Electronic supplementary information

Cyclic dipeptide based cell-penetrating peptidomimetics for effective DNA delivery

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1. Materials & Methods

1.1 General reagents

Wang resin, Rink amide resin, Fmoc/t-But protected amino acids and other starting materials for the peptide/peptidomimetics synthesis were obtained from Nova Biochem, Sigma-Aldrich and Spectrochem India. Dimethylformamide (DMF), N,N-diisopropylethylamine (DIPEA), N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), hydroxybenzotriazole (HOBt), acetonitrile (CH_3CN or ACN) piperidine and all other chemicals were purchased from Spectrochem India. o-Nitrophenol-β-D-galactoside (ONPG) purchased from Sigma-Aldrich. HPLC grade solvents and Milli-Q water were used for reactions and purification. Moisture sensitive reactions were performed under inert nitrogen atmosphere. Thin layer chromatography (TLC) was carried out on silica gel 60 F254 plates obtained from Merck. Spots on the TLC plate were visualized using one of the (or all of the) following methods: (i) spray with a 0.2% (w/v) ninhydrin solution in absolute ethanol followed by charring on hot plate, (ii) UV activity, and (iii) treating with iodine. Column chromatography was carried out on silica gel (100-200 mesh) obtained from Spectrochem India. Laboratory reagent grade solvents were used for purification of compounds by column chromatography. NMR spectra were recorded on a Bruker AV-400 spectrometer with chemical shifts reported as ppm (in DMSO-*d*6/D_2O with tetramethylsilane as internal standard). All the CDP inserted peptidomimetics were purified on Shimadzu reverse-phase (RP) semi-preparative HPLC system using C18 column at 40 °C. The high-resolution mass spectra (HRMS) were obtained from on Agilent Technologies 6538 UHD Accurate-Mass Q-TOF.

HeLa cells (Human epithelial cells from cervical carcinoma) were obtained from Dr. Praveen Vemula, inSTEM, NCBS, Bengaluru. DMEM (Dulbecco's Modified Eagle's Medium), fetal

bovine serum (FBS), penicillin-streptomycin and trypsin were purchased from Gibco (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's phosphate-buffered saline (PBS) and Triton X-100 were purchased from Sigma-Aldrich. Stearyl-R8 and FITC-Stearyl-R8 peptides were obtained from LifeTein, New Jersey, USA.

1.2 Synthesis

Kkd-5 and **Ekd-5** were synthesized following standard 9-fluorenylmethoxycarbonyl (Fmoc) solid phase peptide chemistry protocols. Lys/Glu preloaded Wang resin (Novabiochem) was used as a solid support for the syntheses of cyclic dipeptide (CDP)-inserted peptidomimetic. Amino acids and unnatural CDP-amino acids (**kd**) were coupled using HBTU/HOBt as the activating reagent, DIPEA as the base and DMF as solvent. For deprotection of Fmoc protecting group, 20% piperidine in DMF was used. All compounds were purified using a reverse-phase (RP) semi-preparative HPLC on C18 column at 40 °C. Product purity was 95-98%, as ascertained by the analytical HPLC. The molecular mass of the compounds was verified by HRMS (Q-TOF) analysis. Experimental procedure for each step in the synthesis of **Kkd-5** and **Ekd-5** are described below.

1.2.1 Synthesis of unnatural CDP-amino acid (Fmoc-kd; Scheme 1a)

Synthesis of dipeptide 1

To a stirred solution of Fmoc-Asp(OtBu)-OH (0.83 g, 2 mmol) and DIPEA (0.77 mL, 4.4 mmol) in DMF at 0 °C, HBTU (0.91 g, 2.4 mmol) and HOBt (0.32 g, 2.4 mmol) were added (Scheme 1a). The reaction mixture was kept for stirring about 15 min under nitrogen atmosphere. H-Lys (Boc)-OMe (0.71 g, 2.4 mmol) was added to above solution and the reaction was left to stir for 3

h. After the completion of the reaction (monitored by TLC), solvent was removed and the residue was extracted into EtOAc (50 mL). The combined organic phase was washed with 1N citric acid (2 X 50 mL), 10% Na_2CO_3 (2 X 50 mL), water (1 X 50 mL) and brine (1 X 50 mL). The organic layers were collected, dried over anhydrous Na_2SO_4 and evaporated in *vacuo* to afford the dipeptide **1**.

Cyclization of dipeptide 1 to obtain unnatural CDP-amino acid kd

To the dipeptide **1** (1.5 g, 2.3 mmol), 37.5 mL of 20% piperidine in DCM solution was added and the reaction was stirred for about 8 h at room temperature (Scheme 1a). After the reaction, the precipitate was filtered and the filtrate was washed with 5% citric acid solution (4 X 100 mL), water (3 X 100 mL), brine (1 X 50 mL) and dried over anhydrous Na_2SO_4. Solvent was evaporated and the residue was washed with diethyl ether. The residue was collected and dried to obtain Boc- and tBut-protected cyclic dipeptide **2** in 75% yield.

Boc- and tBut-deprotection of cyclic dipeptide **2** (1 g, 2.5 mmol) was carried out by adding 10 mL solution containing TFA, DCM and TIPS in the ratio of 95:4:1. The reaction mixture was kept for stirring about 4 h at room temperature. After completion of the reaction diethyl ether was added, precipitate was collected and vacuum dried to yield amine and carboxylic acid-deprotected cyclic dipeptide **2** (**kd**) in good yield (80%).

Synthesis of Fmoc-kd

Fmoc-chemistry protocols were followed, for the synthesis of CDP-inserted peptidomimetics in solid phase. Therefore amine functionality of deprotected cyclic dipeptide **2** (**kd**) was re-protected with Fmoc group (Fmoc-**kd**). The amine and carboxylic acid-deprotected cyclic dipeptide **2** (**kd**) from previous step (1 g, 4.4 mmol) was stirred with water and dioxane (10:10)

at 0 °C. To this reaction mixture, Na_2CO_3 (0.93 g, 8.8 mmol) and Fmoc-OSu (1.8 g, 5.3 mmol) were added. After completion of the reaction, diluted with water and aqueous layer was washed with diethyl ether and then neutralized with 5% HCl. The obtained precipitate was filtered and dried to afford Fmoc-**kd** in good yield (75%).

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 1.37 (4H, m); 1.69-1.70 (2H, m); 2.61-2.64 (2H, m); 2.97-2.95 (2H, d, J = 8 Hz); 3.88 (1H, s); 4.18-4.20 (2H, d, J = 8 Hz); 4.28 (2H, s); 7.23-7.89 (9H, m); 8.00 (1H, s); 8.09 (1H, s); 12.31 (1H, s). ^{13}C NMR (400 MHz, DMSO-d_6): δ (ppm): 21.4, 29.2, 31.3, 36.6, 46.7, 50.9, 53.8, 65.1, 66.3, 120.0, 125.1, 127.0, 127.5, 140.7, 143.9, 156.0, 167.6, 168.0, 171.3.

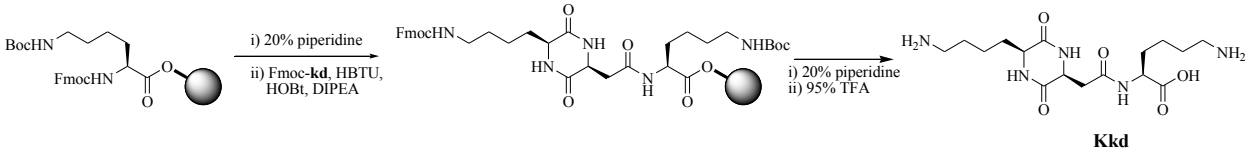
1.2.2 Synthesis of CDP (kd)-peptidomimetics (Kkd-5 and Ekd-5) with lysine and glutamic acid at alternative positions

The CDP-peptidomimetics (**Kkd-5** and **Ekd-5**) were synthesized manually by stepwise coupling of Fmoc-amino acids following the solid phase peptide synthesis protocols. Fmoc-amino acids (K: Lys and E: Glu) were used with their side chains functionalities (-NH_2 and –COOH) protected (with Boc and tBut protecting groups, respectively). (Boc)Lys/(tBut)Glu (K/E)-preloaded Wang resin was allowed to swell in DCM in a peptide synthesis flask and Fmoc group was deprotected. The peptidomimetic chain was grown on (Boc)Lys/(tBut)Glu (K/E)-Wang resin (0.2 g, loading 0.76 mmol/gm). HBTU (2.5 eq), HOBt (2.5 eq), Fmoc-amino acid (2.5 eq) and DIPEA (4 eq) in DMF were added to resin and allowed to swirl for 90 min. The unnatural CDP-amino acid (Fmoc-**kd**) was coupled at alternative positions in peptidomimetics containing K or E, under similar peptide coupling conditions described above. All the couplings were completed in 90-180 min. Coupling and deprotection efficiencies were monitored by the Kaiser test. After

