Supporting Information
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Tuning the Properties of Penetrating Peptides by Oxime Conjugation

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Supporting Information

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1. Supporting methods

1.1. Materials and methods

Briefly, reagents for synthesis were purchased from Fluka, GL Biochem and Aldrich, amino acid derivatives from Iris Biotech and GL Biochem, Trizma buffer (99.9 %) was from Aldrich, salts of the best grade available were from Scharlau, Panreac and Fischer used as received. 8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) was from Sigma and p-xylene-bis-pyridinium bromide (DPX) was from Invitrogen. The aldehyde tested (Tgua) was synthesized following reported protocols.\textsuperscript{1a} The CFR\textsubscript{8} peptide was prepared as reported elsewhere.\textsuperscript{1b} Egg yolk L-α-phosphatidylcholine (EYPC) was from Avanti Polar Lipids. Deoxyribonucleic acid from herring sperm (Herring DNA) was from Aldrich. All reactions were performed under N\textsubscript{2} atmosphere. Unless stated otherwise, column chromatography was carried out on silica gel 60 (Fluka, 40-63 µm). Analytical thin layer chromatography (TLC) was performed in silica gel 60 (Merck, 0.2 mm, F254).

Ultra-high-performance liquid chromatography coupled with mass spectrometry (UHPLC-MS) analyses were carried out on Agilent Technologies 1260 Infinity II associated with a 6120 Quadrupole LC-MS using an Agilent SB-C18 column with Solvent A: Solvent B gradients between 5:95, 95:5 (Solvent A: H\textsubscript{2}O with 0.1 % TFA; Solvent B: CH\textsubscript{3}CN with 0.1 % TFA). High-performance liquid chromatography (HPLC) preparative purification was carried out on Waters 1525 composed by a binary pump with a dual Waters 2489 detector with a Phenomenex Luna C18(2) 100A column. An Agilent 1200 with an Agilent Luna C18(2) 100A column were used for semi-preparative purifications, with gradients between 95:5 and 5:95 (Solvent A: H\textsubscript{2}O with 0.1 % TFA; Solvent B: CH\textsubscript{3}CN with 0.1 % TFA). All the HPLC spectra reported show the absorbance at 222 nm in ordinates axis and the time in minutes in abscises axis.

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker 500 MHz spectrometer. Chemical shifts are reported in ppm (δ units) referenced to the following solvent signals: D\textsubscript{2}O δH 4.79. Accurate mass determinations (HR-MS) using ESI-MS were


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performed on a Sciex QSTAR Pulsar mass spectrometer and are reported as mass-per-charge ratio $m/z$.

Recalculations of concentrations of the labelled peptide (CF extinction coefficient $= 81500 \ \text{M}^{-1} \ \text{cm}^{-1}$) were carried out by ultraviolet-visible (UV-VIS) spectra, which were recorded on a Dynamica HALO XB-10 UV-VIS Single Beam Spectrophotometer. Circular Dichroism (CD) measurements were performed with a Jasco J-1100 CD Spectrometer equipped with a Jasco MCB-100 Mini Circulation Bath.

Vesicles were prepared with a Mini-Extruder from Avanti Polar Lipids (pore size 100 nm). Peptide/aldehyde incubation for oxime bond formation was performed in 500 µl eppendorf vials in a Thermo-shaker TS-100C Biosan. Fluorescence measurements were performed with a FluoroMax-2 spectrofluorometer (Jobin-Yvon Spex) equipped with a stirrer and a temperature controller.

Cell fluorescent images were acquired with an Andor Zyla 4.2 digital camera mounted on a Nikon Eclipse Ti-E fluorescence microscope. Absorbance and fluorescence of cellular extracts were measured using a microplate reader Tecan Infinite F200Pro. $[\alpha]_D^{22}$ values were recorded on a Jasco P-1030 Polarimeter (reported for concentrations c in g / 100 ml solvent). IR spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer (ATR, Golden Gate, unless stated otherwise) and are reported as wavenumbers $\nu$ in cm$^{-1}$ with band intensities indicated as s (strong), m (medium), w (weak), b (broad).

