Electronic Supplementary Material (ESI) for ChemComm.

Versatile symport transporters based on cyclic peptide dimers

Alberto Fuertes, Manuel Amorín* and Juan R. Granja*

Singular Research Centre in Chemical Biology and Molecular Materials, (CIQUS), Organic Chemistry Department, University of Santiago de Compostela (USC), 15782. Santiago de Compostela, Spain

Supporting information

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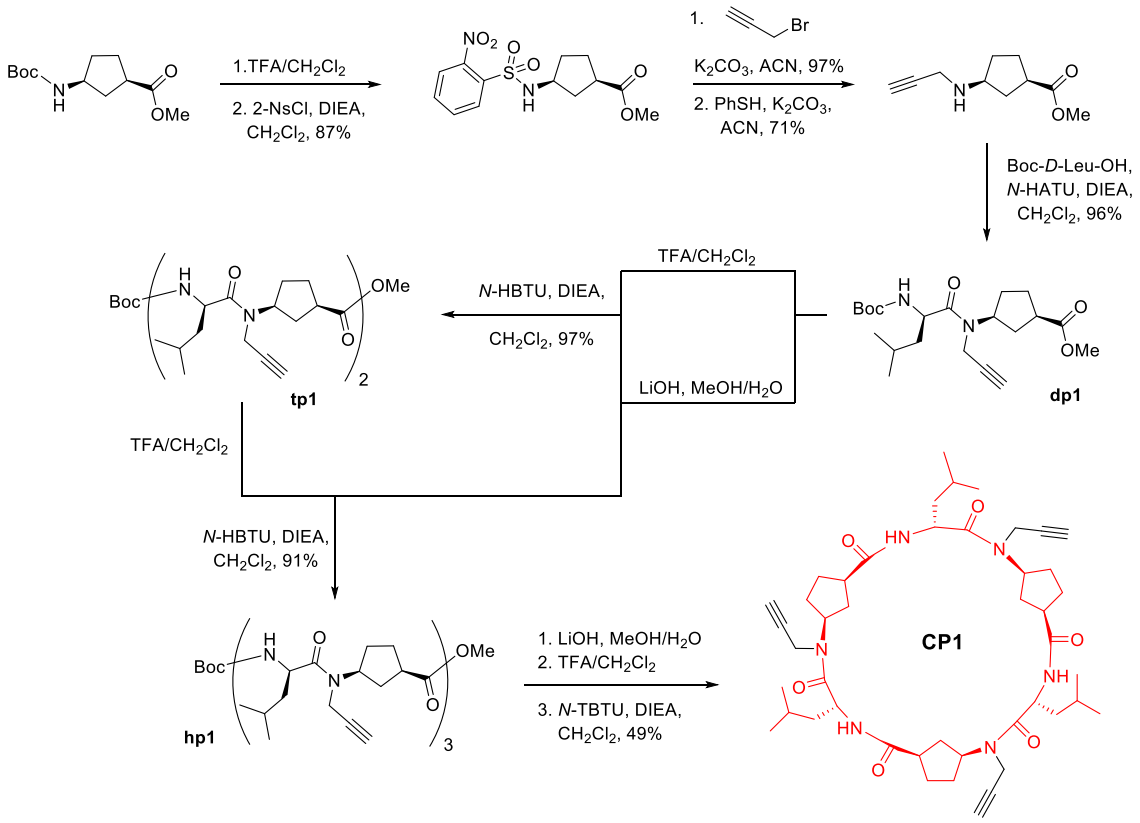
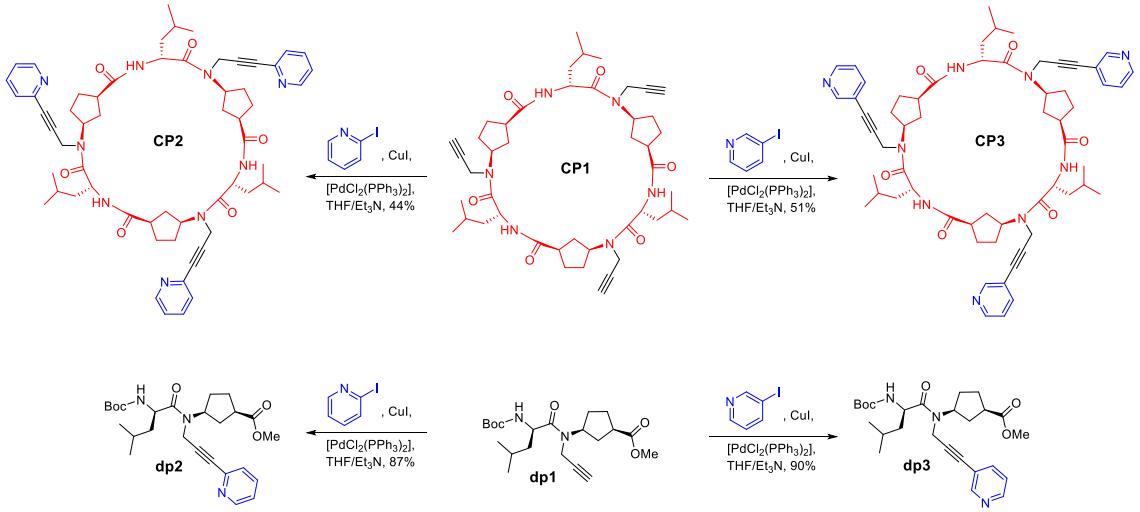
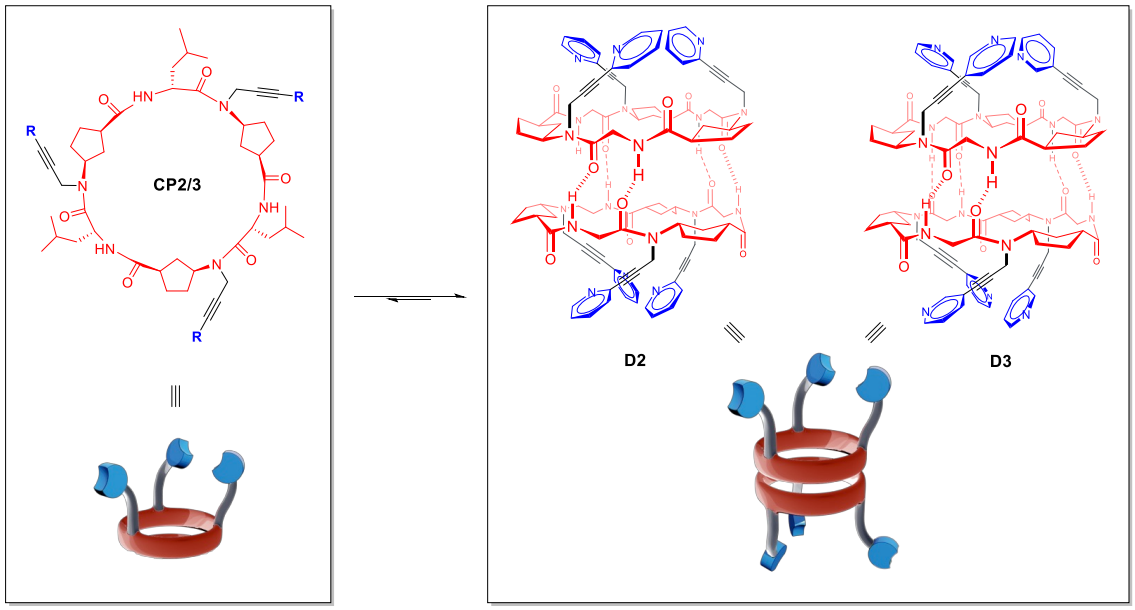


Scheme 1SI. Synthetic route towards precursor **CP1**.



Scheme 2SI. Post-synthetical modifications *via* Sonogashira reaction of **CP1** and **dp1**.



Scheme 3SI. Dimerization process observed for **CP2** and **CP3** in non-polar environments to provide the corresponding dimers (**D2** and **D3**) through the hydrogen bonding interactions between the amide backbone groups of both peptides.

Fluorescence assays

1. CF⊂LUV assay for pH gradient dissipation

Vesicles were prepared by taking 1 mL of an EYPC (Avanti Polar Lipids) $CHCl_3$ solution (25 mg/mL) into a 10 mL round bottom flask and slowly evaporating it on a rotary evaporator to give a thin film that was dried at high vacuum overnight. The film was hydrated with 1 mL of an aqueous solution containing CF (30 µM) in a NaH_2PO_4 buffer (10 mM, pH 5.8). The resulting mixture was subjected to tumbling (rotation) for an hour and, after that, 9 freeze-thaw cycles (N_2 (l) → 40 °C water bath) were carried out. The resulting suspension was extruded 15 times across a 100 nm polycarbonate membrane and finally purified by size exclusion chromatography (Sephadex G-25) using as eluent NaH_2PO_4 buffer (10 mM, pH 5.8). The resulting vesicle suspension was taken up in a total volume of 5 mL (approximate lipid concentration 6.6 mM).

Measurement protocol: 200 µL of the vesicle suspension were taken into a plastic cuvette containing 1800 µL of NaH_2PO_4 buffer (10 mM, pH 7.4). After 70 s, the compound under study (20 µL DMSO solution) was added and the change in CF fluorescence (basification) was measured (λ_{exc} = 493 nm y λ_{em} = 510 nm). After 400 s, Triton-X 100 (50 µL, 10% solution in water) was added to lyse all the vesicles and normalize the fluorescence trace. Experiments ended after 500 s.

The data was normalized using the following equation:

$$F_n = \frac{F_t - F_0}{F_\infty - F_0} \quad (1)$$

Where F_n is the normalized fluorescence, F_t is the fluorescence value at a certain time, F_0 is the fluorescence value before the addition of the compound under study (i.e., 50 s) and F_∞ is the fluorescence intensity after the total lysis of the liposomes (i.e., 450 s).

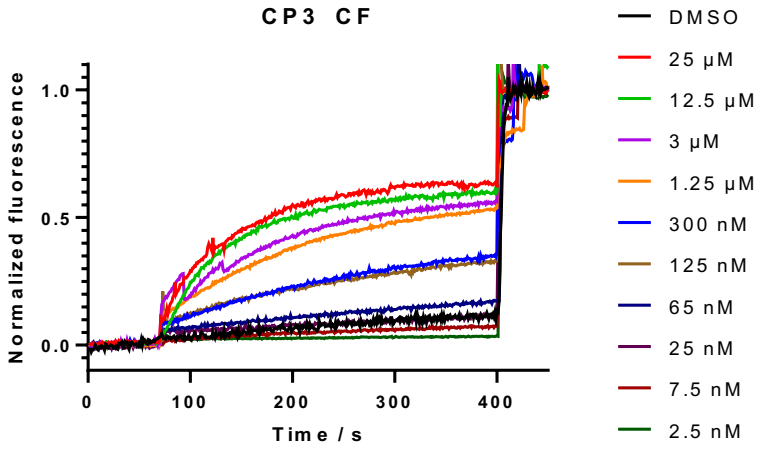
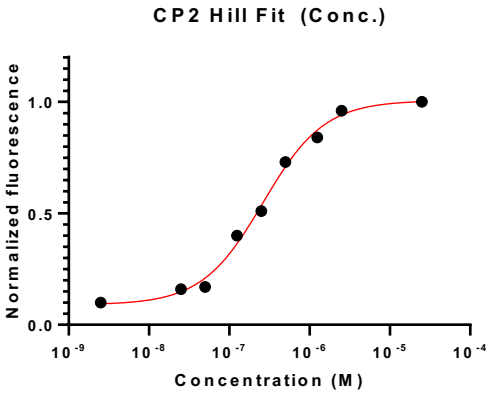
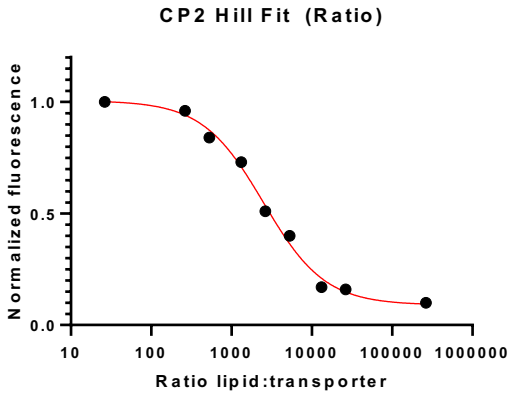


Figure 1SI. Fluorescence traces obtained for **CP3** in the CF⊂LUV assay at a range of concentrations (25 µM-2.5 nM).

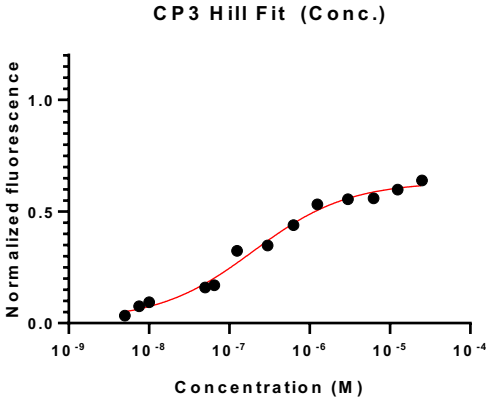


Bottom	0.08945
Hillslope	1.140
Top	1.005
EC50	2.632e-007

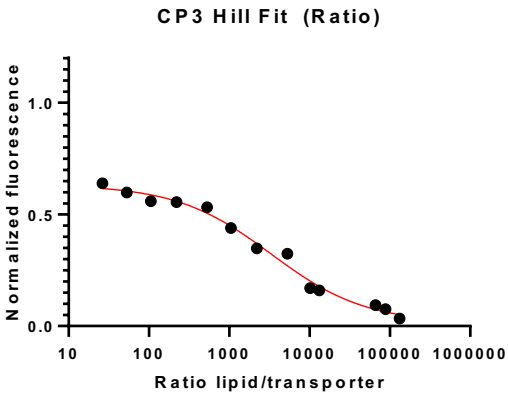


Bottom	1.005
Top	0.08945
IC50	2508
HillSlope	1.140

Figure 2SI. Hill plots obtained in the **CF⊂LUV** assay at different concentrations of **CP2** (mM to nM range). Left: fluorescence plotted against transporter concentration in the cuvette. Right: fluorescence plotted against the ratio between lipid and transporter molecules.



Bottom	0.01230
Hillslope	0.7355
Top	0.6323
EC50	1.988e-007



Bottom	0.6323
Top	0.01230
IC50	3320
HillSlope	0.7355

Figure 3SI. Hill plots obtained in the **CF⊂LUV** assay at different concentrations of **CP3** (mM to nM range). Left: fluorescence plotted against transporter concentration in the cuvette. Right: fluorescence plotted against the ratio between lipid and transporter molecules.

