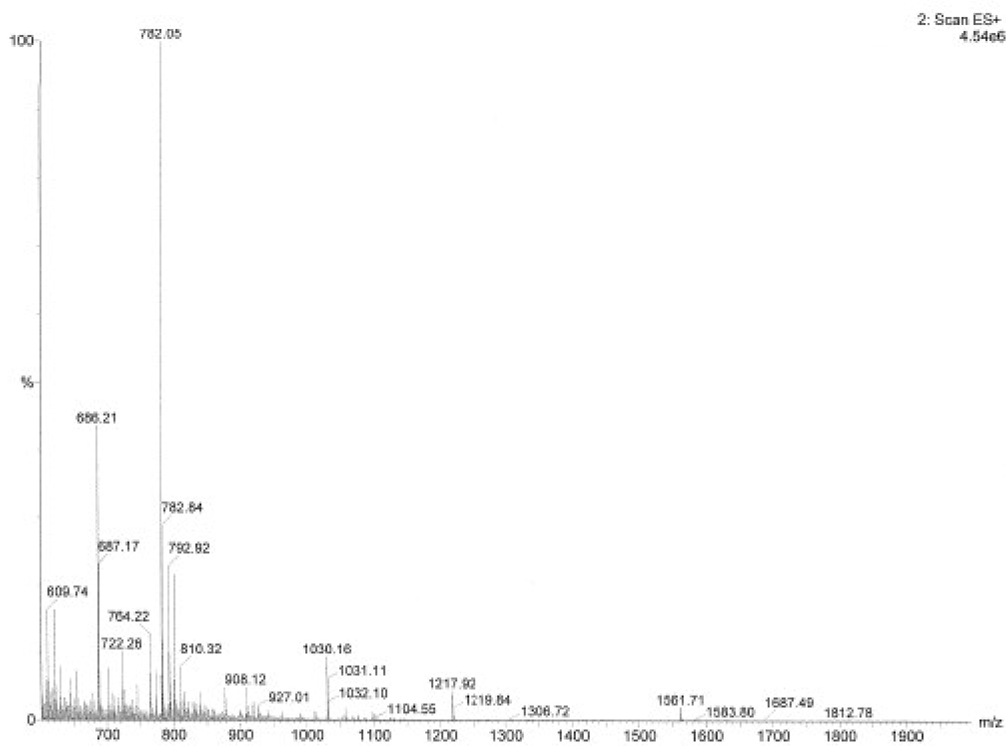

Figure S7. ESI-MS spectrum of **TPP** with characteristic m/z value: 782.05 $(M+2H^{+})^{2+}$.