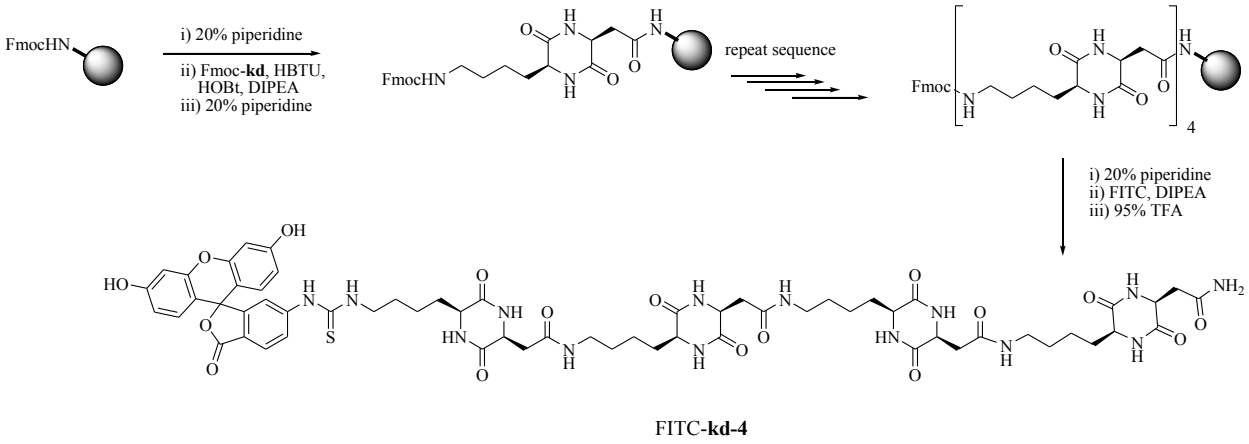
completion of the peptidomimetic chain assembly, resin was thoroughly washed with DMF and DCM. The cleavage of the peptidomimetic from resin and side chain deprotection was performed using cocktail solution containing trifluoroacetic acid (TFA):triisopropyl silane (TIPS):DCM (95:4:1) for 3 h at room temperature. After resin cleavage, the solution was filtered and removed under vacuum and triturated with ether to obtain the white solid. The obtained solid was purified through RP- HPLC using ACN:Water.

1.2.3. Synthesis of FITC labeled Kkd-5 and Ekd-5 (Scheme 1b)

The terminal Fmoc-group of resin bound **Kkd-5**/**Ekd-5** was deprotected and conjugated with fluorescein isothiocyanate (FITC). FITC (1.5 eq) and DIPEA (5 eq) in DMF was added to the resin and swirled for 6 h. After completion of the coupling (as revealed by Kaiser test), excessive reagents were removed by DMF washings and the FITC-labeled peptidomimetics were cleaved from the resin by treating with 95% TFA cocktail solution, purified through RP-HPLC and characterized with HRMS (**Scheme 1b**).



Scheme 1S. Solid phase synthesis of **Kkd**



FITC-**kd-4**

Scheme 2S. Solid phase synthesis of FITC labeled **kd-4**

1.2.4. Synthesis of Kkd (Scheme 1S)

To the lysine preloaded Wang resin, cyclic dipeptide monomer Fmoc-**kd** was coupled using peptide coupling conditions detailed above. The resin bound **Kkd** was cleaved by employing 95% TFA and the crude product was purified through RP-HPLC. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm): 1.71-1.36 (m, 12H), 2.44-2.42 (d, 1H, J = 7.6 Hz), 2.79-2.74 (m, 5H), 3.87 (s, 1H), 4.21-4.18 (t, 2H, J = 4.8 Hz), 7.67 (br, 6H), 7.78 (s, 1H), 8.12 (s, 1H), 8.72-8.25 (d, 1H, J = 8 Hz), 12.67 (s, 1H).

1.2.5. Synthesis of FITC labeled kd-4 (Scheme 2S)

The Rink-amide resin was allowed to swell in DCM for 20 min and Fmoc deprotection was carried out using piperidine (20% in DMF). The monomer Fmoc-**kd** was coupled sequentially to the Rink amide resin using HBTU/HOBt/DIPEA and followed by Fmoc deprotection. After completed the synthesis (tetramer), FITC was conjugated to the resin loaded with tetramer (**kd-4**) and cleaved by 95% TFA and purified through RP-HPLC. Coupling and deprotection reactions were confirmed by Kaiser test. The purified product was characterized by HRMS.

1.3 Serum Stability Analysis

Serum stability of **Kkd-5** and **Ekd-5** was assessed using human blood serum (HBS). **Kkd-5** or **Ekd-5** (100 μM) were incubated in HBS at 37 °C then, 100 μL aliquots were collected at different time points (0, 6, 12, 24, and 48 h) for analysis. Collected samples were added with 50 μL of 6 M urea to precipitate serum proteins and incubated at 4 °C for 10 min. Next, 40 μL of TFA (20%) was added to the above samples and incubated for another 10 min at 4 °C to precipitate the serum proteins. The samples were centrifuged for 10 min and 200 μL of the supernatant was analyzed on RP-HPLC using a linear gradient of ACN (0.3 mL/min flow rate). Equivalent amount of **Kkd-5** or **Ekd-5** in PBS buffer were subjected to the same treatment procedure and used as controls. All the experiments were carried out in triplicate. The percentage recovery of peptidomimetics was analysed by analytical HPLC.[1]

1.4 Cytotoxicity measurements for CDP-peptidomimetics

The quantitative colorimetric MTT assay was employed to evaluate the cytotoxic effects of **Kkd-5** and **Ekd-5** in HeLa cells at 24 and 48 h time points. Briefly, cells were seeded at 12,000 and 8,000 cells/well density in 100 μL media in 96 well plates for 24 and 48 h. Cells were treated with varying concentrations of **Kkd-5** and **Ekd-5** (50-500 μM) in DMEM complete medium to assess their effect on cell viability. Cells were incubated for 24 and 48 h. After completion of indicated incubation period, MTT solution (5 mg/mL prepared in 1X PBS) was added to the cells to a final concentration of 0.5 mg/mL in each well. Plates were further incubated at 37 °C in 95% air/5% CO_2 atmosphere for 3–4 h to produce dark blue formazan crystals. Next, supernatant from each well was carefully removed without disturbing the formazan crystals. Formazan crystals were dissolved by adding 100 μL of methanol:DMSO (1:1) ratio and shaken at 25 °C for 20-30

min. Absorbance values were measured on a microplate reader (Tecan, USA) at 570 nm with background correction at 690 nm. At least three experiments were performed with 4-8 data points in each treatment group. Data was analyzed using Graphpad software (Version 4, SanDiego, CA, USA). The cell viability was defined as percentage reduction in the absorbance of individual groups compared to untreated controls.

1.5 Cell culture and treatments

HeLa Cells were cultured in T-75 flasks (Nest, India) and maintained in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in 95% air/5% CO_2. Stock solutions of **Kkd-5** and **Ekd-5** were prepared in DMSO and aliquots were stored at -20 °C. For all experiments assessing cellular uptake, transfection efficiency with indicated concentrations of peptidomimetics for 4 h and then analyzed unless stated otherwise. Controls were treated with media containing 0.01% DMSO.

1.6 Cellular uptake studies

Cellular uptake studies were carried out in HeLa and PC12 cells employing confocal microscopy and flow cytometry. For confocal microscopy, HeLa cells were seeded onto cover slips placed in 12-well plates at 30,000 cells/well in DMEM complete medium and incubated for 24 h. Cells were treated with FITC-**Kkd-5** at 1 µM and 10 µM concentrations for 4 h in serum-free DMEM medium. After 4 h incubation, cells were washed with 1X PBS and fixed with 4% formaldehyde for 30 min. Cells were further washed with 1X PBS and incubated with DAPI at 300 nM concentration for 15 min. Finally, washed with 1X PBS and cover slips were mounted onto slides using 50% glycerol solution, sealed and used for imaging. Data obtained was processed using LSM 5 Image Examiner software, USA. For flow cytometry, HeLa cells were seeded in 6-

well plates at 2 x 10^5 cells/well and incubated for 24 h. CDP-peptidomimetics and FITC-Stearyl-R8 peptide were incubated with cells at varying concentrations in serum free DMEM media for 4 h. After 4 h, cells were harvested using cell scraper, centrifuged and washed with 1X PBS multiple times to ensure removal of FITC labeled CDP-peptidomimetics from media. Prepared samples were subjected for flow cytometry analysis at 485 nm with 10000 cell count. Data obtained was processed using FCS express software, USA.

1.7 Confocal Microscopy

For cellular uptake studies, after incubation with FITC-**Kkd-5**, FITC-**Ekd-5** and FITC-Stearyl-R8, cells seeded in 24 well plate with cover slips were washed with 1X PBS and fixed with 4% paraformaldehyde for 5-10 min. Cells were washed with 1X PBS and incubated with DAPI (300 nM final concentration) for 5-10 min. Cells are finally washed with 1X PBS (multiple times) and the cover slips were mounted on glass slides with Vectashield mounting medium (Vector Laboratories, CA, USA) and sealed. Images were captured using Delta Vision Elite widefield microscope with FITC and DAPI filters. The collected images were processed using Axiovision or DV SoftWoRX software. The excitation CWL/BP and emission CWL/BP for filters used were 490/20 and 529/38(FITC), 390/18 and 435/48 (DAPI).

1.8 Preparation of lipid model membrane

The biomembrane mimicking liposomal formulation was prepared using dioleyolphosphatidylcholine/ dioleyol- phosphatidylethanolamine/ dioleyolphosphatidylserine/ cholesterol (DOPC/DOPE/DOPS/Chol) at 45:20:20:15, w/w (1mM each) in Milli-Q water. Briefly, 1 mM liposomal model membrane was prepared with each lipid (listed above) in the appropriate mole ratios dissolved in chloroform (500 µL) in a glass vial. The solvent was

removed with a thin flow of moisture free nitrogen gas and the lipid film was kept for drying under high vacuum for 6 h. Sterile Milli-Q water (1 mL) was added to the vacuum dried lipid film and the mixture was allowed to swell overnight. The vial was then vortexed for 2-3 min at room temperature to produce multilamellar vesicles (MLVs). MLVs were then sonicated initially in a water bath followed by ice bath until clarity using a Branson 450 sonifier at 100% duty cycle and 25 W output power to produce small unilamellar vesicles (SUVs).

