Supporting Information

Tuning Electrostatic and Hydrophobic Surfaces of Aromatic Rings to Enhance Membrane Association and Cell Uptake of Peptides

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## Experimental procedures

### Abbreviations

### Computational methods

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**SUPPORTING FIGURES**

**Figure S1.** Calculated Natural Bond Orbitals (NBO7) and orbital overlap energies between sulfur lone pair orbitals and neighbouring antibonding orbitals ($p^*$ and $\sigma^*$) for molecules 3b and 3d. **A)** Sulfur lone pair of electrons orbital and an $p^*$ (anti-bonding) orbital; **B)** Sulfur second lone pair of electrons orbital and a neighbouring C-C antibonding ($\sigma^*$) orbital; **C)** Sulfur second lone pair of electrons orbital and a neighbouring C-F antibonding ($\sigma^*$) orbital. Indicated are the extended interactions of sulfur lone pair of electrons to the $p$ electron system and neighbouring non-bonding orbitals of the phenyl ring for 3d, whereas there were no such interactions detected for 3b.

**Figure S2.** CD spectra for peptide 4 and derivatives 4a, 4j and 4n-s (20 µM) in 20 mM SDS, 10 mM sodium phosphate buffer pH 7.4, showing that incorporation of the different substituents at position 4 had minor impact on the overall helical structure of the lactam-stapled peptide. The percentage helicity measured at 220 nm for each peptide is shown in parentheses.
Figure S3. Correlation between partition coefficient (logD$_{7.4}$) in octanol/aqueous phosphate buffer pH 7.4 with HPLC retention time measured for peptides 4 and 4a-t (A) or with the respective clogP calculated for the sidechain moiety at position 4 (listed in Figs. 1 and 2) (B). This indicated that the peptides showed similar trend in hydrophobicity in the two experiments (logD$_{7.4}$ and HPLC) and that differences in hydrophobicity for the peptides directly reflect the hydrophobic properties of the sidechain located at position 4.

Figure S4. Titration curves for the FITC-labelled peptides 4a-t (100 nM) with increasing concentrations of POPC or POPC:POPS (4:1) LUVs in 10 mM HEPES, 10 mM NaCl pH 7.4 buffer measured by fluorescence polarization assay. Peptide 4 did not bind to the vesicles. Partition coefficient (Kp) calculated from the binding curves (left).
Figure S5. Correlation between the parameters: logD_{7.4} of the peptides (octanol-buffer pH 7.4 partition coefficient) or clogP and polarizability of the sidechain located at position 4 with the partition coefficient (Kp) in the POPC (A) or POPC:POPS (4:1) (B) vesicles or cell uptake in HeLa cells (C). To note, correlations of clogP calculated for the sidechain only (blue graphs here) gave similar results to the entire 12-mer peptide: clogP(FITC-peptide) vs: K_p (POPC), r = 0.72, r^2 = 0.52; K_p (POPC:POPS), r = 0.54, r^2 = 0.29; cell uptake r = 0.76, r^2 = 0.58.
Figure S6. There is no significant correlation between the clogP value and polarizability calculated for the respective sidechain located at position 4 (values in Figs 1 and 2), suggesting that the two parameters (hydrophobicity and polarizability) are independent of each other.

A. MLR Model (HeLa cell uptake)

Predicted vs Actual plot: Multiple linear regression

Model: [Hela cell uptake] = β0 + β1[cLogP] + β2[Polarizability]

| Parameter | Variable     | Estimate | Standard Error | P value | P value summary |
|-----------|--------------|----------|----------------|---------|----------------|
| β0        | Intercept    | -313.5   | 68.26          | 0.0003  | ***            |
| β1        | cLogP        | 93.43    | 19.48          | 0.0002  | ***            |
| β2        | Polarizability | 7.114    | 2.108          | 0.0039  | **             |

Goodness of Fit
Degrees of Freedom: 16
R squared: 0.7817

B. MLR Model (POPC binding)

Predicted vs Actual plot: Multiple linear regression

Model: [POPC affinity] = β0 + β1[cLogP] + β2[Polarizability]

| Parameter | Variable     | Estimate | Standard Error | P value | P value summary |
|-----------|--------------|----------|----------------|---------|----------------|
| β0        | Intercept    | -274.0   | 48.79          | <0.0001 | ****           |
| β1        | cLogP        | 55.83    | 13.92          | 0.0010  | **             |
| β2        | Polarizability | 5.584    | 1.507          | 0.0019  | **             |

Goodness of Fit
Degrees of Freedom: 16
R squared: 0.7594

Figure S7. Multiple linear regression (MRL) analysis: MRL model associating the calculated polarizability and clogP values for the arene incorporated as a sidechain at position 4 of peptides 4b-t with uptake into HeLa cells (A) or the extent of peptide partitioning into POPC LUVs (POPC affinity, B).
MLR validation

Durbin-Watson values from the multiple linear regression analyses. Values below 1 or above 3 suggest that the residuals are not independent, which may affect the reliability of the analyses.

| Compound | POPC affinity | HeLa cell uptake |
|----------|---------------|------------------|
| Durbin-Watson | 2.160         | 1.585            |

Cook’s distances from the multiple linear regression analyses, indicating a lack of outliers (distance > 1), the presence of which may affect the reliability of the analyses.

| Compound | POPC affinity | HeLa cells uptake |
|----------|---------------|------------------|
| 4b       | .00002        | .01342           |
| 4c       | .12866        | .01017           |
| 4d       | .00321        | .00322           |
| 4e       | .06091        | .08263           |
| 4f       | .02701        | .00013           |
| 4g       | .00044        | .01695           |
| 4h       | .09075        | .04270           |
| 4i       | .02803        | .00952           |
| 4j       | .00787        | .02651           |
| 4k       | .02999        | .01761           |
| 4l       | .00881        | .00009           |
| 4m       | .01951        | .38317           |
| 4n       | .01171        | .00813           |
| 4o       | .05569        | .05543           |
| 4p       | .00463        | .00001           |
| 4q       | .00017        | .05652           |
| 4r       | .00000        | .01058           |
| 4s       | .00323        | .15424           |
| 4t       | .52499        | .05339           |

