Electronic Supplementary Material (ESI) for ChemComm.

Unsaturated acyl chains dramatically enhanced cellular uptake by direct translocation of a minimalist oligo-arginine lipopeptide

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Supporting information

S1 – Syntheses - Experimental Procedures

S1.1 - General procedure for solid-phase peptide synthesis

Standard Boc-amino acids, MBHA resin (0.54 mmol.g^{-1}), 1-hydroxybenzotriazole (HOBt), dicyclohexylcarbodiimide (DCC) were purchased from Iris Biotech, GmbH or Novabiochem. 4-Chloro-7-nitrobenz-2-oxa-1,3-diazole, (NBD-Cl), was obtained from Sigma–Aldrich.

The sequence Boc-Arg(Tos)$_4$-Lys(Fmoc)-MBHA was synthesised manually on a MBHA Resin Iris BR 1120 (0.54 mmol g^{-1}, 0.2 mmol) using the SPPS Boc-strategy. For the different couplings, activation was accomplished with DCC/HOBt in NMP. Coupling of NBD-Cl with the amine function of the Lys(Fmoc) side chain at the *C*-terminus of these lipopeptides was performed manually, after orthogonal deprotection of the Fmoc moiety. The coupling to NBD was achieved by addition of NBD-Cl (0.4 mmol) and DIEA (1 mmol) in DMF (2 mL) to the peptidyl resin (0.1 mmol). The solution was stirred overnight and then rinsed with DMF, CH_2Cl_2 and MeOH (5 mL) and dried *under vacuum*.

Acyl chains were coupled to the *N*-terminus of the peptides. Saturated lipopeptides were obtained by on-resin acylation of the peptide followed by NBD-Cl coupling and peptide cleavage from the solid support. Unsaturated lipopeptides were obtained by acylation of the crude H-Arg$_4$-Lys(NBD)-NH$_2$ peptide in solution.

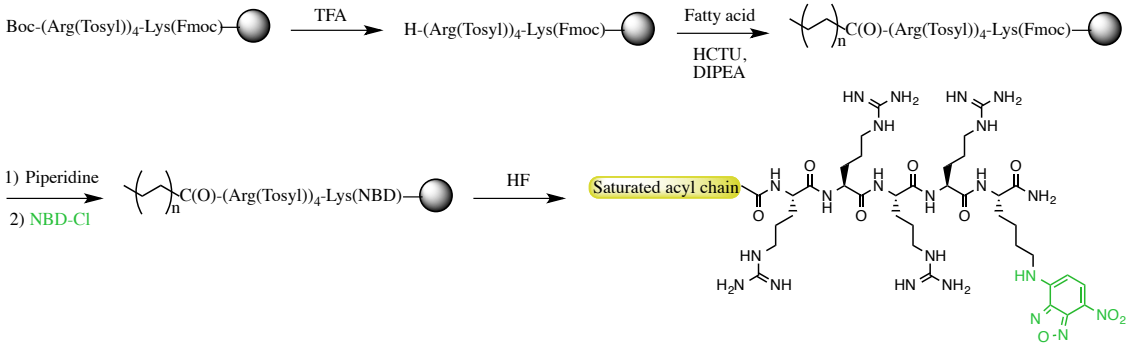
H-Arg$_4$-Lys(NBD)-NH$_2$ and saturated lipopeptides were cleaved from the solid support by treatment with HF (2 h, 0°C) in the presence of anisole (1.5 mL g^{-1} peptidyl-resin) and dimethylsulfide (0.25 mL g^{-1} peptidyl-resin). Crude peptides were precipitated by addition of cold diethyl ether, and the scavengers were removed by filtration. The crude peptide was dissolved in acetic acid aqueous solution (10%, v/v) and lyophilized.

The synthesis of the control peptide NBD-Arg$_9$-NH$_2$, (abbreviated NBD-Arg9) has already been described in ChemBioChem, 2014, 15, 884.

S1.2 - General purification and characterisation procedures of the peptides

C12:0-Arg$_4$-Lys(NBD)-NH$_2$ and C18:0-Arg$_4$-Lys(NBD)-NH$_2$ were purified by FCPC with a two-phase solvent system composed of n-BuOH-AcOH-H_2O (4:1:5) using a Kromaton Technologies (Angers, France) apparatus. FCPC 200 was equipped with a rotor of 20 partition disks, for 1320 partition cells and 200 mL column capacity. The FCPC was connected to a Beta 50 Plus pump (ECOM), a Rheodyne injection valve equipped with 10 ml sample loops, and a Saphire 800 detector (ECOM). Ac-Arg$_4$-Lys(NBD)-NH$_2$, C12:1-Arg$_4$-Lys(NBD)-NH$_2$, C18:1-Arg$_4$-Lys(NBD)-NH$_2$, C18:2-Arg$_4$-Lys(NBD)-NH$_2$, and C22:6-Arg$_4$-Lys(NBD)-NH$_2$ were purified by RP-HPLC with use of solvents A (0.1% TFA in H_2O) and B (0.1% TFA in acetonitrile) in a Waters System with detection at 220 and 340 nm. Semi-preparative RP-HPLC was performed with an ACE 5-C18-300 (4.6 x 250 mm) column, working at 5.0 mL min^{-1} with the optimized gradients. The peptides, (97 to 99% purity), were characterised by analytical RP-HPLC with an Proto 200 C18 (3µm, 100 x 4.6 mm) column working at 1.0 mL.min^{-1} on an automated Dionex system (Ultimate 3000, detection wavelengths were set at 220 nm and 340 nm), and MALDI-TOF-MS with α-cyano-4-hydroxycinnamic acid (HCCA) as the matrix.

S1.3 - Syntheses and characterisation of the saturated lipopeptides



Scheme S1.3. Syntheses of the saturated lipopeptides, general strategy.

