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Antimicrobial activity and mode of action of novel, N-terminal tagged tetra-peptidomimetics

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Materials and Methods

Chemicals: Fmoc-protected amino acids and resins were purchased from Novabiochem. Piperidine, N-hydroxybenzotrizole, trifluoroethanol, trifluoroacetic acid, dimethlyamino pyridine, N-methyl pyrrolidinone, N, N-diisopropylcarbodiimide, dimethyl sulphoxide and tri isopropyl silane were purchased from Sigma Chemical Co. 1-palmitoyl-2-oleoyl-*sn*-Glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-Glycero-3-phospho-(1'-rac-Glycerol) (POPG sodium salt) were purchased from Avanti Polar Lipids. All N-terminal tagging moieties were purchased from Sigma chemicals. All solvents used for the purification were of HPLC grade and obtained from Merck, India. Dimethylformamide (DMF) was obtained from Merck and double distilled prior to use.

Peptide synthesis and purification: All the peptidomimetics were synthesized on solid support based on SPPS on Rink amide MBHA resin using standard Fmoc-chemistry as described previously[1]. Coupling of N-terminal modifying moieties was done using 4 equivalents of tagging moiety with diisopropyl carbodiimide and HOBt overnight. After synthesis peptidomimetics were cleaved from solid support using a cleavage cocktail which consisted of trifluoroacetic acid: triisopropyl silane: phenol: water (95:2:2:1). The crude peptidomimetics were passed through LH-20 column and desired fractions were lyophilized. Purification of peptidomimetics was performed on C-18 semi preparative RP-HPLC column (7.8 x 300 mm, 125 Å, 10-µm particle size) with a linear gradient of acetonitrile (10 to 90 %) in 0.05 % TFA. Peptidomimetics with >85% of purity were obtained for biological activity. The correct peptidomimetic sequences after purification were confirmed by LC-ESI-MS (Quattro micro API, Waters) mass determination.

Antibacterial activity: Antimicrobial susceptibility testing was carried out using a modification of the Clinical Laboratory Standard Institute (CLSI) micro dilution broth assay

(reference 30 in manuscript). Briefly, mid-logarithmic phase bacterial culture was used for inoculum preparation. Each well of a 96-well polypropylene micro titre plate (NUNC) was inoculated with 90 μL of ~10^5 CFU/mL of bacterial suspension per ml of Mueller-Hinton broth (DIFCO). Then 10 μL of appropriate concentration of peptidomimetic conjugates in 0.001% acetic acid and 0.2% bovine serum albumin (SIGMA) was added to the different wells to obtain concentration ranging from 0.5 μg/mL to 1000 μg/mL in the wells. Plates were incubated overnight with shaking (180 rpm) at 37 °C for 18 h and the absorbance was read at 630 nm. Cultures (~10^5 CFU/mL) without peptidomimetics were used as positive control. Uninoculated Mueller-Hinton broth was used as negative control. The tests were carried out in duplicate and the experiments were repeated thrice.

Hemolytic activity: Human red blood cells (hRBC) were used for determining the haemolytic activity of peptidomimetics. Buffy coat of hRBCs was removed by centrifugation for 15 min. The hRBCs were then washed three times with phosphate-buffered saline (35 mM phosphate buffer, 150 mM NaCl, pH 7.2). One hundred micro litres of the diluted hRBC suspended 4% (v/v) in PBS was plated into sterilized 96-well plates and then 100 μL of two fold serially diluted peptidomimetic solution was added to each well. The plates were incubated for 1 h at 37 °C and centrifuged at 1500 rpm for 10 min. Supernatant were transferred to another 96-well plate, where haemoglobin release was monitored using ELISA plate reader (Molecular devices) by measuring the absorbance at 540 nm. Percent hemolysis was calculated by the following formula

$$\% \text{ Hemolysis} = 100[(A-A_0)/ (A_t-A_0)]$$

Where, A represents absorbance of peptidomimetic sample at 540 nm and A_0 and A_t represent zero percent and 100% hemolysis determined in PBS and 1% Triton X-100, respectively.

Proteinase-K stability: Proteinase-K digestion assay was performed using RP-HPLC as described previously (references 32 and 33 in manuscript). Briefly, digestion of peptidomimetics AP-1 and AP-2 was carried out by incubating 100 µg/mL of peptidomimetic sequence with 5 µg/mL of Proteinase-K in 100 mM Tris-HCl buffer (pH 7.6) at 37 °C. The peptidomimetic cleavage after 0 h, 2 h, 6 h, 10 h and 24 h was monitored by RP-HPLC using C18 reverse phase column. Linear gradient of 0.1% aqueous TFA and 0.1% TFA in acetonitrile were run. Absorbance was measured at 220 nm. Digestion was measured as percentage of degraded peptidomimetics calculated from the decrease of the HPLC peak area of the native peptidomimetic peak.