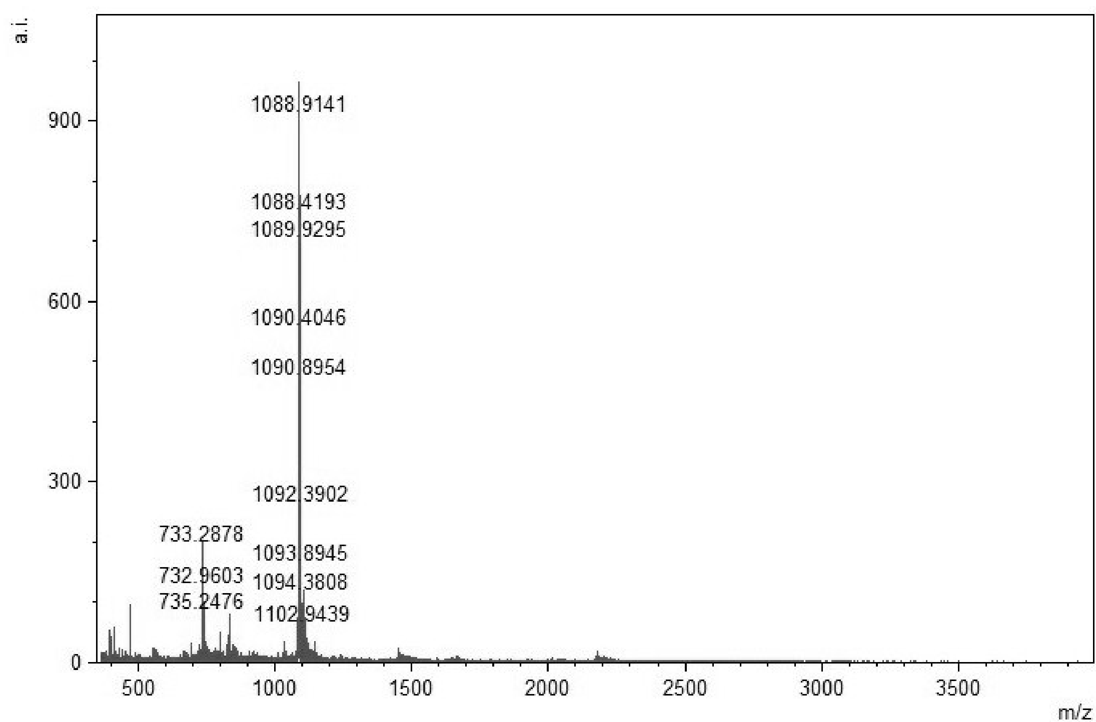

Figure S8. ESI-MS spectrum of **1** with characteristic m/z values: 1088.9141 $(M+2H^+)^{2+}$, 733.2878 $(M+3H^+)^{3+}$.

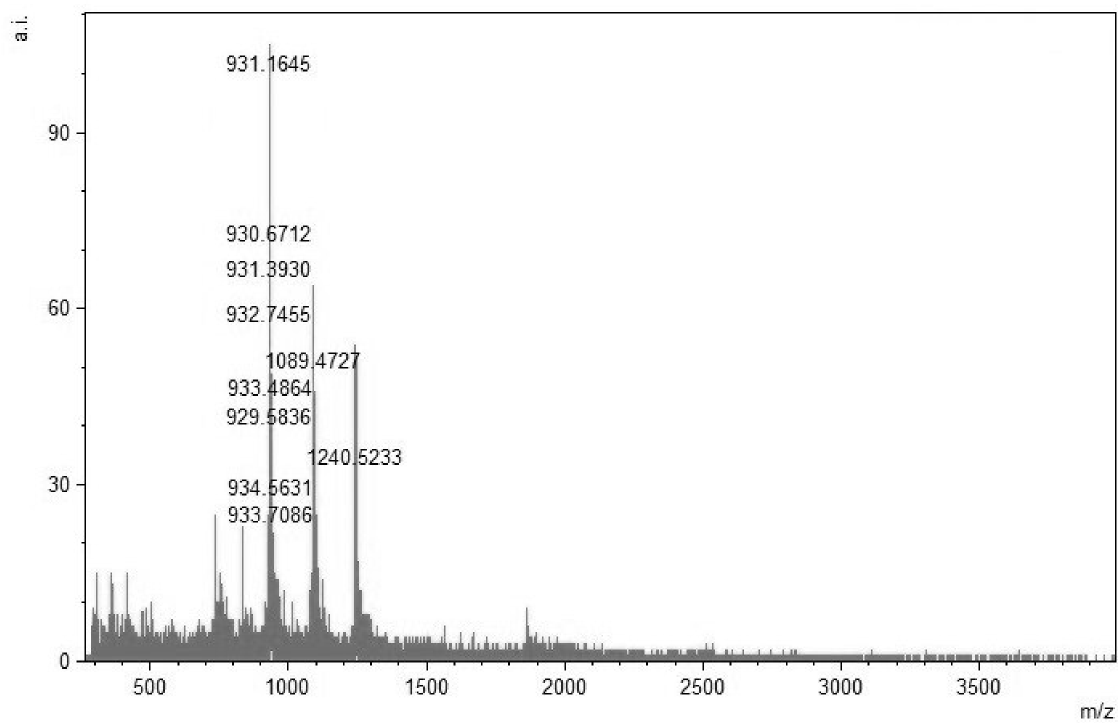

Figure S9. ESI-MS spectrum of **2** with characteristic m/z values: 1240.5233$(M+2H^+)^{2+}$, 931.1645$(M+3H^+)^{3+}$.