The saturated lipopeptides C12:0-Arg_4-Lys(NBD)-NH_2 and C18:0-Arg_4-Lys(NBD)-NH_2 lipopeptides (abbreviated (C12:0-Arg_4 and C18:0-Arg_4, respectively) were synthesised on MBHA resin, as summarized in Scheme 1.3. The last Boc protecting group was removed and the peptidyl-resin (0.1 mmol) was reacted with either lauric acid (C12 chain) or stearic acid (C18 chain), respectively. Dodecanoic acid (0.6 mmol) or stearic acid (0.6 mmol) was activated by HCTU (0.6 mmol) in dichloromethane (5 mL) in the presence of DIPEA (0.6 mmol). The solutions were sonicated before the addition of DIPEA. The couplings were performed for 1 h. The resins were then washed with CH_2Cl_2 (3x), NMP (3x). The Fmoc protecting group was removed and the peptidyl-resin was reacted with NBD-Cl as described above. The peptidyl-resins were then washed with NMP (3x), CH_2Cl_2 (3x), MeOH (3x) and dried under *vacuum*.

At the end of the syntheses, lipopeptides were cleaved from the resin as described in the general protocol. Crude C12:0-Arg_4 and C18:0-Arg_4 were purified by FCPC (36% and 16% yield, respectively). Finally, peptides were characterised by MALDI-TOF MS (m/z) C12:0-Arg_4-Lys(NBD)-NH_2 calcd., 1115.34; found, 1116.0 and C18:0-Arg_4-Lys(NBD)-NH_2 calcd., 1199.5; found, 1199.8.

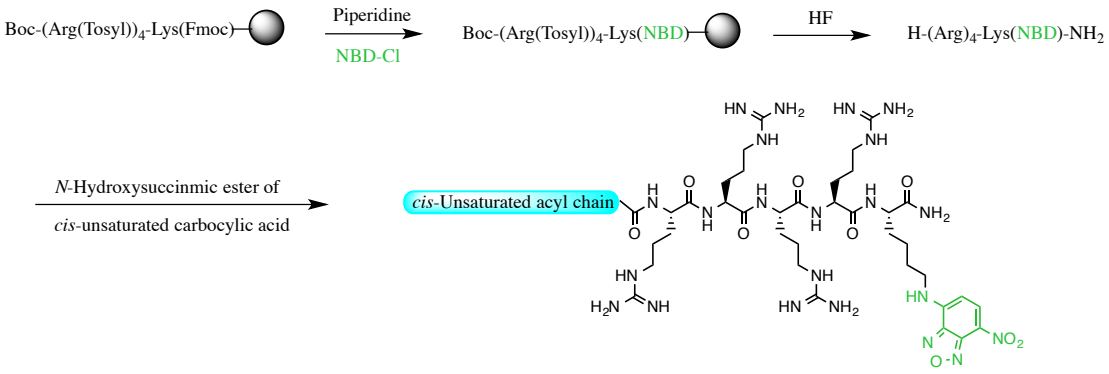
Similarly, the control peptide Ac-Arg_4-Lys(NBD)-NH_2, abbreviated Ac-Arg_4, was prepared by acylation (Ac_2O: 2 mmol, DIPEA: 0.5 mmol, in NMP: 5 mL for 15 min) of the peptidyl-resin, before Fmoc deprotection and NBD coupling, as described above. Crude Ac-Arg_4-Lys(NBD)-NH_2 peptide was purified by RP-HPLC using a 20 min 5 to 25% linear acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA) affording Ac-Arg_4-Lys(NBD)-NH_2 as a white powder. $[M+H]^+$: Ac-Arg_4-Lys(NBD)-NH_2 calcd, 974.54; found, 974.54.

S1.4 - Syntheses of the unsaturated lipopeptides

The unsaturated chain of the lipopeptides C12:1-Arg_4-Lys(NBD)-NH_2 and C18:1-Arg_4-Lys(NBD)-NH2, C18:2-Arg_4-Lys(NBD)-NH2 and C22:6-Arg_4-Lys(NBD)-NH2 lipopeptides (abbreviated (C12:1-Arg_4, C18:1-Arg_4, C18:2-Arg_4, C22:6-Arg_4, respectively) were introduced in solution on the crude peptide Arg_4-Lys(NBD)-NH_2 synthesised on MBHA resin, as summarized in Scheme 1.4.

S1.4.a - In-solution activation of the fatty acids as N-hydroxysuccinimide esters:

To a solution of fatty acid (1 mmol) in anhydrous CH_2Cl_2 (5 mL) were added NHS (1.1 mmol) and EDC.Cl (1.15 mmol). The reaction was stirred at room temperature for 2 h, the course of the reaction being monitored by TLC (CH_2Cl_2). CH_2Cl_2 was removed *under vacuum*. The oily residue was extracted with Et_2O and brine (3x). The organic layer was dried over $MgSO_4$ and filtered. Et_2O was removed *under vacuum* to yield the *N*-hydroxysuccinimide ester as a colorless oil.



Scheme S1.4. Syntheses of the unsaturated lipopeptides, general strategy.

Table S1.4.a. Preparation of the N-hydroxysucciniminic esters.

Unsaturated fatty acid	**12:1**	**18:1**	**18:2**	**22:6**
Yield (%)	62	87	40	52

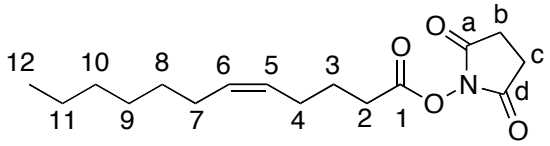
S1.4.b - Coupling of the N-hydroxysucciniminic esters to H-Arg$_4$-Lys(NBD)-NH$_2$:

The unsaturated lipopeptides C12:1-Arg$_4$-Lys(NBD)-NH$_2$, C18:1-Arg$_4$-Lys(NBD)-NH$_2$, C18:2-Arg$_4$-Lys(NBD)-NH$_2$ and C22:6-Arg$_4$-Lys(NBD)-NH$_2$ (abbreviated C12:1-Arg$_4$, C18:1-Arg$_4$, C18:2-Arg$_4$ and C22:6-Arg$_4$) were obtained by coupling the crude H-Arg$_4$-Lys(NBD)-NH$_2$ peptide with the unsaturated *N*-hydroxysuccinmic ester of the following fatty acids: *cis*-5-dodecenoic acid (C12:1), oleic acid (C18:1), linoleic acid (C18:2), and polyunsaturated acid (C22:6), as summarized in Scheme 2.