Tryptophan fluorescence: SUVs were used for the experiment. Similar procedure was used for preparation of SUVs as described earlier (reference 35 in manuscript). Briefly, dry lipids PC/PG (7:3, w/w) as bacterial mimic membranes or PC as mammalian mimic membranes were dissolved in chloroform/methanol mixture in a 150 mL round bottom flask. Solvent was removed by a stream of nitrogen gas, so that a thin lipid film was formed on the walls of the glass vessel. The lipid film thus obtained was then lyophilized overnight. Dried thin films were re-suspended in 10 mM Tris buffer [0.1 mM EDTA and 150 mM NaCl (pH 7.4)] preheated at 60 ºC by vortex mixing. The lipid dispersions were then sonicated in ice for 15–20 min using a titanium-tip ultrasonicator with a burst and halt time of 30 second and 10 second until the solution became opalescent. Titanium debris was removed by centrifugation. Each peptidomimetic (final concentration of 3 µM) was added to 500 µL of 10 mM Tris buffer [0.1 mM EDTA and 150 mM NaCl, pH 7.4] containing 0.3 mM SUVs, and the peptide/lipid mixture was allowed to interact at 25 °C for 2 min. The fluorescence measurements were done using Fluorolog (Jobin Yuvon, Horiba) spectrofluorimeter. Samples were excited at 280 nm, and the emission was scanned from 300 to 400 nm.

Calcein dye leakage assay. Similar procedure was used in order to assess ability of the designed peptidomimetics to cause leakage of liposomal content as described previously[2,3]. Briefly, similar method was used for preparation of LUVs as for SUVs described above except the dry lipid films were rehydrated with 10 mM Tris-HCl [70 mM calcein, 150 mM NaCl, 0.1 mM EDTA]. The liposome suspension obtained after rehydration was freeze thawed for five cycles and extruded 16 times through two stacked polycarbonate filters (100 nm pore size). The free calcein was removed by passing the liposome suspension through a Sephadex G-50 column at 23 °C and eluting with a buffer containing 10 mM Tris-HCl [150 mM NaCl, 0.1 mM EDTA]. After passing the liposome through Sephadex G-50, liposome diameter was measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments). Average diameter of LUVs was found to be in the range of 90-105 nm. Leakage of calcein from the LUVs was monitored by measuring the fluorescence intensity at an excitation wavelength of 490 nm and an emission wavelength of 520 nm on a model Fluorolog (Jobin Yuvon, Horiba) spectrofluorimeter. Different concentrations of peptidomimetics were incubated with bacterial mimic liposome for 5 minutes before exciting the samples. For the determination of 100% dye leakage 10% Triton X-100 (20 μL) in 10 mM Tris-HCl buffer [150 mM NaCl, 0.1 mM EDTA] was added to dissolve the liposome. The percentage of dye leakage caused by the peptidomimetics was calculated using the formula

$$\text{Dye leakage (\%)} = 100[(F-F_0)/ (F_t-F)]$$

Where, F is the fluorescence intensity achieved by addition of peptidomimetic and F_0 and F_t are fluorescence intensities in buffer and with Triton X-100, respectively. All measurements were made in duplicate.

Membrane depolarization: The membrane depolarization activity of peptidomimetics was determined using intact methicillin resistant *S. aureus* (ATCC33591) and membrane potential sensitive fluorescent dye, 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_35$) based on a previously defined method (reference 39 in the manuscript). Briefly, methicillin resistant *S. aureus* was grown at agitation to the mid log phase (OD_{600} ~ 0.4) and then harvested by centrifugation at 4000 rpm for 10 min. Cells were washed twice with respiration buffer (20 mM glucose, 5 mM HEPES, pH 7.4) and re-suspended to an $O.D._{600}$ ~0.05. $DiSC_35$ (0.18 μM prepared in DMSO) was added to a 500 μL aliquot of the re-suspended cells and allowed to stabilize for 1h. Membrane depolarization was then monitored by observing the change in the intensity of fluorescence emission of $DiSC_35$ at an excitation wavelength of 622 nm and an emission wavelength of 670 nm after peptidomimetic addition. Full dissipation of membrane potential was achieved by adding gramicidin A. The membrane potential dissipating activity of peptidomimetics is calculated as follows:

$$\text{Membrane depolarization (\%)} = 100[(F_p\text{-}F_0)/ (F_g\text{-}F_0)]$$

Where, F_p is the fluorescence intensity achieved 5 min after peptidomimetic addition, F_0 is the stable fluorescence value after addition of $DiSC_35$ and F_g is fluorescence intensity after gramicidin A addition.

Circular Dichroism: The Circular Dichroism (CD) spectrum of the AP1 and AP2 was recorded using a Jasco J-815 CD spectrophotometer (Tokyo, Japan) with a 1 cm path length cell. Wavelengths from 200 to 260 nm were scanned with 1.0 nm step resolution, 100 nm/min speed, 0.4 second response time, and 1 nm bandwidth. CD spectra of the peptidomimetics were collected and averaged over two scans in 5 mM sodium phosphate buffer (pH 7.2), bacterial mimic LUVs (POPC:POPG, 7:3, w/w), mammalian mimic POPC LUVs and lipo-polysaccharide micelles in water (1 mg/mL). Spectra were baseline corrected by subtracting a

