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Tripeptide-induced modulation of mesenchymal stem cell biomechanics stimulates proliferation and wound healing

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Abstract: We demonstrate the ability of two tripeptides to promote proliferation and modulate mechanical properties of human mesenchymal stem cells (hMSCs). Notably, Young's modulus of peptide-treated hMSCs was found to be ~ 2 fold higher compared to the control group. These peptides ameliorated wound healing in hMSCs, without osteogenic and adipogenic differentiation, thus showing potential in vascular tissue engineering applications.

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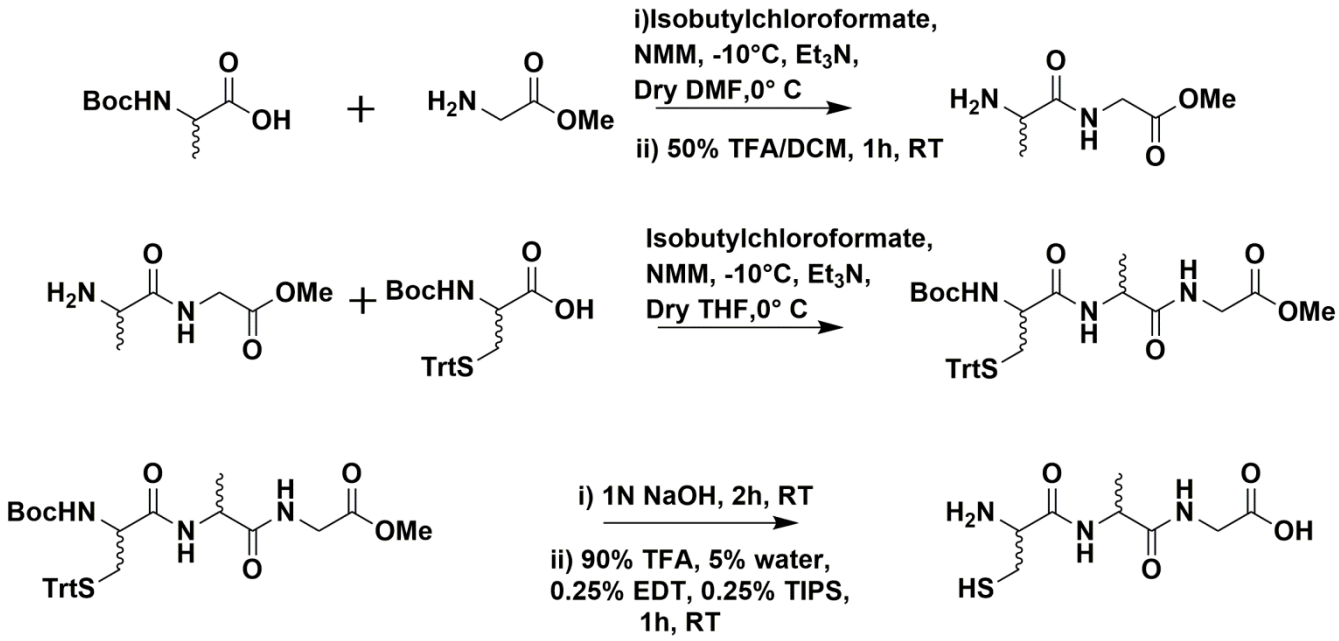
Experimental Procedures

General

Dichloromethane (DCM), N, N'-dimethylformamide (DMF), acetonitrile (CH3CN), triethylamine (TEA), tetrahydrofuran (THF) and methanol (MeOH) were distilled following standard procedures prior to use. Trifluoroacetic acid, hydrochloric acid, isobutylchloroformate, N-methylmorpholine, t-butyloxycarbonyl carbonate, sodium hydroxide, diethylether, L-amino acids were obtained from Spectrochem and used without further purification. D-amino acids were purchased from TCI Chemicals Pvt. Ltd. 1H and 13C NMR spectra were recorded on JEOL-JNM ECS 400 model operating at 400 and 100 MHz, respectively. HRMS spectra were recorded at IIT Kanpur, India, on Waters, Q-Tof Premier Micromass HAB 213 mass spectrometer using capillary voltage 2.6-3.2 Kv.



Scheme S1. Synthetic scheme of **1** (D-CAG) and **2** (L-CAG)

High-performance liquid chromatography (HPLC)

HPLC analyses were performed with a HPLC system (Agilent technologies 1260 infinity) equipped with a quaternary pump (G1311B), auto liquid sampler (G1329B), Diode array detector (G1315D) and analytical scale fraction collector (G1364C). Instrumental control, data acquisition, and processing were performed using ChemStation software (Agilent Technologies, Wokingham, UK). A ZORBAX Eclipse plus C18 (250 x 4.6mm) column with 5 µm particle size at room temperature was used. Acetonitrile/water (10:90) were used as mobile phase and the flow rate was 1 ml/min. Injection volume was 20 µL and effluent was measured at 220 nm.

Cell culture reagents

Human umbilical cord derived mesenchymal stem cells (hMSCs) were purchased from Reliance Life-Sciences (Batch No. 271). The Dulbecco's Modified Eagle's Medium (DMEM), MSC qualified FBS and other cell culture reagents and BrDU kit were purchased from Sigma, USA. AFM cantilevers were purchased from Asylum Research (Oxford Instruments).

Peptide Synthesis: The peptide molecule was synthesised by solution phase peptide synthesis methodology using isobutylchloroformate mediated coupling.[1][2]

Synthesis of N-tert-Butyloxycarbonyl-L-alanyl-glycine methyl ester (A): To a clear solution of N-tertbutyloxycarbonyl-L-alanine (3 gm, 15.9 mmol) in a mixture of 1:1 dry DMF:THF (200 ml) was added N-methylmorpholine (1.7 ml, 15.9 mmol) and the temperature of the mixture was reduced to -15^{O}C under nitrogen atmosphere. Then isobutylchloroformate (2.1 ml, 15.9 mmol) was added to the mixture. To the suspension, a mixture of glycine methyl ester (1.99 gm, 15.9 mmol) with triethylamine (2.2 ml, 15.9 mmol) after 10

min. was added. The reaction mixture was stirred for 3 hrs. at room temperature. Then solvent was evaporated under reduced pressure. The residue was dissolved in DCM and water mixture. The aqueous layer was extracted three times with DCM and the collected organic layer was then washed brine and dried over anhydrous sodium sulphate. The crude compound was purified with column chromatography using 25% ethylacetate/ hexane solvent system to obtain final product. Yield 3.312 gm (80%) R_f : 0.5 (4% MeOH/DCM); ESI-HRMS: $[M+H]^+$, calculated = 261.1445, found = 261.1459; ^{1}H NMR (400 MHz, METHANOL-D3, 25°C, TMS) δ(ppm): 4.84 (d, *J* = 15.0 Hz, 3H), 4.08 (d, *J* = 7.1 Hz, 1H), 3.92 (q, *J* = 17.6 Hz, 2H), 3.69 (s, 3H), 3.28 (dt, *J* = 3.1, 1.7 Hz, 1H), 1.42 (s, 9H), 1.30 (d, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, METHANOL-D3, 25°C, TMS) δ(ppm): 175.17 (s), 170.31 (s), 156.33 (s), 79.30 (s), 51.25 (s), 50.18 (s), 40.46 (s), 27.36 (s), 17.06 (s).

Synthesis of L-Alanyl-glycine methyl ester (B): To a solution of A (3 gm) in 30 ml DCM, 20 ml of trifluoroacetic acid was added in nitrogen atmosphere. The reaction mixture was stirred at room temperature for 2 hrs. The solvent was evaporated in vacuo and the residue was washed with diethyl ether to obtain the final product. The crude compound was purified with column chromatography using 15% MeOH/DCM solvent system to obtain final product. Yield 1.6 gm(85%) R_f : 0.6 (20% MeOH/DCM); ESI-HRMS: $[M+H]^+$, calculated = 161.0921, found = 161.092; ^{1}H NMR (400 MHz, METHANOL-D3, 25°C, TMS) δ(ppm): 4.84 (s, 17H), 4.06 – 3.90 (m, 3H), 3.71 (s, 3H), 3.28 (dt, *J* = 3.1, 1.7 Hz, 4H), 1.51 (d, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, METHANOL-D3, 25°C, TMS) δ(ppm): 170.13 (s), 136.85 (s), 51.40 (s), 40.46 (s), 16.24 (s).

Synthesis of S-trityl-L-cysteine (C): A mixture of L-cysteine (1 gm, 8.2mmol) and triphenylmethanol (2.4 gm, 9 mmol) was dissolved in trifluoroacetic acid (12 ml) and stir at room temperature for 30 min. The solvent was evaporated under reduced pressure. To the residue 30% aqueous ammonium hydroxide was added until complete neutralization. White precipitate obtained was filtered through buchner funnel and washed with cold water and then ethanol. The final product was dried in vaccum. Yield 2.84 gm (95%); ESI-HRMS: $[M+H]^+$, calculated = 363.1293, found = 363.1291, 243.12 [$^+CPh_3$], formation of stable cation due to fragmentation.