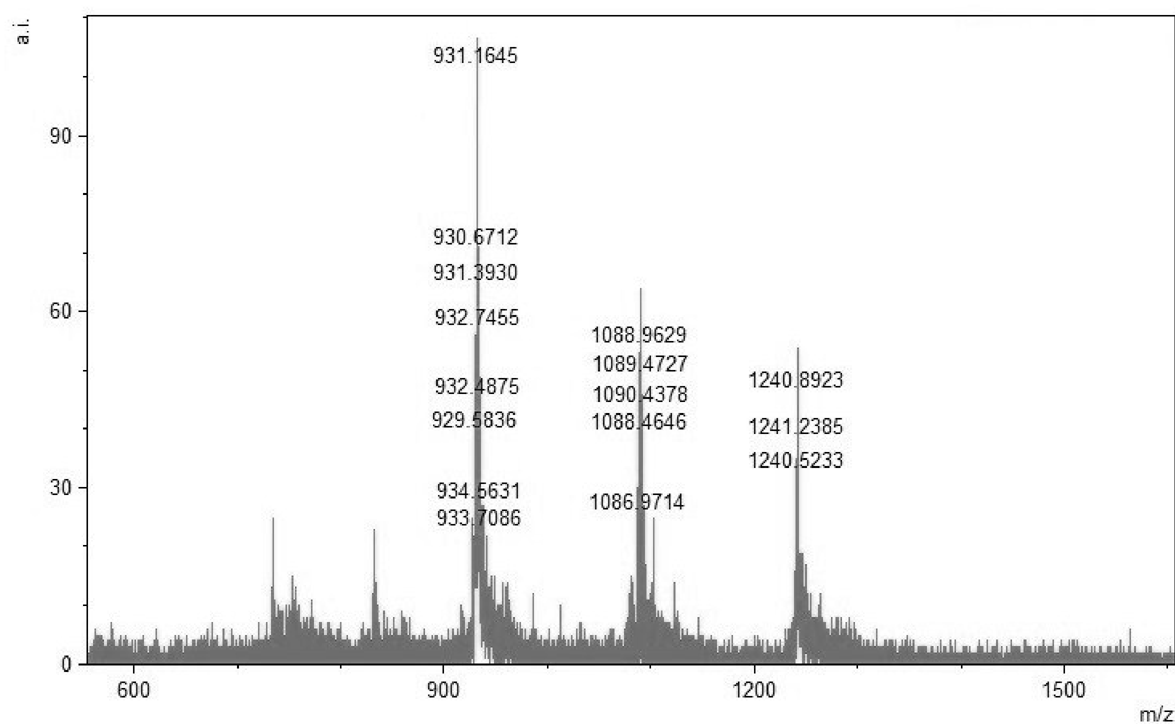

Figure S10. Expanded region between 600-1500 of ESI-MS spectrum of **2** with characteristic m/z values: 1240.5233$(M+2H^+)^{2+}$, 931.1645$(M+3H^+)^{3+}$.

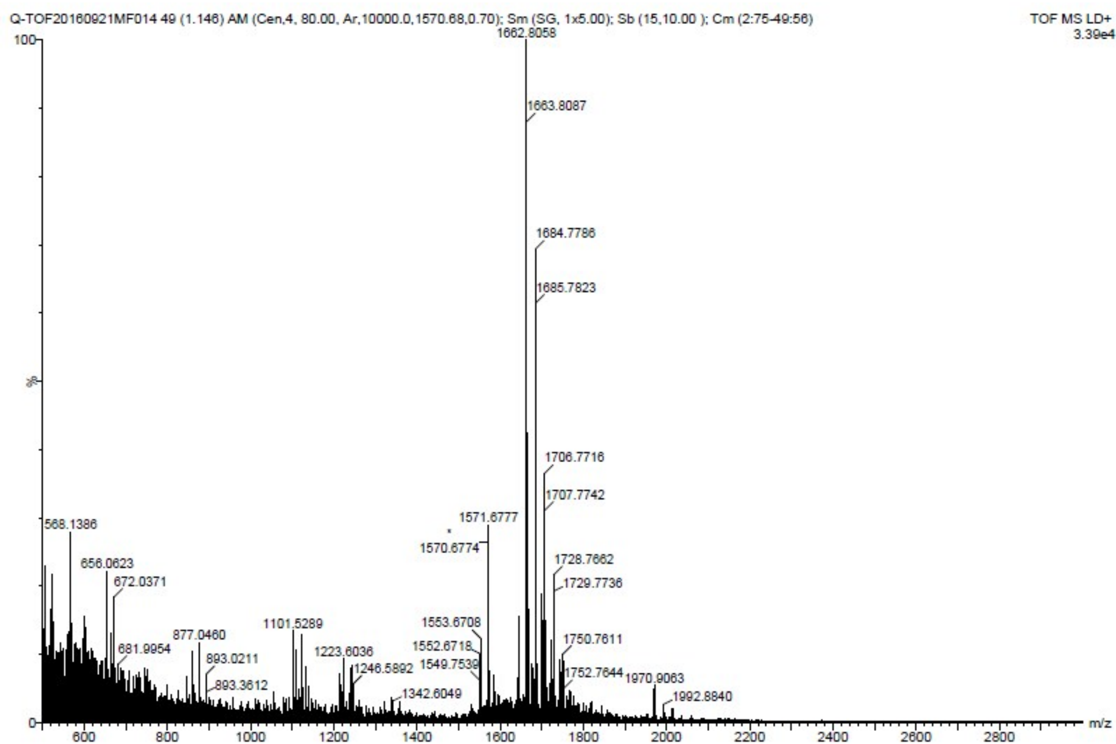

Figure S11. MALDI-TOF MS measurement of **1** with characteristic m/z value: 1662.80; calculated [TPP+succinate(+H)$^+$] 1662.84.