2. Self-quenched CF⊂LUV assay for membrane integrity

Vesicles were prepared by taking 25 µL of an EYPC (Avanti Polar Lipids) EtOH solution (1 g/mL) into a 10 mL round bottom flask containing a mixture of $CHCl_3$/EtOH (1:1, 2 mL), which was slowly evaporated on a rotary evaporator to give a thin film that was dried at high vacuum overnight. Then, the film was hydrated with 1 mL of an aqueous solution containing: CF (50 mM), Tris (10 mM), NaCl (10 mM), $NaNO_3$ (150 mM), pH 7.4. The resulting mixture was subjected to tumbling (rotation) for an hour and after that 9 freeze-thaw cycles (N_2 (l) → 40 °C water bath). The resulting suspension was extruded 15 times across a 100 nm polycarbonate membrane and finally purified by size exclusion chromatography (Sephadex G-25) using as eluent Tris (10 mM), NaCl (10 mM), $NaNO_3$ (150 mM), pH 7.4. The resulting vesicle suspension was taken up in a total volume of 5 mL (approximate lipid concentration 6.6 mM).

Measurement protocol: 100 µL of the vesicle suspension were taking into a plastic cuvette containing 1900 µL of Tris (10 mM), NaCl (107 mM), $NaNO_3$ (150 mM), pH 7.4. After 20 s, the compound under study (DMSO solution) was added and the change in CF fluorescence (due to leakage) was measured (λ_{exc} = 493 nm y λ_{em} = 510 nm). After 400 s, Triton-X (100 µL, 1.2% solution in water) was added to lyse all the vesicles and normalize the fluorescence trace. Experiment ended after 500 s.

Data was normalized using Eq. 1.

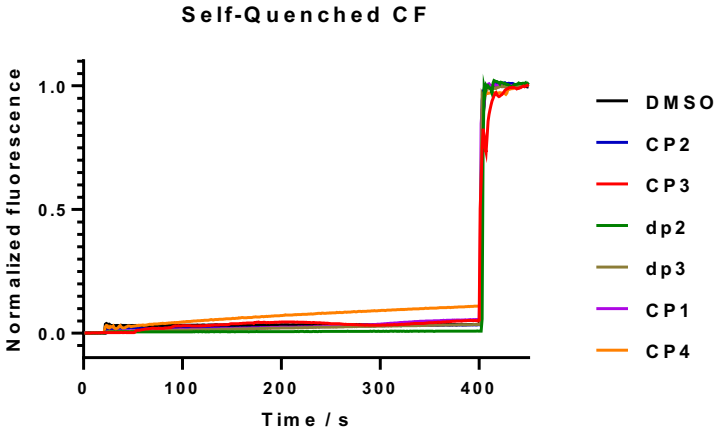


Figure 4SI. Fluorescence traces obtained for all the compounds described in this work in the **SQ-CF⊂LUV** assay. All samples were recorded at 25 µM concentration in cuvette.

3. LG⊂LUV assay for Cl^- transport

a. Nitrate buffer

Vesicles were prepared by taking 25 µL of an EYPC (Sigma Aldrich) EtOH solution (1 g/mL) into a 10 mL round bottom flask containing a mixture of $CHCl_3$/EtOH (1:1, V_t=2 mL), which was slowly evaporated on a rotary evaporator to give a thin film that was dried at high vacuum overnight. Then, the film was hydrated with 1 mL of an aqueous solution containing: *N,N′*-dimethyl-9,9′-bisacridinium dinitrate (lucigenin, 1 mM) and $NaNO_3$ (200 mM). The resulting mixture was subjected to tumbling (rotation) for an hour and after that 9 freeze-thaw cycles (N_2 (l) → 40 °C water bath). The resulting suspension was extruded 15 times across a 200 nm polycarbonate membrane and finally purified by size exclusion chromatography (Sephadex G-25) using $NaNO_3$ (200 mM) as eluent. The resulting vesicle suspension was taken up in a total volume of 5 mL (approximate lipid concentration 6.6 mM).
Measurement protocol: 50 µL of the vesicle suspension were taking into a plastic cuvette containing 1950 µL of $NaNO_3$ (200 mM). After 50 s, NaCl is added (25 µL, 2 M) and then elapsed 100 s the transporter under study (^{i}PrOH solutions) was added and the change in lucigenin fluorescence (nitrate exchanged by chloride; halide collisions cause quenching of fluorophore) was measured (λ_{exc} = 450 nm y λ_{em} = 535 nm). After 400 s, Triton-X 100 was added (50 µL, 10% solution in water) to lyse all the vesicles and normalize the fluorescence trace. Experiment ended after 500 s.

The data was normalized using Eq. 1.

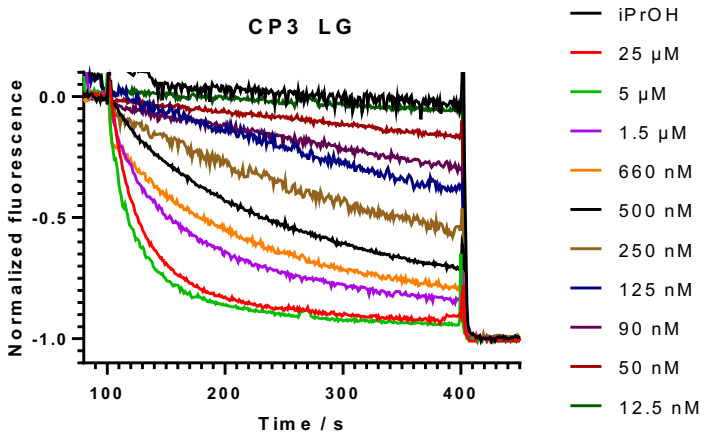
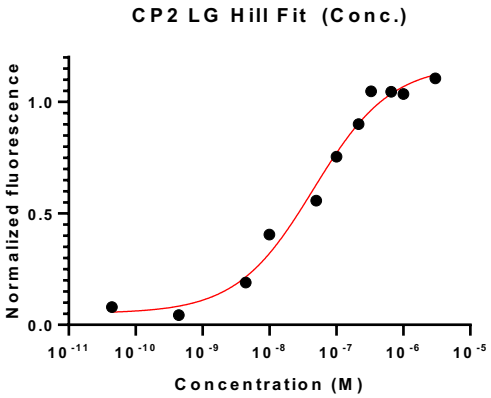
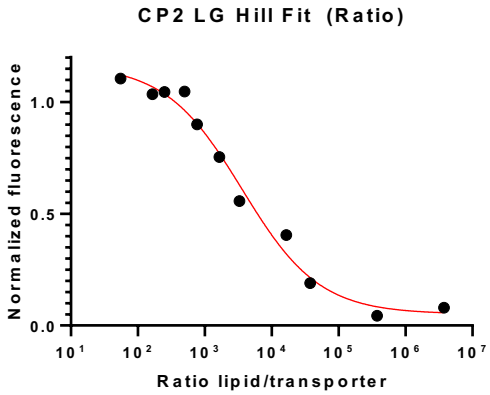


Figure 5SI. Fluorescence traces obtained for **CP3** in the **LG⊂LUV** assay in $NaNO_3$ buffer at a range of concentrations (25 µM-12.5 nM).

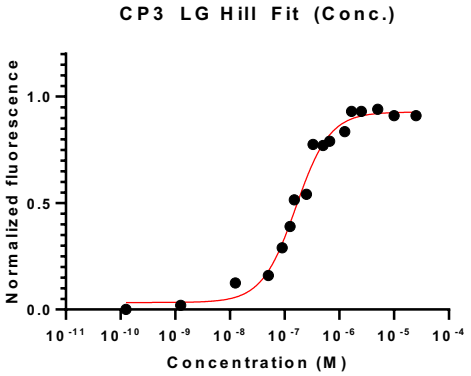


Bottom	0.05121
Hillslope	0.7549
Top	1.166
EC50	4.553e-008

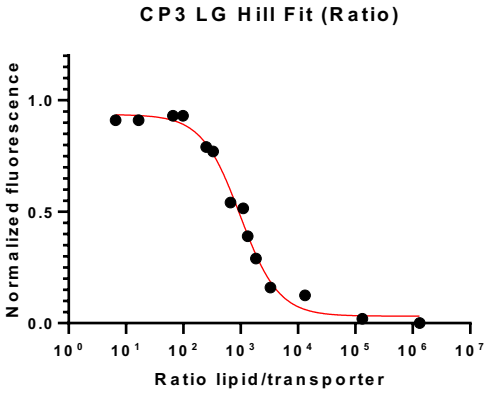


Bottom	1.166
Top	0.05121
IC50	3624
HillSlope	0.7549

Figure 6SI. Hill plots obtained in the **LG⊂LUV** assay in $NaNO_3$ buffer at different concentrations of **CP2** (μM to nM). Left: fluorescence plotted against transporter concentration in the cuvette. Right: fluorescence plotted against the ratio between lipid and transporter molecules.



Bottom	0.03282
Hillslope	1.400
Top	0.9265
EC50	1.594e-007



Bottom	0.9351
Top	0.03142
IC50	974.0
HillSlope	1.299

Figure 7SI. Hill plots obtained in the **LG⊂LUV** assay in $NaNO_3$ buffer at different concentrations of **CP3** (μM to nM). Left: fluorescence plotted against transporter concentration in the cuvette. Right: fluorescence plotted against the ratio between lipid and transporter molecules.

b. Sulfate buffer

25 µL of an EYPC (Sigma Aldrich) EtOH solution (1 g/mL) into a 10 mL round bottom flask containing a mixture of $CHCl_3$/EtOH (1:1, V_t = 2 mL) were slowly evaporated on a rotary evaporator to give a thin film that was dried at high vacuum overnight. Then, the film was hydrated with 1 mL of an aqueous solution containing: *N,N′*-dimethyl-9,9′-*bis*acridinium dinitrate (lucigenin, 1 mM) and Na_2SO_4 (200 mM). The resulting mixture was subjected to tumbling (rotation) for an hour and after that 9 freeze-thaw cycles (N_2 (l) → 40 °C water bath) were performed. The resulting suspension was extruded 15 times across a 200 nm polycarbonate membrane and finally purified by size exclusion chromatography (Sephadex G-25) using Na_2SO_4 (200 mM) as eluent. The resulting vesicle suspension was taken up in a total volume of 5 mL (approximate lipid concentration 6.6 mM).
Measurement protocol: 50 µL of the vesicle suspension were taken into a plastic cuvette containing 1950 µL of Na_2SO_4 (200 mM). After 50 s, NaCl is added (25 µL, 2 M) and then elapsed 100 s transporter aliquots (^{i}PrOH solutions) were added and the change in lucigenin fluorescence (nitrate exchanged by chloride; halide collisions cause quenching of fluorophore) was measured (λ_{exc} = 450 nm and λ_{em} = 535 nm). After 400 s, Triton-X 100 was added (50 µL, 10% solution in water) to lyse all the vesicles and normalize the fluorescence trace. Experiment ended after 500 s.

The data was normalized using Eq. 1.

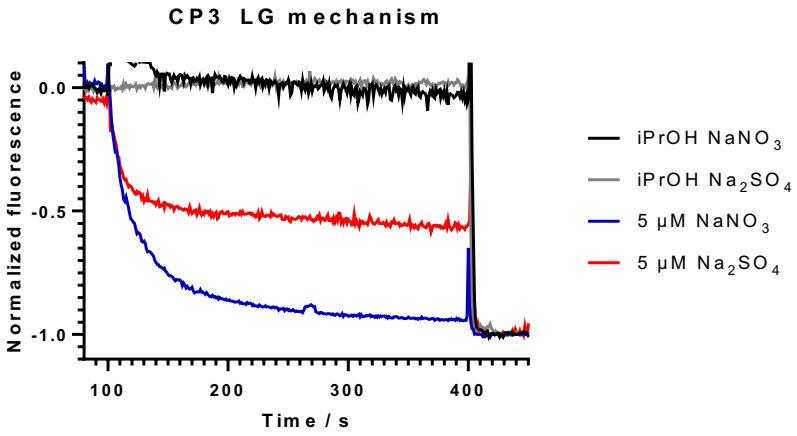


Figure 8SI. Effect of the nature of the intra- and extravesicular buffer on the transport of **CP3** (5µM in cuvette).

4. HPTS⊂LUV assay for ion selectivity

Vesicles were prepared by taking 1 mL of an EYPC (Avanti Polar Lipids) $CHCl_3$ solution (25 mg/mL) into a 10 mL round bottom flask containing a mixture of $CHCl_3$/EtOH (1:1, V_t = 2 mL) and then this mixture was slowly evaporated on a rotary evaporator providing a thin film that was dried at high vacuum overnight. Then, the film was hydrated with 1 mL of an aqueous solution containing: HPTS (1 mM), HEPES (10 mM), NaCl (100 mM), pH 7.0. The resulting mixture was subjected to tumbling (rotation) for an hour and after that 9 freeze-thaw cycles (N_2 (l) → 40 °C water bath). The resulting suspension was extruded 15 times across a 100 nm polycarbonate membrane and finally purified by size exclusion chromatography (Sephadex G-25) using as eluent HEPES (10 mM), NaCl (100 mM), pH 7.0. The resulting vesicle suspension was taken up in a total volume of 5 mL (approximate lipid concentration 6.6 mM).
Measurement protocol: 25 µL of the vesicle suspension were dispersed into a plastic cuvette containing 1980 µL of HEPES (10 mM), MX (100 mM), pH 7.0. After 20 s, NaOH is added (20 µL, 0.5 M) and then elapsed 100 s transporter aliquots (DMSO solutions) were added and the change in HPTS fluorescence (basification) was measured (λ_{exc} = 450 nm and λ_{em} = 510 nm). After 400 s, Triton-X 100 was added (50 µL, 10% solution in water) to lyse all the vesicles and normalize the fluorescence traces. Experiment ended after 500 s.

The data was normalized using Eq. 1.

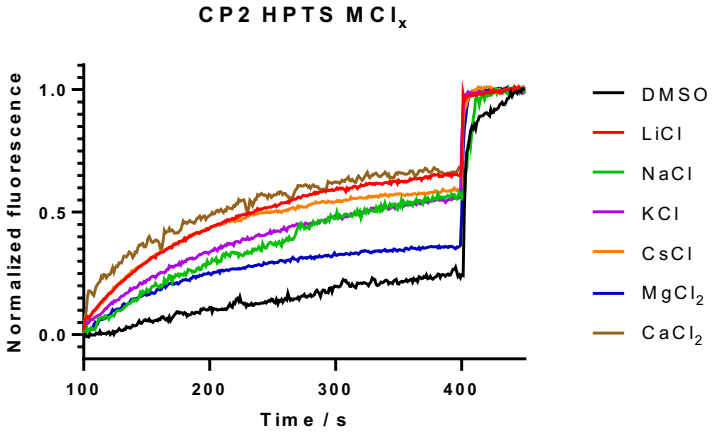


Figure 9SI. Transport efficiency of **CP2** (1µM in cuvette) in the **HPTS⊂LUV** assay under different extravesicular chloride salts.

