Supporting Information

Design of peptide therapeutics as protein-protein interaction inhibitors to treat neurodegenerative diseases

Daryl Ariawan[1†], Kanishka Pushpitha Maha Thananthirige[1†], Ali El-Omar[1], Julia van der Hoven[1], Sian Genoud[1], Holly Stefen[1], Thomas Fath[1], Janet van Eersel[1], Lars Ittner[1], Ole Tietz[1*].

[1] Dementia Research Centre, Macquarie Medical School, Faculty of Medicine, Health and Human Sciences, Macquarie University, North Ryde, Sydney, NSW 2109, Australia.

CONTACT: * telephone: +61 (2) 9850 2649; e-mail: ole.tietz@mq.edu.au

Table of contents

S1. Experimental materials and instruments

Solvents (DMF, DCM, CH_3CN) were obtained from ChemSupply (ChemSupply, Australia). Amino acids, coupling reagents and resins for Fmoc-solid-phase peptide synthesis (SPPS) were obtained from either ChemImpex (ChemImpex International, US) or Sigma Aldrich unless otherwise stated. Automated solid phase peptide synthesis (SPPS) was performed on a CEM Liberty Blue™ Peptide Synthesiser (CEM, USA) according to manufacturer's operating instructions. Manual SPPS work up, as well fluorophore labelling, was performed in polypropylene syringes equipped with porous filter disc, purchased from Torviq.

Analytical and semi-preparative reversed-phase high performance liquid chromatography (HPLC) was performed on a Shimadzu LC20AD series HPLC system (Shimadzu, Japan) equipped with PDA detector (λ = 200 – 800 nm), Autosampler and Fraction Collector. Peptides were analyzed using an analytical Polaris 3 C18-A 150 x 4.6 mm column (Agilent Technologies, USA) at a flow rate of 1.0 mL min^{-1}, and purified using a semi-prep Polaris 3 C18-A 150 x 4.6 mm column (Agilent Technologies, USA) at a flow rate of 5.0 mL min^{-1}. HPLC column was kept at 30°C, chromatograms were collected at 210 nm unless otherwise stated. Gradients for analytical HPLC were run over 17 minutes using a mobile phase composed of 0.1% formic acid (FA) in H_2O (Solvent A) and 0.1% FA in CH_3CN (Solvent B) in a linear gradient as indicated; gradients for semi-preparative HPLC were run over 17 minutes using a mobile phase composed of 0.1% formic acid (FA) in H_2O (Solvent A) and 0.1% FA in CH_3CN (Solvent B) in a linear gradient as indicated. Semi-preparative injections of 0.6 ml to 1.2 mL were carried out onto a 2.0 mL loop using autosampler module. Analysis of the chromatograms was conducted using

LabSolutions proprietary software (Shimadzu, Japan) and retention times (r_t min) of pure peptides reported with the gradients specified.

Peptides were flash frozen in liquid Nitrogen and lyophilized using ScanVac Cool Safe Touch (Labogene, Denmark). Mass spectra were obtained on a Shimadzu LCMS-8050 LCMS system (Shimadzu, Japan) in positive electron spray [ESI+] mode, fitted with a Polaris 3 C18-A 50 x 4.6 mm column (Agilent Technologies, USA). Liquid Chromatography system was operated using LCMS grade solvents and mobile phase of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) and a linear gradient of 5-95% B over 5 min. Data analysis was performed using LabSolutions v5.91 proprietary software.

S2. Peptide Synthesis

S2.1. General Peptide Synthesis

Automated SPPS was carried out using standard Fluorenyl methoxycarbonyl (Fmoc)-solid-phase peptide synthesis on low swell 100 – 200 mesh Wang resin (0.05 mmol reaction scale, 0.5 mmol g^{-1} loading) on a CEM Liberty Blue™ Peptide Synthesiser (CEM, USA). *Initial amino acid loading*: Wang resin (100-200 mesh; 0.65 mmol g^{-1}, 77 mg, 0.05 mmol) was weighed into a 10 mL polypropylene syringe equipped with a porous polypropylene frit, which was used as the reaction vessel. The resin was washed with dichloromethane (3 × 5 mL) before being allowed to swell in dichloromethane (5 mL) for at least 0.5 h prior to the loading of the first amino acid. A solution of Fmoc-AA-OH (4 equiv.) was dissolved in a mixture of dry dichloromethane (2 mL), N,N-dimethylformamide (2 mL), hydroxybenzotriazole (HOBt) (4 equiv.), and N,N'-diisopropylcarbodiimide (DIC) (4 equiv.) and taken up into the syringe with resin and stirred overnight using an orbital shaker. The resin was then capped with acetic anhydride (0.1 mL) and N,N-diisopropylethylamine (DIPEA) (0.1 mL) in dichloromethane (3 mL) for 30 min. The resin was then washed with dichloromethane (3 x 4 mL) and N,N-dimethylformamide (DMF) (3 × 4 mL). *Automated peptide synthesizer:* Resin was pre-swelled in 50/50 dimethylformamide (DMF) and dichloromethane (DCM) for 1 hour. Amino acids were dissolved in DMF at a concentration of 0.2 M before being transferred to the synthesizer. Peptides were synthesized using sequential amid coupling from C to N-terminus for 5 minutes at 75°C, using five equivalents of amino acid with 10 equivalents of activator (0.5 M DIC (N,N′-Diisopropylcarbodiimide) in DMF) and 5 equivalents of activator base (0.5 M Oxyma (Ethyl cyanohydroxyiminoacetate), 0.05 M DIPEA (N,N-Diisopropylethylamine) in DMF), followed by Fmoc deprotection in 20% piperidine in

DMF for 3 minutes at 75°C and 3x resin wash in DMF. Following final Fmoc deprotection, resin was removed from synthesizer, transferred to a syringe fitted with a propylene filter, and either labelled with fluorophore (*see below*) or washed with DMF (x3), DCM (x3), and methanol (x3). Double couplings were performed for arginine residues to ensure complete coupling. *Cleavage*: Peptide was cleaved from resin using cleavage cocktail of 92.5% TFA (trifluoroacetic acid), 2.5% TIPS (triisopropylsilane), 2.5% thioanisole, and 2.5% H_2O for 3 hours at room temperature, precipitated in ice cold diethyl ether, dissolved in H_2O, freeze dried, and purified using a Shimadzu LC-20AD High-performance liquid chromatography (HPLC, Shimadzu, Japan).

S2.2. General TAMRA labelling

To a peptide bound resin in a syringe fitted with a propylene filter, 5-carboxytetramethylrhodamine (5-TAMRA, 2 equiv.), HOBt (3 equiv.), (benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (3 equiv.), and DIPEA (6 equiv.) in DMF (4 mL) was taken up to the syringe and agitated overnight in orbital shaker. Resin was then washed with DMF (x3), DCM (x3), and methanol (x3) and proceeded to cleavage.

