Electronic Supplementary Material (ESI) for ChemComm.

Head to tail cyclisation of cell-penetrating peptides: impact on GAG-dependent internalisation and direct translocation

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Electronic Supplementary Information (ESI)

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1. Structure of the CPP-cargo conjugates used in the biological experiments

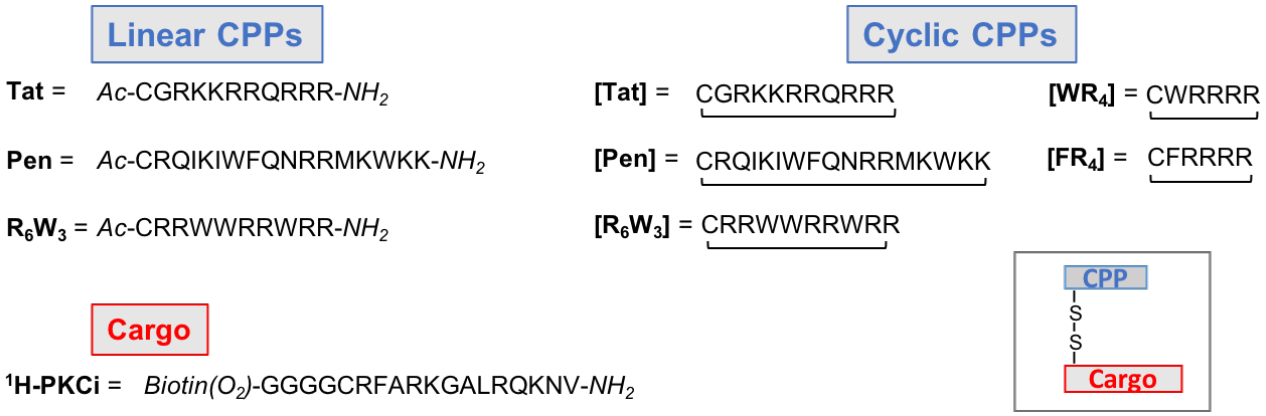


Figure S1. Sequence of the peptide cargo and non lipidated CPPs
Biotin(O_2) = biotine sulfone

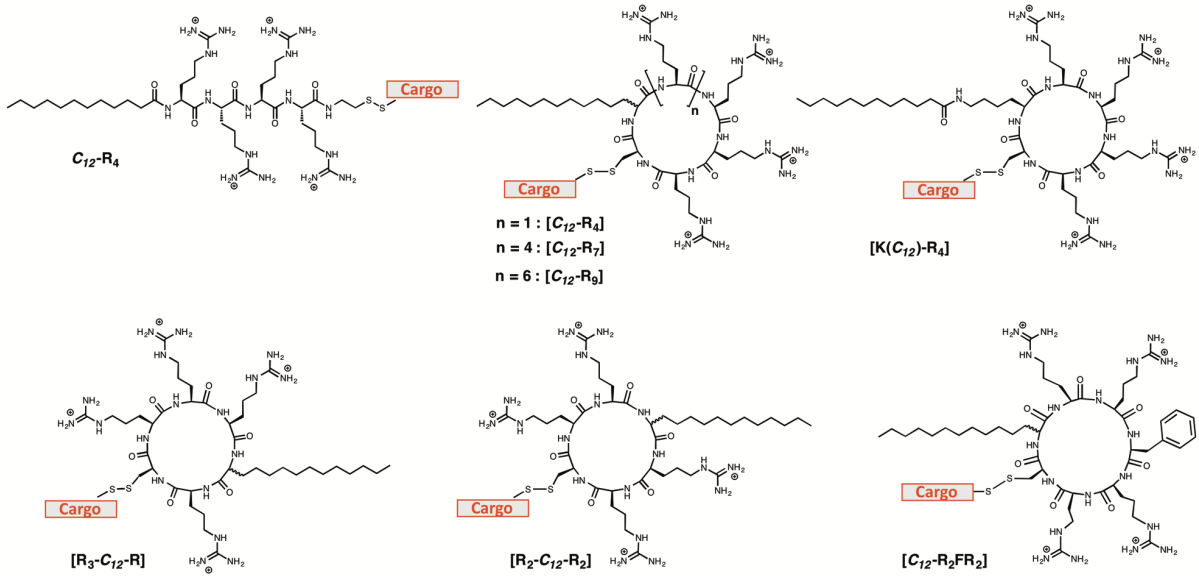


Figure S2. Structure of the lipidated CPP-Cargo conjugates
(Cargo = ^{1}H-PKCi shown in Fig. S1)

Note: For the cyclic lipidated cyclic CPPs, the carbon chain was introduced in the peptide sequence as the (*R*,*S*)-2-amino tetradecanoic acid derivative. A control CPP ([K(C_{12})-R$_4$]) was also synthesised by coupling lauric acid onto the side chain of a Lys residue, its uptake efficiency compared to [C_{12}-R$_4$] is shown Fig S9, page S31.

2. Abbreviations

Boc, *tert*-butoxycarbonyl; **BSA**, bovine serum albumin; ***C*$_{12}$**, (R,S)- 2-amino-tetradecanoic acid; ***C*$_{12:0}$**, lauric acid**; calcd**, calculated; **CHCA**, α-cyano-4-hydroxycinnamic acid; **CHO** cells, Chinese hamster ovary cells; **CPP**, cell-penetrating peptide; **DAPI**, 4',6-diamidino-2-phenylindole; **DCC**, dicyclohexylcarbodiimide; **DCM,** dichloromethane; **DIC**, *N,N'*-diisopropylcarbodiimide; **DMEM**, Dulbecco's modified Eagle medium; **DIEA**, diisopropylethylamine; **DMF**, *N,N*-dimethylformamide; **DTT**, dithiothreitol; **FBS**, fetal bovine serum; **EDT**, 1,2-ethandithiol; **EDTA**, ethylenediamine tetraacetic acid; **Fmoc**, 9-fluorenylmethoxycarbonyl; **HBSS**, Hanks' Balanced Salt solution, **HBTU**, 2-(1H-

benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; **HCTU,** *O*-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, **HOBt**, 1-hydroxybenzotriazole; **^{1}H-PKCi**, non deuterated PKCi; **^{2}H-PKCi**, deuterated PKCi; **HPLC**, high performance liquid chromatography; **MALDI-TOF MS**, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; **MBHA-PS**, 4-methylbenzhydrylamine polystyrene; **MPAA**, 4-mercaptophenylacetic acid, **mpaL**, mercaptopropionic acid leucine; **NCL**, native chemical ligation, **NMP**, *N*-methyl-2-pyrrolydone; **NpyS**, 3-nitro-2-pyridinesulphenyl; **PBS**, phosphate buffered saline; **PKCi**, protein kinase C inhibitor; **rt**, room temperature; **SEM**, standard error of the mean; **SPPS**, solid phase peptide synthesis; **TCEP**, tris(2-carboxyethyl)phosphine; **TES**, triethylsilane; **TFA**, trifluoroacetic acid; **TIPS**, triisopropylsilane.

3. General information

Reagents for peptide synthesis and protected amino acids were purchased from Iris Biotech and Merck Chemicals except for (2,2-D_2, 98%)-Boc glycine, which was obtained from Euriso-top. Solvents (peptide synthesis grade) were obtained from Carlo Erba Reagents. MBHA LL resin was purchased from Bachem. (*R*,*S*)-Boc-2-amino-tetradecanoic acid (CAS 129850-62-0) was purchased from Polypeptides. Tris-HCl, Triton X-100, trypsin inhibitor and bovine serum albumin were obtained from Sigma-Aldrich. DMEM/F-12 (1:1), FBS, trypsin-EDTA (0.05 % trypsin, 0.02 % EDTA) and HBSS were purchased from Gibco. The cell counting kit (CCK8) was from Dojindo Laboratories. Streptavidin-coated magnetic beads (Dynabeads® M-280 Streptavidin) were purchased from Invitrogen. The complete mini tablets of protease inhibitors were from Roche. DAPI was from Pierce. Cells were purchased from ATCC: CHO-K1 (reference ATCC CCL-61) and CHO-pgsA745 (reference CRL-2242). Ultrapure water was obtained using a Milli-Q water system from Millipore. All reagents and solvents were used without further purification.
HPLC purifications were carried out on a Waters system (Pump 600, Absorbance detector 2487) using ACE-5 C4 / C8 (300 Å, 5 µm, 250 × 10 mm, 5 mL/min flow rate) or Kromasil C18 (300 Å, 5 µm, 250 × 10 mm, 5 mL/min flow rate) reverse phase columns. Analytical HPLC were performed either on a Waters system (Pump 1525, Absorbance detector 2487), Agilent 1220 Infinity or Dionex Ultimate 3000. Vydac C4 / C18 (5 µm, 150 × 4.6 mm, 1 mL/min flow rate), Kromasil C18 (5 µm, 250 × 4.6 mm, 1 mL/min flow rate), ACE-5 C4 / C8 (5 µm, 250 x 4.6mm, 1 mL/min flow rate) or PROTO 200 C18 (3 µm, 100 x 4.6 mm, 1 mL/min flow rate) columns were used. Linear gradients of solvent B in A were used with A corresponding to 0.1 % TFA in H_2O and B to 0.1 % TFA in CH_3CN.

MALDI-TOF MS analyses were performed in the linear or reflector positive ion mode on an ABI Voyager DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems) or 4700 Proteomix MALDI-TOF MS/MS (Applied Biosystems), using as matrix a saturated solution of CHCA in CH_3CN/H_2O/TFA (50:50:0.1).

4. General procedures for solid phase peptide synthesis

Amounts of reagent are given in equivalents (equiv.) with respect to the peptidyl-resin. Peptide syntheses were carried out manually using Fmoc or Boc SPPS and standard protected amino acids unless otherwise mentioned. Syntheses were usually performed at a 0.1mmol scale.

***Fmoc SPPS protocol**:* Fmoc-protected amino acids (0.6 mmol, 6 equiv.) were activated with HBTU (0.58 mmol, 5.8 equiv., 0.5 M) and DIEA (1.4 mmol, 14 equiv.) in NMP (3 min)

and added to the peptide-resin (coupling for 30 min). Capping of eventual unreacted amine groups was achieved by treatment with acetic anhydride (10 %) and DIEA (0.5 mmol, 5 equiv.) in DCM for 20 min. Fmoc groups were removed by treatment with 20 % piperidine in NMP (3 x 3 min). Final peptide deprotection and cleavage from the resin were performed by treatment with TFA/H_2O/*i*Pr_3SiH, 95:2.5:2.5 (3 h) (for peptides containing Met or Cys residues, the cocktail TFA/H_2O/*i*Pr_3SiH/EDT, 94:2.5:1:2.5 was used). TFA was sparged under a stream of N_2, peptides were precipitated in ice-cold diethyl ether, recovered by centrifugation and freeze-dried before HPLC purification.

In situ neutralization protocol for Boc SPPS [1]: Boc-protected amino acids (0.6 mmol, 6 equiv.) were activated with HBTU (0.58 mmol, 5.8 equiv., 0.5 M) and DIEA (1.4 mmol, 14 equiv.) in NMP (3 minutes) and added to the peptide-resin (coupling for 30 min). Boc groups were removed by treatment with TFA (2 x 1 min). The resin was carefully washed with NMP. Final peptide deprotection and cleavage from the resin was performed by treatment with anhydrous HF (2 h, 0°C) in the presence of the following scavengers:

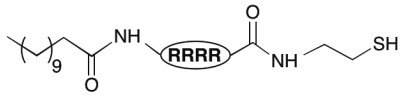
- For *C*-terminal carboxamide peptides with no Cys residues, anisole (1.5 mL/g peptide-resin) and dimethyl sulfide (0.25 mL/g peptide-resin) were used as scavengers.
- For *C*-terminal carboxamide peptides with a Cys residue protected with *p*-methoxybenzyl, *p*-toluenethiol (300 mg/g peptide-resin) was also added.
- For *C*-terminal carboxamide peptides with a Cys residue protected with a NpyS, only anisole was used.
- For peptide-mpaL thioesters, the only scavenger added was *p*-cresol (65 mg/g peptide-resin).

HF was removed under vacuum and the peptide was precipitated in ice-cold diethyl ether. The precipitate was dissolved in degassed acetic acid (10 % in H_2O), the resin eliminated by filtration and the peptide was freeze-dried before HPLC purification.

5. Linear CPPs and peptide cargo

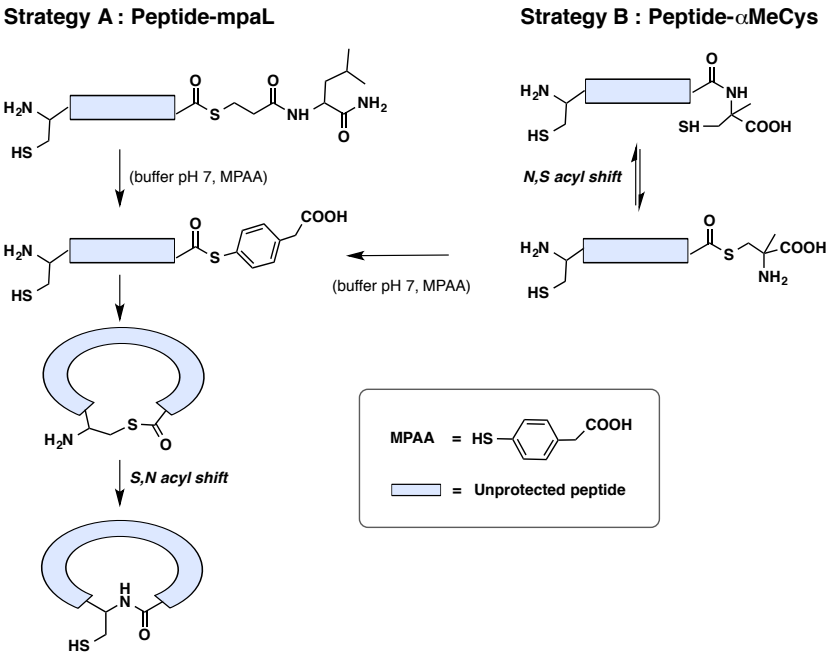
The syntheses of the linear CPPs (Table S1), the PKCi cargo (^{1}H-PKCi) and the deuterated internal standard used for MALDI-TOF MS quantification (^{2}H-PKCi) have been described previously.[2][3]

Table S1. Name and sequences of the peptide cargo and linear CPPs

Name	Sequence
^{1}H-PKCi	Biotin(sulfone)GGGGC(NpyS)RFARKGALRQKNV-*NH_2*
^{2}H-PKCi	Biotin(sulfone)GGGGCRFARKGALRQKNV-*NH_2**
Tat	*Ac*-CGRKKRRQRRR-*NH_2*
R_6W_3	*Ac*-CRRWWRRWRR-*NH_2*
Pen	*Ac*-CRQIKIWFQNRRMKWKK-*NH_2*
C_{12}-R_4	

Ac = acetyl. NpyS, 3-nitro-2-pyridinesulphenyl. *Gly residues shown in red in the sequence are bideuterated.

6. Cyclic CPPs



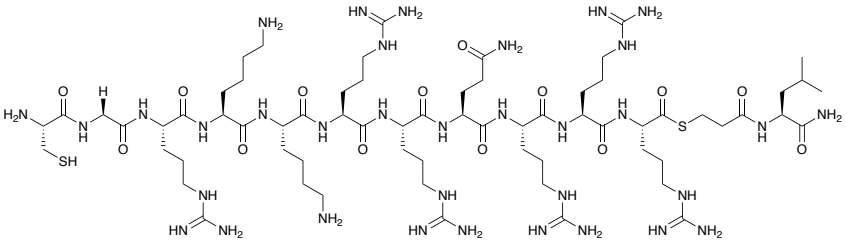
Scheme S1. Strategies used for peptide cyclisation

a. Strategy A: Synthesis of cyclic CPPs by native chemical ligation (NCL) using peptide-mpaL thioesters

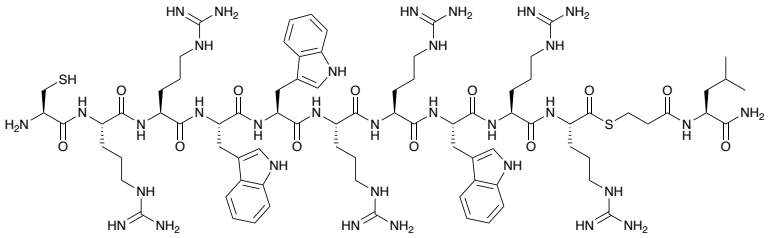
Synthesis of peptide-mpaL thioesters

The procedure described by Hackeng *et al.* was applied.[4] Briefly, Boc-Leu was first coupled on MBHA-PS resin (0.54 mmol/g, 0.1 mmol) (see the in situ neutralization protocol for Boc SPPS p. S4). *S*-trityl-mercaptopropionic acid (0.2 mmol, 2 equiv.) was then coupled using HBTU (0.18 mmol, 1.8 equiv.) and DIEA (0.4 mmol, 4 equiv.) in NMP for 1h30. The peptide-resin was washed with NMP. Removal of the trityl protecting group was performed by 15 min continuous flow with a mixture of TFA/H_2O/TES, 95:2.5:2.5 followed by washing with NMP. The next Boc-protected amino acid was immediately coupled (HBTU/DIEA activation, 45 min coupling) and the peptides elongated using the in situ neutralization Boc SPPS protocol. Peptides were then deprotected and cleaved from the resin by HF treatment (protocol p. S4) and purified by semi-preparative RP-HPLC.