1.9 Small angle X-ray scattering (SAXS) measurements

The small angle X-ray scattering (SAXS) system was used to decipher the physical and structural interactions of CDP-peptidomimetics (CDP-*CPP*s) with model membrane in solution. SAXS experiments were performed at CSIR-Centre for Cellular and Molecular Biology (CSIR-CCMB), Hyderabad. The SAXS facility at CSIR-CCMB is S3-MICRO Point-Focus system, Hecus X-ray systems, GmbH with 50 Watt source power and Cu-Kα (λ=1.54 Å) X-rays. The beam size at sample is 50 x 200 μm^2 with photon flux upto 2 X 10^8 photons per sec. Pilatus 100k detector with a pixel size of 172*172 μm^2 with resolution range of 2000 Å to 30 Å was used. Sample-detector distance was maintained at 300 mm and Q-range of 0.003 to 0.6 $Å^{-1}$ that enables to cover a distance range of 2000 to 11 Å. Samples were filled into glass capillaries and flame-sealed. Scans were performed at 22 °C with exposure time of 1 h. Data was collected by using a Pilatus 100K Detector. Diffraction intensity vs Q plots were obtained by radial integration of the 2D patterns by using the interactive data evaluating program FIT2D followed by ATSAS program suite.

1.10 Gel retardation assay

The DNA binding ability of the CDP-*CPP*s was assessed by gel retardation assay on a 1% agarose gel (pre-stained with ethidium bromide) with plasmid DNA and varying peptidomimetics (**Kkd-5** or **Ekd-5**) concentration ratios (1:0.5, 1:1, 1:2, 1:4 and 1:8). pCMV-β-gal (0.30 μg) was complexed with the varying amount of CDP-*CPP*s in a total volume of 30 μL in HEPES buffer (pH 7.4) and incubated at room temperature for 20-25 min. The samples were loaded into the agarose gel (1%) and electrophoresed at 80 V for 80 min. The DNA bands were visualized in the Gel documentation unit.

1.11 EtBr displacement assay

DNA complexation and condensation by **Kkd-5** is assessed through EtBr displacement assay. Fluorescence of EtBr (λ_{ex} 526 nm and λ_{em} = 605 nm) was monitored. To the solution of calf thymus DNA (10 μM) in PBS buffer (10 mM, pH 7.4) EtBr (2 μM) was added. Then the solution was titrated with increasing concentrations of **Kkd-5** (0, 2, 4, 6, 8, 10, 20, 30, 50, 100, 150 and 200 μM) and the emission of EtBr was monitored (Fig. S9).

1.12 Transfection experiment

A reporter gene (pDNA) encoding p-CMV-SPORT-β-gal for β-galactosidase (β-Gal) enzyme was used to evaluate and quantify the transfection efficiency of **Kkd-5, Ekd-5** and Stearyl-R8 in HeLa and HEK293 cell lines. The β-Gal expression was quantified by measuring its enzymatic activity on a known substrate o-nitrophenyl-β-D-galactoside (ONPG). In this assay, ONPG is cleaved into galactose and o-nitrophenol (yellow color), and intensity of the yellow color is directly proportional to the concentration of β-Gal expressed, which in turn, correlates with the transfection efficiency of **Kkd-5, Ekd-5** and Stearyl-R8. Cells were seeded at a density of 10000

cells per well in a 96-well plate and allowed to adhere for 24 h before the transfection. 300 ng of pDNA encoding β-galactosidase enzyme was complexed with varying amounts of peptidomimetics (300, 600, 1200, 2400 ng) in plain DMEM medium (total volume made up to 100 μL) for 30 min. The solution containing pDNA/peptidomimetic complexes were then added to the cells and incubated for 3-4 h. After incubation media was removed and complete medium (media containing 10% serum) was added to the cells and incubated. The reporter gene activity was estimated between 36 and 48 h. The cells were washed with PBS (2 x 100 μL) and lysed with 50 μL lysis buffer [0.25 M Tris-HCl, pH 8.0, 0.5% NP40]. The β-galactosidase activity per well was estimated by adding 50 μL of substrate solution [1.33 mg/mL of ONPG, 0.2 M sodium phosphate (pH 7.3), and 2 mM magnesium chloride] to the lysate in a 96-well plate and read at 405 nm. A calibration curve was constructed with pure commercial β-galactosidase enzyme and absorption values were converted to β-galactosidase units using the calibration curve. Each transfection experiment was carried in triplicate with 3-6 data points. The transfection efficiency values shown are the average of triplicate experiments performed.

1.13 Statistical analysis

ANOVA One-way analysis of variance followed by Tukey's multiple comparison post hoc test was performed to establish the statistical significance. P values < 0.05 was considered significant for experiments.

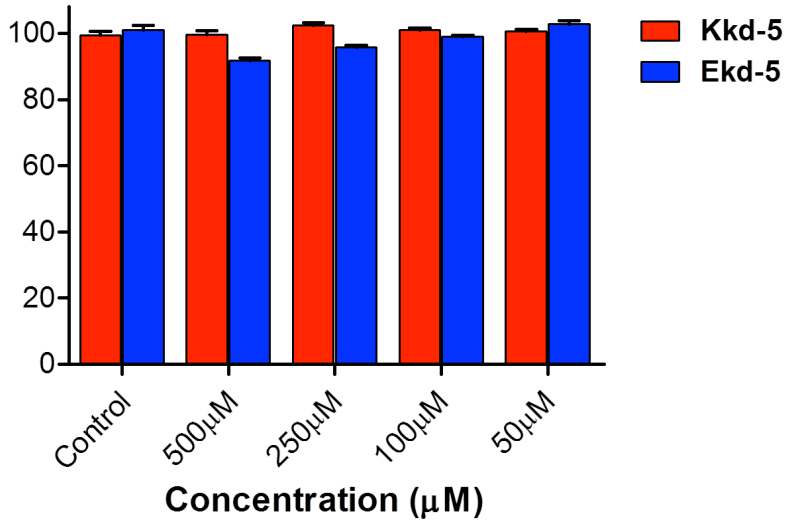


Fig. S1 Cytotoxicity evaluation of **Kkd-5** and **Ekd-5** in HeLa cells at 24 h.

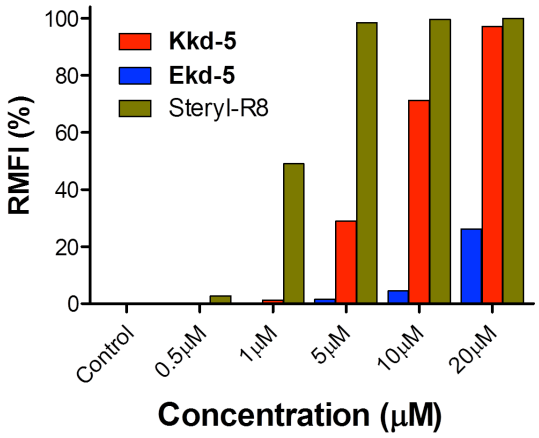


Fig. S2 Cellular uptake studies in HeLa cells. Quantitative cellular uptake of **Kkd-5**, **Ekd-5** and Stearyl-R8 were assessed by the flow cytometry analysis. RMFI= Relative mean fluorescence intensity.

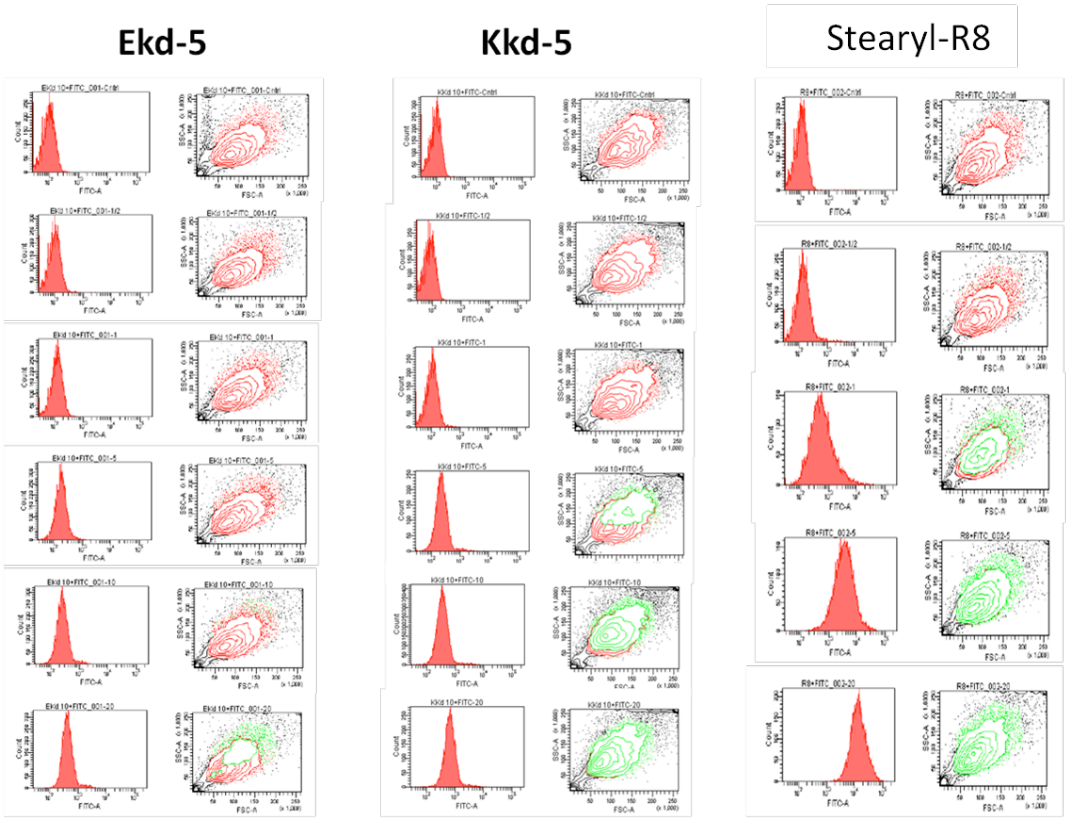


Fig. S3 Cellular uptake studies in HeLa cells. Quantitative cellular uptake of FITC labeled **Ekd-5**, **Kkd-5** and positive control Stearyl-R8 was assessed by the flow cytometry analysis. Cells were treated with peptidomimetics at varying concentrations for 4 h and subjected for flow cytometric analysis.

