Synthesis, characterization and cytolytic activity of α-helical amphiphilic peptide nanostructures containing crown ethers†

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Many natural α-helical amphiphilic peptides are known to have lytic activity toward different cells. Herein, we describe the synthesis and the characterization of synthetic α-helical amphiphilic peptide nanostructures containing crown ethers, as well as the modulation of their cytolytic activity by adding different acidic dipeptide chains at the N- or C-terminus.

Introduction

α-Helical amphiphilic peptides have been shown to have lytic activity towards a wide range of cells: gram-positive and gram-negative bacteria, fungi, eukaryotic mammalian and cancer cells.[1] Lytic and antimicrobial activities of these peptides is still not completely understood and these activities depend on many factors: capacity to form amphiphilic structures, net charge, hydrophobicity/hydrophilicity balance, molecule length and amino acid sequence.[2] To obtain lytic peptides, research groups have exploited different methods: synthesis of natural peptide analogues,[3] use of β-peptides,[4] use of cyclic peptides composed of D and L amino acids,[5] and using simple amphiphilic peptides.[6]

Previously, we have reported functional studies on a series of peptides with a repeating sequence of seven amino acids.[7–13] The repeating sequence contains polar but uncharged residues bearing a benzo-21-crown-7 ring spaced in such a way that upon formation of an α-helical secondary structure, the crown ether side chains are aligned on one side of the peptidic backbone.[14] The opposite face of such nanoscale peptide structures is lined by hydrophobic side-chains of leucine (Leu), an amino acid recognized for its strong propensity to induce α-helical conformation, therefore confering an amphiphilic character to such peptide nanostructures.

Results showed that the peptide consisting of three repeating sequences of the seven amino acids (21-mer) acts as an artificial ion channel[8–11,13] capable of facilitating the transport of monovalent cations across bilayer membrane in different assays (pH-stat, patch clamp and ^{23}Na NMR).[13,15] The peptide consisting of one repeating sequence (7-mer) acted as a typical ion carrier and not as an ion channel.[8] A 14-residue peptide with two heptapeptide sequences such as **1** interacts with bilayer membranes in a very different way; it is a powerful membrane-disrupting agent that promotes the leakage of large fluorescent dyes from vesicles, and haemoglobin from erythrocytes.[7] In an attempt to develop improved peptide nanostructures with tunable lytic ability, we describe herein the synthesis of a family of 14-residue peptide analogues, as well as studies of their conformational and lytic activity behaviors.

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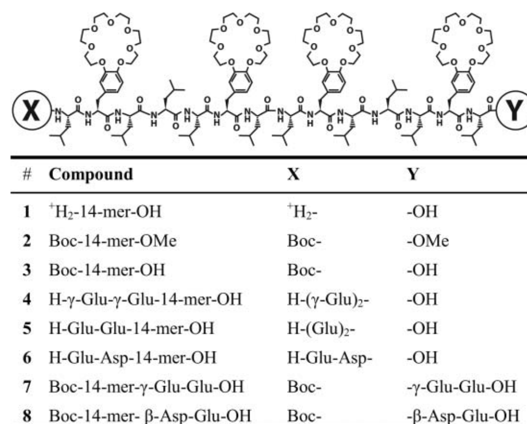
Results

Design of peptide nanostructures

Past studies have shown that terminal groups on 14-mer molecules have an effect on leakage induced on lipid bilayers.[7] Therefore to modulate lytic activity of such compounds, we have added at the N- or C-terminus different short negatively-charged dipeptide chains (**1–8**, Fig. 1). These chains are formed by a combination of glutamic (Glu) and/or aspartic (Asp) acid, linked by an α, β or γ peptidic bond.



#	Compound	X	Y
1	$^{+}H_2$-14-mer-OH	$^{+}H_2$-	-OH
2	Boc-14-mer-OMe	Boc-	-OMe
3	Boc-14-mer-OH	Boc-	-OH
4	H-γ-Glu-γ-Glu-14-mer-OH	H-(γ-Glu)$_2$-	-OH
5	H-Glu-Glu-14-mer-OH	H-(Glu)$_2$-	-OH
6	H-Glu-Asp-14-mer-OH	H-Glu-Asp-	-OH
7	Boc-14-mer-γ-Glu-Glu-OH	Boc-	-γ-Glu-Glu-OH
8	Boc-14-mer- β-Asp-Glu-OH	Boc-	-β-Asp-Glu-OH

Fig. 1 Peptide nanostructures used in the present investigation.

