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Design, synthesis, and characterization of peptide nanostructures having ion channel activity

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Abstract—We report the synthesis and the functional studies of multiple crown α-helical peptides designed to form artificial ion channels. The approach combines the versatility of solid phase peptide synthesis, the conformational predictability of peptidic molecules, and the solution synthesis of crown ethers with engineerable ion-binding abilities. Several biophysical methods were employed to characterize the activity and the mode of action of these crown peptide nanostructures. The 21 residue peptides bearing six 21-EC-7 turned out to facilitate the translocation of ions in a similar fashion to natural ion channels.

1. Introduction

Nanotechnology and the development of analytical devices for the detection of important analytes at the molecular level constitute one of the most active area of research in bioanalytical chemistry and a formidable challenge to scientists. However, before getting full benefits from nanotechnology, scientists need to devise novel approaches to the preparation of well defined molecular architectures in the nanometre range that will serve as discrete components in the construction of functional and practical devices, such as for example diagnostic kits. As in many areas of research, Nature, with an infinite number of functional nanostructures, serves as a source of inspiration. For instance, channel proteins exemplify beautifully nanopore technology. These proteins are large and complex membrane proteins that regulate the flow of ions across cell membranes. Due to their involvement in several diseases and their enormous potential in nanotechnology and single molecule detection,[1–6] ion channel proteins have been the subject of intense investigation in the past 15 years. Truly remarkable results have been obtained by using natural channel proteins, in particular α-hemolysin. However, there are several problems associated with natural ion channel proteins that severely limit their utility as both component of biosensors and potential therapeutics. These transmembrane proteins are difficult to isolate in their pure and functional form and they tend to denature easily. Also, their chemical modification into more elaborated molecular systems is almost impossible owing to their complex multidomain structure. To overcome these difficulties, numerous studies have focused on the development of artificial molecules designed to mimic natural ion channel proteins.[7–19] Important achievements have been made by many groups around the world and some successful approaches gave compounds that showed single-channel activity. [7–19] However, there are still several issues that need to be further addressed before artificial channels and nanopores can be used in practical applications: these include ion selectivity, preparation on larger scales, complete characterization of the mode of action, selective modifications into more elaborated systems for recognition of relevant analytes, and implementation into workable devices.

Our group has devised a unique strategy for the preparation of artificial ion channels.[20] The general concept is to use α-helical peptidic structures as scaffold to orient multiple macrocyclic ligands on a top of each other. Thus, to create membrane active channels, we have designed and prepared 21-residue peptides bearing six

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crown ether modified amino acids at position 2, 6, 9, 13, 16, and 20. When the peptidic framework adopts an α-helical conformation, the crown rings are proposed to align to form a polar pore long enough to allow the passage of ions across a lipid bilayer membrane (Fig. 1). Because the peptides contain 15 L-leucine residues, they are sufficiently hydrophobic to incorporate in a lipid bilayer environnement.[21] In addition, the strong helix induction propensity of leucine suggests that the peptidic backbone should adopt preferentially an α-helical conformation necessary to bring the crown ring in the proposed supramolecular array.[22,23]

There are several advantages to this strategy. First, ion selectivity can be easily modified by changing the size of the crown ether rings. Second, since they are designed to operate as monomer and not through aggregation, their detailed mechanism and functional structure should be more easily assessed. Third, post synthesis modifications and end group engineering can be easily and efficiently done selectively. Finally, solid-phase peptide synthesis is well developed and allows rapid, parallel preparation of numerous analogues on a relatively large scale. Herein, we report synthesis and characterization of hexacrown peptide **1** and its analogues **2**–**5** as well as studies on their activity, selectivity, mode of action, and cytotoxicity.

2. Results and discussion

2.1. Synthesis

Molecule **1** represents a prototype of our approach towards artificial ion channels. It is a 21 amino acid peptide composed of 15 L-leucines and six 21-crown-7 L-phenylalanines. Incorporation of the modified amino acids at positions 2, 6, 9, 13, 16, and 20 of the sequence allows the crown ligands to be on the same side of the peptidic framework when it adopts an α-helical structure. As mentioned in the introduction, we aimed at exploiting the unique ion binding ability of crown ethers. The binding selectivity of such neutral macrocyclic ligands depends on their size and on the number of oxygen atoms. On that basis, we devised a general synthetic strategy toward enantiopure crown ether aminoacids. The strategy starts with 3,4-dihydroxy-L-phenylalanine (L-DOPA) commercially available and is illustrated in Figure 2. Many analogues have been prepared using the synthetic scheme which turned out to be quite general using Cs_2CO_3 in DMF as base. The best yield was obtained with the 21-crown-7 analogue known to bind Cs^+ very tightly, hence the templating effect being best in this case.

Having access to a series of crown ether amino acids, we devised an efficient synthetic strategy for the peptide nanostructure. To simplify the synthesis of nanostructures like **1**, we envisioned that the 21 amino acid peptide could come from the trimerization of a heptapeptide, where crown ether amino acids are in positions 2 and 6. The choice of a repeating heptad minimizes the effort in the synthesis if the desired peptide is to be prepared by segment condensation.[24,25] Indeed, to avoid the tedious purification steps associated with a stepwise synthesis of a 21-residue peptide, we adopted a strategy involving selective coupling of peptide segments. The basic idea was to prepare first a seven-residue peptide **28** on oxime resin, and then proceed to its self-condensation, also on solid support, to afford a 14-mer, then the desired 21-mer (Fig. 3).

Coupling reactions to prepare heptapeptide **28** were performed using *N*-BOC amino acids and DIC/HOBT

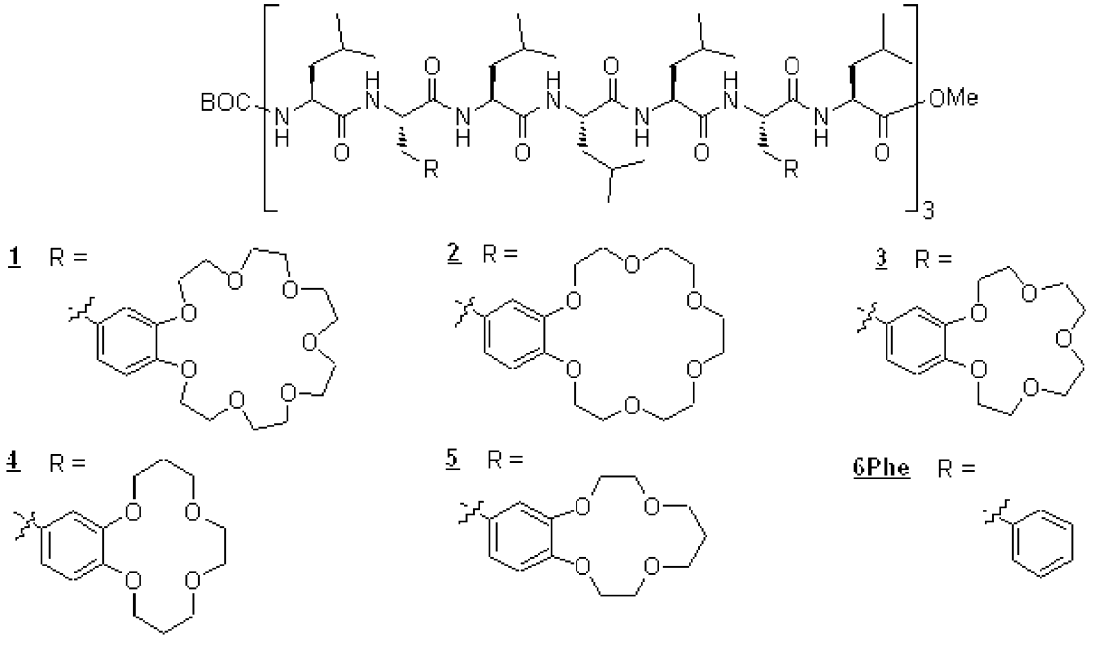

Figure 1. Crown peptide nanostructures synthesized and used in the present studies.