1.2. Abbreviations

Aa: Amino acid; Arg: Arginine; Boc: tert-Butoxycarbonyl; Calcd: Calculated; Cbz: (Benzyloxy)carbonyl; CF: 5(6)-Carboxyfluorescein; DCM: Dichloromethane; DIEA: N,N-Diisopropylethylamine; DMF: N,N-Dimethylformamide; DMSO: Dimethylsulfoxide; DPX: p-Xylene-bispyridinium bromide; EYPC: Egg yolk phosphatidyleholine; HFIP: 1,1,1,3,3,3-Hexafluoro-2-propanol; HPTS: 8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt; HR-MS (ESI): High resolution mass spectrometry (electrospray ionization); LUVs: Large unilamellar vesicles; Lys: Lysine; Tris: Tris(hydroxymethyl)aminomethane; Mtt: 4-Methyltrytill; N-HATU: N-[(Dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridine-1-
ylmethylene]-N-methylmethanaminium-hexafluorophosphate \( N \)-oxide; \( N \)-HBTU: \( N \)-[(1H-Benzotriazol-1-yl)-(dimethylamino)methylene]-N-methylmethanaminium hexafluorophosphate \( N \)-oxide; Ox: Oxime; Pbf: 2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl; SPPS: solid phase peptide synthesis; TFE: Trifluoroethanol; TIS: Trisopropylsilane; TNBS: 2,4,6-Trinitrobenzenesulfonic acid.

1.3 Synthesis

1.3.1. General protocol for the solid phase synthesis

The peptide was synthesized by manual Fmoc solid-phase peptide synthesis (SPPS)\(^2\) using Rink amide solid support (140 mg, load: 0.71 mg/mmol) previously swelled in DMF (peptide synthesis grade) for 30 min and then filtered and washed with DMF (3 x 4 ml, 3 min). Coupling cycle consisted of Fmoc group deprotection with a solution of piperidine in DMF (20%, 2 x 5 ml, 15 min) and DMF wash (3 x 3 ml, 1 min) followed by coupling with \( \alpha \)-amino acids (4 equiv), \( N \)-HBTU (4 equiv) and DIEA (4 equiv, 0.195 M solution in DMF) for 30 min. Finally the resin was washed with DMF (3 x 3 ml, 1 min). Each peptide coupling and deprotection was monitored employing the TNBS test.\(^3\) Once the lineal peptide was finished the acetylation capping of \( N \)-terminal group was performed by standard Fmoc deprotection conditions (20% piperidine in DMF (2 x 5 ml, 15 min)) followed by treatment with a solution of 2,6-lutidine/acetic anhydride (1:1, 3 ml). The resin suspension was mechanically shaken for 40 min and washed with DMF (3 x 3 ml, 1 min) and DCM (3 x 3 ml, 5 min). The Mtt protective group was selectively removed by mechanically stirring the resin in a mixture of DCM/HFIP/TFE/TIS (6.5:2:1:0.5, 2 x 3 ml, 3 h). After Mtt deprotection, the resin was washed with DCM (3 x 3 ml, 1 min) and DMF (3 x 3 ml, 10 min). A solution of (Boc-aminoxy)acetic acid (2.5 equiv per free Lysine) and \( N \)-HATU (2.5 equiv per free Lysine) in DMF (1 ml) was added to resin followed by the dropwise addition of a solution of a DIEA (2.5 equiv per free Lysine) in DMF (1 ml). The resin was then mechanically stirred for 45 min and finally washed with DMF (3 x 3 ml, 5 min) and DCM (3 x 3 ml, 5 min). The peptides were deprotected and cleaved from the resin.