Synthesis

The synthetic approach used exploits a combination of solution and solid phase synthesis. The general synthetic strategy is divided in four parts: the solution synthesis of benzo-21-crown-7 ether modified amino acid[12] (*N*-Boc-3-(3,4-(21-crown-7)-phenyl)-L-alanine; CE), the solid phase synthesis of a 14-mer peptide, the solution synthesis of Asp/Glu chains, and the chain coupling followed by the cleavage-deprotection of the desired peptides.

There are several advantages to this strategy. First, 14-mer peptides can be prepared by dimerization of a heptapeptide segment on a solid support, facilitating synthesis and purification. Second, post-synthesis modifications of the core 14-mer peptide and end group engineering[16] can be selectively, easily and efficiently done. Third, the Asp/Glu chains synthesis is convergent, so each

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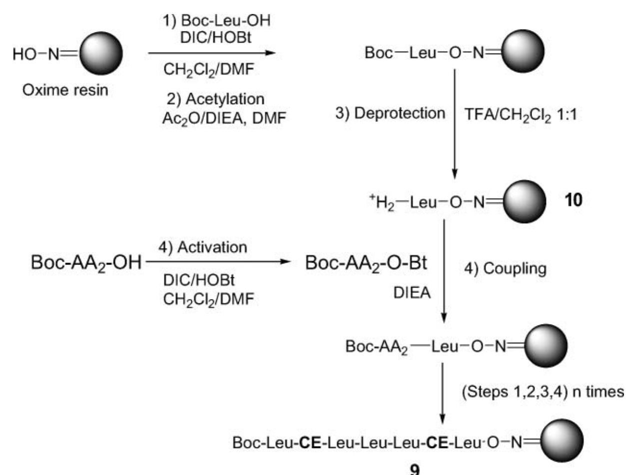
chain can be coupled to the N- or C-terminus of the core peptide. Finally, solid phase synthesis is well developed and allows rapid, parallel preparation of analogues on a relatively large scale.

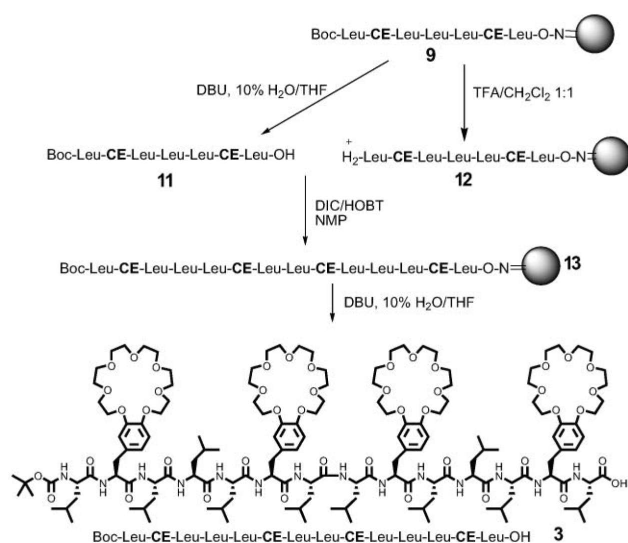
Synthesis of the 14-mer core peptide

The 14-mer peptide was obtained by condensation of two heptameric segments on the oxime resin, used as solid support. Coupling reactions to prepare the heptapeptide **9** were performed using *N*-Boc amino acids and *N*,*N*′-diisopropylcarbodiimide–2-hydroxybenzotriazole (DIC–HOBt) as reagents in CH_2Cl_2–DMF (1 : 1), as shown in Scheme 1. The first leucine was activated with DIC at 0 °C and then HOBt was added and the resulting solution was stirred for 30 min. The solution was added to the resin swollen in DMF and was shaken mechanically for 24 h at room temperature. After acetylation of an unsubtituted oxime group by acetic anhydride, the *N*-Boc group was removed by using 50% CF_3COOH (TFA) in CH_2Cl_2 to yield **10**. The substitution level was determined by a quantitative nynhydrin test[17] and ranged from 0.5 to 0.6 mmol of oxime group per gram of resin. The second amino acid bearing a crown ether ring and the subsequent amino acids were coupled under the same conditions but with a 2 h shaking time. The completion of couplings was monitored by a qualitative nynhydrin test.



Scheme 1 Synthesis of *N*-Boc protected 7-mer on the oxime resin. (CE = 21-crown-7-L-Phe).