Figure S8. Validation of the multiple linear regression model associating the calculated polarizability and clogP values for the arene incorporated as a sidechain at position 4 of peptides 4b-t with the extent of peptide partitioning into POPC LUVs (POPC affinity) or uptake into HeLa cells.
Figure S9. Variation of free energy of insertion of molecules 3a-d into model zwitterionic POPC lipid bilayers over time determined by MD simulations.
**Figure S10.** Number of contacts between peptides 7a-d and lipids in POPC bilayer models measured over 200 ns MD simulations. Movie file for 7d is also provided as a separate file (MD simulation for 7d in POPC bilayer models at 298 K constructed between frames 1270 to 1490 (0.27 sec/frame) from a 200 ns MD simulations using Maestro/Schrodinger package version 2020-3).
| Properties                        | 7d(Ac) | 8(Ac) |
|----------------------------------|--------|-------|
| clogP (peptide)                  | 1.25   | 1.94  |
| Calculated hydrophobic surface area | 477 Å² | 486 Å² |
| HPLC retention time              | 16.4 min | 16.6 min |
| Helicity in buffer 7.4           | 58 %   | 62 %  |
| HDM2 affinity (Kᵢ)              | 25.0 nM | 10.7 nM |

**Figure S11.** Comparison of biophysical properties between N-terminal acetylated peptides 7d and 8 (not displaying FITC-βAla at the N-terminus). HDM2 affinity was measured by fluorescence polarization competition assays according to de Araujo et al.¹

**Figure S12.** Percentage HeLa cell death (7-AAD⁺ stained cells) after incubating peptides (5 µM) for 1 h at 37 °C. Ethanol was used as 100 % cell death control. Error bars represent mean ± SEM.
Figure S13. LDH release$^1$ after incubation of peptide 7d at different concentrations in HeLa cells for 1h indicating no significant lysis up to 50 µM peptide concentration.
EXPERIMENTAL PROCEDURES

ABBREVIATIONS

7-AAD, 7-amino-actinomycin D; Ac, acetyl; ACN, acetonitrile; DCE, dichloroethane; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMEM, Dulbecco's modified eagle medium; DMF, dimethylformamide; DODT, 3,6-dioxo-1,8-octanedithiol; DTNP, 2,2'-dithiobis(5-nitropyridine); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EIPA, 5-(N-ethyl-N-isopropyl)amiloride; ESI-MS, electron spray mass spectroscopy; FBS, fetal bovine serum; Fmoc, 9-fluorenylmethoxycarbonyl; FITC, fluorescein isothiocyanate; HATU, 2-(7-Aza-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HCTU, 2-(1H-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Hcy, homocysteine; HOBr, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; HR-MS, high-resolution mass spectroscopy; MBHA, 4-methylbenzhydromine; Mtt, 4-methyltrityl; OPip, 2-phenylisopropyl ester; PBS, phosphate-buffered saline; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-choline; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, R8, (R)-2-(7′octenyl)alanine; RCM, ring-closing metathesis; S5, 2-(4′-pentenyl) alanine; SDS, sodium dodecyl sulfate; TCEP, dithiolthreitol; EDTA, ethylenediaminetetraacetic acid; TIS, trisopropylsilane; TRIS, tris(hydroxymethyl)aminomethane; UPLC, ultra-performance liquid chromatography.

clogP

clogP values were calculated using ChemDraw version 20.0 software.

Peptide molecular volume

Peptide volumes were estimated using Molinspiration Cheminformatics free web services, https://www.molinspiration.com.

DFT Calculations

All compounds were examined using ab initio density functional theory (DFT) calculations for optimization, frequency and energy calculations, at the B3LYP/6-311+ +G(2d,2p) level of theory using Gaussian 16. Total electron density was mapped onto electrostatic potential surface on the scale of -7.0 kcal/mol (red) to +7.0 kcal/mol (blue) and density isovalue of 0.002. All dipole moments and electronic surface potentials (ESP) were displayed by GaussView 9 graphical interface.

NBO Calculations

Natural Bond Orbital (NBO) calculations were performed for all compounds using ab initio density functional theory (DFT) calculations at the B3LYP/6-311+ +G(2d,2p) level of theory using NBO7.0 program. All calculations were performed using Gaussian 16 through the graphical interface GaussView 9. All structures were optimized in Gaussian (version 16) prior to NBO calculations. The orbital interaction energy was derived from the second order perturbation theory analysis of fock matrix in NBO basis.

Molecular Dynamics Simulations

All simulations were performed using Maestro/Schrodinger simulation package version 2020-3. The force field topologies of the small molecules and the cyclic tetrapeptides were derived from the OPLS3e parameter set. The small molecules and cyclic peptides were placed outside the modeled POPC bilayer membrane, in a pre-equilibrated truncated octahedral box filled with SPC water molecules. The simulation was carried out at 298 K. After a steepest descent minimization step, the cyclic peptides were subjected to 100 ps of simulation with position restraints on the peptide to relax the solvent. The systems were subsequently equilibrated for 1 ns without restraints. The simulations were performed at constant temperature (298 K) and pressure (1 atm). This was achieved using a Berendsen thermostat with a coupling time of 0.1 ps and a Berendsen barostat with a coupling time of 0.5 ps. Nonbonded interactions were calculated using a twin-range cut-off. Interactions within the short-range cut-off of 0.8 nm were updated every time step. Interactions within the longer-range cut-off of 1.4 nm were updated every 5 time-steps together with the pairlist. All bonds were constrained using the SHAKE algorithm with a geometric tolerance of 0.0001.
Initial velocities were taken from the Maxwell-Boltzmann distribution at 300 K and were acquired for 200 ns. Interactions between small molecules/cyclic peptides and membranes were calculated, plotted and movies were made using the Maestro program version 2020-3.