S2.3. Peptide 1 - Tat-NR2B9c (nerinetide)

Tat-NR2B9c (1)

Sequence: YGRKKRRQRRRKLSSIESDV-OH

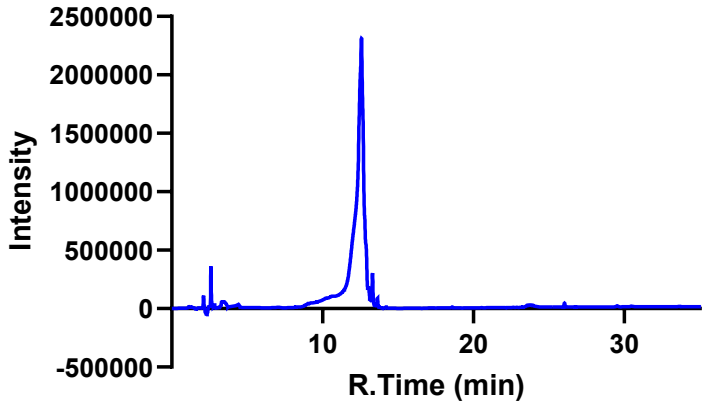
Molecular Weight: 2518.88 g mol^{-1}

LCMS [ESI+]: 1260.20 (m/z_2; calculated: 1260.44), 840.45 (m/z_3; calculated: 840.63), 630.75 (m/z_4; calculated: 630.72), 504.95 (m/z_5; calculated: 504.78), 420.90 (m/z_6; calculated: 420.81)

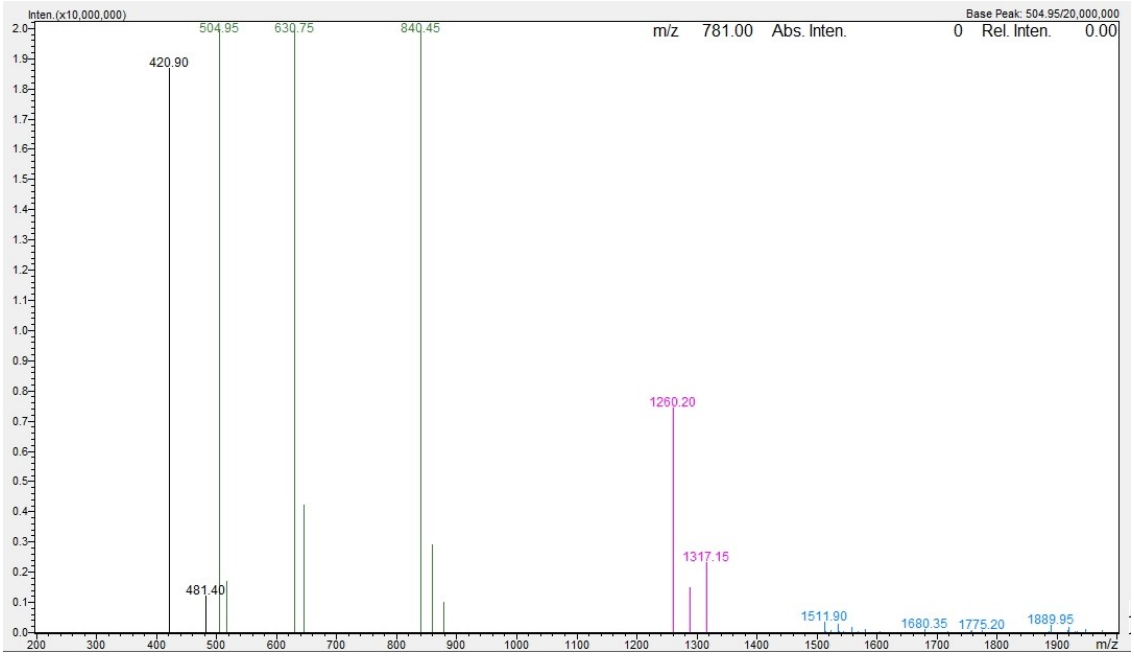
HPLC retention time r_t = 12.5 min

Purity > 95%

Yield: 28% (35 mg, 0.014 mmol)



HPLC trace of Tat-NR2B9c (**1**) at 210 nm



Non-deconvoluted LCMS mass spectra [ES+] of Tat-NR2B9c (**1**)

S2.4. Peptide 2 – TAMRA-Tat

TAMRA-Tat (2)

Sequence: *TAMRA*-YGRKKRRQRRR-OH

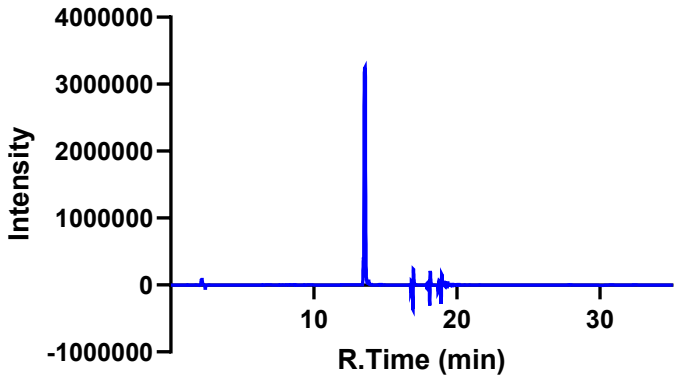
Molecular Weight: 1972.27 g mol-1

LCMS [ESI+]: 986.90 (m/z_2; calculated: 987.14), 658.50 (m/z_3; calculated: 658.42), 494.20 (m/z_4; calculated: 494.07), 395.65 (m/z_5; calculated: 395.45), 329.95 (m/z_6; calculated: 329.71)

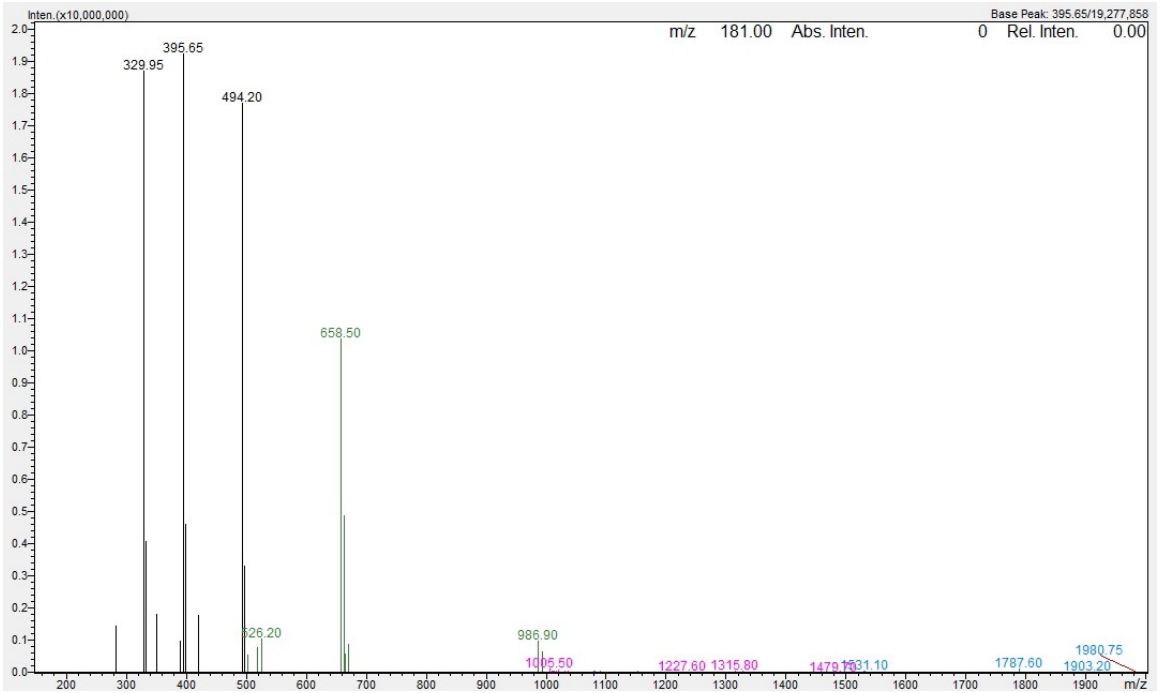
HPLC retention time r_t = 13.5 min

Purity > 95%

Yield: 14% (14 mg, 0.0071 mmol)



HPLC trace of TAMRA-Tat (**2**) at 210 nm



Non-deconvoluted LCMS mass spectra [ES+] of TAMRA-Tat (**2**)

S2.5. Peptide 3 – TAMRA-NR2B9c

TAMRA-NR2B9c (3)