Synthesis of S-trityl-N-tert-butyloxycarbonyl-L-cysteine (D): A solution of C (2 gm, 5.5 mmol) in a mixture of MeOH (25 ml) and triethylamine (1.5 ml, 11 mmol) was stirred and cooled in ice bath. Di-tert-butylcarbonic anhydride (1.89 ml, 8.25 mmol) was added to it and stirring was continued for 6 hrs. at room temperature. The mixture was concentrated to vaccum and mixture of DCM and water was added. The aqueous layer was washed with DCM three times. Then the organic layer was extracted with $NaHCO_3$ and dried over anhydrous sodium sulphate. The crude compound was purified with column chromatography using 20% ethyl acetate/hexane solvent system. Yield 2.08 gm (81.5%); ESI-HRMS: $[M+Na]^+$, calculated = 486.1715 , found = 486.1719, 243.12 [$^+CPh_3$], formation of stable cation due to fragmentation. ^{1}H NMR (400 MHz, METHANOL-D3, 25°C, TMS) δ(ppm): 7.44 – 7.12 (m, 15H), 4.85 (s, 17H), 4.05 – 3.91 (m, 1H), 3.32 – 3.25 (m, 2H), 2.62 – 2.46 (m, 2H), 1.41 (s, 9H). ^{13}C NMR (101 MHz, METHANOL-D3, 25°C, TMS) δ(ppm): 172.87 (s), 144.66 (s), 129.39 (s), 127.66 (s), 126.57 (s), 79.39 (s), 66.57 (s), 52.97 (s), 33.56 (s), 27.38 (s).

Synthesis of S-trityl-N-tert-butyloxycarbonyl-L-cysteinyl-L-alanyl-glycine methyl ester (E): To a clear solution of D (2 gm, 4.3 mmol) in a mixture of 1:1 dry DMF:THF (100 ml) was added N-methylmorpholine (0.5 ml, 4.3 mmol) and the temperature of the mixture was reduced to -15 ^{o}C under nitrogen atmosphere. Then isobutylchloroformate (0.6 ml, 4.3 mmol) to the mixture was added. To the suspension, a mixture of B (0.7 gm, 4.3 mmol) with triethylamine (0.6 ml, 4.3 mmol) after 10 min. was added and stirred for 3 hrs. Then solvent was evaporated under reduced pressure. The residue was dissolved in DCM and water mixture. The aqueous layer was extracted three times with DCM and the collected organic layer was then washed brine and dried over anhydrous sodium sulphate. The crude was purified by column chromatography using 30% ethyl acetate/hexane solvent system. Yield 2.33 gm (89.5%); R_f = 0.6 (6% ethyl acetate/hexane); ESI-HRMS: $[M+Na]^+$, calculated = 628.2457, found = 628.2455, 243.12 [$^+CPh_3$], formation of stable cation due to fragmentation; ^{1}H NMR (400 MHz, METHANOL-D3, 25°C, TMS) δ(ppm): 7.40 – 7.17 (m, 15H), 4.85 (s, 10H), 4.40 – 4.29 (m, 1H), 3.91 (t, *J* = 6.4 Hz, 1H), 3.83 – 3.72 (m, 2H), 3.65 (s, 3H), 2.59 – 2.43 (m, 2H), 1.40 (s, 9H), 1.31 (d, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, METHANOL-D3, 25°C, TMS) δ(ppm): 171.29 (s), 170.11 (s), 156.16 (s), 144.56 (s), 129.37 (s), 127.72 (s), 126.67 (s), 66.67 (s), 53.72 (s), 51.29 (s), 48.83 (s), 40.29 (s), 33.83 (s), 27.32 (s), 16.65 (s).

Synthesis of S-trityl-N-tert-butyloxycarbonyl-L-cysteinyl-L-alanyl-glycine (F): E (2 gm, 33 mmol) was dissolved in MeOH (40 ml) and 1N NaOH (4 ml, 36.3 mmol) was added. The reaction mixture was stirred for 2 hrs. at room temperature. Then solvent was evaporated in vacuo. The crude was purified by column chromatography using 40% ethyl acetate/hexane solvent system. Yield 1.85 gm (95%); Rf = 0.4 (4% MeOH/DCM); ESI-HRMS: $[M+Na]^+$, calculated = 614.2301, found = 614.2306, 243.12 [$^+CPh_3$], formation of stable cation due to fragmentation; 1H NMR (400 MHz, METHANOL-D3, 25°C, TMS) δ(ppm): 7.44 – 7.12 (m, 15H), 4.85 (s, 17H), 4.05 – 3.91 (m, 1H), 3.32 – 3.25 (m, 2H), 2.62 – 2.46 (m, 2H), 1.41 (s, 9H). 13C NMR (101 MHz, METHANOL-D3, 25°C, TMS) δ(ppm): 172.87 (s), 144.66 (s), 129.39 (s), 127.66 (s), 126.57 (s), 79.39 (s), 66.57 (s), 52.97 (s), 33.56 (s), 27.38 (s). ^{1}H NMR (396 MHz, METHANOL-D3, 25°C, TMS) δ(ppm): 7.39 – 7.18 (m, 15H), 4.39 (ddd, *J* = 17.5, 10.2, 4.2 Hz, 1H), 3.76 (ddd, *J* = 38.9, 18.3, 11.8 Hz, 3H), 2.63 – 2.38 (m, 2H), 1.50 – 1.22 (m, 12H). ^{13}C NMR (100 MHz, METHANOL-D3, 25°C, TMS) δ(ppm): 171.55 (s), 144.59 (s), 129.37 (s), 127.69 (s), 126.61 (s), 48.88 (s), 27.32 (s).

Synthesis of D-cysteinyl-D-alanyl-glycine (1): The procedure followed is same as the synthesis method of G starting with D amino acids. ^{1}H NMR (400 MHz, D_2O, 25°C, TMS) δ(ppm): 4.66 (s, 13H), 4.31 (q, *J* = 7.2 Hz, 1H), 4.10 (t, *J* = 5.9 Hz, 1H), 3.85 (d, *J* = 1.1 Hz,

2H), 3.03 – 2.89 (m, 2H), 1.32 (d, *J* = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, D_2O, 25°C, TMS) δ(ppm): 174.94 (s), 173.70 (s), 167.85 (s), 54.48 (s), 49.85 (s), 41.48 (s), 24.78 (s), 16.71 (s).

Synthesis of L-cysteinyl-L-alanyl-glycine (2): The final deprotection F (1 gm, 16.8 mmol) was achieved by using a 20ml of scavenging mixture of TFA/water/ethanediithiol (EDT)/triisopropylsilane(TIPS) in 10/0.5/0.5/0.25 ratio for 2 hrs. The mixture was evaporated in vacuo and the residue was washed with diethyl ether to obtain the final product. The peptide was purified by preparative RP-HPLC (Agilent Technologies) at 12 ml/min. on a Agilent 10 Prep-C18 column (250x30 mm) using a gradient of A [water + 0.1% TFA] and B [acetonitrile + 0.1% TFA]: 0% B to 40% B for 30 min., 40% to 95% for 5 min. and 10% B for 5 min. detection at 220 nm; t_r = 5 min. The acetonitrile was evaporated under reduced pressure and the aqueous solution was freeze-dried to give a white solid. Yield 344 mg (82%) ESI-HRMS: $[M+H]^+$, calculated = 250.0862, found = 250.0868; ^{1}H NMR (400 MHz, D2O, 25°C, TMS) δ(ppm): 4.66 (s, 10H), 4.31 (q, *J* = 7.2 Hz, 1H), 4.13 (t, *J* = 5.5 Hz, 1H), 3.90 – 3.73 (m, 2H), 2.99 (qd, *J* = 15.0, 5.5 Hz, 2H), 2.10 (s, 0.2H), 1.32 (d, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-D6, 25°C, TMS) δ(ppm): 172.77 (s), 171.54 (s), 166.92 (s), 54.26 (s), 48.92 (s), 41.12 (s), 25.74 (s), 18.52 (s).