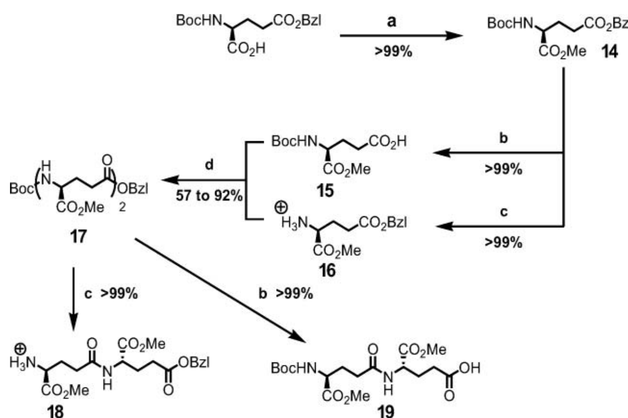
As shown in Scheme 2, 70% of the resin that contained heptapeptide **9** on solid support was cleaved by a mixture of THF and water (9 : 1) in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to generate the *N*-Boc heptapeptide acid **11**. Coupling of this segment on the deprotected heptapeptide on solid support (**12**) was performed using DIC–HOBt in 1-methyl-2-pyrrolidone (NMP) to yield the 14-mer on solid support **13**. Half of the 14-mer on solid support was cleaved to obtain Boc–14-mer–OH **3**, using the previously described procedure, and then different Asp/Glu chains were coupled to the C-terminus. The remaining 14-mer on the solid support was used for subsequent Asp/Glu chains coupling to the N-terminus after deprotection of the *N*-Boc group. It should be noted that segment coupling reactions under the conditions used proceed with low levels of epimerization.[12] Indeed, analytical HPLC demonstrated that less than 5% epimerization occurs during the coupling of **11** to **12**.



Scheme 2 Synthesis of *N*-Boc protected 14-mer **3** by a heptapeptide segment condensation on solid support.

Synthesis of Asp/Glu chains

Using a convergent strategy, several Asp/Glu chains were synthesized. The general pathway for the synthesis of all dipeptides is shown in Scheme 3. The procedure was applied for each Asp/Glu chain used in this investigation. As mentioned previously, chains are composed of two residues, glutamic and/or aspartic acid, linked by an α, β or γ peptidic bond, leading to several possibilities.



Scheme 3 A representative synthesis of a dipeptide chain: (a) CH_2N_2, Et_2O; (b) H_2, 10% Pd/C, MeOH; (c) 4M HCl, dioxane; (d) EDC·HCl, HOBt, NEt_3, CH_2Cl_2.

The first step in the synthesis is the methylation of the free carboxylic acid of the diprotected peptide by diazomethane (CH_2N_2) formed *in situ* from Diazald©.[18] The product **14**, obtained in a quantitative yield, was then divided in two parts. A catalytic hydrogenation using 10% palladium on activated carbon (Pd/C) was used on one part to liberate the carboxylic acid of the side chain (**15**). The *N*-Boc group of the second part was removed using 4M HCl in dioxane to yield **16**. Reaction of **15** with **16** using 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC·HCl) as the condensing agent resulted in the fully protected dipeptide compound **17**. To allow the coupling

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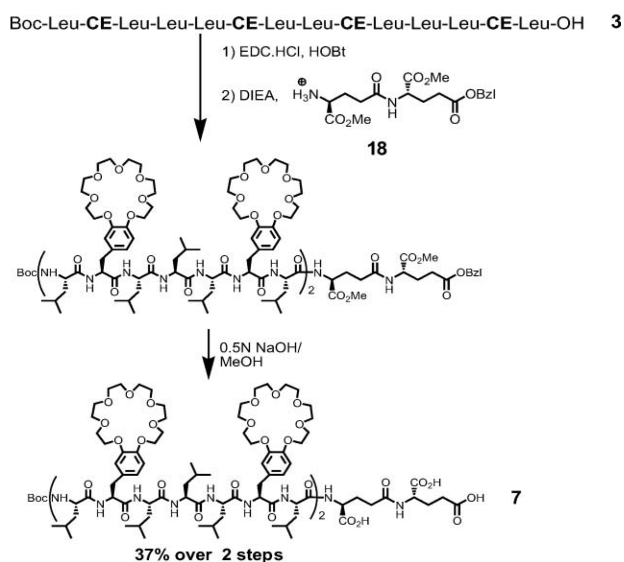
at the N- or C-terminus of the 14-mer core peptide, half of the product **17** obtained was treated with HCl in dioxane to cleave the Boc group (**18**). Likewise, hydrogenation of **17** led to the free carboxylic acid **19**, which is ready to be coupled to the N-terminus of the 14-mer peptide.

