End group engineering of artificial ion channels

François Otis,[a] Normand Voyer,*[a] Ange Polidori[b] and Bernard Pucci[b]

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Toward developing nanostructures for single molecule detection, we report the synthesis of new derivatives of artificial devices that possess ion channel activity. The membrane stability and the ion transport ability of these multiple crown α-helical peptides have been optimized by modulating the polarity of *N*- and *C*-terminal groups with incorporation of hydrophilic non-ionic units. Circular dichroism and ATR studies confirmed the α-helical conformation and membrane incorporation of new nanostructures while ^{23}Na NMR assays shown a significant increase of their ion transport ability.

Introduction

The potential miscellaneous applications of artificial compounds mimicking the conductivity properties of ion channel proteins have stirred considerable efforts towards developing such compounds. Many successful approaches have been devised leading to a variety of artificial ion channels with their own advantages and disadvantages.[1] Our approach exploits nanoscale peptidic frameworks that orient multiple crown ethers to form a polar transmembrane channel for ions. Herein, we report the molecular engineering of the end groups of a prototype crown peptide channel in order to improve their apolar membrane incorporation and active orientation.

We have reported the synthesis of various peptides endowed with six aligned crown ethers[2] and demonstrated their single-channel activity in planar lipid bilayers and ion transport ability in vesicle experiments.[3,4] It is noteworthy that the chosen synthetic pathway provides several advantages: rapid synthesis by using solid phase technique, easy preparation of a collection of analogs and, above all, facile purification of the targeted compounds. For example, a series of synthetic helical peptides that are oligomers of a repeating unit with five leucine residues and two 21-crown-7 phenylalanines appropriately positioned, exhibit an α-helical structure with the crown ethers aligned on one side of the helix axis. Under an α-helical conformation, the 21 residue peptide ($n = 3$) can span a bilayer membrane and act, under certain conditions, as artificial ion channel.[5]

In order to provide even more efficient functional ion channels, the active orientation of peptides must be enhanced, *i.e.* the helical structure should reside most often in the transmembrane orientation and the adsorption at the surface of the membrane must be disfavoured (Fig. 1).

If we consider that such an adsorption could be due to a hydrophobic interaction between the leucine side chains of crown peptides and the lipid chains, it can be assumed that increasing the hydrophilicity of the terminal head groups by means of non-ionic moieties should stabilize the transmembrane active form. Herein, we report the synthesis of a series of crown modified peptides **2**, **3**, **4**, analogs of **1**, having variable non-ionic polyhydroxylated groups derived from tris(hydroxymethyl)aminomethane (Fig. 2). By using CD, FTIR and ATR techniques the incorporation and orientation of these different amphiphilic peptides in model membranes have been studied.

Results and discussion

Synthesis

The synthesis of polar groups starts with commercially available tris(hydroxymethyl)methylamine (TRIS), which was first protected with *tert*-butoxydimethylsilyl groups (Scheme 1) to give **5**.[6,7] Reaction of **5** with succinic anhydride leads to the *N*-terminal end group **6**, ready to be coupled. On the other hand, coupling *N*-benzyloxycarbonyl aminohexanoic acid[8,9] with **5** provided **8**, which after hydrogenation yielded the *C*-terminal end group **9** (Scheme 2).

Preparation of 21mer peptide from *N*-BOC leucine and (21-C-7)-**L**-Phe on oxime resin was described previously.[5] Introduction of polar end groups is illustrated in Scheme 3. Cleavage of the 21mer peptide from resin using the amine **9** as nucleophile provided the desired *C*-terminal polar head peptide **3**, after deprotection of alcohols with TBAF. Deprotection of *N*-BOC group from 21mer peptide using 50% trifluoroacetic acid (TFA) in CH_2Cl_2 followed by the coupling of acid **6** using DIC/HOBt as reagent in DMF/CH_2Cl_2 (1 : 1), lead to the formation of the intermediate **10**, still attached to the resin. A portion of the resin was used to obtain *N*-terminal polar head peptide **2** by cleavage with DBU in MeOH/THF (1 : 9), followed by deprotection of hydroxyl groups with acetic acid.[10] The remaining of resin **10** was cleaved with amine **9** and deprotected with TBAF to produce peptide **4** with polar heads at both extremities. Purification of peptide **1** was achieved by reversed-phase HPLC, while peptides **2–4**