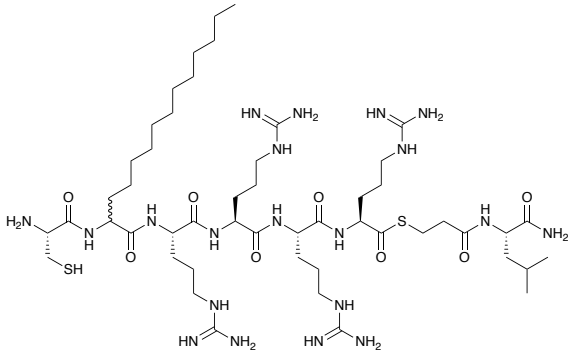
Synthesized peptide-mpaL thioesters:



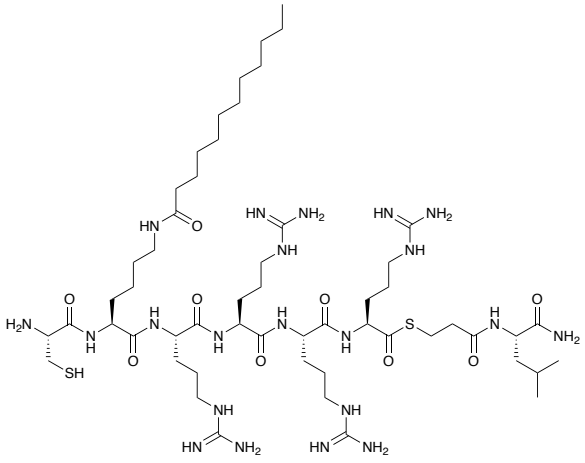
Tat-mpaL (CGRKKRRQRRR-mpaL)
MALDI-TOF *m/z* $[M+H]^+$ calcd.: 1701.1, found: 1701.4



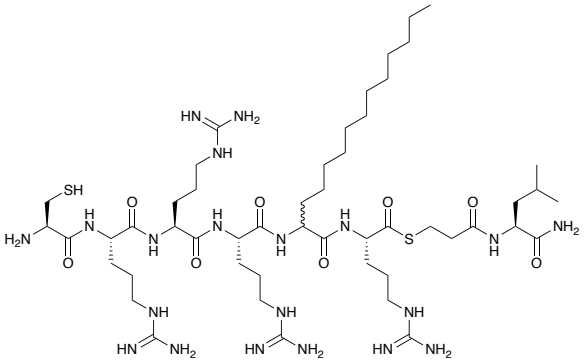
R_6W_3-mpaL (CRRWWRRWRR-mpaL)
MALDI-TOF (*m/z*) $[M+H]^+$ calcd.: 1817.9, found: 1818.2



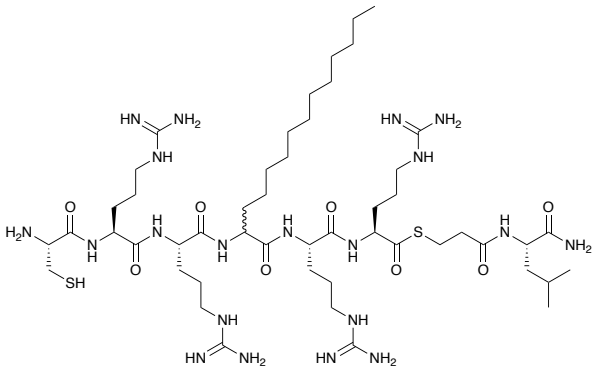
***C_{12}*-R_4-mpaL** (C*C_{12}*RRRR-mpaL)
MALDI-TOF (*m/z*) $[M+H]^+$ calcd.: 1171.7, found: 1771.6



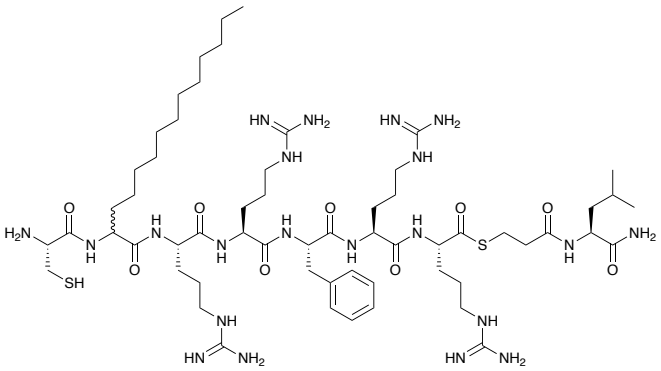
K(*C_{12}*)-R_4-mpaL (CK(*$C_{12:0}$*)RRRR-mpaL)
MALDI-TOF (*m/z*) $[M+H]^+$ calcd.: 1257.8, found: 1258.9



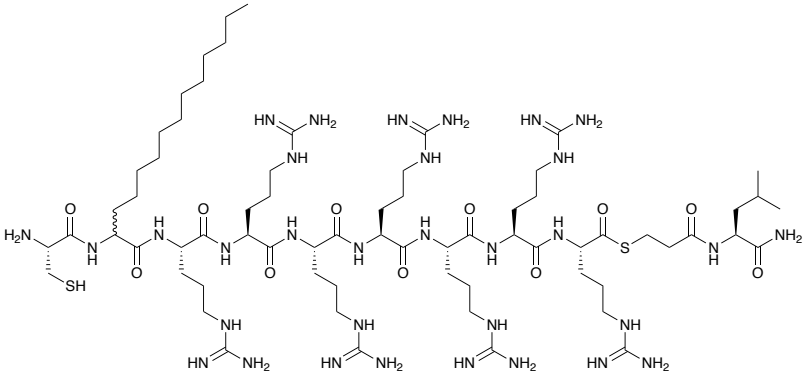
R$_3$-*C$_{12}$*-R-mpaL (CRRR*C$_{12}$*R-mpaL)
MALDI-TOF (*m/z*) [M+H]$^+$ calcd.: 1171.7, found: 1173.4



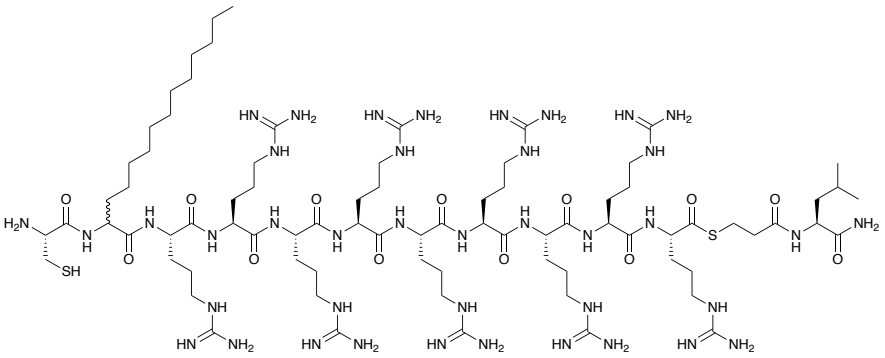
R$_2$-*C$_{12}$*-R$_2$-mpaL (CRR*C$_{12}$*RR-mpaL)
MALDI-TOF (*m/z*) [M+H]$^+$ calcd.: 1171.6, found: 1174.7



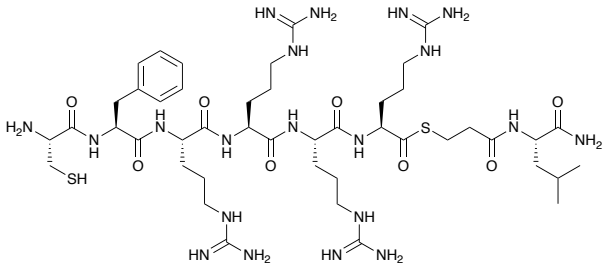
***C$_{12}$*-R$_2$FR$_2$-mpaL** (C*C$_{12}$*RRFRR-mpaL)
MALDI-TOF (*m/z*) [M+H]$^+$ calcd.: 1318.8, found: 1320.5



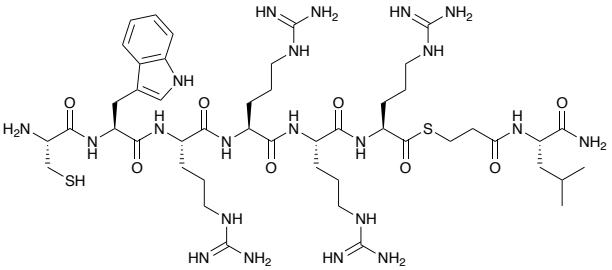
***C$_{12}$*-R$_7$-mpaL** (C*C$_{12}$*RRRRRRR-mpaL)
MALDI-TOF (*m/z*) [M+H]$^+$ calcd.: 1640.0, found: 1638.2



***C_{12}*-R_9-mpaL** (C*C_{12}*RRRRRRRRR-mpaL)
MALDI-TOF (*m/z*) $[M+H]^+$ calcd.: 1952.2, found: 1954.6



FR_4-mpaL (CFRRRR-mpaL)
MALDI-TOF (*m/z*) $[M+H]^+$ calcd.: 1093.6, found: 1093.7



WR_4-mpaL (CWRRRR-mpaL)
MALDI-TOF (*m/z*) $[M+H]^+$ calcd.: 1132.6, found: 1132.8

Cyclisation of the mpaL thioesters

A carefully degassed 0.2 M sodium phosphate buffer containing 2 mM EDTA, 3 or 6 M guanidine hydrochloride (see below), 3 mM MPAA and 3.5 mM TCEP was added to the peptide mpaL thioester (concentration 1 mM, final pH 6.5 or 7).The ligations were carried out at room temperature and monitored by RP-HPLC (Fig S3). For this purpose 10 µL aliquots of the reaction mixture were injected in HPLC. After completion, the reaction mixtures were diluted with 0.1 % TFA in water and purified by RP-HPLC to afford the desired cyclic products (Fig S1 and S2).
NCL was performed in buffer containing 3 M Guanidine for mpaL derivatives of Tat, R_6W_3, *C_{12}*-R_7, *C_{12}*-R_9, FR_4 and WR_4, which showed good solubility in these conditions. For the other derivatives, 6 M guanidine was used.

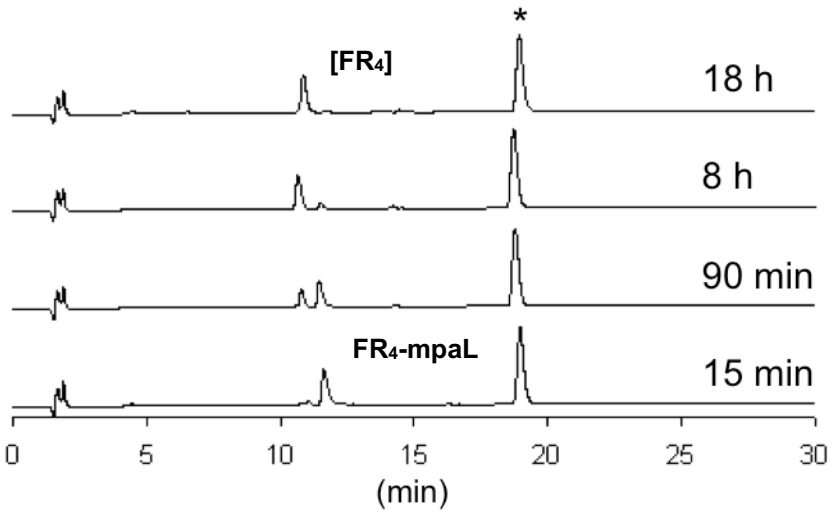
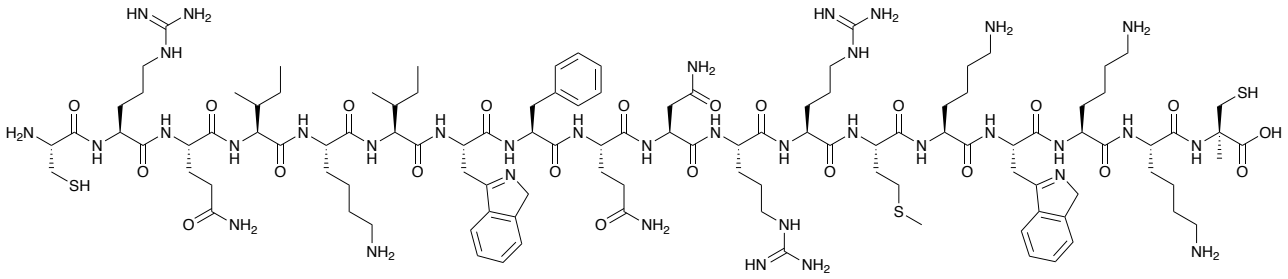


Figure S3. HPLC monitoring of the cyclisation of FR_4-mpaL to give [FR_4]
The reaction was performed in 200 mM sodium phosphate buffer containing 2 mM EDTA, 3 M Guanidine, 3.5 mM TCEP, 3 mM MPAA, final pH 6.5 using 1 mM FR_4-mpaL. HPLC gradient: 0-30 % B in 30 min; column: Vydac C18; detection wavelength: 214 nm. *MPAA.

b. Strategy B: Synthesis of cyclic CPP by NCL using a peptide with a C-terminal α-methylcysteine (αMeCys)

The *C*-terminal α-methylcysteine-containing peptide was synthesised by standard Fmoc SPPS on Wang resin functionalised by an α-methylcysteine (0.20 mmol/g, 0.1mmol) as described previously.[5] After completion of elongation, peptide-resin was deprotected and cleaved using the standard cleavage procedure (protocol p S3) and the peptide-αMeCys was purified by RP-HPLC.



Pen-αMeCys (CRQIKIWFQNRRMKWKK-αMeC)
MALDI-TOF (*m/z*) $[M+H]^+$ calc.: 2465.3, found: 2465.7

Cyclisation of Pen-αMeCys
To optimise the conditions of cyclisation, we first evaluated the effect of different parameters (pH, guanidine and MPAA concentration) on the reaction rate. Pen-αMeCys (final concentration 1 mM) was dissolved in degassed ligation buffer containing 200 mM sodium phosphate, 2 mM EDTA, 50 mM TCEP and the guanidine and MPAA concentrations and pH indicated Table S2. The reaction was monitored by HPLC injecting aliquots of 10 µL of the ligation mixture at different reaction times (Fig S4). The extent of conversion of Pen-αMeCys into [Pen] was deduced from the relative areas of both species HPLC peaks, considering that both compounds have the same extinction coefficient at 220 nm. The

reaction was repeated in larger scale at pH 6 using 3 M Guanidine and 450 mM MPAA and the compound purified by reverse phase HPLC.