SYNTHETIC METHODS

Materials
All solvents and reagents used during peptide chain assembly were peptide synthesis grade and purchased from commercial suppliers. Unless stated in the text, all crosslinkers used were commercially available.

General solid-phase peptide synthesis (SPPS)
Linear peptides were assembled using Fmoc-based chemistry on a peptide synthesizer (Symphony, Protein Technologies) and Rink-amide MBHA resin (Novabiochem or ChemImpex). Peptides undergoing on-resin cyclization were assembled on a low-loading resin Rink-amide resin (0.35 mmol/g, Novabiochem). Standard Fmoc-protected amino acids were used for all peptides, unless otherwise stated (see procedure for each individual peptide). Usually, 4 equiv. of Fmoc-protected amino acid, 4 equiv of HCTU and 4 eq of DIPEA were used in 2 x 30 min coupling cycles. Fmoc deprotection was achieved by treatment with 3:7 piperidine:DMF for 2 x 3 min. For pDI-peptides featuring FITC, the N-terminus was first coupled to a Fmoc-beta-alanine residue, followed by Fmoc-group removal and labeling with FITC by treating the free amine resin with FITC (4 equiv.) and DIPEA (8 equiv.) in DMF overnight. For non-fluorescent pDI peptides (7d(Ac) and 8(Ac) in Fig. S10), the N-terminus was acetylated with Ac2O:DIPEA:DMF (0.87:0.47:15 mL) for 10 min.

Cleavage from solid support (TFA acidolysis)
Peptides were cleaved from the resin with TFA:TIS:HOAc (95:2.5:2.5) for 2.5 h. Peptides containing Cys/Hcy were cleaved with TFA:TIS:HOAc:DIPEA (91:3:3:3) for 2.5 h. The crude peptides were precipitated and washed with cold Et2O, redissolved in 75% acetonitrile/0.05 % TFA in water and lyophilized. Incomplete decarboxylation of the Trp residue was observed in all crude material, characterized by the presence of a -44 Da adduct (+CO2) in ESI-MS analysis. Because spontaneous decarboxylation occurred under basic conditions, crude mixtures containing the carboxylated adduct were directly used for subsequent thiolation reactions under basic conditions. For the other peptides, the crude was redissolved in 70% acetonitrile/10% acetic acid and let stand at room temperature to allow complete decarboxylation of the Trp residue (usually overnight), as monitored by ESI-MS. After that, the crude material was again freeze-dried and subjected to HPLC purification.

Peptide purification by HPLC
Crude peptides were purified on a Shimadzu preparative HPLC System using a Phenomenex Luna C18 column eluting at a flow rate of 20 mL/min (or 4 mL/min for smaller samples < 5 mg); and a gradient of 20 to 80 % buffer B (90 % ACN/10 % HOAc/0.1% TFA in buffer A, 0.1% TFA in water) over 30 min (PDI-peptides) or 0 to 50 % buffer B over 30 min (CPP9-peptides). Purity and identity of collected peptide fractions were analysed by analytical HPLC, UPLC-MS and/or HR-MS.

FITC-βAla-Leu-Thr-Phe-Glu-Glu-Tyr-Trp-c[Lys-Gln-Leu-Thr-Asp]-CONH2 (4)

Lactam stapling was carried out by orthogonal deprotection of Fmoc-Lys(Mtt)-OH and Fmoc-Asp(OPip)-OH residues located at stapling positions, followed by PyBOP-assisted lactamization. Briefly, the sequence Fmoc-Glu(Bu)Tyr(Bu)-Trp(Boc)-Lys(Mtt)-Gln(Trt)-Leu-Thr(Bu)-Asp(OPip) was assembled by the general SPPS procedure. Afterwards, the resin was washed with DCM and treated repeatedly with 3 % TFA in DCM (10 x 1 min). After washing with DCM and DMF, a solution of PyBOP (4 eq) and DIPEA (6 eq) in DMF was added to the resin and the reaction was agitated overnight. Next, the Fmoc group was removed from the N-terminus and assembly continued to construct the final sequence: FITC-[βAla-Leu-Thr(Bu)-Phe-Glu(Bu)-Glu(Bu)-Tyr(Bu)-Trp(Boc)-c[Lys-Gln(Trt)]-Leu-Thr(Bu)-Asp]. The peptide was cleaved from the resin and purified by HPLC. Analytical HPLC (Method C): 12.5 min. ESI-MS: deconvoluted mass 2013.9 (expected 2014.2).
FITC-βAla-Leu-Thr-Phe-Cys-Glu-Tyr-Trp-c[Lys-Gln-Leu-Thr-Asp]-CONH₂ (4a)

The sequence FITC-βAla-Leu-Thr(tBu)-Phe-Cys(Trt)-Glu(tBu)-Tyr(tBu)-Trp(Boc)-c[Lys-Gln(Trt)-Leu-Thr(Trt)-Asp] was assembled following the same procedure as 4. The Cys-peptide was cleaved by TFA and used as a crude mixture for the next conjugation step or partially purified by HPLC. Analytical HPLC (Method A): 9.9 min. ESI-MS: deconvoluted mass 1988.0 (expected 1988.2).