[a] *Département de Chimie and Centre de Recherche sur la fonction, la structure et l'ingénierie des protéines, Faculté des sciences et de génie, Université Laval, Québec, Canada G1K7P4. E-mail: normand.voyer@chm.ulaval.ca; Fax: +1-418-656-7916; Tel: 1-418-656-3613*
[b] *Laboratoire de Chimie Bioorganique et des Systèmes Moléculaires Vectoriels, Faculté des sciences, Université d'Avignon, 33, rue Louis Pasteur, Avignon, France*

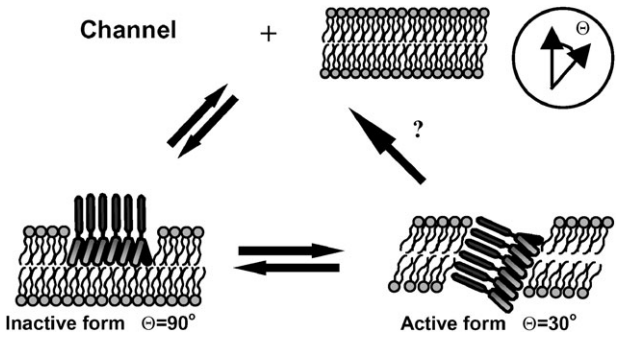


Fig. 1 Proposed mode of action for the channel activity of peptide **1**.

were purified on Sephadex LH-20. All peptides were successfully characterized by ^{1}H NMR and mass spectroscopy and purity was checked by reversed-phase HPLC.

Conformational studies

Conformational studies performed by circular dichroism (CD) and FT-IR provided strong evidence that peptide nanostructures such as **1** exist mainly under a helical conformation and in a monomeric, unaggregated state in several solvents (MeOH, TFE and 1,2-dichloroethane), as well as in bilayer membranes prepared with egg yolk lecithin.[4,11] To verify the influence of the polar head groups on the required helical conformation, CD experiments have been performed with **2**, **3**, and **4**. Results, illustrated in Fig. 3, reveal an increase of peptide helicity in the cases of the engineered peptide **2–4** as compared to **1**. This indicates that increasing polarity at one or both ends of the 21mer peptidic framework does not interfere with the correct conformation of the chain and, indirectly, with the alignment of crown rings.

Membrane incorporation

Activity of channel compounds such as **1** depends greatly on their orientation in the bilayer membrane. Using polarized infrared ATR spectroscopy, measurement of the orientation of peptidic framework within bilayer membrane has previously

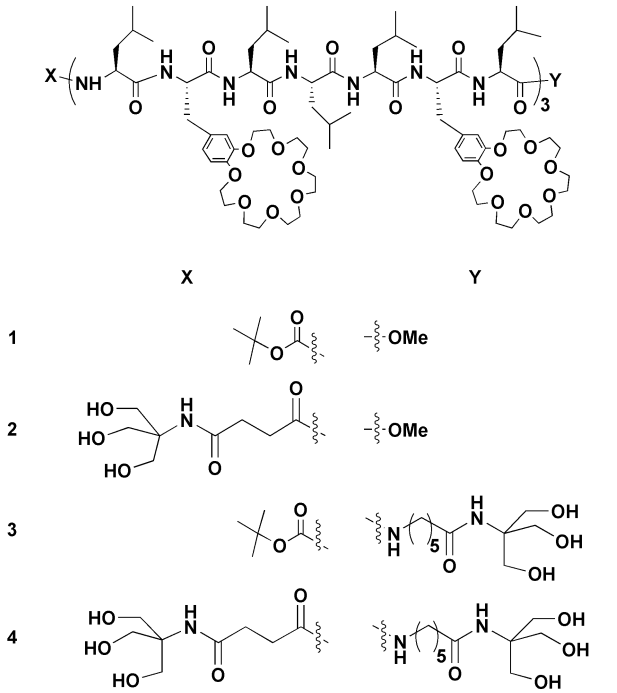
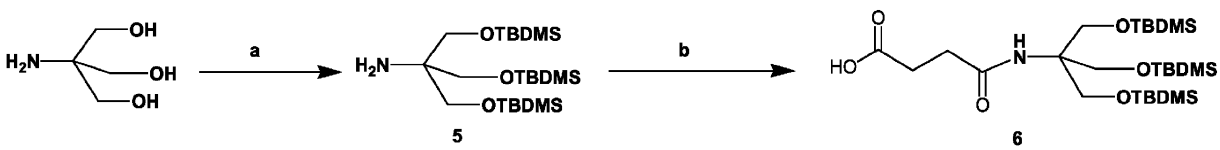
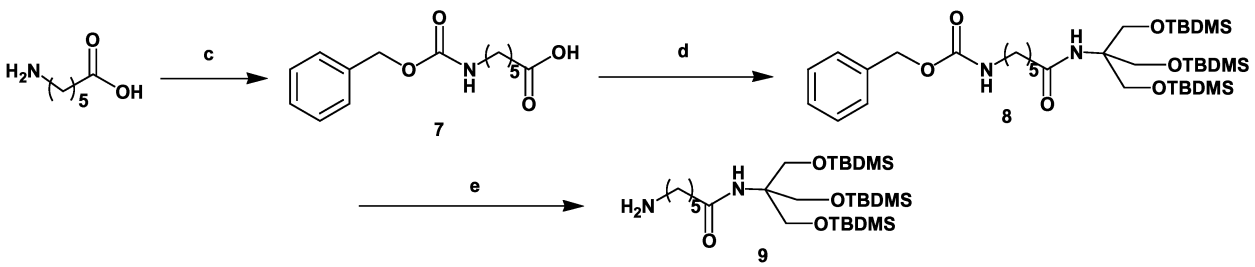