Sequence: *TAMRA*-KLSSIESDV-OH

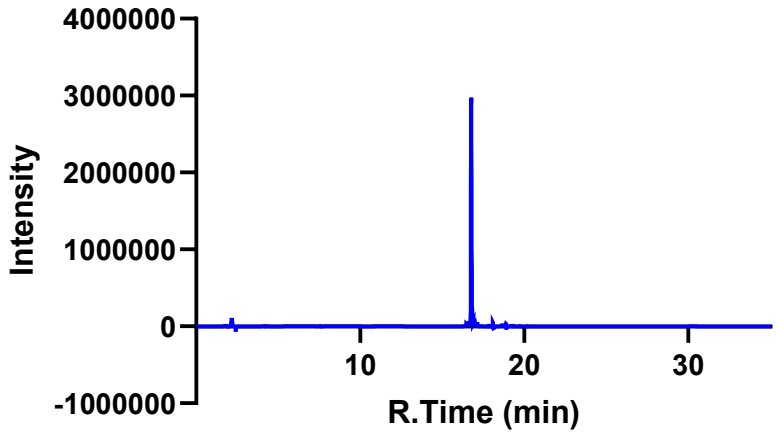
Molecular Weight: 1389.50 g mol-1

LCMS [ESI+]: 1389.80 (m/z_1; calculated: 1390.50), 695.70 (m/z_2; calculated: 695.75)

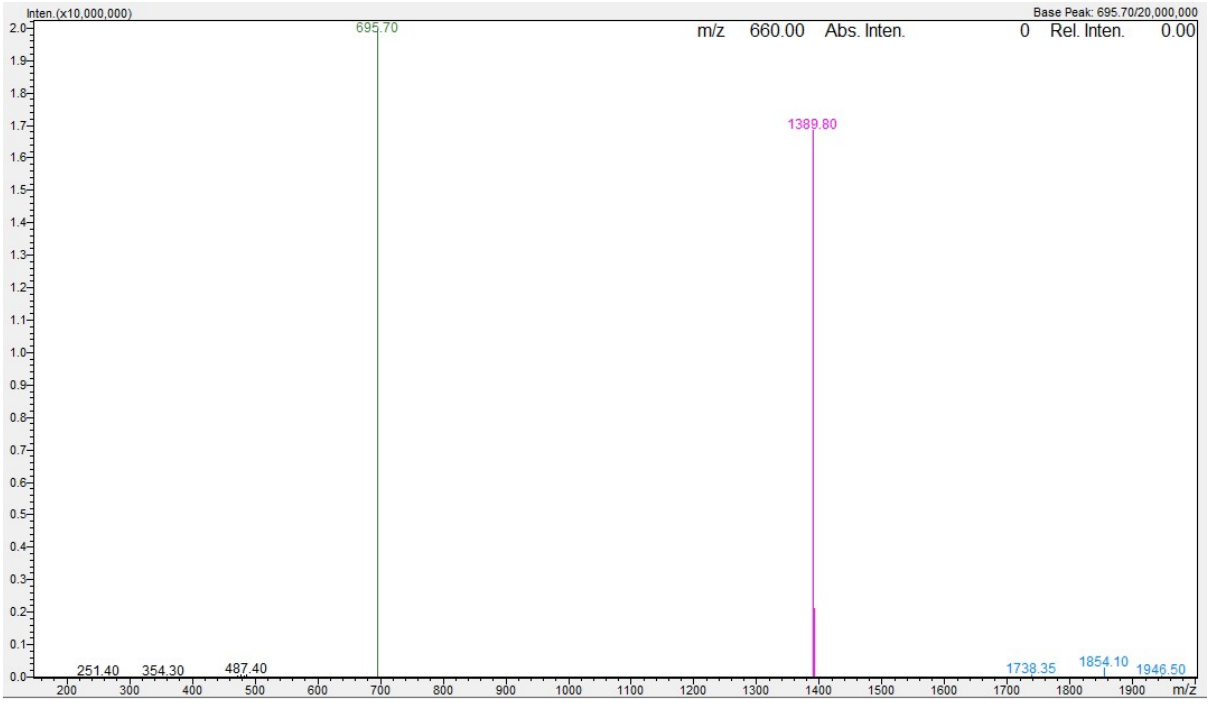
HPLC retention time r_t = 16.8 min

Purity > 95%

Yield: 50% (35 mg, 0.025 mmol)



HPLC trace of TAMRA-NR2B9c (**3**) at 210 nm



Non-deconvoluted LCMS mass spectra [ES+] of TAMRA-NR2B9c (**3**)

S2.6. Peptide 4 – TAMRA-D-Tat

TAMRA-D-Tat (4)

Sequence: *TAMRA*-ygrkkrrqrrr-OH

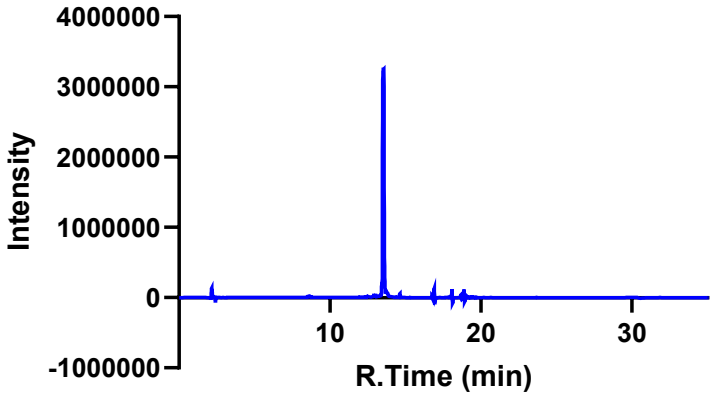
Molecular Weight: 1972.27 g mol-1

LCMS [ESI+]: 987.20 (m/z_2; calculated: 987.14), 658.50 (m/z_3; calculated: 658.42), 494.15 (m/z_4; calculated: 494.07), 395.65 (m/z_5; calculated: 395.45), 329.95 (m/z_6; calculated: 329.71)

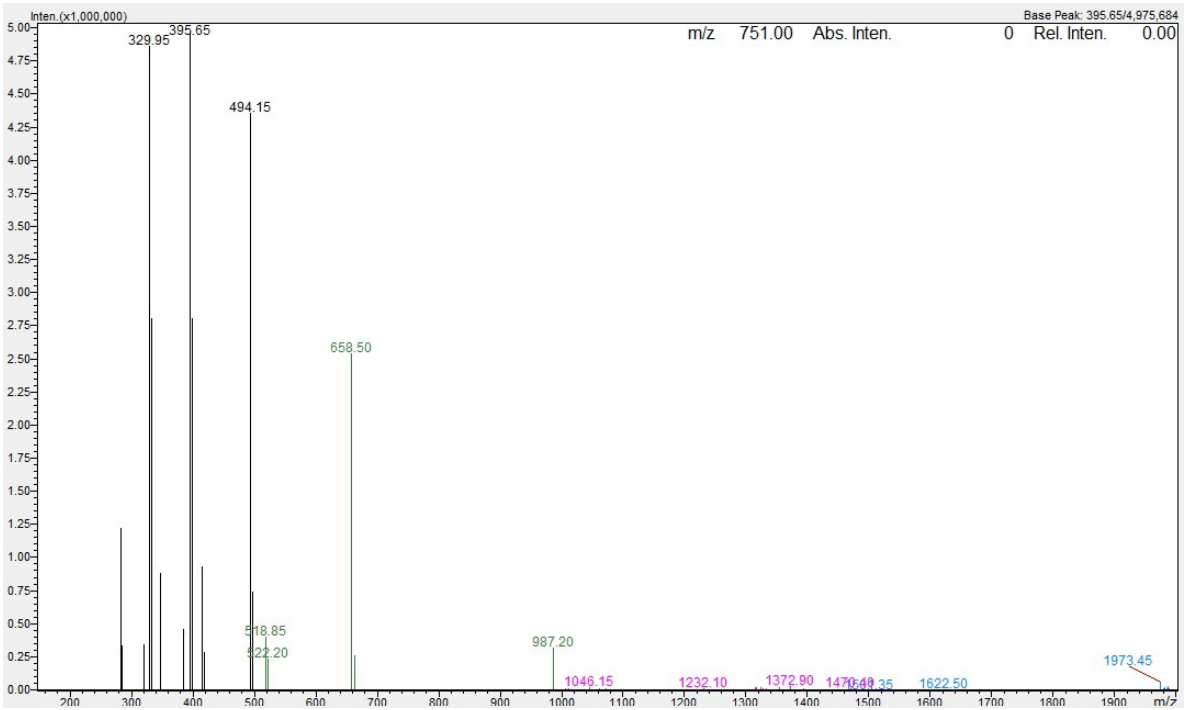
HPLC retention time r_t = 13.6 min

Purity > 95%

Yield: 14% (14 mg, 0.0071 mmol)