by standard TFA cleavage at room temperature (TFA/DCM/H₂O/TIS, 90:5:2.5:2.5, 2 h) as shown in the scheme in fig. S1. Then, the mixture was filtered and the peptides were precipitated in ice-cold Et₂O and filtered. The precipitate was centrifuged and washed with Et₂O (2 x 10 mL). Crude peptides were dissolved in CH₃CN/H₂O (1:1, 1 mL) and purified using a C18 reverse-phase HPLC [Nucleosil 100-7 C18, H₂O (0.1% TFA)/CH₃CN (0.1% TFA) with a binary gradient of Solvent A and Solvent B, the collected fractions were lyophilized and stored at -20 °C. Purity and characterization were confirmed by analytical HPLC, ¹H NMR and mass spectrometry.

1.3.2. Synthesis of AcP(Ox)₂

Following the general protocol for the solid phase synthesis, the AcP(Ox)₂ was obtained after HPLC purification with an overall yield of 12 %. Rₜ = 16.7 min. RP-HPLC [Nucleosil 100-7 C18, H₂O (0.1% TFA)/CH₃CN (0.1% TFA) 95:5→5:95 (5→35 min), 0:100 (>35 min)]. [α]D: -6.3 (c = 0.0525 in H₂O/CH₃CN, 1:1). IR (neat): 3292 (m), 3250 (m), 2957 (m), 2805 (w), 1542 (m), 1451 (w), 1340 (w), 1224(w), 1200 (w), 1178 (m), 1136 (m), 1096 (w), 1012 (w), 836 (w), 725 (w). ¹H-NMR (500 MHz, D₂O): 4.4-4.1 (m, 17H), 3.3-3.2 (m, 10H), 2.1 (s, 3H), 1.9-1.5 (m, 40H), 1.5-1.4 (m, 11H), 1.0-0.85 (m, 32H). MS (ESI, H₂O): 1709 (10, [M+H]+), 855 (50, [M+2H]²⁺), 571 (100, [M+3H]³⁺), 428 (10, [M+4H]⁴⁺) (Fig. S2). HR-MS (ESI) Calcd for: C₇₁H₁₃₅N₂₅O₁₄ 1709.1026 found: 1709.1021.

1.3.3. Synthesis of the fluorophore labelled peptide

Following the general protocol for the solid phase synthesis, CFP(Ox)₂ was obtained after the last Fmoc group deprotection with a solution of piperidine in DMF (20%, 2 x 5 ml, 15 min) and DMF wash (3 x 3 ml, 1 min) followed by coupling with 5(6)-carboxyfluorescein (2 equiv), N-HBTU (2 equiv) and DIEA (0.25 equiv) for 4 hours (Fig. S5). The CFP(Ox)₂ was directly reacted with the corresponding aldehydes and the resulting oximes were purified by HPLC.
1.3.4. Preparation of AcP(T_{Iso})_2 and AcP(T_{Gua})_2

5 µL of AcP(Ox)_2 (50 mM) in H_2O/DMSO/AcOH (1:1:0.05) were mixed with 2 equivalents of the different aldehyde for each aminooxyacetate of the peptide (e.g., 10 µl of 100 mM hydrophobic aldehyde in DMSO and adjusted to a total volume to 100 µl with H_2O/DMSO/AcOH (1:1:0.05). The mixture was stirred at 60°C for 1 hour and the resulting O-alkyloximes (upon corresponding dilution) were then used for DNA activation experiments. Oxime-bond formation was confirmed by HPLC-MS. RP-HPLC [Nucleosil 100-7 C18, H_2O (0.1% TFA)/CH_3CN (0.1% TFA) 95:5→5:95 (5→35 min), 0:100 (>35 min)]. AcP(T_{Gua})_2: R_t: 20.5 min; MS (ESI, H_2O/CH_3CN): 1194 (90, [M+3TFA+2H]^{2+}), 720 (20, [M+TFA+3H]^{3+}), 512 (100, [M+4H]^{4+}) (Fig. S4). AcP(T_{iso})_2: R_t: 22.5 min; MS (ESI, H_2O): 1846 (65, [M+H]^+) , 924 (100, [M+2H]^{2+}), 616 (80, [M+3H]^{3+}) (Fig. S3).