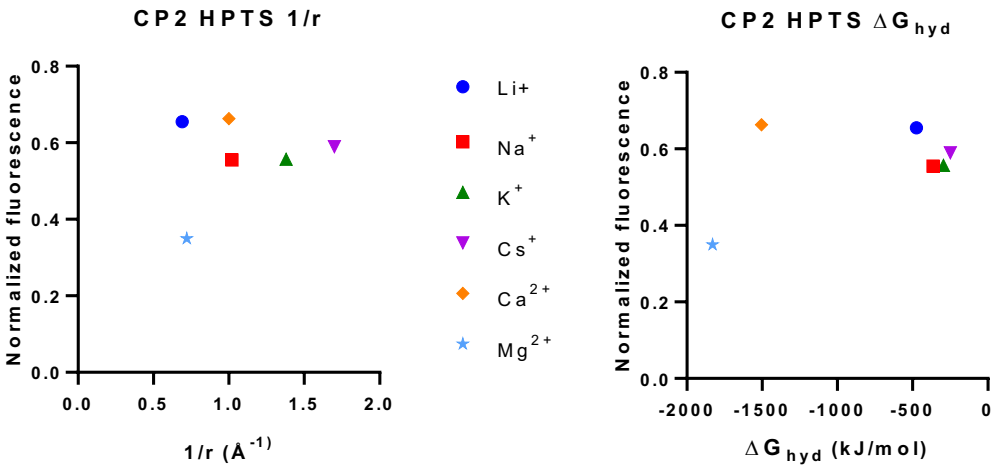


Figure 10SI. Plots of transport intensity of **CP2** (1μM in cuvette) in the **HPTS⊂LUV** assay against the inverse of the ionic radius (1/r, left) and the hydration energy (ΔG_{hyd}, right) of selected cations.

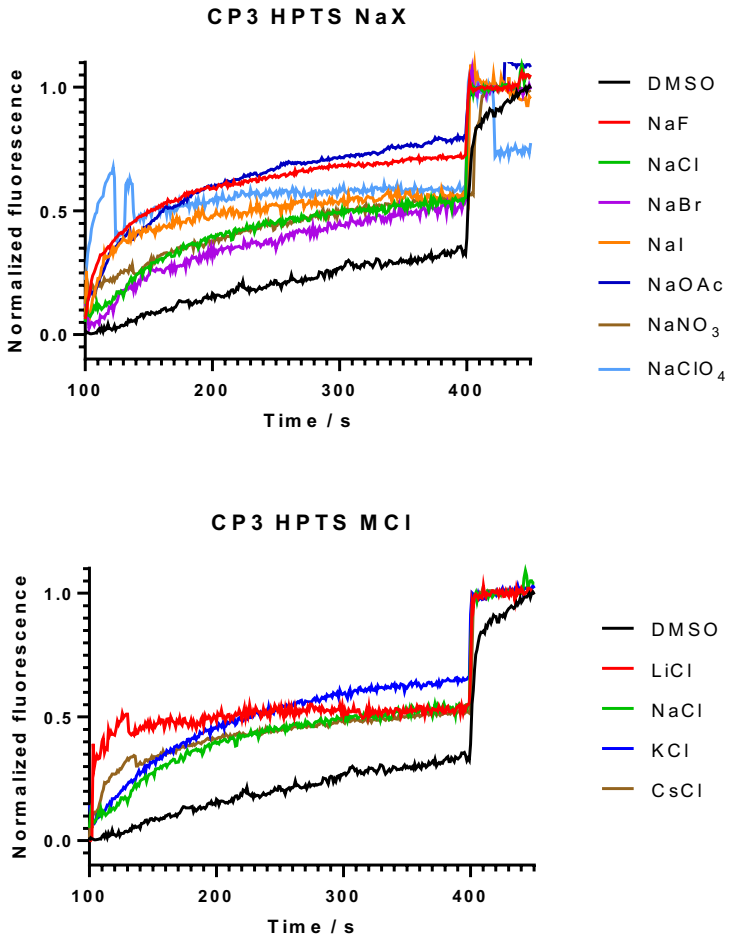


Figure 11SI. Transport efficiency of **CP3** (1μM in cuvette) in the **HPTS⊂LUV** assay under different extravesicular sodium (top) and chloride (bottom) salt.

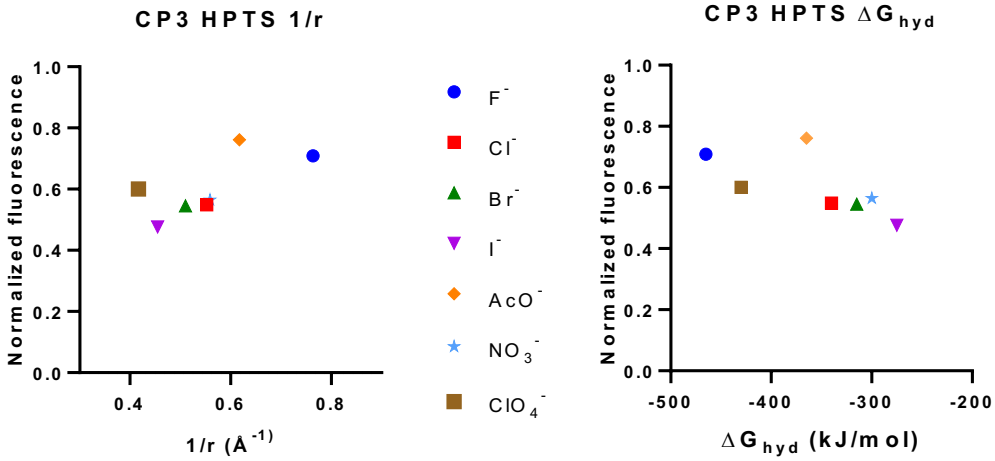


Figure 12SI. Plots of transport intensity of **CP3** (1μM in cuvette) in the **HPTS⊂LUV** assay against the inverse of the ionic radius (1/r, left) and the hydration energy (ΔG_{hyd}, right) of selected anions.

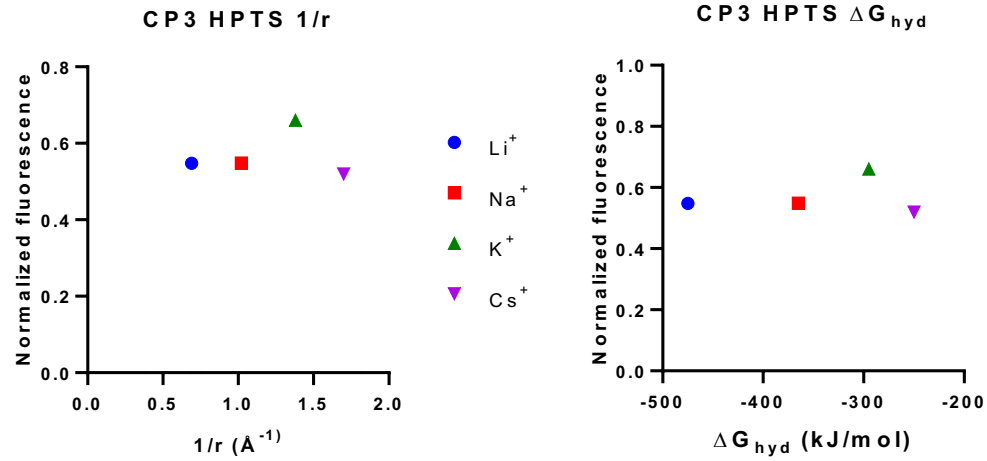


Figure 13SI. Plots of transport intensity of **CP3** (1μM in cuvette) in the **HPTS⊂LUV** assay against the inverse of the ionic radius (1/r, left) and the hydration energy (ΔG_{hyd}, right) of selected cations.

5. ^{23}Na NMR assay for ion

Vesicles were prepared by taking 1 mL of an EYPC (Avanti Polar Lipids) $CHCl_3$ solution (25 mg/mL) into a 10 mL round bottom flask and slowly evaporating it on a rotary evaporator to give a thin film that was dried at high vacuum overnight. The film was hydrated with 1 mL of an aqueous solution containing LiCl (80 mM). The resulting mixture was subjected to tumbling (rotation) for an hour and, after that, 9 freeze-thaw cycles (N_2 (l) → 40 °C water bath) were carried out. The resulting suspension was extruded 15 times across a 200 nm polycarbonate membrane and finally purified by size exclusion chromatography (Sephadex G-25) using as eluent an aqueous solution of LiCl (80 mM). The resulting vesicle suspension was taken up in a total volume of 5 mL (approximate lipid concentration 6.6 mM).

240 µL of this vesicle suspension were taken in a 5mm NMR tube, together with 40 µL of an aqueous solution of [$Na_7Dy(PPP)_2$] (10 mM)[1] and NaCl (30 mM). After recording a ^{23}Na NMR spectrum, 40 µL of a **CP2** solution (2.5 mM, DMSO) were added and shacked mechanically for 5 minutes, after which a new ^{23}Na NMR spectrum was recorded.

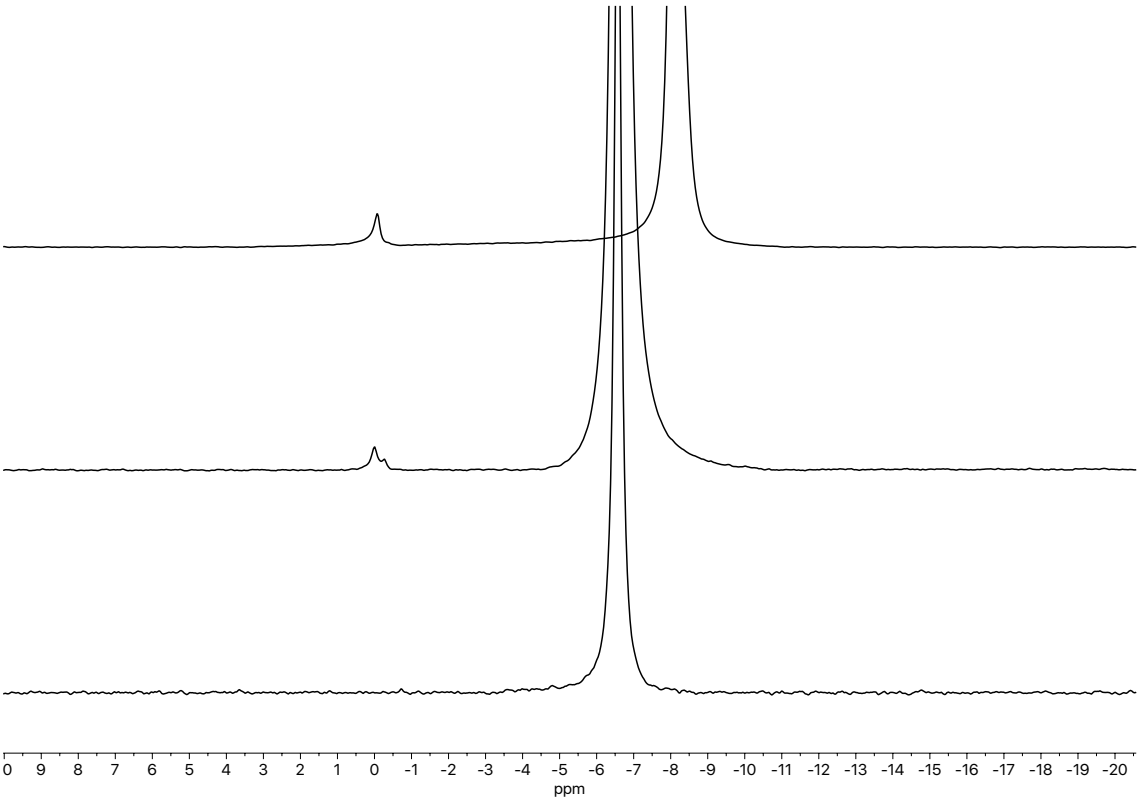


Figure 14SI. Bottom: ^{23}Na NMR of the sample prepared with the mixture of LiCl vesicles and the DyPPP-containing extravesicular solution, where only the peak that corresponds to the Na-Dy complex (-6.5 ppm*) is observed. Middle: ^{23}Na NMR obtained after the addition of **CP2** over the vesicle dispersion, where a new peak at 0 ppm appears after the transport of Na^+ ions into the intravesicular media. Top: ^{23}Na NMR of the sample prepared with the mixture of NaCl vesicles and the DyPPP-containing extravesicular solution, where both peaks that correspond to the Na-Dy complex (-9.0 ppm*) and the intravesicular NaCl (0.0 ppm) are observed. * The chemical shift of sodium nuclei in the presence of DyPPP depends on the ratio between the ion and the dysprosium salt.

[1] M. J. Pregel, L. Julien, J.-M. Lehn, *Angew. Chem. Int. Ed. Eng.* **1992**, *31*, 1637-1640.

6. Calcein⊂LUV assay for Ca^{2+} transport

Vesicles were prepared by taking 1 mL of an EYPC (Avanti Polar Lipids) $CHCl_3$ solution (25 mg/mL) into a 10 mL round bottom flask and slowly evaporating it on a rotary evaporator to give a thin film that was dried at high vacuum overnight. The film was hydrated with 1 mL of an aqueous solution containing Calcein (30 μM) in a HEPES buffer (10 mM, pH 11.5). The resulting mixture was subjected to tumbling (rotation) for an hour and, after that, 9 freeze-thaw cycles (N_2 (l) → 40 °C water bath) were carried out. The resulting suspension was extruded 15 times across a 100 nm polycarbonate membrane and finally purified by size exclusion chromatography (Sephadex G-25) using as eluent HEPES buffer (10 mM, pH 11.5). The resulting vesicle suspension was taken up in a total volume of 5 mL (approximate lipid concentration 6.6 mM).

Measurement protocol: 200 μL of the vesicle suspension were taken into a plastic cuvette containing 1800 μL of HEPES buffer (10 mM, pH 11.5). At t = 50 s, $CaCl_2$ was added (25 μL, 2M) and at t=100 s the compound under study (20 μL DMSO solution) was added .and the change in CF fluorescence (basification) was measured (λ_{exc} = 494 nm y λ_{em} = 520 nm). At t = 400 s, Triton-X 100 (50 μL, 10% solution in water) was added to lyse all the vesicles and normalize the fluorescence trace. Experiment ended at t = 500 s.

The data was normalized using Eq. 1.