HPLC trace of TAMRA-D-Tat (**4**) at 210 nm



Non-deconvoluted LCMS mass spectra [ES+] of TAMRA-D-Tat (**4**)

S2.7. Peptide 5 – TAMRA-NR2Baa

TAMRA-NR2Baa (5)

Sequence: *TAMRA*-KLSSIEADA-OH

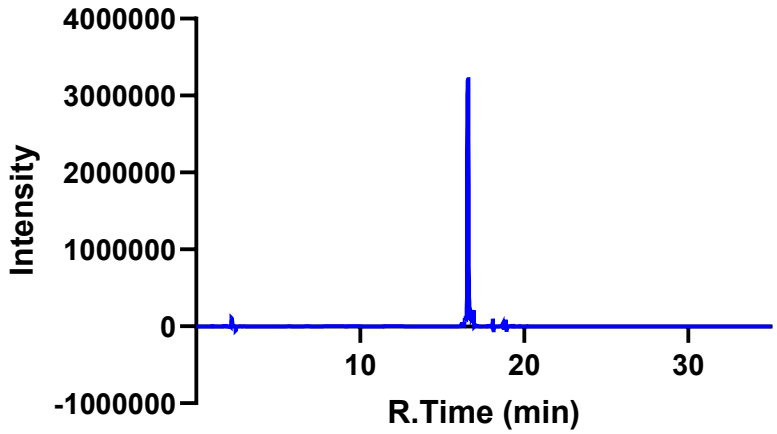
Molecular Weight: 1345.45 g mol-1

LCMS [ESI+]: 1345.70 (m/z_1; calculated: 1346.45), 673.70 (m/z_2; calculated: 695.73)

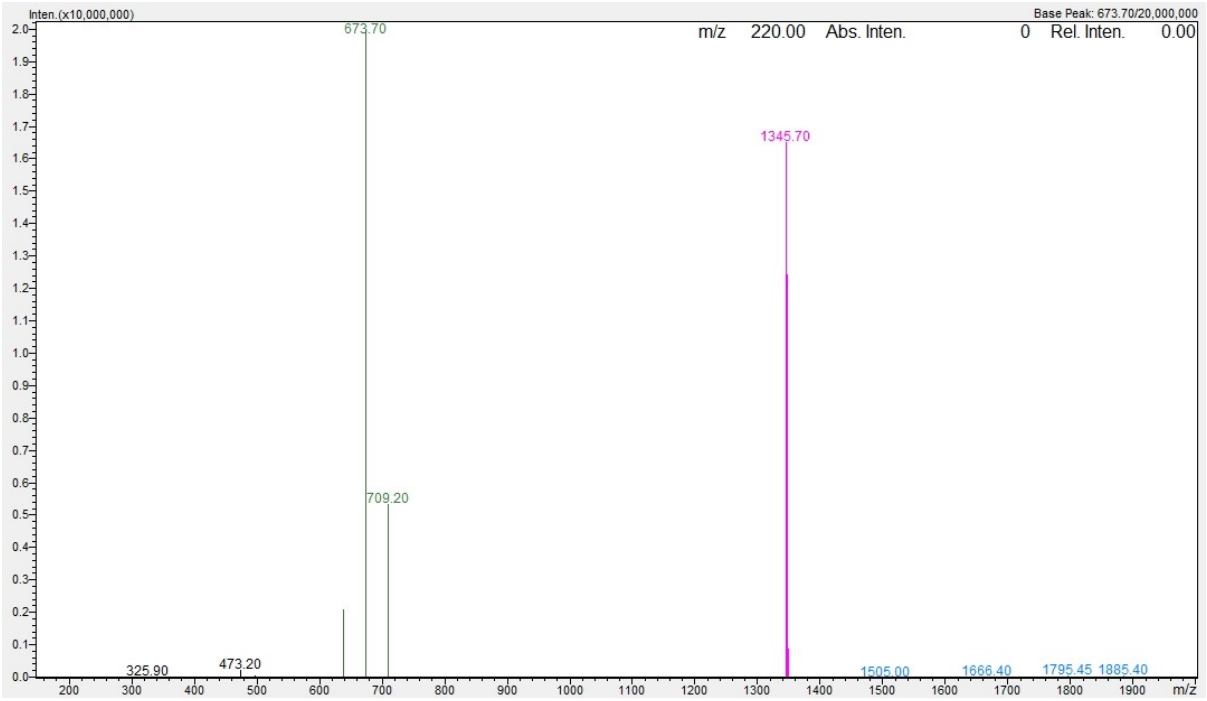
HPLC retention time r_t = 16.5 min

Purity > 95 %

Yield: 61 % (41 mg, 0.030 mmol)



HPLC trace of TAMRA-NR2Baa (**5**) at 210 nm



Non-deconvoluted LCMS mass spectra [ES+] of TAMRA-NR2Baa (**5**)

S2.8. Peptide 6 – TAMRA-Tat-NR2B9c

TAMRA-Tat-NR2B9c (6)

Sequence: *TAMRA*-YGRKKRRQRRRKLSSIESDV-OH

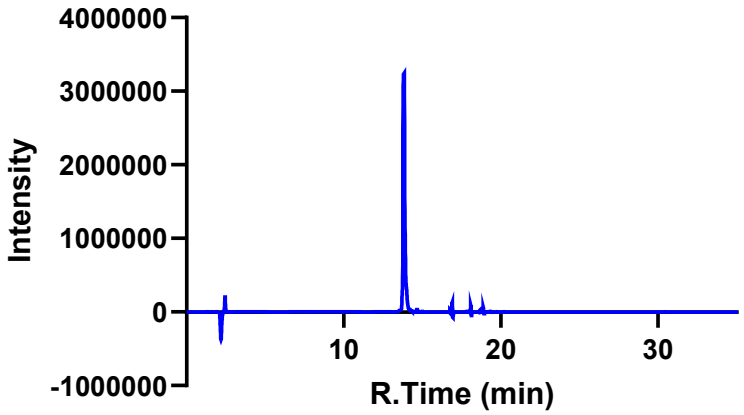
Molecular Weight: 2931.32 g mol-1

LCMS [ESI+]: 977.80 (m/z_3; calculated: 978.11), 733.95 (m/z_4; calculated: 733.83), 587.50 (m/z_5; calculated: 587.26), 489.80 (m/z_6; calculated: 489.55)