1.3.5 Preparation of CFP(T_{Gly})_2, CFP(T_{Gua})_2 and CFP(T_{Acet})

Reactive peptides of CFP(Ox)_2 in H_2O (5 mM) were mixed with 2 equivalents of T_{Gly} (60 mM), T_{Gua} (60 mM) and T_{Acet} (60 mM) and adjusted the total volume to 500 µl with H_2O/DMSO/AcOH (1:1:0.05). The mixture was stirred at 60°C for 1 hour and the resulting O-alkyloximes were then directly used, after dilution in HKR buffer, in HeLa cells experiments. Oxime-bond formation was confirmed HPLC-MS. RP-UHPLC [SB-C18, H_2O (0.1% TFA)/CH_3CN (0.1% TFA) 95:5→5:95 (5→15 min), 0:100 (>15 min)]. CFP(T_{Gly})_2: R_t: 11.1 min; MS (ESI, H_2O/CH_3CN): 724 (100, [M+3H]^{3+}) (Fig S6). CFP(T_{Gua})_2: R_t: 10.5 min; MS (ESI, H_2O/CH_3CN): 788 (100, [M+3H]^{3+}), 592 (15, [M+4H]^{4+}), 473 (10, [M+5H]^{5+}) (Fig S7). CFP(T_{Acet})_2: R_t: 12 min; MS (ESI, H_2O/CH_3CN): 703 (100, [M+3H]^{3+}), 528 (10, [M+4H]^{4+}) (Fig. S8).
1.4. Circular Dichroism

Circular dichroism measurements were carried out with the following settings: acquisition range: 300-190 nm; band width: 1.0 nm; accumulation: 3 scans; data pitch: 1 nm; CD scale 200 mdeg/1.0 dOD; D.I.T. (Data Integration Time): 1 s; scanning mode: continuous; scanning speed: 200 nm/min. Measurements were done from 10 °C to 60 °C (data interval: 10 °C; temp. gradient 5 °C/min) in a quartz cell of 0.2 cm path length at a final volume of 0.5 mL (H₂O or TFE) with a final peptide concentration of 200 µM. The results are expressed as the mean residue molar ellipticity \([\theta]_{MRI}\) with units of degrees·cm²·dmol⁻¹ and calculated using the following equation S1.

\[
[\Theta]_{MRI} = \frac{0.1 \times \theta}{C \cdot l \cdot \text{number of residues}} \quad \text{(deg · cm}^2 \cdot \text{dmol}^{-1}) \quad \text{(S1)}
\]

Equation S1: Formula to calculate the ellipticity. \(\theta\) is the ellipticity (mdeg), \(C\) is the peptide concentration (M) and \(l\) is the cell path length (cm).

1.5. General procedures for vesicle experiments

1.5.1. Vesicle preparation

A thin lipid film was prepared by evaporating a solution of EYPC (25 mg) in a mixture of MeOH and CHCl₃ (1:1, 1 ml) on a rotary evaporator (room temperature) and then in vacuo overnight. The resulting film was hydrated with 1.0 ml buffer (5 mM HPTS, 16.5 mM DPX, 10 mM Tris, 72 mM NaCl, pH 7.4) for more than 30 min, subjected to freeze-thaw cycles (7 times) and extrusions (15 times) through a polycarbonate membrane (pore size 100 nm). Extravesicular components were removed by gel filtration (Sephadex G-50) with 10 mM Tris, 107 mM NaCl, pH 7.4. Final conditions: ~5 mM EYPC; inside: 5 mM HPTS, 16.5 mM DPX, 10 mM Tris, 72 mM NaCl, pH 7.4; outside: 10 mM Tris, 107 mM NaCl, pH 7.4.
1.5.2. DNA transport experiments in vesicles