FITC-βAla-Leu-Thr-Phe-Hcy-Glu-Tyr-Trp-[Lys-Gln-Leu-Thr-Asp]-CONH₂ (precursor 4a(Hcy))

The sequence FITC-βAla-Leu-Thr(tBu)-Phe-Hcy(Trt)-Glu(tBu)-Tyr(tBu)-Trp(Boc)-c[Lys-Gln(Trt)-Leu-Thr(tBu)-Asp] was assembled following the same procedure as 4. The Hcy-peptide was cleaved by TFA and used as a crude mixture for the next conjugation step. ESI-MS: deconvoluted mass 2001.6 (expected 2002.2).

FITC-βAla-Leu-Thr-Phe-HSec-Glu-Tyr-Trp-[Lys-Gln-Leu-Thr-Asp]-CONH₂ (precursor 4a(HSec))

The sequence FITC-βAla-Leu-Thr(tBu)-Phe-HSec(pMeBzl)-Glu(tBu)-Tyr(tBu)-Trp(Boc)-c[Lys-Gln(Trt)-Leu-Thr(tBu)-Asp] was assembled following the same procedure as 4. Fmoc-homoselenocysteine(pMeBzl)-OH was used to couple the HSec residue following the standard coupling procedure. The seleno-containing peptide was cleaved from resin using 20 eq DTNP in TFA:TIS:H₂O (95:2.5:2.5) for 2.5 h. After Et₃O precipitation, the crude was isolated as a mixture of selenide monomers, diselenide dimers and TNP-Sec labelled peptide.³ To remove TNP-adducts, the crude mixture was dissolved in 50 mM TCEP: ACN (1:2) and led standing for 10 min. The selenide product was then isolated by HPLC. Upon freeze-drying of the purified fractions, the monomer selenide spontaneously dimerized to a diselenide. ESI-MS: deconvoluted mass 2047.1 (expected 2048.1, monomer).
FITC-$\beta$Ala-Leu-Thr-Phe-Aoc-Glu-Tyr-Trp-c[Lys-Gln-Leu-Thr-Asp]-CONH$_2$ (4b)

The sequence FITC-$\beta$Ala-Leu-Thr(tBu)-Phe-Aoc-Glu(tBu)-Tyr(tBu)-Trp(Boc)-c[Lys-Gln(Trt)-Leu-Thr(tBu)-Asp] was assembled following same procedure as 4. The peptide was cleaved by TFA and purified by HPLC. Analytical HPLC (Method A): 18.7 min. ESI-MS: deconvoluted mass 2026.0 (expected 2026.3).

FITC-$\beta$Ala-Leu-Thr-Phe-Glu-Tyr-Trp-c[Lys-Gln-Leu-Thr-Asp]-CONH$_2$ (4c)

The sequence FITC-$\beta$Ala-Leu-Thr(tBu)-Phe-Phe-Glu(tBu)-Tyr(tBu)-Trp(Boc)-c[Lys-Gln(Trt)-Leu-Thr(tBu)-Asp] was assembled following same procedure as 4. The peptide was cleaved by TFA and purified by HPLC. Analytical HPLC (Method A): 16.4 min. ESI-MS: deconvoluted mass 2031.7 (expected 2032.3)

FITC-$\beta$Ala-Leu-Thr-Phe-(4-CF$_3$-Phe)-Glu-Tyr-Trp-c[Lys-Gln-Leu-Thr-Asp]-CONH$_2$ (4d)

The sequence FITC-$\beta$Ala-Leu-Thr(tBu)-Phe-(4-CF$_3$-Phe)-Glu(tBu)-Tyr(tBu)-Trp(Boc)-c[Lys-Gln(Trt)-Leu-Thr(tBu)-Asp] was assembled following same procedure as 4. The peptide was cleaved by TFA and purified by HPLC. Analytical HPLC (Method A): 17.4 min. ESI-MS: deconvoluted mass 2099.6 (expected 2100.3)

FITC-$\beta$Ala-Leu-Thr-Phe-(4-NO$_2$-Phe)-Glu-Tyr-Trp-c[Lys-Gln-Leu-Thr-Asp]-CONH$_2$ (4e)

The sequence FITC-$\beta$Ala-Leu-Thr(tBu)-Phe-(4-NO$_2$-Phe)-Glu(tBu)-Tyr(tBu)-Trp(Boc)-c[Lys-Gln(Trt)-Leu-Thr(tBu)-Asp] was assembled following same procedure as 4. The peptide was cleaved by TFA and purified by HPLC. Analytical HPLC (Method A): 15.9 min. ESI-MS: deconvoluted mass 2076.6 (expected 2077.3).
The sequence FITC-βAla-Leu-Thr-Phe-(pentfluoro-Phe)-Glu-Tyr-Trp-[Lys-Gln-Leu-Thr-Asp]-CONH$_2$ (4f) was assembled following same procedure as 4. The peptide was cleaved by TFA and purified by HPLC. Analytical HPLC (Method A): 16.6 min. ESI-MS: deconvoluted mass 2121.7 (expected 2122.2).

The sequence FITC-βAla-Leu-Thr-(tBu)-Phe-(pentfluoro-Phe)-Glu-(tBu)-Tyr-(tBu)-Trp-(Boc)-c[Lys-Gln(Trt)-Leu-Thr(tBu)-Asp] was assembled following same procedure as 4. The peptide was cleaved by TFA and purified by HPLC. Analytical HPLC (Method A): 15.8 min. ESI-MS: deconvoluted mass 2070.9 (expected 2071.3).

The sequence FITC-βAla-Leu-Thr-(tBu)-Phe-Trp-(Boc)-Glu-(tBu)-Tyr-(tBu)-Trp-(Boc)-c[Lys-Gln(Trt)-Leu-Thr(tBu)-Asp] was assembled following same procedure as 4. The peptide was cleaved by TFA and purified by HPLC. Analytical HPLC (Method A): 17.2 min. ESI-MS: deconvoluted mass 2081.9 (expected 2082.3).