Synthesis of L-Alaninyl-L-cysteinyl-glycine (SP): 2-Chlorotrityl chloride resin (152 mg, 0.2 mmol, having substitution 1.32 mmol/gm) was swollen for 30 minute in dry DCM (3 mL). Resin was then washed with dry DCM (3x3 mL). Solution of Fmoc–Gly-OH (178.4 mg, 0.6 mmol) and DIPEA (209 µL, 1.2 mmol) in 4 mL of dry DCM was added to the resin and reaction mixture was stirred for 3 h by purging nitrogen gas. To endcap the reactive group on resin HPLC grade methanol (1 mL) was added to the solution and stirred for 15 minutes. After that, the resin was washed with DMF (3x3 mL) and DCM (3x3 mL). The resin was then treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen and filtered. The resin was then washed with DMF (3x3 mL). To this resin a solution of Fmoc-Cys(Trt)-OH (351.4 mg, 0.6 mmol) and HOBt (81 mg, 0.6 mmol) in 4 mL of DMF was added followed by addition of DIC (94 µL, 0.6 mmol) and the reaction mixture was stirred by purging nitrogen gas for 3 h under nitrogen. Coupling was monitored by Kaiser's test. If the test is found positive, stirring was continued till Kaiser's test is negative. After complete reaction, the resin was washed with DMF (3x3 mL). The resin was then treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen. The resin was washed with DMF (3x3 mL). After proper washing of resin a solution of Fmoc-Ala-OH (186.8 mg, 0.6 mmol) and HOBt (81 mg, 0.6 mmol) in 4 mL of DMF was added followed by addition of DIC (94 µL, 0.6 mmol) and the reaction mixture was stirred by purging nitrogen gas for additional 4 h under nitrogen. Sample of resin was tested for monitoring the coupling by Kaiser's test. If the test is found positive, stirring was continued till Kaiser's test is negative. The resin after proper washing was treated with 20% piperidine/DMF (3 mL) and stirred for 5 minute and filtered. The resin was again treated with 20% piperidine/DMF (5 mL) and stirred for 25 minutes under nitrogen and filtered. After completion of reaction, the resin was then washed with DMF (3x3 mL) and DCM (3x3 mL). The resin obtained was then dried under vacuum. The resin after proper drying was then treated with 10 mL mixture of TFA/TIPS/EDT/Water/DCM (30/2.5/2.5/2.5/62.5%) and the reaction mixture was stirred for 1 h. The resin was removed by filtration and the filtrate was reduced to half by evaporation under reduced pressure below 45 °C. Peptide was precipitated with diethyl ether. Precipitate obtained was filtered and further washed three times with diethyl ether. The crude peptide was further purified by reversed phase HPLC (RP-HPLC) using solvent system 0.1 % TFA in water/acetonitrile with C-18 column and then the sample was lyophilized to give the desired tripeptide ACG as a white powder. Yield (37.4 mg, 0.15 mmol, 75%); white colour solid; ESI-MS: $[M+H]^+$, calculated = 250.0862, found = 250.2; ^{1}H NMR (400 MHz, D2O, 25°C, TMS) δ(ppm): 4.51 – 4.43 (m, 1H), 4.04 (q, *J* = 7.1 Hz, 1H), 3.98 – 3.84 (m, 2H), 2.83 (qd, *J* = 14.2, 6.3 Hz, 2H), 1.43 (d, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, D_2O) δ 172.96 (s), 171.95 (s), 170.88 (s), 55.68 (s), 48.99 (s), 41.15 (s), 25.23 (s), 16.51 (s).

Cell Culture: hMSC has been purchased from Reliance Life-Sciences (Batch No. 271) and characterized for positive MSC markers CD90 (99.5%), CD105 (98.8%), CD73 (99.1%), and negative MSC markers CD34 (0%), CD14 (0%), CD19 (0%) and HLADR (0.1%). They have been cultured in DMEM with 10% FBS, glutamax (2mg/ml) and 1% penicillin-streptomycin.

Cell viability assay:

The effect of small tripeptide **1**, **2** and **SP** on hMSCs viability was determined by MTT assay. Briefly, 2×10^3 cells/well were seeded in complete growth medium in 96-well plate and kept in a humidified tissue culture incubator at 37°C with 5% CO_2. After 24 h, cells were exposed to different concentrations of peptides ranged from 10nm to 1µm in reduced serum (0.5% FBS) medium for 24 h. The cells were then incubated with 10µl/well MTT (5mg/ml) for 4h and insoluble formazan crystals (formed from reduction of MTT in viable cells) were dissolved by adding DMSO, and absorbance at 540 nm wavelength was recorded.

Cell proliferation assay:

hMSCs were seeded as described above and treated with small tripeptide **1**, **2** and **SP** for 24 hrs. in complete growth medium. Then cells were treated with bromodeoxyuridine (BrdU) for the last 4 h before termination. BrdU was assessed by ELISA using kit from Roche Diagnostics by following the manufacturer's instructions. Absorbance of the samples was read in an ELISA reader at 450 nm with reference wavelength at 690 nm.

MSC differentiation to osteoblasts and adipocytes

1×10^4 cells/well were seeded in six-well plates and cultured in complete medium. At 60-70% confluence, medium was changed into osteoblast- differentiation medium (10nM dexamethasone, 10mM β-glycerophosphate and 50ug/ml ascorbic acid) and adipo-differentiation medium (0.5mM IBMX, 100µM indometacin, 10µg/ml. insulin and 1µM dexamethasone. At 60-70% confluence cells were treated with 10nM of 1 & 2 and after 21 days, osteoblast differentiation was identified by calcium deposition (mineralised nodules) by staining with 1% alizarin red S for 10 min. Quantification of the mineralized nodules was done by extraction of the stain. Adipocyte differentiation was assessed by the presence of fat globules visualized by staining with 0.18% oil red O solution for 5 min. Each assay was performed in triplicate.

Scratch wound healing assay:

1×10^4 hMSCs/ well were seeded on a 12-well culture plate for scratch assay and cultured at 37°C and 5% CO_2 for 24 hrs. Next day, the monolayer cultures were scratched with a sterile 200µl tip and treated with control (Vehicle receiving DMSO), **1**, **2** and **SP** at 10nm of concentration. The cell culture media were replaced immediately with fresh culture medium. After 24 hrs., wound closure was measured with the EVOS FL Auto time-lapse imaging system (Thermo Fisher Scientific, Waltham, MA, USA).

Western Blotting:

hMSC were treated with Control (Vehicle receiving DMSO) , 1, 2, and SP, at 10nM concentration for 72h to determine vimentin and α-tubulin cytoskeleton protein expression. Total protein from the cells was isolated as per described by China et al (Bone 105:75-86). 30 µg of total protein was resolved by 12% SDS PAGE, transferred to PVDF membranes (0.22 µm pore size). For immunoblotting α-tubulin (#A11126; 1:5000) from Thermo Fisher Scientific; vimentin (#5741S; 1:1000) from Cell Signalling Technologies and β-actin (#A3854, 1:50,000) from Sigma Aldrich were purchased. Secondary anti-rabbit antibody (#A0545, 1:3000) and secondary anti-mouse antibody (#515-035-062, 1:5000) were purchased from Sigma Aldrich and Jackson ImmunoResearch Laborotories, Inc. respectively.

Atomic Force Microscopy Experiments

Cell preparation

2^x10^3 hMSCs were seeded in 16 mm coverslip at 37°C for 24 hrs. Next, cells were treated with Control (Vehicle receiving DMSO), **1** and **2** peptide at 10nM concentration. Next day cells were slowly washed with PBS. Procedure has been repeated for three times to remove all the dead cells and floating debris. The cover slip has been fixed on the liquid cell stage for AFM measurements.

Atomic Force Microscopy

Force mapping was performed with an Asylum MFP-3D Origin and Molecular Force Probe 3D controller (Asylum Research). The driver program MFP-3D Xop was written in IGOR Pro software (version 6.37, USA). Asylum TR400PB silicon nitride probes of resonant frequency 32 kHz and spring constant 0.09 N/m was used. The probes have V shape tip of 42 nm radius. The force maps of samples were recorded in contact mode in liquid medium with average loading force less than 1nN at room temperature. Samples were prepared at 60% - 70% confluency of cells. The cells preserved their viability for 3h consistent with literature data.

The AFM experiments were conducted after the hMSC has been checked for their confluency by an inverted microscope. AFM's greatest strength in cell biology is its ability to provide accurate and quantitative mechanical measurements in near- physiological conditions, i.e. cell culture medium at 37^0C. This microscopy technique is based on the detection of attractive and repulsive forces acting between a sharp probe and the cell surface. AFM was employed to investigate the elastic modulus of hMSC and CAG treated hMSC by recording a force curve as it has advantages to characterize mechanical properties of living cells. It has been reported that under a change of the external conditions, the change in the elasticity of cell membranes is much stronger. We, therefore, maintained the natural environment of cells in fresh exchange medium and did not use the substrate treatment and cell fixation.

After placing the coverslip with hMSCs in the AFM liquid cell, the liquid cell was allowed to stabilize for 30 minutes to attain thermal equilibrium, before force curve measurements. Special care was taken to assure the stability and repeatability of the measurements by diminishing the thermal shift of the consecutive measurements to almost zero. At the time of analysis, cantilever probe and cells

were in PBS. The AFM scan of 40x40 μm^2 generally showed 2-3 cells at the cell confluency of 60-70%. The observed maximum cell height was about 3 µm which was also seen by optical profilometry (Fig.5). Using the in-built module of the AFM, force between the AFM probe and a cell was recorded by bringing the probe in contact with the cell from a separation distance of (2 µm) and then retracting it again after compression.

This model is mostly used by many researchers as it is compatible for extremely low modulus materials like cell walls [ref]. Force-distance curves reveal changes in the cell elasticity by force map at different points. Force mapping was done at 100 data points over 10µmx10µm area. Out of the 100 data points minimum 60 random points were used to measure elasticity of a single cell allowing error minimization in modulus measurements due to local variations of the cell structure.

Data acquisition and analysis

All the measurements have been taken in liquid contact mode by using a sharp tip mounted on a cantilever of spring constant 0.01N/m. The AFM was operated in constant deflection for imaging mode or in force mode during force mapping. Force curve were typically recorded at a scan rate of 1Hz, corresponding to maximum loading rate of 1nN/s and a maximum force (1nN) to cells during each force curve.

Typically, force maps of 60 x 60 force curves were recorded in a selected area of a cell of 50um x 50um. From each force curve the elastic modulus was calculated and plotted. Data was analysed in a force range of 100-500pN, where it followed the Hertz-Sneddon model.

