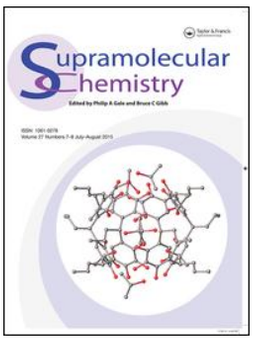

Supramolecular Chemistry

ISSN: 1061-0278 (Print) 1029-0478 (Online) Journal homepage: https://www.tandfonline.com/loi/gsch20

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To cite this article: Alberto Fuertes, Manuel Amorín & Juan R. Granja (2020): Membrane spanners based on a dimeric α,γ-cyclic peptide core, Supramolecular Chemistry, DOI: 10.1080/10610278.2020.1731510

To link to this article: https://doi.org/10.1080/10610278.2020.1731510

Published online: 27 Feb 2020.

SUPRAMOLECULAR CHEMISTRY
https://doi.org/10.1080/10610278.2020.1731510

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Membrane spanners based on a dimeric α,γ-cyclic peptide core

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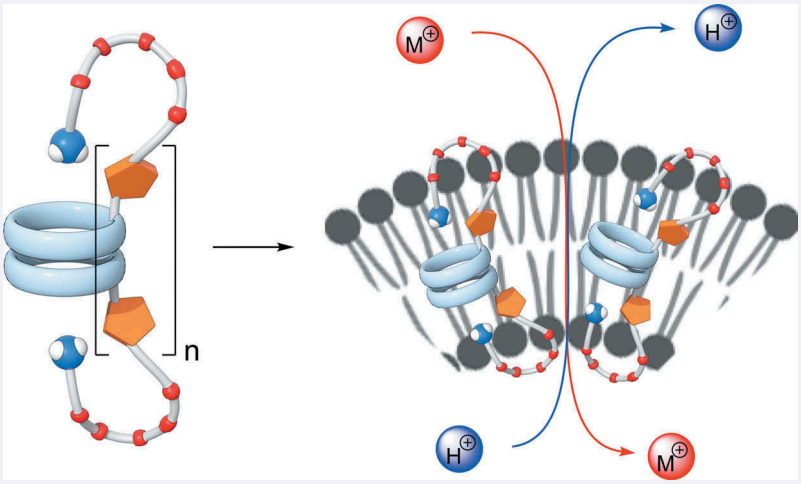
ABSTRACT

In this article, we describe the design, synthesis and transmembrane ion transport properties of a small library of molecular membrane-spanning ionophores based on dimer-forming cyclic peptide scaffolds decorated with flexible pendants. Thanks to the modular synthetic approach, it is possible to obtain a variety of α,γ-cyclic peptides with different internal diameters as well as a number of pendant groups that are perpendicularly projected from the peptide backbone. These groups are incorporated by a copper-catalysed cycloaddition between azide-modified oligo-ethylene glycol-based tentacles of different lengths and properties and cyclic peptides bearing a different number of propargyl groups attached at the amide skeleton. This methodology allowed the preparation of different 'spanners' and the identification of new artificial transporters that can rectify transmembranal pH gradients with EC50 values in the medium μM range.



ARTICLE HISTORY
Received 10 November 2019
Accepted 10 February 2020

KEYWORDS
Membrane spanners; α,γ-Cyclic Peptide; ion transport

Introduction

The transport of ions across biological membranes is a key step in many relevant biological processes, such as homoeostasis, cell communication or energy production [1]. For these tasks Nature has developed different tools such as membrane proteins or ionophores [2]. The latter can sometimes bind a particular ion with a remarkable selectivity over other structurally similar counterparts [3]. The preparation of artificial ionophores or channels that emulate the transport properties of the natural occurring molecules has attracted much attention from the scientific community, designing both supramolecular and covalent receptors to mimic natural functions [4–6].