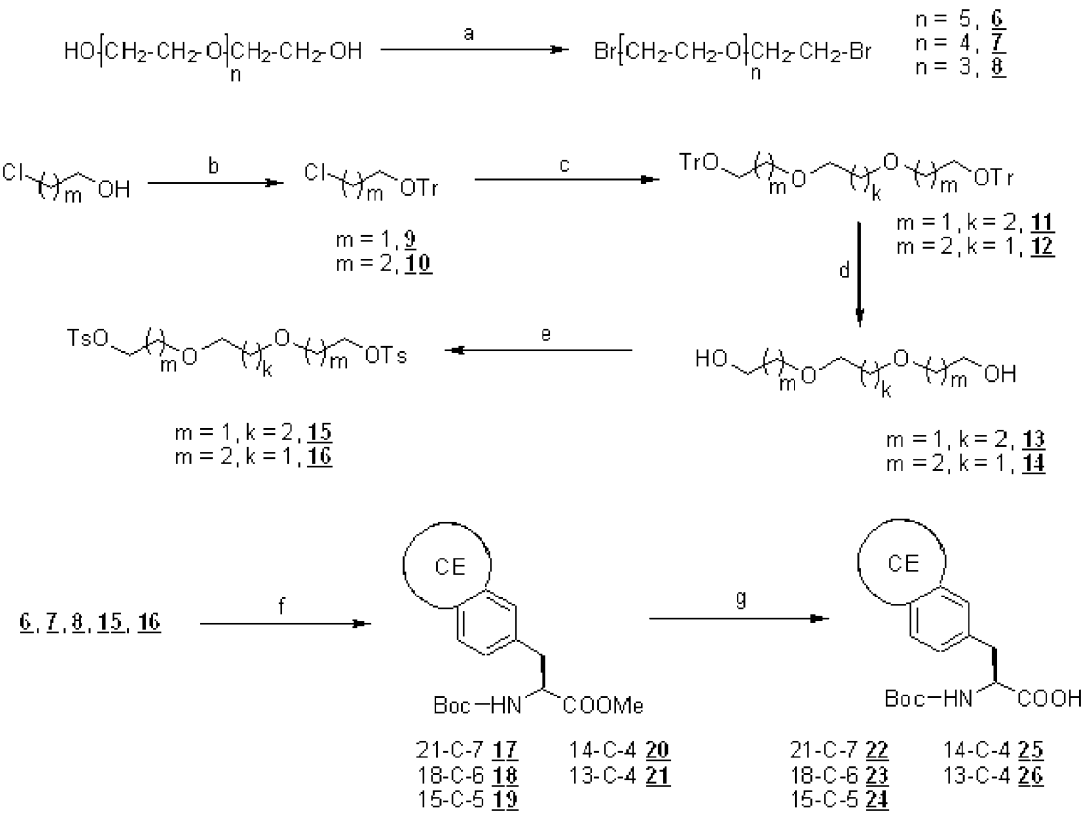

Figure 2. Synthesis of *N*-*t*-BOC protected crown ether amino acids: (a) PPh_3, Br_2, CH_3CN; (b) Et_3N, TrCl, DMAP, CH_2Cl_2; (c) NaH, HO–CH_2–$(CH_2)_k$–OH ($k = 1$, 2), DMF; (d) 6 N HCl/dioxane, CH_2Cl_2; (e) TsCl, Py; (f) BOC-L-DOPA-OMe, Cs_2CO_3, DMF; (g) 1 N NaOH/MeOH (CE, crown ether ring).

as reagents in DCM/DMF (1:1). The *N*-BOC group was removed between each coupling by using 50% trifluoroacetic acid (TFA) solution in DCM. After preparation of the key heptapeptide **28** on solid support, a fraction (3/4) of the total amount of resin was cleaved with water and DBU in THF to generate the *N*-BOC heptapeptide acid **29**. On the remaining resin, the *N*-BOC group was removed by treatment with TFA. Coupling of *N*-BOC heptapeptide acid **29** was performed using DIC/HOBT in DMF to yield the 14-residue intermediate. It is noteworthy that these coupling conditions are the ones that gave the lowest racemization (<10%), as determined by RP-HPLC after cleaving an analytical amount of peptides from the resin. Indeed, any other conditions attempted (HBTU, HATU, BOP) led to more epimerization. TFA deprotection of the 14-mer followed by another coupling of segment **29** generated efficiently the desired 21-residue peptide still attached to the resin. Finally, the peptide was cleaved from the resin using MeOH in the presence of 2 equivalent of DBU to yield the 21 amino acid peptide **1**. The use of DBU in MeOH for cleavage reactions was first reported for the cleavage of peptides from Wang resin and turned out to be very useful to the construction of peptide ester from oxime resin.[26] The purification of **1** and its analogues (**2**–**5**, **31**) was easily achieved by reversed-phase HPLC, and they were characterized successfully by ^{1}H NMR and mass spectrometry. Thus peptide nanostructures bearing six crown ethers were efficiently prepared in pure overall yields higher than 10% generally.

2.2. Conformational studies

Circular dichroism (CD) studies have previously shown that peptides **1**–**5** adopt an α-helical conformation in different media, protic (TFE, MeOH) as well as hydrophobic (1,2-dichloroethane), and at different concentrations in TFE (Fig. 4 left).[27,28] We have also shown that these peptides keep their α-helical conformation when incorporated in lipid bilayers (Fig. 4 left).[27,28] These studies were supported by FTIR spectroscopy studies using DPPC vesicles and egg yolk lecithin (Fig. 4 right).[28] These results suggest that the poly-L-leucine framework dictates the conformation of these nanostructures and also that the crown rings are on the same side of the peptidic framework as predicted initially. They also point out that compound **1** and its analogues does not tend to aggregate in low polarity environment as demonstrated by the lack of change in the ellipticity at 222 nm at different concentrations (0.1 mM–0.1 μM).[27] Likewise, it is noteworthy that CD studies showed that the conformation of **2** is not affected by the presence of metal ions or water (data not shown). These observations indicate clearly the possibility of describing accurately peptide nanostructures from basic fundamental principles.

2.3. Ion transport studies

The ion transport ability of hexacrown peptides were investigated using different techniques. Our first attempt used the pH stat method.[29] In this assay, a sample of

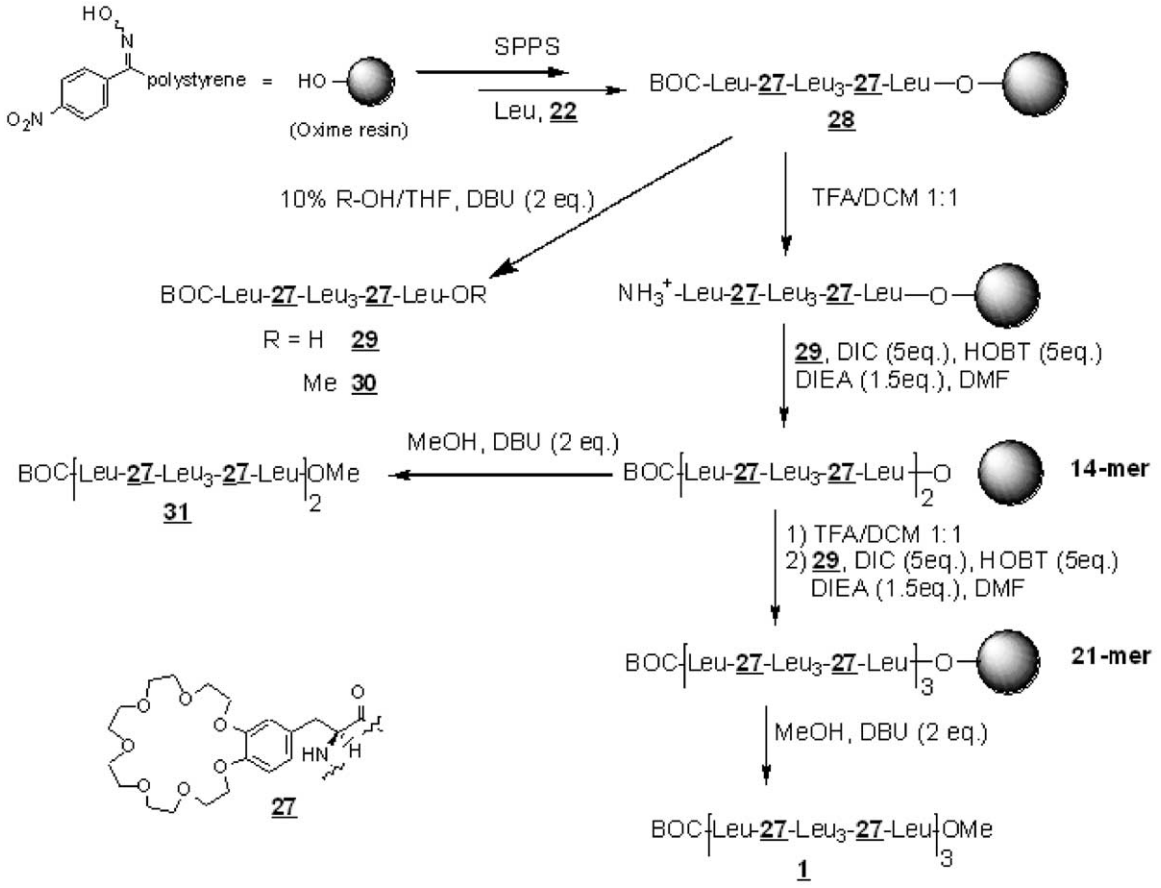

Figure 3. General synthetic strategy for the preparation of crown peptide nanostructures on oxime resin illustrated by the synthesis of the hexacrown peptide **1**.