The sequence FITC-βAla-Leu-Thr-(tBu)-Phe-nal-Glu-(tBu)-Tyr-(tBu)-Trp-(Boc)-c[Lys-Gln(Trt)-Leu-Thr(tBu)-Asp] was assembled following same procedure as 4. The peptide was cleaved by TFA and purified by HPLC. Analytical HPLC (Method A): 17.8 min. ESI-MS: deconvoluted mass 2059.8 (expected 2060.3).
FITC-βAla-Leu-Thr-Phe-[S-benzyl-Cys]-Glu-Tyr-Trp-c[Lys-Gln-Leu-Thr-Asp]-CONH₂ (4j)

The crude peptide precursor 4a (8.0 mg, 4 µmol) was dissolved in 50 mM TRIS base in DMF (3 mL) and benzyl bromide (2 eq) was added. After 30 min, the reaction mixture was acidified by addition of 1 % TFA solution and the product was purified by HPLC. Isolated yield: 2.0 mg (24 % from crude). Analytical HPLC (Method A): 16.7 min. ESI-MS: deconvoluted mass 2078.0 (expected 2078.3).

FITC-βAla-Leu-Thr-Phe-[S-pMeBzl-Cys]-Glu-Tyr-Trp-c[Lys-Gln-Leu-Thr-Asp]-CONH₂ (4k)

The crude peptide precursor 4a (15 mg, 7.5 µmol) was dissolved in 50 mM TRIS base in DMF (5 mL) and p-methyl-benzyl bromide (2 eq) was added. After 60 min, the reaction mixture was acidified by addition of 1 % TFA solution and the product was purified by HPLC. Isolated yield: 4.2 mg. Analytical HPLC (Method A): 17.5 min.

FITC-βAla-Leu-Thr-Phe-[S-NO₂-Cys]-Glu-Tyr-Trp-c[Lys-Gln-Leu-Thr-Asp]-CONH₂ (4l)

The crude peptide precursor 4a (16 mg, 8 µmol) was dissolved in 50 mM TRIS base in DMF (5 mL) and p-NO₂-benzyl bromide (2 eq) was added. After 30 min, the reaction mixture was acidified by addition of 1 % TFA solution and the product was purified by HPLC. Isolated yield: 4.8 mg (30 % from crude). Analytical HPLC (Method A): 16.5 min. ESI-MS: deconvoluted mass 2122.7 (expected 2123.4).
FITC-βAla-Leu-Thr-Phe-[^S-pSF₃-Cys]-Glu-Tyr-Trp-c[Lys-Gln-Leu-Thr-Asp]-CONH₂ (4m)

The crude peptide precursor 4a (22 mg, 11 µmol) was dissolved in 50 mM TRIS base in DMF (7 mL) and p-SF₃-benzyl bromide (2 eq) was added. After 60 min, the reaction mixture was acidified by addition of 1 % TFA solution and the product was purified by HPLC. Isolated yield: 6.7 mg (30% from crude). Analytical HPLC (Method A): 19.0 min. ESI-MS: deconvoluted mass 2203.8 (expected 2204.4).

FITC-βAla-Leu-Thr-Phe-[^S-FBzl-Cys]-Glu-Tyr-Trp-c[Lys-Gln-Leu-Thr-Asp]-CONH₂ (4n)

The crude peptide precursor 4a (9.8 mg, 4.9 µmol) was dissolved in 50 mM TRIS base in DMF (4 mL) and 2,3,4,5,6-pentafluorobenzyl bromide (2 eq) was added. After 40 min, the reaction mixture was acidified by addition of 1 % TFA solution and the product was purified by HPLC. Isolated yield: 2.7 mg (25% from crude). Analytical HPLC (Method A): 11.7 min. ESI-MS: deconvoluted mass 2167.8 (expected 2168.3).

FITC-βAla-Leu-Thr-Phe-[^S-pNO₂Ph-Hcy]-Glu-Tyr-Trp-c[Lys-Gln-Leu-Thr-Asp]-CONH₂ (4o)

The crude peptide precursor 4a(Hcy) (10 mg, 5.0 µmol) was dissolved in DMF (5 mL) and 1,4-dintrobenezene (30 eq) and DIPEA (30 eq) were added. After 30 min, the mixture was acidified by addition of 1 % TFA and the product was purified by HPLC. Isolated yield: 1.6 mg (15% from crude). Analytical HPLC (Method B): 17.5 min. ESI-MS: deconvoluted mass 2123.3 (expected 2123.3).
FITC-βAla-Leu-Thr-Phe-[S-SFPh-Hcy]-Glu-Tyr-Trp-c[Lys-Gln-Leu-Thr-Asp]-CONH₂ (4p)

The crude peptide precursor 4a(Hcy) (12.3 mg, 6.1 µmol) was dissolved in 50 mM TRIS base in DMF (5 mL) and hexafluorobenzene (100 eq) was added. After 4 h, an additional 50 eq hexafluorobenzene was added. After 6 h, the mixture was acidified by addition of 1 % TFA solution and the product was purified by HPLC. Isolated yield: 3.5 mg (26 % from crude). Analytical HPLC (Method A): 12.1 min. ESI-MS: deconvoluted mass 2168.0 (expected 2168.3).