Fig. 2 21Mer crown peptide derivatives with polar head groups subject to investigation.

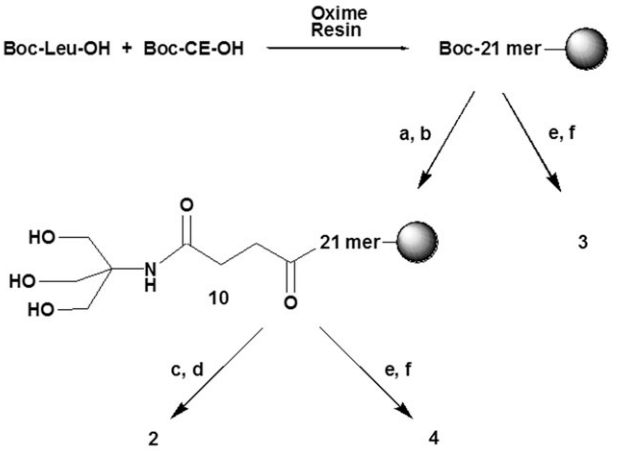
shown that peptide **1** is partially incorporated in DMPC membranes.[11] On the basis of previous biophysical studies, we proposed that the channel peptides are in a two-state incorporation equilibrium, one active at 30° parallel to the lipid hydrocarbon chains and the other inactive at 90° (see Fig. 1).[11] In order to verify the effect of increasing end group polarity and hydrophilicity on the active orientation of hexacrown peptide in membranes, we performed ATR experiments on compounds **2**, **3**, and **4**. For each sample, orientation of lipid chains and peptide were calculated from the symmetric CH_2 stretching band of the lipid hydrocarbon tails and C=O stretching amide I band of the peptide.[12–14] Table 1 compares dichroic ratios (R) and mean angles of incorporation θ



Scheme 1 Synthesis of *N*-terminal polar head group: a) TBDMS-Cl, imidazole, DMF; b) succinic anhydride, DMAP, CH_2Cl_2.



Scheme 2 Synthesis of *C*-terminal polar head group: c) NaOH, H_2O, Benzyloxycarbonyl chloride; d) **5**, DCC, CH_2Cl_2; e) H_2, Pd/C, MeOH.



Scheme 3 Synthesis of crown peptide derivatives with hydrophilic head groups: a) 50% TFA/CH_2Cl_2; b) **6**, DIC, HOBt, DIEA, CH_2Cl_2; c)THF/MeOH (9 : 1), DBU, LiBr; d) AcOH; e) **9**, CH_2Cl_2, AcOH (cat); f) 1M TBAF/THF.

calculated for each crown peptides. On the basis of a two-state incorporation equilibrium, it is possible to evaluate the participation of each state to the resulting incorporation angle and obtain a percentage of incorporation of peptides in the membrane.[11] First, the value obtained for the orientation angle of pure DMPC (25.0°) is in good agreement with those reported in the literature.[12]