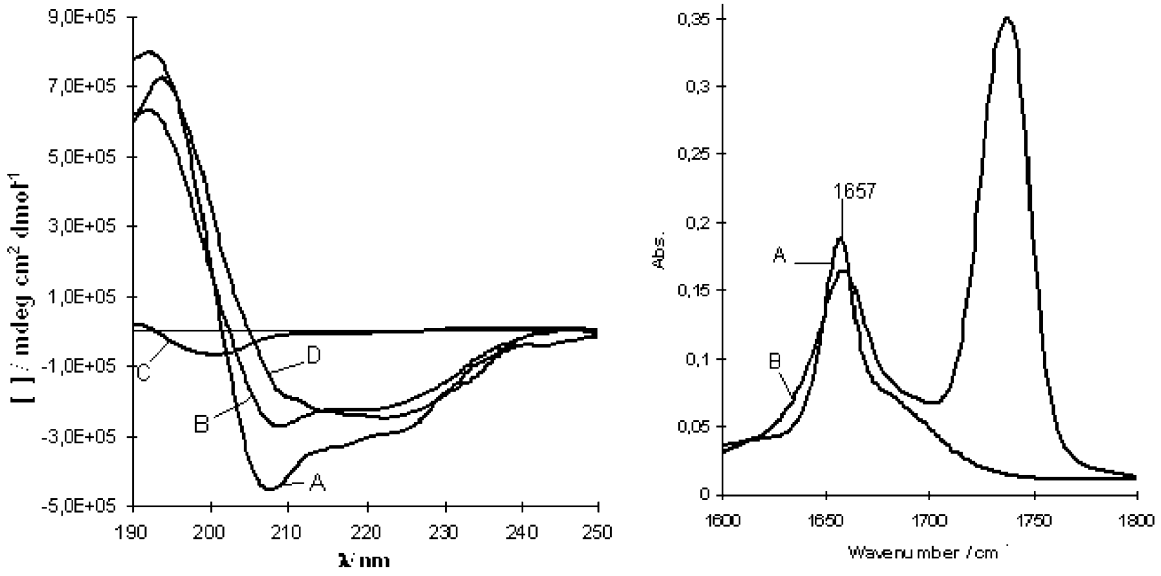

Figure 4. Left: CD spectra of (A) 21-mer **1**, (B) 14-mer **31**, (C) 7-mer **30** in TFE, as well as (D) **1** in egg yolk lecithin (80:1 w/w lipid/peptide) (5×10^{-5} M) in MOPS 10 mM, 20% TFE pH 7.4. Right: FTIR spectra of **1** in TFE (A), and in lecithin vesicles (B).

unilamellar vesicles with an internal pH of 6.6 is diluted with an external solution, and the pH is raised to 7.6 to create a proton gradient.[30] Then, ions and FCCP,[31] a proton carrier, are added. Upon addition of a functional transporter, its transport ability can be monitored by the release of protons required to maintain the electroneutrality across the vesicle wall (Fig. 5a). The protons are neutralized continuously to maintain a constant solution pH of 7.6. The graph of the volume of base added versus time allows the determination of the transport mode.[30,32] A typical result obtained is illustrated in Figure 5b for the case of Cs^+. As it can be

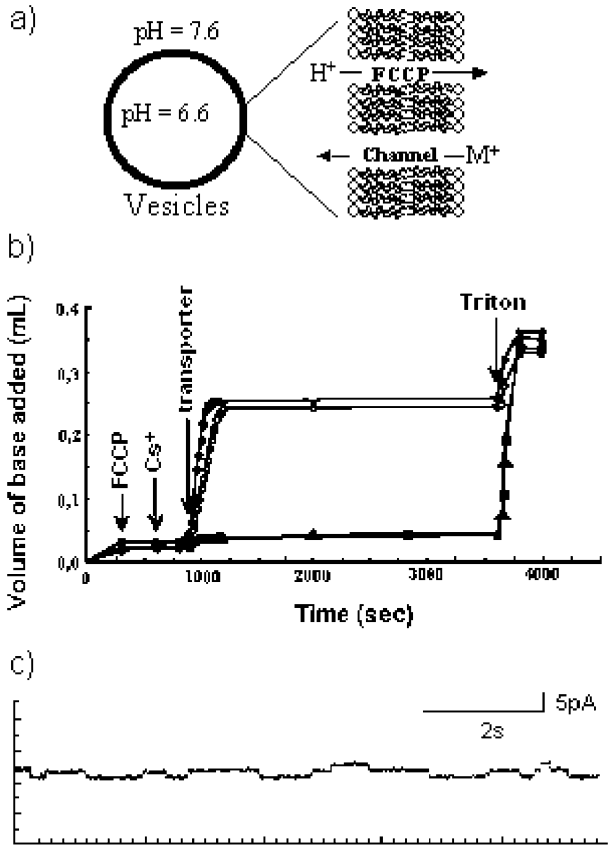

Figure 5. (a) Schematic generalization of the pH-stat method. The transport ability of test compounds is monitored by the release of protons from the vesicles as a function of time. (b) Illustrative example of results obtained using the pH stat technique. Cs^+ transport ability of monomeric crown ether **17** (△), heptapeptide **30** (■), hexacrown peptide **1** (●), and Gramicidin A (○) (reprinted with permission of ACS).[29] (c) Single-channel conductance recordings of peptide **1** in 1 M KCl (at −100 mV, filtered at 200 Hz) in a DPPC bilayer (current level 3.5±0.2 pA, average lifetimes of open states >1 s (reprinted with permission of Wiley-VCH).[27]

seen, the monomeric crown ether (**17**) and the heptapeptide analogue (**30**), too short to span the membrane, act as typical carriers by slowly and constantly transporting Cs^+ at roughly similar rate. However, addition of the hexa-crown peptide **1** led to a very rapid release of protons that reached saturation after less than 2 min. For comparison purposes, we studied Gramicidin A, a natural compound known to form exceptionally efficient channels. As it can be seen, hexacrown peptide **1** is as efficient as Gramicidin A for the transport of Cs^+. This result suggests that **1** functions as an artificial ion channel in a manner analogous to Gramicidin A. Furthermore, the hexa-crown peptide **1** exhibited the same transport activity with Li^+, Na^+, K^+ and Rb^+. Although these results support the channel mechanism of **1** and that ions with diameter smaller than Cs+ are expected to travel the channel formed by the large 21-C-7 ring array, it clearly indicates the deficiency of the pH stat technique to assess the ion selectivity of artificial, membrane active compounds. Several control experiments were performed to confirm the channeL-like mechanism of **1**. Changing the order of addition of the components (i.e., salts, FCCP, transporter) gave the same results. Studies using **6Phe**, the analogue of **1** lacking the crown ring, did not result in any significant transport. Even though, this is in agreement with the channeL-like mechanism of **1** and ions travelling the crown channel, the absence of ion transport with **6Phe** could also be the result of its insolubility in the media. Interestingly, addition of Triton X-100 at the end of every experiment led to complete lysis of vesicles and release of entrapped H^+. This phenomenon demonstrates that vesicles were still intact at the end of experiments, which strongly suggests that 21-mer peptide **1** is not acting as a sophisticated surfactant. This is further supported by the physical observation that addition of **1** to a vesicle suspension does not altered the opalescent look of the solution. By contrast, addition of 5 μL of a 0.5 mM Triton X-100 solution immediately clarifies the solution.

It is noteworthy that in the case of Gramicidin A and **1**, proton release is not complete. Indeed, in every case with these functional compounds transport reaches a plateau at roughly 80% of maximum theoretical transport. This can be explained by the fact that some of the vesicles are multilamellar and hence addition of transporter can effect the release of protons only from the outer layer and not from the inner ones.

In order to characterize further the transport ability of **1**, we carried out single-channel measurements[33] using a modified patch clamp technique.[34] To demonstrate the ion channel activity, 2 μL of a 0.1 mM solution of **1** in methanol was introduced in a 2-mL cell containing an unbuffered 1 M KCl solution (pH 5.8); the bilayer was constructed on a pipette tip from zwitterionic DPPC (5 mg/mL in decane). As initial experiments, only K^+ transport was studied as this ion is weakly bound by 21-C-7 rings and therefore should traverse with great facility channel formed by the crown ether array. The conductance recordings shown in Figure 5c are characteristic of the results obtained with **1** and typical of ion channel activity observed with natural proteins.[27,35] Typical transitions ('steps') between closed and open states were evident throughout experiments. The discrete conductance depend on the number of channels in the open state.[33] In DPPC bilayers, compound **1** has a current level of 3.5±0.2 pA with lifetimes for the open states of around 1 s. These results corroborate findings from the pH stat studies. However, more experiments are required with different ions and analogues **2**–**5** to quantify accurately their ion channel activity.

In search of a practical method to screen many analogues for channel activity and to further confirm the activity of peptide nanostructure **1**, we used a dynamic NMR method. Developed by Riddell and Hayer[36] and devoided of any driving force, this method permit the assessment of the efficiency of sodium cation transport. In this method, sodium ions trapped inside vesicles, composed of phosphatidylcholine and phosphatidylglycerol, are distinguished from external sodium cations by adding a shift reagent (usually Dy^{3+}). Addition of a functional transporter leads to an alteration of the signal width caused by an exchange of sodium cations

across the bilayer. These variations can be related to the transport rate. This methodology, successfully applied to Gramicidin D,[37] was also exploited to evaluate the efficiency of artificial hydraphile channels.[38] One benefit of this method is the obtention of reliable relative rates. The NMR experiments reported here are normalized to a relative rate of 100 representing activity of Gramicidin D, our standard in this study. The results of ^{23}Na NMR transport experiments are shown in Table 1.