Table S2: Effect of pH, guanidine and MPAA concentrations on the half-time of ligation

Guanidine (M)	MPAA (mM)	pH	t ½ (h)
6	300	6.5	> 15
3	300	6.5	8
3	300	5.5	6
3	450	5.5	5
3	450	7	6
3	450	5	3.5

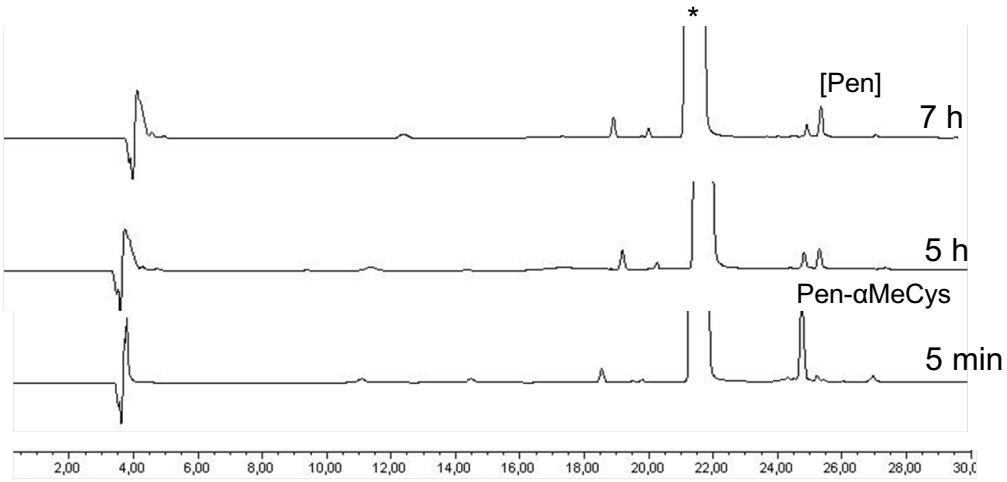


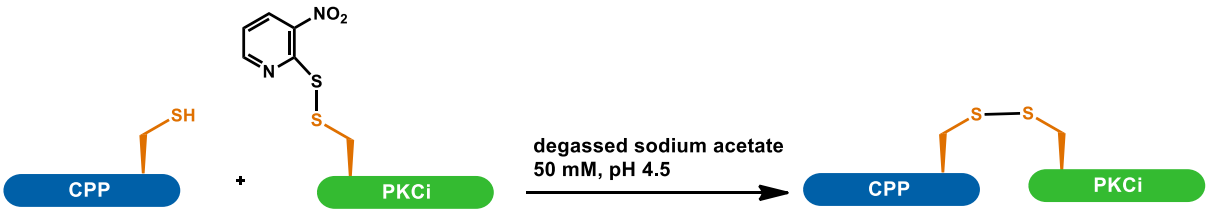
Figure S4. HPLC monitoring of the cyclisation of Pen-αMeCys to give [Pen]. The reaction was performed in 200 mM sodium phosphate buffer containing 2 mM EDTA, 3 M Guanidine, 50 mM TCEP, 450 mM MPAA, pH 6. HPLC gradient: 5-55 % B in 30 min; column: ACE-5 C8; detection wavelength: 214 nm. *MPAA

Table S3. MALDI-TOF MS characterisation of the cyclic CPPs

Peptide	$[M+H]^+$ (calc)	$[M+H]^+$ (found)
[Tat]	1481.8	1481.6
[R_6W_3]	1598.9	1597.6
[Pen]	2331.1	2330.9
[C_{12}-R_4]	953.2	953.4
[K(C_{12})-R_4]	1038.4	1039.0
[R_3-C_{12}-R]	953.2	953.4
[R_2-C_{12}-R_2]	953.2	953.8
[C_{12}-R_2FR_2]	1100.4	1100.9
[C_{12}-R_7]	1420.7	1421.7
[C_{12}-R_9]	1734.2	1733.5
[FR_4]	875.1	875.5
[WR_4]	913.8	913.7

7. Synthesis and characterisation of the CPP-PKCi disulfide conjugates used in the biological experiments

The CPP was dissolved in degassed sodium acetate buffer (50 mM, pH 4.5 to 5) and mixed with 1.1 eq. of ^{1}H-PKCi (final peptide concentrations: 1 to 5 mM). The reaction was monitored by HPLC. At the end of the reaction, conjugates were purified by reverse phase HPLC.

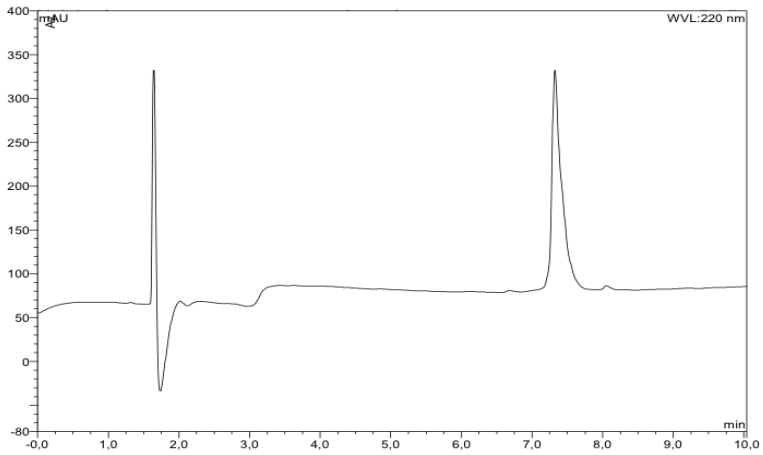


Scheme S2. Disulfide bridge formation between the CPP and the cargo

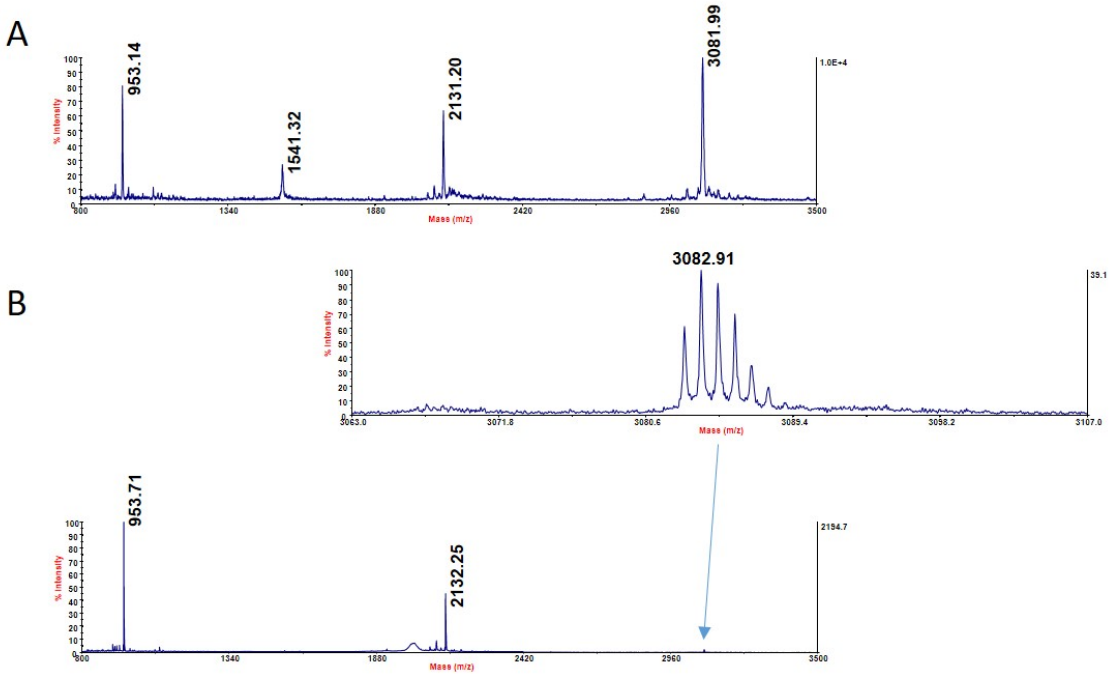
The conjugates were characterized by MALDI-TOF MS on a Voyager DE-PRO (Applied Biosystems) or on a 4700 Proteomix MALDI-TOF/TOF (Applied Biosystems) in the linear positive ion modes and reflector positive ion mode. The disulfide bond is fragile and a fragmentation is observed at this position during MALDI-TOF MS analysis, the $[M+H]^+$ peak being more difficult to detect in the reflector mode compared to the linear one.

Conjugate C_{12}-R_4-PKCi has been previously described.[3]

[C_{12}-R_4]-PKCi

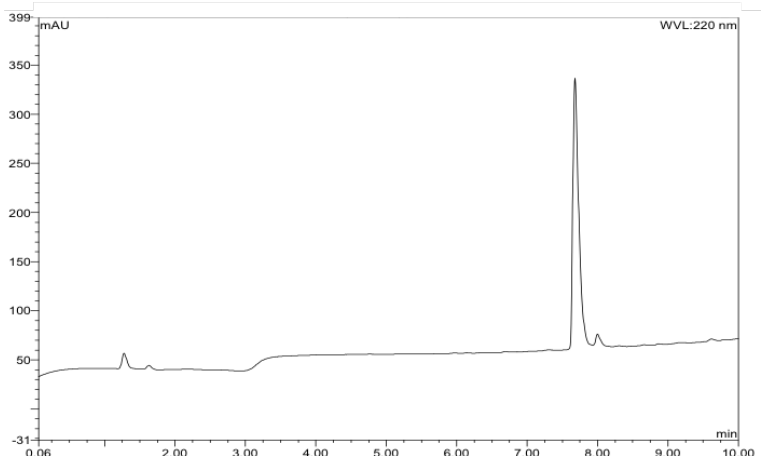


HPLC trace of [C_{12}-R_4]-PKCi
Column: PROTO 200 C18 3μm, 100 x 4.6 mm, Higgins Analytical, Inc
Gradient: 0-70 % B in 10 min, 1 mL/min
HPLC: Dionex Ultimate 3000



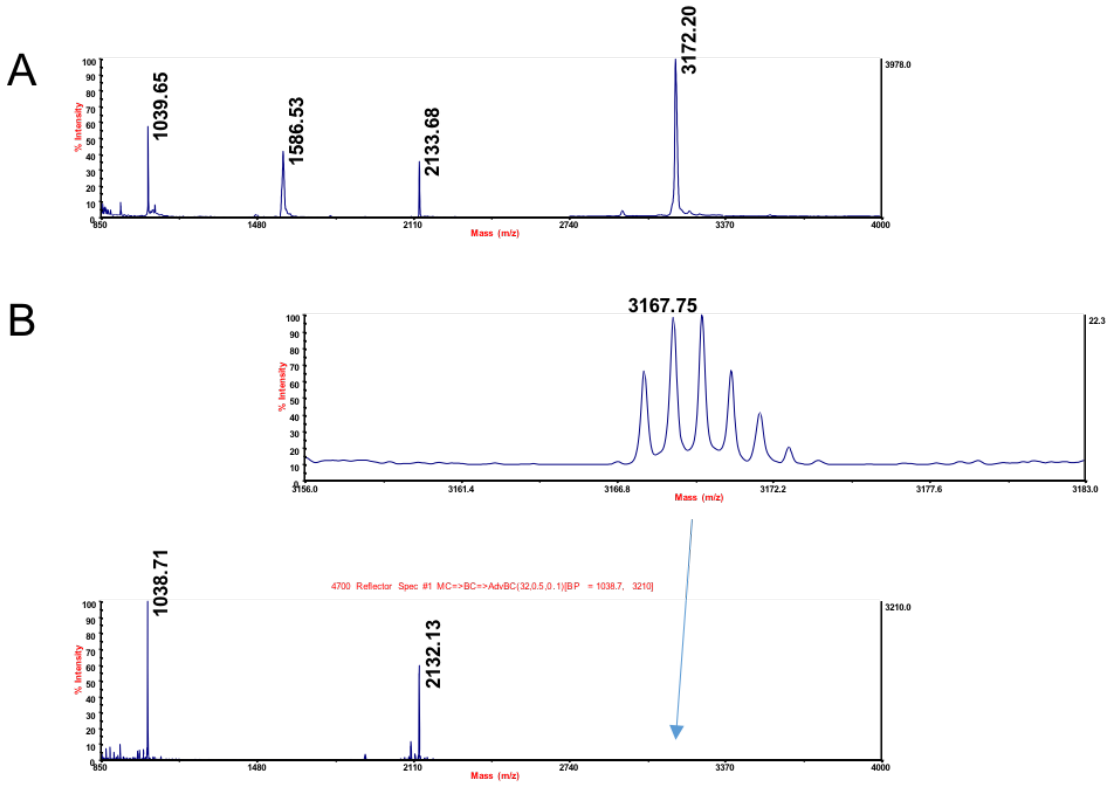
MALDI-TOF MS analysis of the [C_{12}-R_4]-PKCi disulfide conjugate: A) in the linear positive ions mode and **B)** in the reflector positive ions mode (top shows a zoom on the $[M+H]^+$ peak).
Reflector mode mass spectrum:
m/z 3082.9 (monoprotonated ion of [C_{12}-R_4]-PKCi, calc. 3083.8)
Fragmentation of the disulfide bond gives two ions at m/z 2132.2 (PKCi) and m/z 953.7 ([C_{12}-R_4]).

[K(C_{12})-R_4]-PKCi



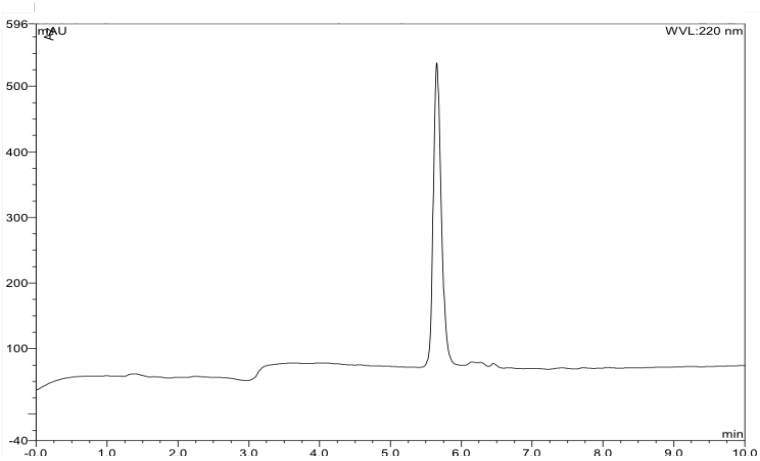
HPLC trace of [K(C_{12})-R_4]-PKCi

Column: PROTO 200 C18 3 μm, 100 x 4.6 mm, Higgins Analytical, Inc
Gradient: 0-70 % B in 10 min, 1 mL/min
HPLC: Dionex Ultimate 3000

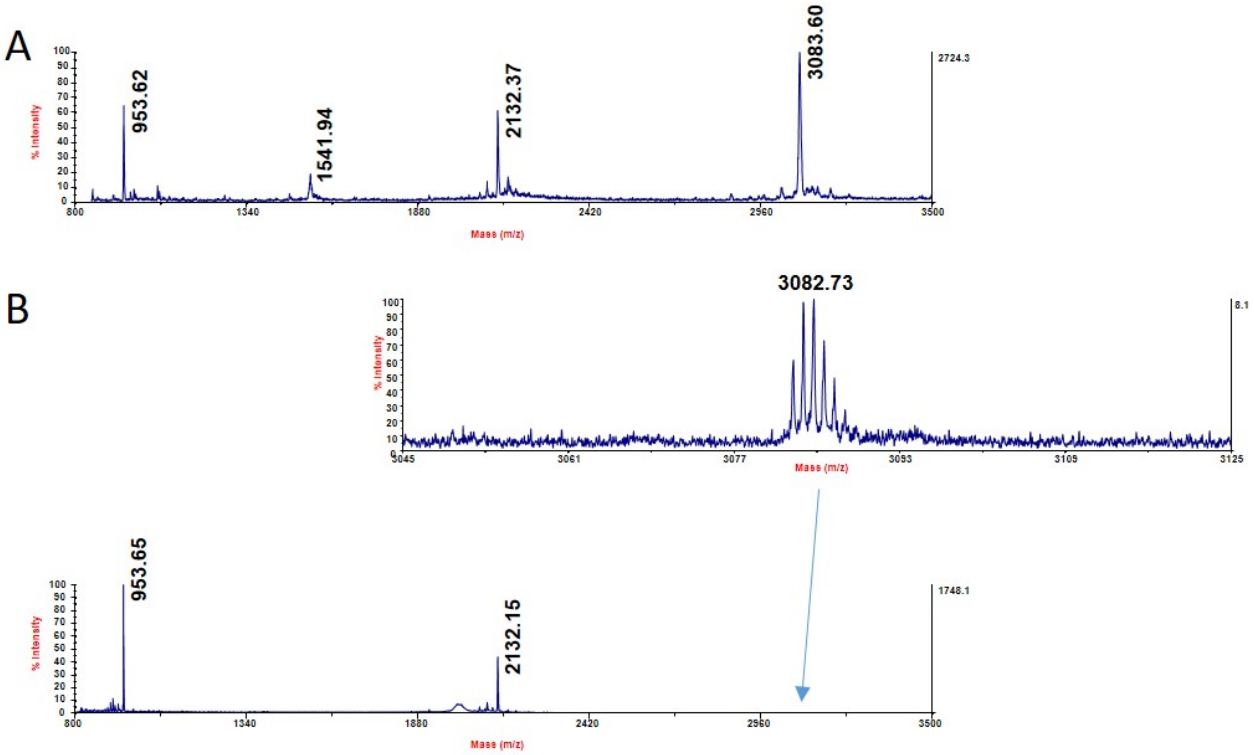


MALDI-TOF MS analysis of the [K(C_{12})-R_4]-PKCi disulfide conjugate: A) in the linear positive ions mode and **B)** in the reflector positive ions mode (top shows a zoom on the $[M+H]^+$ peak).
Reflector mode mass spectrum:
m/z 3167.7 (monoprotonated ion of [K(C_{12})-R_4]-PKCi, calc. 3168,9)
Fragmentation of the disulfide bond gives ions at m/z 2132.1(PKCi) and m/z 1038.7 ([K(C_{12})-R_4]).