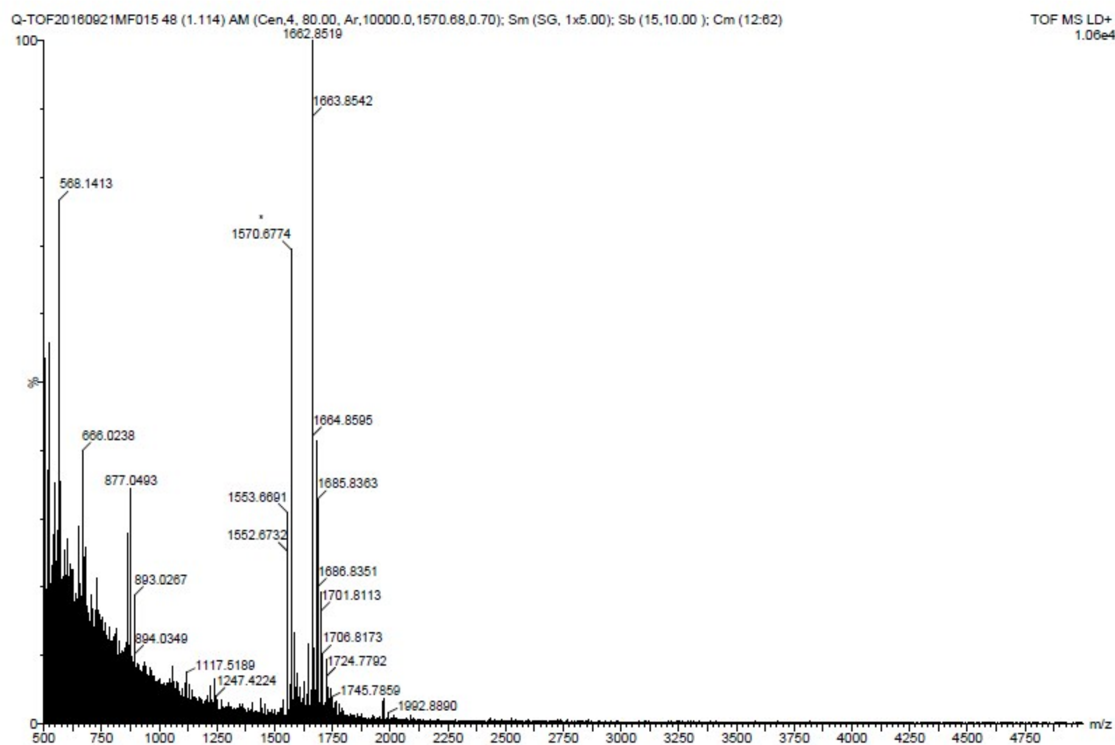

Figure S12. MALDI-TOF MS measurement of **2** with characteristic m/z value: 1662.85; calculated [TPP+succinate(+H)$^+$] 1662.84.