The crude H-Arg$_4$-Lys(NBD)-NH$_2$ peptide (~ 6.5 μmol) was reacted with the corresponding unsaturated fatty acid activated as its *N*-hydroxysuccinimide ester (~ 7 μmol) in a 1-mL vial in DMSO (200 μL). DIPEA (~ 2 μL) was added to adjust the pH to ~ 10. The reaction was stirred at room temperature until completion. The reaction was monitored by analytical RP-HPLC on the Dionex system at flow rate of 1.0 mL min^{-1} with a gradient 0 to 100 % acetonitrile (0.1 % TFA) in 15 min using the PROTO 200 C18 column. The sample was prepared by dissolving in 5 μL of DMSO and 45 μL of 0.1% TFA in water. Crude unsaturated lipopeptides were purified by RP-HPLC. C12:1-Arg$_4$, C18:1-Arg$_4$, were purified with a 35 min multi-step acetonitrile (0.1% TFA) in an aqueous solution (0.1% TFA) gradient: 0% for 5min, then 0 to 25% in 5min, 25 to 75% in 15 min and finally 75 to 100% in 10 min, affording C12:1-Arg$_4$, C18:1-Arg$_4$ as a bright orange powder. C18:2-Arg$_4$ and C22:6-Arg$_4$ were purified with a 35 min multi-step acetonitrile (0.1% TFA) gradient in an aqueous solution (0.1% TFA): 0% for 5min, then 0 to 30% in 5min, 30 to 60% in 15 min and finally 60 to 100% in 10 min, leading to C18:2-Arg$_4$ and C22:6-Arg$_4$ as a bright orange powder. The purity of all these peptides ranged from 97% to 99%, with overall yields ranging from 6 to 12%, depending on the purity of the crude precursor H-Arg$_4$-Lys(NBD)-NH$_2$, which might contain from 7% to 15% of the corresponding H-Arg$_3$-Lys(NBD)-NH$_2$ analogue, when acylated by a fatty acyl chain the two analogues (containing three or four arginines) were readily separated/isolated by HPLC or FCPC.

S1.4.c - Characterisation of the N-hydroxysucciniminic esters

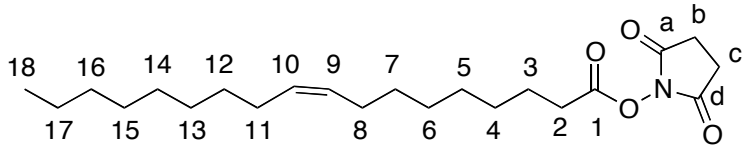
N-hydroxysuccinimide ester of *cis*-5-dodecenoic acid (C12:1):



^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.35 (m, 2H, H5+H6), 2.81 (s, 4H, Hb+Hc), 2.58 (t, J = 7.5 Hz, 2H, H2), 2.13 (m, 2H, H4), 2.00 (m, 2H, H7), 1.78 (m, 2H, H3), 1.27 (m, 8H, H8+H9+H10+H11), 0.85 (t, J = 6.6 Hz, 3H, H12).

^{13}C-NMR (75 MHz, $CDCl_3$) δ: 169.35-168.80 (Ca+Cd), 132.10 (C6), 127.71 (C5), 31.95 (C8 or C9 or C10 or C11), 30.50 (C2), 29.82 (C8 or C9 or C10 or 11), 29.15 (C8 or C9 or C10 or 11), 27.42 (C7), 26.37 (C4), 25.78 (Cb+Cc), 24.74 (C3), 22.83 (C8 or C9 or C10 or 11), 14.30 (C12).

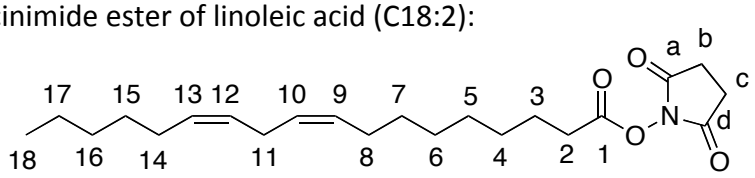
N-hydroxysuccinimide ester of oleic acid (C18:1):



^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.31 (ddd, J = 5.7 Hz, 3.5 Hz, 2.3 Hz, 2H, H9+H10), 2.80 (d, J= 1.8 Hz, 4H, Ha+Hd), 2.57 (t, J = 7.5, 2H, H2), 1.98 (m, 4H, H8+H11), 1.71 (q, 2H, H3), 1.29 (m, 20H, H4+H5+H6+H7+H12+H13 +H14+H15+H16+H17), 0.85 (t, J = 6.7 Hz, 3H, H18).

^{13}C-NMR (75 MHz, $CDCl_3$) δ: 169.40-168.86 (Ca+Cb+C1), 130.22-129.88 (C9+C10), 32.09 (C4 or C5 or C6 or C7 or C12 or C13 or C14 or C15 or C17), 31.11 (C2), 29.95-29.82-29.71-29.50-29.18-28.95 (C4 or C5 or C6 or C7 or C12 or C13 or C14 or C15 or C17), 27.40-27.32 (C8+C11), 25.77 (Cb+Cc), 24.74 (C3), 22.87 (C4 or C5 or C6 or C7 or C12 or C13 or C14 or C15 or C17), 14.31 (C18).