As shown in Table 1, all peptides bearing a polar end group demonstrate higher membrane incorporation. However, addition of a polar group at the *N*-terminal position has the most pronounced effect on the stability of incorporated peptide. Indeed, compound **2** demonstrates the best improvement in incorporation, increasing from 28% for the fully protected peptide **1** to 54% with **2** having polar end group at the *N*-terminal position. Orientation of lipid chains varies in the presence of **2** (27.1°) compared with pure DMPC (25.0°), confirming that an important amount of the peptidic nanostructure is incorporated into the membrane. Insertion of a hydrophilic group at the *C*-terminal position also leads to a significant enhancement of incorporation, 38% for peptide **3**, but to a lower extent as compared with **2**. For crown peptide **4** bearing the polar head group at both termini, a 48% of membrane incorporation is observed. This result points out the lack of additivity of two polar head groups in the membrane incorporation/stabilization processes. It is also possible that the grafting of a second polar head, not only increases the water solubility of **4**, but also impedes slightly membrane incorporation.

Table 1 ATR orientation studies on peptide nanostructures **1**, **2**, **3**, and **4** in planar DMPC bilayers

Sample	ν_{C-H} sym		$\nu_{amide\ 1}$		Incorporation (%)[a]
	R	*θ*	*R*	θ	
DMPC	1.08	25.0°			
1	1.13	28.0°	2.03	54.2°	28
2	1.12	27.1°	2.71	41.8°	54
3	1.15	28.9°	2.28	49.0°	38
4	1.14	28.3°	2.54	44.4°	48

[a] Relates to the amount of peptide nanostructures parallel to the lipid chain (Fig. 1)

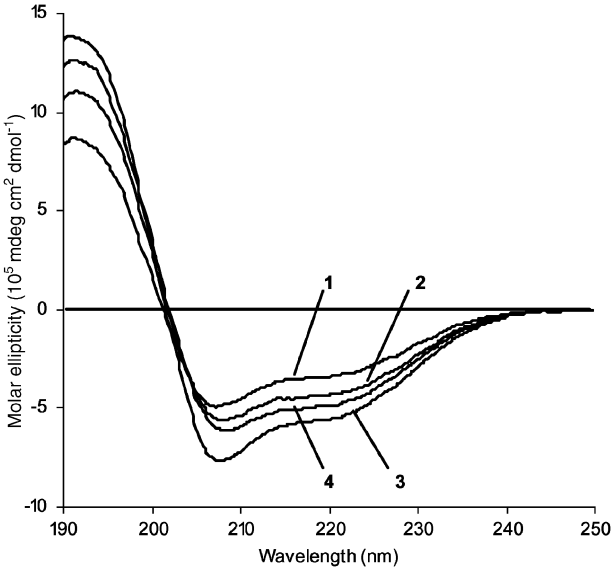


Fig. 3 CD spectra in TFE of peptide derivatives **1–4** at a concentration of 0.2 mM.

Ion transport studies

In order to obtain information on the ability of these new channels to conduct ions, membrane transport studies were done using the ^{23}Na NMR method.[15] This method permits a good assessment of channel transport activity. Using Dy^{3+} as shift reagent, this technique provides the exchange rate of sodium ions between the two sides of model membranes in the presence of a functional channel and has been successfully applied to Gramicidin D[16] and artificial ion channels.[5,17] Previous studies have demonstrated that transport activity of peptide **1** represents 3% of the activity of Gramicidin D.[5] Moreover, they have shown the importance of crown ether size for sodium ion transport while supporting the monomolecular channel mechanism of hexacrown peptides such as **1**. To verify the impact of polar terminal groups on transport abilities, compounds **1**, **2**, **3** and **4** were tested. The results are reported in Table 2 and are expressed as relative rates compared to peptide **1** (100%).

The main conclusion from theses studies is the increase of transport activity for compound **2**, the one with polar head at the *N*-terminal position, which has an activity corresponding to 271% of the one of fully protected peptide **1**. This result is in agreement with ATR results that demonstrated better membrane incorporation for **2**. Moreover, the enhancement of transport activity for **4**, even if more modest than the one

Table 2 Rate constant (*k*) and relative transport rates for Na^+ across a DMPC/DMPG bilayer membrane by ^{23}Na NMR

Sample	$k/s^{-1}\ \mu M^{-1}$	Relative rate (%)
1	2.4	100
2	6.5	271
3	1.9	80
4	2.8	117