FITC-βAla-Leu-Thr-Phe-[S-SFPh-HSec]-Glu-Tyr-Trp-c[Lys-Gln-Leu-Thr-Asp]-CONH₂ (4q)

The deselenide precursor 4a(HSec) (2.6 mg) was dissolved in 50 mM TRIS base in DMF (0.3 mL) and TCEP (1.0 mg, 3 eq) and hexafluorobenzene (15 µL, 100 eq) were added. After 16 h, the mixture was acidified by addition of 1 % TFA and the product was purified by HPLC. Isolated yield: 1.2 mg (43 %). Analytical HPLC (Method A): 12.1 min. ESI-MS:

FITC-βAla-Leu-Thr-Phe-[S-(4-SMe-2,3,5,6-4F-Ph)-Hcy]-Glu-Tyr-Trp-c[Lys-Gln-Leu-Thr-Asp]-CONH₂ (4r)

Peptide 4p (0.70 mg, 0.32 µmol) was dissolved DMF (80 µL) and sodium thiomethoxide (110 µL, 300 mM in H₂O, 100 eq) was added. After 20 min, the mixture was acidified by addition of 1 % TFA and the product was purified by HPLC. Isolated yield: 0.58 mg (83 %). Analytical HPLC (Method B): 18.6 min. ESI-MS: deconvoluted mass 2195.4 (expected 2196.4).
Peptide 4p (1.0 mg, 0.47 µmol) was dissolved in 100 mM TRIS base in DMF (50 µL) and mercaptoethane (50 µL of a 200 mM solution in DMF, 21 eq) was added. After 4 h, the mixture was acidified by addition of 1 % TFA and the product was purified by HPLC. Isolated yield: 0.7 mg (68 %). Analytical HPLC (Method A): 12.8 min. ESI-MS: deconvoluted mass 2210.0 (expected 2210.4).

Peptide 4p (2.0 mg, 0.92 µmol) was dissolved in 100 mM TRIS base in DMF (300 µL) and NHMe₂ (30 µL of a 7.9 M solution in water, 100 eq) was added. After 13 h, the mixture was acidified by addition of 1 % TFA and the product was purified by HPLC. Isolated yield: 0.7 mg (68 %). Analytical HPLC (Method A): 19.1 min. ESI-MS: deconvoluted mass 2192.8 (expected 2193.4).

Peptide 6a

Peptide 6a is an analogue of CPP9, differing by having a Asp/Asn at the site of Lys(FITC) attachment instead of Glu/Gln reported for CPP9. Compound 6a was assembled based on the procedure reported by Pei et al. Briefly, Fmoc-Lys(Mtt) was first attached to the Rink amide resin, followed by standard Fmoc deprotection. Fmoc-Asp(COOH)-OAll (4 eq) was then coupled via its free side-chain acid using HATU (4 eq) and DIPEA (4 eq) in DMF for 60 min. After that, the remaining sequence was assembled accordingly to the general SPPS procedure to construct Fmoc-phe-Val-AAsp(Hcy)-Arg(Pbf) (4 eq) and DIPEA (4 eq) in DMF for dry DCM (3 x 15 min), following by removal of N-terminal Fmoc group. Cyclization was subsequently promoted by PyBOP (5 eq), HOBt (5 eq) and DIPEA (10 eq) in DMF (1 x 6 h, 1x overnight). Finally, the Mtt group was removed by treatment with 1 % TFA in DCM (10 x 1 min) and fluorescein was coupled to the side-chain of Lys with FITC (4 eq) and DIPEA (8eq) in DMF for 4 h. Finally, the cyclic peptide was cleaved by TFA acidolysis and purified by HPLC. Analytical HPLC (Method D): 9.8 min. ESI-MS: deconvoluted mass 1600.8 (expected 1600.8).
Peptides 6b-d

Peptide 6b
The 6(Hcy) precursor was prepared as reported for 6a, but using Hcy(Trt) instead of NaI and purified by HPLC to ~80-90 % purity. The 6(Hcy) precursor (5.0 mg, 3.2 µmol) was dissolved in 50 mM TRIS in DMF (3 mL) and 200 µL water followed by addition of hexafluorobenzene (190 µL, 200 eq). After 4 h at 30 °C, the reaction was acidified by addition of 1 % TFA:ACN (1:1) and the product was purified by HPLC. Isolated yield: 3.6 mg (68 %). Analytical HPLC (Method D): 10.4 min. ESI-MS: deconvoluted mass 1686.7 (expected 1686.8).

Peptide 6c
Peptide 6b (1.6 mg, 0.95 µmol) was dissolved in DMF (250 µL) and sodium thiomethoxide (316 µL, 300 mM in DMF, 10 % H2O, 100 eq) was added. After 15 min, the mixture was acidified by addition of 1 % TFA:ACN (1:1) and the product was purified by HPLC. Isolated yield: 1.1 mg (69 %). Analytical HPLC (Method D): 10.7 min. ESI-MS: deconvoluted mass 1714.7 (expected 1714.9).
Peptide 6d
Peptide 6b (1.0 mg, 0.6 µmol) was dissolved in 50 mM TRIS base in DMF (120 µL) and mercaptoethane (16 µL of 2M solution in DMF, 85 eq) was added. After 3 h, the mixture was acidified by addition of 1 % TFA:ACN (1:1) and the product was purified by HPLC. Isolated yield: 0.9 mg (87 %). Analytical HPLC (Method D): 11.1 min. ESI-MS: deconvoluted mass 1728.7 (expected 1728.9).

Peptide 7a
The sequence FITC-βAla-Leu-Thr(tBu)-Phe-Cys(Trt)-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Cys(Trt)-Gln(Trt)-Leu-Thr(tBu)-Ser(tBu) was assembled on solid support as described in the general SPPS procedure. The crude linear di-Cys peptide (20 mg, 10.2 µmol) was then combined to 1,4-Bis(bromomethyl)benzene (4.3 mg, 1.5 eq) in 100 mM TRIS base (10 mL). After 15 min, the mixture was acidified by addition of 1 % TFA and the product was purified by HPLC. Isolated yield: 4.2 mg (21 %). Analytical HPLC (Method A): 10.7 min. ESI-MS: deconvoluted mass 2054.9 (expected 2055.3).