EYPC-LUV stock solutions (5 µl) were diluted with a buffer (10 mM Tris, 107 mM NaCl, pH 7.4) and placed in a thermostated fluorescence cuvette (25 °C) and gently stirred (total volume 2000 µl; final lipid concentration 13 µM). HPTS efflux was monitored at λ 511 nm (λex 413 nm) as a function of time after addition of oxime-peptide [(20 µl in DMSO) at variable concentrations 0.1-5 µM] at t = 25 s, DNA (20 µl stock solution in buffer, at fixed concentration 70 µM) at t = 50 s and aqueous Triton X-100 (1.2%, 40 µl, 370 µM final concentration) at t = 225 s. Fluorescence intensities were normalized to fractional emission intensity I(t) using equation S2.

\[ I(t) = \frac{(I(t) - I_0)}{(I_\infty - I_0)} \]  

**Equation S2:** Formula to normalize transport kinetics. \( I_0 \) = It at DNA addition, \( I_\infty \) = It at saturation after lysis.

1.6. Cell assays

1.6.1. Cell culture and live cell imaging

HeLa cells were grown at 37 °C, 5 % CO₂, in Dulbecco’s Modified Eagle’s Medium (4500 mg/l glucose, L-glutamine, sodium pyruvate and sodium bicarbonate; Sigma-Aldrich), supplemented with 10 % fetal bovine serum (Sigma-Aldrich) and 1 % of Penicillin-Streptomycin-Glutamine Mix (Fischer).

For live cell imaging, HeLa cells grown on glass bottom dishes were washed with HEPES-Krebs-Ringer (HKR) buffer (5 mM HEPES, 137 mM NaCl, 2.68 mM KCl, 2.05 mM MgCl₂, 1.8 mM CaCl₂, pH 7.4) and incubated for 30 min with 1 µM Hoechst 33342 (ThermoFisher) in HKR to stain the nucleus. This solution was removed and cells were incubated for another 30 min with 4 µM of each (CFP(T)₂) diluted in HKR buffer, washed twice with HKR and examined on an epifluorescence microscope (Nikon Eclipse Ti-E).

For co-localization studies with lysosomes, cells were washed and then incubated with the different peptides in HKR for 30 min. After three HKR washes, pre-warmed complete culture media was added to the cells and they were incubated for 3 h at 37 °C. For
lysosomal staining, the cells were washed again and incubated with 100 nM LysoTracker Deep Red (ThermoFisher) in HKR buffer for 10 min, followed by three washes and immediately imaged.

1.6.2. Uptake quantification by fluorometry

Cellular uptake of the CFP(T)$_2$ was determined by fluorometry following the procedure described by Holm et al.$^4$ with minor modifications. HeLa cells seeded the day before in 12-well plates were washed with HKR and incubated with 4 µM of each CFP(T)$_2$ diluted in HKR buffer for 30 min at 37 ºC. After incubation, cells were washed twice with HKR buffer, trypsinized and 1 ml of HKR buffer was added before pelleting cells by centrifugation at 500 g for 5 min. The supernatant was removed and the cell pellet was lysed by incubation with 300 µl of 0.1 M NaOH at 4 ºC for 1 h.

The fluorescence of the CFP(T)$_2$ in the lysates was measured in an Infinite F200Pro microplate reader (Tecan) using $\lambda_{ex}$ 485 nm and $\lambda_{em}$ 535 nm and the concentration of the CFP(T)$_2$ was calculated by comparison with a calibration curve. Protein concentration was determined after neutralization of the NaOH in the lysates with a volume of 0.1 M HCl using a Coomassie (Bradford) assay kit (ThermoFisher) and measuring the absorbance at 570 nm (Infinite F200Pro, Tecan). Cellular uptake of the peptide was expressed as pmoles of peptide per mg of total protein.