Optical Profilometry:

The images of the cells were taken by optical profilometer of NanoMap-D (aep Technology) at 20X field view and processed by SPIP imaging software 5.1.11.

Statistical Analysis

Data are expressed as mean ± S.E.M. The significant difference was analysed by one-way ANOVA followed by tukey's Multiple comparison test of significance using GraphPad Prism 5 software.

Results and Discussion

HPLC spectrum

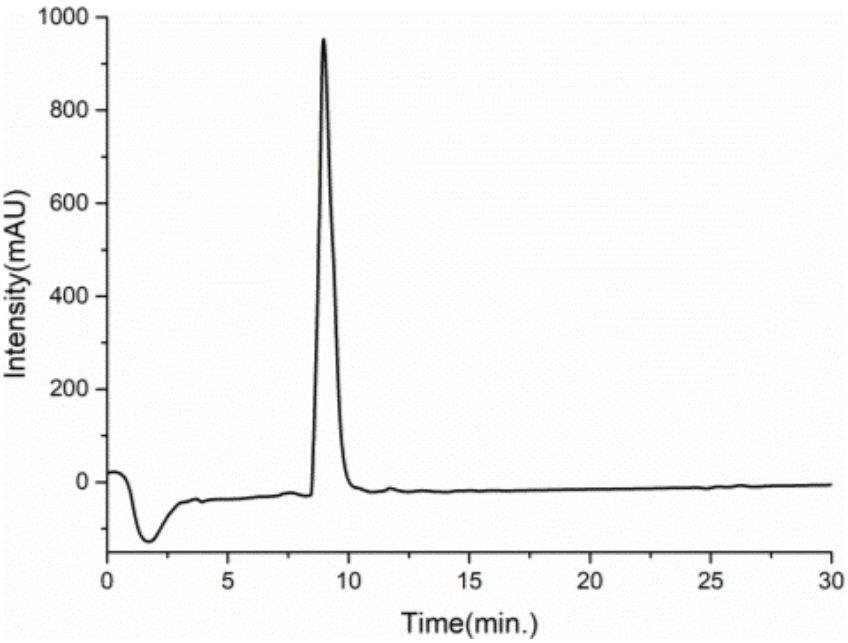


Fig. S1 HPLC spectrum of 1**.**

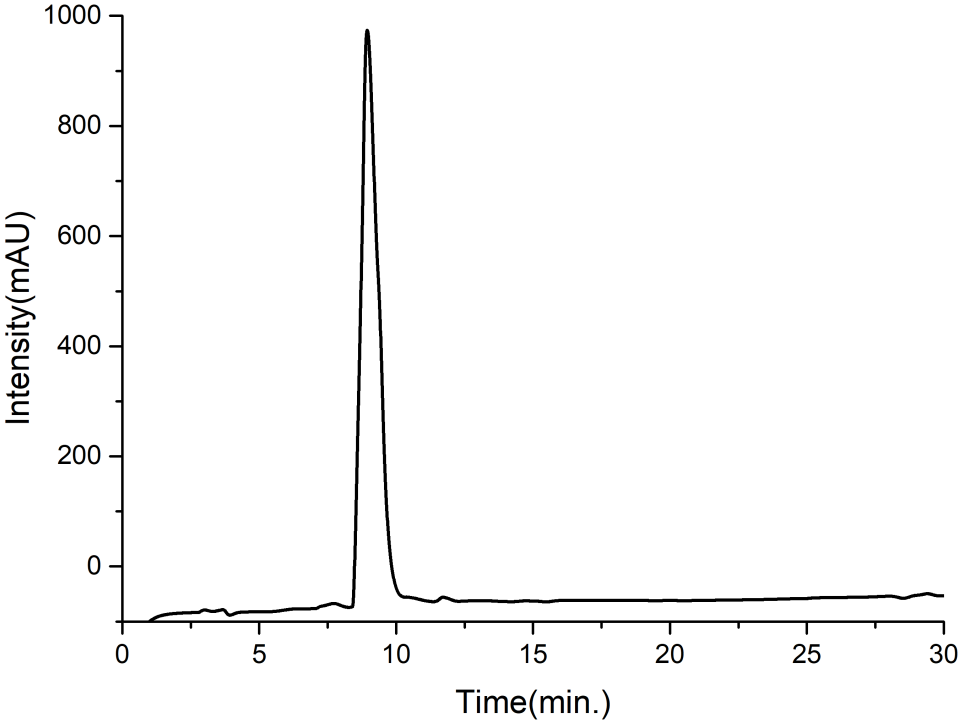


Fig. S2 HPLC spectrum of 2.

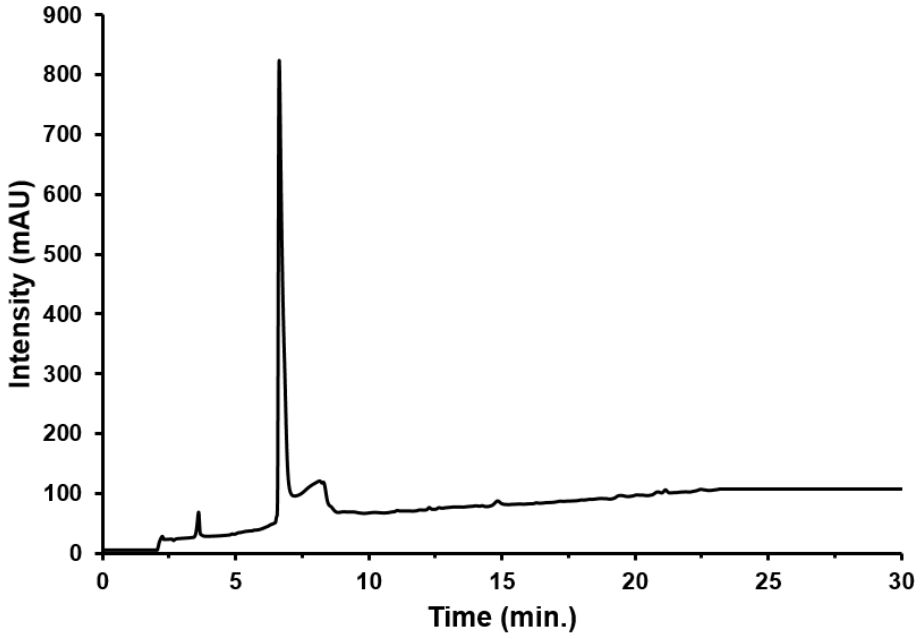


Fig. S3 HPLC spectrum of **SP.**

Cell viability assay

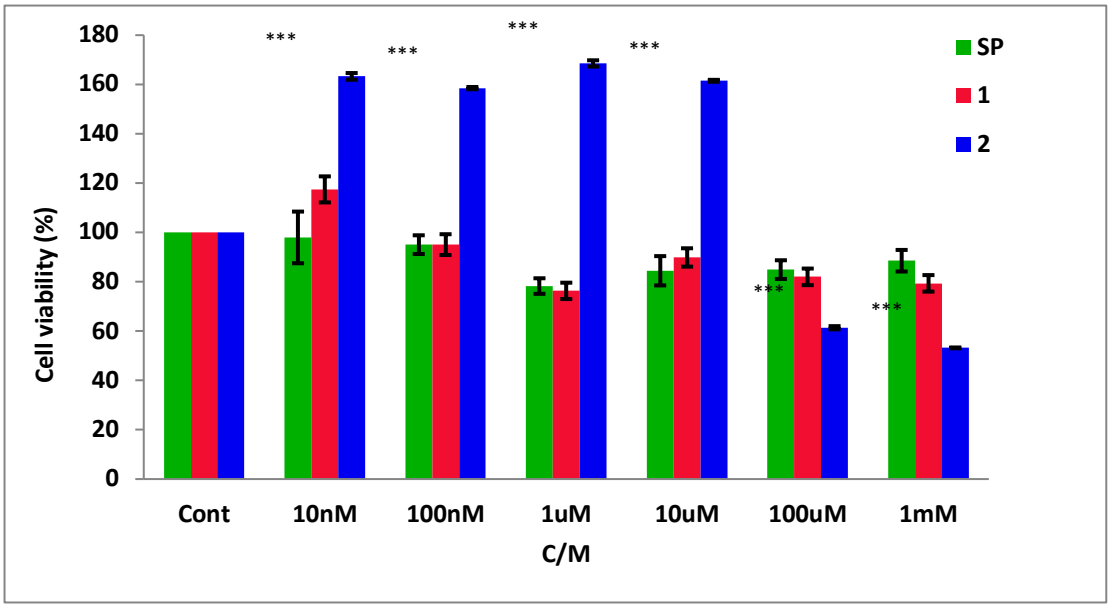


Fig. S4 hMSCs treated with vehicle (DMSO), SP, 1 and 2 for 24h at concentrations from 10nM to 1mM to assess cell viability using MTT assay. Data represented is mean value ± SE of three independent experiments in triplicate; **p < 0.01, ***p < 0.001 versus control (receiving vehicle).