[R_3-C_{12}-R]-PKCi

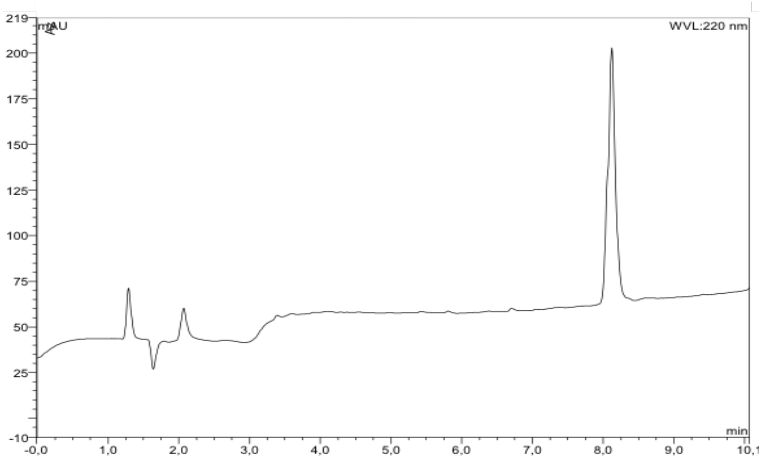


HPLC trace of [R_3-C_{12}-R]-PKCi
Column: PROTO 200 C18 3 μm, 100 x 4.6 mm, Higgins Analytical, Inc
Gradient: 0-70 % B in 10 min, 1 mL/min
HPLC: Dionex Ultimate 3000

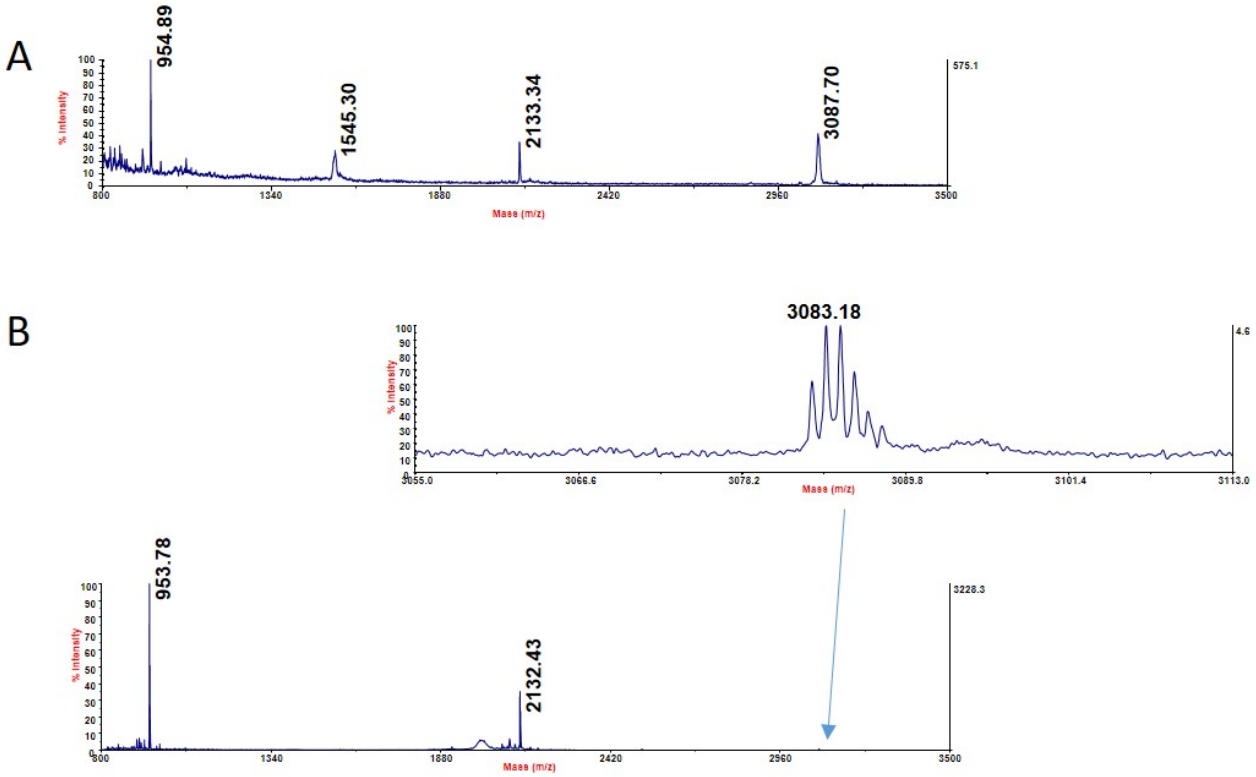


MALDI-TOF MS analysis of the [R_3-C_{12}-R]-PKCi disulfide conjugate: A) in the linear positive ions mode and **B)** in the reflector positive ions mode (top shows a zoom on the $[M+H]^+$ peak).
Reflector mode mass spectrum:
m/z 3082.7 (monoprotonated ion of [R_3-C_{12}-R]-PKCi, calc. 3083.8)
Fragmentation of the disulfide bond gives ions at m/z 2132.1 (PKCi) and m/z 953.6 ([R_3-C_{12}-R]).

[R_2-C_{12}-R_2]-PKCi

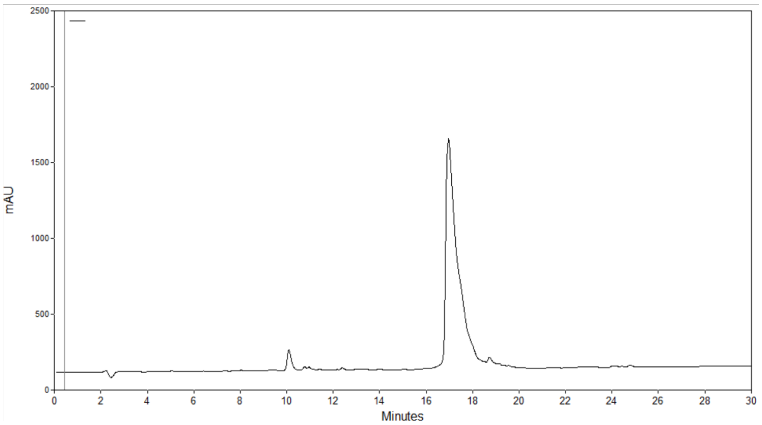


HPLC trace of [R_2-C_{12}-R_2]-PKCi
Column: PROTO 200 C18 3 μm, 100 x 4.6 mm, Higgins Analytical
Gradient: 0-70 % B in 10 min, 1 mL/min
HPLC: Dionex Ultimate 3000

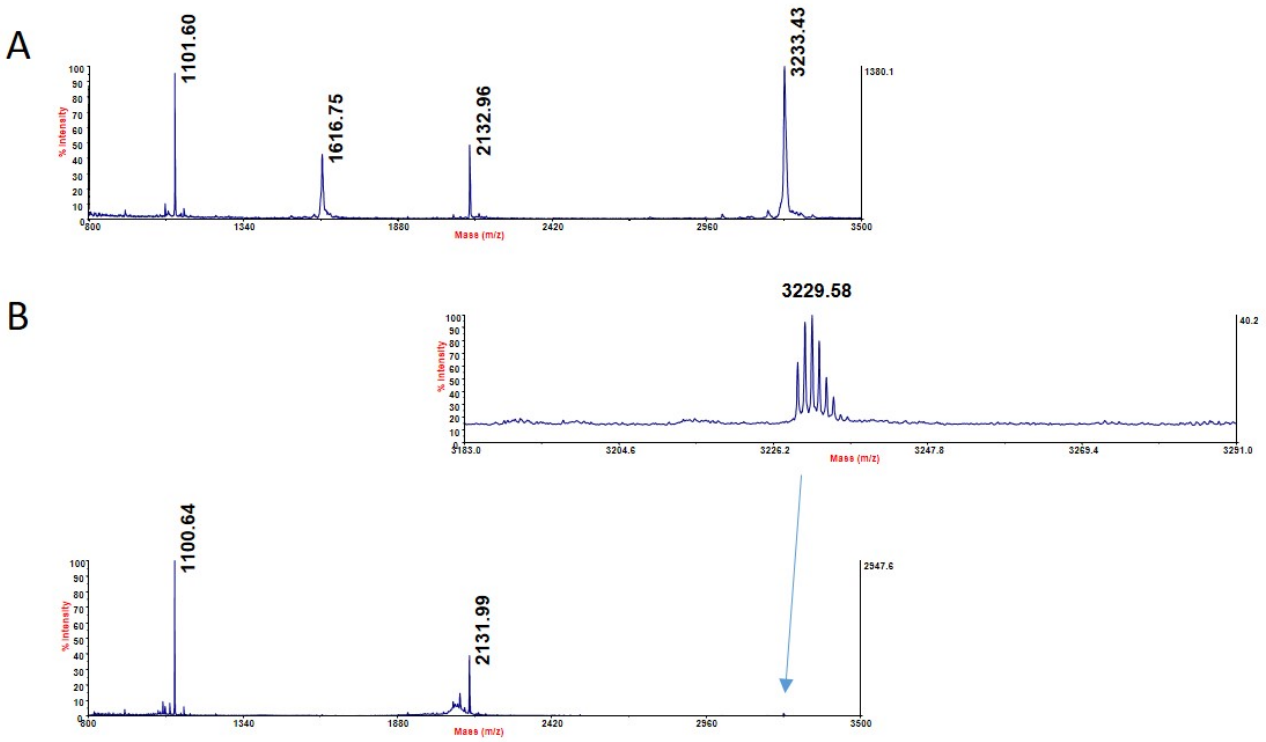


MALDI-TOF MS analysis of the [R_2-C_{12}-R_2]-PKCi disulfide conjugate: A) in the linear positive ions mode and **B)** in the reflector positive ions mode (top shows a zoom on the $[M+H]^+$ peak).
Reflector mode mass spectrum:
m/z 3083.2 (monoprotonated ion of [R_2-C_{12}-R_2]-PKCi, calc. 3083.8)
Fragmentation of the disulfide bond gives ions at m/z 2132.4 (PKCi) and m/z 953.8 [R_2-C_{12}-R_2]).

[C_{12}-R_2FR_2]-PKCi

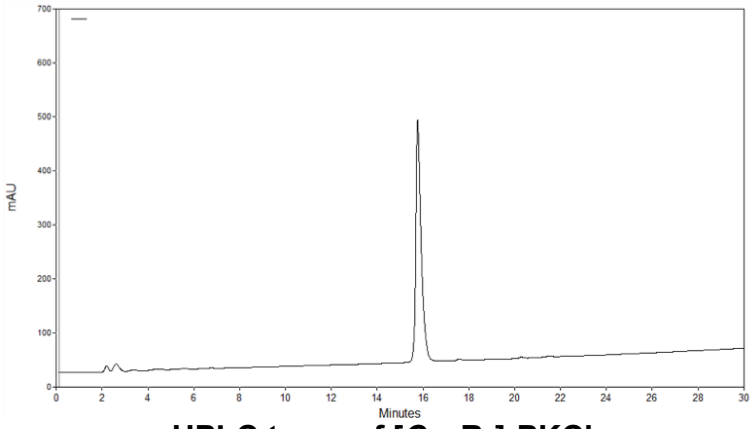


HPLC trace of [C_{12}-R_2FR_2]-PKCi
Column: Vydac C18 5 μm, 150 x 4.6 mm
Gradient: 5-60 % B in 30 min, 1 mL/min
HPLC: Agilent 1220 Infinity

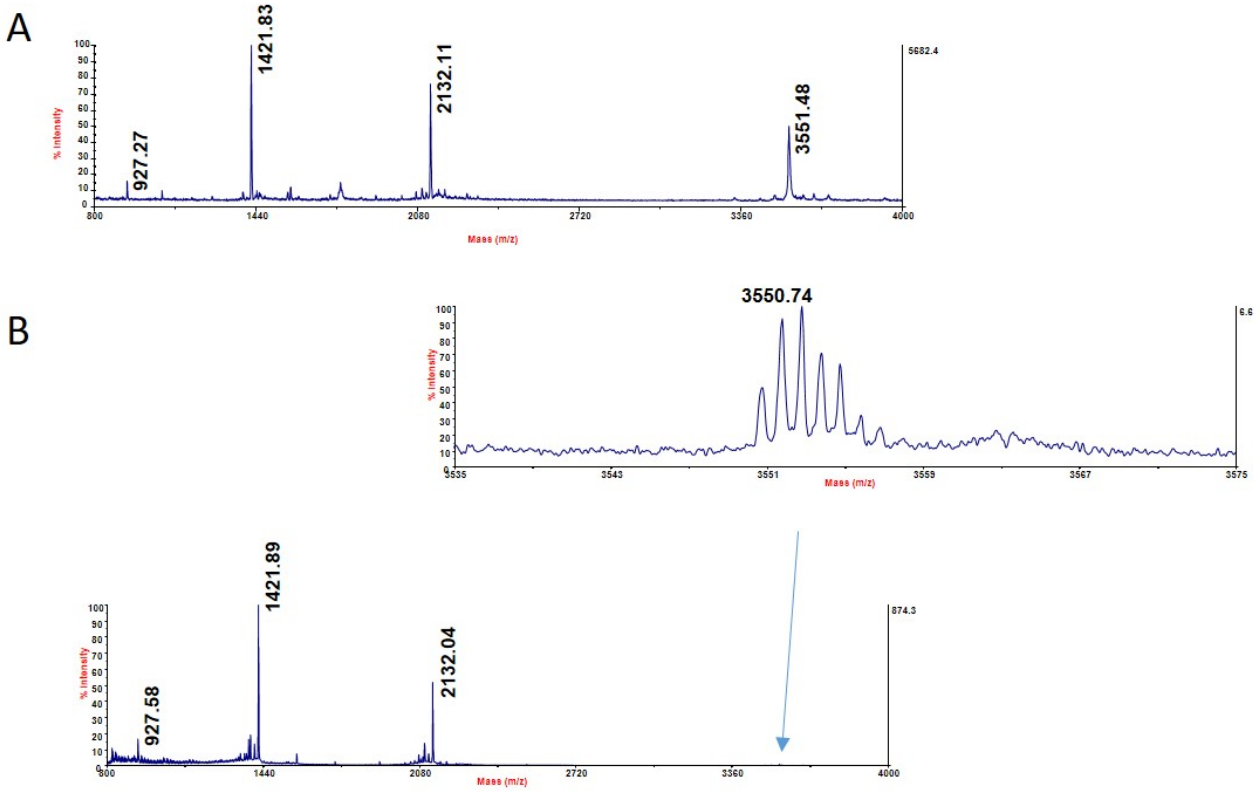


MALDI-TOF MS analysis of the [C_{12}-R_2FR_2]-PKCi disulfide conjugate: A) in the linear positive ions mode and **B)** in the reflector positive ions mode (top shows a zoom on the $[M+H]^+$ peak).
Reflector mode mass spectrum:
m/z 3229.6 (monoprotonated ion of [C_{12}-R_2FR_2]-PKCi, calc. 3230.9)
Fragmentation of the disulfide bond gives ions at m/z 2132 (PKCi) and m/z 1100.6 ([C_{12}-R_2FR_2]).