Apart from the carriers and synthetic channels whose design comes from the mere imitation of the natural transporters, there are other molecules that have not attracted as much attention, such as the membrane spanners [4,7]. This type of molecules is characterised by the combination of a rigid component to which one or several flexible pendants crowned by a polar group are attached and, generally, their length in the fully extended conformation is enough to reach both membrane sides. Namely, they can span both lipidic leaflets. Even though the mechanism by which ions can flow across lipid bilayers in the presence of these transporters is not entirely known, it is believed that they can either induce a transient permeable state in the membranes by preventing the

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independent mobility of the two phospholipid layers or shorten a local region of a membrane, increasing its permeability (Figure 1). Cyclodextrins [8] and other macrocycles [9] have been used for this purpose as the rigid central component through the incorporation of different chains at both sides of the macrocycle. Normally, this approach requires very demanding synthetic efforts to prepare them. In order to simplify these synthetic demands, we envisage that the use of a supramolecular approach, in which small constituents could be used combined to form the active component upon interacting with the lipidic media of membranes.

Self-assembling cyclic *D,L*-α-peptides (*D,L*-α-CPs) are very interesting macrocyclic building blocks that present unique supramolecular properties when they are composed by an even number of amino acids. These ring-shaped molecules are prearranged to adopt a flat conformation that projects the amide backbone perpendicularly to the plane of the ring, allowing the formation of a network of hydrogen bond interactions (β-sheet-like structure) that enables the successive stacking of different subunits on top of each other, thus giving rise to a hollow nanocylinder. Thanks to these design principles, the diameter of the cavity of the supramolecular stack can be precisely tailored by modifying the number of residues incorporated on the CP. Additionally, the side chains of the amino acids are projected in a pseudo equatorial conformation decorating the outer surface of the supramolecular aggregate [10].

Our research group has developed a new class of nanotube forming CPs known as α,γ-cyclic peptides, an evolution of the *D,L*-α-CPs, in which all the homoquiral residues of the original design are substituted by cyclic γ-amino acids [11]. This substitution, which does not

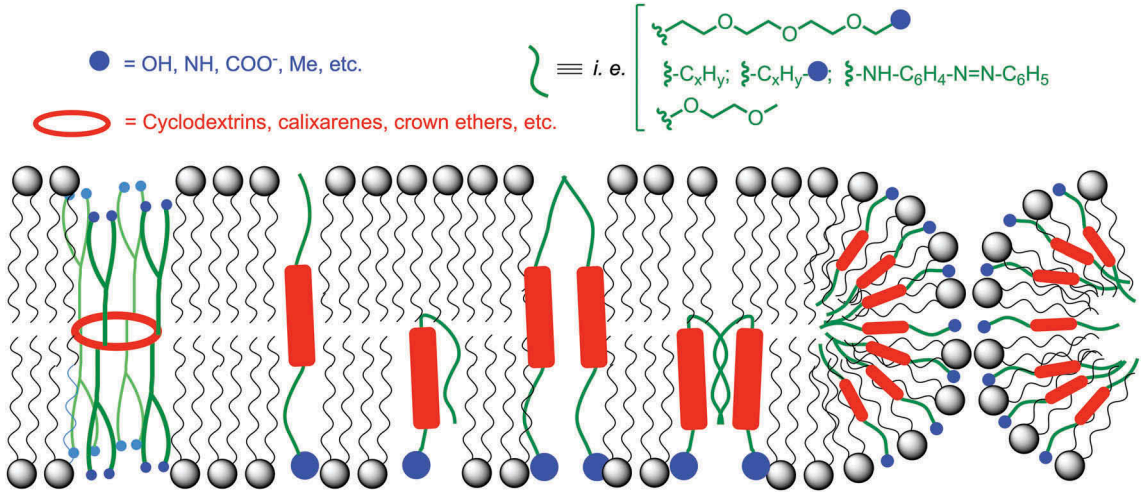


Figure 1. General scheme on the composition and transmembrane disposition of different artificial molecular spanning transporters described in literature.