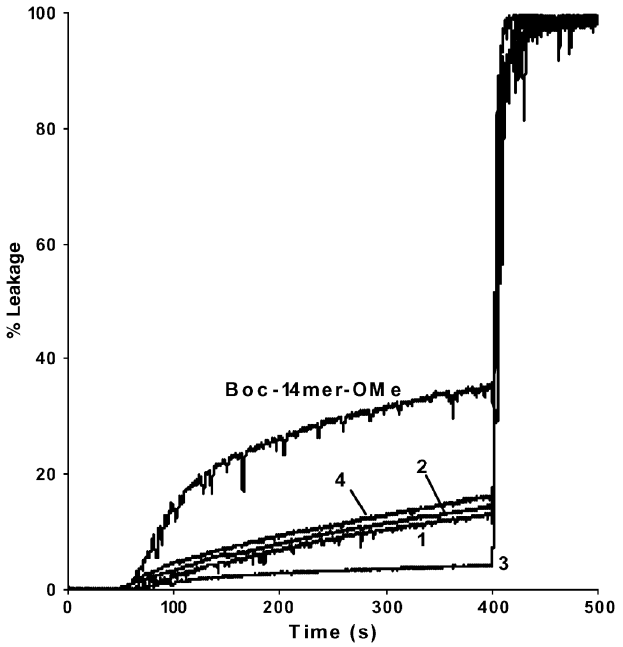


Fig. 4 Calcein leakage induced by addition of peptide nanostructures **1–4** and Boc-14mer-OMe. Complete leakage (100%) was obtained by addition of a solution of Triton X-100 at 400 s.

for **2**, indicates that hydrophilic non-ionic end groups can penetrate the vesicle membrane. Indeed, in these experiments, vesicles were formed before addition of crown peptides, whereas, in ATR studies, phospholipid membranes were prepared in the presence of peptides. Introduction of polar end group only at the *C*-terminal position does not result in increase transport ability and the rate is even lower for **3** than for **1**. Difficulty of incorporation and lower stability in membrane may explain this lower activity, although more work is necessary to understand the molecular details involved in this case.

To verify that the activity observed by the ^{23}Na NMR assay is not due to lysis of vesicles, a control assay was performed. The vesicle lysis assay is based on the increase of self quenching fluorescence of calcein upon lysis.[18] Fig. 4 reports calcein release profiles induced by addition of **1**, **2**, **3**, **4**, and Boc-14mer-OMe, precursor of **1**, known to have an important lytic activity.[5,18] It appears that all 21mer analogs induced very little lysis compared to the control, Boc-14mer-OMe. The lower effect on the membrane induced by **3** reflects its apparent feeble interactions with membranes and is in agreement with ATR and NMR results. On the other hand, the very low level of lysis observed confirmed that results obtained in the ^{23}Na NMR assay reflect the transport ability of artificial channels **2**, **3**, and **4**.

Conclusion

We have described a simple and efficient approach to stabilize in phospholipid membranes artificial ion channels based on crown peptide nanostructures. Utilization of non-ionic hydrophilic groups derived from TRIS represents an efficient alternative to increase the polarity at transmembrane peptide extremities. The ion transport efficiency and enhanced membrane incorporation observed of *N*-monosubstituted peptide **2** allow the possibility of further engineering of the *C*-terminal position of such peptide nanostructures in order to incorporate various recognition elements to target important biological analytes. The results reported also open the door to the use of more sophisticated head groups such as carbohydrate derivatives for increased interaction with specific cell membranes. Work is in progress along these lines.

Experimental

General

The oxime resin was prepared according to a reported procedure using polystyrene beads (100–200 mesh 1% DVB, Advanced ChemTech, Louisville, KY).[19] 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), diethyl ether and THF (distilled from sodium and benzophenone). Water used throughout the studies was distilled and deionized using a Barnstead NANOpurII system (Boston, MA) with four purification columns. Phosphatidylglycerol (20 mg mL^{-1} solution in $CHCl_3$), phosphatidylcholine (20 mg mL^{-1} 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). Solid phase peptide synthesis was performed manually using solid-phase reaction vessels equipped with a coarse glass frit (ChemGlass, Vineland, NJ). ^{1}H and ^{13}C NMR spectra were recorded on a Varian 400 MHz spectrometer. Sonication was done using a Branson water bath model 3510. Analyses by reverse phase HPLC were done with a Vydac C-4 column (0.45 cm × 25 cm). All solvents were degassed and gradients of A (H_2O/0.1% TFA) and B (49.95% CH_3CN/49.95% isopropanol/0.1% TFA) were used. CD measurements, infrared ATR analyses, ^{23}Na NMR measurements and fluorescence vesicle lysis experiments have been carried out as described previously.[5,11]