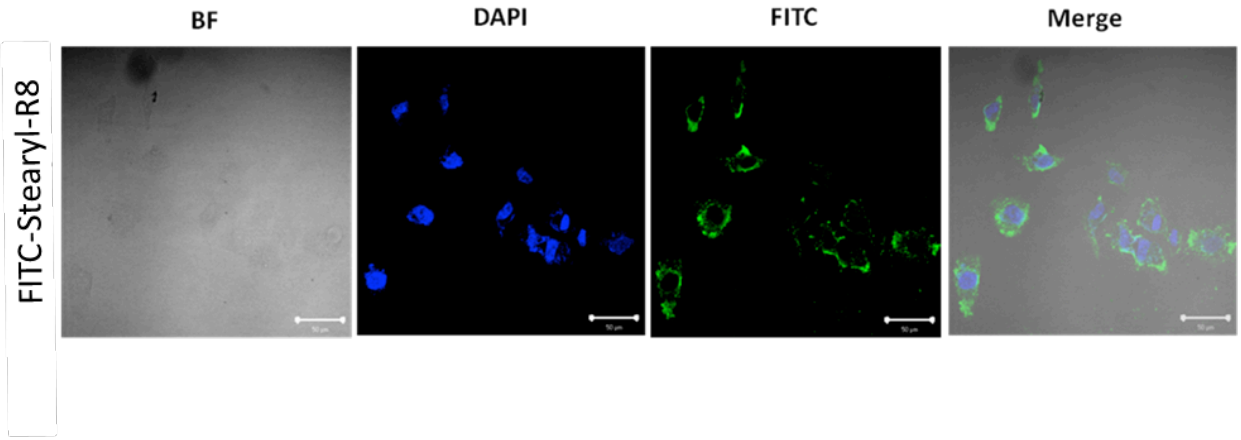


Fig. S4 Confocal microscopy images for cellular translocation and localization of FITC-Stearyl-R8.

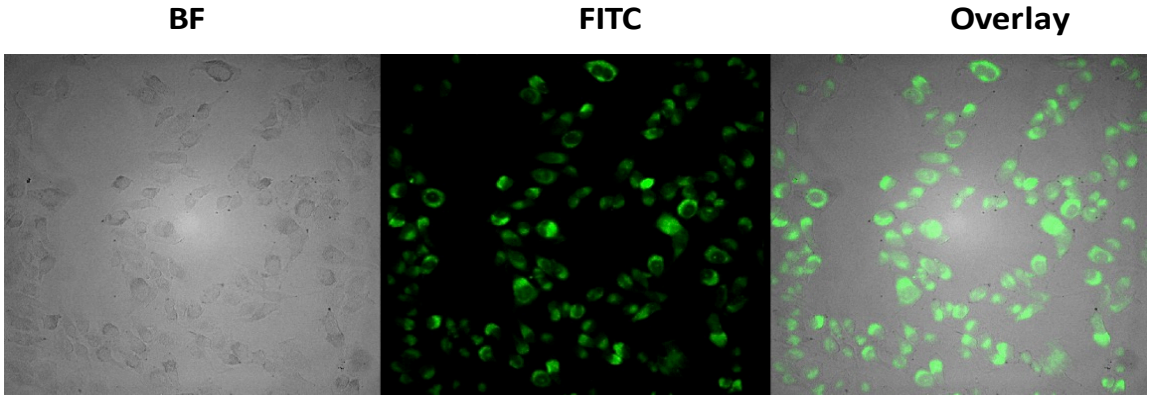


Fig. S5 Fluorescence microscopy images of FITC-**kd-4** (50 μM) in PC12 cells (live) which show cellular translocation and localization.

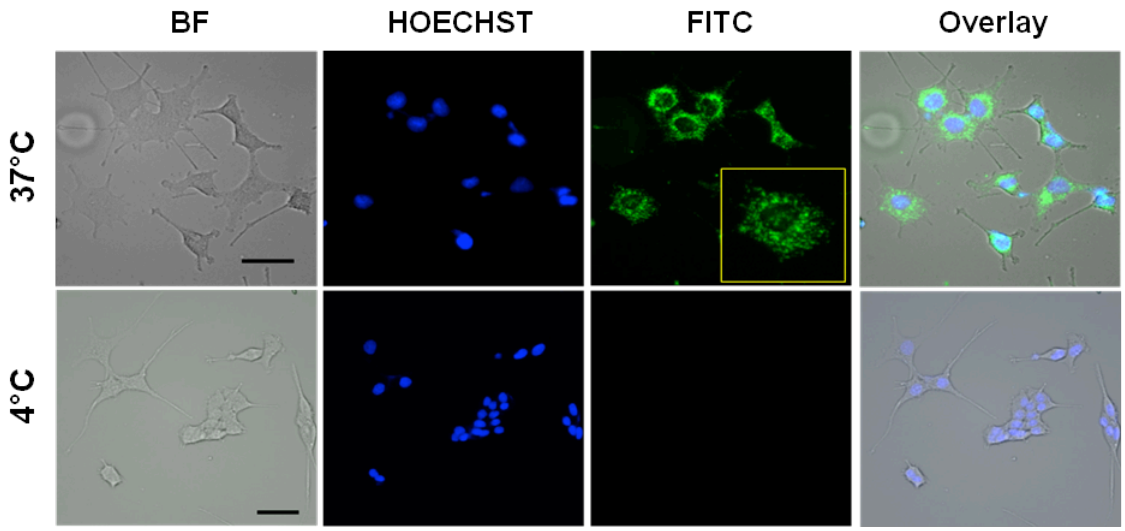


Fig. S6 **Kkd-5** internalization into the PC12 cells at 37 °C and 4 °C. Scale bar = 50 µM.

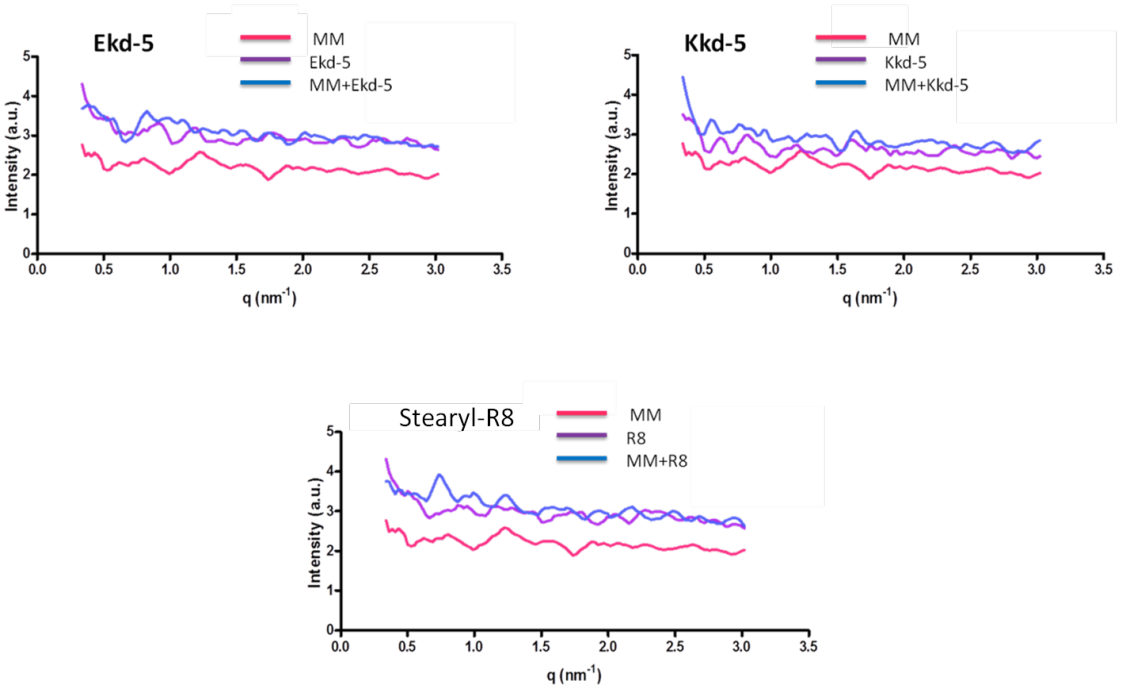


Fig. S7 SAXS data of **Ekd-5**, **Kkd-5** and Stearyl-R8, alone and co-incubation with model membrane (MM) at 1:1 molar ratio. Radially integrated scattering intensities of **Ekd-5**, **Kkd-5** and Stearyl-R8, with and without the MM. MM is a phospholipid mixture of DOPC:DOPE:DOPS:Chol (45:20:20:15), w/w content that closely mimics mammalian cell membrane. Curve smoothing was done using GraphPad Prism software.