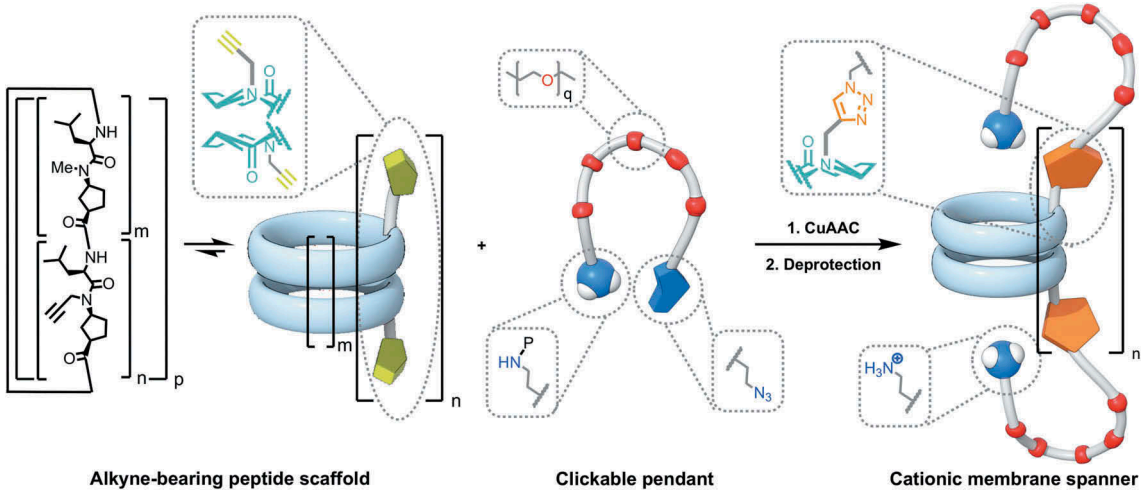


Figure 2. Strategy for the synthesis of a library of membrane spanner ionophores based on the modification of the peptide structure (variation of n and m units) and the type of pendants decorated with amino and azido groups at different ends.

alter the self-assembling properties, increases the rigidity of the peptide backbone, due to the cyclic character of the γ-amino acids, which enhances the conformational stability of larger cyclic scaffolds [12]. Also, the methylene group of the β-position in these γ-amino acids is pointing towards the lumen, which grants amphipathic properties to the assemblies. In addition, these groups can be further functionalised to change the tube properties [13]. The easy substitution of the amide proton of these residues by an alkyl group (typically a methyl group) prevents the formation of the extended hydrogen bond networks of the nanotube to generate only dimeric aggregates. This methodology grants access to a wide variety of dimeric scaffolds with substituted γ-residues, whereas the methylation protocol of previous *D,L*-α-CPs could only be applied onto Ala residues [14], due to the strong steric hindrance with the alkyl chains and the added methyl group. This procedure has allowed the incorporation of different substituents pointing perpendicularly to the disc structure and towards the entrance of the cavity, leading to the development of covalent CP dimers or supramolecular capsules [15].