Cell differentiation assay

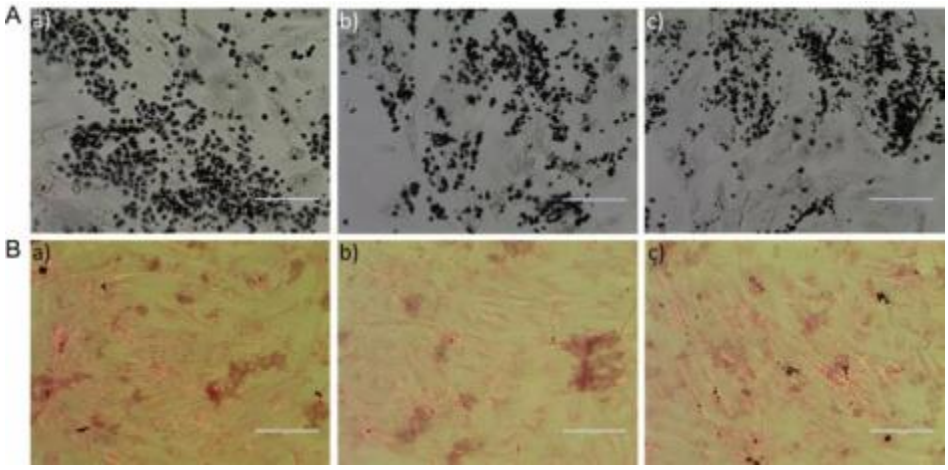


Fig. S5 Effect of 1 and 2 on osteogenic and adipogenic differentiation of hMSCs. A) Alizarin red S-stained cells. a) Control (received vehicle) b) 1 at 10nM and c) 2 at 10nM **B)** Oil red O-stained cells. a) Control (received vehicle) b) 1 at 10nM and c) 2 at 10nM (Scale bar=200µm). 1 and 2 have no effect on osteogenic and adipogenic differentiation.

Quantification of the mineralized nodules and fat globules formation

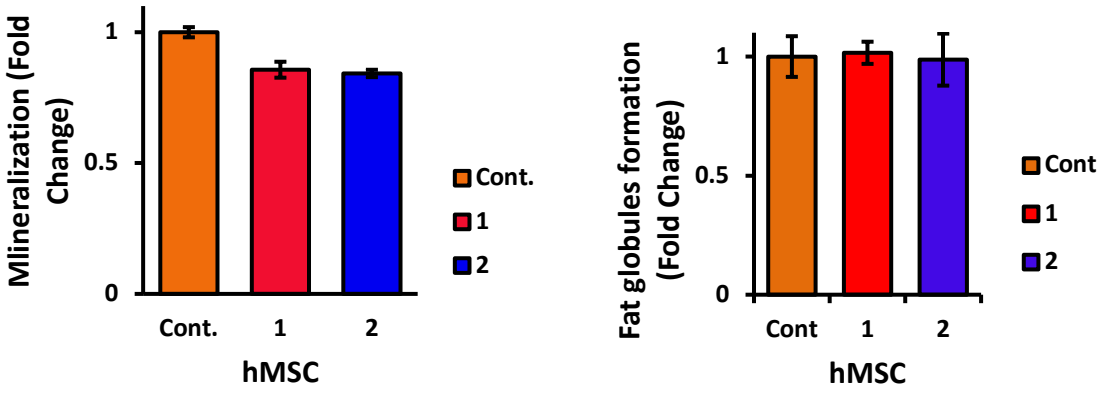


Fig. S6 hMSC was treated with 1 and 2 at 10nM concentration. Nodules and adipocytes formation was assessed by Alizarin red-S and Oil red O staining. Quantification of the mineralized nodules and fat globules formation was done by extraction of the stain.

Optical Profilometry

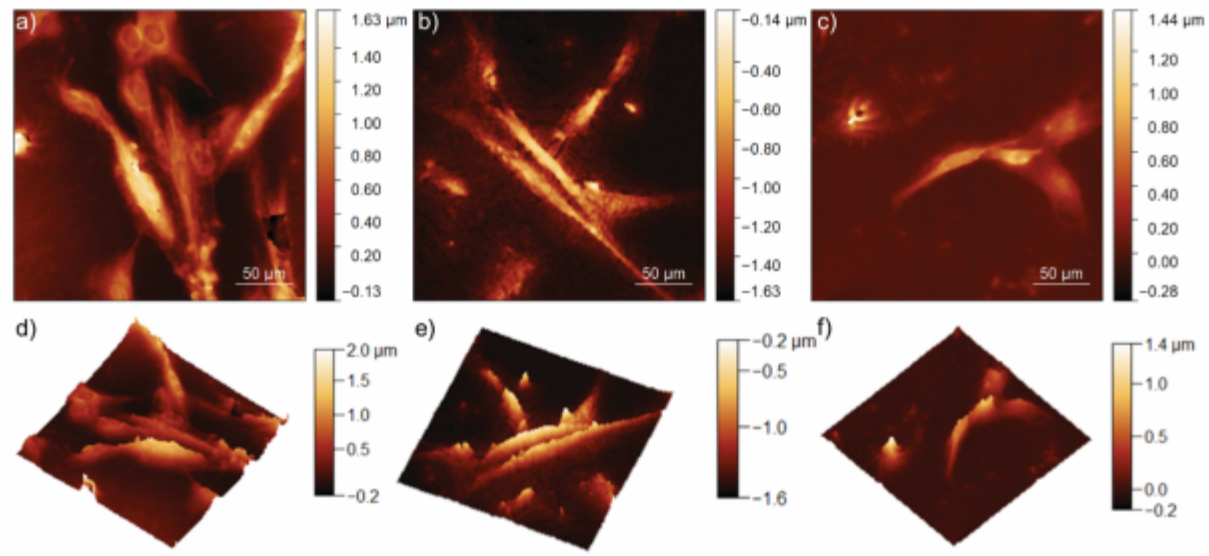


Fig. S7 Optical profiler images of a) control cells b) **1**, c) **2** treated showing X and Y range, d), e), and f) 3D image of control, **1** and **2** at 10nM treated showing z-range of surface respectively. (Scale bar = 50 µm).

Table S1. Young's modulus values (kP) of control and **2** treated hMSC

Control	hMSC+ **1**	hMSC+ **2**
4.99 ± 0.36	8.92 ±1.17	10.35±1.38

^{1}H NMR, ^{13}C and ESI-MS Spectra

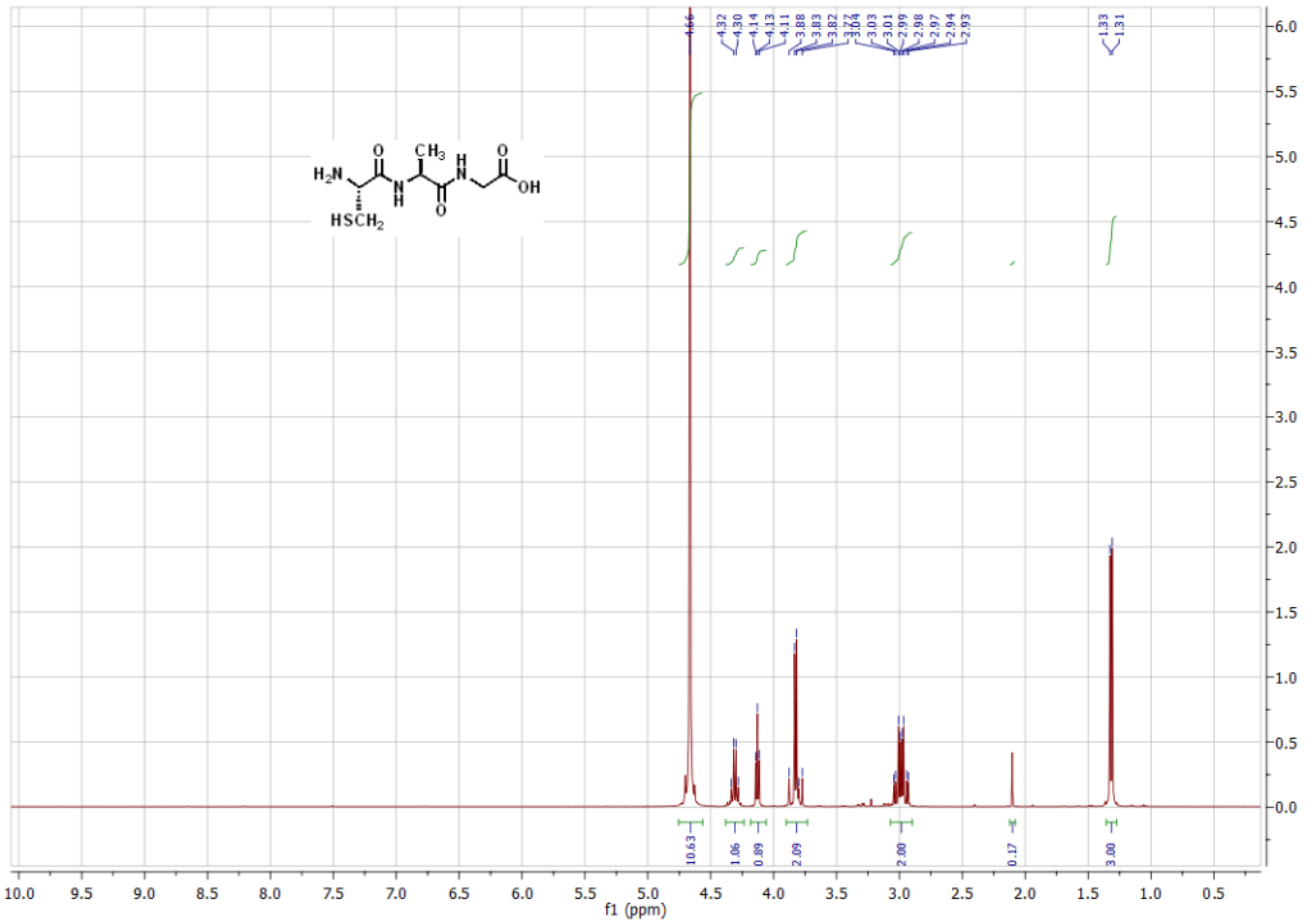


Fig S8. ^{1}H NMR spectrum of **2**.