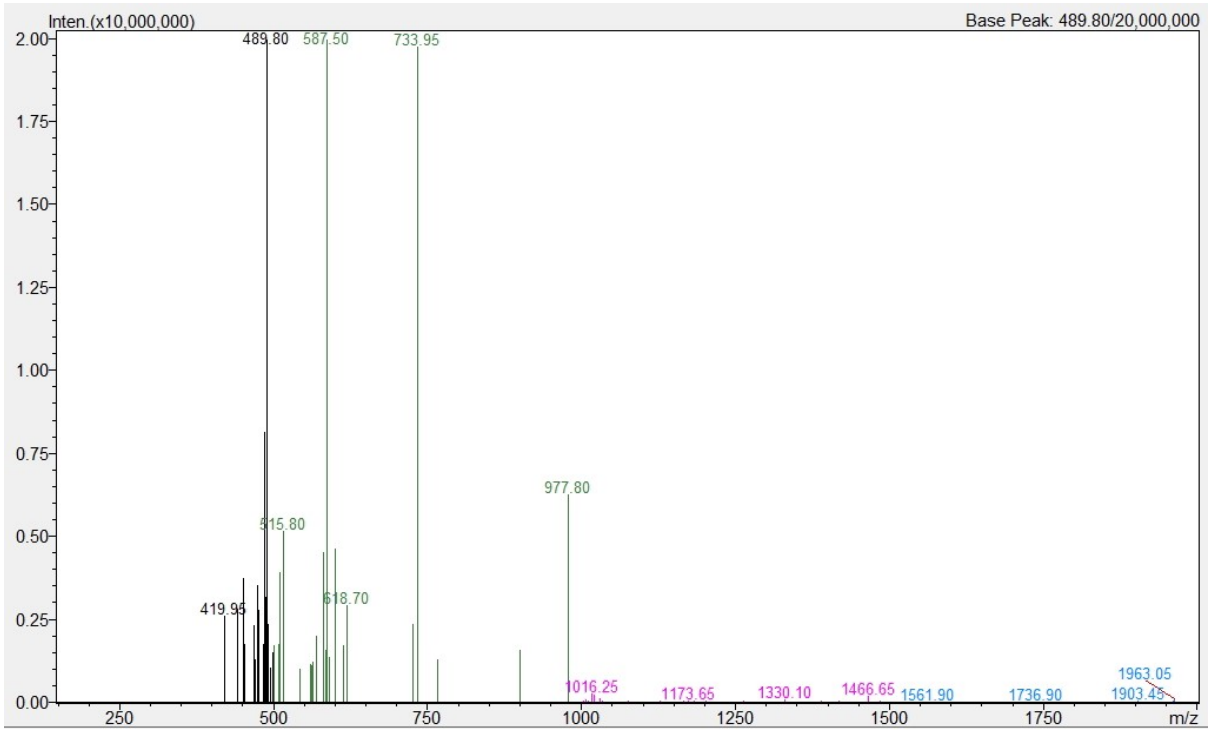
HPLC retention time r_t = 13.7 min

Purity > 95 %

Yield: 25 % (36 mg, 0.012 mmol)



HPLC trace of TAMRA-Tat-NR2B9c (**6**) at 210 nm



Non-deconvoluted LCMS mass spectra [ES+] of TAMRA-Tat-NR2B9c (**6**)

S2.9. Peptide 7 – TAMRA-Tat-NR2Baa

TAMRA-Tat-NR2Baa (7)

Sequence: *TAMRA*-YGRKKRRQRRRKLSSIEADA-OH

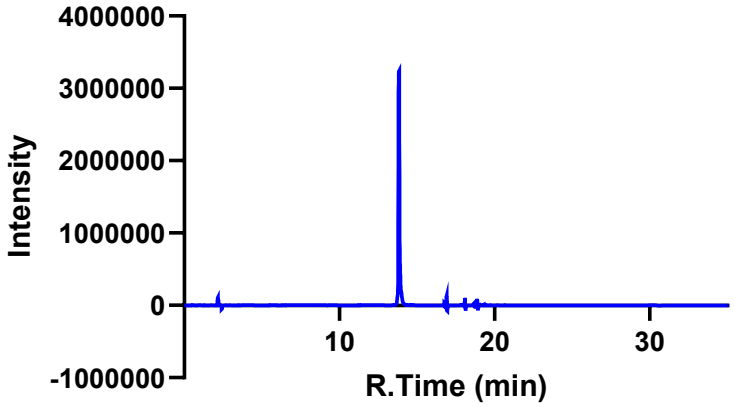
Molecular Weight: 2887.27 g mol-1

LCMS [ESI+]: 1444.60 (m/z_2; calculated: 1444.64), 963.40 (m/z_3; calculated: 963.42), 722.90 (m/z_4; calculated: 722.82), 578.65 (m/z_5; calculated: 578.45), 482.45 (m/z_6; calculated: 482.21)

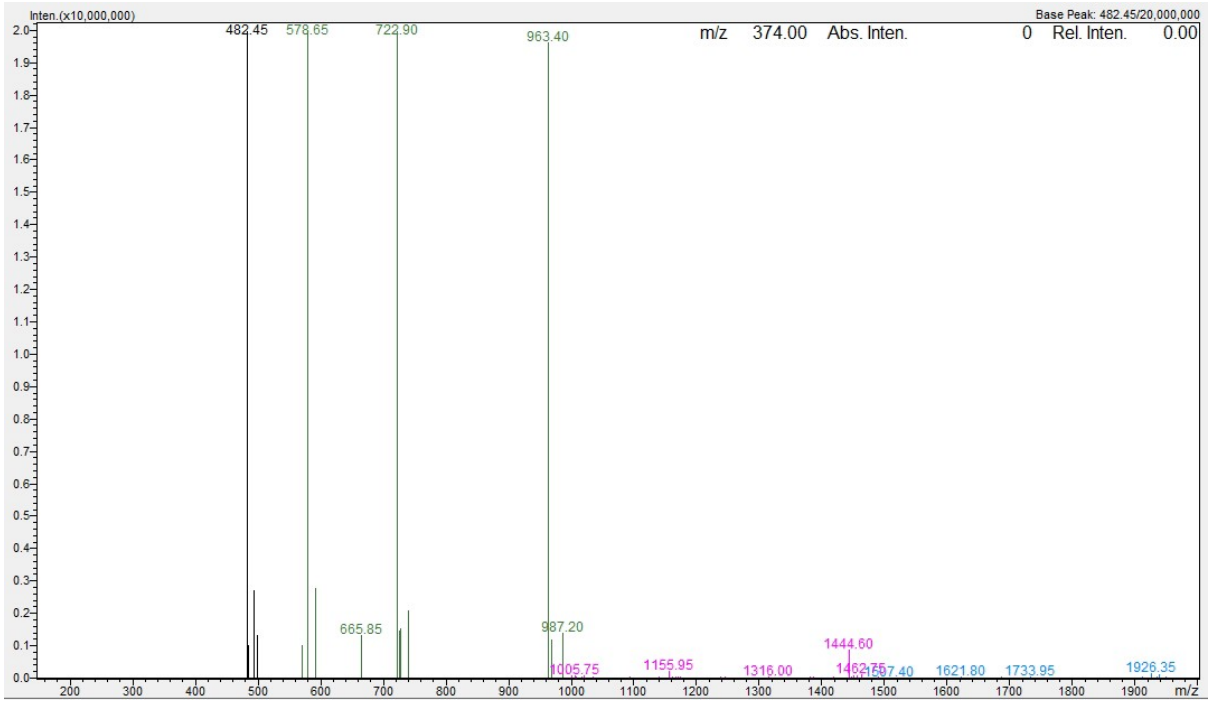
HPLC retention time r_t = 13.7 min

Purity > 95 %

Yield: 24 % (35 mg, 0.012 mmol)



HPLC trace of TAMRA-Tat-NR2Baa (**7**) at 210 nm



Non-deconvoluted LCMS mass spectra [ES+] of TAMRA-Tat-NR2Baa (**7**)

S2.10. Peptide 8 – TAMRA-D-Tat-NR2B9c

TAMRA-D-Tat-NR2B9c (8)

Sequence: *TAMRA*-ygrkkrrqrrrKLSSIESDV-OH

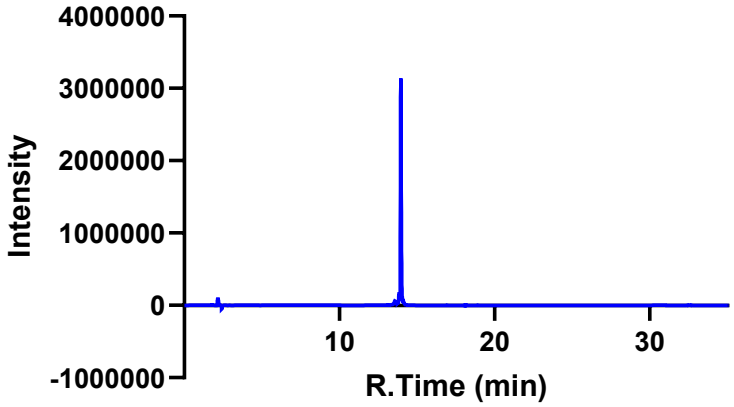
Molecular Weight: 2931.32 g mol-1

LCMS [ESI+]: 1466.65 (m/z_2; calculated: 1466.66), 977.50 (m/z_3; calculated: 978.11), 733.60 (m/z_4; calculated: 733.83), 587.30 (m/z_5; calculated: 587.26), 489.65 (m/z_6; calculated: 489.55)