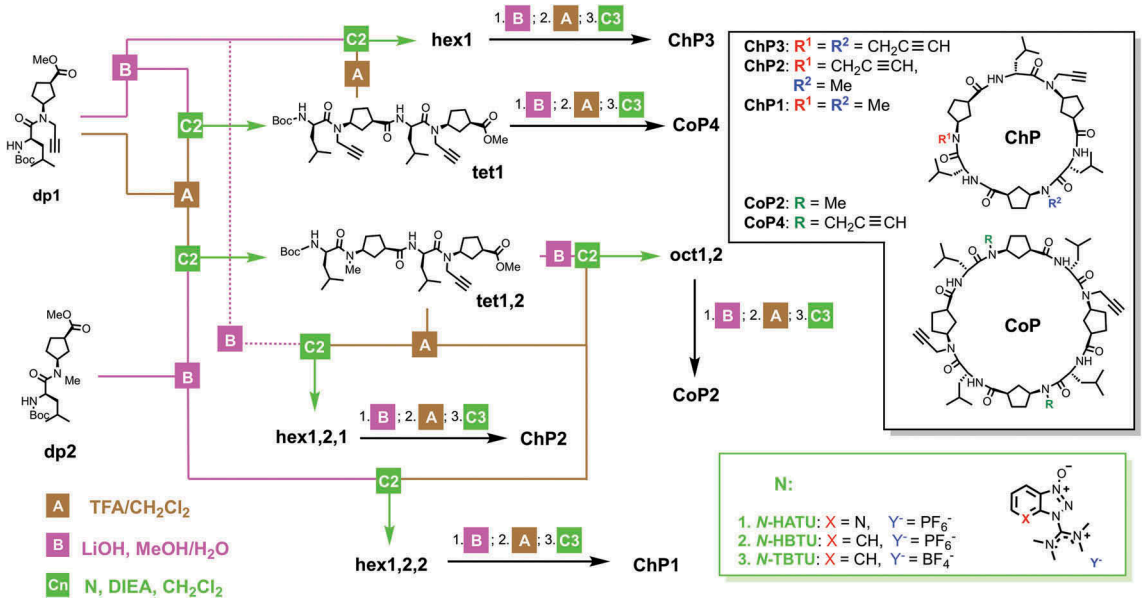
Results and discussion

Considering all these precedents, we envisioned the use of dimer-forming α,γ-cyclic peptides as a versatile nanotechnological platform for the rapid screening of potential membrane transporter agents based on the spanner model. In our design, we propose a convergent synthetic protocol to create a variety of peptides (hexamers or octamers) with a different number of anchoring positions oriented perpendicular to the dimerisation plane, but without interfering in the assembly process (Figure 2). The use of a click-type transformation, in this case a copper-catalysed alkyne-azide cycloaddition (CuAAC) [16], should allow the incorporation of different types of flexible amphipathic chains. This method has an enormous potential to carry out structure-activity-based studies, in which not only the peptidic core can be modified but also the 'pendants' attached to it. This strategy could generate a library of membrane spanners whose ion transport efficiencies across model membranes (LUVs) can be measured utilising the fluorescence properties of stimuli-responsive probes [17].

One of the key steps on our strategy was the introduction of the terminal acetylene-bearing chain (*i.e.* propargyl group) onto the amino group of the cyclic γ-amino acid (Acp, 3-aminocyclopentanecarboxylic acid) [11]b, which was performed by means of a Fukuyama functionalisation (See SI, Scheme S1) [18]. This strategy involves the incorporation of a 2-nitrobenzenesulfonyl moiety (nosyl group, Ns) on the deprotected amino group of Acp, a reaction that is carried out over the C-terminal protected residue (methyl ester). The incorporation of this electron-withdrawing group increases the acidity of the proton attached to the N-sulphonamide, which allows its abstraction under mild basic conditions, such as K_2CO_3. This method prevents the epimerisation on the α-carbon atom, which preserves the self-assembling properties of the final macrocycle. The addition of good electrophiles, such as propargyl bromide, under these mild conditions, produces the N-alkylated product in excellent yields (>95%). The last step is the release of the terminal amine group to allow the elongation of the peptide chain, which is accomplished using thiophenol in the presence of K_2CO_3, providing **Aa1** in good yield. Additionally, synthetic amino acid **Aa2** was also prepared (see SI, Scheme S1), which bears an inert methyl group that is incorporated exclusively for preventing undesired aggregation. In this case, the alkylation step can be carried out directly over the Boc-protected acid-free *L*-Acp residue. Finally, this N-methylated residue was protected by means of methyl ester formation to guarantee the orthogonality during the peptide synthesis protocol.

After securing **Aa1** and **Aa2**, they were coupled with Boc-*D*-Leu-OH, using *N*-HATU as a coupling agent and DIEA as a base. Under these conditions **dp1** and **dp2** were prepared in very good yields (>90%, see SI) [15]a. These are the only two building blocks implemented in the whole library of peptides. By employing solely two 'monomers', the synthetic protocol can be optimised, reducing the number of reactions in the route to the minimum, while maintaining the chemical diversity in the library to extract reliable structure–activity relationship (Scheme 1). In this regard, we focused our attention on the preparation of α,γ-CPs made of 3 (cyclic hexamers, ChP) or 4 (cyclic octamers, CoP) building blocks, each one containing a variable number of terminal alkynes (coming from **dp1**). This route provides, in a simple manner (less than 4 peptide couplings), access to a number of derivatives that ensures the understanding on how the effect of the ring-size (7 Å and 10 Å, respectively) [12] and the degree of functionalisation (1, 2, 3, or 4 alkyne groups) affect the transport efficiency. Particularly, the fine tuning of the orthogonal deprotection (TFA for N-terminal group, and LiOH in MeOH/H_2O for the C-terminal end) and coupling (*N*-HBTU or *N*-TBTU, DIEA in CH_2Cl_2) sequences (see Scheme 1 for detailed synthetic route designs) allowed the efficient formation of cyclic hexapeptides **ChP1, ChP2** and **ChP3** (bearing 1, 2, and 3 propargyl groups, respectively) and cyclic octapeptides **CoP2** and **CoP4** (incorporating 2 and 4 alkyne moieties, respectively) [19].