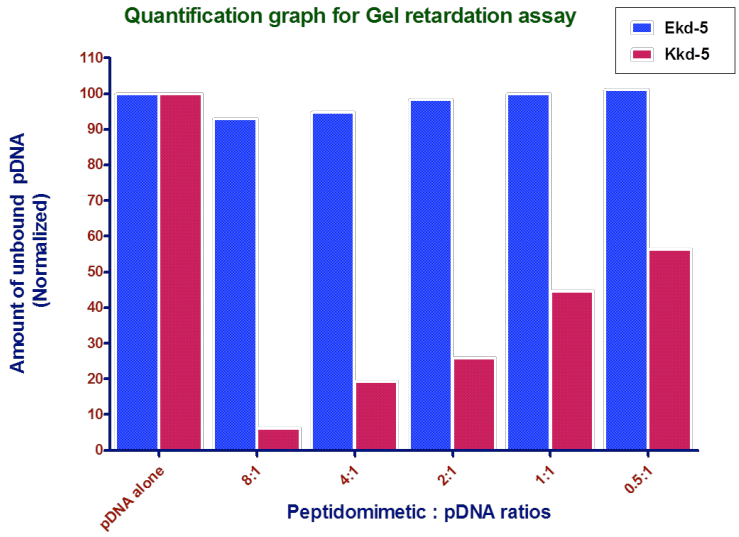


Fig. S8 Quantification graph for gel retardation assay as shown in Figure 3b (Normalized to 100% with unbound pDNA alone).

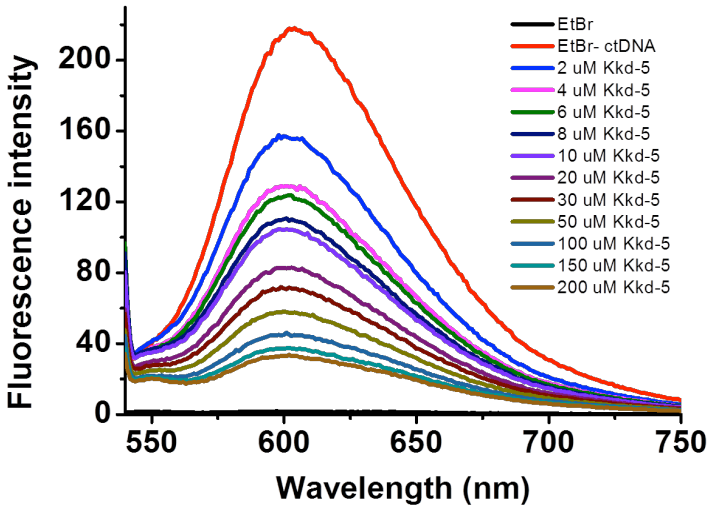


Fig. S9 Fluorescence titration of calf thymus DNA (ctDNA) (10 μM) and EtBr (2 μM) complex with increasing concentration of **Kkd-5** (0, 2, 4, 6, 8, 10, 20, 30, 50, 100, 150 and 200 μM).

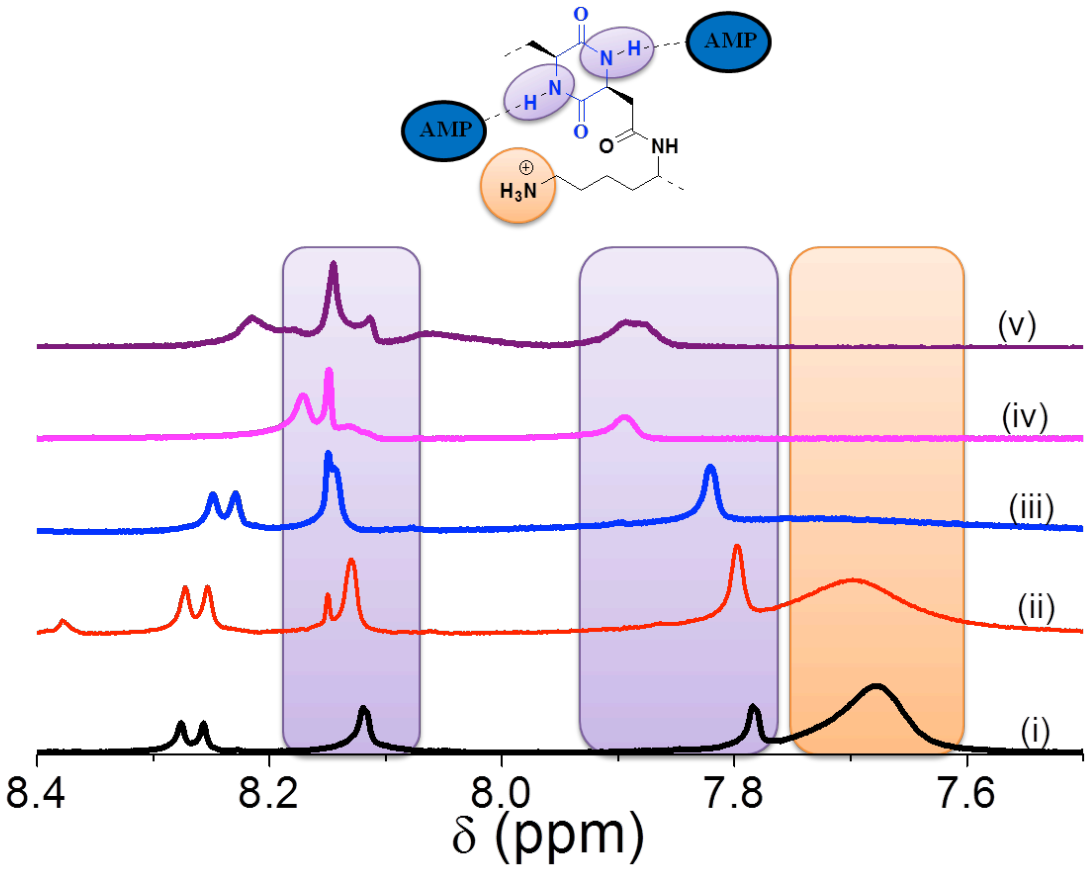
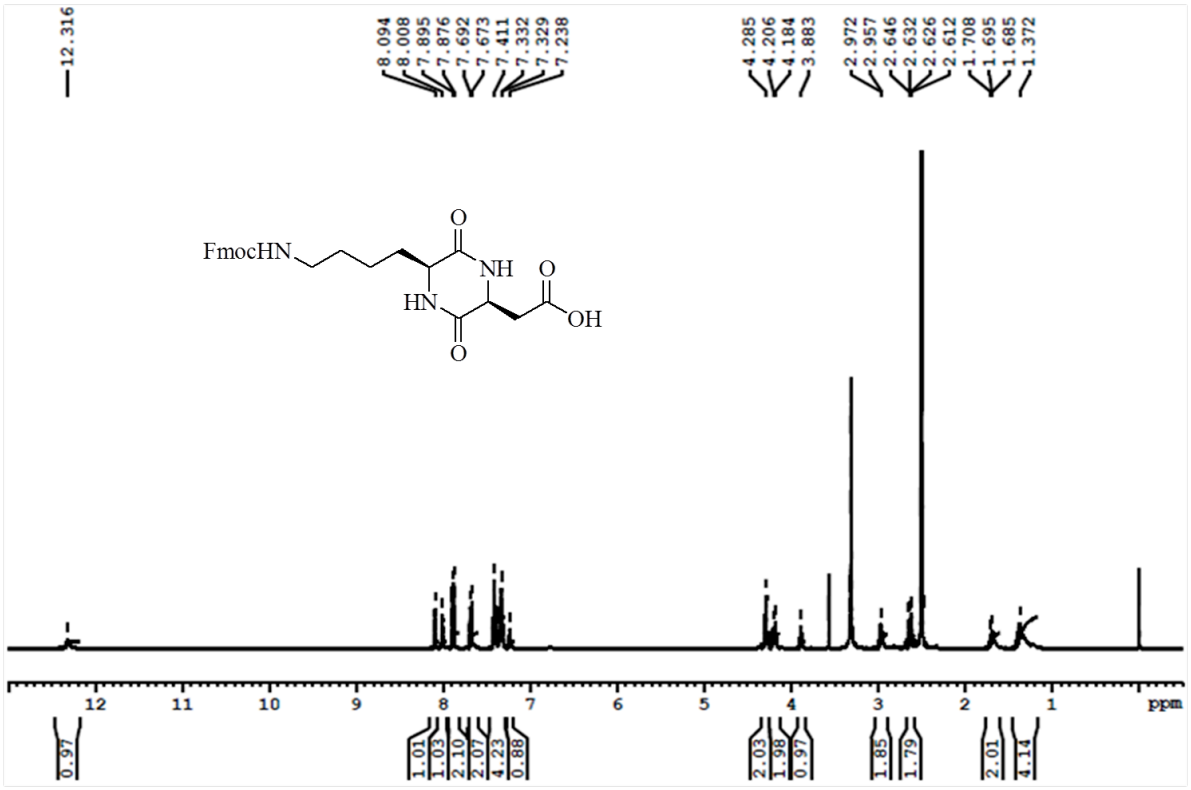