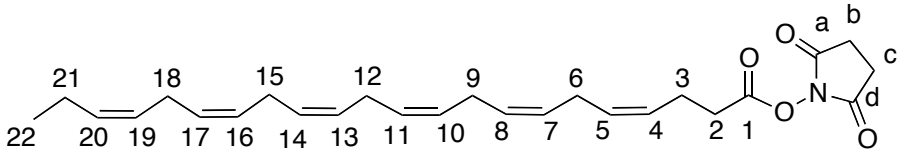
N-hydroxysuccinimide ester of linoleic acid (C18:2):



^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.33 (m, 4H, H9+H10+H12+H13), 2.76 (m, 6H, H11+Hb+Hc), 2.57 (t, J = 7.5 Hz, 2H, H2), 2.02 (m, 4H, H8+H14), 1.71 (m, 2H, H3), 1.29 (m, 14H, H4+H5+H6+H7+H15+H16+H17), 0.86 (t, J = 6.8 Hz, 3H, H18).

^{13}C-NMR (75 MHz, $CDCl_3$) δ: 169.41 (Ca+Cd), 168.87 (C1), 130.29-128.09 (C9 or C10 or C12 or C13), 31.70 (C4 or C5 or C6 or C7 or C15 or C16 or C17), 31.11 (C2), 29.73-29.53-29.18-28.94 (C4 or C5 or C6 or C7 or C15 or C16 or C17), 27.37 (C8+C14), 25.79 (C11 or Cb+Cc), 24.74 (C3), 22.76 (C4 or C5 or C6 or C7 or C15 or C16 or C17), 14.26 (C18).

N-hydroxysuccinimide ester of docosahexaneoic acid (C22:6):



^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.35 (m, 12H, H4+H5+H7+H8+H10+H11 +H13+H14+H16 +H17+H19+H20), 2.83 (m, 9H, H6+H9+H12+H15+H18+Hb+Hc), 2.65 (t, J = 7.9 Hz, 2H, H2), 2.49 (q, J = 6.9 Hz, 2H, H3), 2.05 (p, J = 7.5 Hz, 2H, H21), 0.95 (t, J = 7.5 Hz, 3H, H22).

^{13}C NMR (75 MHz, $CDCl_3$) δ: 169.29 (Ca+Cd), 168.37 (C1), 132.26-130.58-128.78-128.66-128.48-128.30-128.10-127.23-126.71 (C4 or C5 or C7 or C8 or C10 or C11 or C13 or C14 or C16 or C17 or C19 or C20), 31.15 (C2), 25.75 (C6+C9+C12+C15+C18+Cb+Cc), 22.55 (C3), 20.77 (C21), 14.49 (C22).

S1.4.d - Characterisation of the unsaturated lipopeptides

Peptides were purified by HPLC and characterised by Maldi-Tof mass spectrometry (Table S1.4.d).

Table S1.4.d. Characterisation of the peptides.

Peptide	**Analytical RP-HPLC gradient[a]**	**R_T (min)**	**$[M+H]^+$ Calc. (m/z)**	**$[M+H]^+$ Found (m/z)**
Ac-Arg_4	5 to 25% B in 20 min	17.0	974.54	974.54
C12:0-Arg_4	0 to 100% B in 30 min	17.5	1115.34	1116.00
C18:0-Arg_4	0 to 100% B in 40 min	30.3	1199.50	1199.8
C12:1-Arg_4	0 to 100% B in 15 min	11.7	1113.32	1113.67
C18:1-Arg_4	0 to 100% B in 15 min	14	1197.48	1197.8
C18:2-Arg_4	0 to 100% B in 15 min	13.6	1195.47	1195.7
C22:6-Arg_4	0 to 100% B in 15 min	11.2	1243.51	1243.7

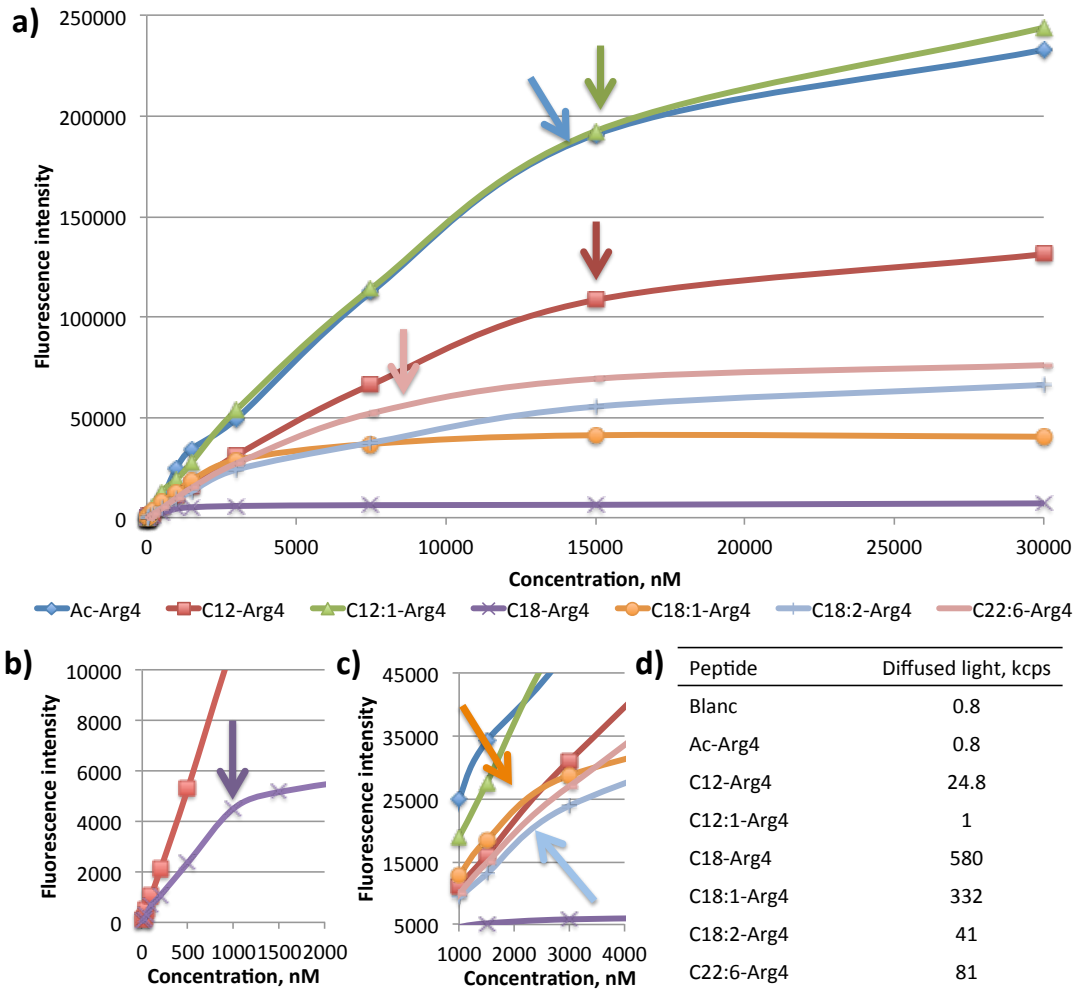
[a] Analytical and semi-preparative RP-HPLC gradients, solvent system A: 0.1% TFA in H_2O, B: 0.1% TFA in CH_3CN. [b] Proto 200 C18 (3µm, 100 x 4.6 mm) column at flow rate of 1.0 mL.min-1 on an automated Dionex system (Ultimate 3000).