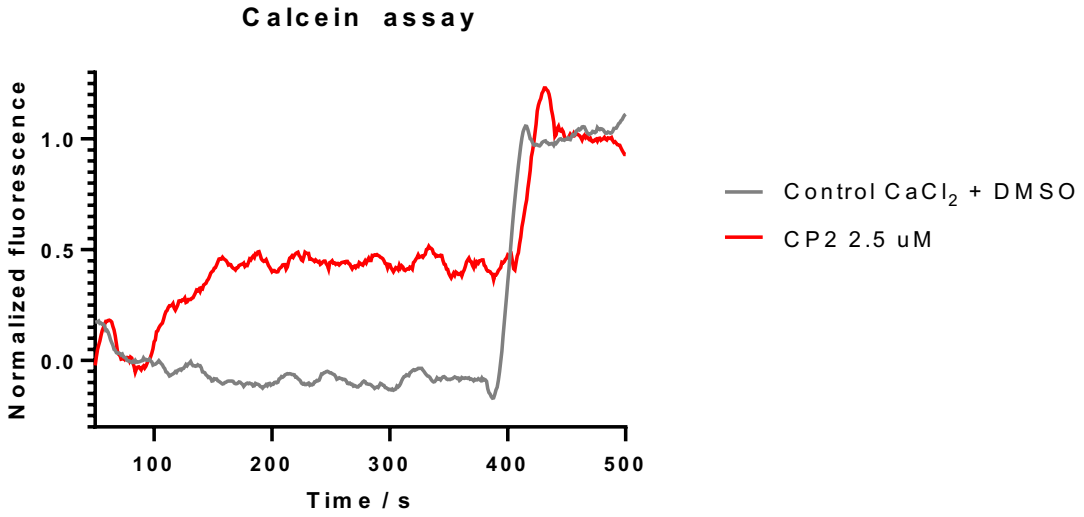


Figure 15SI. Fluorescence traces obtained in the ionophore-mediated Ca^{2+} transport assay with Calcein⊂LUVs.

7. DPPC assay at variable temperature to unveil the transport mechanism

DPPC (25 mg, 1 equiv) and **CP3** (1.7 mg, 0.05 Equiv) were dissolved in $CHCl_3$ (1 mL). The resulting mixture was slowly evaporated under vacuum. The lipid-transporter film was dried overnight under high vacuum and then it was hydrated for an hour at 50 °C with 1 mL of an aqueous solution containing: HPTS (1 mM), HEPES (10 mM), NaCl (100 mM), pH 7.0. After that time, the mixture was sonicated for 30 s (degass mode) and subjected to 6 freeze-thaw cycles (N_2 (l) → 50 °C water bath). The resulting mixture was extruded through a 400 nm polycarbonate membrane (19 times) at 50 °C and finally purified by size exclusion chromatography (Sephadex G-25; eluant HEPES (10 mM), NaCl (100 mM), pH 7.0) at 50 °C to remove all extravesicular HPTS (final volume of vesicle suspension is 3 mL).
Measurement protocol: 400 μL of the vesicle suspension were taken into a plastic cuvette containing 2600 μL of HEPES (10 mM), NaCl (100 mM), pH 7.0 and left to stir for 5 min at 45 °C inside a thermostatic chamber before start recording any data. After 150 s the experiment starts, NaOH was added (20 μL, 0.5 M) and the change in HPTS fluorescence (basification) was measured (λ_{exc} = 450 nm and λ_{em} = 510 nm). After 400 s, Triton-X was added (50 μL, 10% solution in water) to lyse all the vesicles and normalize the fluorescence trace. Experiment ended after 500 s.

The data was normalized using Eq. 1.

NMR analysis of heterodimer formation (D2-4)

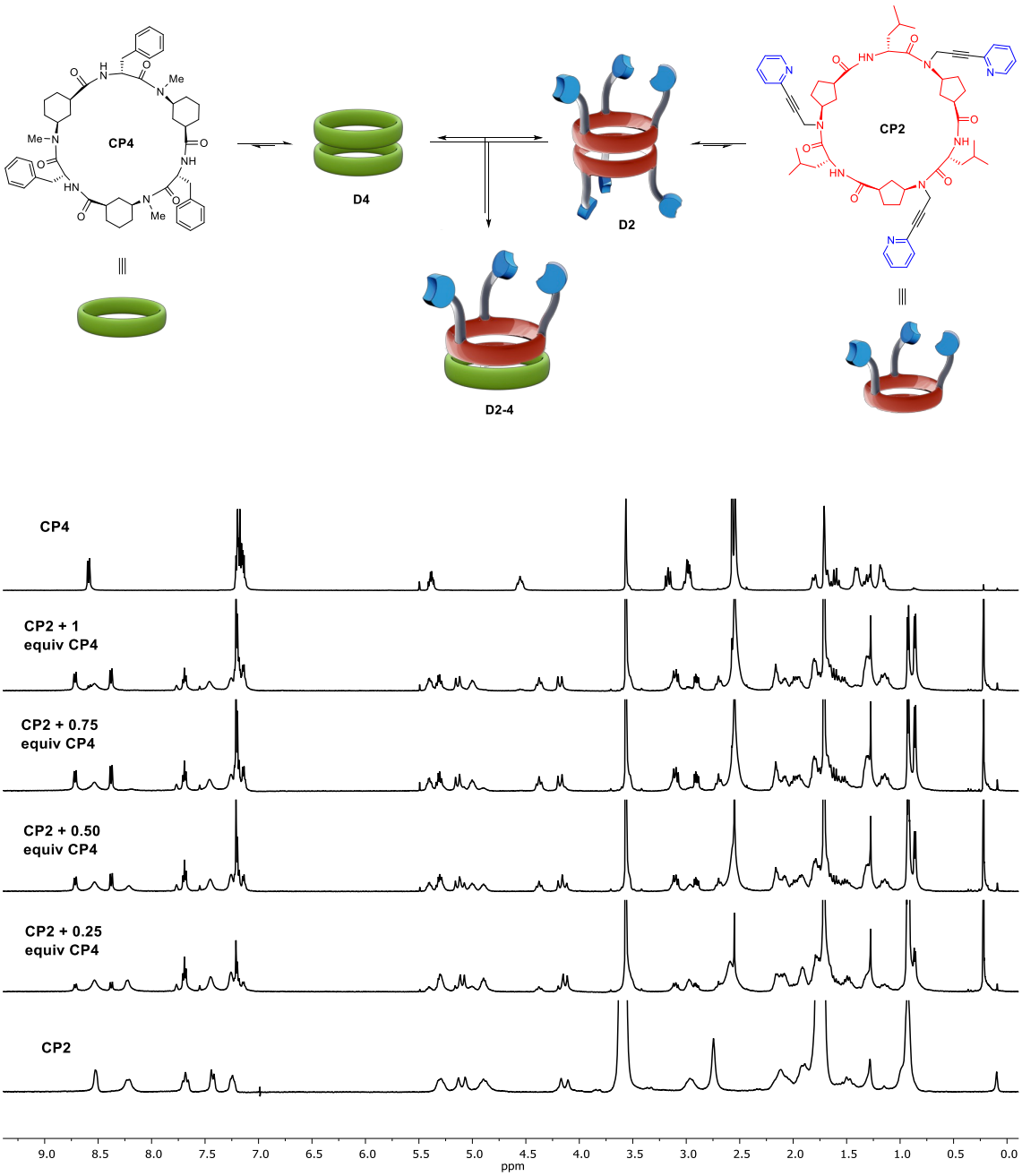
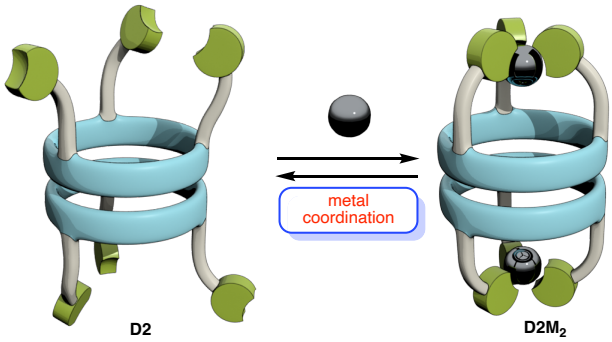


Figure 16SI. Top: Schematic representation of the selective formation of heterodimer **D2-4** from monomers **CP2** and **CP4** in non-polar media. Bottom: ^{1}H NMR traces of the successive additions of increasing amounts of **CP4** over a solution of **CP2** (5 mM in THF d_8).

NMR analysis of metal coordination of D2



a. CuI

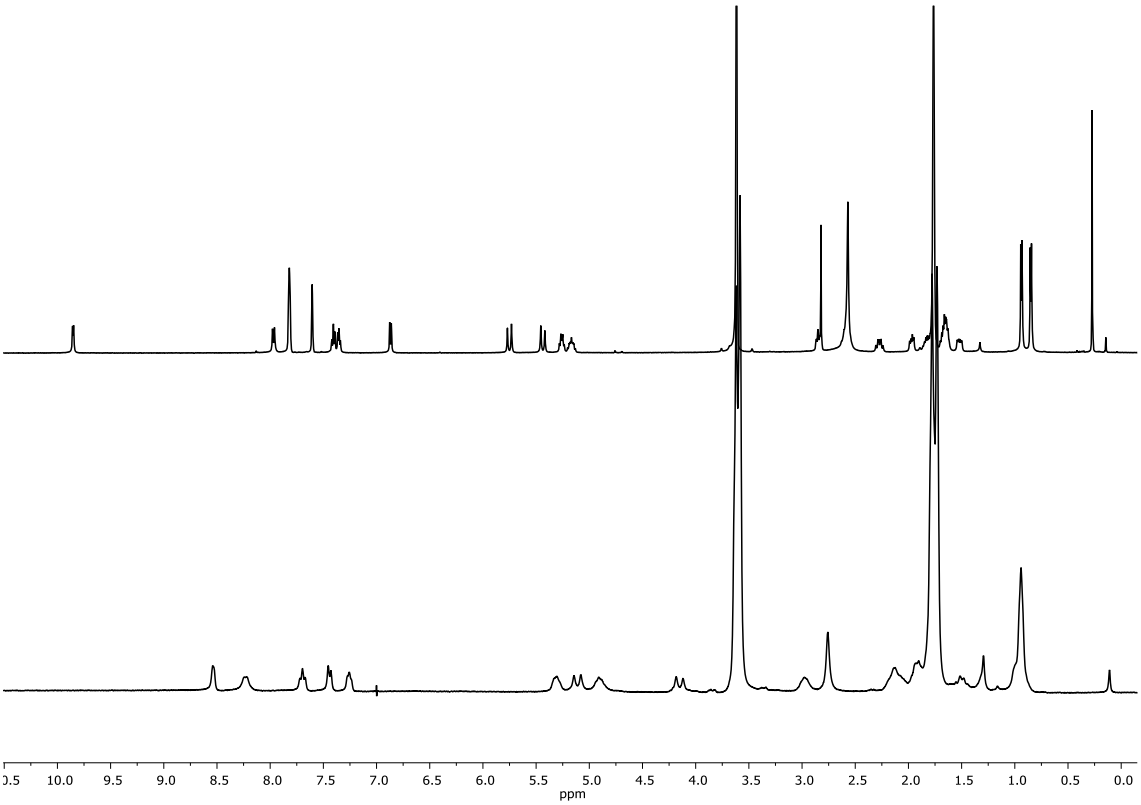


Figure 17SI. Bottom: **CP2** (5 mM in THF d_8). Top: Addition of 1 equiv. of CuI over a solution of **CP2** in THF d_8 (singlets at 7.83 and 7.60 ppm come from the addition of $Na(B(ArF)_4$ salt and singlet at 0.22 ppm is the signal of TMSS.

b. $Zn(OTf)_2$

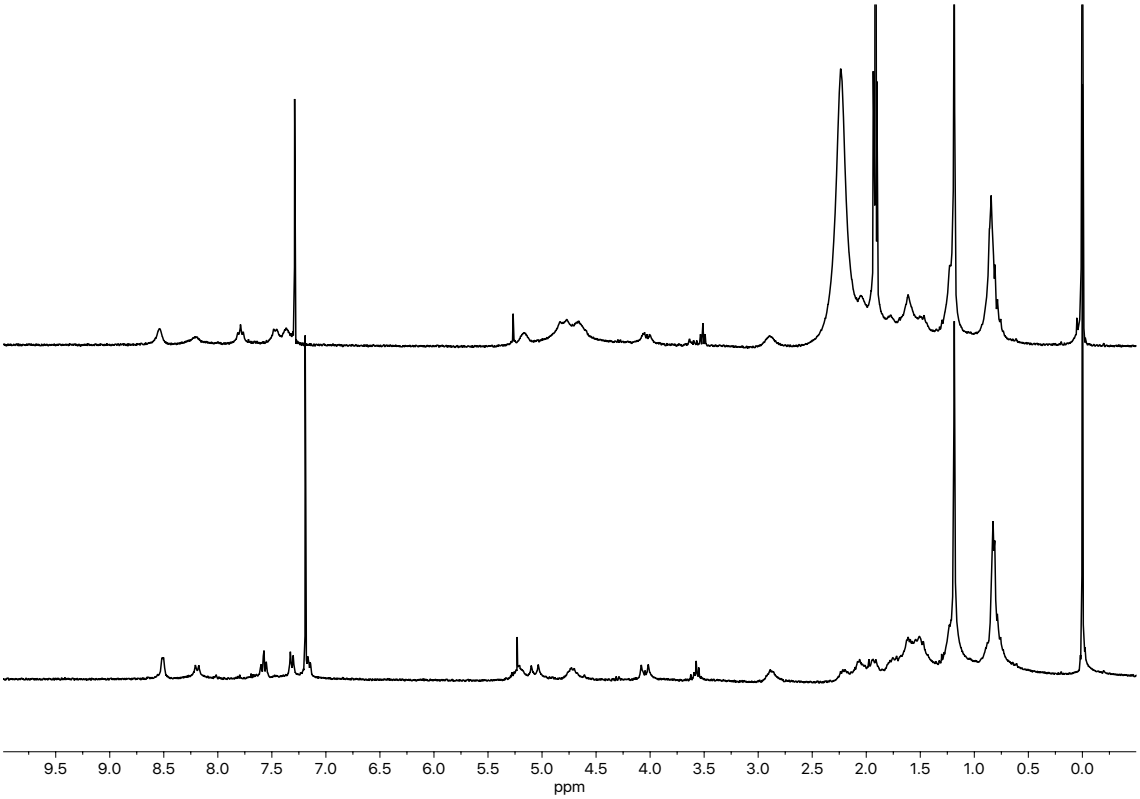


Figure 18SI. Bottom: **CP2** (5 mM in $CDCl_3$). Top: Addition of 1 equiv. of $Zn(OTf)_2$ (CD_3CN stock solution) over a solution of **CP2** in $CDCl_3$.

Materials and methods

All reagents and solvents were purchased from Iris Biotech, Fischer Scientific, Alfa Aesar, Sigma-Aldrich, Avanti Polar Lipids or GL Biochem (Shanghai) Ltd, China and were used without further purification unless otherwise stated. CH_2Cl_2 and THF were distilled from CaH_2 and Na/benzophenone, respectively, immediately prior to their use when anhydrous conditions were required. All solvents used were HPLC or synthesis grade.

Analytical thin-layer chromatography was performed on E. Merck silica gel 60 F_{254} plates. Silica-gel flash chromatography was performed using E. Merck silica gel (type 60SDS, 230-400 mesh). Mixtures for chromatography are reported as v/v ratios of the solvents noted for each compound. Size exclusion chromatography was carried out using Sephadex® G-25 as stationary phase.