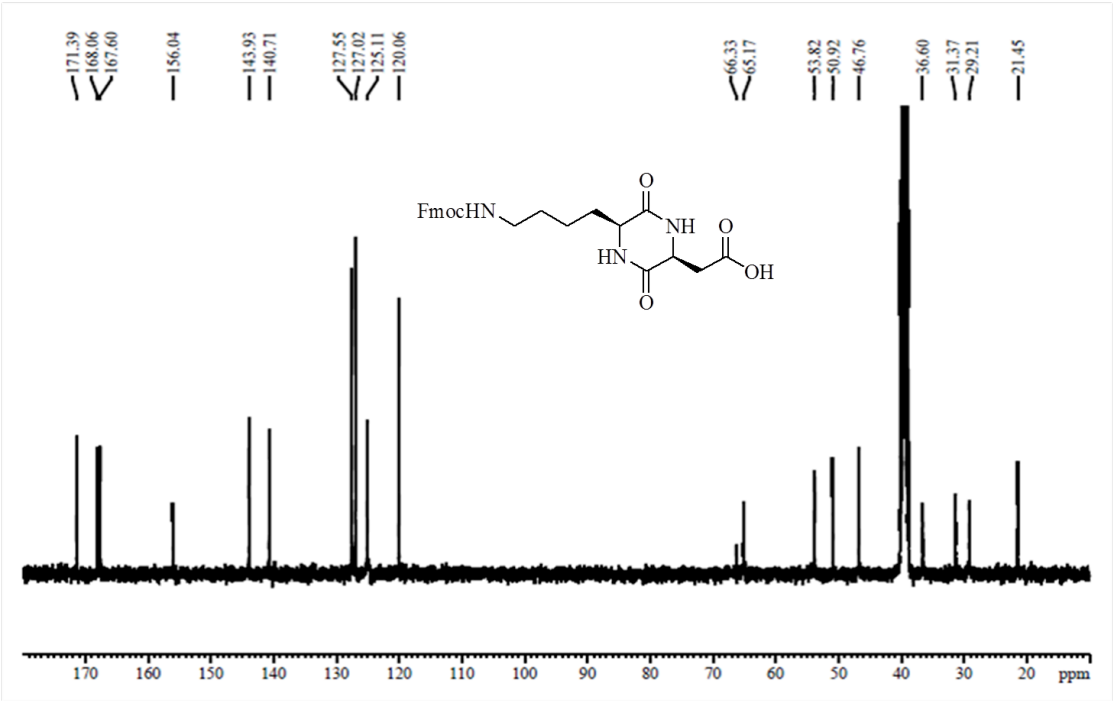
Fig. S10 NMR titration data for **Kkd** with increasing adenosine monophosphate (AMP) concentration (mM) 0 (i), 1 (ii), 2 (iii), 4 (iv), 8 (v).

NMR Data

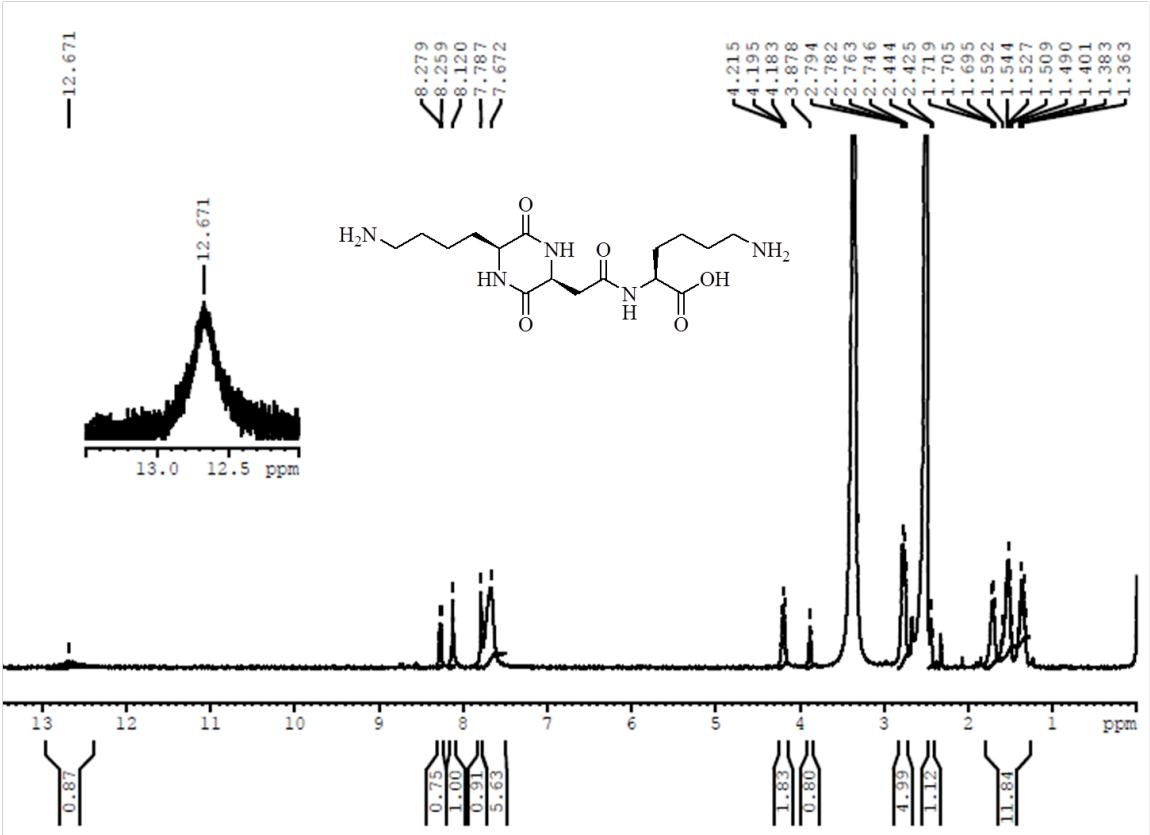
^{1}H NMR spectrum of Fmoc-**kd**



^{13}C NMR spectrum of Fmoc-**kd**



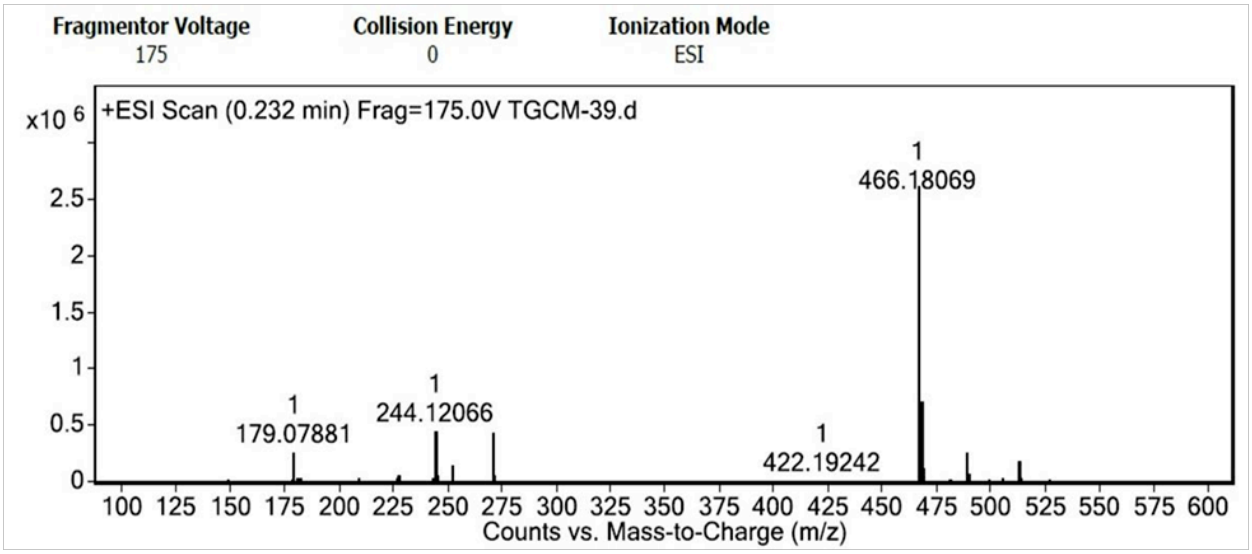
^{1}H NMR spectrum of **Kkd**



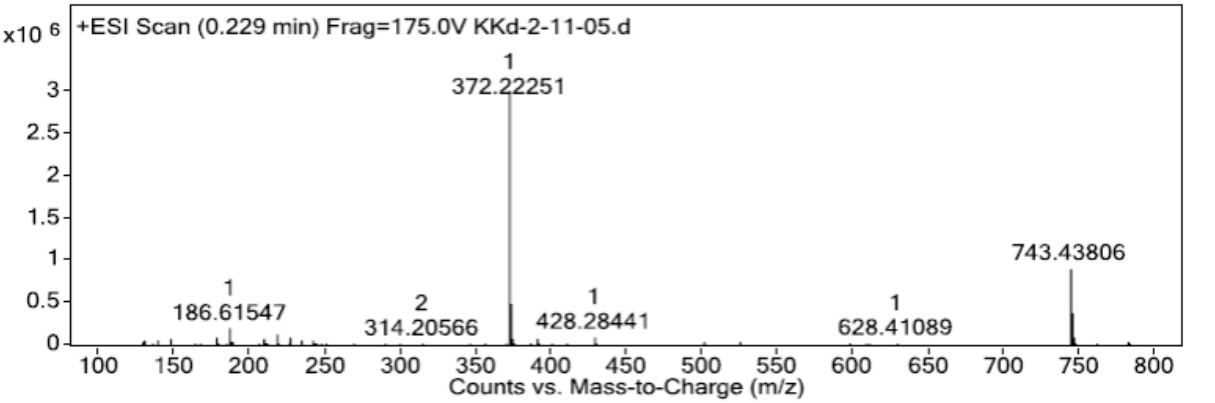
HRMS data

Compound	Calcd. mass	Obtained mass
Fmoc-**kd**	465.1900	466.1807 [M+H]
Kkd	371.2169	372.2225
Kkd-5	1784.0421	893.5279 [M^{+}/2], 595.6887 [M^{+}/3]
Ekd-5	1788.7802	895.3952 [M^{+}/2], 597.2663 [M^{+}/3]
FITC-**Kkd-5**	2173.0779	1088.0433 [M^{+}/2]
FITC-**Ekd-5**	2177.816	1098.4131 [M^{+}/2]
FITC-**kd-4**	1306.5073	1307.4981[M+H], 654.2555 [M^{+}/2]