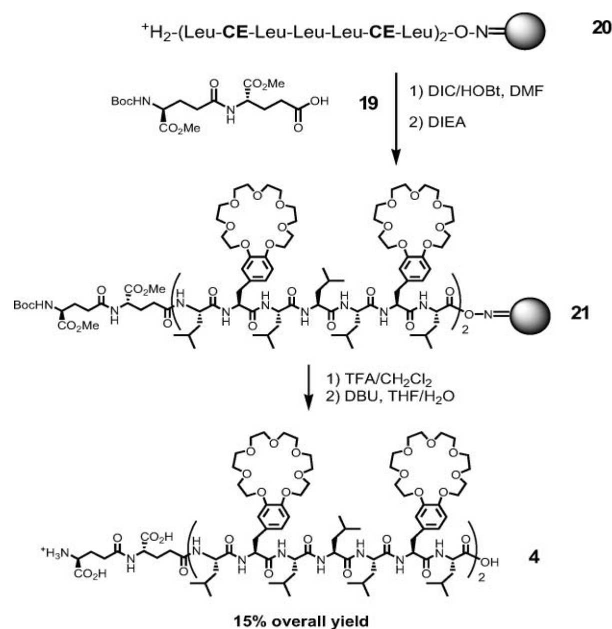
Coupling of Asp/Glu chains to 14-mer, cleavage and deprotection

Coupling of the Asp/Glu chain **18** at C-terminus of the core 14-mer **3** was done in solution after activation of the carboxylic acid with EDC·HCl (Scheme 4). Hydrolysis of methyl and benzyl esters with 0.5N NaOH in MeOH led to **7** with a 37% yield for the last two steps.



Scheme 4 Coupling of γ-Glu-Glu chain at the C-terminus of the 14-mer **3** and final deprotection.

Coupling at the N-terminus was done on solid phase with usual conditions. Dipeptide acid **19** was activated with DIC/HOBt in DMF and then added to the 14-mer on solid support, previously deprotected with 50% TFA in CH_2Cl_2 **20**. The N-Boc group of the 16-mer peptide obtained was removed by treatment with TFA. Finally, deprotected 16-mer peptide was cleaved and the methyl ester group was hydrolyzed in one step by the action of 10% water in THF with DBU to give **4** with a 15% overall yield as shown in Scheme 5. In this case, NaOH treatment was not necessary. Purification of all peptide nanostructures presented in Fig. 1 was achieved by reversed-phase HPLC and they were characterized successfully by ^{1}H NMR and mass spectrometry.



Scheme 5 Coupling of a γ-Glu-γ-Glu chain to the N-terminus of the 14-mer core peptide, cleavage and deprotection.

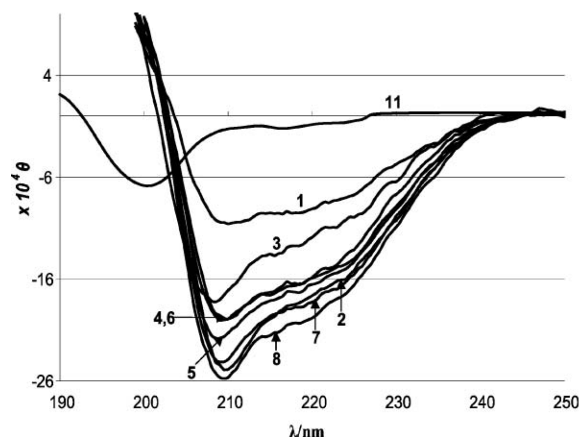


Fig. 2 CD spectra of peptide nanostructures **1–8** and **11** in 2,2,2-trifluoroethanol (TFE) at 25 °C at 0.28 mM. (θ = mdeg cm^2 dmol^{-1}).

Conformational studies

The solution conformation of peptide nanostructures was evaluated by circular dichroism spectropolarimetry (CD). Previous studies have shown that Boc–14-mer–OH **3** adopts an α-helical conformation in different media and at different concentrations. The fully protected, Boc–14-mer–OMe **2** CD spectrum (Fig. 2) indicates also a preference for an α-helical conformation, under which the crown ether units are aligned on the same side of the peptidic framework.

Likewise, results show that all peptide nanostructures synthesized preferentially adopt an α-helical conformation but not the shortest, Boc–7-mer–OH **11**. As seen by the enhanced absorption at 222 nm as compared with **3**, presence of a glutamic and/or aspartic acid chain at an N- or C-terminus of the 14-mer core structure increases helicity. This increase of helicity can be attributed to the additional hydrogen bond formed by the Glu/Asp residues added. Also, in the case of **4–6**, this could be due to a favorable electrostatic effect between the negatively charged residues at the N-terminus and the helix macrodipole.[19] Capping the N-terminus with a methyl ester (**2**) also enhances helicity as demonstrated by an increasing band at 222 nm on CD spectra. However, deprotection of the amine protecting group (**1**) leads to a slight decrease in the helical conformation, probably in favor of a β-sheet. The latter results can also be the result of an unfavorable electrostatic effect of the positively charged N-terminus with the helix macrodipole and the loss of one stabilizing hydrogen bond.