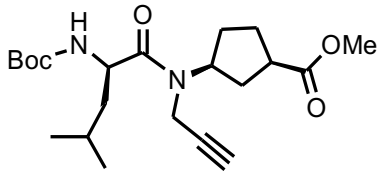
^{1}H NMR and ^{13}C NMR spectra were recorded on Varian Inova 500, Bruker AVIII-500, Varian Mercury 300 or Agilent VNMRS-300. Chemical shifts (δ) are reported in parts per million (ppm), relative to the deuterated solvent in which the spectrum was recorded. ^{1}H NMR splitting patterns are designated as singlet (s), doublet (d), triplet (t) or quartet (q). All first-order splitting patterns were assigned based on the appearance of the multiplet. Non-easily interpreted signals are designated as multiplet (m) or broad (br). Carbon resonances were assigned using either Distortionless Enhancement by Polarization Transfer (DEPT) spectra obtained with phase angles of 135° or by Heteronuclear Single Quantum Coherence experiments (HSQC). The signals of the ^{1}H NMR spectra of the peptides were identified from the corresponding double-quantum-filled 2D: COSY, TOCSY and NOESY or ROESY spectra acquired at a concentration between 2 and 100 mM and at room temperature unless otherwise specified (mixing times for NOESY and/or ROESY ranged from 150 to 300 ms, but were not optimized).

Accurate mass determination (HRMS) using ESI-MS were performed on a Sciex QSTAR Pulsar spectrometer.

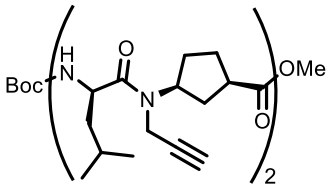
FT-IR measurements were made on a JASCO FT/IR-400 spectrophotometer placing the sample on a CaF_2 pellet or on a Perkin Elmer Spectrum Two ATR-FTIR, directly depositing the sample as a thin film over its diamond plate (neat).

Fluorescence assays were performed on a FluoroMax-2 spectrofluorometer (Jobin-Yvon Spex) equipped with a stirrer and a temperature controller (all experiments were carried out at 25 °C unless otherwise stated). All measurements were carried out as duplicates or triplicates.

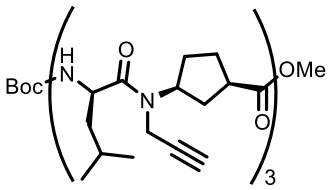
LUV extrusion procedure was carried out utilizing a Mini-Extruder set purchased from Avanti Polar Lipids, as well as filter supports and polycarbonate membranes of the different diameters (100, 200 and 400 nm).



Boc-*D*-Leu-^{prop}*N*-*L*-γ-Acp-OMe (dp1).[2] This compound, as well as all of its precursors, were prepared following a previously described protocol. **^{1}H NMR** ($CDCl_3$, 300 MHz, δ): 5.49 and 5.11 (2d, *J* = 8.92, 1H), 4.88 - 4.57 (m, 1H), 4.40 (m, 1H), 4.21 - 3.83 (m, 2H), 3.67 (d, *J* = 6.5 Hz, 3H), 3.06 - 2.71 (m, 1H), 2.43 - 1.07 (m, 19H), 1.05 - 0.81 (m, 6H).

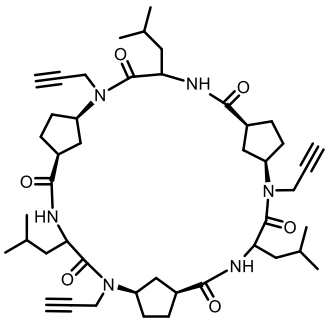


Boc-(*D*-Leu-^{prop}*N*-*L*-Acp)$_2$-OMe (tp1). A solution of **dp1** (1.11 g, 2.83 mmol) in MeOH/H_2O (V_t = 20 mL, 4:1) was treated with LiOH (340 mg, 14.1 mmol) and stirred for 1 h. After this time, MeOH was concentrated under reduced pressure and the resulting solution was acidified until pH 2 (HCl 5% aq.) and extracted with CH_2Cl_2 (4x15 mL). The combined organic layers were dried with anh. $MgSO_4$, filtered and concentrated under reduced pressure to give **Boc-*D*-Leu-^{prop}*N*-*L*-Acp-OH** as a white foam. Parallelly, a different fraction of **dp1** (550 mg, 1.40 mmol) was dissolved in CH_2Cl_2 (10 mL) and treated with TFA (4 mL). After stirring for 15 min, the solvent was removed under reduced pressure and the resulting oil was redissolved in CH_2Cl_2 and further evaporated, repeating this process up to three times. The free-acid fraction (533 mg, 1.40 mmol) was dissolved in dry CH_2Cl_2 (5 mL) and subsequently DIEA (0.49 mL, 2.80 mmol) and *N*-HBTU (634 mg, 1.75 mmol) were added. The TFA salt was dissolved in dry CH_2Cl_2 (5 mL) and DIEA was added (0.98 mL, 5.60 mmol). Finally, both fractions were mixed and reacted under Ar for 2 h. This solution was washed with HCl 5% (2x15 mL) and sat. aq. $NaHCO_3$ (2x15 mL), dried with anh. $MgSO_4$, filtered and concentrated under reduced pressure. The crude mixture was purified by flash column chromatography (25-50% AcOEt/hexane). To give the title compound as a foam. [897 mg, 97%, R_f = 0.30 (50% AcOEt/hexane)]. **HRMS (ESI)** Calculated for $C_{36}H_{57}N_4O_7$: 657.4222; found: 657.4222.

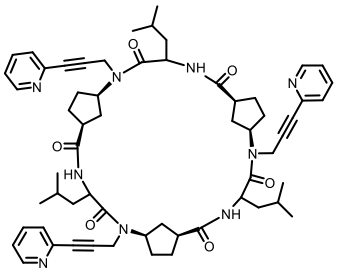


Boc-(*D*-Leu-^{prop}*N*-*L*-Acp)$_3$-OMe (hp1). Tp1 (692 mg, 1.05 mmol) was dissolved in CH_2Cl_2 (5 mL) and treated with TFA (5 mL). After stirring for 15 min, the solvent was removed under reduced pressure and the resulting oil was redissolved in CH_2Cl_2 and further evaporated, repeating this process thrice. Free-acid **Boc-*D*-Leu-^{prop}*N*-*L*-Acp-OH** obtained in the previous step was dissolved in dry CH_2Cl_2 (5 mL) and then DIEA (0.37 mL, 2.10 mmol) and *N*-HBTU (478 mg, 1.26 mmol) were added. The TFA salt was dissolved in dry CH_2Cl_2 (10 mL) and DIEA was added (0.73 mL, 4.20 mmol). Finally, both fractions were mixed and reacted under Ar for 2 h. This solution was washed with HCl 5% (2x15 mL) and sat. aq. $NaHCO_3$ (2x15 mL), dried with anh. $MgSO_4$, filtered and concentrated *in vacuo*. The crude mixture was purified by flash column chromatography (0-4 % MeOH/CH_2Cl_2), to give the linear hexapeptide as a foam. [874 mg, 91%, R_f = 0.42 (5% MeOH/CH_2Cl_2)]. **HRMS (ESI)** Calculated for $C_{51}H_{79}N_6O_9$: 919.5903; found: 919.5904.

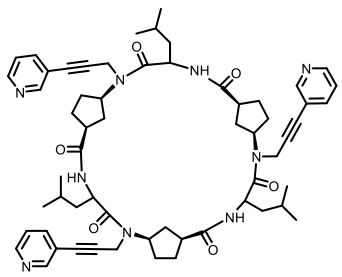
[2] A. Fuertes, H. L. Ozores, M. Amorín, J. R. Granja, *Nanoscale,* 2017, **9**, 748-753.



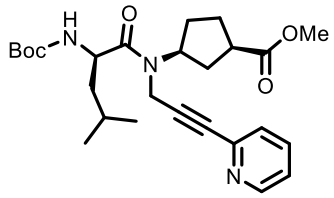
***c*-[(*D*-Leu-^{prop}*N*-*L*-Acp)$_3$] (CP1)**. To a solution of **hp1** (804 mg, 0.876 mmol) in MeOH/H_2O (V_t = 8.7 mL, 3:1), LiOH (63.0 mg, 2.63 mmol) was added and the resulting mixture was stirred for 2 h. Then, the organic solvent was concentrated under reduced pressure and the remaining aqueous solution was acidified until pH 2 (HCl 5% aq.), extracted with CH_2Cl_2 (4x10 mL), dried with anh. $MgSO_4$ and filtered. After solvent evaporation, the free acid was obtained as white foam. This foam was further dissolved in CH_2Cl_2 (4 mL) and treated with TFA (4 mL). After stirring for 15 min, the solvent was removed under reduced pressure and the resulting oil was redissolved in CH_2Cl_2 (*i.e.*, 5 mL) and successively evaporated (3x). This residue was dissolved in dry CH_2Cl_2 (876 mL; 2 mM) and then DIEA (0.92 mL, 5.26 mmol) and *N*-TBTU (338 mg, 1.05 mmol) were added. After 16 h of stirring under Ar, this solution was concentrated to i.e., one tenth of its initial volume and washed with HCl 5% (2x10 mL) and sat. aq. $NaHCO_3$ (2x10 mL), dried with anh. $MgSO_4$, filtered and concentrated by rotary-evaporation. The crude mixture was purified by flash column chromatography (0-4 % MeOH/CH_2Cl_2). To give the desired cyclic peptide as white solid. [341 mg, 49%, R_f = 0.35 (5% MeOH/CH_2Cl_2)].**^{1}H NMR** ($CDCl_3$, 300 MHz, δ): 8.11 (d, *J* = 9.5 Hz, 3 H), 5.10 (m, 3H), 4.80 (AB, *J* = 20.1 Hz, 3H), 4.66 (m, 3H), 3.82 (AB, *J* = 20.1 Hz, 3H), 2.86 (m, 3H), 2.27 (s, 3H), 2.21 - 1.28 (m, 27H), 0.94 - 0.84 ppm (overlapped 2d, 18H). **^{13}C NMR** ($CDCl_3$, 75 MHz, δ): 175.7 (C), 173.6 (C), 80.2 (C), 72.4 (CH), 54.9 (CH), 47.2 (CH), 41.4 (CH), 41.4 (CH_2), 35.9 (CH_2), 32.7 (CH_2), 27.7 (CH_2), 27.5 (CH_2), 24.6 (CH), 23.3 (CH_3), 22.0 ppm (CH3). **FTIR** (CaF_2 pellet): *ν* = 3301 (amide A), 1665, 1620 (amide I), 1533 cm-1 (amide II). **HRMS (ESI)** Calculated for $C_{45}H_{67}N_6O_6$: 787.5117; found: 787.5116.



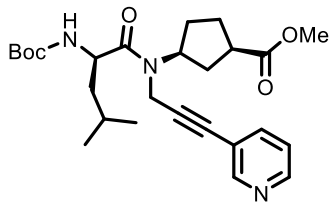
***c*-[(*D*-Leu-$^{py\text{-}o}$*N*-*L*-Acp)$_3$] (CP2). CP1** (30.0 mg, 0.0382 mmol), 2-iodopyridine (37 µL, 0.344 mmol) and CuI (2.2 mg, 0.0115 mmol) were dissolved in a THF/Et_3N mixture (V_t = 3.8 mL, 4:1) and degassed for 15 min with a steady Ar bubbling. After this time, $Pd(PPh_3)_2Cl_2$ (4.0 mg, 0.00573 mmol) was added and the mixture was stirred under Ar for 12 h. The solvent was evaporated, and the resulting mixture was dissolved in CH_2Cl_2 (5 mL) and washed with sat. aq. NH_4Cl (2 x 5 ml). The organic layer was dried over anh. $MgSO_4$, filtered and concentrated under reduced pressure. Finally, the crude mixture was purified by flash column chromatography (0-10% MeOH/CH_2Cl_2), which afforded the title compound as white solid. [17.0 mg, 44%, R_f = 0.27 (10% MeOH/CH_2Cl_2)]. **^{1}H NMR** ($CDCl_3$, 300 MHz, δ): 8.58 (d, *J* = 4.4 Hz, 3H), 8.26 (d, *J* = 9.4 Hz, 3H), 7.64 (t, *J* = 6.4 Hz, 3H), 7.38 (d, *J* = 7.7 Hz, 3H), 7.23 (t, *J* = 5.0 Hz, 3H), 5.28 (m, 3H), 5.13 (AB, *J* = 19.0 Hz, 3H), 4.79 (m, 3H), 4.12 (AB, *J* = 19.0 Hz, 3H), 2.95 (m, 3H), 2.36-1.32 (m, 27H), 1.02 - 0.78 ppm (m, 18H). **^{13}C NMR** ($CDCl_3$, 75.4 MHz, δ): 175.7 (CO), 173.9 (CO), 150.1 (CH), 142.7 (C), 136.1 (CH), 127.0 (CH), 123.0 (CH), 85.8 (C), 83.6 (C), 55.0 (CH), 47.6 (CH), 42.0 (CH), 41.6 (CH_2), 36.3 (CH_2), 33.6 (CH_2), 28.0 (CH_2), 27.8 (CH_2), 24.8 (CH), 23.4 (CH_3), 22.1 (CH_3) ppm. **FTIR** (CaF_2 pellet): *ν* = 3307 (amide A), 1665, 1627 (amide I), 1530 cm^{-1} (amide II). **HRMS (ESI)** calculated for $C_{60}H_{76}N_9O_6$: 1018.5913, found: 1018.5920.



***c*-[(*D*-Leu-$^{py\text{-}m}$*N*-*L*-Acp)$_3$] (CP3). CP1** (30.0 mg, 0.0382 mmol) was dissolved in a dry and degassed mixture of THF/Et_3N (V_t = 3.8 mL, 3:1), with 3-iodopyridine (70.5 mg, 0.344 mmol) and CuI (2.2 mg, 0.0115 mmol). Then, $Pd(PPh_3)_2Cl_2$ (4.0 mg, 0.00573 mmol) was added and the resulting solution was stirred under Ar for 12 h. The solvent was evaporated, and the oily mixture was dissolved in CH_2Cl_2 (5 mL) and washed with sat. aq. NH_4Cl (2 x 5 ml). The organic layer was dried over anh. $MgSO_4$, filtered and concentrated. Finally, the crude mixture was purified by flash column chromatography (0-10% MeOH/CH_2Cl_2), which afforded the title compound as a pale yellow solid. [20.0 mg, 51%, R_f = 0.25 (10% MeOH/CH_2Cl_2)]. **^{1}H NMR** ($CDCl_3$, 300 MHz, δ): 8.62 (br s, 3H), 8.54 (br d, 3H), 8.28 (d, *J* = 9.3 Hz, 3H), 7.67 (d, *J* = 7.7 Hz, 3H), 7.24 (t, *J* = 5.0 Hz, 3H), 5.26 (m, 3H), 5.18 (d, *J* = 18.7 Hz, 3H), 4.78 (m, 3H), 4.15 (d, *J* = 18.7 Hz, 3H), 2.99 (m, 3H), 2.37 - 1.43 (m, 27H), 0.97 - 0.86 ppm (2d, 18H). **^{13}C NMR** ($CDCl_3$, 75.4 MHz, δ): 176.0 (CO), 173.9 (CO), 152.2 (CH), 149.0 (CH), 138.5 (CH), 123.2 (CH), 119.7 (C), 89.2 (C), 80.9 (C), 55.2 (CH), 47.6 (CH), 41.8 (CH_2), 41.7 (CH), 36.1 (CH_2), 33.7 (CH_2), 28.1 (CH_2), 27.9 (CH_2), 24.9 (CH), 23.4 (CH_3), 22.2 ppm (CH_3). **FTIR** (CaF_2 pellet): ν = 3307 (amide A), 1665, 1627 (amide I), 1530 cm^{-1} (amide II). **HRMS (ESI)** calculated for $C_{60}H_{76}N_9O_6$: 1018.5913, found: 1018.5920.