We can observe that activity of peptide nanostructure **1** having 21-crown-7 macrocycles (3% of Gramicidine D) is 30 times higher than its analogue **2**, with smaller macrocycles, and more than 600 times higher than **4** and **5**. This strongly supports the hypothesis that cations travel through the channel formed by the array of macrocycles in order to cross the bilayer membrane. In fact, cavity of 14-crown-4 and 13-crown-4 is too small to let sodium cations passing through, while 18-crown-6 seems to have enough binding affinity for Na^+ to diminish partially the channel activity of nanostructure **2**. The two most active compounds are **1** and **31**, respectively the 21-mer and the 14-mer built with 21-C-7 amino acids. Surprisingly, shorter analogue **31** is not long enough to span entirely the lipid bilayer. We reasoned that this unexpected result could be explained by the ability of **31** to induce vesicle lysis, which indeed can present in ^{23}Na NMR experiments, results similar to those obtained for cation transport. To verify this possibility, control experiments were performed using a fluorescent vesicle lysis assay.[39] Shown in Figure 6 are the calcein release profiles induced by addition of 7-mer **30**, 14-mer **31** and 21-mer **1** to unilamellar vesicles prepared from egg phosphatidylcholine and egg phosphatidylglycerol (1:2). Clearly the 21-mer **1** and 7-mer **30** induce very little leakage. Beside, the 14-mer **31** induces rapid and significant lysis. This unique behaviour of **31** may be the result of an hydrophobic mismatch[39] that introduces weaknesses in the bilayer and lead to lysis.

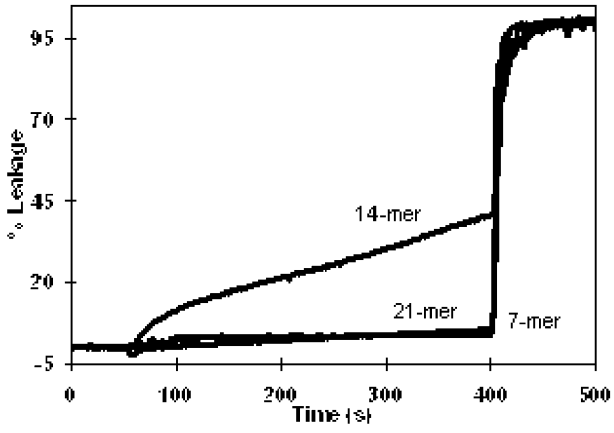

Figure 6. Calcein leakage induced by addition of 7-mer **30**,14-mer **31**, and 21-mer **1** (5 mM) to phosphatidylglycerol/phosphatidylcholine (1:1) vesicles in HEPES 100 mM, NaCl 170 mM, EDTA 5 mM at pH 7.4. Vesicles were lysed with Triton X-100 at 400 s.

Table 1. Rate constants (k) and relative transport rates for Na^+ across a phospholipid bilayer membrane by ^{23}Na NMR

Compd	k (s^{-1} μM^{-1})	Relative rate (%)
Gramicidin D	240	100
Boc-21mer-Ome (21-C-7) **1**	6.6	3
Boc-14mer-Ome (21-C-7) **31**	9.2	4
Boc-21mer-Ome (18-C-6) **2**	0.2	0.1
Boc-21mer-Ome (14-C-4) **4**	<0.1	<0.05
Boc-21mer-Ome (13-C-4) **5**	<0.1	<0.05

Contrarily, compound **1** has a definite transport activity but does not induce lysis confirming that the observation made in the ^{23}Na NMR experiments is indeed due to ion transport activity. These results show that caution should be exerted when using ^{23}Na NMR to probe ion channel activity, as it can not discriminate between transport and lysis. Finally, the Na^+ transport activity of **1**, even though much weaker than Gramicidin D, is encouraging as this compound correspond to our starting point in channel development and that it can be easily optimized, particularly with termini modifications with polar heads.

2.4. Mechanism of ion transport

In order to characterize in more details the ion transport mechanism of **1**, we undertook a thorough biophysical investigation using ATR spectroscopy. Polarized infrared ATR spectra of oriented films of DMPC and DPPC in the absence and presence of crown peptide **1** and analogues, and at different concentrations, were recorded.[28] These experiments led us to conclude that hexacrown peptides are in a two-state incorporation equilibrium, one active parallel to the lipids chains and the other inactive at 90° of the same chains. These data combined with the ones previously obtained by CD and FTIR spectroscopy allow us to propose a model for the formation of active ion channels by crown peptide **1** (Fig. 7).[28] First, when added to the bilayer system from MeOH solution, crown peptide **1** and its analogues precipitate and form solid aggregates. Then, one crown peptide from the aggregates adsorbs at the surface of the membrane, most probably through electrostatic interactions between the crown ether units and the lipid polar groups. It is also possible that hydrophobic interactions between leucine side chains and the lipid chains

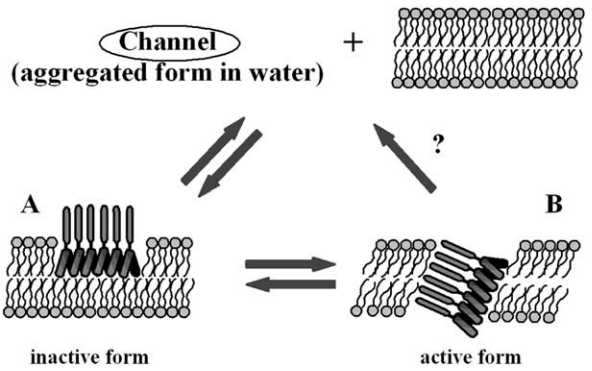

Figure 7. Proposed mode of action for the channel activity of peptide **1**. From the aggregates formed in the bulk water solution, one crown peptide adsorbs at the bilayer surface (A) and is in the adsorbed inactive form. From this partially incorporated form, peptide reorients itself into the active form B (reprinted with permission of Wiley-Interscience).[28]

act as driving force for the initial adsorption. Finally, under the influence of an electrical potential or an ionic gradient, one of the adsorbed peptide nanostructure flips over parallel to the lipid chains and channel activity can be observed. This mechanism is very close to the one described by DeGrado et al. for self assembly helical peptides[40,41] and to the barrel-stave mechanism of some antimicrobial peptides.[42]

2.5. Biological activity

As hexacrown compounds mimic properties of natural membrane active peptides and proteins, we sought to investigate their bioactivity. Specifically, we were interested in verifying their antimicrobial activity and cytotoxicity. Hence, biological activity of peptide nanostructures **1** and **3** was tested against bacteria and mammalian cells. No antimicrobial activity was found for peptide **1** with several bacteria, both gram+ and gram− bacteria. It appears that presence of positive charges along the peptide backbone is necessary for antimicrobial activity.

On the other hand, cytotoxicity of different crown peptides was measured against breast cancer cells (MDA) and mouse leukemia cells (P388). Results are reported in Table 2. Interestingly, 21-mer **1** and **3** peptides showed cytotoxic activities against MDA and P388 cells, while **30**, the 7-residue analogue of **1**, and **17**, the monomeric crown ether modified amino acid, were totally inactive. These results point out the necessity of having nanostructures of appropriate length (3–4 nm) in order to efficiently form a membrane pore and become cytotoxic agents. Noteworthy of mention, the deprotected analogue of **1** bearing charged head groups is much less active than **1** itself (last two entries of Table 2). These charged compounds were also shown not to incorporate easily bilayer membrane (unpublished results).

3. Conclusion

We have described a simple and efficient strategy to prepare new nanoscale devices with well defined molecular architectures that serve as functional synthetic ion channels. The results reported illustrate well the utility of peptide nanostructures as scaffolds for the construction of molecular devices.[43] The studied hexacrown peptides **1**–**5** keep an helical conformation independently of the crown ether size or the environment (MeOH, TFE, DCE or bilayer membranes). Furthermore, they displayed variable ion transport abilities across membranes in different experiments (pH-stat, patch clamp and ^{23}Na NMR). Also some ion selectivity was observed in the ^{23}Na NMR experiments, strongly suggesting that ions pass through the channel formed by crown array and not through defects in bilayers caused by undefined peptide aggregates. Vesicles lysis assays have demonstrated that the activity observed does not come from membrane disruption. Conformational and orientational studies led us to propose a gating mechanism for the channel activity of compound **1** and its analogues. On the other hand, peptides **1** and **3** showed very interesting cytotoxic activities towards cancer cells. Taking advantage of the simplicity and versatility of our synthetic strategy, we are currently synthesizing improved analogues of **1** with different polar heads and reporter groups at C- and N-termini. Finally, in addition to our ongoing fundamental investigation of these peptide nanostructures, we are also working on the engineering of **1** into workable devices for the detection of important analytes at the molecular level and continuing their bioactivity investigation.