Table 1 Calcein release from unilamellar vesicles[a] induced by peptide nanostructures at three different concentrations

	Compound	% Release 0.72 µM	1.25 µM	12.5 µM
—	Blank	1	2	2
1	$^{+}H_2$–14-mer–OH	74	77	80
2	Boc–14-mer–OMe	14	30	58
3	Boc–14-mer–OH	89	90	90
4	H–γ-Glu-γ-Glu–14-mer–OH	31	52	65
5	H–Glu-Glu–14-mer–OH	46	63	68
6	H–Glu-Asp–14-mer–OH	39	53	68
7	Boc–14-mer–γ-Glu-Glu–OH	85	86	95
8	Boc–14-mer–β-Asp-Glu–OH	75	89	95
11	Boc–7-mer–OH	2	1	3

[a] DOPC: Chol (70:30) vesicles.

Lytic activity

The membrane disruption ability of peptide nanostructures **1–8** and Boc-7-mer-OH **11** was investigated using a fluorescent vesicle lysis assay.[20] Table 1 reports the calcein release induced by addition of peptide nanostructures to unilamellar vesicles prepared from 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC) and cholesterol (Chol) (70:30), at three different concentrations.

Results can be compared to the model compound Boc–14-mer–OH **3**, which was our most studied and active cytolytic compound, showing strong lysis (90%) at the lowest concentration investigated. First, Boc–7-mer–OH **11** did not induce calcein release. When the Boc group in **3** was substituted by aspartic and/or glutamic chains (**4–6**), the calcein leakage decreased substantially. When the substitution is at the C-terminus (**7**, **8**), there is also a decrease in calcein release, but to a lower extent. A decrease of the vesicle permeability is also observed when negative charges at C-terminus are absent as in the case of **2**. Finally, zwitterrionic peptide **1** induces calcein leakage slightly less than was induced by model compound **3**, but **1** is also quite active. The concentration of the peptide nanostructure is also important for membrane disruption activity as expected. The leakage rate at any peptide concentration slows down with time as shown in Fig. 3. These results are consistent with a barrel-stave mechanism of cytolytic action.[21]

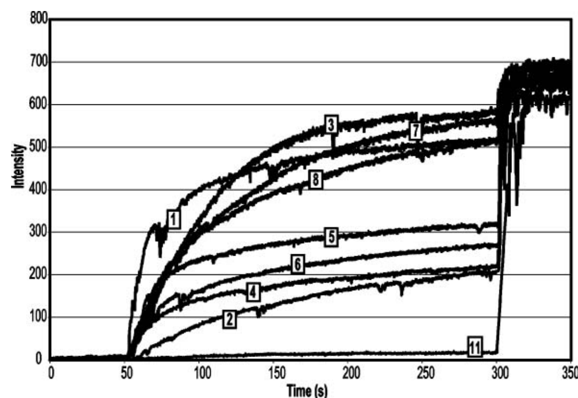


Fig. 3 Calcein release induced by addition of peptide nanostructures **1** to **8** and **11** (0.72 µM) to DOPC : Chol (70 : 30) vesicles (50 µM lipid) in 10 mM HEPES–100 mM NaCl at pH 7.4. Vesicles lysed with detergent (Triton X) at 300 s.

Overall, results demonstrate that an increase of membrane permeability induced by crown peptides depends on many factors. First, results obtained with Boc–7-mer–OH **11** show that helicity is required for activity, even if it appears that there is no direct correlation between helical content and lytic activity. For example, $^{+}H_2$–14-mer–OH **1** possesses the lowest α-helix content, and has more than 70% lytic activity at the lowest concentration tested. However, for core peptides modified with aspartic and/or glutamic acid chains, the one with the highest α-helix content (**8**) induced the greatest lysis at 1.25 and 12.5 µM.

Second, a positively charged N-terminal group has a minor influence on the activity. This is confirmed by the similar membrane permeability profile of **1** and **3**, though the latter induced more lysis than the former (Fig. 3). On the contrary, the polarity of the C-terminus position greatly influences the lytic activity. We observed significantly more lytic activity with crown peptides having one or more negative charges at the C-terminus as compared to the neutral analogue **2**. The increase in hydrophilicity by adding anionic amino acids at the C-terminus could also explain the enhanced membrane lysis observed with peptides **3–8** as compared with **2**.