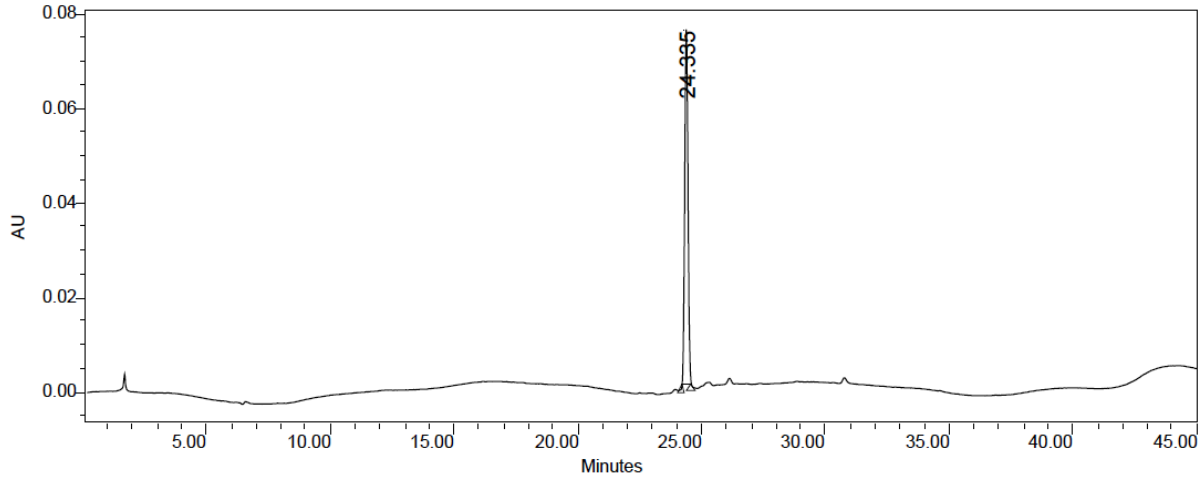
blank spectrum containing only buffer or LUVs. The spectra were expressed as ellipticity [Θ] vs. wavelength.

For the CD experiment LUVs were prepared as described previously [references 33 and 37 in manuscript]. Briefly, desired amounts of lipids [POPC: POPG (7:3, w/w)] were mixed in 2 mL chloroform/methanol mixture, in a 150 mL round bottom flask. The solvent was removed under a stream of nitrogen and the lipid film obtained was lyophilized overnight to remove organic solvent. The thin lipid film was rehydrated with 5 mM sodium phosphate buffer and vortex mixed for half an hour. The multilamellar vesicles thus obtained were extruded 16 times through a 100 nm polycarbonate membrane extruder (Avanti mini extruder apparatus). Mean diameter of LUVs was determined by dynamic light scattering, using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Mean diameter of LUVs was found to be in the range of 95-115 nm.

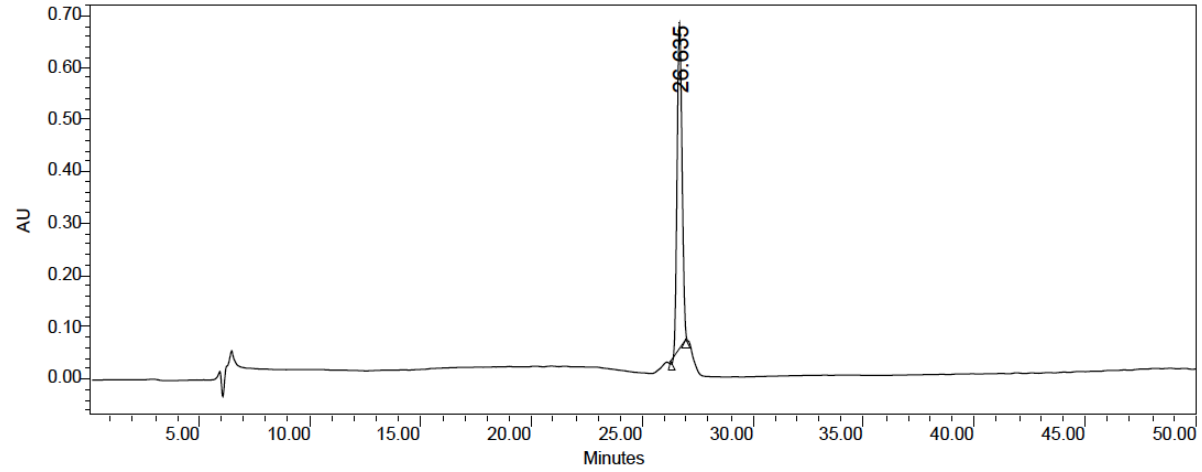
Scanning electron microscopy: For electron microscopy samples were prepared as described previously (reference 37 in manuscript). Methicillin resistant *S. aureus* (ATCC 33591) was grown on Mueller Hinton Agar and an isolated colony was picked to inoculate 25 mL of Mueller Hinton broth. The bacterial suspension was incubated overnight at 37 ºC. A volume of 100 µL of this culture was used to freshly inoculate 5 mL of MHB. The suspension was then allowed to grow till OD 600 of 0.5 (corresponding to 10^8 CFU/mL). Bacterial cells were then spun down at 4000 rpm for 15 min, washed thrice in PBS (20 mM, 150 mM NaCl) and re-suspended in equal volume of PBS. The cultures were then incubated with peptidomimetics at concentrations 5 X MIC for 30 min. Controls were run in the absence of peptidomimetics. After 30 min, the bacterial pallet was washed thrice with PBS. The pallet was re-suspended in 1 mL of 2.5% gluteraldehyde in PBS for cell fixation and was incubated at 4 ºC for 4 h. Cells were then spun down and washed with PBS thrice. Further the samples were dehydrated in series of graded ethanol solutions (30% to 100%), and finally

dried in a desiccators under vacuum. An automatic sputter coater (Polaron OM-SC7640) was used for coating the specimens with 20-nm gold particles. Then samples were viewed via scanning electron microscopy (SEM) (EVO 40, Carl Zeiss, Germany)