Table 2. Cytotoxicity of different peptide nanostructures on cancer cells

Compd	MDA LD_{50} (μM)	P388 LD_{50} (μM)
Gramicidin D	0.2	4.0
BOC-7mer-OMe(21-C-7) **30**	ND[a]	35.0
BOC-21mer-Ome(21-C-7) **1**	15.0	8.5
BOC-21mer-Ome(15-C-5) **3**	10.0	2.5
BOC-21-C-7-OMe **17**	ND	ND
H-21mer-EDA(21-C-7)[b]	15.0	ND
Pyr-21mer-OH(21-C-7)[c]	>30.0	>40.0

[a] ND, not determined due to low toxicity.
[b] Pyr, pyromellitic acid.
[c] EDA, ethylene diamine.

4. Experimental

4.1. General

Oxime resin was prepared according to a reported procedure[44] using polystyrene beads (100–200 mesh, 1% DVB, Advanced ChemTech, Louisville, KY, USA). Resins with substitution levels of around 0.5 mmol per gram of oxime group were used. Boc-protected amino acids were purchased from Advanced ChemTech. All solvents were Reagent, Spectro, or HPLC grade quality purchased commercially and used without any further purification except for DMF (degassed with N_2), dichloromethane (distilled), and diethyl ether (distilled from sodium and benzophenone). Water used throughout the studies was distilled and deionized using a Barnstead NANOpurII system (Boston, MA, USA) with four purification columns. Phosphatidylglycerol (20 mg/mL solution in $CHCl_3$), phosphatidylcholine (20 mg/mL solution in $CHCl_3$) were purchased from Avanti Polar-Lipids and used without further purification. D_2O was purchased from CDN Isotopes (Pointe-Claire, QC, Canada) and used without further purification. All other reagents were purchased from Sigma Aldrich Co. (Milwauke, WI, USA). Solid phase peptide synthesis was performed manually using solid-phase reaction vessels equipped with a coarse glass frit (ChemGlass, Vineland, NJ, USA). Purification of peptides was performed by reversed phase HPLC with a C_4 semi-preparative column (Phenomenex, Torrance, CA, USA). All solvents were degassed and gradients of A (H_2O/0.1% TFA) and B (49.9% CH_3CN/49.9% isopropanol/0.1% TFA) were used. ^{1}H NMR spectra were recorded on a Bruker AC-F-300 spectrometer. Sonication was done using a Branson water bath model 3510.

Mass spectra were obtained from the Mass Spectrometry Laboratory of the Faculty of Medecine at the University of Toronto, Toronto, Canada.

4.2. Crown ether synthesis

4.2.1. 1,17-Dibromo-3,6,9,12,15-pentaoxaheptadecane 6. Triphenylphosphine (92.9 g, 354 mmol) was suspended in acetonitrile (400 mL) under N_2 at 0 °C. Bromine (56.6 g, 354 mmol) was added during 45 min. Hexaethylene glycol (50.0 g, 177 mmol) was dissolved in acetonitrile (60 mL) and added dropwise. Reaction was stirred during 48 h at room temperature. The white residue was eliminated by filtration and the solvent was evaporated. The resulting pasty orange-coloured residue was extracted several times with hexane. After evaporation of the combined extracts, the dibromide was obtained as colourless oil (60.5 g, 84%). ^{1}H NMR ($CDCl_3$), δ 3.71–3.66 (t, J = 6.5 Hz, 4H, C$\underline{H_2}$–CH_2–Br), 3.53 (m, 16H, O–CH_2–CH_2–O), 3.37–3.33 (t, J = 6.5 Hz, 4H, CH_2–Br).

4.2.2. *N-t*-BOC-21-C-7-OMe 17. BOC-L-DOPA-OMe[45] (25.0 g, 80.3 mmol) was dissolved in DMF (800 mL) under N_2. Anhydrous cesium carbonate (26.2 g, 80.4 mmol) was added and the solution was brought to 60 °C. Dibromide **6** (32.8 g, 80.4 mmol) was added during 2 h and the suspension was stirred for 24 h at 60 °C. DMF was evaporated in vacuo and brown residue was dissolved in CH_2Cl_2 (200 mL) and washed with 1 N NaOH and NaCl satd. The organic phase was dried over $MgSO_4$ and filtered. The oil obtained was purified by flash column chromatography (silica gel; 2–6% MeOH in CH_2Cl_2). The compound was precipitated as a beige powder (20.5 g, 46%) by triturating in petroleum ether. ^{1}H NMR ($CDCl_3$), δ 6.80 (m, 1H, H Ar (H_2)), 6.75–6.65 (m, 2H, H Ar (H_5 + H_6)), 5.02 (d, J = 7.9 Hz, 1H, NH), 4.52 (m, 1H, αCH), 4.08–4.03 (m, 4H, CH_2–OAr), 3.86–3.82 (m, 4H, C$\underline{H_2}$–CH_2–OAr), 3.78–3.55 (m, 16H, O–CH_2–CH_2–O), 3.45 (s, 3H, OCH_3), 3.05–3.01 (m, 2H, βCH_2), 1.40 (s, 9H, H *t*-Bu). ESMS m/z (M^+) 558.

4.2.3. *N-t*-BOC-21-C-7-OH 22. The crown ether amino ester **17** (20.4 g, 36.6 mmol) was dissolved in MeOH (50 mL) and cooled to 0 °C. 1 N NaOH (60 mL) was added and the mixture was stirred at room temperature for 3 h. The alkaline solution was washed with diethyl ether and acidified to pH 2–3 with 1 N HCl. The product was extracted with CH_2Cl_2, dried over $MgSO_4$ and filtered. The compound was precipitated as a beige powder (17.6 g, 88%) by triturating in petroleum ether. ^{1}H NMR ($CDCl_3$), δ 6.78 (m, 1H, H Ar (H_2)), 6.65–6.60 (m, 2H, H Ar (H_5 + H_6)), 4.95 (d, J = 7.9 Hz, 1H, NH), 4.48 (m, 1H, αCH), 4.07 (m, 4H, CH_2–OAr), 3.85 (m, 4H, C$\underline{H_2}$–CH_2–OAr), 3.78–3.60 (m, 16H, O–CH_2–CH_2–O), 2.99 (m, 2H, βCH_2), 1.40 (s, 9H, H *t*-Bu). ESMS m/z (M^+) 543.

4.2.4. 1,14-Dibromo-3,6,9,12-tetraoxatetradecane 7. Prepared as described for compound **6** from pentaetylene glycol (66.2 g, 88%). ^{1}H NMR ($CDCl_3$), δ 3.82–3.77 (t, J = 6.5 Hz, 4H, C$\underline{H_2}$–CH_2–Br), 3.65 (m, 12H, O–CH_2–CH_2–O), 3.48–3.44 (t, J = 6.5 Hz, 4H, CH_2–Br).

4.2.5. *N-t*-BOC-18-C-6-OMe 18. Prepared as described for compound **17** using the dibromide **7** (44.5 g, 56%). ^{1}H NMR ($CDCl_3$), δ 6.80 (m, 1H, H Ar (H_2)), 6.69–6.61 (m, 2H, H Ar (H_5 + H_6)), 4.98 (d, J = 7.9 Hz, 1H, NH), 4.52 (m, 1H, αCH), 4.10 (m, 4H, CH_2-OAr), 3.88 (m, 4H, C$\underline{H_2}$–CH_2–OAr), 3.78-3.63 (m, 12H, O–CH_2–CH_2–O), 3.68 (s, 3H, OCH_3), 3.01 (m, 2H, βCH_2), 1.40 (s, 9H, H *t*-Bu). ESMS m/z ($M + Na^+$) 536.

4.2.6. *N-t*-BOC-18-C-6-OH 23. Prepared as described for compound **22** from crown ether amino ester **18** (38.9 g, 90%). ^{1}H NMR ($CDCl_3$), δ 6.77–6.70 (m, 3H, H Ar), 5.42 (m, 1H, OH), 5.20 (d, J = 7.9 Hz, 1H, NH), 4.48 (m, 1H, αCH), 4.22–3.92 (m, 4H, CH_2–OAr), 3.91–3.78 (m, 4H, C$\underline{H_2}$–CH_2–OAr), 3.77–3.68 (m, 12H, O–CH_2–CH_2–O), 3.01 (m, 2H, βCH_2), 1.40 (s, 9H, H *t*-Bu). ESMS m/z (M^+) 499.

4.2.7. 1,11-Dibromo-3,6,9-trioxaundecane 8. Prepared as described for compound **6** from tetraetylene glycol (51.5 g, 88%). ^{1}H NMR ($CDCl_3$), δ: 3.72–3.68 (t, J = 6.5 Hz, 4H, C$\underline{H_2}$–CH_2–Br), 3.56 (m, 8H, O–CH_2–CH_2–O), 3.39–3.34 (t, J = 6.5 Hz, 4H, CH_2–Br).