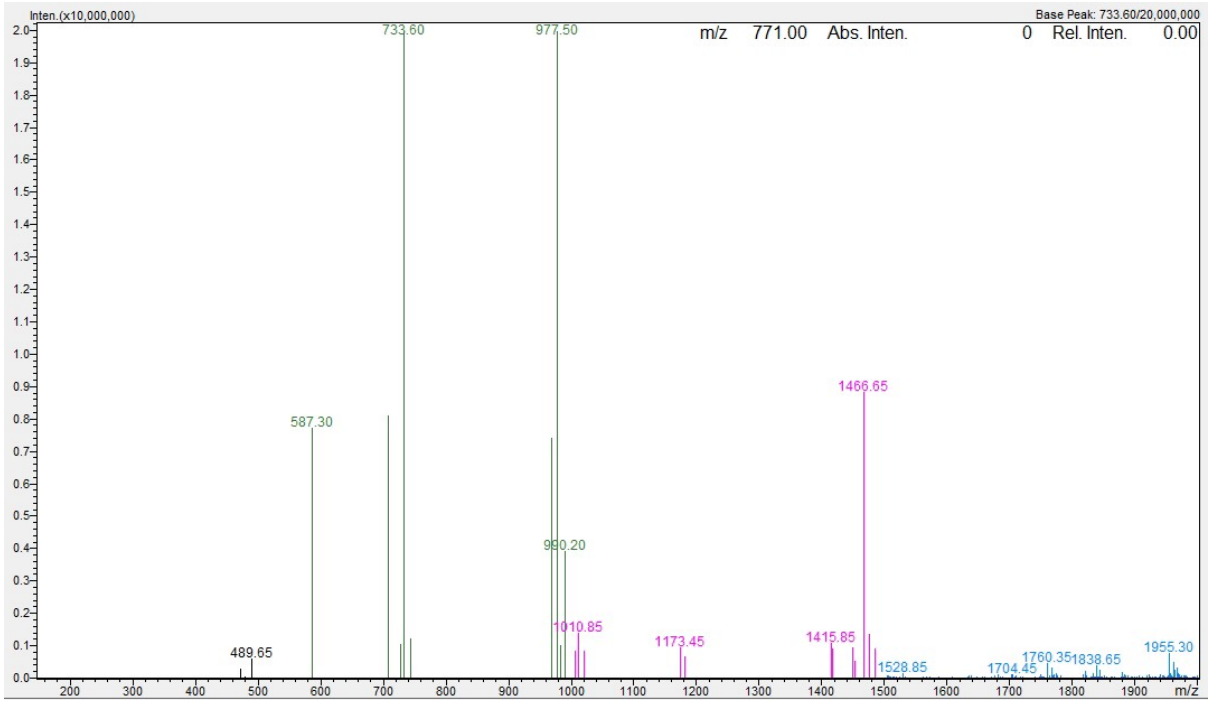
HPLC retention time r_t = 13.9 min

Purity > 95 %

Yield: 22 % (32 mg, 0.011 mmol)



HPLC trace of TAMRA-D-Tat-NR2B9c (**8**) at 210 nm



Non-deconvoluted LCMS mass spectra [ES+] of TAMRA-D-Tat-NR2B9c (**8**)

S2.11. Peptide 9 - Tat-NR2Baa

Tat-NR2Baa (9)

Sequence: YGRKKRRQRRRKLSSIEADA-OH

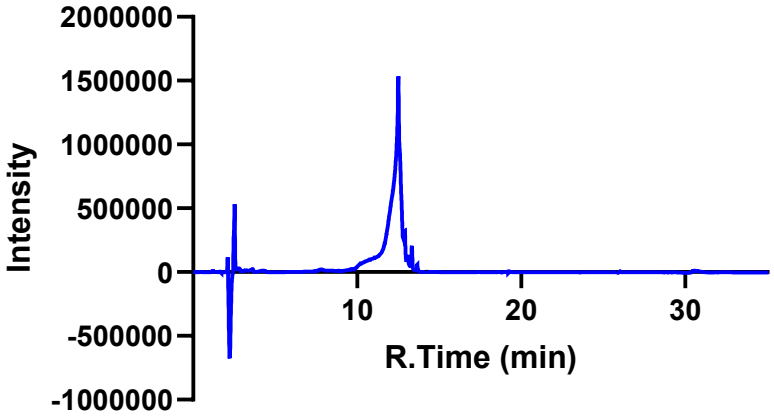
Molecular Weight: 2474.83 g mol-1

LCMS [ESI+]: 1238.35 (m/z_2; calculated: 1238.42), 825.95 (m/z_3; calculated: 825.94), 619.90 (m/z_4; calculated: 619.71), 496.20 (m/z_5; calculated: 495.97), 413.65 (m/z_6; calculated: 413.47)

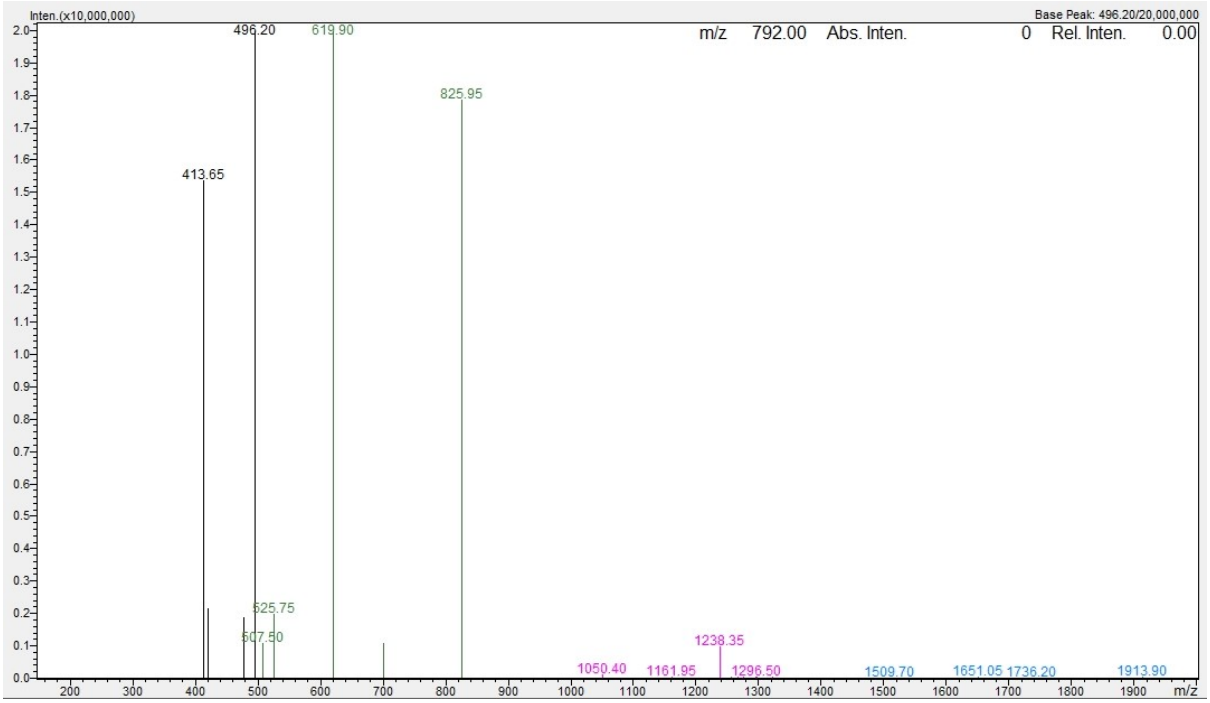
HPLC retention time r_t = 12.3 min

Purity > 95 %

Yield: 23 % (29 mg, 0.012 mmol)



HPLC trace of Tat-NR2Baa (**9**) at 210 nm



Non-deconvoluted LCMS mass spectra [ES+] of Tat-NR2Baa (**9**)

S2.12. Peptide 10 – D-Tat-NR2B9c

D-Tat-NR2B9c (10)