Peptide 7b
The sequence FITC-βAla-Leu-Thr(tBu)-Phe-Cys(Trt)-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Cys(Trt)-Gln(Trt)-Leu-Thr(tBu)-Ser(tBu) was assembled on solid support as described in the general SPPS procedure. The crude linear di-Cys peptide (21 mg, 10.7 µmol) was then combined to 1,4-bis(bromomethyl)-2,3,5,6-tetrafluorobenzene (5.3 mg, 1.5 eq) in 100 mM TRIS base (10 mL). After 15 min, the mixture was acidified by addition of 1 % TFA and the product was purified by HPLC. Isolated yield: 5.6 mg (25 %). Analytical HPLC (Method A): 11.0 min. ESI-MS: deconvoluted mass 2126.9 (expected 2127.3).
Peptide 7c

The sequence FITC-β-Ala-Leu-Thr(tBu)-Phe-Hcy(Trt)-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Hcy(Trt)-Gln(Trt)-Leu-Thr(tBu)-Ser(tBu) was assembled on solid support as described in the general SPPS procedure. The stapling reaction was attained following the palladium-mediated S-arylation approach reported by Rojas et al.7. The crude linear di-Hcy peptide (30 mg, 10.7 µmol) was then combined to 1,4-bis(bromomethyl)-2,3,5,6-tetrafluorobenzene (5.3 mg, 1.5 eq) in 100 mM TRIS base (10 mL). After 15 min, the mixture was acidified by addition of 1% TFA and the product was purified by HPLC. Isolated yield: 0.5 mg (2%). Analytical HPLC (Method A): 11.1 min. ESI-MS: deconvoluted mass 2054.9 (expected 2055.3).

Peptide 7d

The sequence FITC-β-Ala-Leu-Thr(tBu)-Phe-Cys(Trt)-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Cys(Trt)-Gln(Trt)-Leu-Thr(tBu)-Ser(tBu) was assembled on solid support as described in the general SPPS procedure. The crude linear di-Cys peptide (21 mg, 10.7 µmol) was then combined to 1,4-bis(bromomethyl)-2,3,5,6-tetrafluorobenzene (5.3 mg, 1.5 eq) in 100 mM TRIS base (10 mL). After 15 min, the mixture was acidified by addition of 1% TFA and the product was purified by HPLC. Isolated yield: 5.6 mg (25%). Analytical HPLC (Method A): 11.8 min. ESI-MS: deconvoluted mass 2127.0 (expected 2127.3).

Peptide 8

Hydrocarbon stapling was performed via on-resin RCM of α,α-disubstituted olefin residues (S5 or R8).8 The sequence Fmoc-Leu-Thr(tBu)-Phe-S5-Glu(tBu)-Tyr(tBu)-Trp(Boc)-S5-Gln(Trt)-Leu-Thr(tBu)-Ser(tBu) was assembled on SPPS. The resulting resin was washed with DCM and dried under high vacuum overnight. The dry resin was then placed in the synthesizer apparatus and swollen in dry DCE under N2 stream for 10 min and drained. The RCM reaction was performed by treating the resin with a 10 mM solution of Grubbs catalyst 1st generation in dry DCE (2 mL per 50 µmol resin) under N2 bubbling for 2h. The catalyst solution was drained and a fresh 10 mM Grubbs catalyst solution was added to the resin and reacted for 2h. After that, the resin was washed with DCE and DMF. Next, the Fmoc group was removed and the N-terminus was coupled to beta-alanine and FITC. Finally, the peptide was cleaved by TFA acidolysis and purified by HPLC. A major product peak (> 95%) was isolated exhibiting the expected mass. The cis/trans configuration of the olefin double bond was not determined. Analytical HPLC (Method C): 15.2 min. ESI-MS: [M+H]+ 1995.8 (expected 1995.9).
**ANALYTICAL METHODS**

**Peptide concentration determination**
The concentration of peptide stock solutions was determined by measuring the FITC absorbance signal (495 nm) of a diluted peptide solution (~ 200 µM) at pH 8 (10 mM sodium phosphate buffer for PDI-peptides; 10 mM Tris, 2M Urea for CPP9-peptides) using Nanodrop spectrophotometer.

**Analytical HPLC methods**
Analytical HPLC was performed on an Shimadzu system, using a Phenomenex Luna C18 (150 x 4.60 mm, 3 micron, 100A) column eluting at a flow rate of 1 mL/min and the following gradient of buffer B (90% ACN/10% H2O/0.1% TFA) in buffer A (0.1% TFA in water): Method A, 25 to 70 % B over 12 min; Method B, 25 to 70 % B over 20 min; Method C, 30 to 80 % B over 20 min; Method D, 0 to 60 % B over 12 min.

**ESI-MS methods**
UPLC-MS was performed on a Shimadzu Nexera UPLC system connected to Shimadzu LCMS-2020 single quadrupole mass spectrometer using an Agilent Eclipse Plus C18 RRHD 1.8 µm (2.1 x 100 mm) column and a gradient of 20 to 80 % buffer B (90% ACN/10% H2O/0.1% formic acid in buffer A, 0.1% formic acid in water) over 6 min. The observed ion masses were deconvoluted using Esiprot software.

**BIOPHYSICAL METHODS**

**Circular Dichroism Spectroscopy**
Peptide stock solutions were diluted in 10 mM phosphate buffer pH 7.4 (for acetylated peptides), 20 mM SDS in 10 mM sodium phosphate, 100 mM sodium fluoride buffer pH 7.4 or 0.4 mM POPC vesicles in 10 mM HEPES, 150 mM NaCl pH 7.4 to a final concentration of 20 µM. CD measurements were performed using a Jasco model J-710 spectropolarimeter which was routinely calibrated with (1S)-(+)10-camphorsulfonic acid. Spectra were recorded at room temperature (298K), with a 0.1 cm Jasco quartz cell over the wavelength range 260-185 nm at 50 nm/min, with a bandwidth of 1.0 nm, response time of 1 s, resolution step width of 1 nm and sensitivity of 20-50 Mdeg. Each spectrum represents the average of 5 scans. Spectra were analyzed using the Spectra Analysis software and smoothed using ‘adaptive smoothing’ function. Percentage helicity of peptides was calculated from the residue-molar ellipticity found at 220 nm using reported procedures.