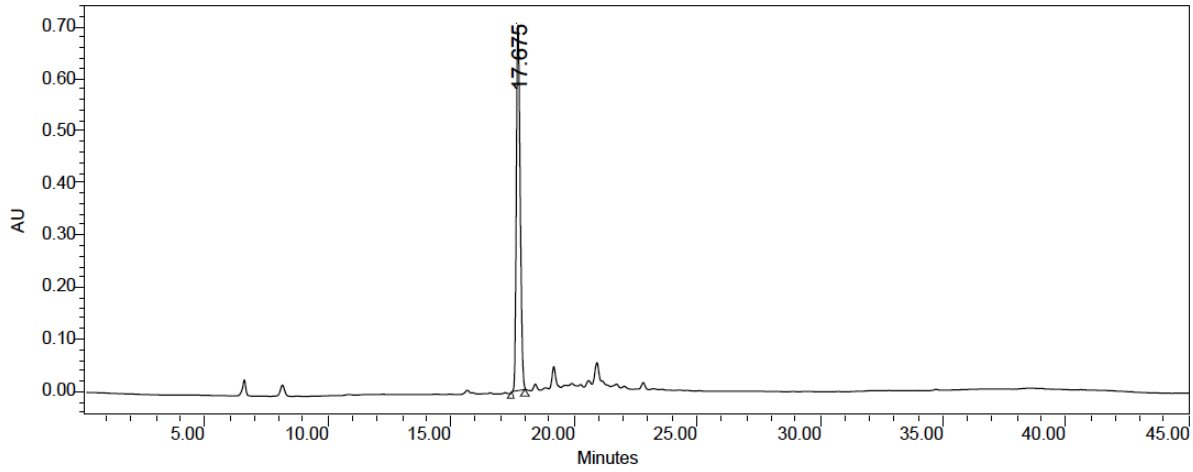
A) RP-HPLC profiles of sequences AP1, AP2, AP3, AP4, AP5, and AP6 (absorbance at 220 nm). For AP1 and AP3-AP6 HPLC gradient of 10 to 90% buffer 2 was run where, buffer 1 was water (0.05 % TFA) and buffer 2 was acetonitrile (0.05 % TFA) over 45 minutes. For AP2, gradient of 30 to 70% buffer 2 was run over 45 minutes where, buffer 1 was water (0.1 % TFA) and buffer 2 was acetonitrile (0.1 % TFA)