[C_{12}-R_7]-PKCi

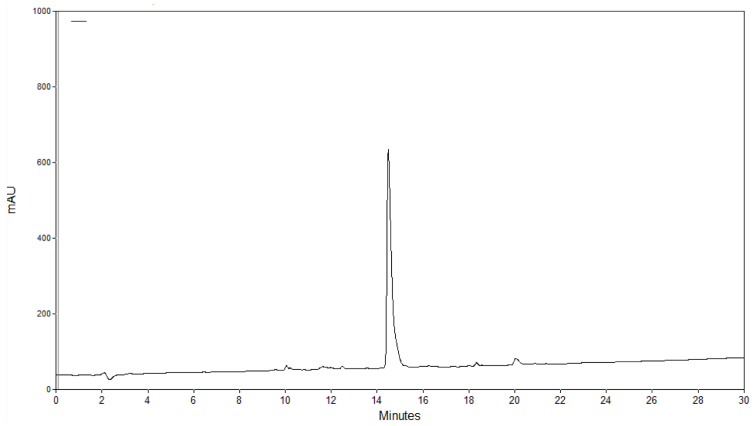


HPLC trace of [C_{12}-R_7]-PKCi
Column: Vydac C18 5 μm, 150 x 4.6 mm
Gradient: 5-60 % B in 30 min, 1 mL/min
HPLC: Agilent 1220 Infinity

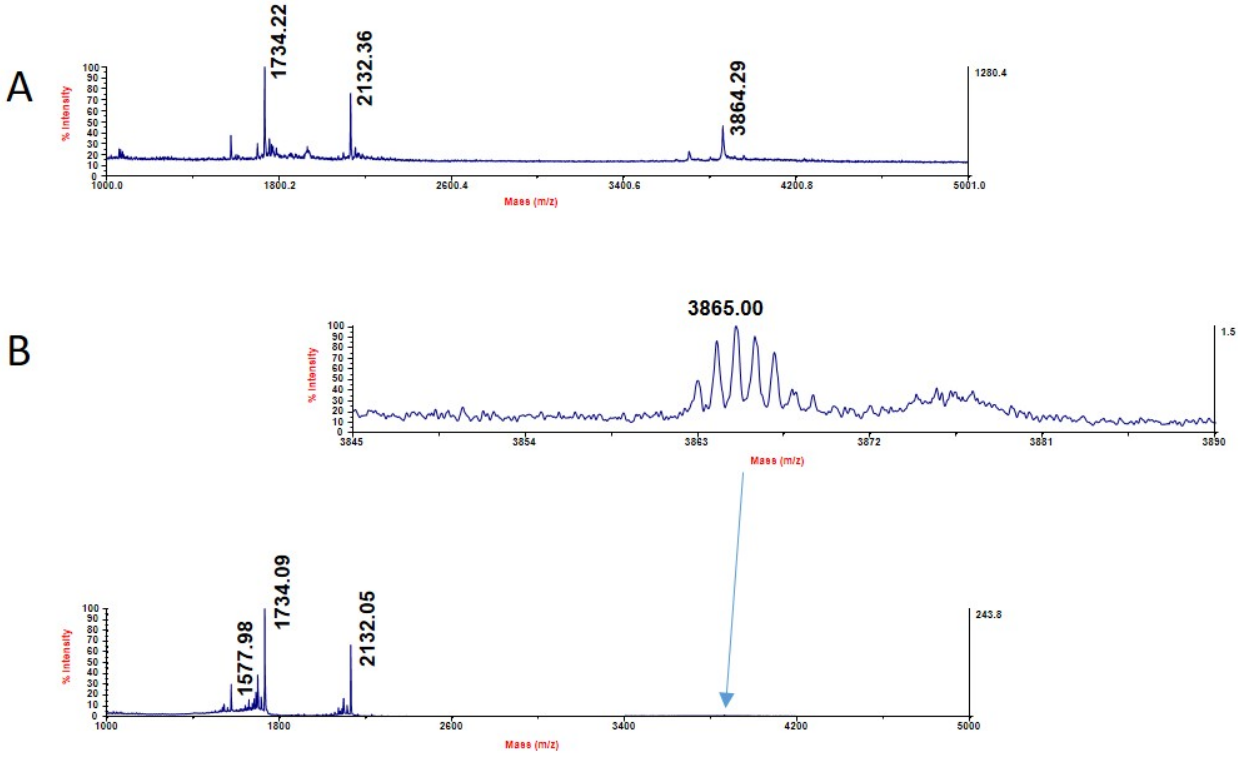


MALDI-TOF MS analysis of the [C_{12}-R_7]-PKCi disulfide conjugate: A) in the linear positive ions mode and **B)** in the reflector positive ions mode (top shows a zoom on the $[M+H]^+$ peak).
Reflector mode mass spectrum:
m/z 3550.7 (monoprotonated ion of [C_{12}-R_7]-PKCi, calc. 3551.2).
Fragmentation of the disulfide bond gives ions at m/z 2132.0 (PKCi) and m/z 1421.9 ([C_{12}-R_7]).

[*C*$_{12}$-R$_9$]-PKCi

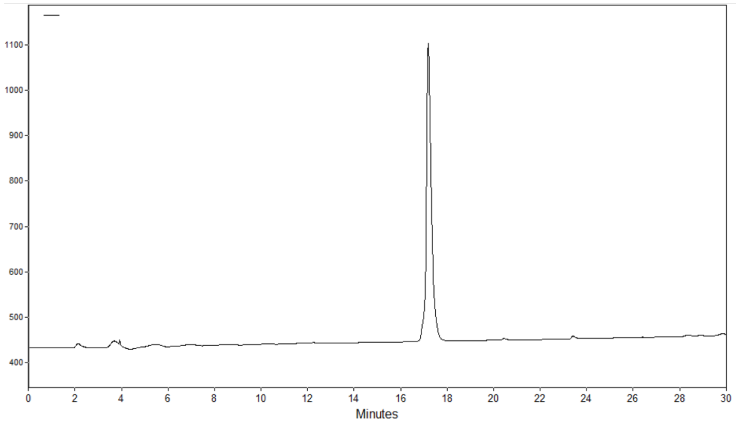


HPLC trace of [*C*$_{12}$-R$_9$]-PKCi
Column: Vydac C18 5 μm, 150 x 4.6 mm
Gradient: 5-65 % B in 30 min, 1 mL/min
HPLC: Agilent 1220 Infinity

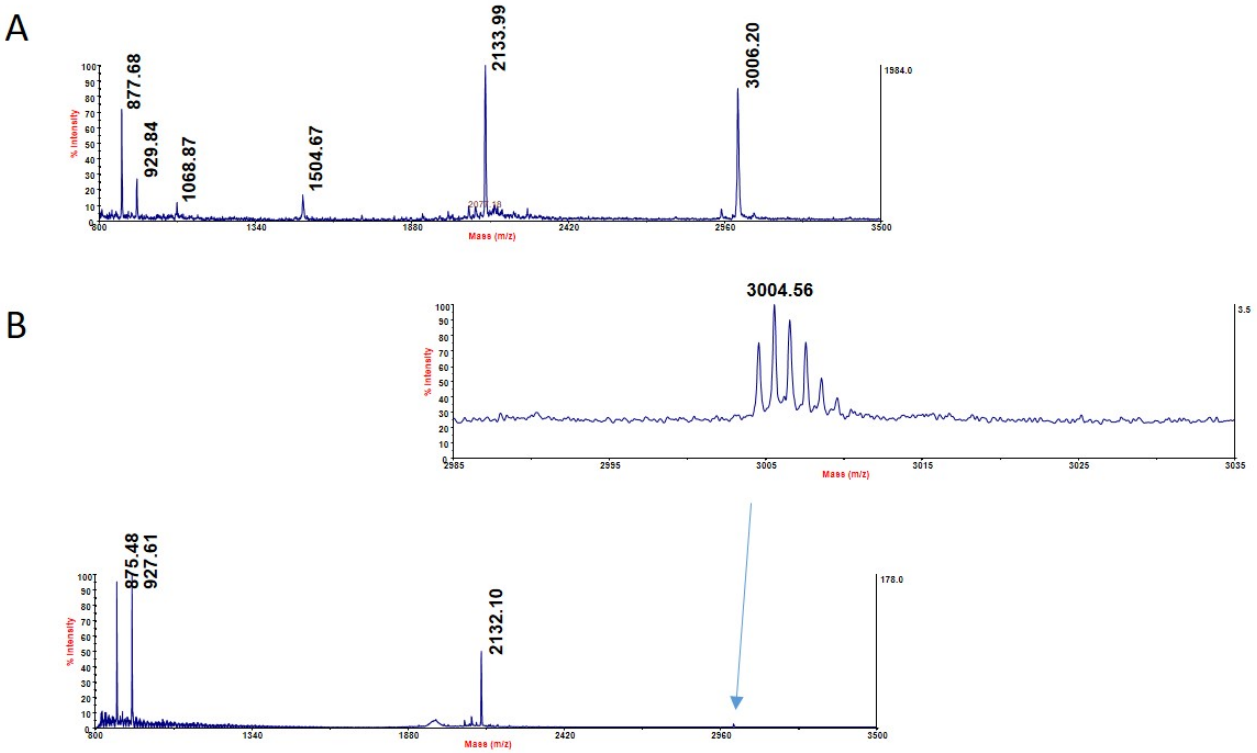


MALDI-TOF MS analysis of the [*C*$_{12}$-R$_9$]-PKCi disulfide conjugate: A) in the linear positive ions mode and **B)** in the reflector positive ions mode (top shows a zoom on the $[M+H]^+$ peak).
Reflector mode mass spectrum:
m/z 3865.0 (monoprotonated ion of [*C*$_{12}$-R$_9$]-PKCi, calc. 3864.7)
Fragmentation of the disulfide bond gives ions at m/z 2132.0 (PKCi) and m/z 1734.1 ([*C*$_{12}$-R$_9$]).

[FR$_4$]-PKCi

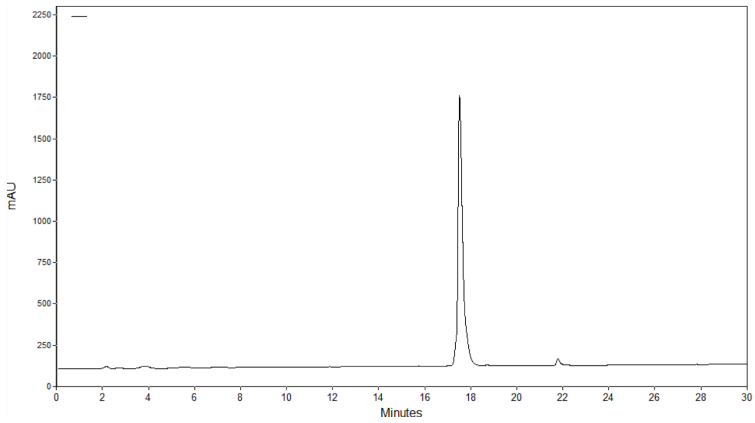


HPLC trace of [FR$_4$]-PKCi
Column: Vydac C18 5 μm, 150 x 4.6 mm
Gradient: 0-40 % B in 30 min, 1 mL/min
HPLC: Agilent 1220 Infinity

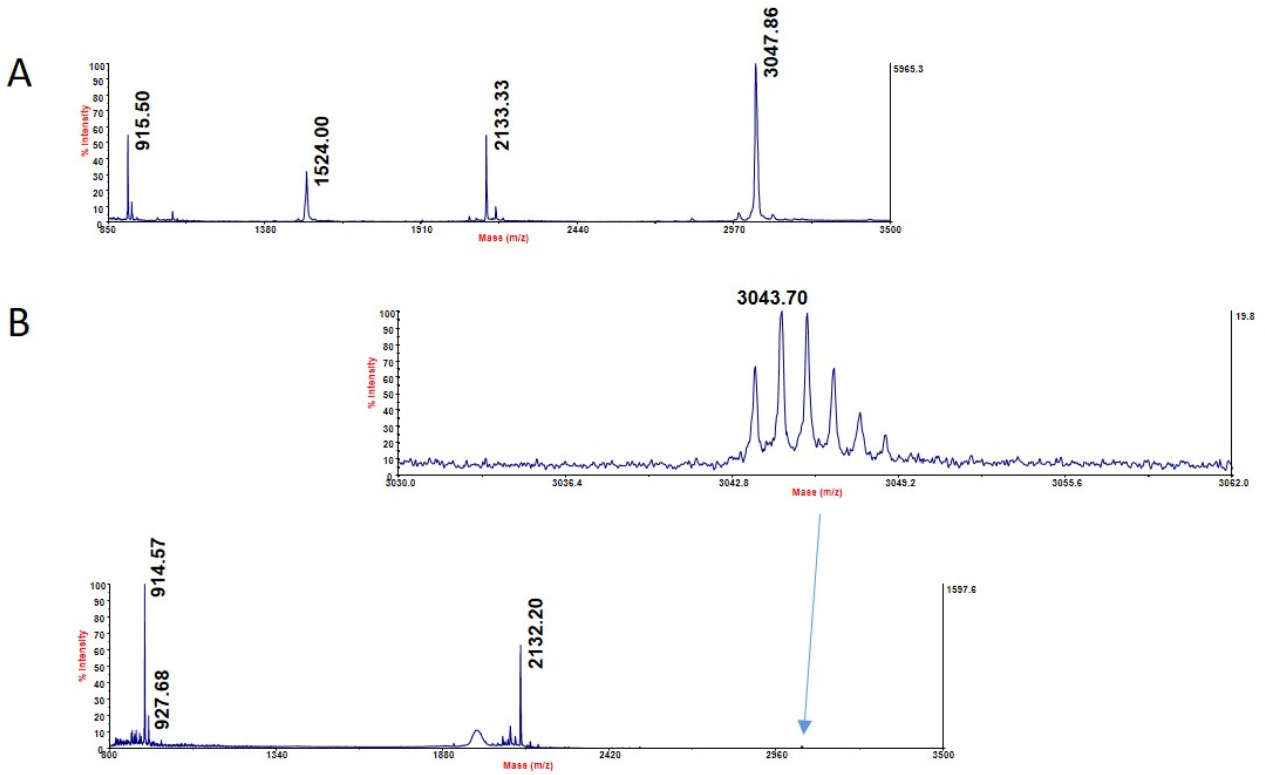


MALDI-TOF MS analysis of the [FR$_4$]-PKCi disulfide conjugate: A) in the linear positive ions mode and **B)** in the reflector positive ions mode (top shows a zoom on the $[M+H]^+$ peak).
Reflector mode mass spectrum:
m/z 3004.6 (monoprotonated ion of [FR$_4$]-PKCi, calc. 3005.6).
Fragmentation of the disulfide bond gives ions at m/z 2132.1 (PKCi) and m/z 875.5 [FR$_4$]).

[WR$_4$]-PKCi

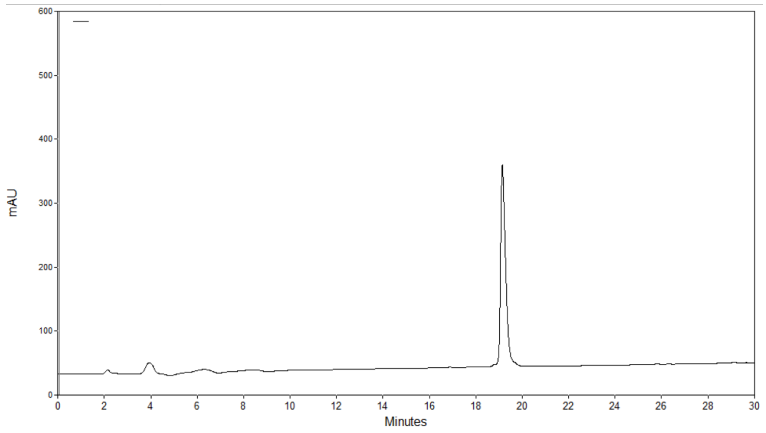


HPLC trace of [WR$_4$]-PKCi
Column: Vydac C18 5 μm, 150 x 4.6 mm
Gradient: 0-40 % B in 30 min, 1 mL/min
HPLC: Agilent 1220 Infinity

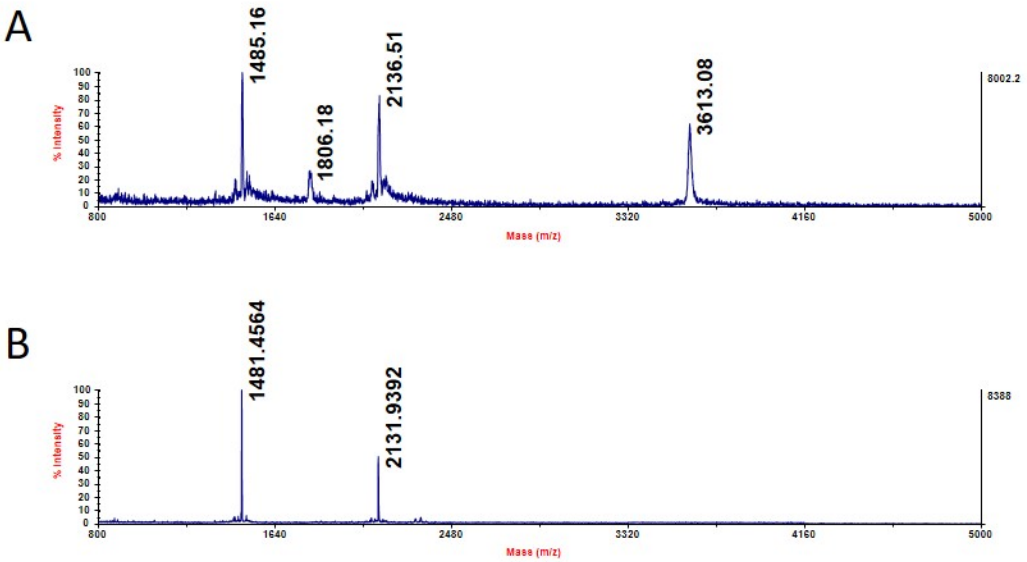


MALDI-TOF MS analysis of the [WR$_4$]-PKCi disulfide conjugate: **A)** in the linear positive ions mode and **B)** in the reflector positive ions mode (top shows a zoom on the $[M+H]^+$ peak).
Reflector mode mass spectrum:
m/z 3043.7 (monoprotonated ion of [WR$_4$]-PKCi, calc. 3044.3).
Fragmentation of the disulfide bond gives ions at m/z 2132.2 (PKCi) and m/z 914.6 [WR$_4$]).