4.2.8. *N-t*-BOC-15-C-5-OMe 19. Prepared as described for compound **17** using the dibromide **8** (55.0 g, 78%). ^{1}H NMR ($CDCl_3$), δ 6.81 (m, 1H, H Ar (H_2)), 6.64–6.61 (m, 2H, H Ar (H_5 + H_6)), 4.95 (d, J = 7.9 Hz, 1H, NH), 4.53 (m, 1H, αCH), 4.11–4.08 (m, 4H, CH_2–OAr), 3.90–3.88 (m, 4H, C$\underline{H_2}$–CH_2–OAr), 3.75–3.69 (m, 8H, O–CH_2–CH_2–O), 3.68 (s, 3H, OCH_3), 2.98 (m, 2H, βCH_2), 1.41 (s, 9H, H *t*-Bu). ESMS m/z (M^+) 469.

4.2.9. *N-t*-BOC-15-C-5-OH 24. Prepared as described for compound **22** from crown ether amino ester **19** (43.7 g, 82%). ^{1}H NMR ($CDCl_3$), δ 6.78–6.69 (m, 3H, H Ar), 5.37 (m, 1H, OH), 5.03 (d, J = 7.7 Hz, 1H, NH), 4.51 (m, 1H, αCH), 4.09 (m, 4H, CH_2–OAr), 3.88 (m, 4H, C$\underline{H_2}$–CH_2–OAr), 3.75–3.73 (m, 8H, O–CH_2–CH_2–O), 3.02 (m, 2H, βCH_2), 1.41 (s, 9H, H *t*-Bu). ESMS m/z (M^+) 455.

4.2.10. 2-Triphenylmethoxy-1-chloroethane 9. Chloroethanol (21.8 mL, 326 mmol) was added to a flask containing CH_2Cl_2 (500 mL). Triethylamine (50 mL, 358 mmol), chlorotriphenylmethane (100.0 g, 358 mmol), and 4-dimethylaminopyridine (catalytic amount) were added and this resulting mixture was stirred 24 h at room temperature. The organic phase was washed with $NaHCO_3$ 5% and H_2O, then was dried over $MgSO_4$ and filtered. CH_2Cl_2 was evaporated and the solid obtained was recrystallized in EtOH to give white crystals (103.3 g, 98%). ^{1}H NMR ($CDCl_3$), δ 7.49–7.45 (d, J = 7.2 Hz, 6H, H Ar (H_2,H_6)), 7.33–7.22 (m, 9H, H Ar (H_3,H_4,H_5)), 3.60–3.57 (t, J = 5.9 Hz, 2H, CH_2–Cl), 3.40–3.36 (t, J = 5.9 Hz, 2H, CH_2–OTr). ESMS m/z (M^+) 322.

4.2.11. 1,9-Di(triphenylmethoxy)-3,7-dioxanonane 11. Sodium hydride (8.3 g, 346.3 mmol) was added to 1,3-propanediol (10 mL, 138.5 mmol) previously dissolved in DMF (250 mL). The mixture was stirred during 30 min at 50 °C. The temperature was lowered to 45 °C and

the protected chloroethanol **9** (98.4 g, 304.7 mmol), dissolved in DMF by sonication, was added to the reaction along with sodium iodide (catalytic amount). The mixture was stirred 48 h at 45 °C, then the reaction was quenched with water. The crude product was extracted three times with a solution of hexanes/diethyl ether 1:1. The organic phase was washed with satd NaCl and H_2O, dried over $MgSO_4$, filtered, and evaporated in vacuo to obtain a white solid (76.4 g, 85%). ^{1}H NMR ($CDCl_3$), δ 7.49–7.46 (d, J = 7.0 Hz, 12H, H Ar (H_2,H_6)), 7.30–7.18 (m, 18H, H Ar (H_3,H_4,H_5)), 3.64-3.59 (m, 8H, O–CH_2–CH_2–O), 3.23–3.20 (t, J = 5.3 Hz, 4H, O–C<u>H</u>$_2$–CH_2–C<u>H</u>$_2$–O), 1.95–1.87 (qn, J = 6.4 Hz, 2H, O–CH_2–C<u>H</u>$_2$–CH_2–O). ESMS m/z (M^+) 648.

4.2.12. 3,7-Dioxanonane-1,9-diol 13. Diprotected polyether **11** (75 g, 115 mmol) was dissolved in CH_2Cl_2. A solution of 6 N HCl/dioxane was added and the solution was stirred for 30 min. MeOH was added and the mixture was stirred until appearance of a white residue, which was filtered off. Evaporation led to yellow oil which was first purified by flash chromatography (silica gel; CH_2Cl_2/acetone). The resulting beige oil was distilled in vacuo (bp 118 °C at 1 mm Hg) to give **13** as a colourless oil (17.2 g, 90%). ^{1}H NMR ($CDCl_3$), δ 3.67–3.64 (t, J = 4.5 Hz, 4H, O–CH_2–C<u>H</u>$_2$–OH), 3.56–3.52 (t, J = 6.1 Hz, 4H, O–C<u>H</u>$_2$–CH_2–OH), 3.50–3.47 (t, J = 4.6 Hz, 4H, O–C<u>H</u>$_2$–CH_2–C<u>H</u>$_2$–O), 3.06 (s, 2H, OH), 1.86–1.77 (qn, J = 6.1 Hz, 2H, O–CH_2–C<u>H</u>$_2$–CH_2–O). ESMS m/z ($M + H^+$) 165.

4.2.13. 1,9-Ditosyl-3,7-dioxanonane 15. Diol **13** (17 g, 103 mmol) was dissolved in pyridine at 0 °C and tosyl chroride (43.2 g, 227 mmol) in pyridine was added. The mixture was stirred 4 h at 4 °C, then was transferred in a beaker filled with ice. 6 N HCl (500 mL) was added. The resulting mix was extracted with CH_2Cl_2 and the organic phase was washed with 2 N HCl and H_2O, dried over $MgSO_4$, and filtered. After evaporation of the solvent, **15** was obtained as a beige oil (48.1 g, 99%). ^{1}H NMR ($CDCl_3$), δ 7.78–7.77 (d, J = 8.4 Hz, 4H, H Ar (H_2,H_6)), 7.34–7.32 (d, J = 8.1 Hz, 4H, H Ar (H_3,H_5)), 4.14–4.11 (t, J = 4.7 Hz, 4H, O–CH_2–C<u>H</u>$_2$–OTs), 3.60–3.56 (t, J = 4.7 Hz, 4H, O–C<u>H</u>$_2$–CH_2–OTs), 3.44–3.40 (t, J = 6.3 Hz, 4H, O–C<u>H</u>$_2$–CH_2–C<u>H</u>$_2$–O), 2.43 (s, 6H, CH_3–Ar), 1.74–1.66 (qn, J = 6.1 Hz, 2H, O–CH_2–C<u>H</u>$_2$–CH_2–O). ESMS m/z (M^+) 472.

4.2.14. *N*-*t*-BOC-13-C-4-OMe 21. Prepared as described for compound **17** using the ditosylate **15** (24.6 g, 56%). ^{1}H NMR ($CDCl_3$), 6.85 (m, 1H, H Ar (H_2)), 6.70–6.65 (m, 2H, H Ar (H_5 + H_6)), 4.95 (d, J = 7.7 Hz, 1H, NH), 4.55 (m, 1H, αCH), 4.16 (t, 4H, O–C<u>H</u>$_2$–CH_2–C<u>H</u>$_2$–O), 4.05 (m, 4H, CH_2–OAr), 3.75 (m, 4H, C<u>H</u>$_2$–CH_2–OAr), 3.62 (s, 3H, OCH_3), 3.00 (m, 2H, βC<u>H</u>$_2$), 1.85–1.78 (qn, 2H, O–CH_2–C<u>H</u>$_2$–CH_2–O), 1.35 (s, 9H, H *t*-Bu). ESMS m/z (M^+) 439.

4.2.15. *N*-*t*-BOC-13-C-4-OH 26. Prepared as described for compound **22** using the crown ether amino ester **21** (21.1 g, 90%). ^{1}H NMR ($CDCl_3$), δ 6.88 (m, 1H, H Ar (H_2)), 6.79–6.75 (m, 2H, H Ar (H_5 + H_6)), 4.93 (d, J = 7.7 Hz, 1H, NH), 4.53 (m, 1H, αCH), 4.11 (t, 4H, O–C<u>H</u>$_2$–CH_2–C<u>H</u>$_2$–O), 3.77–3.75 (m, 8H, 2 CH_2–OAr + 2 C<u>H</u>$_2$–CH_2–OAr), 3.07 (m, 2H, βC<u>H</u>$_2$), 1.82–1.78 (qn, 2H, O–CH_2–C<u>H</u>$_2$–CH_2–O), 1.35 (s, 9H, H *t*-Bu). ESMS m/z (M^+) 425.

4.2.16. 3-Triphenylmethoxy-1-chloropropane 10. Prepared as described for compound **9** from 3-chloropropanol (104.3 g, 95%). ^{1}H NMR ($CDCl_3$), δ: 7.45–7.41 (d, J = 7.5 Hz, 6H, H Ar (H_2,H_6)), 7.33–7.21 (m, 9H, H Ar (H_3,H_4,H_5)), 3.73–3.68 (t, J = 6.7 Hz, 2H, CH_2–Cl), 3.24–3.21 (t, J = 5.9 Hz, 2H, CH_2–OTr), 2.07–1.99 (qn, J = 6.2 Hz, 2H, CL–CH_2–C<u>H</u>$_2$–CH_2–O).