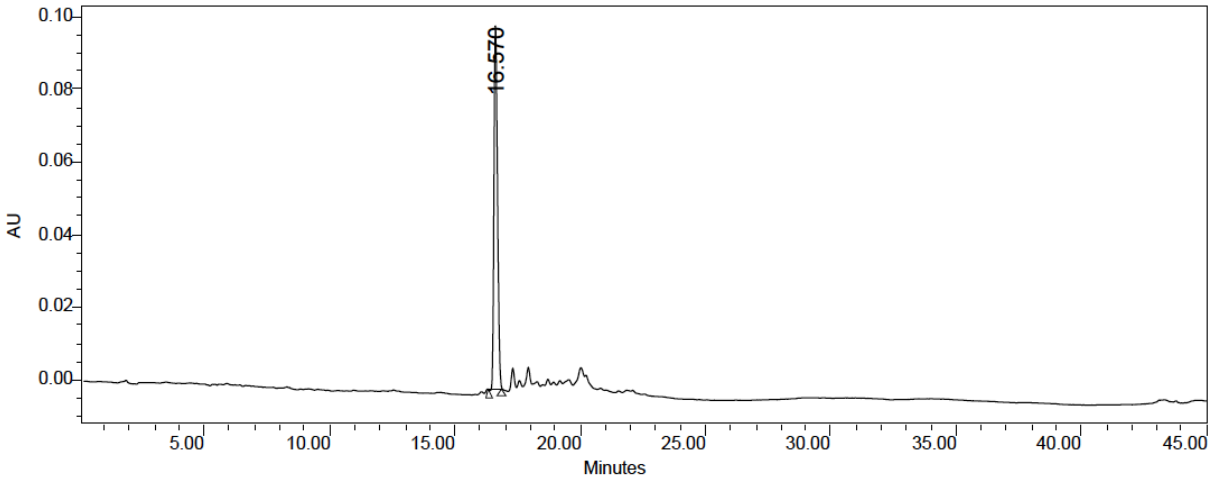
AP1



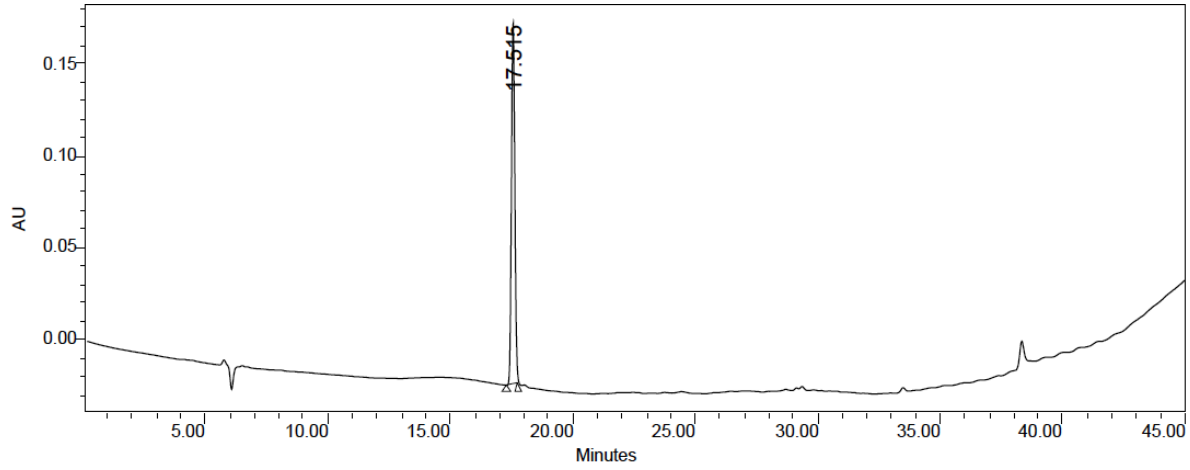
AP2



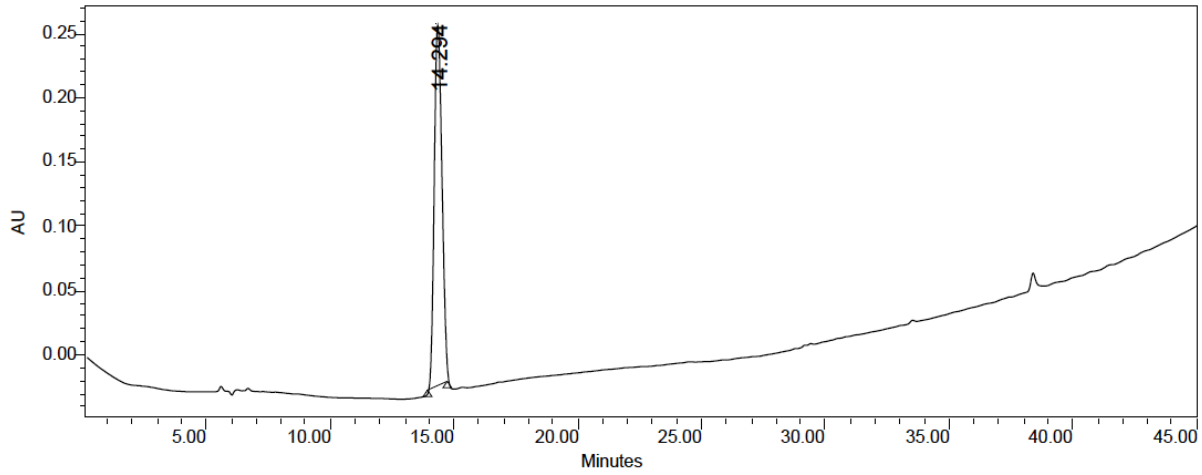
AP3



AP4

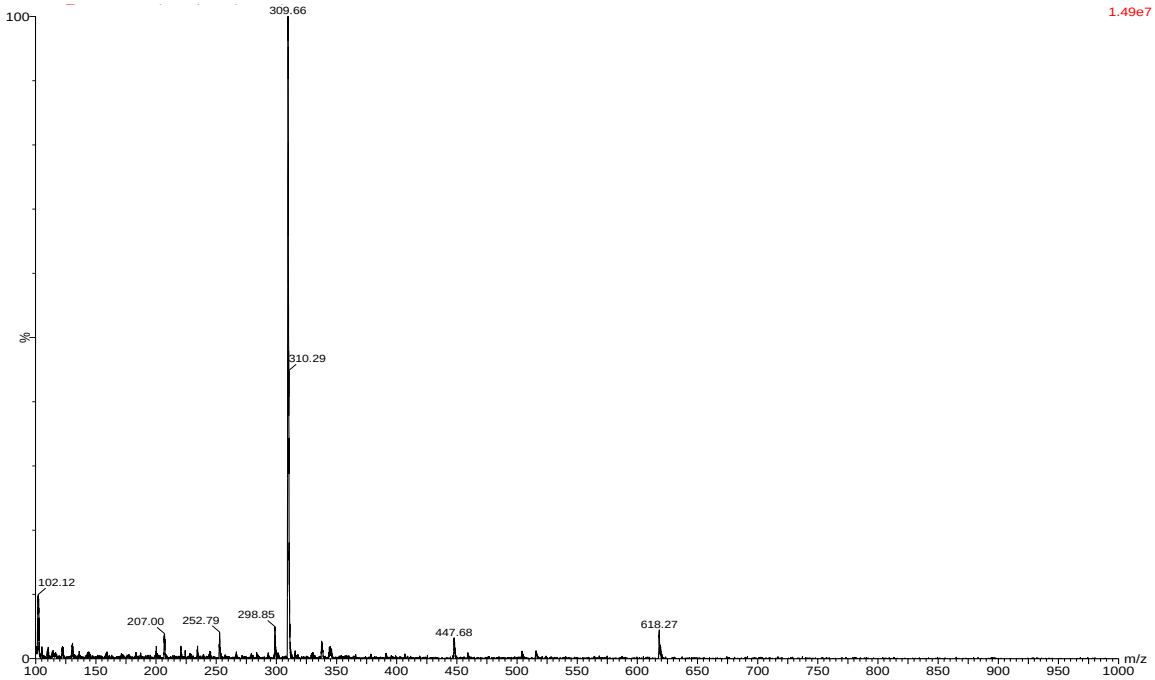


AP5