To compare the uptake of the different CFP(T)$_2$, the distribution of each CFP(T)$_2$ in live cells was studied by microscopy and the amount of peptide internalized quantified by fluorometry as described in materials and methods. Cells incubated with the CFP(T$_{Gly}$)$_2$ showed a punctate cytoplasmic fluorescence, suggesting the presence of the peptide inside vesicles. Although the punctate pattern is similar to the one observed for octaarginine, the number of fluorescent vesicles seemed higher in the case of the CFP(T$_{Gly}$)$_2$. This was confirmed by quantification of the uptake by fluorometry, where it was observed a much higher uptake for the CFP(T$_{Gly}$)$_2$ than for octaarginine.

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1.6.3. Cell viability: MTT assay

Cell viability was established by a standard MTT assay. One day before the assay, a suspension of HeLa cells was plated in 96-well tissue culture plates (Costar 96 Flat Bottom Transparent Polystyrol) by adding 100 µl (~10,000 cells) per well. The next day, the medium was aspirated and cells were incubated in the presence of CFP(T)₂ (50 µl/well) in HKR buffer at different concentrations. After 30 min of incubation at 37 ºC, the HKR buffer was changed to DMEM containing 10% Fetal Bovine Serum (FBS) and the viability was measured by quantifying the cellular ability to reduce the water-soluble tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) to its insoluble formazan salt as follows. MTT (5 mg/ml in PBS, 10 µl/well) was added to the wells and the cells were further incubated for 4 h. The supernatant was carefully removed and the water-insoluble formazan salt was dissolved in DMSO (100 µl/well). The absorbance at 560 nm was measured. Data points were collected in triplicate and expressed as normalized values for untreated control cells (100%).
2. Supporting Figures

![Diagram of peptide synthesis]

**Fig. S1:** General synthetic scheme for the acetylated reactive peptide.
Fig. S2: A) Structure of AcP(Ox)$_2$; B) RP-HPLC [Nucleosil 100-7 C18, H$_2$O (0.1% TFA)/CH$_3$CN (0.1% TFA) 95:5→5:95 (5→35 min), 0:100 (>35 min)]. $R_t$ 16.7 min; C) $^1$H-NMR in D$_2$O; D) ESI-MS.
Fig. S3: A) Contracted structure of AcP(T_{iso})_2; B) RP-HPLC [Nucleosil 100-7 C18, H_2O (0.1% TFA)/CH_3CN (0.1% TFA) 95:5→5:95 (5→35 min), 0:100 (>35 min)]. R_t 22.5 min; C) ESI-MS.
Fig. S4: A) Contracted structure of AcP(T_{Gua})₂; B) RP-HPLC [Nucleosil 100-7 C18, H₂O (0.1% TFA)/CH₃CN (0.1% TFA) 95:5→5:95 (5→35 min), 0:100 (>35 min)]. Rₜ 20.5 min; C) ESI-MS.
Fig. S5: General synthetic scheme for the reactive peptide labeled with fluorophore.
Fig. S6: A) Contracted structure of CFP(T_{Gly})_2; B) RP-UHPLC [SB-C18, H_2O (0.1% TFA)/CH_3CN (0.1% TFA) 95:5→5:95 (5→15 min), 0:100 (>15 min)]. R_t 11.1 min. The double peak (both with equal mass) could be due to CF regioisomers; C) ESI-MS.
Fig. S7: A) Contracted structure of CFP(T_{Gua})$_2$; B) RP-UHPLC [SB-C18, H$_2$O (0.1% TFA)/CH$_3$CN (0.1% TFA) 95:5→5:95 (5→15 min), 0:100 (>15 min)]. $R_t$ 10.5 min. The double peak (both with equal mass) could be due to CF regioisomers; C) ESI-MS.
Fig. S8: A) Contracted structure of CFP(T\textsubscript{Acet})\textsubscript{2}; B) RP-UHPLC [SB-C18, H\textsubscript{2}O (0.1% TFA)/CH\textsubscript{3}CN (0.1% TFA) 95:5→5:95 (5→15 min), 0:100 (>15 min)]. R\textsubscript{t} 12 min; The double peak (both with equal mass) could be due to CF regioisomers; C) ESI-MS.