4.2.17. 1,10-Di(triphenylmethoxy)-4,7-dioxadecane 12. Prepared as described for compound **11** from ethylene glycol and the protected chloropropanol **10** (59.1 g, 60%). ^{1}H NMR ($CDCl_3$), δ 7.49–7.46 (d, 12H, H Ar (H_2,H_6)), 7.30–7.18 (m, 18H, H Ar (H_3,H_4,H_5)), 3.64–3.59 (t, 4H, O–CH_2–CH_2–O), 3.35–3.30 (t, 4H, O–C<u>H</u>$_2$–CH_2–CH_2–OTr), 3.23–3.20 (t, 4H, O–CH_2–CH_2–C<u>H</u>$_2$–OTr), 1.95–1.90 (qn, 4H, O–CH_2–C<u>H</u>$_2$–CH_2–O).

4.2.18. 4,7-Dioxadecane-1,10-diol 14. Prepared as described for compound **13** using the diprotected polyether **12** (12.7 g, 80%). ^{1}H NMR ($CDCl_3$), δ 3.67–3.64 (t, 4H, O–CH_2–CH_2–O), 3.56–3.52 (t, 4H, O–CH_2–CH_2–C<u>H</u>$_2$–OH), 3.50–3.47 (t, 4H, O–C<u>H</u>$_2$–CH_2–CH_2–OH), 3.06 (s, 2H, OH), 1.95–1.90 (qn, 4H, O–CH_2–C<u>H</u>$_2$–CH_2–O).

4.2.19. 1,10-Ditosyl-4,7-dioxadecane 16. Prepared as described for compound **15** from diol **14** (31.0 g, 95%). ^{1}H NMR ($CDCl_3$), δ 7.79–7.76 (d, 4H, H Ar (H_2,H_6)), 7.33–7.30 (d, 4H, H Ar (H_3,H_5)), 4.14–4.11 (t, 4H, O–CH_2–CH_2–O), 3.60–3.56 (t, 4H, O–CH_2–CH_2–C<u>H</u>$_2$–OTs), 3.44–3.40 (t, 4H, O–C<u>H</u>$_2$–CH_2–CH_2–OTs), 2.43 (s, 6H, CH_3–Ar), 1.72–1.64 (qn, 4H, O–CH_2–C<u>H</u>$_2$–CH_2-O).

4.2.20. *N*-*t*-BOC-14-C-4-OMe 20. Prepared as described for compound **17** using the ditosylate **16** (14.2 g, 49%). ^{1}H NMR ($CDCl_3$), δ 6.83 (m, 1H, H Ar (H_2)), 6.70–6.63 m, 2H, H Ar ($H_5 + H_6$)), 4.98 (d, J = 7.7 Hz, 1H, NH), 4.58 (m, 1H, αCH), 4.26 (t, 4H, O–CH_2–CH_2–O), 4.05 (m, 4H, CH_2–OAr), 3.75 (m, 4H, C<u>H</u>$_2$–CH_2–CH_2–OAr), 3.62 (s, 3H, OCH_3), 3.00 (m, 2H, βC<u>H</u>$_2$), 1.84–1.78 (qn, 4H, O–CH_2–C<u>H</u>$_2$–CH_2–O), 1.35 (s, 9H, H *t*-Bu).

4.2.21. *N*-*t*-BOC-14-C-4-OH 25. Prepared as described for compound **22** using the crown ether amino ester **20** (11.5 g, 85%). ^{1}H NMR ($CDCl_3$), δ 6.83 (m, 1H, H Ar (H_2)), 6.70–6.63 (m, 2H, H Ar ($H_5 + H_6$)), 5.00 (d, J = 7.7 Hz, 1H, NH), 4.59 (m, 1H, αCH), 4.27 (t, 4H, O–CH_2–CH_2–O), 4.09 (m, 4H, CH_2–OAr), 3.76 (m, 4H, C<u>H</u>$_2$–CH_2–CH_2–OAr), 3.00 (m, 2H, βC<u>H</u>$_2$), 1.84–1.76 (qn, 4H, O–CH_2–C<u>H</u>$_2$–CH_2–O), 1.35 (s, 9H, H *t*-Bu). ESMS m/z ($M + NH_4^+$) 457.

4.3. Solid-phase synthesis

4.3.1. Typical procedure for amino acid coupling on solid support. The amino acid (5 equiv) was activated with

DIC/HOBt during 30 min at 0C in CH_2Cl_2/DMF (1:1), then added to the resin swollen in DMF, followed by addition of 1.5 equiv of DIEA. The mixture was shaken mechanically for 2 h at room temperature. The resin was filtered and washed thoroughly with DMF (3×50 mL), MeOH (3×50 mL), DMF (3×50 mL), MeOH (3×mL) and then dried in vacuo. The *N*-BOC group was deprotected by a 30 min treatment with a 50% CF_3COOH solution in DCM. The completion of coupling reactions was monitored by the ninhydrin test. When necessary, a second coupling was performed under the same conditions.

4.3.2. Crown peptide nanostructures. All hexacrown peptides have been synthesized, purified, and characterized in the same manner using appropriate crown ether amino acids. The following synthesis of nanostructure **1** is illustrative of all synthesis.

4.3.3. *N*-*t*-BOC-L-27-L-L-L-27-L-Resin 28. The key heptapeptide was prepared using 5 g of oxime resin. Coupling reactions were performed as described above with *N*-*t*-BOC-Leu-OH and *N*-*t*-BOC-**6**-OH.[20]

4.3.4. *N*-*t*-BOC-L-27-L-L-L-27-L-OH 29. Cleavage of the key heptapeptide from 4 g of resin was realized using a 10% H_2O/THF solution with 2 equiv of DBU at room temperature for 2 h. The solution was recovered and after evaporation of solvent in vacuo, the crude peptide was dissolved in DCM and washed twice with 0.5 N HCl and water. After drying with $MgSO_4$, solvent was evaporated and residue triturated with anhydrous ether and petroleum ether to give 2.1 g of a beige powder pure enough by HPLC to be used as is in the next step. ^{1}H NMR (DMSO-d_6), δ 8.15 (d, J = 8.2 Hz, 1H, NH), 8.05 (d, J = 8.3 Hz, 1H, NH), 7.98 (d, J = 7.8 Hz, 1H, NH), 7.86 (d, J = 8.0 Hz, 1H, NH), 7.72 (d, J = 7.9 Hz, 1H, NH), 7.65 (d, J = 7.8 Hz, 1H, NH), 6.97 (d, J = 8.1 Hz, 1H, NH-BOC), 6.77–6.63 (m, 6H, H Ar), 4.50–4.48 (m, 2H, αCH 21-C-7), 4.33–4.20 (m, 4H, Leu αCH), 4.10–3.90 (m, 8H, CH_2–OAr), 3.84 (m, 1H, Leu-BOC αCH), 3.79-3.65 (m, 8H, CH_2–CH_2–OAr), 3.64–3.44 (m, 32H, CH_2–O), 3.00–2.59 (m, 4H, βCH_2 21-C-7), 1.63–1.22 (m, 24H, 5 Leu βCH_2 + 5 Leu γCH + 9H *t*-Bu), 0.88–0.75 (m, 30H, Leu CH_3). ESMS m/z ($M + H^+$) 1535.

4.3.5. *N*-*t*-BOC-L-27-L-L-L-27-L-OMe 30. Cleavage from 0.2 g of resin **28** was performed using the same protocol as above but using MeOH as solvent. Cleavage yielded 55 mg of **30** as a beige powder. ^{1}H NMR (DMSO-d_6), δ 8.31–7.62 (m, 6H, NH), 6.97 (m, 1H, NH-BOC), 6.80–6.63 (m, 6H, H Ar), 4.55–4.47 (m, 2H, αCH 21-C-7), 4.33–4.20 (m, 4H, Leu αCH), 4.10–3.90 (m, 8H, CH_2–OAr), 3.84 (m, 1H, Leu-BOC αCH), 3.79–3.65 (m, 8H, CH_2–CH_2–OAr), 3.64–3.44 (m, 35H, 16 CH_2-O + CH_3–O), 3.00–2.59 (m, 4H, βCH_2 21-C-7), 1.63–1.22 (m, 24H, 5 Leu βCH_2 + 5 Leu γCH + 9H *t*-Bu), 0.88–0.75 (m, 30H, Leu CH_3). ESMS m/z ($M + H)^+$ 1549.

4.3.6. *N*-*t*-BOC-(L-27-L-L-L-27-L)$_2$-resin. The key heptapeptide **29** (1.1 g, 0.72 mmol) was activated with DIC/HOBt during 45 min at 0 °C in CH_2Cl_2/DMF (1:1), and then added with 1.5 equiv of DIEA to the deprotected heptapeptide on resin **28** (0.8 g, 0.32 mmol) swollen with DMF. The mixture was shaken mechanically for 4 h at room temperature. The resin was drained and washed as described above.