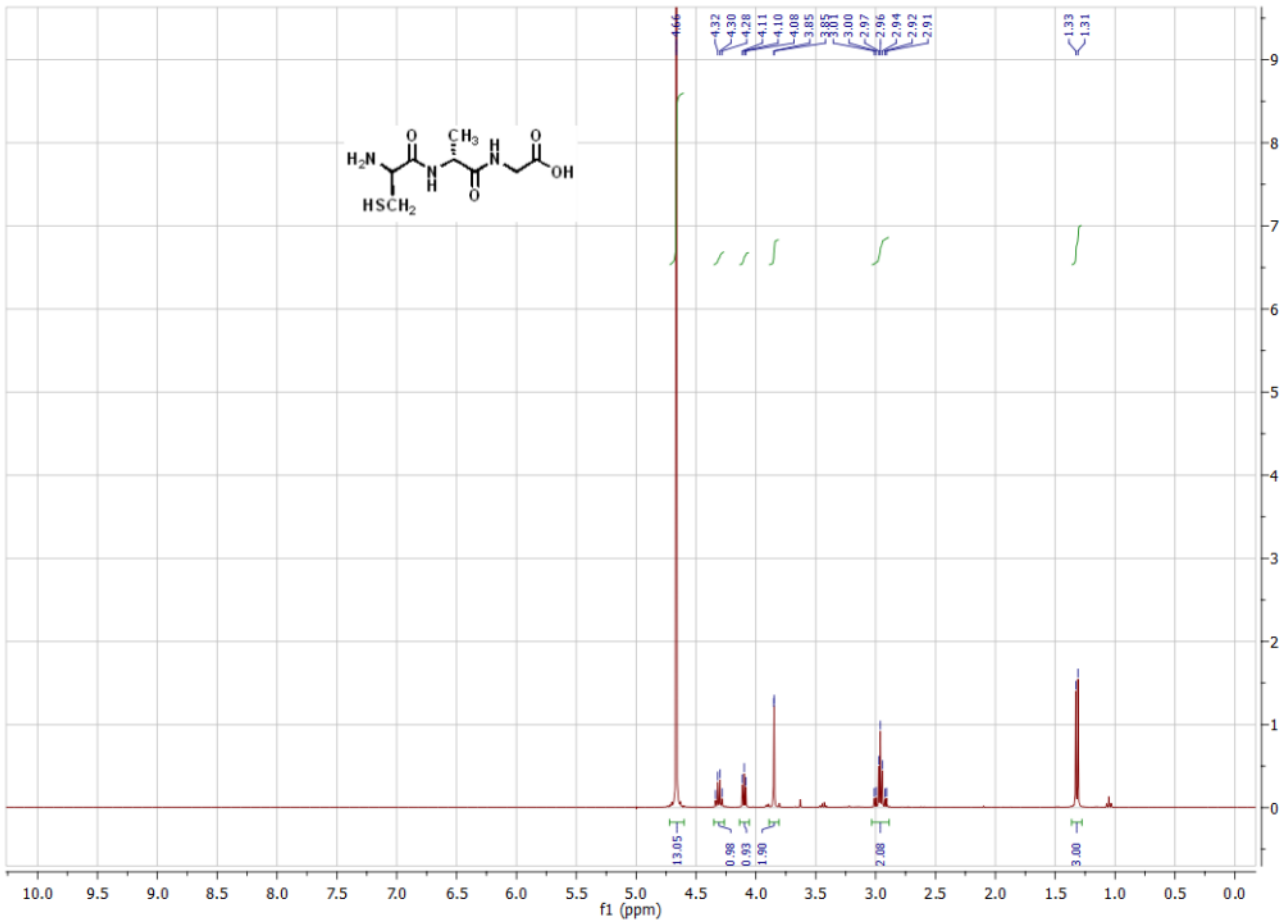


Fig. S9 ^{1}H NMR spectrum of **1**.

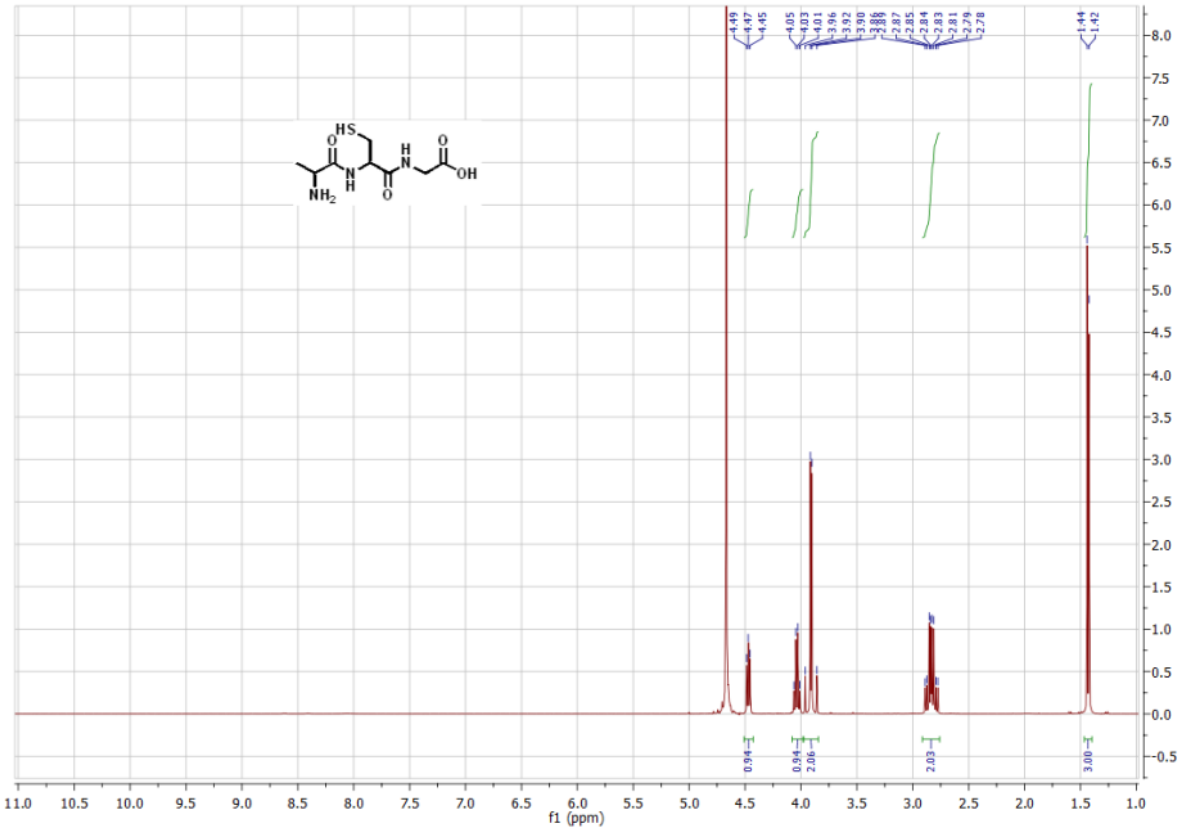


Fig. S10 ^{1}H NMR spectrum of **SP**.