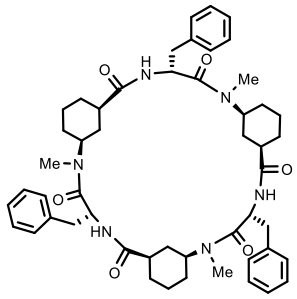


Boc-(*D*-Leu-$^{py\text{-}o}$*N*-*L*-Acp)-OMe (dp2). dp1 (40.0 mg, 0.1 mmol), 2-iodopyridine (32 µL, 0.3 mmol) and CuI (3.0 mg, 0.015 mmol) were dissolved in a THF/Et_3N mixture (V_t = 5 mL, 4:1) and degassed for 15 min with Ar flow. After this time, $Pd(PPh_3)_2Cl_2$ (4.0 mg, $5.73\cdot10^{-3}$ mmol) was added and the mixture was stirred under Ar for 12 h at 50 ºC. The solvent was evaporated, and the resulting mixture was dissolved in CH_2Cl_2 (5 mL) and washed with sat. aq. NH_4Cl (2 x 5 ml). The organic layer was dried over anh. $MgSO_4$, filtered and concentrated under reduced pressure. Finally, the crude mixture was purified by flash column chromatography (25-100% AcOEt/hexane), which afforded the title compound as transparent oil. [41.4 mg, 87%, R_f = 0.27 (50% AcOEt/hexane)]. **^{1}H NMR** ($CDCl_3$, 300 MHz, δ): 8.62 - 8.51 (m, 1H), 7.74 - 7.59 (m, 1H), 7.52 - 7.36 (m, 1H), 7.32 - 7.17 (m, 1H), 5.39 - 5.27 (m, 0.5H), 5.15 (d, J = 9.2 Hz, 0.5H), 5.00 - 4.63 (m, 2H), 4.48 - 4.14 (m, 2H), 3.78 - 3.63 (m, 3H), 2.94 - 2.77 (m, 1H), 2.39 - 1.53 (m, 9H), 1.53 - 1.37 (m, 9H), 1.10 - 0.84 (m, 6H). **^{13}C NMR** ($CDCl_3$, 75.4 MHz, δ): 176.3 and 175.8 (CO), 174.0 and 172.59 (CO), 149.92 and 149.58 (CH), 136.19 (CH), 132.2 and 132.0 (C), 127.4 and 127.3 (CH), 123.1 and 122.9 (CH), 86.6 (C), 83.6 (C), 79.6 (C), 57.8 and 55.2 (CH), 52.0 and 51.91 (CH_3), 49.6 and 48.9 (CH), 43.4 and 42.5 (CH_2), 41.2 (CH_3), 33.61 and 32.87 (CH_2), 31.96 and 31.58 (CH_2), 29.2 (CH_2), 28.3 (CH_3), 27.68 and 27.34 (CH_2), 3.51 and 23.42 (CH), 22.02 and 21.61 (CH). **HRMS (ESI)** calculated for $C_{26}H_{37}N_3O_5Na$: 494.2625, found: 494.2621.



Boc-(*D*-Leu-$^{py\text{-}m}$*N*-*L*-Acp)-OMe (dp3). dp1 (40.0 mg, 0.1 mmol), 3-iodopyridine (61 mg, 0.3 mmol) and CuI (3.0 mg, 0.015 mmol) were dissolved in a THF/Et_3N mixture (V_t = 5 mL, 4:1) and degassed for 15 min with an Ar flow. After this time, $Pd(PPh_3)_2Cl_2$ (4.0 mg, $5.73\cdot10^{-3}$ mmol) was added and the mixture was stirred under Ar for 12 h at 50 ºC. The solvent was evaporated, and the resulting mixture was dissolved in CH_2Cl_2 (5 mL) and washed with sat. aq. NH_4Cl (2 x 5 ml). The organic layer was dried over anh. $MgSO_4$, filtered and concentrated under reduced pressure. Finally, the crude mixture was purified by flash column chromatography (25-100% AcOEt/hexane), which afforded the title compound as an oil. [43.2 mg, 90%, R_f =0.51 (10% MeOH/CH_2Cl_2)]. **^{1}H NMR** ($CDCl_3$, 300 MHz, δ): 8.70 - 8.57 (m, 1H), 8.55 - 8.41 (m, 1H),

7.77 - 7.64 (m, 1H), 7.23 (m, 1H), 5.29 (d, *J* = 9.1 Hz, 0.5H), 5.15 (d, *J* = 9.2 Hz, 0.5H), 4.95 - 4.60 (m, 2H), 4.44 - 4.12 (m, 2H), 3.73 - 3.64 (m, 3H), 2.92 - 2.76 (m, 1H), 2.30 - 1.44 (m, 9H), 1.43 - 1.38 (m, 9H), 0.93 (m, 6H). **^{13}C NMR** ($CDCl_3$, 75.4 MHz, δ): 176.5 and 175.9 (CO), 174.0 and 172.6 (CO), 155.6 (C), 152.1 (CH), 148.8 and 148.3 (CH), 138.9 (CH), 123.0 (CH), 89.7 and 89.0 (C), 81.0 (C), 79.6 (C), 57.8 and 55.0 (CH), 52.1 and 51.9 (CH), 49.5 and 48.9 (CH), 43.2 and 42.7 (CH_2), 41.2 (CH_3), 33.6 and 32.8 (CH_2), 31.9 and 31.6 (CH_2), 29.1 and 28.3 (CH_2), 28.1 (CH_3), 27.7 and 27.4 (CH_2), 24.7 (CH), 23.5 and 23.4 (CH), 22.1 and 21.7 (CH). **HRMS (ESI)** calculated for $C_{26}H_{37}N_3O_5Na$: 494.2625, found: 494.2622.



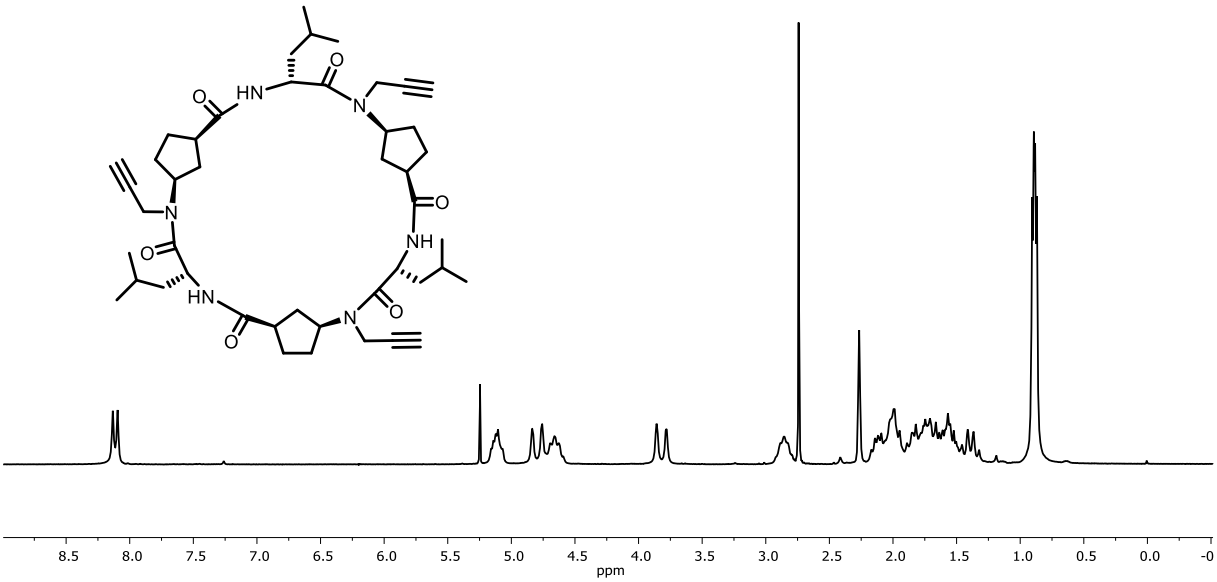
***c*-[(*D*-Phe-^{Me}*N*-*L*-Ach)$_3$] (CP8)**. This compound was prepared following a previously described protocol in our group.[3] **^{1}H NMR** (THF d_8, 500 MHz) δ 8.59 (d, *J* = 9.4 Hz, 3H), 7.24 - 7.07 (m, 15H), 5.39 (m, 3H), 4.55 (m, 3H), 3.17 (m, 3H), 3.05 - 2.91 (m, 6H), 2.57 (s, 9H), 1.84 - 1.09 (m, 24H).

[3] R. J. Brea, M. Amorín, L. Castedo, J. R. Granja, *Angew. Chem. Int. Ed.*, 2005, **44**, 5710-5713.

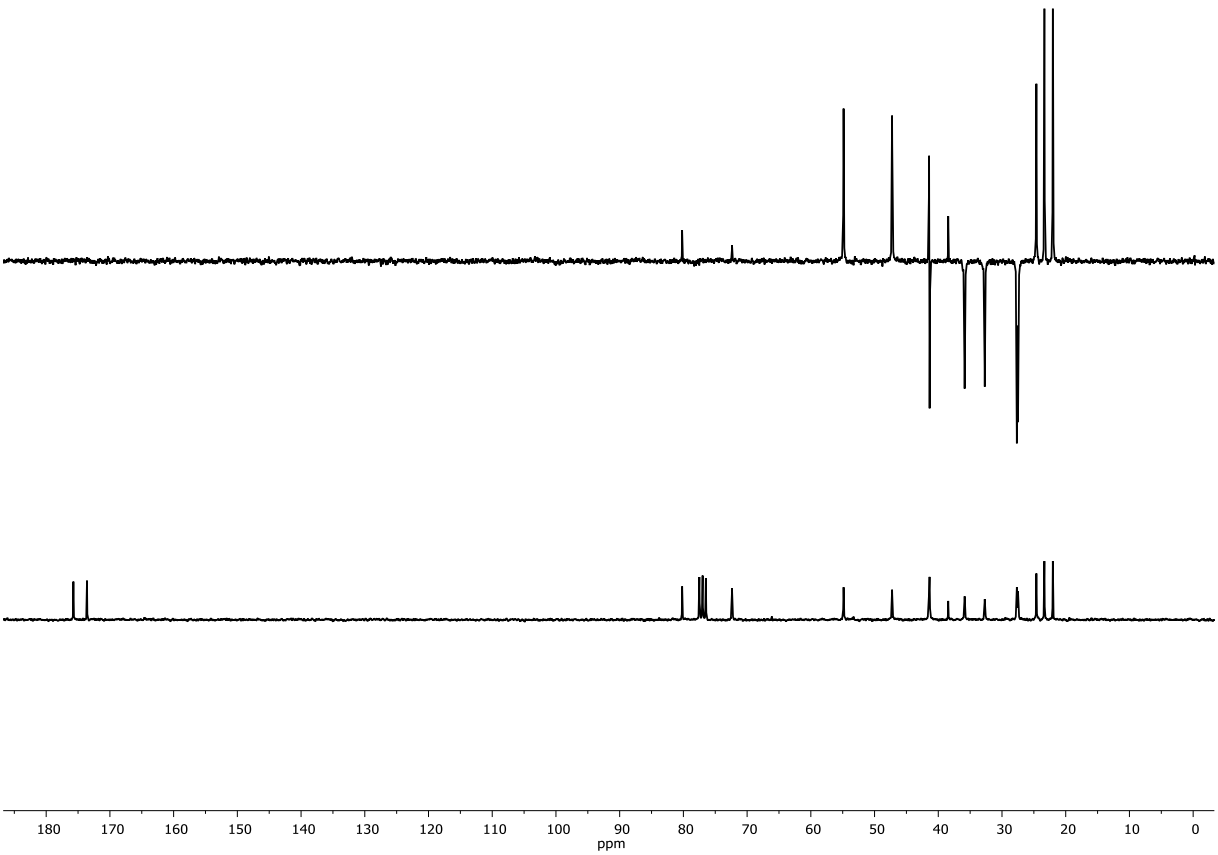
NMR and FT-IR spectra

c-[(D-Leu-^{prop}*N*-*L*-Acp)$_3$] (CP1)

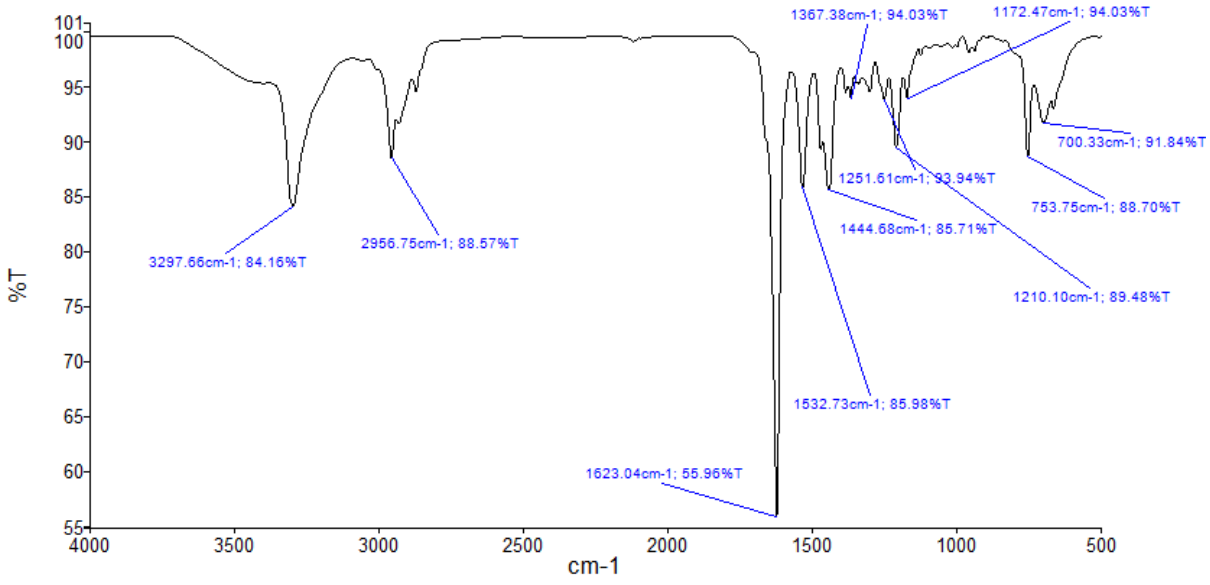
^{1}H NMR(50 mM in $CDCl_3$, 298 K, 300 MHz)