[Tat]-PKCi

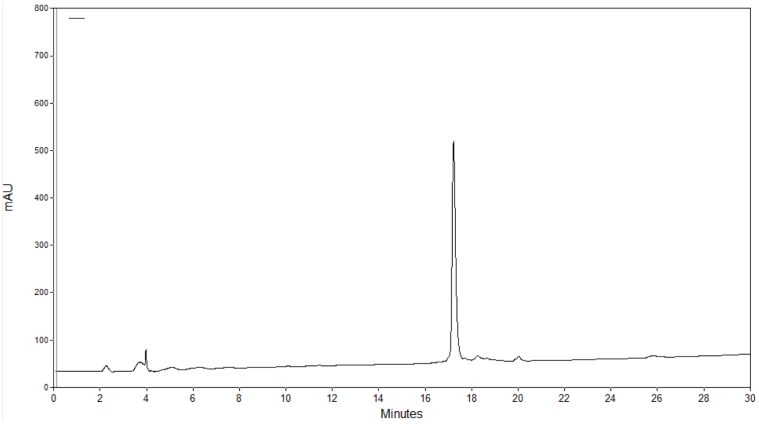


HPLC trace of [Tat]-PKCi
Column: Vydac C18 5 μm, 150 x 4.6 mm
Gradient: 0-30 % B in 30 min, 1 mL/min
HPLC: Agilent 1220 Infinity

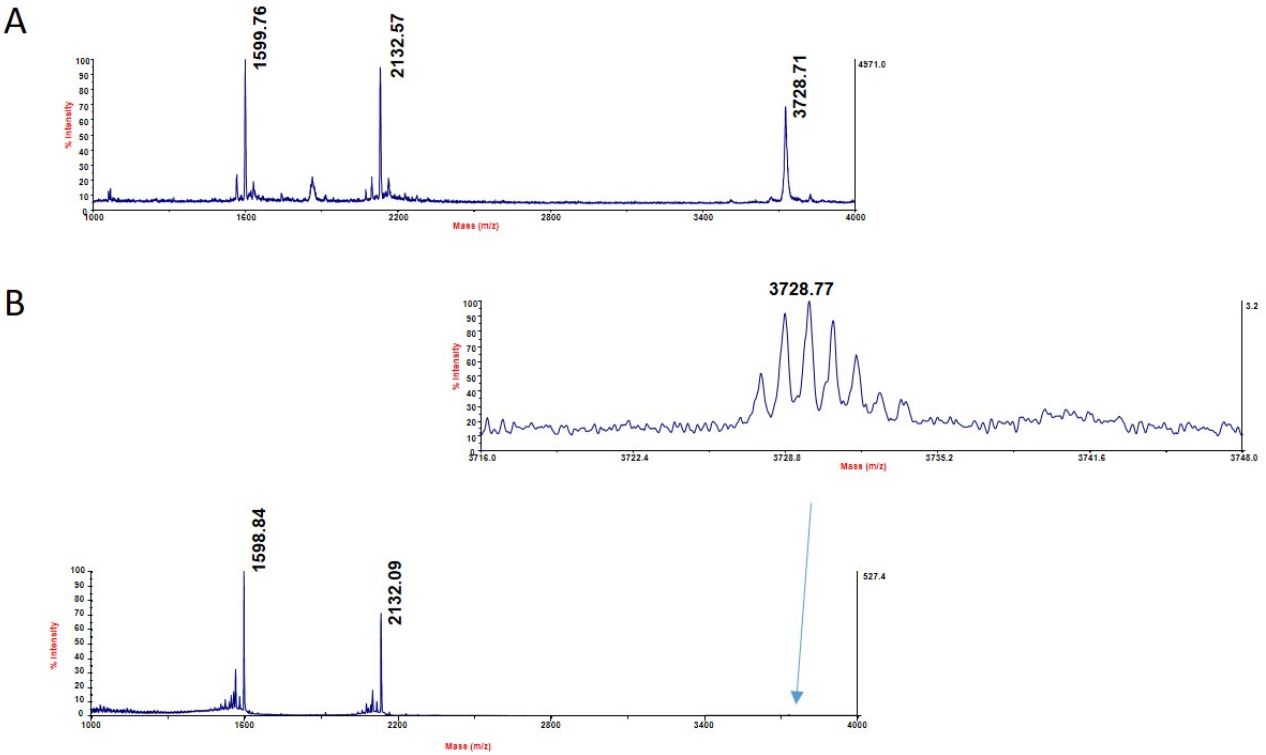


MALDI-TOF MS analysis of the [Tat]-PKCi disulfide: A) in the linear positive ions mode and **B)** in the reflector positive ions mode (the $[M+H]^+$ peak was not detected here).
Linear mode mass spectrum:
m/z 3613.1 (monoprotonated ion of [tat]-PKCi, calc. 3612.3).
Fragmentation of the disulfide bond gives ions at m/z 2136.5 (PKCi) and m/z 1485.2 [Tat]).

[R_6W_3]-PKCi

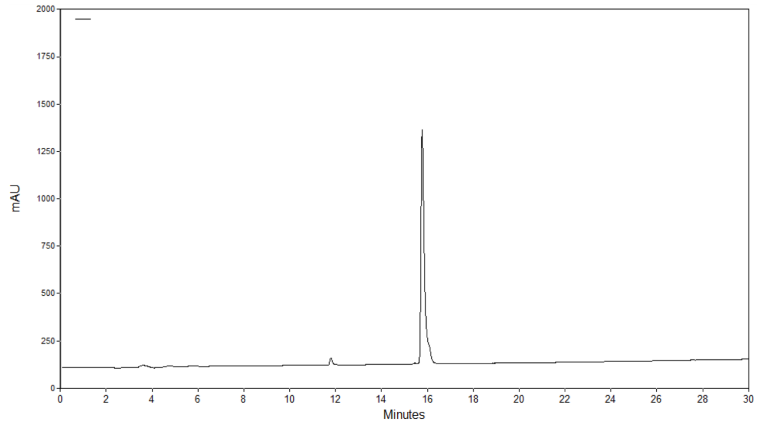


HPLC trace of [R_6W_3]-PKCi
Column: Vydac C18 5 μm, 150 x 4.6 mm
Gradient: 0-50 % B in 30 min, 1 mL/min
HPLC: Agilent 1220 Infinity

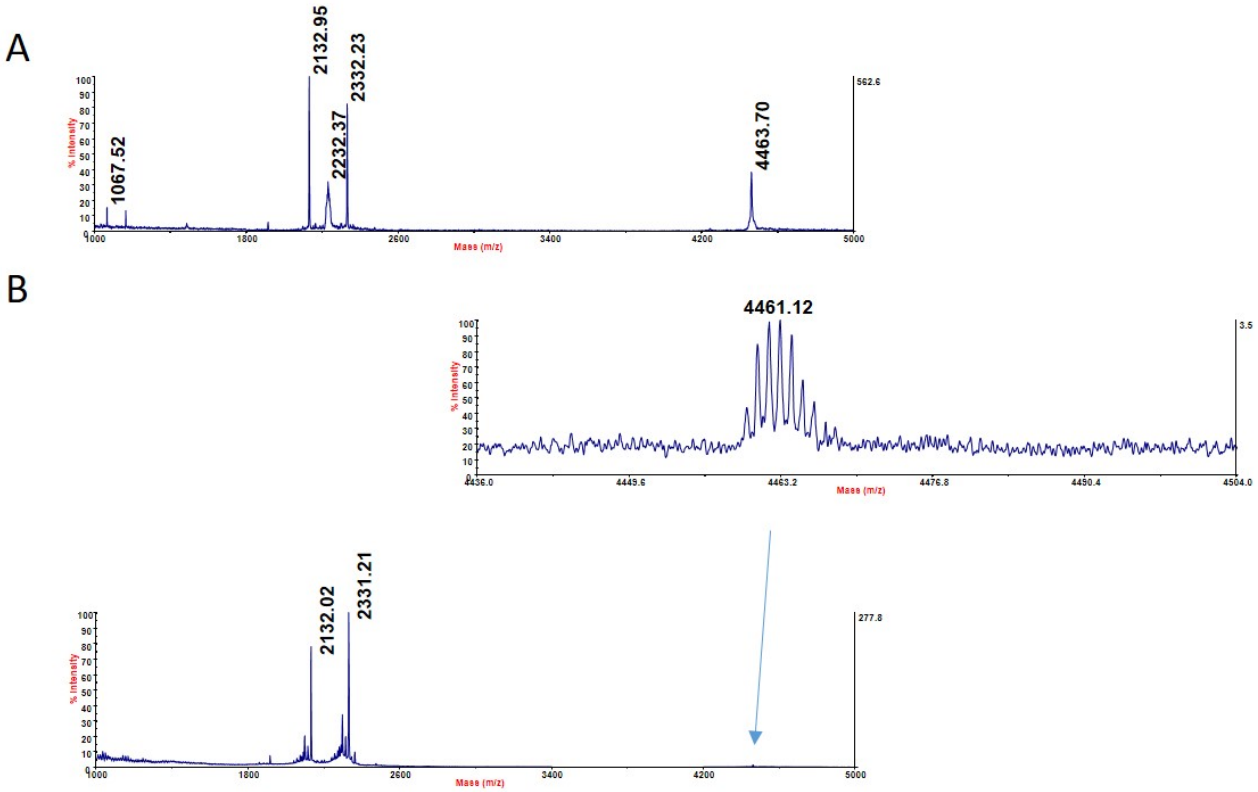


MALDI-TOF MS analysis of the [R_6W_3]-PKCi disulfide conjugate: A) in the linear positive ions mode and **B)** in the reflector positive ions mode (top shows a zoom on the $[M+H]^+$ peak).
Reflector mode mass spectrum:
m/z 3728.8 (monoprotonated ion of [R_6W_3]-PKCi, calc. 3729.4).
Fragmentation of the disulfide bond gives ions at m/z 2132.1 (PKCi) and m/z 1598.8 [R_6W_3]).

[Pen]-PKCi

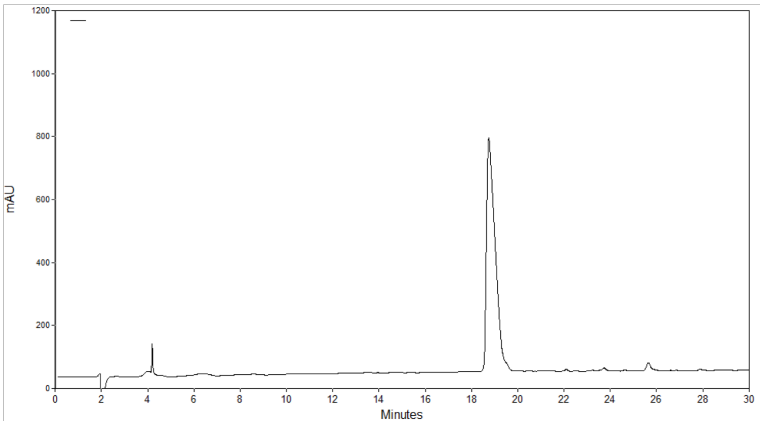


HPLC trace of [Pen]-PKCi
Column: Vydac C18 5 μm, 150 x 4.6 mm
Gradient: 0-60 % B in 30 min, 1 mL/min
HPLC: Agilent 1220 Infinity

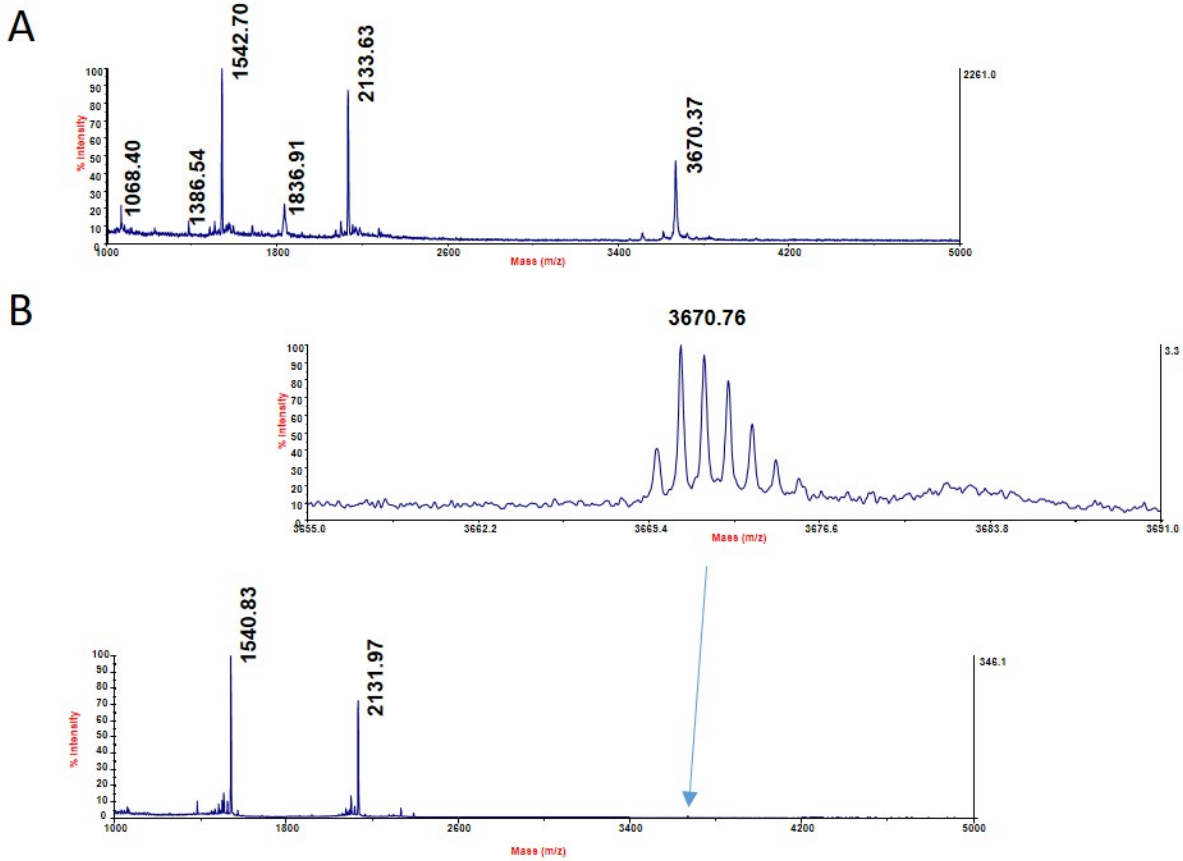


MALDI-TOF MS analysis of the [Pen]-PKCi disulfide conjugate: A) in the linear positive ions mode and **B)** in the reflector positive ions mode (top shows a zoom on the $[M+H]^+$ peak).
Reflector mode mass spectrum:
m/z 4461.1 (monoprotonated ion of [Pen]-PKCi, calc. 4461.6).
Fragmentation of the disulfide bond gives ions at m/z 2132.0 (PKCi) and m/z 2331.2 [Pen]).