The synthesis and characterisation of NBD-Arg_9 was described in Swiecicki, J.-M.; Bartsch, A.; Tailhades, J.; Di Pisa, M.; Heller, B.; Chassaing, G.; Mansuy, C.; Burlina, F.; Lavielle, S. ChemBioChem, 2014, *15*, 884-91.

S2 – Self-association of the peptide

S2.1 - Quenching of fluorescence

The fluorescence emission spectra have been recorded for 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 1.5, 3,7.5, 15, 30 μM solutions of each peptide on a Jasco Fluorescence Spectrophotometer at 20 °C controlled by a Jasco MCB-100 mini circulation bath. A Xenon lamp was used as excitation source and the excitation and emission slid-widths were set at 5 nm. These concentrations were obtained directly in polystyrene to be replaced by polymethacrylate cuvettes (Sigma) by dilution with PBS buffer of the 10, 100, 500 μM stock solutions, previously prepared. The buffer was supplied from Sigma-Aldrich as tablet and prepared by dissolving one tablet in 200 mL of MilliQ water leading to a 10 mM phosphate buffer (2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4). Measurements on a stirred solution (800 rpm) were recorded at the excitation wavelength corresponding to the maximum emission of the NBD, obtained after analysing first the excitation of NBD for a 0.1 μM solution of each peptide.



Peptide	Diffused light, kcps
Blanc	0.8
Ac-Arg4	0.8
C12-Arg4	24.8
C12:1-Arg4	1
C18-Arg4	580
C18:1-Arg4	332
C18:2-Arg4	41
C22:6-Arg4	81

Figure S2.1. Self-association of the peptides.

Fluorescence intensity as a function of CPP concentration, a) all peptides from 0.1 to 30 μM in PBS buffer, b) and c) zoom for the different peptides, and d) DLS measurements in water. The respective arrow shows the inflexion point for each peptide, indicative of the loss of linearity and thus of peptide aggregation at this concentration, from 1 to 10 μM, depending of the lipopeptide, the unsaturated lipopeptides aggregating less (at higher concentrations) than the saturated ones.

S2.2 - Dynamic light scattering

Solutions in water of 2.5, 5 and 10 μM for each peptide were prepared from stock solutions. DLS experiments were carried out on a Brookhaven system, equipped with a Brookhaven multiple τ digital correlator, and a laser with an output power of 30 mW, at a wavelength of 637 nm. The data were collected at 90° angle and 25 °C. The mean intensity of scattered light was measured over a 1 min acquisition time. For DLS analysis, the homodyne intensity–intensity correlation function G(q,t) was related to the correlation function of the scattered field. Inversion of the autocorrelation function was performed using the program NNLS. The intensity of the diffused light is summarized above in Figure S2.1.d).

S3 – General procedure for the preparation of symmetric LUVs

DOPG and DPPG were purchased from Avanti Polar Lipids, as a solution in chloroform.

Depending on the desired LUV composition, the appropriate amount of phospholipids (PLs), was introduced in a round-bottomed flask and chloroform was slowly evaporated under *vacuum* at 40 °C using a rotary evaporator and then for at least 2 h under *high-vacuum*. The lipids were hydrated with phosphate-buffered saline [phosphate (10 mM), potassium chloride (2.7 mM), sodium chloride (137 mM), pH 7.4] to a concentration of 1 mM. Depending on the phase transition temperature of the phospholipids, the buffer was pre-warmed or not for the gentle hydration step. For high temperature transition phospholipid DPPG (41°C), the extruder was heated using a thermocontrolled circulation bath. The turbid suspension of multilamellar vesicles was subsequently extruded 7 times through a 200 nm polycarbonate track-etch membrane and 10 times through a 100 nm polycarbonate track-etch membrane (Whatman) using a 10 mL Thermobarrel extruder (Lipex Biomembranes). The LUV solutions were used from the day after their preparation and within one week. LUVs made of DOPG were stored at 4 °C, whereas LUVs made DPPG were stored at room temperature.

S4 – Fluorescence experiments

Sample preparation: the concentration of the NBD-labelled lipopeptides was determined by UV at 460 nm with ε = 27000 L mol^{-1} cm^{-1}.

S4.1 - Fluorescence experiment

Emission spectra and time-course fluorescence measurements were recorded with a Jasco Fluorescence Spectrophotometer at 20°C (controlled with a Jasco MCB-100 mini circulation bath). As an excitation source a Xenon lamp was used, and the excitation and emission slide widths were set at 5 nm. To avoid peptide adsorption and subsequent fluorescence quenching, polymethacrylate cuvettes were used. During the whole measurement the solution was stirred at 800 rpm.