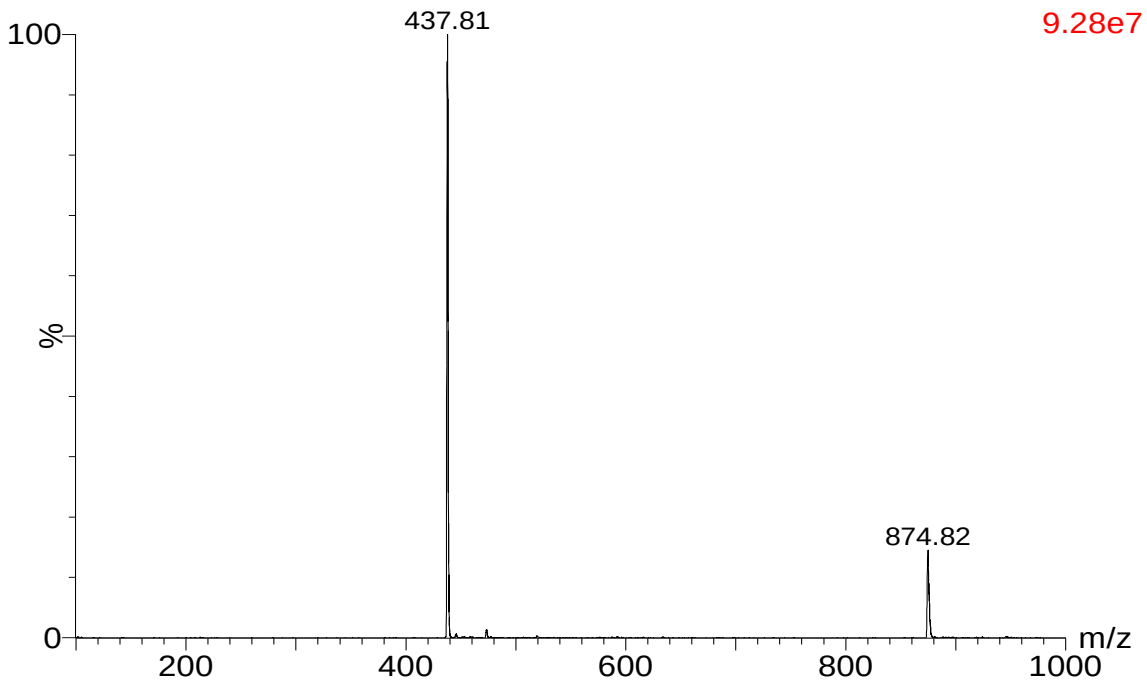


AP6

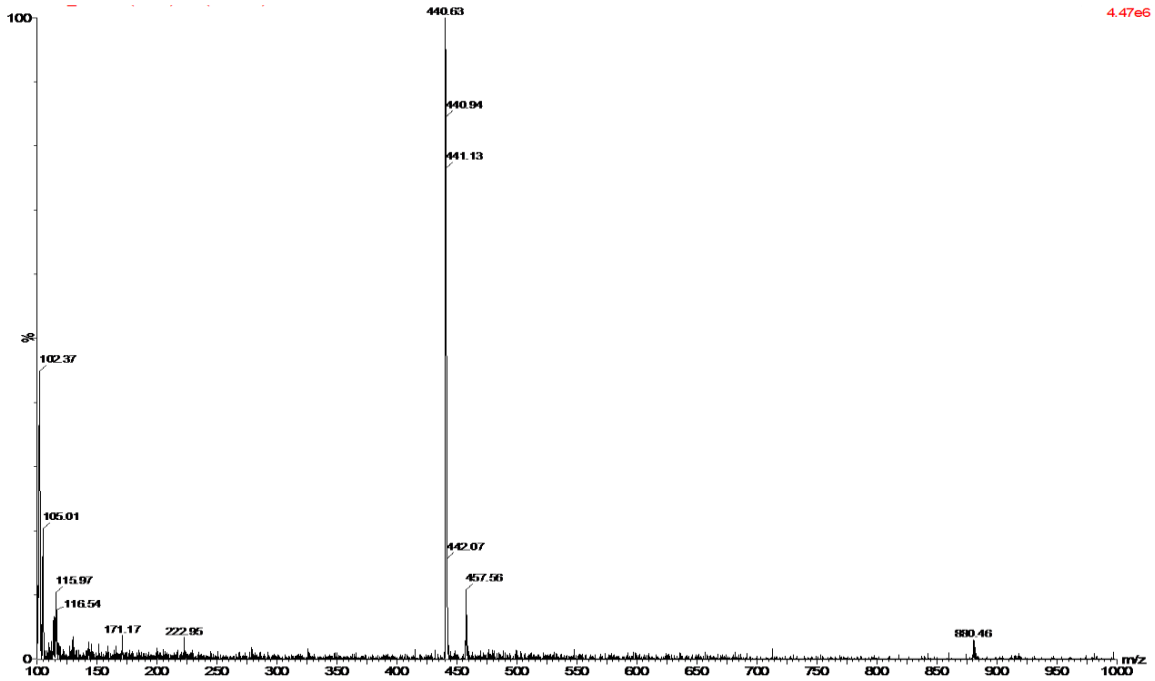
B) ESI-LCMS spectra of AP, AP1, AP2, AP3, AP4, AP5 and AP6 using LC-ESI-MS on UPLC (Acquity UPLC, Waters) and Quattro micro API (Waters).