Finally, it is worth nothing that the lytic activity behaviors of **1–8** depend on the number of charged end groups. Indeed, the most active compounds have one end group free and one protected. This suggest that one end of the peptide nanostructures needs to incorporate the bilayer membrane to be active. This behavior supports the barrel stave pore-forming mechanism proposed for the action of crown peptide nanostructures such as **1–8**. This behavior has been also observed for certain cationic peptide antibiotics, synthetic melittin analogues, and rigid push–pull rod model compounds.[22,23]

Conclusions

The present experimental data demonstrate that cytolytic activity of crown peptide nanostructures may be modulated by engineering the N- or C-terminus groups. Hydrophobicity/hydrophilicity of the N- or C-terminus of the peptide nanostructures has a major impact on their membrane lysing ability. Due to their sequence simplicity and their ease of preparation, crown ether modified peptides such as **1–8** constitute a novel and interesting family of compounds that could be used to understand the mechanism of action of many natural bioactive peptides. Also, based on this groundwork, we are currently investigating the potential use of these peptide nanostructures as prodrugs that could be activated by a specific proteolytic enzyme to selectively target and destroy undesirable cells.

Experimental

General

Oxime resin was prepared according to a reported procedure[24] using polystyrene beads (100–200 mesh, 1% divinyl benzene (DVB), Advanced ChemTech, Louisville, KY). Resins with substitution levels 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

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except for DMF (degassed with N_2), dichloromethane (distilled), and diethyl ether (distilled from sodium and benzophenone). Water used throughout studies was distilled and deionized using a Barnstead NANOpurII system (Boston, MA) with four purification columns. 1,2-dioleoyl-sn-glycerol-3-phosphocholine and cholesterol were purchased from Avanti Polar-Lipids and used without further purification. All other reagents were purchased from Sigma Aldrich Co. (Milwaukee, WI). Solid phase peptide synthesis was performed manually using solid-phase reaction vessels equipped with a coarse glass frit (ChemGlass, Vineland, NJ). Purification of peptides was achieved by reversed phase HPLC with a C_4 semipreparative column (Phenomenex, Torrance, CA). 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. ^1H NMR spectra were recorded on a Varian 400 MHz spectrometer. Sonication was done using a Branson water bath model 3510. Mass spectra were obtained from the Mass Spectrometry Laboratory of the Faculty of Medicine at the University of Toronto, ON, Canada. *N*-Boc-3-(3,4-(21-crown-7)-phenyl)-L-alanine was prepared acording to the procedure we have reported.[12]

Asp/Glu Chains synthesis

The following examples illustrate the synthesis of all dipeptides. The procedures were applied for each Asp/Glu chain described herein. Spectroscopic data are reported in the supporting information.

Protection of carboxylic acid with diazomethane. For this procedure, a flask with a Clear-Seal joint must be used. To a solution of potassium hydroxide (5.0 g, 89 mmol) in water (8 mL) in a reaction vessel, 10 mL of 95% ethanol were added. A 100 mL receiving flask containing the carboxylic acid to be protected was attached to the condenser. The flask was placed in an ice bath. A separatory funnel was installed over the reaction vessel and charged with a solution of Diazald© (5.0 g, 23 mmol) in diethyl ether (45 mL). The reaction vessel was warmed to 65° C and the Diazald© solution was added over a period of 20 min, with the rate of distillation approximating the rate of addition. At the end, nitrogen was bulled into the solution to remove excess diazomethane. When the yellow coloration disappeared, the solvent was evaporated to obtain a white solid in a quantitative yield.

Deprotection of *N*-Boc group. To a solution of a *N*-Boc-amino acid in CH_2Cl_2, 4N HCl in dioxane was added at 0 °C and the resulting mixture was stirred at room temperature for 3 h. At the end, the solvent was evaporated to obtain the deprotected compounds as white solids in quantitative yields.

Deprotection of benzyl esters. An amino acid with benzylic ester as protecting group was dissolved in a minimum of ethanol in a hydrogenation vessel. A catalytic amount of 10% Pd on activated carbon was delicately added to the solution. The system was purged three times with hydrogen. Then the mixture was shaken mechanically for 2 h at room temperature under a pressure of 40 psi of H_2. The reaction mixture was filtered on Celite© and the solvent was evaporated to yield the free carboxylic acid as a white solid in a quantitative yield.