**Determination of octanol/buffer pH 7.4 partition coefficient (logD7.4)**
The experimental partition was carried out using the shake flask method. The two liquid phases were presaturated with which other by rigorously shaking a 10 mM sodium phosphate pH 7.4 buffer solution to n-octanol for 10 minutes and let stand overnight to allow full separation of the two phases. The peptides were diluted in the presaturated buffer solution to a concentration of 20 µM. 500 µL aliquots of the peptide solution were transferred into two 1.5 mL HPLC glass vials. 500 µL n-octanol (presaturated) was then added to one of the vials, while the other was taken as reference for the partition. Both vials were vortexed for 30 s, and placed onto a shaker for 1 h (at 700 rpm, room temperature), then left resting for 2 h. After that, the vials were centrifugated (10 min, 4000 rpm) until clear visual separation of the phases was observed. The vials were submitted to HPLC analysis, where 20 µL samples were injected into the system, making sure the needlestroke permitted for sampling of the aqueous phase only. The absorbance intensity...
(Abs) of the peptide peak at 214 nm UV spectrum was used to calculate the rate of partition, accordingly to the equation: \[ \log D_{7.4} = \log \left( \frac{\text{Abs}_{\text{ref}}}{\text{Abs}_{\text{partition}}} - 1 \right) \], where \( \text{Abs}_{\text{ref}} \) is the measured absorbance intensity of the peptide reference sample and \( \text{Abs}_{\text{partition}} \) is the UV peptide absorbance intensity of the partition sample. \( \log D_{7.4} \) values are described in the manuscript as mean of at least two measurements.

**Preparation of lipid vesicles**

Model phospholipid membranes were prepared using synthetic POPC and POPC lipids (Avanti Polar Lipids). Lipid films were prepared by evaporation of chloroform lipid solutions (10 mg/mL of POPC alone or POPC:POPS (4:1)) with a \( \text{N}_2 \) stream followed by vacuum overnight. The lipid film was suspended in buffer (10 mM HEPES, 150 mM NaCl pH 7.4), vortexed and freeze-thawed 8-times and extruded 21-times through a 100 nm pore sized polycarbonate filter, affording uniform large unilamellar vesicles (LUVs) solutions used for CD and FP assays.

**Binding to phospholipid LUVs determined by fluorescence polarization assays**

In a 96-well plate, the FITC-labelled peptides (100 nM) were titrated with a serial dilution of POPC or POPC/POPS (4:1) LUVs in 10 mM HEPES, 150 mM NaCl pH 7.4 ranging from 0 to 800 \( \mu \)M concentrations. After 15 min incubation, fluorescence polarization to LUV concentration response was measured and the anisotropy values were fitted into a saturation binding curve using Prism software as depicted in Fig. S4. The partition coefficient \( K_p \) was calculated from the binding curves following the procedure reported by Henriques et al.\(^{10}\)

**CELL ASSAYS**

**Materials**

All cell culture reagents were purchased from Thermo Fisher Scientific, unless otherwise stated.

**Peptide uptake in HeLa cells**

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 10 U/mL penicillin, 10 \( \mu \)g/mL streptomycin and 1x GlutaMAX. HeLa cells were plated at a density at 2.5 x 10\(^5\) cells in a 24-well plate and allowed to incubate overnight. Media were removed and cells were washed with phosphate-buffered saline (PBS) then treated with 5 \( \mu \)M of fluorescein-labelled peptides diluted in serum-free DMEM for 1 h at 37\(^\circ\)C. Cells were then washed twice with PBS and dissociated with TrypLE for 10 min at room temperature. Flow cytometry were performed as described\(^{11}\) with minor modifications, cells were centrifuged at 500 g for 5 min at 4\(^\circ\)C and resuspended in ice-cold PBS supplemented with 1% FBS. 7-AAD viability staining solution (BioLegend) and trypan blue were added to the cells prior to flow cytometric analysis using CytoFLEX (Beckman Coulter). Cellular uptake of fluorescein-labelled peptides was analyzed using CytExpert (Beckman Coulter) as median fluorescence intensity and presented as percentage uptake, normalized to the fluorescence intensity of TAT (FITC-\( \mu \)Ala-RKKRRQRRRR). Cell death was quantified as 7-AAD positive stained cells, and ethanol was used as 100% cell death control.

**Endocytosis inhibition**

For endocytosis inhibition experiments, cells were pre-incubated with different endocytosis inhibitor (50 \( \mu \)M EIPA, 50 \( \mu \)M Dynasore, 10 \( \mu \)M chlorpromazine or 500 \( \mu \)g/\( \mu \)L genistein) for 2 h at 37\(^\circ\)C in serum-free DMEM prior to the treatment of 5 \( \mu \)M 7d. For 4\(^\circ\)C experiments, cells were placed on ice for 2 h prior to the treatment of 5 \( \mu \)M 7d in ice-cold serum-free DMEM. Cell harvesting and flow cytometric analysis are performed as mentioned above.

**Statistical analysis**

All graphs were plotted and analyzed using Prism 9 for macOS (Graphpad). Flow cytometry data were analyzed as median fluorescence intensity using CytExpert and presented as mean ± SEM of at least three independent experiments. Statistical significance was analyzed using one-way ANOVA.
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