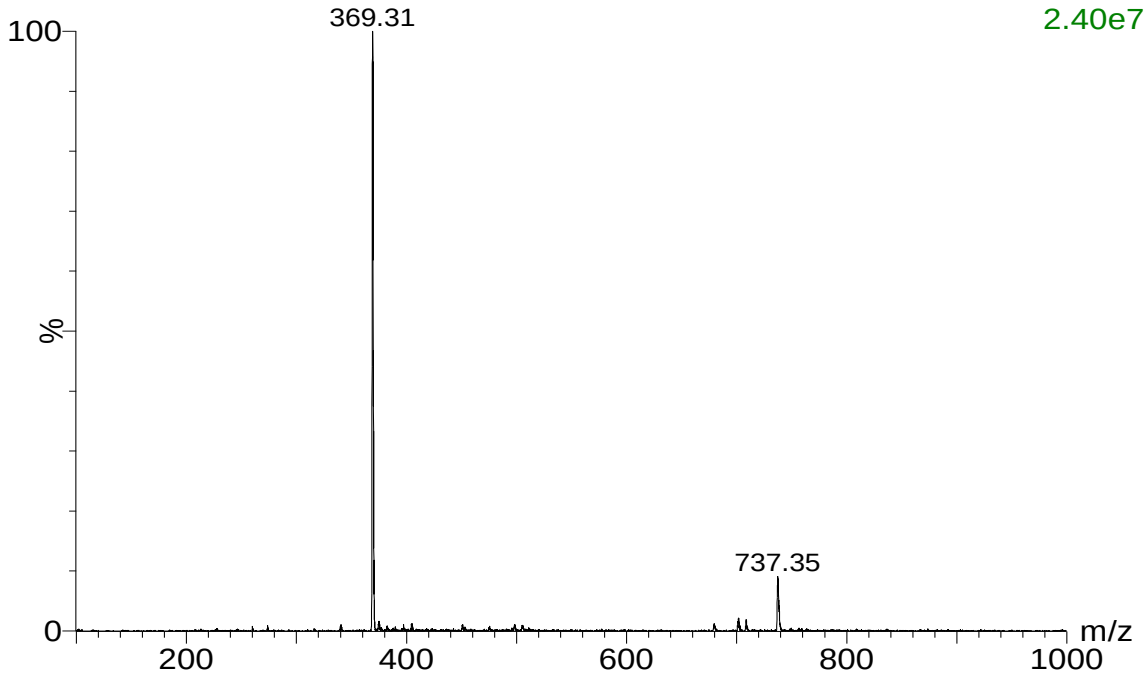
AP



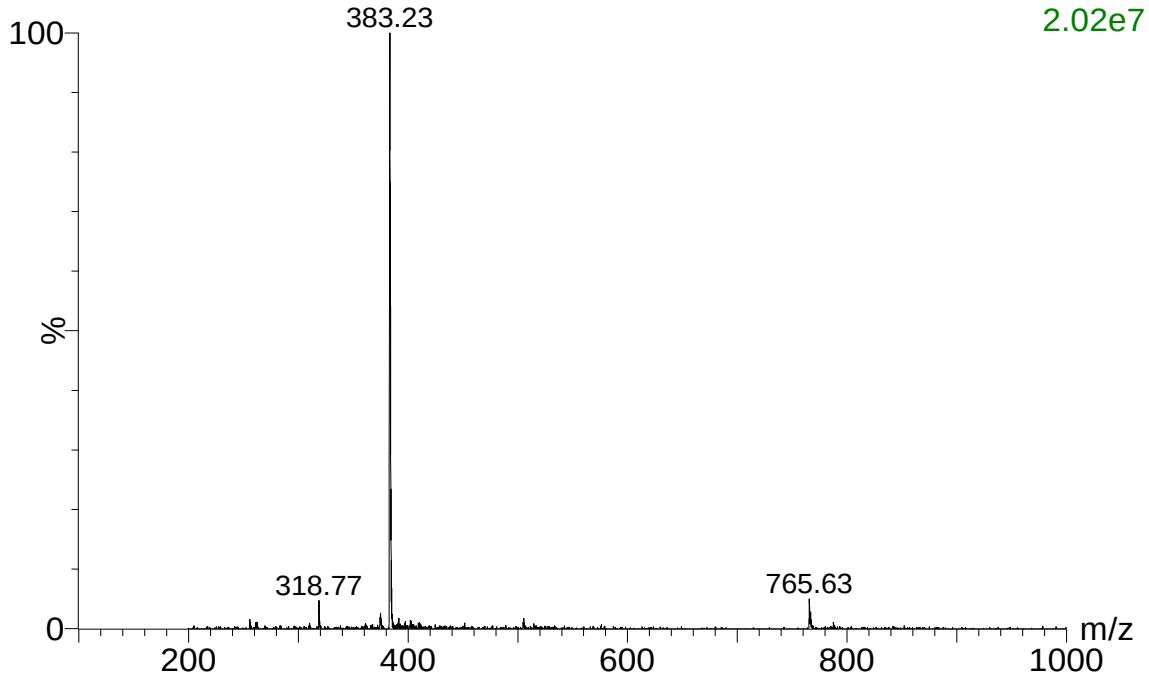
AP1



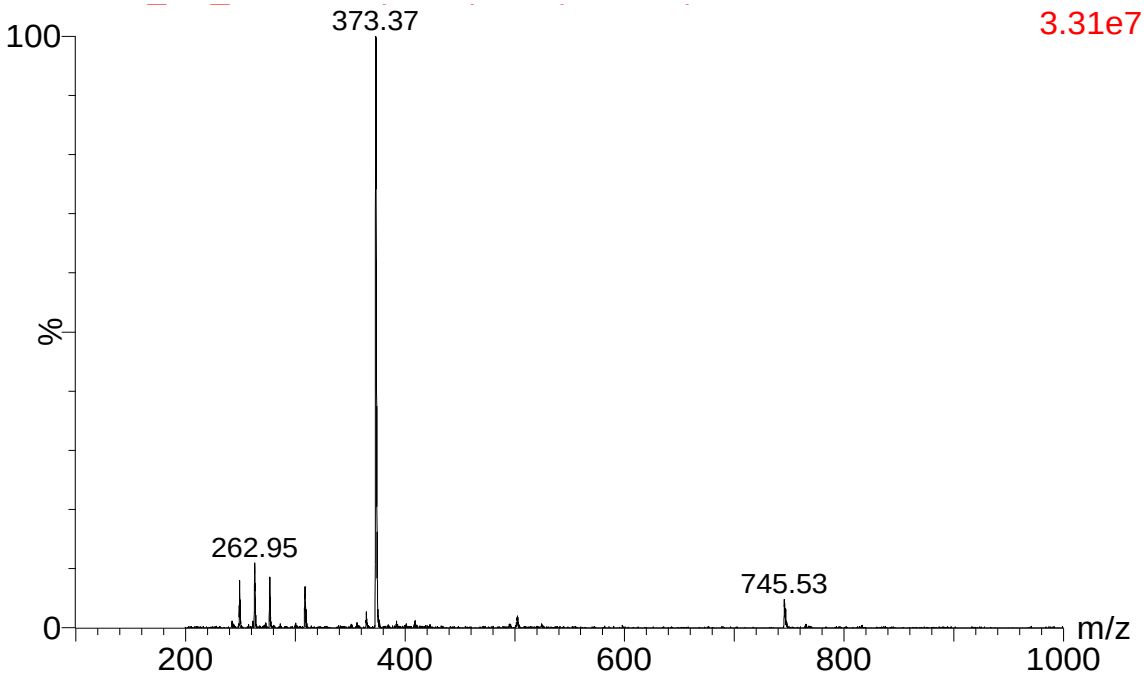
AP2



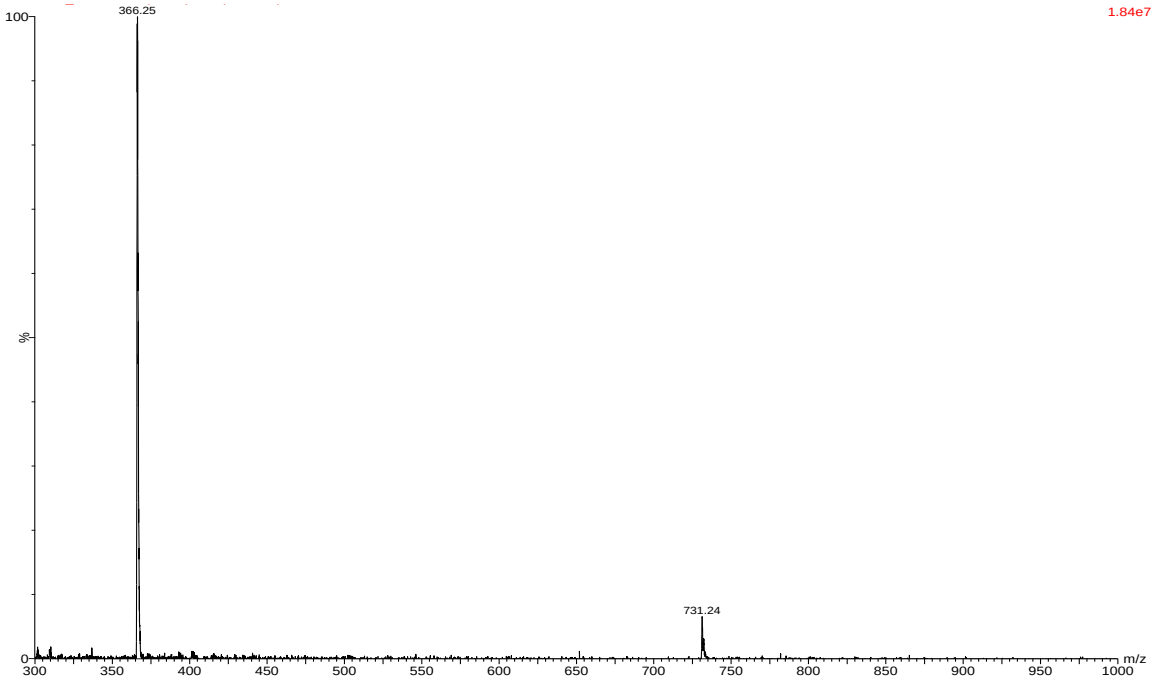
AP3



AP4



AP5



AP6

Table S1: Calcein leakage fluorescence intensity data

Concentration (µg/mL)	Fluorescence Intensity (a.u.)						
	AP	**AP1**	**AP2**	**AP3**	**AP4**	**AP5**	**AP6**
0.00E+00	1.21E+06	1.11E+06	1.41E+06	1.76E+06	1.02E+06	931730	1.86E+06
5.68E+00	1.25E+06	2.04E+06	2.19E+06	2.17E+06	1.91E+06	1.00E+06	1.90E+06
1.41E+01	1.33E+06	2.10E+06	2.21E+06	2.33E+06	2.06E+06	2.05E+06	1.92E+06
2.78E+01	1.34E+06	2.15E+06	2.23E+06	2.40E+06	2.10E+06	2.05E+06	1.98E+06
4.10E+01	1.41E+06	2.17E+06	2.17E+06	2.47E+06	2.17E+06	2.04E+06	2.04E+06
TX-100	2.44E+06	2.33E+06	2.48E+06	2.78E+06	2.54E+06	2.57E+06	2.28E+06

References

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