S4.2 - Internalisation experiments

Internalisation experiments: unlabelled LUVs were used with NBD-labelled CPPs. The excitation wavelength was fixed at 460 nm, and the emission wavelength was fixed at 555 nm, as schematically presented in Figure S4.2., and described in details in Swiecicki, J.-M.; Bartsch, A.; Tailhades, J.; Di Pisa, M.; Heller, B.; Chassaing, G.; Mansuy, C.; Burlina, F.; Lavielle, S. ChemBioChem, 2014, *15*, 884-91.

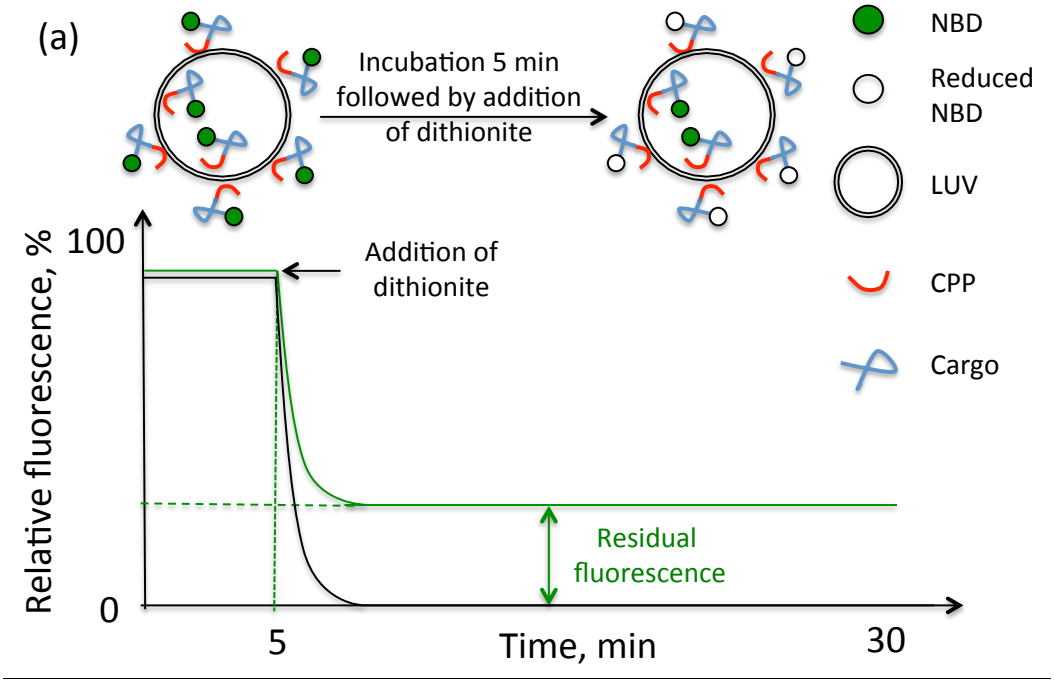


Figure S4.2. Schematic representation of the internalisation experiment.

S5 – Cell culture

The cell lines MA-104, RAW 264.7, HepG2 and Caco-2 were used and maintained in Eagle's minimal essential medium (EMEM), supplemented with 5 to 10 % foetal calf serum (Gibco) and antibiotics in culture flasks at 37 °C under an atmosphere of 5 % CO_2 in air and 100 % relative humidity. Cells were transferred to the appropriate culture dish at the desired density (see below) and grown 24 h (MA-104, Caco-2, and RAW 264.7) or 48 h (HepG2) in DMEM at 37 °C under an atmosphere of 5 % CO_2 in air and 100 % relative humidity. The day of the experiments, cells were washed 2x with Opti-MEM reduced serum medium (this medium does not contain phenol red; Life Technologies).

The viability assays were performed with 10^4 cells *per* well, while the FACS and confocal microscopy analysis were performed using 10^5 cells per dish (10 cm^2).

S6 – Cell-viability in the presence of labelled peptides

Cytotoxicity assays were performed in transparent 96-well plates (Thermo Scientific) using the CCK-8 assay (Dojindo Molecular Technologies) by measuring the absorbance of each well at 450 nm using a microplate reader (Molecular Devices).

MA-104, RAW 264.7, HepG2 or Caco-2 cells were seeded in 96-well plates in DMEM. The day after, cells (10^4 *per* well) were washed twice with Opti-MEM and incubated for 60 min with NBD-labelled peptides (1 to 10 μ M) in Opti-MEM (100 μL) at 37 °C. Controls were performed with incubation in pure Opti-MEM (positive control) or 0.1 % SDS in Opti-MEM (negative control). The supernatant was removed and the cells were washed once with Opti-MEM. CCK-8 solution was diluted 10 times in Opti-MEM and 100 μL of this solution was distributed in each well. After 2 h incubation at 37 °C, cell cytotoxicity was determined by measuring the absorbance of each well.

Cell viability was defined as followed (where A stands for the optical density at 450 nm):

$$cell\ viability = \frac{A(considered\ well) - A(negative\ control)}{A(positive\ control) - A(negative\ control)}$$

Adherent cells (10^4 *per* well) were incubated for 1 h at 37°C with various concentrations of NBD-lipopeptides, from *left* to *right*: 10, 5, 2, and 1 µM, in Opti-MEM (100 µL). After incubation, the supernatant was removed and a 1:10 solution of CCK-8 in Opti-MEM was dispensed in each well (100 µL). Cell viability was determined after 2 h incubation at 37 °C. Viability data are expressed as a percentage of viable cells (a means of 3-4 experiments ± s.d.) compared to the positive control incubated with only Opti-MEM (100 µL) for 60 min with at 37 °C.