4.3.7. *N*-*t*-BOC-(L-27-L-L-L-27-L)$_2$-OMe 31. Cleavage of the 14mer peptide from 300 mg of resin was done in MeOH using 2 equiv of DBU. After recovering the solution and evaporation, purification was effected by reversed-phase HPLC (0–100% B in 45 min). Solvents were removed in vacuo to give a colorless oil that was dissolved in glacial acetic acid, and lyophilized to yield 52 mg of a fluffy white solid. ^{1}H NMR (DMSO-d_6), δ 8.20–7.30 (m, 13H, NH), 6.85–6.50 (m, 12H, H Ar), 4.50 (m, 4H, αCH 21-C-7), 4.25–3.95 (m, 26H, 10 Leu αCH + 8 CH_2–OAr), 3.95–3.50 (m, 83H, 40 CH_2–O + CH_3–O), 3.35–3.00 (m, 8H, βCH_2 21-C-7), 2.05–1.35 (m, 39H, 10 Leu βCH_2 + 10 Leu γCH + 9H *t*-Bu), 1.05–0.70 (m, 60H, Leu CH_3). ESMS m/z ($M + Na)^+$ 2988.

4.3.8. *N*-*t*-BOC-(L-27-L-L-L-27-L)$_3$-OMe 1. The key heptapeptide **29** (0.8 g, 0.5 mmol) was activated and added with 1.5 equiv of DIEA to the deprotected 14mer on resin (0.5 g, 0.2 mmol) swollen in DMF. The mixture was shaken mechanically for 4 h at room temperature. After washing and drying, cleavage of the 21mer peptide from 500 mg of resin was performed in MeOH using 2 equiv of DBU. The cleavage solution was recovered and evaporated to give crude **1**. Purification was done by reversed-phase HPLC (0–100% B in 45 min). Solvents were removed in vacuo to give a colorless oil that was dissolved in acetic acid, and lyophilized to yield 31 mg of **1** as a fluffy white solid. ^{1}H RMN (300 MHz, $CDCl_3$), δ 8.30–7.40 (m, 21H, NH), 6.85–6.60 (m, 18H, H Ar), 4.35–4.25 (m, 6H, αCH 21-C-7), 4.10–3.85 (m, 24H, 8 CH_2–OAr), 3.75–3.60 (m, 24H, 40 CH_2–CH_2–OAr), 3.60–3.40 (m, 114H, 48 CH_2–O + 15 Leu αCH + CH_3–O), 3.10–2.90 (m, 12H, βCH_2 21-C-7), 1.65–1.35 (m, 54H, 15 Leu βCH_2 + 15 Leu γCH + 9H *t*-Bu), 0.95–0.65 (m, 90H, Leu CH_3). ESMS m/z ($M + Na)^+$ 4406, ($M + Na)^+$ 4406.

4.4. Ion transport assays

4.4.1. pH stat experiments. The pH stat method and the preparation of vesicles have been described in details by Fyles et al.[25,30] Briefly, vesicles were prepared by the sonication of an ethereal solution of an 8:1:1 mixture of egg phosphatidyl choline/egg phosphatidic acid/cholesterol in a pH 6.6 buffer solution (0.2 M choline sulphate and 0.054 M D-mannitol in water). After evaporation of ether, vesicles were suspended in an unbuffered solution and purified by filtration through a 0.45-μm syringe filter to remove large aggregates and by gel filtration on Sephadex G-25. Vesicles are predominantly unilamellar with a diameter of around 150 nm and contain a small portion of smaller (50 nm) unilamellar vesicles and multilamellar vesicles. In a typical experiment, 0.2 mL of a fresh vesicle dispersion was added to 3.8 mL of the external solution (0.11 M choline sulphate and 0.093 M D-mannitol in water) and the pH was raised to 7.6 by

the addition of a choline hydroxide solution. At 5-min intervals, the following solutions were added: 10 μL of a 1-mM MeOH solution of FCCP,[31] 0.5 mL of Cs_2SO_4 (0.5 M), and 10 μL of a 0.275 mM MeOH solution (2.75 mmol) of a transporter. Release of protons starts at this point and pH is maintained at 7.5 by addition of 4.75 mM choline hydroxide solution using an automated titrator. After 1 h, the remaining entrapped protons are released by lysis of the vesicles by addition of Triton X-100. The experiments were performed under a stream of N_2.

4.4.2. Na^+ transport experiments using ^{23}Na NMR. To prepare vesicles, the procedure described by Gokel[38] was followed with some modifications. At room temperature, 13 mg of phosphatidylglycerol and 5.2 mg of phosphatidylcholine were diluted in 25 mL of $CHCl_3$. The solvent was evaporated in vacuo and lipids were dried for 12 h. Under nitrogen, the lipid film was dissolved in 25 mL of diethyl ether. 10 mL of buffer solution was added. The buffer solution has a total Na^+ concentration of 100 mM and is composed of NaCl (98.8 mM), KCl (0.26 mM), Na_2HPO_4 (0.64 mM) and KH_2PO_4 (0.14 mM) in water at pH 7.3. The two-phase suspension was placed in water bath sonicator at 2–5 °C and sonicated during 40 min. The organic phase was evaporated under reduced pressure. The resulting vesicle solution was filtrated through a polycarbonate membrane (Poretics, 0.4 μm). In a Eppendorf tube, 0.8 mL of vesicle solution was mixed with an appropriate amount (1–10 μL) of a TFE solution of transporter (5–40 mM). Sample was warmed at 60 °C during 60 min. Then, 0.1 mL of D_2O and 0.1 mL of the shift reagent solution (sodium tripolyphosphate (0.133 M) and $DyCl_3$ (0.033 M) in water) were added. After stirring, the sample was transferred in a NMR tube for study.

4.4.3. NMR measurements and rate constant. ^{23}Na NMR spectra were recorded on a Varian XL-200 NMR spectrometer operating at 52.9 MHz. Typically, 1800 FID transients were accumulated per data set at a probe temperature of 25 °C. For each potential transporter incorporated into phospholipid membranes, any line broadening was determined. The rate constant $k = 1/\tau$, is directly proportional to the observed line broadening, $= \pi(\Delta\nu - \Delta\nu_0)$, and is determined from the slope of a plot of $1/\tau$ versus transporter concentration.

4.4.4. Fluorescence vesicle lysis experiments. For fluorescence measurements used to study vesicle lysis, the procedure described by Lafleur et al.[46] was followed with some modifications. A calcein solution was prepared by dilution calcein in internal buffer (100 mM HEPES, 30 mM NaCl and 5 mM EDTA) to obtain a calcein concentration of 80 mM. The pH of the solution was adjusted to 7.4 with a 1 N NaOH solution. At room temperature, 13 mg of phosphatidylglycerol and 5.2 mg of phosphatidylcholine were diluted in 25 mL of $CHCl_3$. Solvent was evaporated in vacuo and lipids were dried for 12 h. Calcein solution (5 mL) was added to the lipid film. The resulting mixture was placed in water bath sonicator at 2–5 °C and sonicated during 40 min. Vesicles were filtrated through a polycarbonate membrane (Poretics, 0.4 μm). Calcein-containing vesicles were separated from the free calcein by exclusion chromatography using a column filled with Sephadex G-50 superfine gel swollen in the external buffer (100 mM HEPES, 170 mM NaCl and 5 mM EDTA at pH 7.4). Fluorescence measurements were performed on a Varian Cary Eclipse spectrometer. Fluorescence intensity of calcein was monitored at emission wavelength of 513 nm (response time of 0.5 s) after excitation at 490 nm. The temperature was fixed to 25 °C. In a quartz cuve of 1 cm, 4 mL of external buffer solution and 50 μL of vesicle solution were introduced. Stirring and acquisition were started. After 50 s, an appropriate amount (1–10 μL) of a TFE solution of transporter (5–40 mM) was added. Then, after 400 s, a 10% Triton X-100 solution was added and experiment was terminated after 500 s.

4.5. Biological activity assays

4.5.1. Antimicrobial assays. Bacterial strains used were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27855 and *Streptococcus pneumoniae* ATCC 6303. After determination of cell tolerance to DMSO or MeOH, solutions of crown peptides were prepared in these solvents. Water was added to obtain an amount of 2% of DMSO, or 4% of MeOH. Compounds were added to Petri dish infected with bacterial strains and incubated for 24 h at 37 °C. Visual inspection of dish showed no significant antimicrobial activity.

4.5.2. Cytotoxicity experiments. Using the same compounds solutions, serial dilutions of stock solutions were realized. Cancer cells used were MDA MB1231 (breast cancer) and P388 D1 (mouse leukemia). Cells were disposed into wells of microtiter plates that contained RPMI 1640 culture medium. Then, the different compound solutions were added. After incubation during 72 h at 37 °C, compound efficiency was determined by treatment with 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and absorbance measurement at 570 nm.

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