^{13}C & DEPT (50 mM in $CDCl_3$, 298 K, 300 MHz)

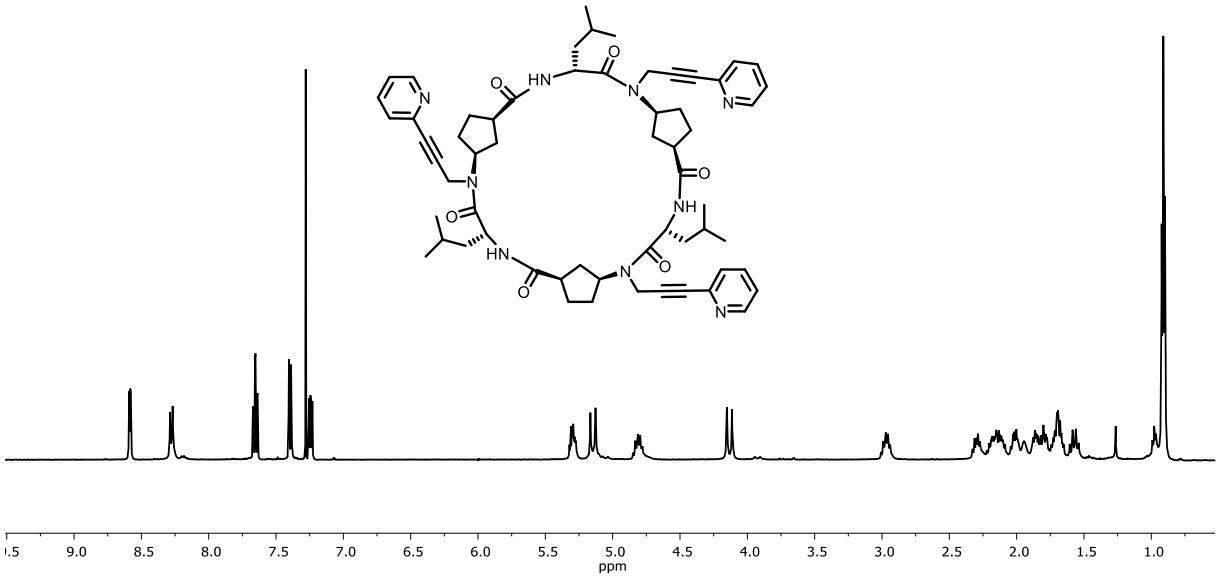


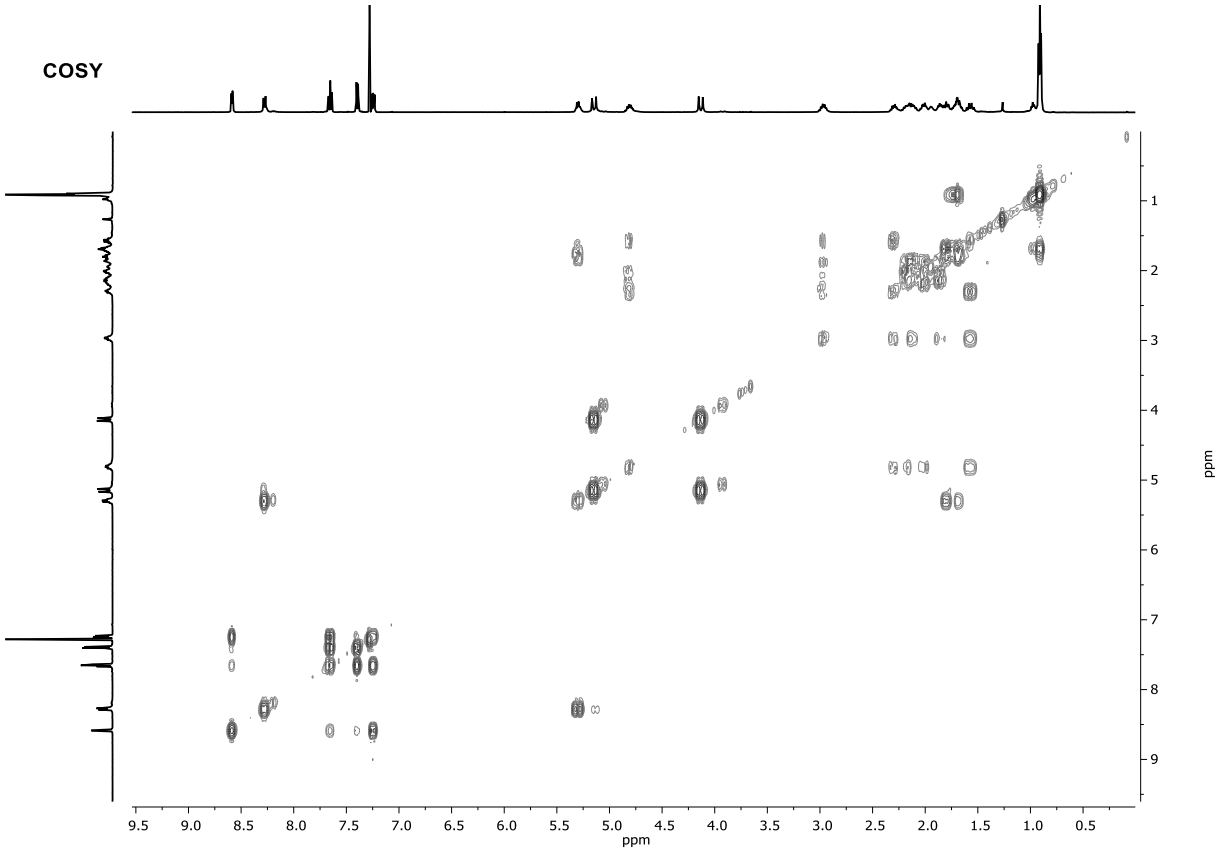
FT-IR (neat, 298 K)

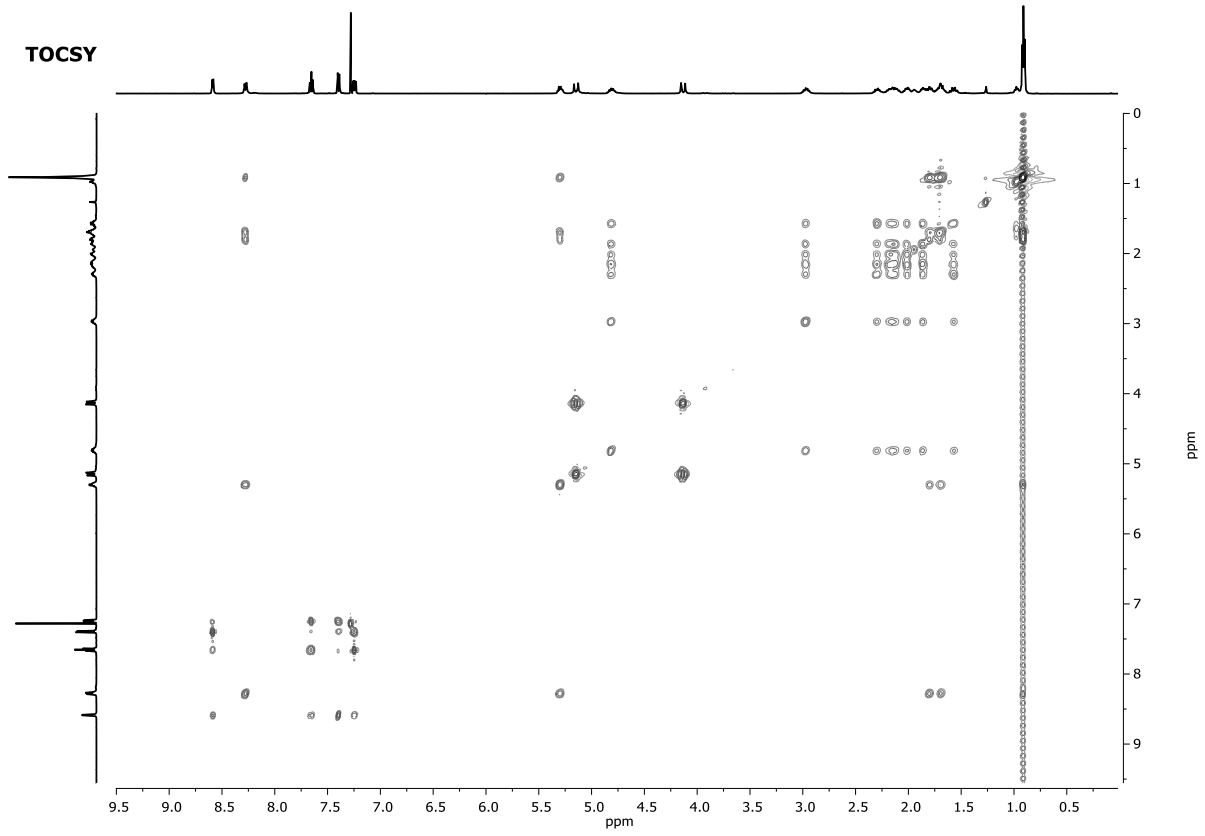


***c*-[(D-Leu-*$^{py\text{-}o}$N*-*L*-Acp)$_3$] (CP2)**

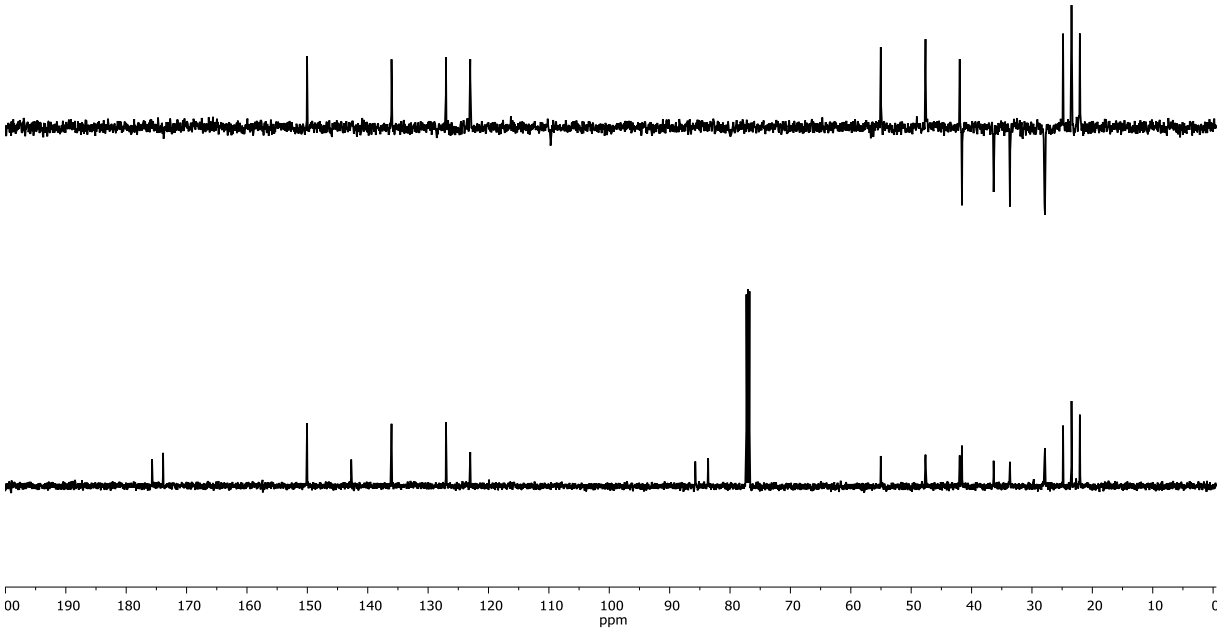
^{1}H NMR, COSY, TOCSY (8 mM in $CDCl_3$, 298 K, 500 MHz)



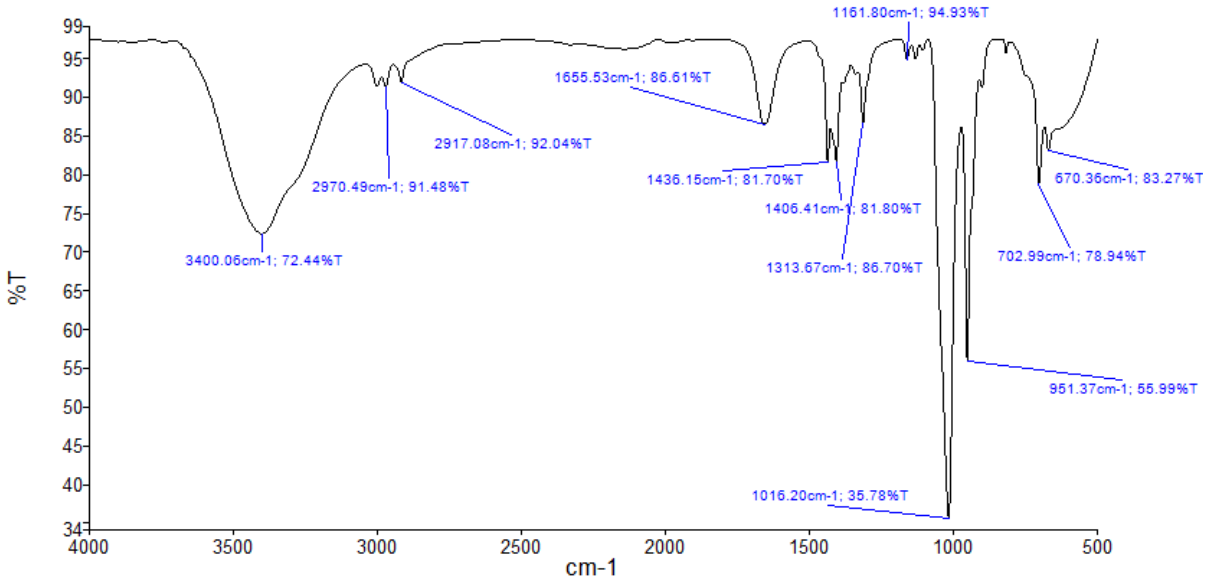




^{13}C & DEPT (8 mM in $CDCl_3$, 298 K, 125 MHz)