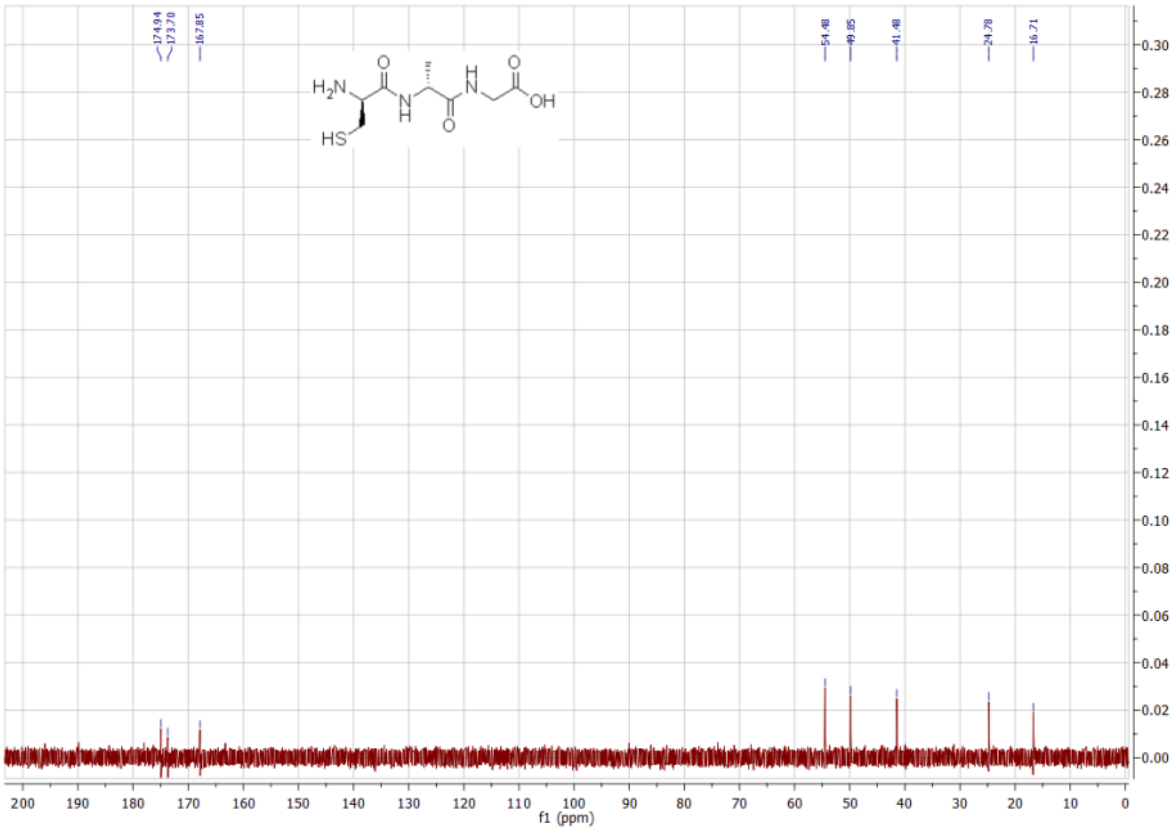


Fig. S11 ^{13}C NMR spectrum of **1**

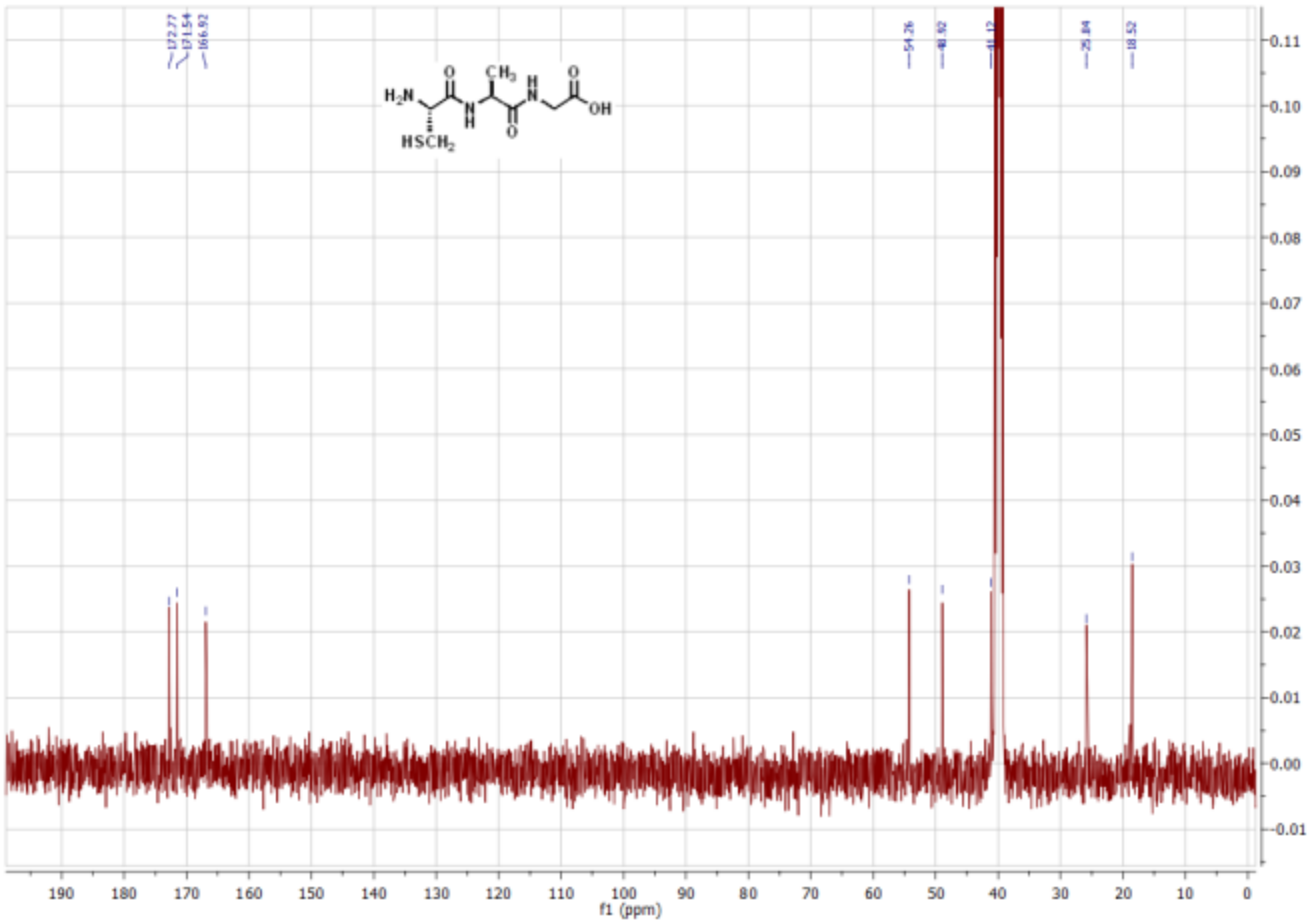


Fig S12 ^{13}C NMR spectrum of **2**.