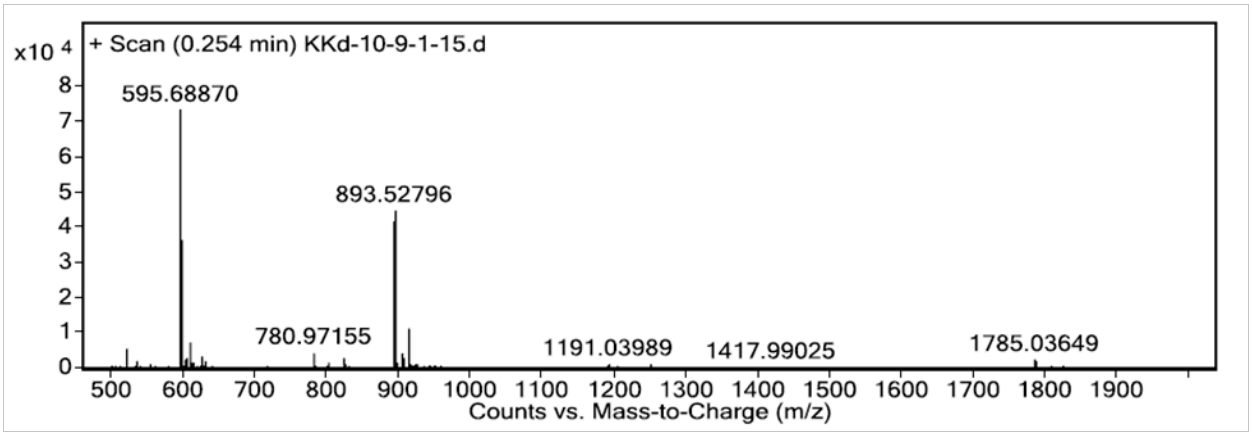
HRMS spectrum of Fmoc-**kd**



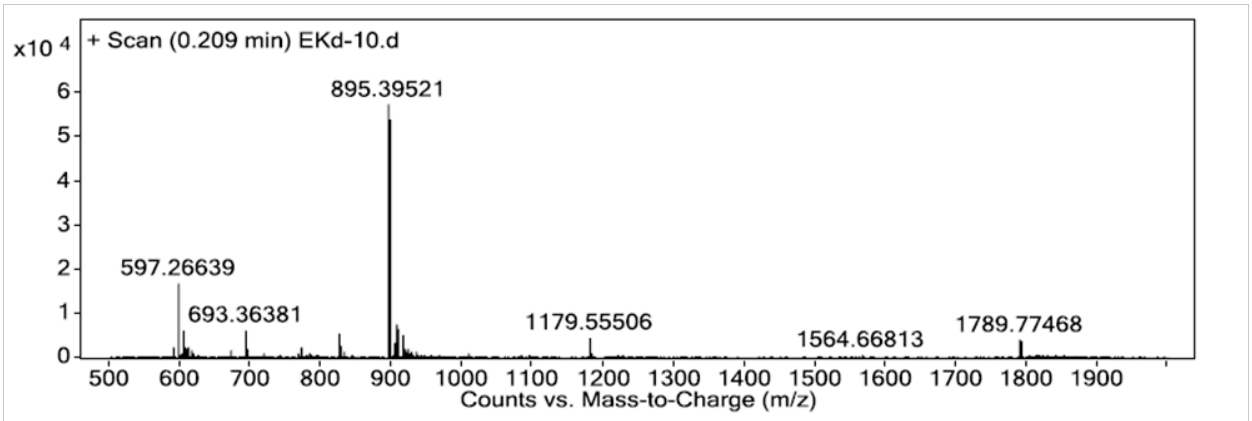
HRMS spectrum of **Kkd**



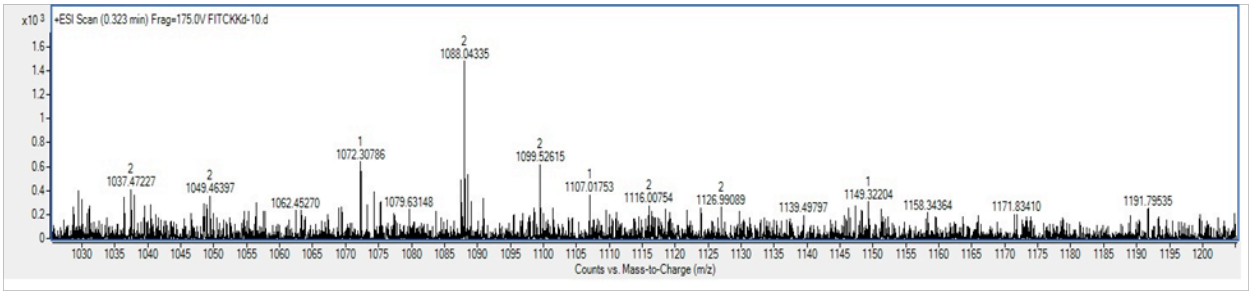
HRMS spectrum of **Kkd-5**



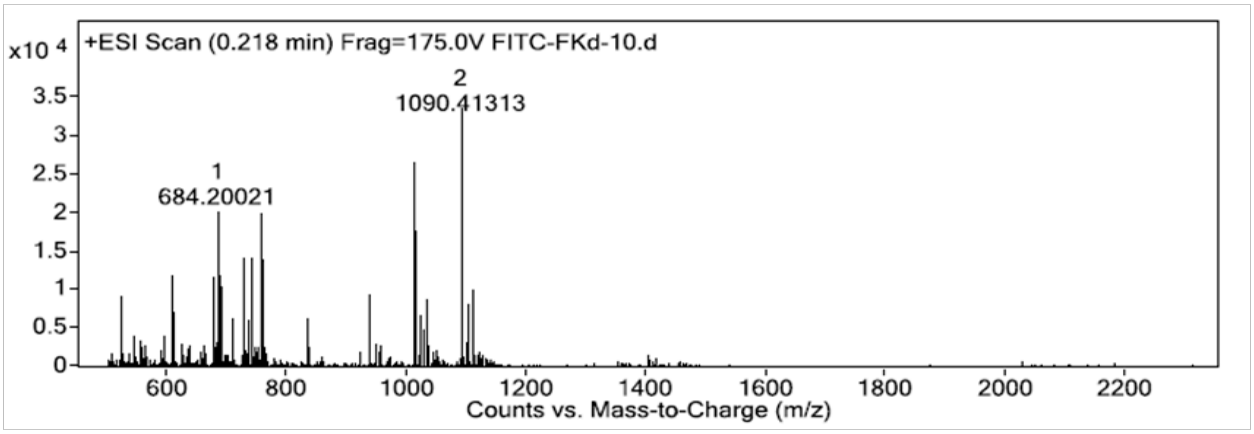
HRMS spectrum of **Ekd-5**



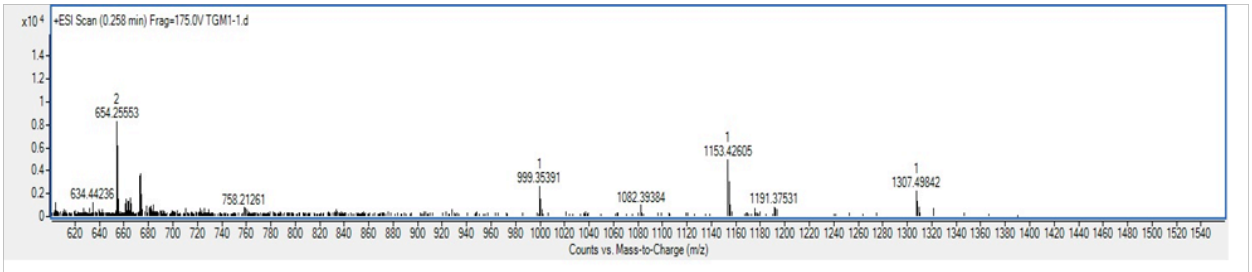
HRMS spectrum of FITC-**Kkd-5**



HRMS spectrum of FITC-**Ekd-5**

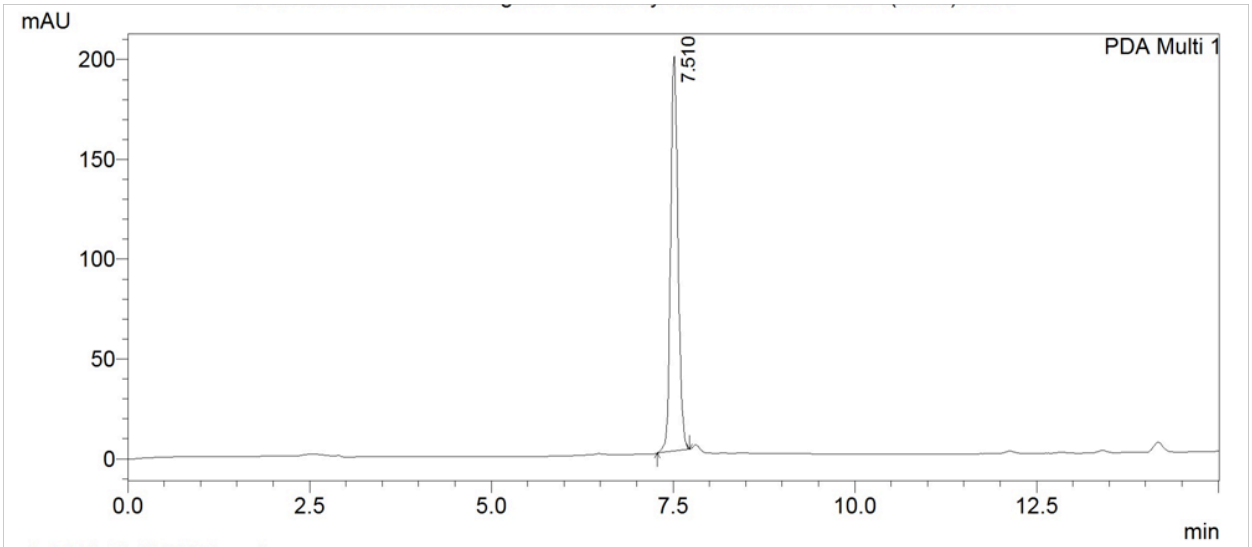


HRMS spectrum of FITC-**kd-4**



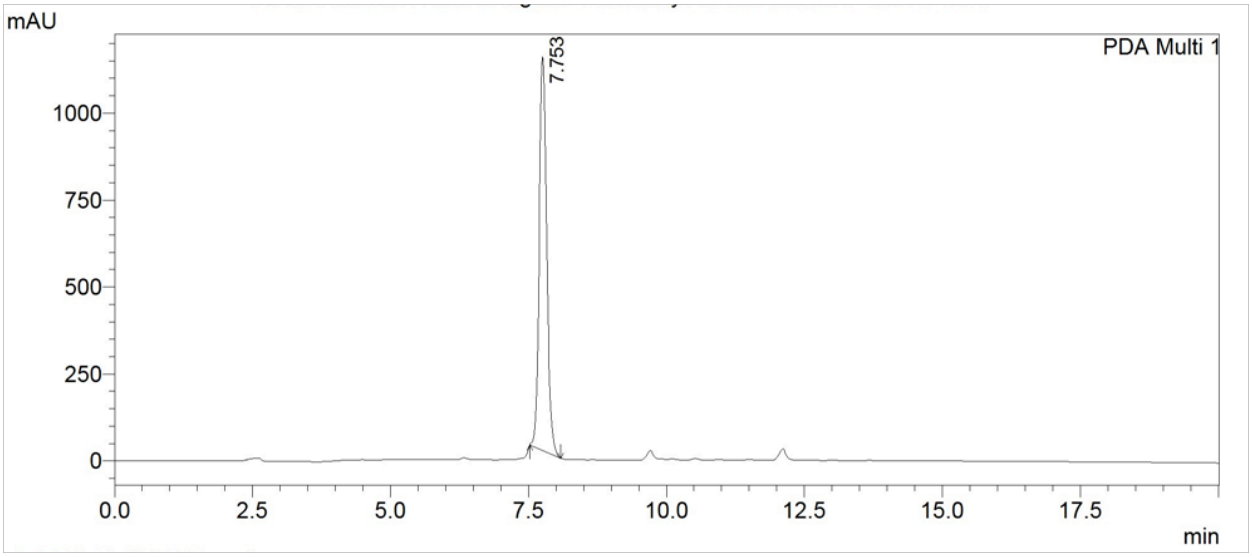
HPLC Data

HPLC chromatogram of **Kkd-5**



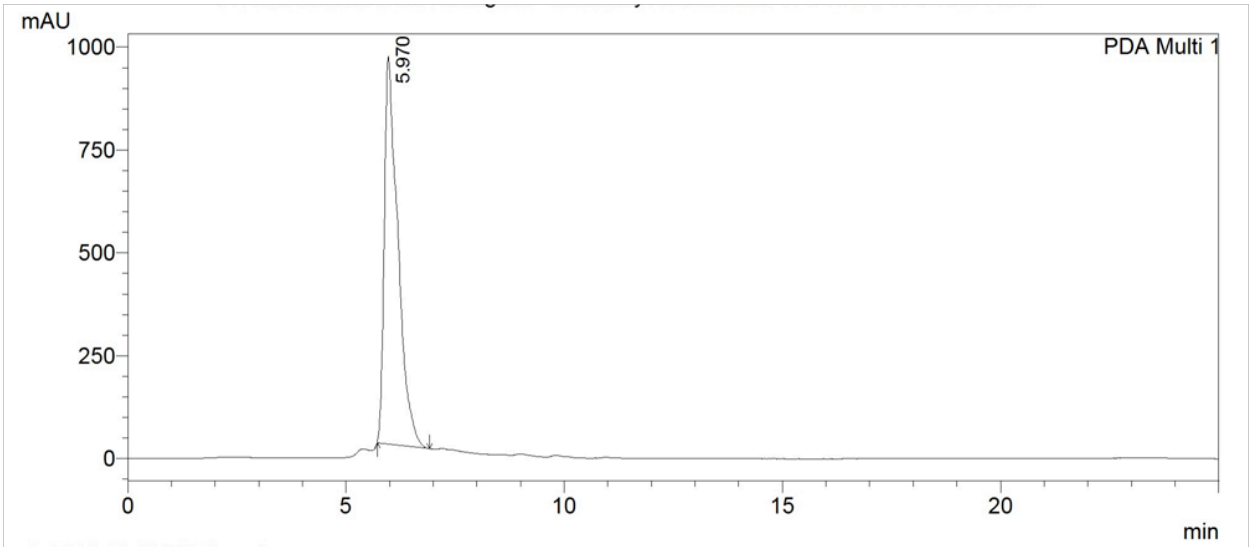
HPLC Conditions: System: Shimadzu, Column: Phenomenex, RP-C18 (10 µm), Mobile Phase: Binary gradient (Acetonitrile: Water; at 0 min 0% acetonitrine and at 15 min 100% acetonitrile), Flow Rate: 8 mL/min, Detection: UV@220 nm.