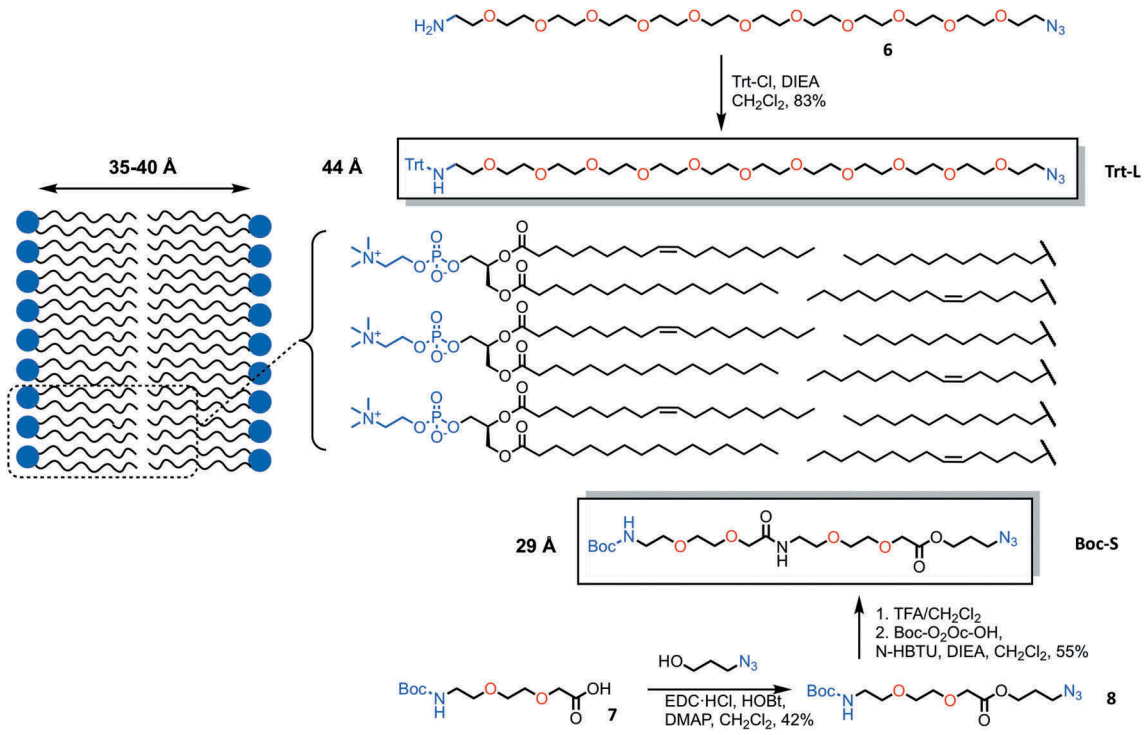
All these peptide scaffolds self-assemble into dimers in non-polar media (*i.e.* $CHCl_3$), as confirmed by NMR and FT-IR experiments (see SI for details). Therefore, the



Scheme 1. Synthetic strategy followed for the obtention of the different families of α,γ-CPs. Colour code is used for the synthetic strategy, brown for the deprotection of the N-terminous group (TFA/CH_2Cl_2), magenta for the removal of the C-terminus protecting group (LiOH, MeOH/H_2O) and green for the coupling conditions in which the 'n' number is used to differenciate the different coupling reagents used in this process (1 for *N*-HATU, 2 for *N*-HBTU and 3 for *N*-TBTU).

incorporation of the reactive alkyne groups, which lay perpendicular to the planarised macrocycle, does not interfere in the peptide stacking.

We considered two different types of tentacles as pendants to be attached to the central core, where the length of the flexible moiety was the parameter at which we focus our study. On one hand, undecaethylene glycol decorated with an azide at one end and trityl protected-amino group at the other end (**Trt-L**) was selected because its length (*i.e.* 44.5 Å) should span the whole membrane (Scheme 2) [20]. The protection of the primary amine in commercially available **6** proved to be crucial for



Scheme 2. Synthetic protocol and length comparison with a typical lipid bilayer of both spanning arms **Boc-S** and **Trt-L**.