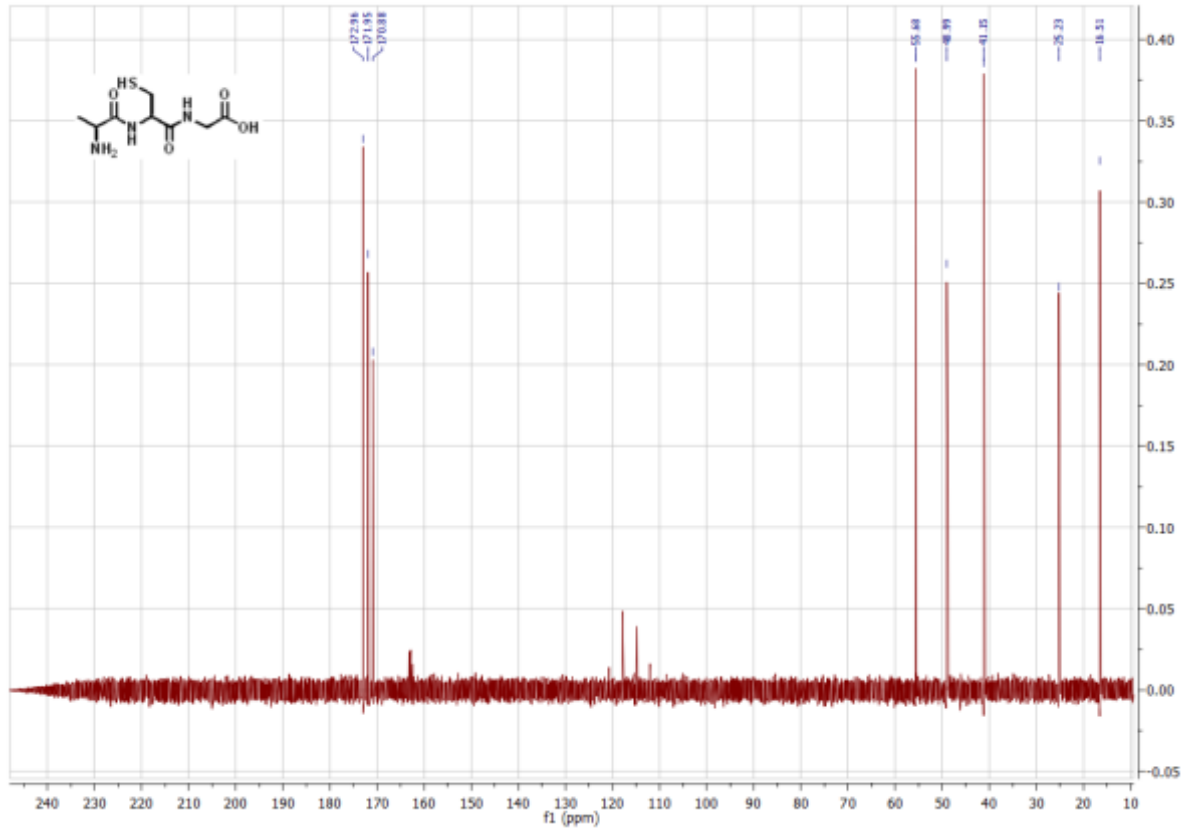


Fig S13 ^{13}C NMR spectrum of **SP.**

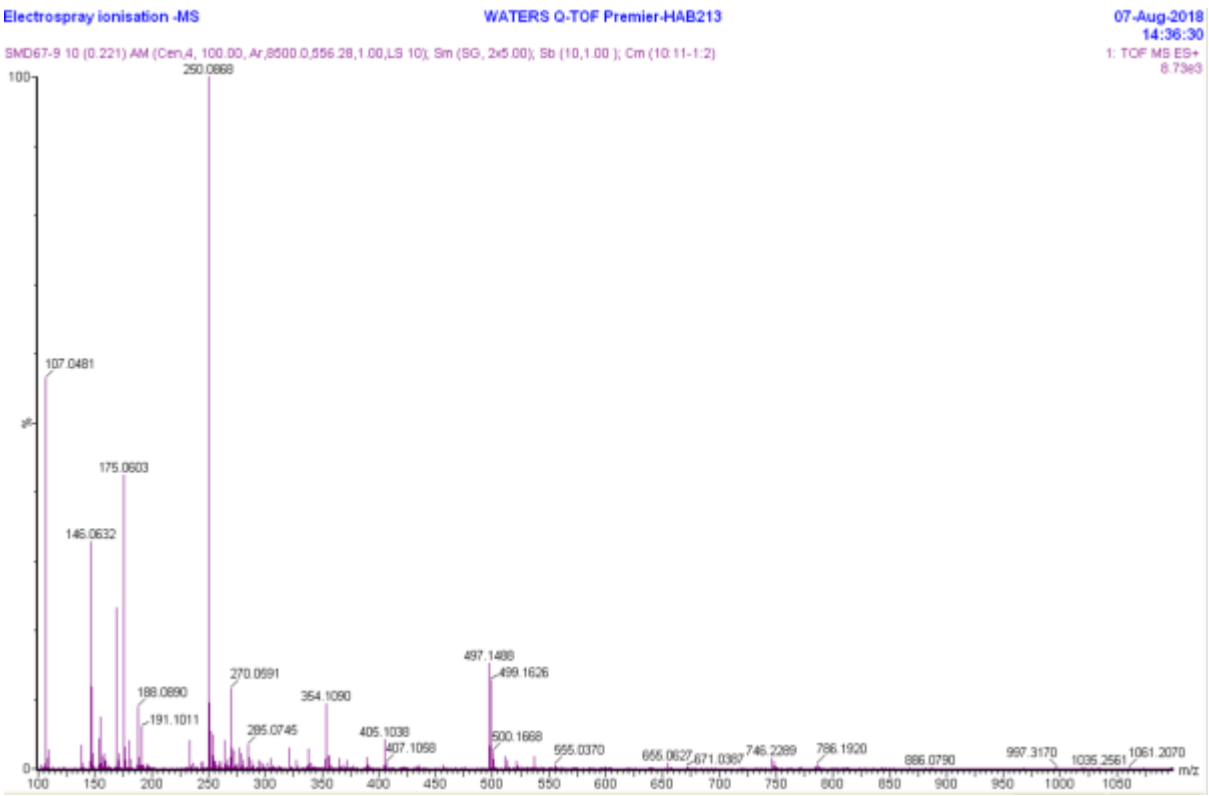


Fig. S14 ESI-MS spectrum of **1**.

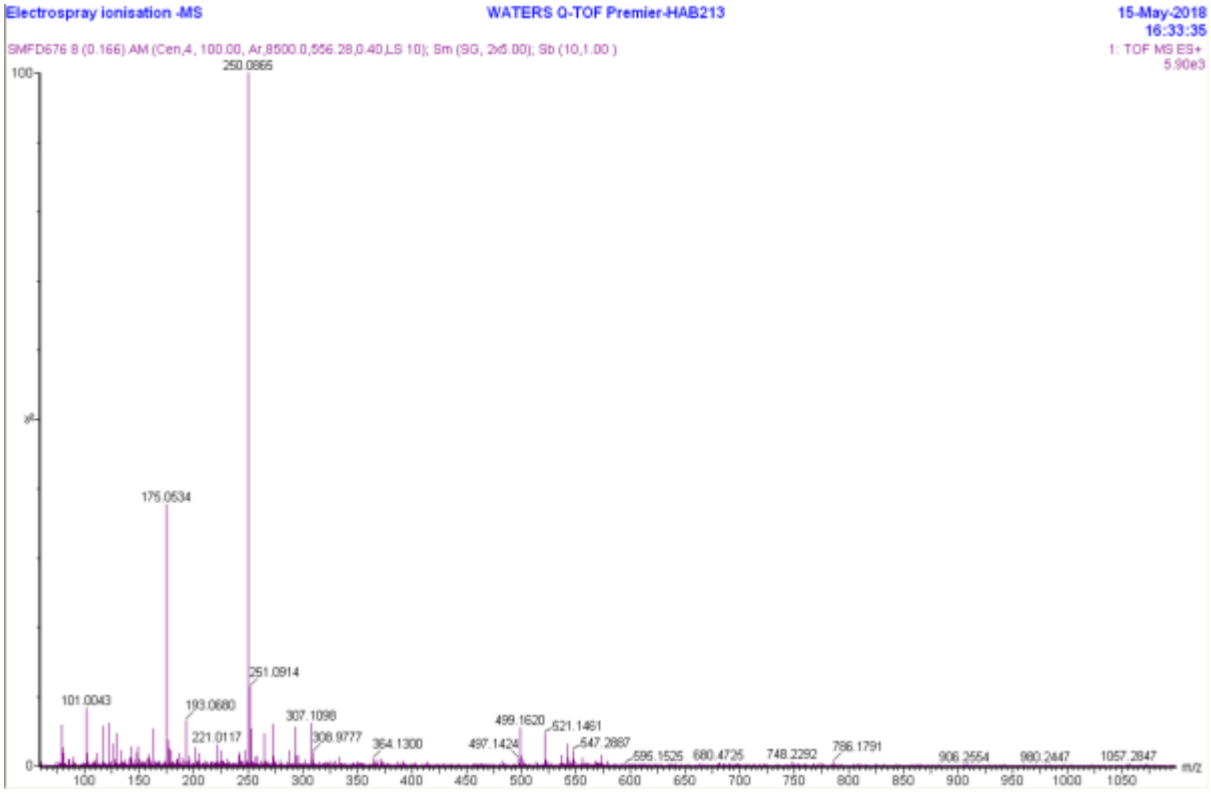


Fig. S15 ESI-MS spectrum of **2**.

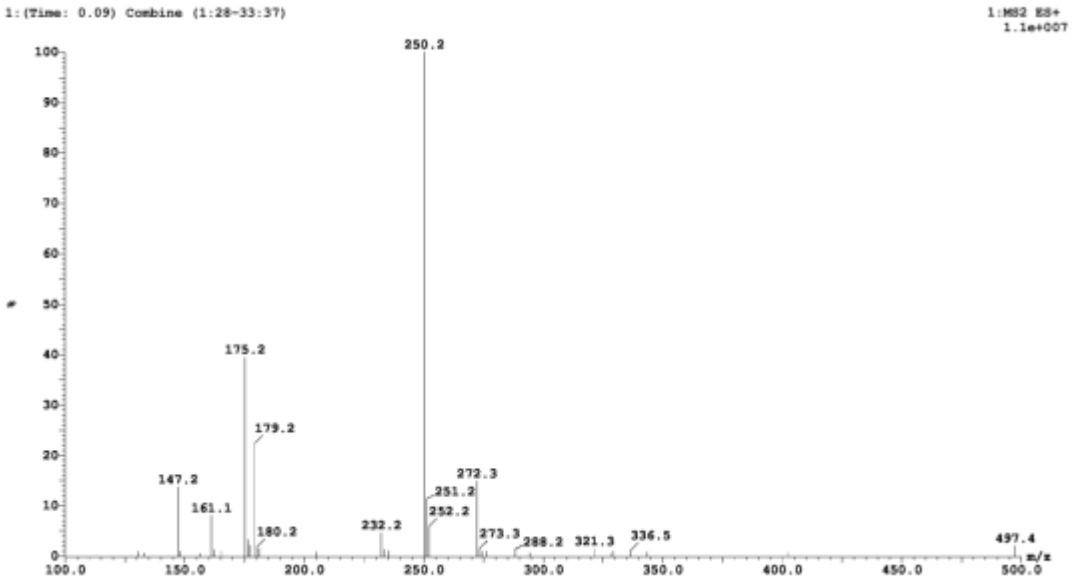


Fig. S16 ESI-MS spectrum of **SP**.

References

[1] J. Nanda, B. Adhikari, S. Basak, A. Banerjee, *J. Phys. Chem. B* **2012**, *116*, 12235–12244.

[2] G. W. Anderson, J. E. Zimmerman, F. M. Callahan, *J. Am. Chem. Soc.* **1967**, *89*, 5012–5017.

Author Contributions

SV and DT conceptualized the study; SS, DT, CK, MK, RA, and KP, performed experiments; SV and DT designed and supervised experiments; all authors analyzed data and contributed to the discussion. SV, DT and SS wrote the paper. NC supervised cell culture studies and contributed to discussion. SS, DT, CK, MK take responsibility for data integrity.