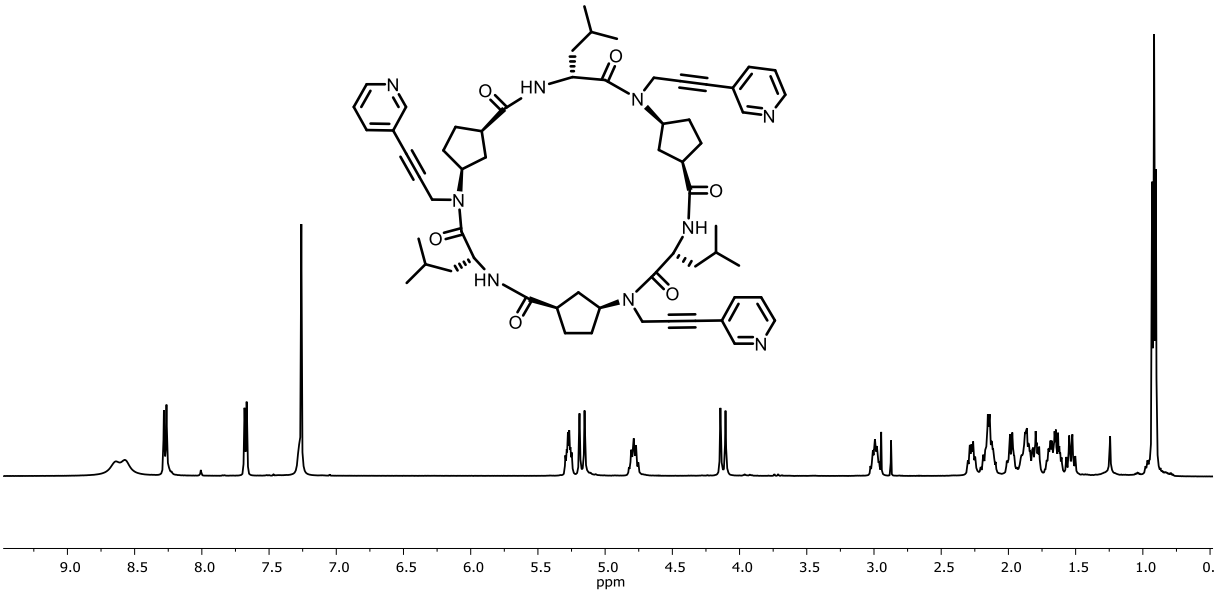


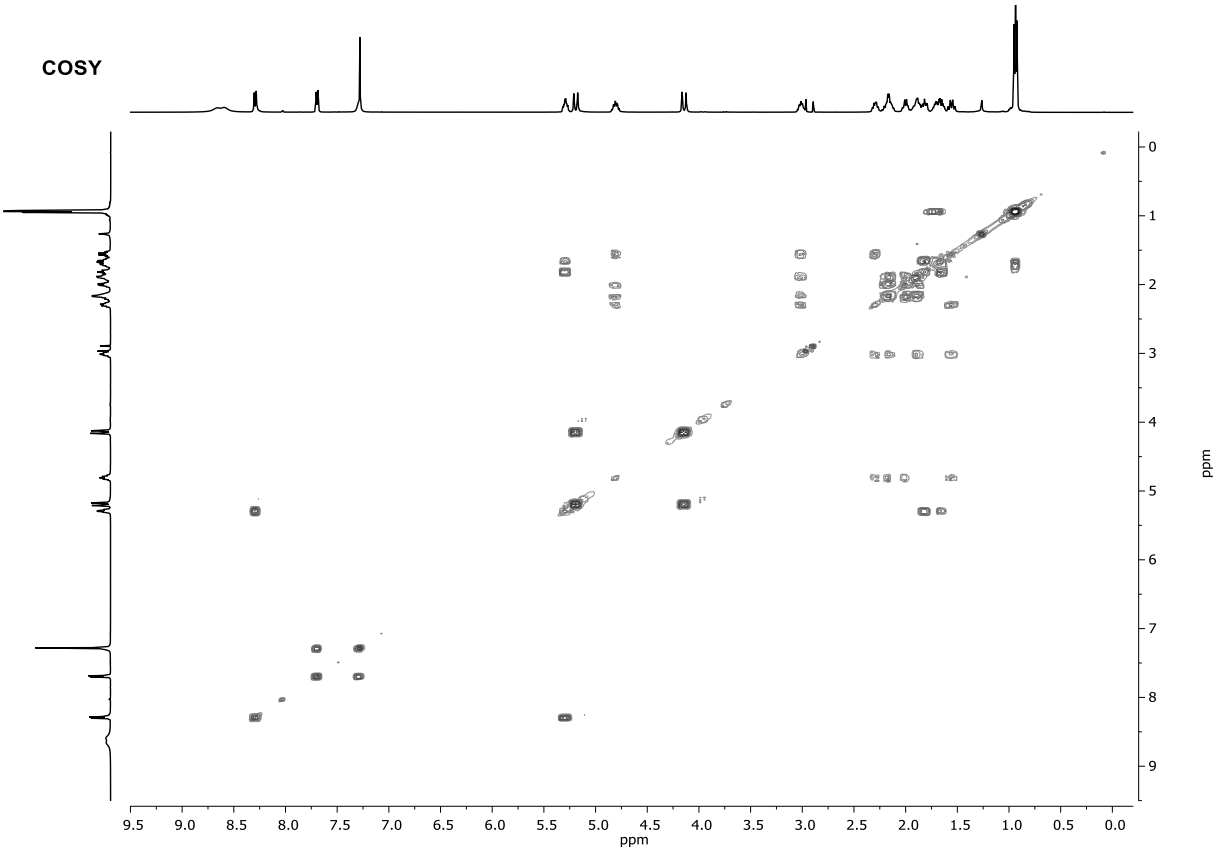
FT-IR (neat, 298 K)

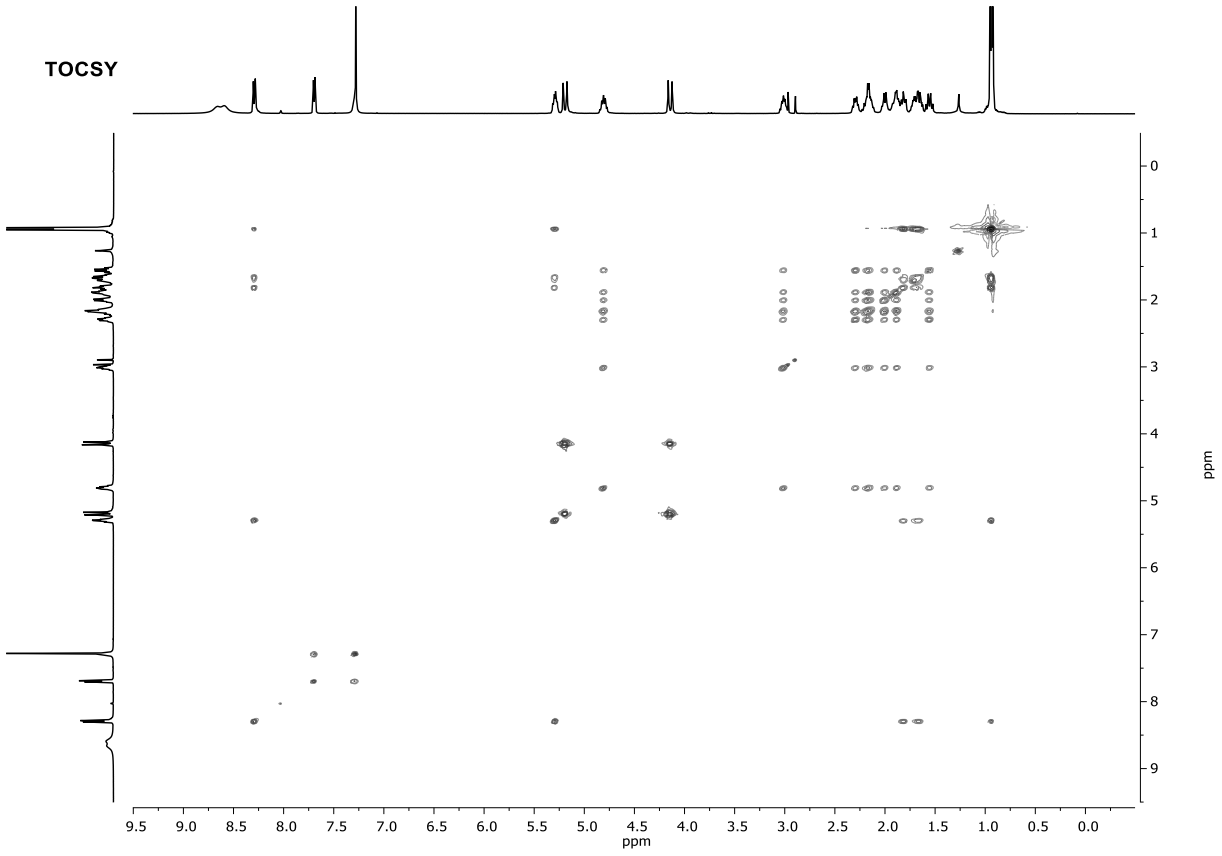


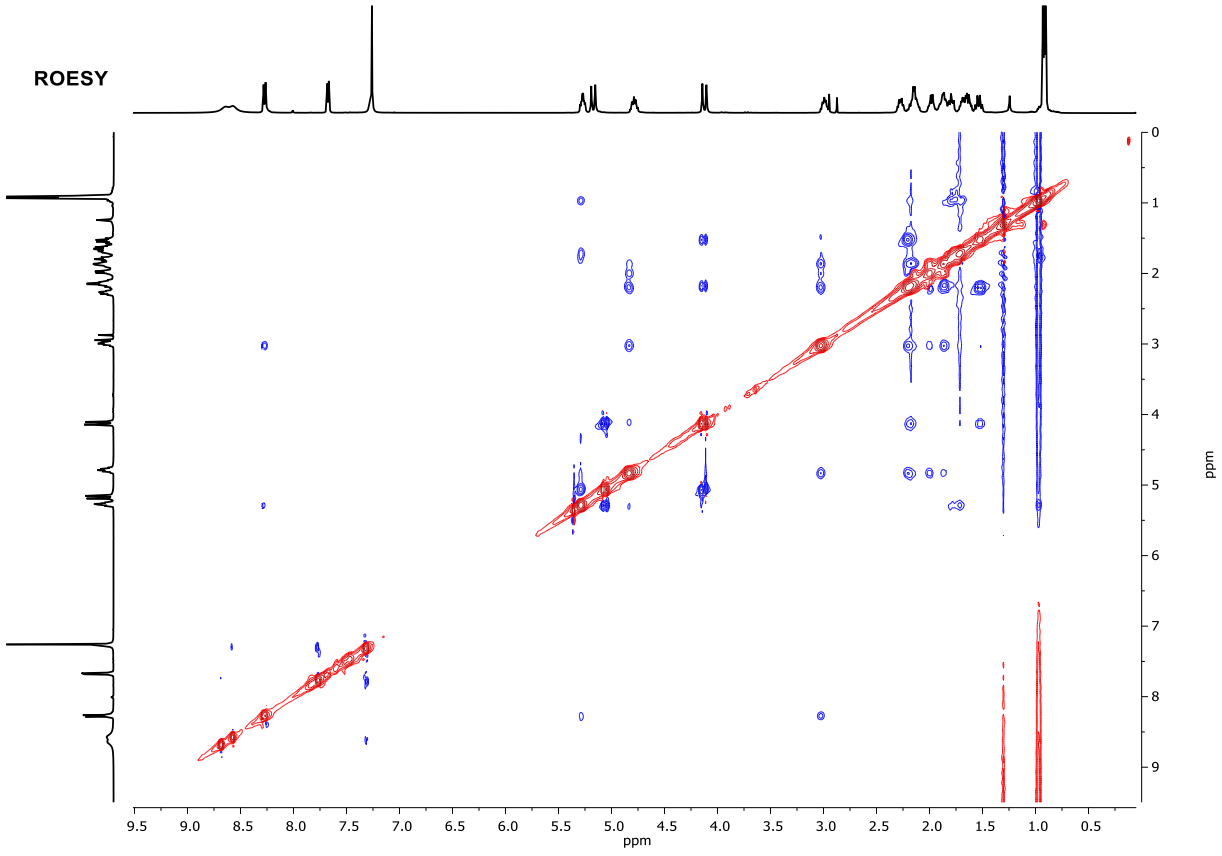
***c*-[(D-Leu-*$^{py\text{-}m}$N*-*L*-Acp)$_3$] (CP3)**

^{1}H NMR, COSY, TOCSY and ROESY (10 mM in $CDCl_3$, 298 K, 500 MHz)

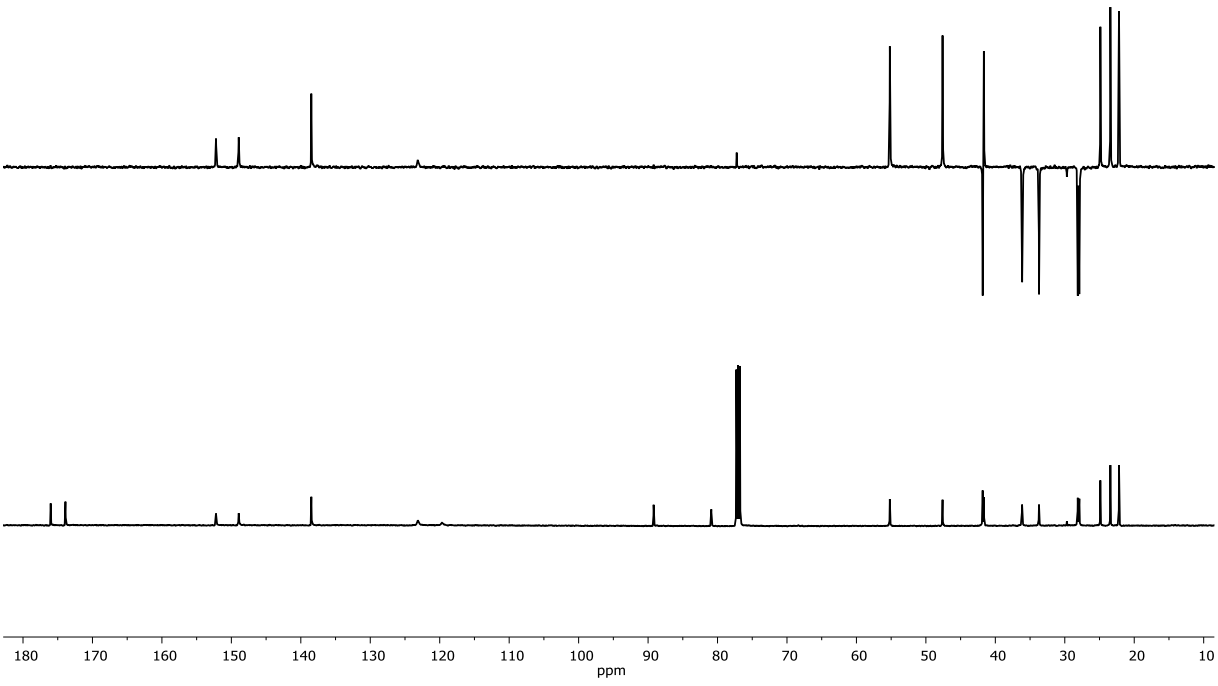





TOCSY
ppm
ppm


ROESY
ppm
ppm

^{13}C y DEPT (10 mM en $CDCl_3$, 298 K, 125 MHz)



FT-IR (neat, 298 K)