Solution coupling for the formation of Asp/Glu chains. To a solution containing a compound with a free carboxylic acid (1.0 eq) in CH_2Cl_2 (1 M), EDC·HCl (1.2 eq) and HOBt (1.0 eq) were added at 0 °C. The mixture was stirred at room temperature for 30 min. Then, the free amino compound (1.0 eq) and triethylamine (1.2 eq) were added to the solution. The reaction was stirred for a period of 5 to 24 h depending on the reaction. The reaction mixture was washed with saturated $NaHCO_3$ aqueous solution, water, 10% citric acid solution, and water, then dried over anhydrous $MgSO_4$. The solvent was evaporated and the crude dipeptide compound thus obtained was recrystallized in a mixture of diethyl ether–hexane to yield a white powder in yields ranging from 57% to 92%, depending on the compound prepared.

Solid phase synthesis

Deprotection of the *N*-Boc group. The *N*-Boc group was deprotected by a 30 min treatment with a 50% TFA solution in CH_2Cl_2.

Procedure for amino acid coupling on solid support. The amino acid (5 eq) was activated with DIC–HOBt for 30 min at 0 °C in CH_2Cl_2–DMF (1 : 1), then added to the resin swollen in DMF, followed by addition of 1.5 eq of *N*,*N*-diisopropylethylamine (DIEA). The mixture was shaken mechanically for 2 h at room temperature. The completion of coupling reactions was monitored by the ninhydrin test. 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.* When necessary, a second coupling was performed under the same conditions.

***N*-Boc–Leu-CE-Leu-Leu-Leu-CE-Leu–Resin 9.** The key heptapeptide was prepared using 5 g of oxime resin. Deprotection and coupling reactions were performed as described above with *N*-Boc–Leu–OH and *N*-Boc–21-C-7–OH.

***N*-Boc–Leu-CE-Leu-Leu-Leu-CE-Leu–OH 11.** Cleavage of the key heptapeptide **9** from 4 g of resin was realized using a 10% H_2O–THF solution with 2 eq of DBU at room temperature for 2 h. The solution was recovered by filtration and after evaporation of solvent *in vacuo*, the crude peptide was dissolved in CH_2Cl_2 and washed twice with 0.5M HCl and water. After drying with $MgSO_4$, solvent was evaporated and residue triturated with anhydrous diethyl ether and petroleum ether to give 2.1 g of a white powder pure enough (>95%) by HPLC to be used as-is in the next step.

MALDI TOF-MS m/z (M + H^+) 1535, (M + Na^+) 1558

HPLC : $R_t = 24.04$ min

***N*-Boc–(Leu-CE-Leu-Leu-Leu-CE-Leu)$_2$–Resin 13.** The key heptapeptide **13** (1.1 g, 0.72 mmol) was activated with DIC–HOBt for 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 **12** (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.

***N*-Boc–(Leu-CE-Leu-Leu-Leu-CE-Leu)$_2$–OH 3.** Cleavage of the 14-mer peptide **13** from 1.4 g of resin was done in 10% THF–H_2O using 2 eq of DBU. After recovering the solution and evaporation, purification was effected by reversed-phase HPLC. Solvents and water were removed *in vacuo* to give a colorless oil that was

dissolved in glacial acetic acid, and lyophilized to yield 700 mg of a fluffy white solid.

MALDI TOF-MS m/z (M + H^+) 2952, (M + Na^+) 2974, (M + K^+) 2990

HPLC: R_t : 39.1 min

***N*-Boc–(Leu-CE-Leu-Leu-Leu-CE-Leu)$_2$–OMe 2.** Cleavage of the 14-mer peptide **13** from 140 mg of resin was done in MeOH using 2 eq 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 75 mg of a fluffy white solid.

MALDI TOF-MS m/z (M + Na^+) 2988, (M + K^+) 3006

HPLC: R_t : 40.8 min

$^+H_2$–(Leu-CE-Leu-Leu-Leu-CE-Leu)$_2$–OH 1. Peptide **3** (20 mg) was dissolved in 4M HCl in dioxane. The mixture was shaken for 3 h at room temperature. Solvents were removed *in vacuo* to give a colorless oil that was dissolved in glacial acetic acid, and lyophilized to yield 18 mg of a fluffy white solid.

MALDI TOF-MS m/z (M + H^+) 2851, (M + Na^+) 2874, (M + K^+) 2890

HPLC: R_t : 34.4 min

N-terminal coupling of Asp/Glu chains on Boc–14-mer–resin 13 cleavage and final deprotection

Dipeptide chain Asp/Glu with a free carboxylic group was activated with DIC–HOBt for 45 min at 0 °C in CH_2Cl_2–DMF (1 : 1), and then added with 1.5 eq of DIEA to the deprotected 14-mer on resin **20** (0.8 g, 0.32 mmol) swollen with DMF. The mixture was shaken mechanically for 6 h at room temperature. The completion of coupling reactions was monitored by the ninhydrin test. When necessary, a second coupling was performed under the same conditions. The resin was drained and washed as described above. The *N*-Boc group was deprotected by a 30 min treatment with a 50% TFA solution in CH_2Cl_2. The resin was drained and washed as described above. Cleavage and deprotection of the 16-mer peptide from resin was done in 10% H_2O–THF using 2 eq of DBU. Purification was effected by exclusion chromatography using a column filled with Sephadex LH-20 using methanol as eluent. Solvent was removed *in vacuo* to give a colorless oil that was dissolved in glacial acetic acid, and lyophilized to obtain **4–6** as fluffy white solid.