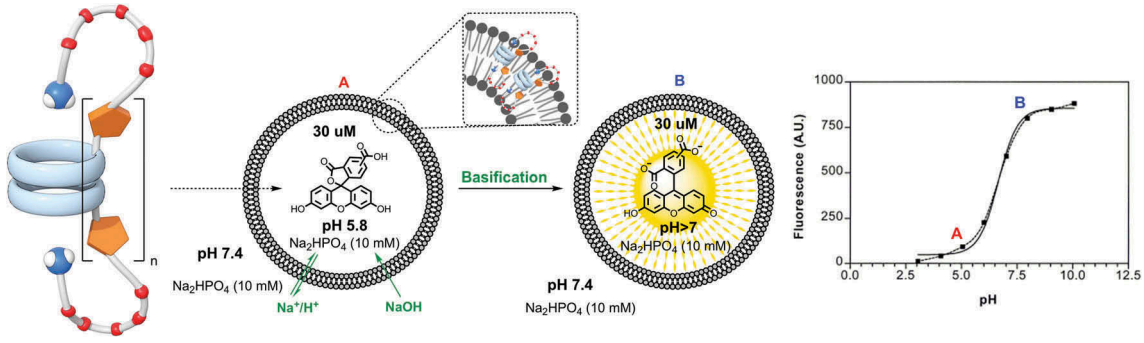
a successful reaction with the cyclic peptide core (*vide infra)*. Additionally, shorter pendant **Boc-S** was prepared using commercially available Boc protected 3,6-dioxa-8-aminooctanoic acid (**Boc-O_2Oc-OH**) as basic unit. In this direction, the first step consisted of an esterification with 3-azido-propan-1-ol, followed by acidic cleavage of the Boc group and then coupling with an extra unit of **Boc-O_2Oc-OH** to afford **Boc-S**. Apart from the length (29 Å in the fully extended form, similar to that of a phospholipid like POPC, Scheme 2), the main difference between **L** and **S** is the presence of two carbonyl (one amide and one ester) groups in the chain of **S** compared to the pure ethylene glycol skeleton in **L**.

Once the CPs and the azido-spanning arms were prepared, the covalent attachment of both units and, hence, the obtention of the target chemical library, was carried out by means of the CuAAC reaction [16]. For this purpose, the use of catalytic amounts of $[Cu(MeCN)_4PF_6]$ (10 mol% per alkyne), DIEA and TBTA ligand in CH_2Cl_2 afforded the clicked amine-protected transporters containing from 1 to 4 triazole rings in good to excellent yields (57-95%, see SI). In all the cases, NMR studies revealed that the products presented the typical singlet derived from the successful formation of the triazole rings that connect the core and the spanning arms (δ 7.60–7.70 ppm), as well as amide NH chemical shifts (*i.e.* 8.30–8.40 ppm) that suggest the equilibrium remains shifted towards the dimeric species in non-polar media. Finally, both protecting groups (Boc and Trt in **S** and **L**, respectively) were removed employing a TFA cocktail, which afforded the cationic membrane spanner compounds, whose transport properties were tested on model membranes (egg yolk phosphatidyl choline, EYPC-LUVs).

Due to its simplicity, a pH rectification assay based on the use of pH-responsive 5(6)-carboxyfluorescein (CF) was envisioned to test whether the synthetic molecules could induce the translocation of cations. Because of the pseudo-linear increase in the fluorescence emission intensity between pH 6 and 7 that this fluorescent probe presents (Scheme 3, right), it is possible to correlate the variation on the pH in the intravesicular medium of liposomes with a fluorescence intensity change of the entrapped CF (**CF⊂LUVs**) [21]. Hence, LUVs that present an intravesicular acidic (pH 5.8) character at the beginning of the experiment were suspended on a solution buffered at more basic conditions (*i.e.* pH 7.4) and treated with the spanners. The increase in the fluorescence intensity of the fluorophore was expected if the transporter is capable of promoting the translocation of ions and, hence, facilitate the equilibration of intra and extravesicular pH values (Scheme 3). The basification mechanism can proceed either by electroneutral symport pathways in which the cotransport of Na^+ and OH^- towards the interior or the movement of Na^+ and H^+ in opposite directions (antiport).

To test this hypothesis, all the compounds in the transporter library were added (DMSO stock solutions) onto the vesicular suspension in a range of concentrations (100–1 μM; ratio lipid/transporter 66/1-6600/1) and the increase in the fluorescence intensity of the CF was recorded. After 400 s a detergent (Triton X-100) was added to release the vesicular content and induce the full basification of the system, which allowed the normalisation of the transport data. Transport results for all the chemical library are presented in Figure 3.