Sequence: ygrkkrrqrrrKLSSIESDV-OH

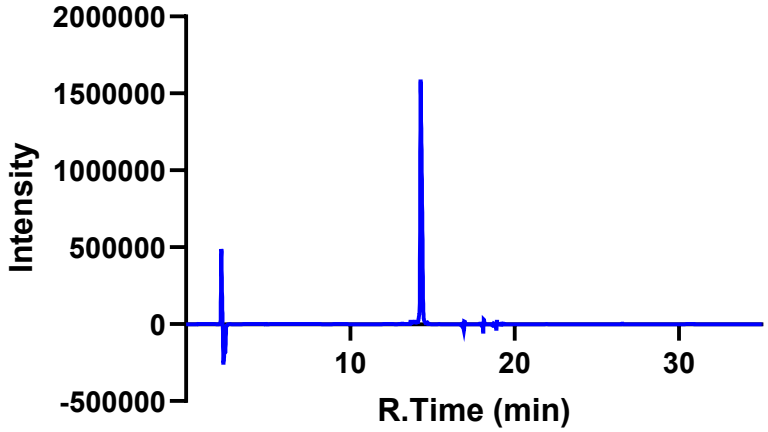
Molecular Weight: 2518.88 g mol-1

LCMS [ESI+]: 1260.65 (m/z_2; calculated: 1260.44), 840.75 (m/z_3; calculated: 840.63), 631.00 (m/z_4; calculated: 630.72), 505.00 (m/z_5; calculated: 504.78), 421.10 (m/z_6; calculated: 420.81)

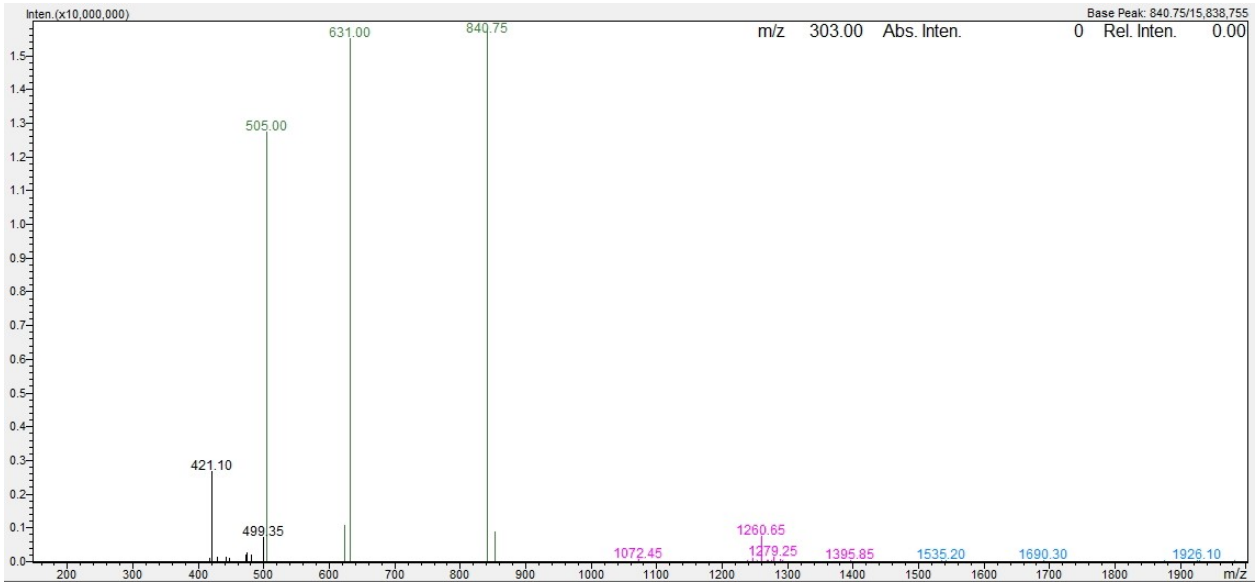
HPLC retention time r_t = 14.0 min

Purity > 95 %

Yield: 12 % (15 mg, 0.0060 mmol)



HPLC trace of D-Tat-NR2B9c (**10**) at 210 nm



Non-deconvoluted LCMS mass spectra [ES+] of D-Tat-NR2B9c (**10**)

S2.13. Peptide 11 – NR2B9c

NR2B9c (11)

Sequence: KLSSIESDV-OH

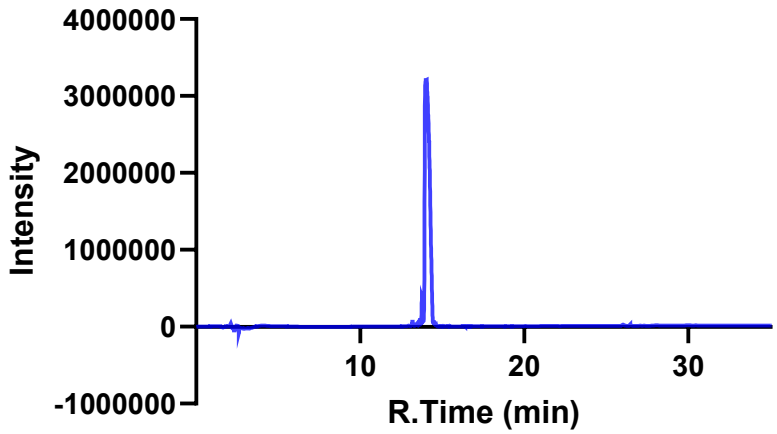
Molecular Weight: 977.07 g mol-1

LCMS [ESI+]: 977.65 (m/z_1; calculated: 978.07), 489.65 (m/z_4; calculated: 489.54)

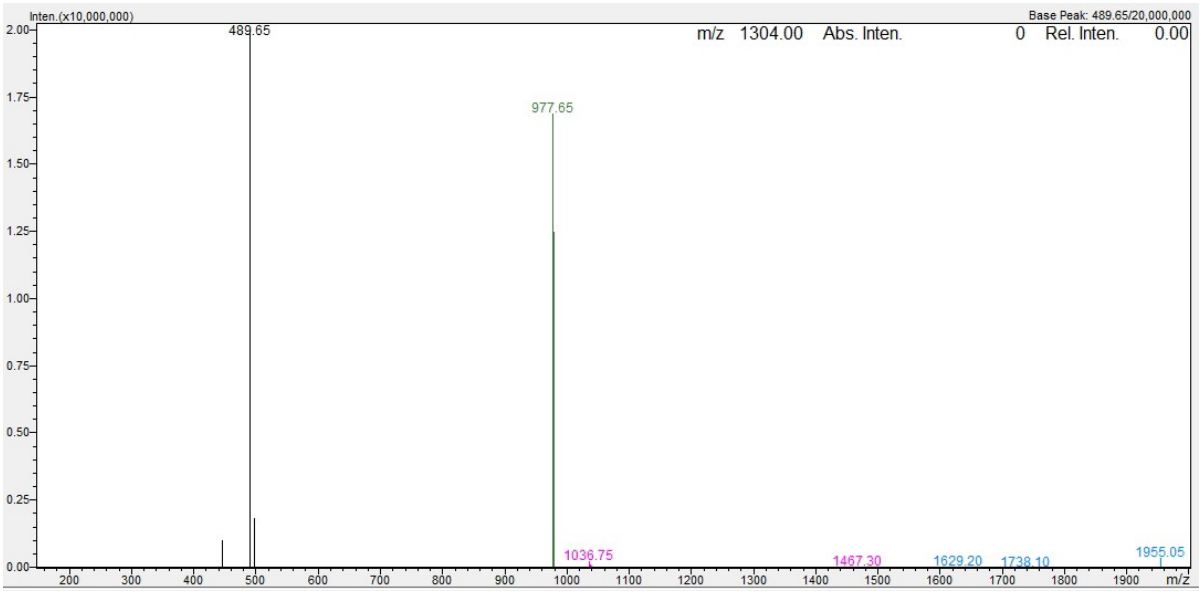
HPLC retention time r_t = 14.1 min

Purity > 95 %

Yield: 47 % (23 mg, 0.024 mmol)



HPLC trace of NR2B9c (**11**) at 210 nm



Non-deconvoluted LCMS mass spectra [ES+] of NR2B9c (**11**)

S3. Peptide Plasma Stability

S3.1. Plasma Extraction

Plasma was collected at mouse ages ranging between 2-4 months of age. Following anaesthetization (ketamine/xylazine 100/10 mg/kg i.p.), blood was collected by cardiac puncture, rapidly transferred to Microvette® 500 K3 EDTA microtubes and centrifuged at 2000 x g for 15 minutes at 4°C to separate plasma from platelets and blood cells. Collected plasma was stored at -30°C until required for experiments.