Salient data.

H–γ-Glu-γ-Glu-(Leu-CE-Leu-Leu-Leu-CE-Leu)$_2$–OH **4**.

MALDI TOF-MS m/z (M + H^+) 3110, (M + Na^+) 3133

HPLC: R_t : 34.2 min

H–Glu-Glu-(Leu-CE-Leu-Leu-Leu-CE-Leu)$_2$–OH **5**.

MALDI TOF-MS m/z (M + H^+) 3110, (M + Na^+) 3132, (M + K^+) 3153

HPLC: R_t : 31.1 min

H–Glu-Asp-(Leu-CE-Leu-Leu-Leu-CE-Leu)$_2$–OH **6**.

MALDI TOF-MS m/z (M + H^+) 3096, (M + Na^+) 3117, (M + K^+) 3133

HPLC: R_t : 37.8 min

C-terminal coupling of Asp/Glu chains on Boc–14-mer–OH 3

To a solution containing the 14-mer **3** in CH_2Cl_2, EDC·HCl was added at 0° C. The mixture was stirred at room temperature for 30 min. Then a dipeptide with free N-terminus and triethylamine (Et_3N) were added to the solution. The reaction was stirred for a period of 5 to 24 h, depending on the compound prepared. The reaction mixture was washed with saturated $NaHCO_3$ solution, water, 10% citric acid solution, and water, then dried over anhydrous $MgSO_4$. Evaporation and drying *in vacuo* gave the crude peptide. For the deprotection of carboxylic acid, the crude peptide was dissolved in a minimum of methanol. The temperature was adjusted to 60° C and a solution of 0.5M sodium hydroxide was added. The mixture was stirred for 6 h. The pH was then lowered to between 2 and 3, and the product was extracted with with CH_2Cl_2 (3 times) and washed with water (3 times). The organic phase was dried over anhydrous $MgSO_4$ and solvent was evaporated to obtain a colorless oil. Purification was effected by exclusion chromatography with a column filled with Sephadex LH-20 using methanol as eluant. Solvent was removed *in vacuo* to give a colorless oil that was dissolved in glacial acetic acid, and lyophilized to give a fluffy white solid.

Salient data.

N-Boc–(Leu-CE-Leu-Leu-Leu-CE-Leu)$_2$-γ-Glu-Glu–OH **7**.

MALDI TOF-MS m/z (M + H^+) 3208, (M + Na^+) 3232, (M + K^+) 3249

HPLC: R_t : 39.6 min

N-Boc–(Leu-CE-Leu-Leu-Leu-CE-Leu)$_2$-β-Asp-Glu–OH **8**.

MALDI TOF-MS m/z (M + Na^+) 3218, (M + K^+) 3236

HPLC: R_t : 39.2 min

Vesicle lysis experiments using fluorescence

For fluorescence measurements used to study vesicle lysis, the procedure described by Benachir and Lafleur[21] was followed with some modifications. A calcein solution was prepared by solubilizing calcein in the internal buffer [100 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 30 mM NaCl and 5 mM of ethylenediaminetetraacetic acid (EDTA)] to obtain a calcein concentration of 80 mM. The pH of the solution was adjusted to 7.4 with a 1M NaOH solution. 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 under vacuum for 12 h. 5 mL of calcein solution were added to the lipid film. The resulting mixture was placed in water bath sonicator at 2–5 °C and sonicated for 40 min. Vesicles thus obtained were filtrated through a polycarbonate membrane (Poretics, 0.4 microns). Calcein-containing vesicles were separated from the free calcein by size exclusion chromatography using a column filled with Sephadex G-50 super-fine 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 spectrofluorometer. 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 set to 25 °C. In a 1 cm quartz cuvette, 4 mL of external buffer solution and 50 μL of vesicle solution were introduced. Stirring and measurement were started. After 50 s, an appropriate amount (1–10 μL) of a

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TFE solution of the crown peptide nanostructures (5–40 mM) was added. Then, after 400 s, a 10% Triton X-100 solution was added and the experiment was terminated after 500 s.

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