Tat-PKCi

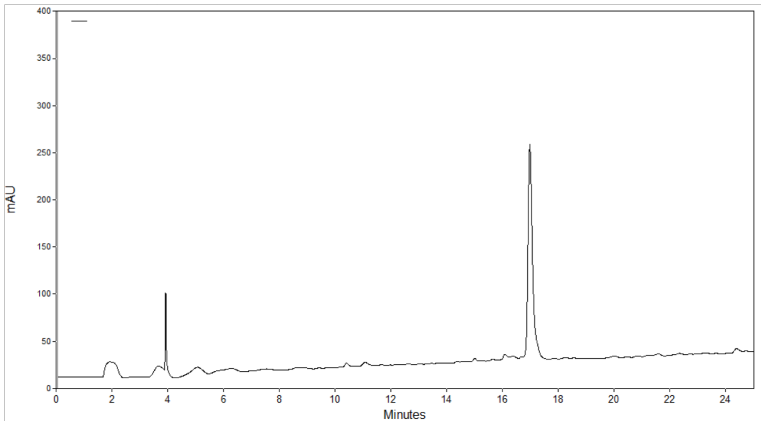


HPLC trace of Tat-PKCi
Column: Vydac C18 5 μm, 150 x 4.6 mm
Gradient: 0-30 % B in 30 min, 1 mL/min
HPLC: Agilent 1220 Infinity

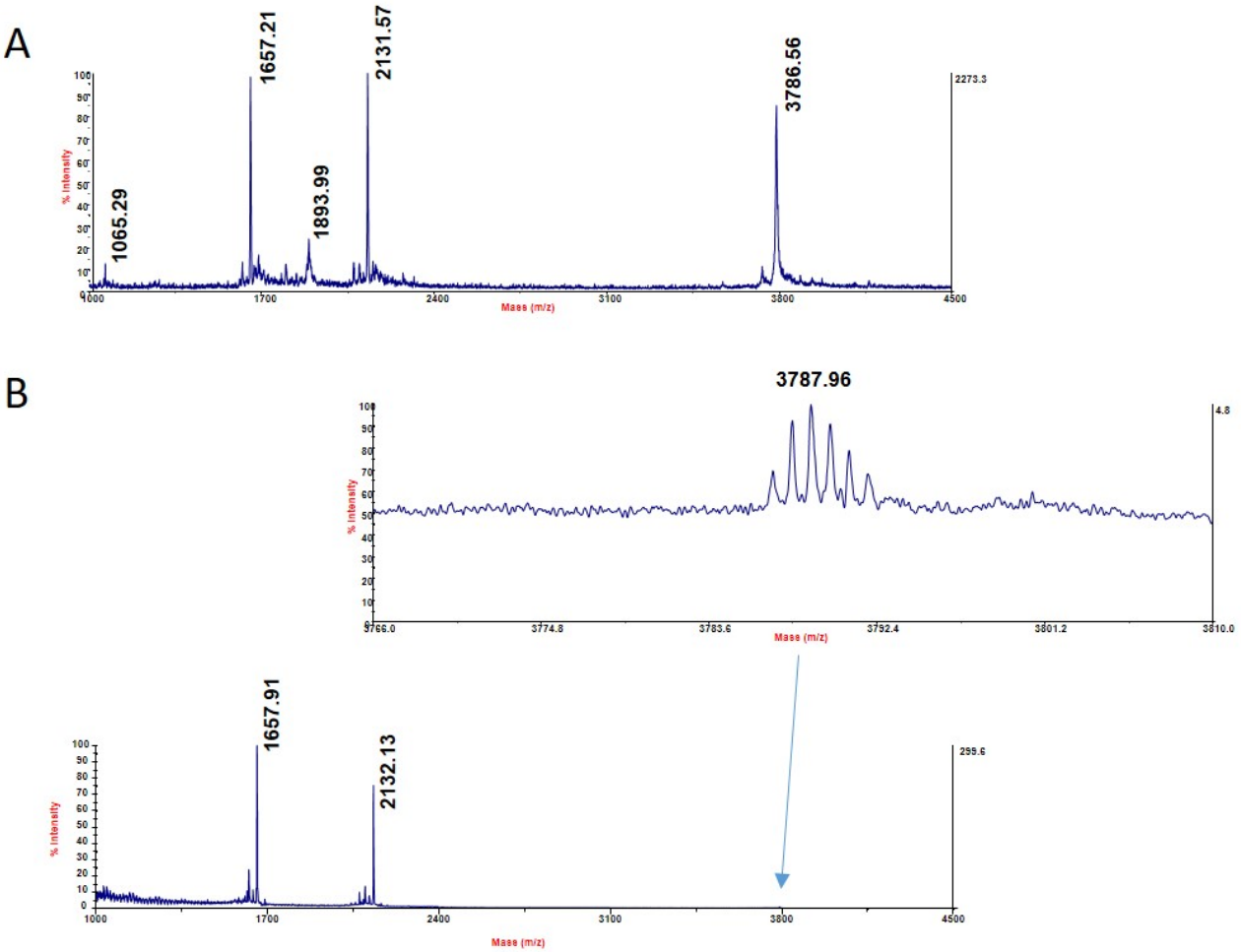


MALDI-TOF MS analysis of the Tat-PKCi disulfide conjugate: A) in the linear positive ions mode and **B)** in the reflector positive ions mode (top shows a zoom on the $[M+H]^+$ peak).
Reflector mode mass spectrum:
m/z 3670.8 (monoprotonated ion of Tat-PKCi, calc. 3671.4).
Fragmentation of the disulfide bond gives ions at m/z 2131.9 (PKCi) and m/z 1540.8 (Tat).

R_6W_3-PKCi

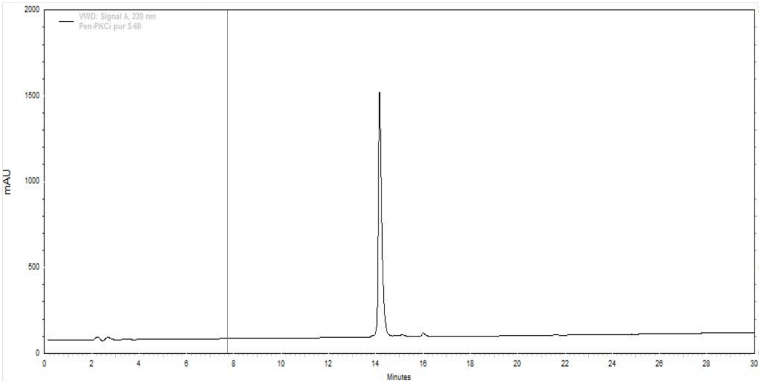


HPLC trace of R_6W_3-PKCi
Column: Vydac C18 5 μm, 150 x 4.6 mm
Gradient: 0-50 % B in 30 min, 1 mL/min
HPLC: Agilent 1220 Infinity

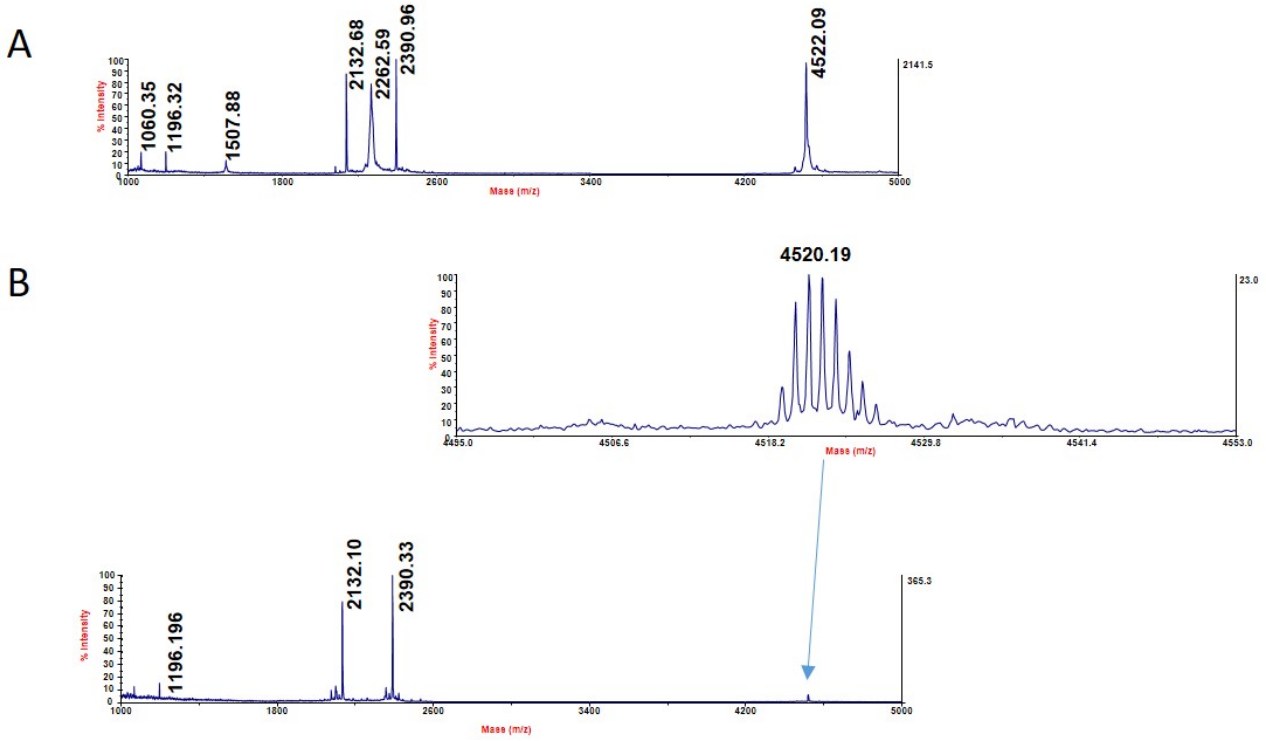


MALDI-TOF MS analysis of the R_6W_3-PKCi disulfide conjugate: A) in the linear positive ions mode and **B)** in the reflector positive ions mode (top shows a zoom on the $[M+H]^+$ peak).
Reflector mode mass spectrum:
m/z 3787.9 (monoprotonated ion of R_6W_3-PKCi, calc. 3788.5).
Fragmentation of the disulfide bond gives ions at m/z 2132.1 (PKCi) and m/z 1657.9 (R_6W_3).

Pen-PKCi



HPLC trace of Pen-PKCi
Column: Vydac C18 5 μm, 150 x 4.6 mm
Gradient: 0-60 % B in 30 min, 1 mL/min
HPLC: Agilent 1220 Infinity



MALDI-TOF MS analysis of the Pen-PKCi disulfide conjugate: A) in the linear positive ions mode and **B)** in the reflector positive ions mode (top shows a zoom on the $[M+H]^+$ peak).
Reflector mode mass spectrum:
m/z 4520.2 (monoprotonated ion of Pen-PKCi, calc. 4521.4).
Fragmentation of the disulfide bond gives ions at m/z 2132.1 (PKCi) and m/z 2390.3 (Pen).

8. Cell culture and cell viability assays

CHO cells were cultured in sterile conditions in DMEM/F-12 medium supplemented with 10 % heat-inactivated FBS in a humidified atmosphere of 5 % CO_2, at 37 °C. Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8). Cells were seeded in 96-well plates to obtain 5 000 cells/well the day of the cell viability experiment. Cells were incubated for 75 min at 37 °C with 100 µL of the conjugate solution (7.5 µM in DMEM) and for the control experiment with 100 µL of DMEM. The supernatant was removed and 100 µL of CCK8 (10 % in DMEM) was added. After 2 h incubation at 37 °C, the absorbance was measured at 450 nm using a microplate reader (FLUOstar OPTIMA, BMG LABTECH) with a reference wavelength at 620 nm. The experiments were performed in triplicates and repeated twice independently. For each conjugate, the percentage of viable cells compared to the control conditions (no conjugate) was calculated. Mean values +/- SEM are given in the histogram.

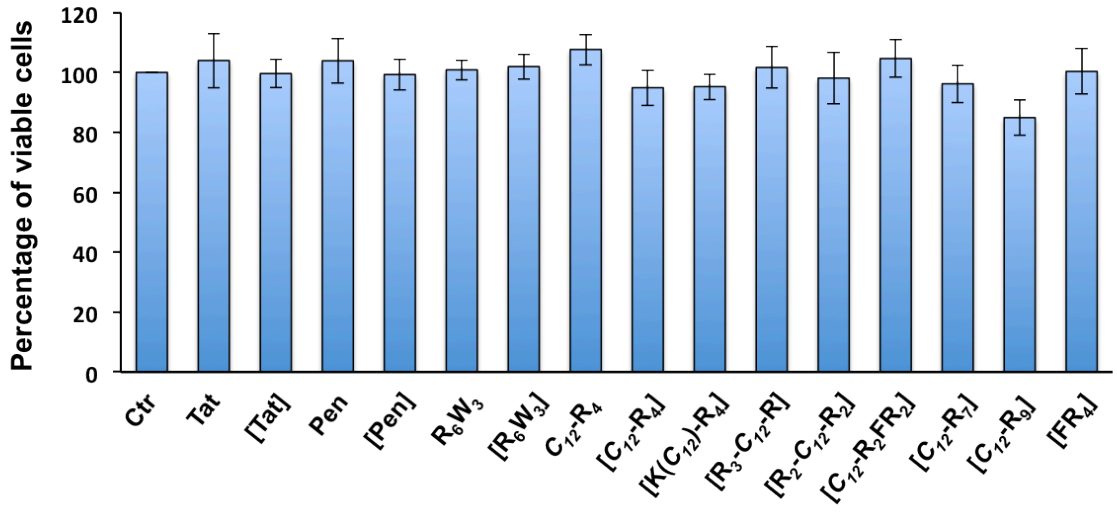


Figure S5. Viability of cells incubated with the different CPP-PKCi conjugates
(Ctr = control, no conjugate added to the cells)

9. Quantification of the internalised cargo by MALDI-TOF MS

CHO-K1 cells (named in the manuscript WT cells) or CHO-pgsA745 (GAG^{neg} cells) were seeded in sterile conditions in 12-well plates 24 h before the internalisation experiment. Internalisation experiments were performed on confluent cells (10^6 cells/well). Cells were incubated with the free ^{1}H-PKCi peptide (no carrier) or with the ^{1}H-PKCi-CPP conjugates (7.5 µM in DMEM, 500 µL) for 75 min at 37 °C (temperature allowing conjugate entry by endocytosis and direct translocation) or at 4 °C (temperature allowing internalisation only by direct translocation).

Elimination of the membrane-bound peptide:
After incubation, cells were washed 3 times with 1 mL HBSS, treated for 3 min at rt with 200 µL TCEP (2 mM in 50 mM Tris-HCl buffer pH 7.5) to reduce all cell-surface disulfide conjugates and washed again once with 1 mL HBSS.
- When internalisation experiments had been performed at 37 °C: membrane-bound peptide was eliminated by cell treatment for 5 min at 37 °C with 500 µL trypsin-EDTA. Trypsin inhibitor (100 µL, 5 mg/mL) and BSA (100 µL, 1 mg/mL) were then added and the cell suspension was transferred to a micro-tube.

- When internalisation experiments had been performed at 4°C: membrane-bound peptide was eliminated by cell treatment for 10 min with pronase (0.5 mg/mL in 100 mM Tris-HCl pH 7.5). A cocktail of protease inhibitors (100 µL of a 7x solution of Complete mini, Roche) and BSA (100 µL, 1 mg/mL) were then added and the cell suspension was transferred to a micro-tube.
In both cases, wells were washed with 500 µL of 50 mM Tris-HCl buffer (pH 7.5). The combined suspensions were centrifuged for 2 min at 640 g. The pellet was washed with 1 mL 50 mM Tris-HCl pH 7.5, 0.1 % BSA (buffer A) and centrifuged again in the same conditions.

Cell lysis and extraction of the biotinylated peptide:
The cell pellet was mixed with a known amount of ^{2}H-PKCi internal standard (typically between 0.1 to 10 pmol) and 150 µL of a solution containing 0.3 % Triton X-100, 1 M NaCl, 2 mM DTT. The mixture was immediately heated for 15 min at 100 °C. The lysate was centrifuged for 5 min at 7080 g. The supernatant was mixed with 850 µL of buffer A containing 2 mM DTT. Streptavidin-coated magnetic beads (10 µL of Dynabeads® M-280 Streptavidin, Invitrogen) were added to the sample and the mixture was incubated for 1 h at rt to capture the biotinylated peptides (intact ^{1}H and ^{2}H-PKCi and potential digests). After bead immobilisation with a magnet, the supernatant was removed and beads were washed twice with 200 µL buffer A, they were incubated 10 min at 50 ºC with 20 µL Laemmli buffer (d10)*, then washed twice with 200 µL buffer A containing 1 M NaCl, twice with 200 µL H_2O, incubated 2 min with 50 µL biotin (10 µM in H_2O), washed with 50 µL H_2O and finally 50 µL CH_3CN/H_2O (1:1). The supernatant was removed and 3 µL of matrix (saturated solution of CHCA in CH_3CN/H_2O/TFA, 50:50:0.1) was added. The beads were incubated 10 min at rt to elute the biotinylated peptides and 1 µL of the supernatant was deposited on the MALDI-TOF plate.
*Diluted (d10) Laemmli buffer composition: 1 % SDS, 6 % glycerol, 1 % ß-mercaptoethanol, 0.025 % bromophenol blue, 25 mM Tris-HCl, pH 6.8.
MALDI-TOF MS analysis:
MALDI-TOF MS analyses were performed in the ion positive reflector mode on an ABI Voyager DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems). On the MALDI-TOF mass spectrum corresponding to the average of several hundreds laser shots, the area of the $[M+H]^+$ signals (including all isotopes) of the ^{1}H-PKCi and ^{2}H-PKCi signals were measured and the amount of intact internalised cargo was calculated from the area ratio. All internalisation experiments were performed in triplicates and repeated at least twice independently.