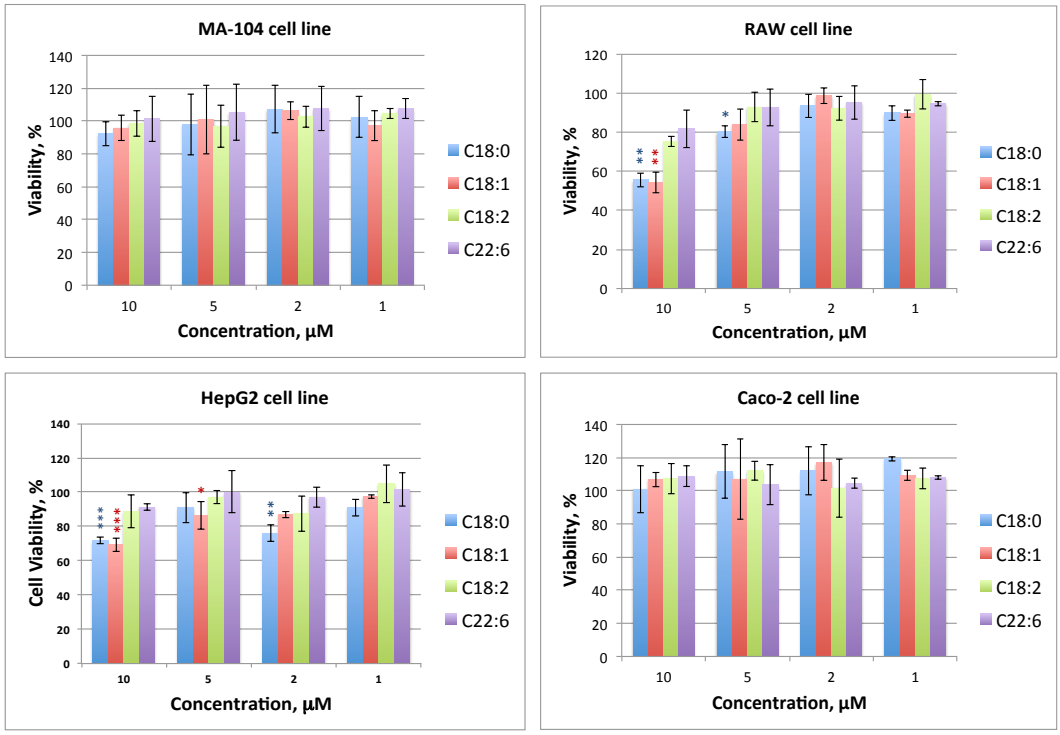


Figure S5.1. Normalized cell viability of the four cell lines MA-104, RAW264.7, HepG2 and Caco-2 cells.

Cell cytotoxicity was determined in transparent 96-well plates using the CCK-8 assay, (with 10^4 cells per well). Data are means of 2-4 experiments ± s.d., Student test: with *: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.001$, respectively.

S7 – Flow cytometry analysis

Flow cytometry requires cell dissociation and cell detachment. This has been done with accutase (accutase treatment is known to preserve more the integrity of cells than trypsin; Life Technologies). The samples were prepared in 5-mL polystyrene round-bottom tubes (Fisher Scientific). Flow cytometry was performed with a LSR II analyser (Becton-Dickinson). The significance of the data was determined by calculating the p-value with the software InStat (GraphPad).

To analyse the internalisation of the fluorescently labelled peptides by flow cytometry, exponentially growing MA-104 cells in culture flask were dissociated with accutase. Cells were concentrated by centrifugation at (800 rpm for 3 min) and the supernatant was discarded. Cells were washed with Opti-MEM, centrifuged and resuspended in Opti-MEM. Cells (10^5 *per* tube) in Opti-MEM (1 mL) were then incubated at 37 °C or 4 °C with the peptide at 5 μM. After 10 or 60 min incubation, dithionite was added to final concentration of 10 mM in order to quench the fluorescence of the non-internalised NBD (3 min). The quenching of the fluorescence was quantitative as demonstrated by total reduction/quenching of the control NBD-labelled peptide, Ac-Arg4. Fluorescence analysis was performed with a LSR II cell analyser and at least 5.10^3 events were recorded. Cell fragments (as evaluated by the forward scatter) were discarded from the analysis.

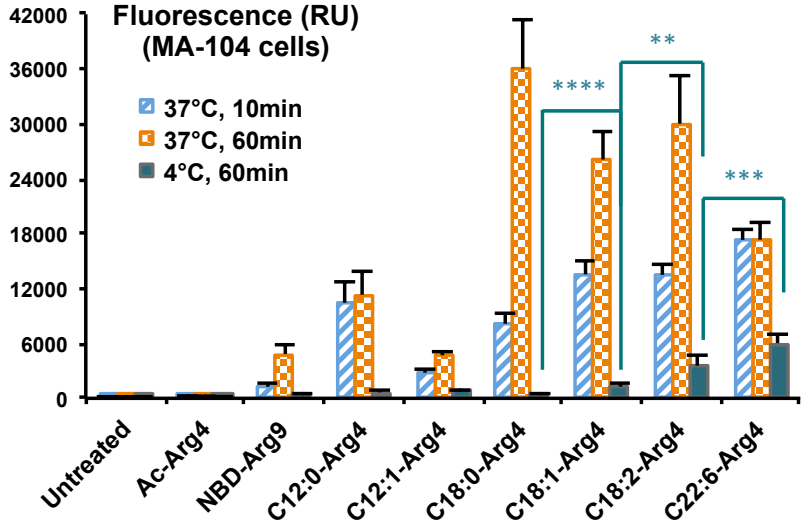


Figure S7. Uptake into MA-104 cells (10^5 cells) at 4°C or 37°C of the indicated NBD-labelled peptides (5 μM) analysed by FACS.

At the end of the incubation (10 or 60 min), dithionite was added to quench the fluorescence of the non-internalised NBD-peptides. Data are means of 3-4 experiments ± s.d.; Student test: with **: $p \leq 0.01$, ***: $p \leq 0.001$ and ****: $p \leq 0.0001$.

S8 – Confocal laser scanning microscopy and image analysis

Confocal microscopy acquisition was performed using an inverted SP2 setup (DM IRBE2 microscope equipped with a TCS SP2 detector; Leica). Lectin WGA labelled with tetramethylrhodamine isothiocyanate (TRITC), Lyso tracker deep red, Mito tracker deep red, and Golgi tracker (BODIPY TR-ceramide) were purchased from Life Technologies.