It was found that some of the compounds, such as **ChP1 L, ChP3 L, CoP2 L** and **CoP2 S** (underlined in Figure 3) presented a poor solubility in the aqueous media, causing noisy and erratic fluorescence transport traces, resulting in the appearance of turbidity in the cuvette, making difficult to fully analyse their transport capabilities. This result was unexpected for us, considering that oligoethylene glycol moieties are supposed to confer solubility in both aqueous and lipophilic media and can be found in several previously reported synthetic transporters in literature [22]. Perhaps, this result suggests that the strong amphipathic character of the transporters studied in this communication, due to the presence of the hydrophobic peptide scaffold and the cationic primary amine



Scheme 3. **CF⊂LUVs** assay based on membrane-spanner induced basification of the intravesicular pH, which promotes an increase in the fluorescence intensity of CF fluorophore (right).

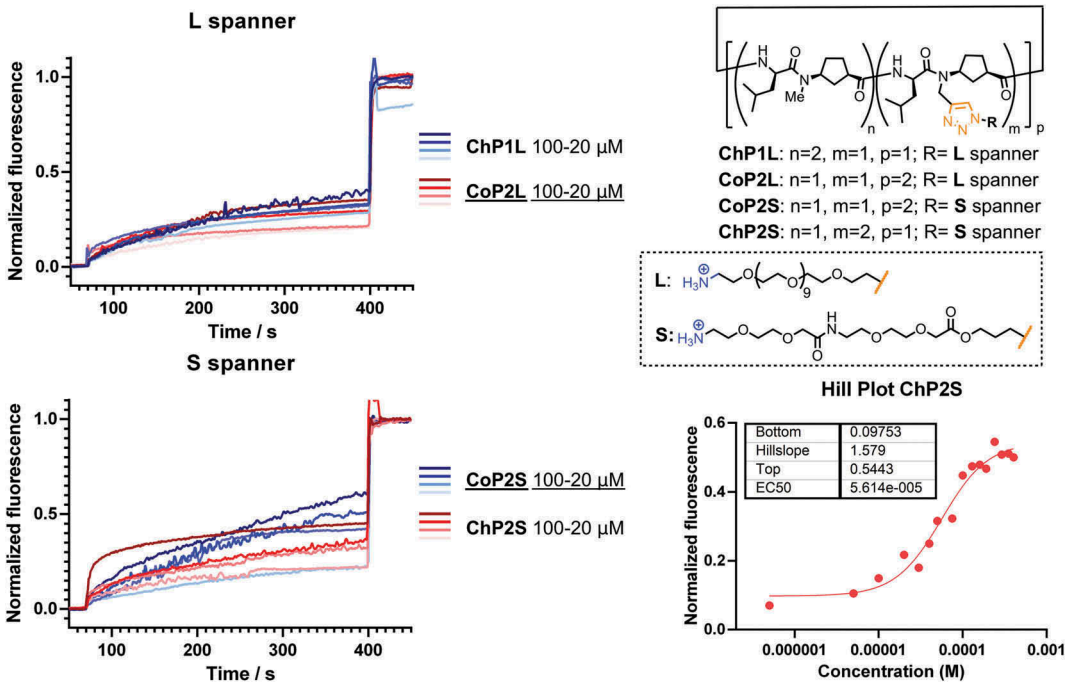


Figure 3. Fluorescence traces obtained in the transport experiment carried out on **CF⊂LUVs** (EYPC) with the most active compounds in the chemical library at a range of concentrations (100–20 μM or ratio lipid/transporter 66:1-330/1), traces of less active compounds can be found in the SI). Underlined traces represent the samples in which turbidity was observed at high concentrations.

region on opposite ends, generates some kind of aggregates that crash out of the aqueous solution. Apart from these, there is a clear difference between the transporters, where the trend among them, **ChP2 S**> **ChP1 L**> **ChP1 S**≫ **ChP3 S**> **CoP4 S**, shows that the efficiency is reduced when the number of pendants is increased. In this regard, transporters comprised either by 1 or 2 pendants presented the best results, where **ChP2 S** is close to reaching a 50% pH rectification efficiency (see detailed Hill Plot [23] in Figure 3, bottom right and SI) at its higher concentration. These results suggest that molecules equipped with **S** pendants present more attractive transport properties that their **L** derivative counterparts, as suggested by the higher initial rate of transport (see, for instance, the slope after the addition of **ChP2 S** in Figure 3) and, also, by the fact that none of the compounds in the **L** series reach a rectification activity of 40%. Additionally, the results with **CoP2 S** and **CoP2 L**, even though they have a low solubility in the aqueous media at the highest concentration tested and therefore the observed results should be taken with care due to the turbidity observed in the cuvette at the end of the fluorescence experiment, suggest that transporters bearing two oligoethylene glycol chains are the most efficient scaffold studied in this work.