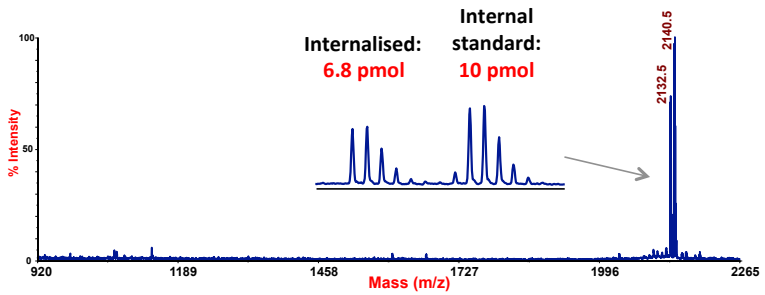
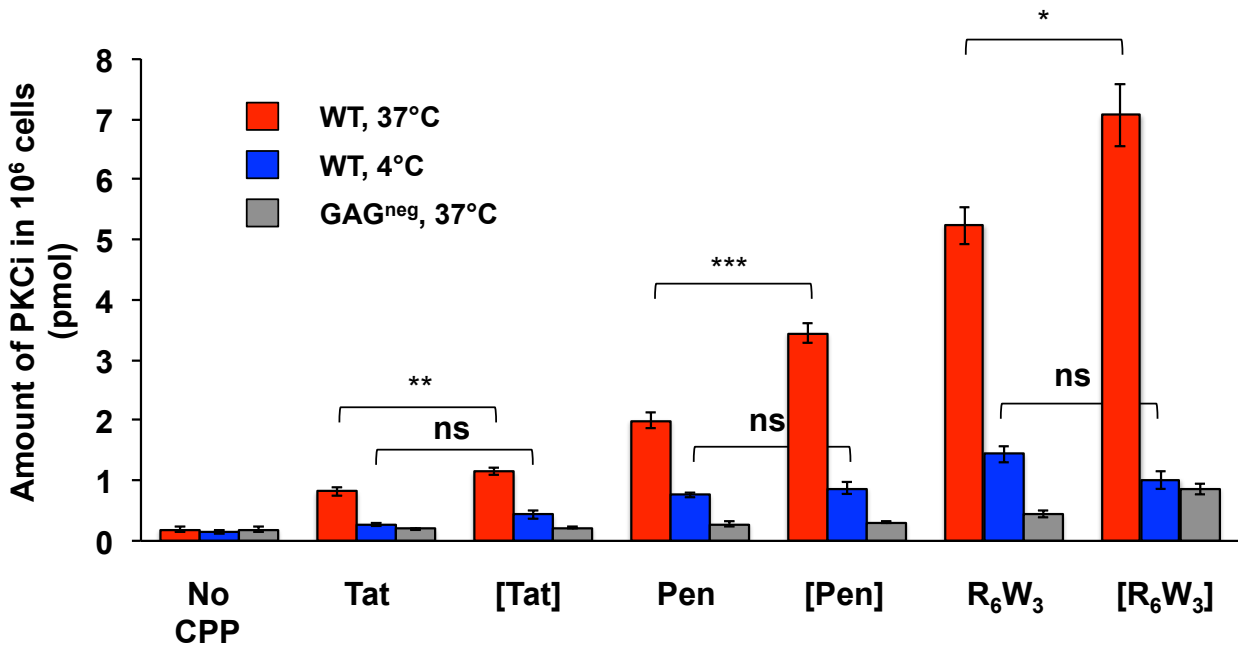


Figure S6. Mass spectrum obtained for the cellular uptake of [R_6W_3]-PKCi conjugate at 37 °C in CHO-K1 cells. Insert: zoom on the intact internalised cargo (^{1}H-PKCi) and the internal standard (^{2}H-PKCi) peaks, which are used for quantification.



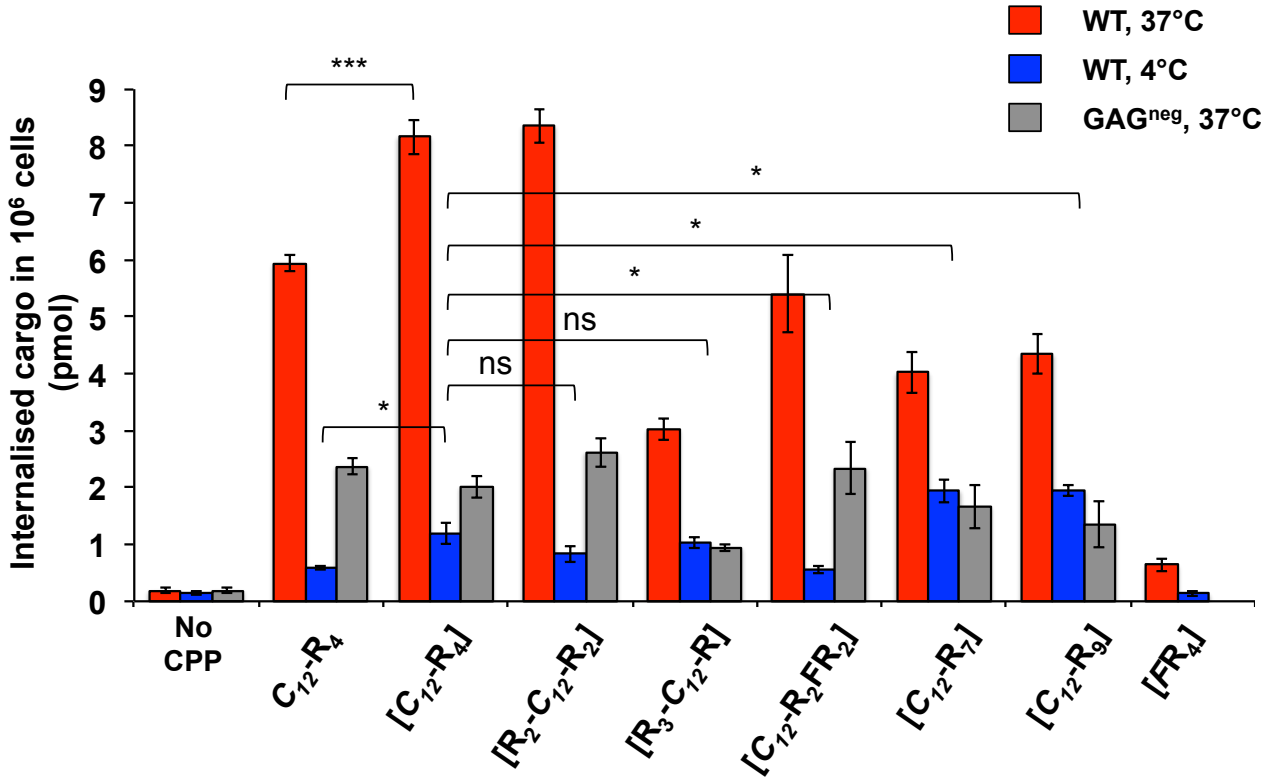
	No CPP	Tat	[Tat]	Pen	[Pen]	R_6W_3	$[R_6W_3]$
WT 37°C	0.19	0.81	1.15	1.99	3.44	5.24	7.06
WT 4°C	0.14	0.27	0.43	0.77	0.87	1.44	1.01
GAG^{neg} 37°C	0.15	0.20	0.22	0.28	0.30	0.44	0.87

Figure S7. Amount of intact PKCi cargo delivered inside cells by the linear classical CPPs and their cyclic derivatives. The cyclic derivatives are marked under square brackets. Conjugates CPP-PKCi (7.5 µM) or unconjugated PKCi (No CPP) were incubated with 10^6 WT (CHO-K1) or GAG^{neg} (CHO 745) cells for 75 min at 4°C or 37°C. Mean values +/- SEM are given in the histogram. Mean values are also shown in the table just below the histogram (pmol for 10^6 cells).
Significance was tested using a Welch's corrected t test (^{ns} $p > 0.05$, * $0.05< p > 0.01$, ** $0.01< p > 0.001$, *** $p<0.001$)

Table S4. Effect of cyclisation on the efficiency of endocytosis, translocation and GAG-dependent entry

Route of internalisation	Tat	[Tat]	Pen	[Pen]	R_6W_3	$[R_6W_3]$
Endocytosis[1]	0.54	0.72	1.22	2.57	3.8	6.05
Translocation[2]	0.27	0.43	0.77	0.87	1.44	1.01
GAG-dependent entry[3]	0.61	0.93	1.71	3.14	4.8	6.19

[1] The efficiency of endocytosis was estimated by subtracting the amount of intact PKCi cargo (pmol) delivered in 10^6 WT cells at 4°C from the values obtained in WT cells at 37°C.
[2] The efficiency of translocation is directly given by the amount of intact PKCi cargo measured in WT cells at 4°C.
[3] The efficiency of GAG-dependent entry was estimated by subtracting the amount of intact PKCi cargo (pmol) delivered in 10^6 GAG^{neg} cells at 37°C from the values obtained in WT cells at 37°C.



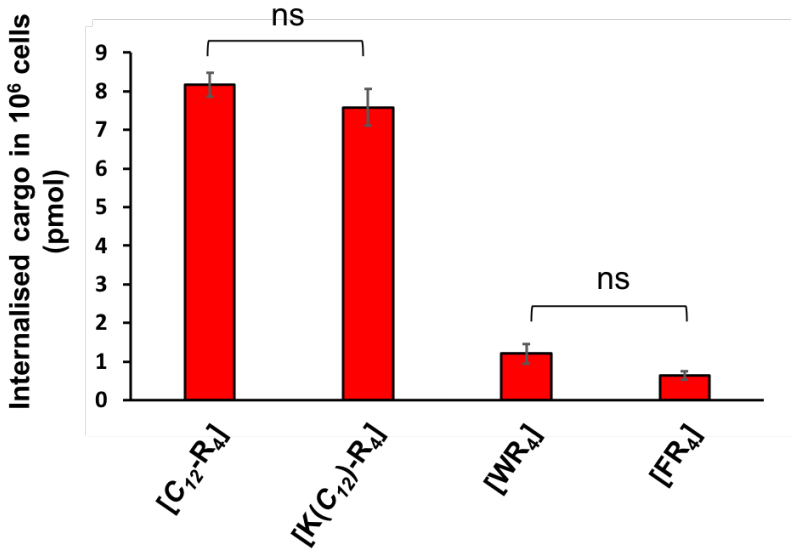
	No CPP	C_{12}-R$_4$	[C_{12}-R$_4$]	[R$_2$-C_{12}-R$_2$]	[R$_3$-C_{12}-R]	[C_{12}-R$_2$FR$_2$]	[C_{12}-R$_7$]	[C_{12}-R$_9$]	[FR$_4$]
WT 37°C	0.19	5.94	8.16	8.36	3.02	5.40	4.02	4.34	0.64
WT 4°C	0.14	0.59	1.10	0.83	1.03	0.56	1.95	1.94	0.13
GAG^{neg}37°C	0.15	2.37	2.01	2.61	0.95	2.34	1.66	1.35	ND

Figure S8. Amount of intact PKCi cargo delivered inside cells by the lipidated CPP and its cyclic derivatives. The cyclic derivatives are marked under square brackets. Conjugates CPP-PKCi (7.5 µM) and unconjugated PKCi (No CPP) were incubated with 10^6 WT (CHO-K1) or GAG^{neg} (CHO 745) cells for 75 min at 4°C or 37°C. Mean values +/- SEM are given in the histogram. Mean values are also shown in the table below (pmol for 10^6 cells). ND: not determined.
Significance was tested using a Welch's corrected t test (^{ns} $p > 0.05$, * $0.05< p > 0.01$, ** $0.01< p > 0.001$, *** $p<0.001$).

Table S5. Effect of cyclisation on the efficiency of endocytosis, translocation and GAG-dependent entry

Route of internalisation	C_{12}-R$_4$	[C_{12}-R$_4$]	[R$_2$-C_{12}-R$_2$]	[R$_3$-C_{12}-R]	[C_{12}-R$_2$FR$_2$]	[C_{12}-R$_7$]	[C_{12}-R$_9$]
Endocytosis[1]	5.35	7.06	7.53	1.99	4.84	2.07	2.4
Translocation[2]	0.59	1.10	0.83	1.03	0.56	1.95	1.94
GAG-dependent entry[3]	3.57	6.15	5.75	2.07	3.06	2.36	2.99

[1] The efficiency of endocytosis was estimated by subtracting the amount of intact PKCi cargo (pmol) delivered in 10^6 WT cells at 4°C from the values obtained in WT cells at 37°C.
[2] The efficiency of translocation is directly given by the amount of intact PKCi cargo measured in WT cells at 4°C.
[3] The efficiency of GAG-dependent entry was estimated by subtracting the amount of intact PKCi cargo (pmol) delivered in 10^6 GAG^{neg} cells at 37°C from the values obtained in WT cells at 37°C.



	[C_{12}-R_4]	[K(C_{12})-R_4]	[WR_4]	[FR_4]
WT 37°C	8.16	7.59	1.2	0.64

Figure S9: Impact of the anchoring position of the lipid chain and of its replacement by an aromatic amino-acid. Amount of intact PKCi cargo delivered inside cells. Conjugates CPP-PKCi (7.5 µM) were incubated with 10^6 WT (CHO-K1) at 37°C. Mean values +/- SEM are given in the histogram. Mean values are also shown in the table below (pmol for 10^6 cells). Significance was tested using a Welch's corrected t test (^{ns} $p > 0.05$).

10. Confocal microscopy experiments

CHO-K1 cells were seeded in sterile conditions in 12-well plates 24 h before the internalisation experiment. After washing with DMEM, cells were incubated with the CPP-PKCi conjugates (7.5 µM in DMEM) or with the unconjugated PKCi (no CPP) for 75 min at 37 °C (Fig 3) or 4 °C (Fig S10). Cells were washed 3 times with DMEM, incubated for 10 min at rt with 200 µL unlabelled avidin (10 µM) and washed with PBS. Cells were then incubated with 3 % paraformaldehyde (4 °C, 10 min), permeabilised with 0.1 % Triton X-100 in PBS (rt, 5 min) and incubated with 10 % FCS in PBS (rt, 30 min). Cells were incubated with streptavidin-Alexa 488 (1 µg/mL) for 1 h at rt, washed again with PBS and treated with DAPI (1.5 µg/mL) for 10 min at rt. Coverslips were mounted in Fluoromount mounting medium and imaged with a Nikon Eclipse Ti equipped with a Scan head CSUX1-A1 and Camera Evolve Metamorph 63 X.

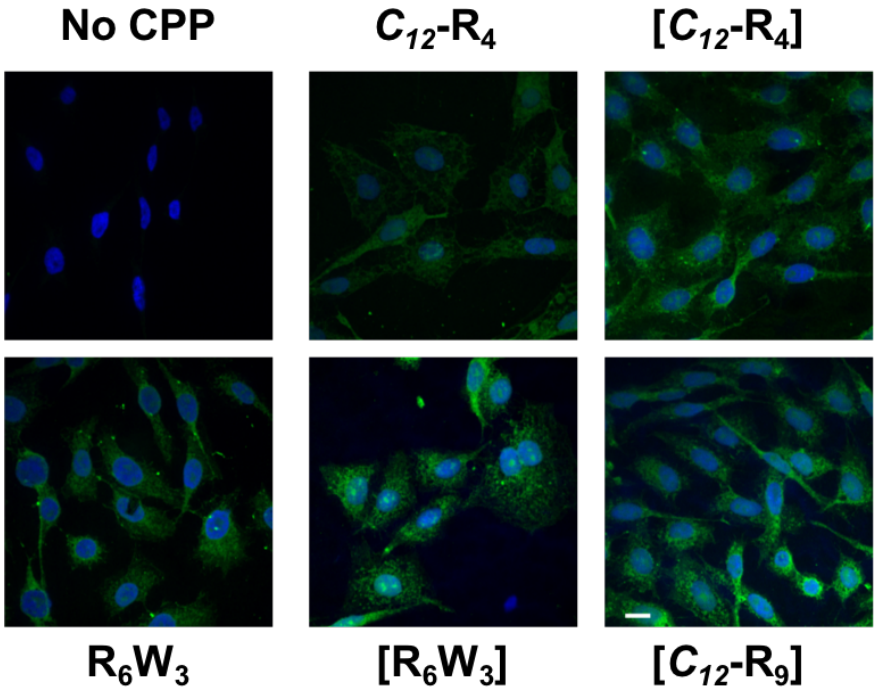


Figure S10. **Intracellular distribution of the delivered PKCi cargo at 4°C.** CPP-PKCi conjugates or unconjugated PKCi (No CPP) (7.5 µM) were incubated for 75 min with WT cells (CHO-K1) at 4°C. The cargo biotin moiety was revealed with streptavidin-Alexa 488 (green). Nuclei were stained with DAPI (blue). The scale bar corresponds to 15 µm.

11) References

1. M. Schnölzer, P. Alewood, A. Jones, D. Alewood, S.B. Kent, *Int. J. Pept. Protein Res.,* 1992*,* ***40***, 180.
2. B. Aussedat, E. Dupont, S. Sagan, A. Joliot, S. Lavielle, G. Chassaing, F. Burlina, *Chem. Commun.*, 2008, 1398.
3. S. A. Bode, M. Thevenin, C. Bechara, S. Sagan, S. Bregant, S. Lavielle, G. Chassaing, F. Burlina, *Chem. Commun.,* 2012, ***48***, 7179.
4. T. M. Hackeng, J. H. Griffin, P. E. Dawson, *Proc. Natl. Acad. Sci. USA,* 1999, ***96***, 10068.
5. F. Burlina, G. Papageorgiou, C. Morris, P. D. White, J. Offer, *Chem. Sci.,* 2014, ***5***, 766.