Cells were grown in 35 mm μ-dishes (tissue culture treated, ibiTreat, Biovalley). MA-104, RAW 264.7 and Caco-2 cells were seeded at a density of $5x10^4$ cells *per* dish and cultured for 24 h at 37 °C in order to have 10^5 cells *per* dish the day of the experiment. HepG2 cells were seeded at a density of $2.5x10^4$ cells *per* dish and cultured over 48 h. Cells were washed twice with Opti-MEM and incubated in 1mL Opti-MEM with the lipopeptides (5 μM), under different experimental conditions: temperature (4 or 37°C) and incubation time (10 or 60 min). Co-incubation with various staining agents was performed as indicated in Table S7. Different combinations were tested, provided that no spectral overlap occurs between the fluorophores.

Table S8. Incubation conditions with staining agents, to identify the plasma membrane, endosomes, mitochondria, lipid droplets or lysosomes.

T (°C)	Cell line	Staining agent	Experimental condition
4	MA-104	Rhodamine-lectin	0.02 mg.mL^{-1}, 60 min 4°C
		LysoTracker deep red	100 nM, 30 min 37°C
		BODIPY-TR ceramide	5 μM, 30 min 4°C, 30 min 37°C
	Caco-2, HepG2, RAW 264.7	Rhodamine-lectin	0.02 mg.mL^{-1}, 60 min 4°C
37	MA-104	Rhodamine-lectin	0.02 mg.mL^{-1}, 10 min or 60 min
		LysoTracker deep red	100 nM, 30 min 37°C
		BODIPY-TR ceramide	5 μM, 30 min 4°C, 30 min 37°C

The supernatant was discarded and the cells were washed three times with Opti-MEM. Finally, Opti-MEM (1 mL) was added to hydrate the cells during the experiment, which were immediately observed by confocal microscopy at room temperature before and after the addition of sodium dithionite (DT, 10 mM final concentration in 1 mL) to reduce the NBD moiety and thus quench the fluorescence of the non-internalised NBD-labelled peptide. Cells (10^5 *per* dish) were imaged soon, and snapshots were recorded once signal stabilization was reached (about 3 to 5 min after the addition of DT). All images were corrected for background by using the freeware image analysis program Fiji (http://fiji.sc/Fiji).

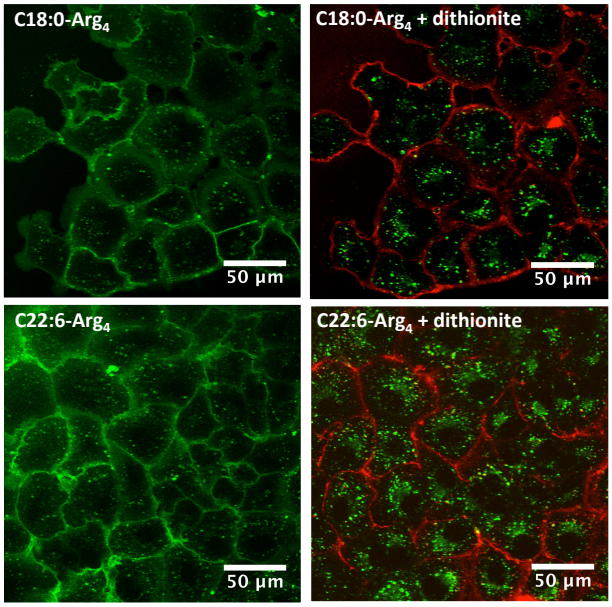


Figure S8.1. Confocal laser scanning microscopy, reduction of the non-internalised NBD-labelled peptide by dithionite, DT.
Incubation of MA-104 cells (10^5 *per* dish) with C18:0-Arg$_4$ or C22:6-Arg$_4$ (5 μM, in green) at 37°C for 10 min, without (left) or with (right) reduction by dithionite. (Table S8). On the left panels the fluorescence was dense on the plasma membranes for both peptides, whereas after incubation with DT only the intracellular NBD-labelled peptides were detected while reduction by DT quenched the NBD-labelled non-internalised peptides. The plasma membrane was labelled by Rhodamine-lectin (in red, only shown on the right panels).

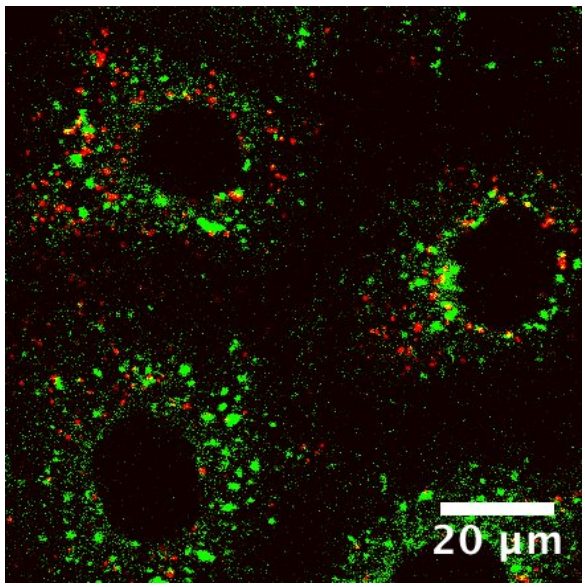


Figure S8.2. Confocal laser scanning microscopy, colocalisation studies with Lyso Tracker of the NBD-labelled C22:6-Arg_4.
Incubation of MA-104 cells (10^5 *per* dish) for 30 min at 37°C with Lyso Tracker Deep Red (100 nM), then C22:6-Arg_4, at 5 μM, was added and cells were further incubated for 60 min at 37°C. At the end, MA-104 cells were immediately treated with dithionite (3 min) to quench the staining of labelled peptides remaining at the cell surface. For this typical merged picture no colocalisation is observed for the C22:6-Arg_4 is (in green) and the Lyso Tracker (in red).