Next, we checked the membrane integrity of the LUVs during transport experiments. For that purpose, we carried out experiments with LUVs containing CF at a high self-quenching concentration (**SQCF⊂LUV**). In this regard, the fluorescence intensity of the probe would only increase if it is released and gets diluted, meaning that CF molecules are liberated into the extravesicular medium as, for example, it is observed when a detergent-like Triton X-100 was added. Most of the transporters tested showed low leaking activity (Figure 4) and almost no changes in the fluorescence emission were observed. Only **ChP2 S** showed some leaking activity, giving rise to a maximum increase of 20% of vesicle emission.

In conclusion, we have developed a highly modular synthetic strategy that allows the straightforward obtention of a variety of α,γ-CPs of different diameters and a different number of alkyne moieties on top of the peptide scaffold. The click reaction of the aforementioned peptides with different azide-functionalised ethylene glycol amines afforded a small chemical library whose ion transport capabilities were tested. In this regard, transporters bearing one and two spanning pendants were, in general, more active than highly substituted analogues, with activities around 10–100 μM in a **CF⊂LUVs** pH rectification assay for the best candidates. These results demonstrate the versatility that functionalised CP scaffolds present for the development of structure-activity-based studies in the bionanotechnological area and open the possibility to use them in further studies.

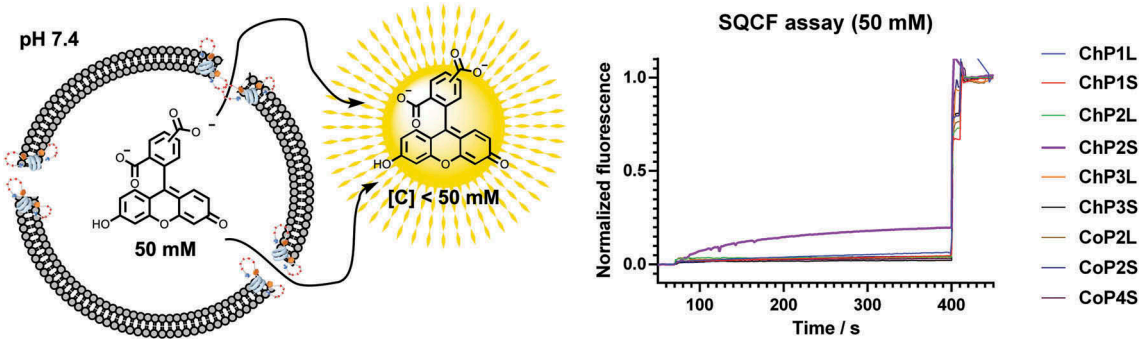


Figure 4. Fluorescence traces obtained in the spanner-induced leakage assay on SQCF (50 mM) loaded LUVs. The compounds were tested at a concentration of 100 μM (ratio lipid/transporter 66/1).

Acknowledgments

This work was supported by the Spanish Agencia Estatal de Investigación (AEI) and the ERDF (CTQ2016-78423-R), and by the Xunta de Galicia and the ERDF (ED431C 2017/25 and Centro singular de Investigación de Galicia accreditation 2016-2019, ED431G/09). We also thank the ORFEO-CINCA network and Mineco (CTQ2016-81797-REDC). A.F. thanks the Spanish Ministry of Education for his FPU fellowship.

Disclosure Statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by Centro singular de Investigación de Galicia accreditation 2016-2019, ED431G/09; Centro singular de investigación de Galicia accreditation 2019-2022, ED431G 2019/03.

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[19] In the nomenclature of the compounds, lowercase letter indicates ring size (**h** stands for hexa- and **o** stands for octa-), whereas the digit (1-4) accounts for the number of alkyne positions in the peptide scaffold. The last letter relates to the type of spanner chain attached.

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