S3.2. Plasma Stability Assay

To determine the peptide stability, peptides (100 μM) without or with alteplase (22.5 mg/mL) were spiked to mice plasma and incubated at 37 °C.[1] At 0-, 15-, 30-, 60-, 120-, and 300-min time point, 100 μL of plasma was collected and stored at −80°C until analysed. Peptide contents were analysed with general analytical HPLC method employing 0.1% formic acid (FA) in H2O (Solvent A) and 0.1% FA in CH3CN (Solvent B) in a linear gradient described in S1 detecting at 550 nm for TAMRA wavelength.

S4. Uptake into Live Neurons

S4.1. Animal Experiments General

Male and female C57Bl/6 mice were obtained from Ozgene, Australia. Mice were group-housed on a 12 h light/dark cycle with ad libitum access to food and water. All procedures involving animals were approved by the Macquarie University Animal Ethics Committee and conducted in accordance with national and international guidelines.

S4.2. Murine Primary Neurons

Primary cortical and hippocampal neurons were cultured from E16.5 embryonic mouse brains as previously described.[2] Neurons were seeded on greiner 75/25 mm glass bottom cell view cell culture slides (Greiner Bio-One). To promote cell growth and adhesion to the plate's bottom, 200 µL of Poly-D-Lysine (PDL) (Sigma, Merk, Sydney, Australia) were added to the each well. After an overnight incubation, PDL was removed, and the wells were washed three times with sterile Milli-Q water. Once the wells were dried, cell seeding was carried out. 1.5 x 10^4 cells / well were seeded in Neurobasal Medium (NBM) (Gibco, Thermofisher, MA, USA) enriched with 2% (v/v) B27 (Gibco, Thermofisher) and 0.25% (v/v) GlutaMAX (Gibco, Thermofisher). The plates were then incubated at 37°C with 5% CO_2 for 11 days. In preparation for the experiment, half of the cell culture media was removed (conditioned media), stored for post-experimental use, and replaced with fresh culture media.

S4.3. Live cell confocal microscopy

Peptides were dissolved in PBS, added to culture media, and neurons incubated at 37 °C in an atmosphere of 5% CO_2 for 60 min. Culture media was removed, a 50/50

mixture of pre-experiment culture media and fresh culture media added to the cell, and slides transferred microscope fitted with incubation chamber (microscope incubation chamber at 37 °C, 5% CO_2). Samples were visualized using a Zeiss LSM 880 inverted confocal microscope fitted with a XLmultiS1 DARK LS incubation chamber and Plan-Apochromat 63x / 1.40 Oil DIC M27 objective. TAMRA (λex = 552 nm) was excited using 561nm laser line and collected at 405 - 561 nm. Images were acquired at laser strength of 1% with a gain of 650. Bright field images were collected using a transmission detector and the 488nm laser line. Images were analyzed using ImageJ software. Digital adjustments and image processing are consistent for equivalent treatment concentrations throughout the manuscript, unless otherwise stated. Statistical analysis was carried out using GraphPad Prism 10 software and all data used for statistical analysis consists of measurements from distinct cells. Where applicable a non-parametric, unpaired, two-tailed (Mann-Whitney) test was used for statistical testing. The transduction efficiency data presented in Figures 3A, 4A, 4C, 5A, 5C were generated by randomly selecting multiple images from at least three separate experiments and counting the total number of cells in the image as well as the number of transduced cells. Transduced cells were scored as positive when they showed homogenous cytoplasmic and nucleolar fluorescence due to the presence of peptide containing Tat sequence (for NR2B9c / NR2Baa experiments homogenous cytoplasmic staining was taken to be indicative of positive transduction). The ratio of transduced cells:total number of cells was calculated for each image with mean and standard deviation of ratios shown in graphs. As such, the standard deviation represents the variation in transduction efficiency between different groups of cells.

S5. Toxicity and Excitotoxicity Assay

Toxicity and Excitotoxicity assay was conducted as previously described.[3] Murine primary neurons were obtained and cultured as described above.

S5.1. NMDA and peptide treatment of primary neurons

Neurons were seeded on cover slip coated with PdL in 24-well plate at a density of 100,000 cells cm-2 in 1 mL NBM media and cultured at 37 °C in an atmosphere of 5% CO_2 for 11 days. Prior to treatment, 500 μL of media (conditioned media) from each well were taken up and incubated at 37 °C for washing after NMDA administration. For toxicity assay, primary neurons were treated with peptides (100 nM) for 1 hour. For excitotoxicity assay, primary neurons were pre-treated with peptides (100 nM) for 1 hour prior to NMDA (100 μM) exposure. After 60 min, media were replaced with neuron conditioned media. Cells were incubated at 37 °C in an atmosphere of 5% CO_2 for 24 hours. Dead cells were visualized using propidium iodide (10 μM) uptake that was added to the medium 5 min prior to fixation with 4% PFA.

S5.2. Imaging and Image Analysis

Fixed neurons were stained with DAPI to visualise the cell nuclei. Images were visualized using ZEISS Axioscan 7 Microscope Slide Scanner imaging at 475 nm for DAPI and 555 nm for propidium iodide. The cell viability data presented in Figure 6 were counted by randomly selecting multiple images from three replicate experiments. The ratio of death cells:total number of cells was calculated for each image with mean and standard deviation of ratios shown in graphs.

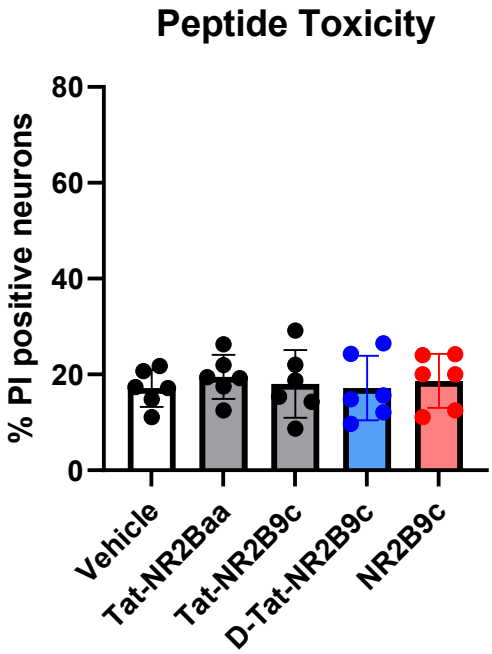


Figure S1. Quantification of the percentage of propidium iodide-positive neurons in neurons treated with 100 nM of peptide (no NMDA treatment).

S6. References

[1] Mayor-Nunez D, Ji Z, Sun X, Teves L, Garman JD, Tymianski M. Plasmin-resistant PSD-95 inhibitors resolve effect-modifying drug-drug interactions between alteplase and nerinetide in acute stroke. Science Translational Medicine. 2021 Apr 7;13(588):eabb1498.

[2] Fath T, Ke YD, Gunning P, Götz J, Ittner LM. Primary support cultures of hippocampal and substantia nigra neurons. Nature protocols. 2009 Jan;4(1):78-85.

[3] Ittner LM, Ke YD, Delerue F, Bi M, Gladbach A, van Eersel J, Wölfing H, Chieng BC, Christie MJ, Napier IA, Eckert A. Dendritic function of tau mediates amyloid-β toxicity in Alzheimer's disease mouse models. Cell. 2010 Aug 6;142(3):387-97.