HPLC chromatogram of **Ekd-5**

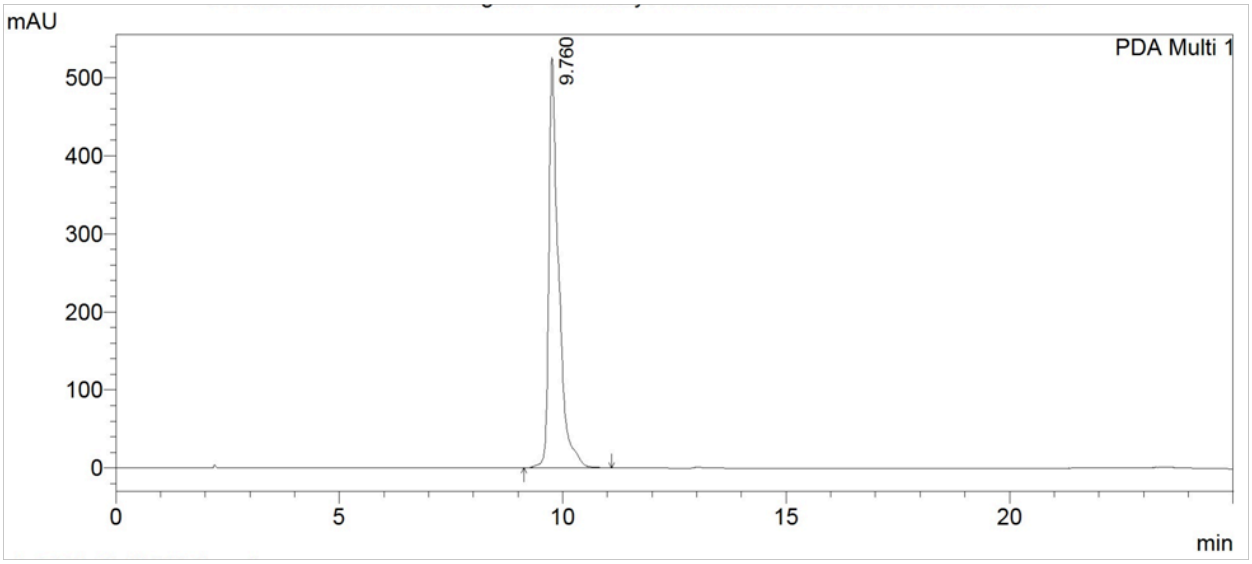


HPLC Conditions: System: Shimadzu, Column: Phenomenex, RP-C18 (10 µm), Mobile Phase: Binary gradient (Acetonitrile: Water; at 0 min 10% acetonitrile and at 20 min 100% acetonitrile), Flow Rate: 8 mL/min, Detection: UV@220 nm

HPLC chromatogram of FITC-**Kkd-5**



HPLC chromatogram of FITC-**Ekd-5**



HPLC Conditions:

System: Shimadzu, Column: Phenomenex, RP-C18 (10µm), Mobile Phase: Binary gradient (Acetonitrile: Water; at 0 min 10% acetonitrile and at 25 min 100% acetonitrile), Flow Rate: 8 mL/min, Detection: UV@254 nm

References

1 R. Halai, B. Callaghan, N. L. Daly, R. J. Clark, D. J. Adams and D. J. Craik, *J. Med. Chem*., 2011, **54**, 6984-6992.