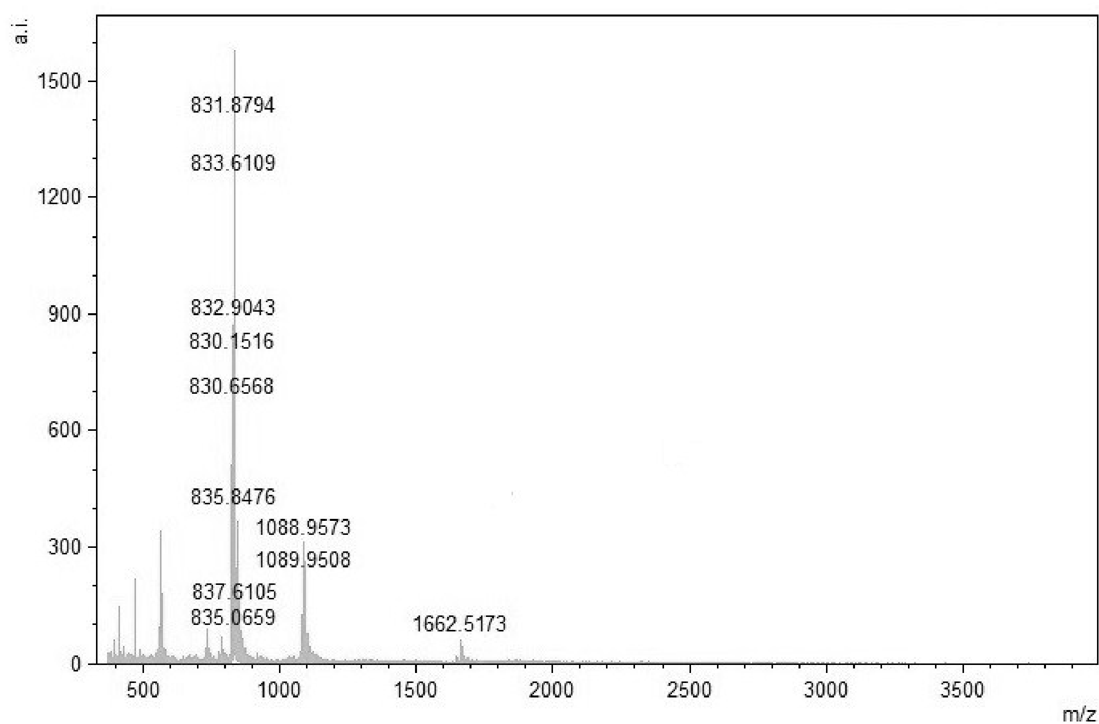

Figure S13. ESI-MS spectrum of **3** with characteristic m/z values: 1662.5173 [ScP+succinate(+H)$^+$], 1088.9573 $(M+2H^+)^{2+}$, [ScP+succinate(+2H)$^{2+}$] 831.8794.

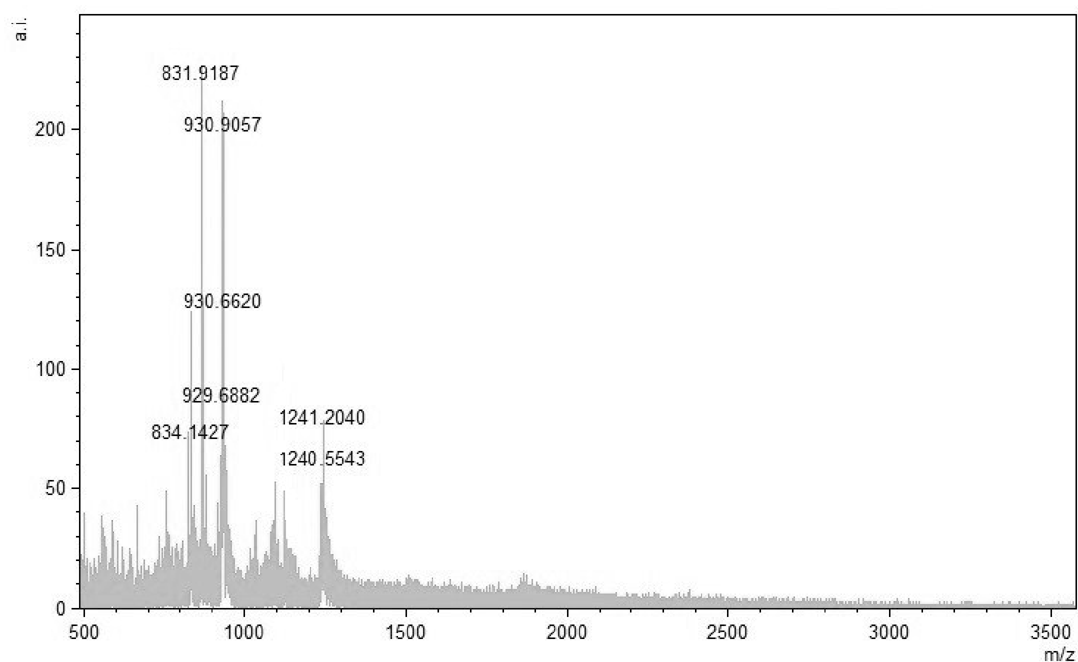

Figure S14. ESI-MS spectrum of **4** with characteristic m/z values: 1240.5543 $(M+2H^+)^{2+,}$ 930.9057$(M+3H^+)^{3+}$, [ScP+succinate(+2H)$^{2+}$] 831.9187.

References:

1. S. Abramkin, S. M. Valiahdi, M. A. Jakupec, M. Galanski, N. Metzler-Nolte and B. K. Keppler, *Dalton Trans.*, 2012, **41**, 3001-3005.