Synthesis

Tris(*tert*-butyldimethylsilyloxymethyl)aminomethane 5. *tert*-Butyldimethylsilyl chloride (6.7 g, 44.6 mmol) and imidazole (6.3 g, 92.9 mmol) were dissolved in 5 mL DMF. Tris(hydroxymethyl)methyl amine (1.5 g, 12.4 mmol) was added and the mixture was stirred at room temperature for 4 h. The product was washed with H_2O, extracted with CH_2Cl_2, dried over $MgSO_4$ and filtered. Solvent was removed *in vacuo* to give a white powder (5.4 g, 95%): mp 32–34 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.55 (s, 6H, CH_2–O), 0.89 (s, 27H, *t*-Bu), 0.05 (s, 18H, Si–CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 64.3, 57.6, 26.1, 18.4, −5.3. ESMS *m*/*z* (M + H$^+$) 465.

[Tris(*tert*-butyldimethylsilyloxymethyl)methyl]amidosuccinic acid 6. Succinic anhydride (0.71 g, 7.1 mmol) and **5** (3.0 g, 6.5

mmol) were dissolved in CH_2Cl_2. DMAP (60 mg, 0.49 mmol) was added and the mixture was stirred at room temperature for 2 h. The product was extracted with 5% $NaHCO_3$ solution, acidified and extracted with CH_2Cl_2. The organic phase was dried over $MgSO_4$, filtered, and solvent was removed *in vacuo* to give a white powder (2.0 g, 55%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.75 (s, 1H, NH), 3.78 (s, 6H, CH_2–O), 2.60 (t, 2H, C*H*$_2$–COOH), 2.44 (t, 2H, C*H*$_2$–CONH), 0.84 (s, 27H, *t*-Bu), 0.00 (s, 18H, Si–CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 175.9, 172.3, 62.4, 60.7, 31.6, 30.6, 26.0, 18.4, −5.4. ESMS *m*/*z* ($M + H^+$) 564.

6-(*N*-Benzyloxycarbonylamino)hexanoic acid 7. 6-Aminohexanoic acid (5.0 g, 38.1 mmol) was dissolved in H_2O and cooled to 0 °C. Alternatively, benzylchloroformate (8.1 g, 56.7 mmol) and 5N NaOH (60 mL) was added in portions, keeping the reaction mixture at pH 10. The mixture was stirred at room temperature for 1 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, and dried over $MgSO_4$. After filtration, solvent was removed *in vacuo* to give a white powder (8.6 g, 85%): mp 48–49 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.01 (s, 1H, COOH), 7.47–7.30 (m, 5H, Ar), 7.25 (t, 1H, NH), 5.02 (s, 2H, C*H*$_2$-Ar), 2.99 (q, 2H, C*H*$_2$–NH), 2.20 (t, 2H, C*H*$_2$–COOH), 1.50 (m, 2H, C*H*$_2$–CH_2–NH), 1.41 (m, 2H, C*H*$_2$–CH_2–COOH), 1.26 (m, 2H, C*H*$_2$–CH_2–CH_2–NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 179.3, 156.7, 134.8–128.3, 66.9, 41.0, 34.1, 29.8, 26.3, 24.4. ESMS *m*/*z* ($M + H^+$) 266.

[Tris(*tert*-butyldimethylsilyloxymethyl)methyl]6-(*N*-benzyloxycarbonylamino) hexanamide 8. At 0 °C, **7** (1.2 g, 4.5 mmol) and DCC (0.93 g, 4.5 mmol) were dissolved in CH_2Cl_2. The amine **5** (2.1 g, 4.5 mmol) dissolved in CH_2Cl_2 was added and the mixture was stirred overnight at room temperature. Urea was eliminated by filtration and the product was washed with $NaHCO_3$ 5%, H_2O, 1N HCl, H_2O, and dried over $MgSO_4$. After filtration, solvent was removed *in vacuo* and purification on silica gel (25% AcOEt/hexanes) gave a white powder (1.5 g, 47%): mp 88–89 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.43–7.30 (m, 5H, Ar), 7.22 (t, 1H, NH(Z)), 6.73 (s, 1H, NH(Tris)), 5.00 (s, 2H, CH_2–Ar), 3.67 (s, 6H, C*H*$_2$–OSi), 2.96 (q, 2H, C*H*$_2$–NH), 2.10 (t, 2H, C*H*$_2$–CO), 1.45 (m, 2H, C*H*$_2$–CH_2–NH), 1.38 (m, 2H, C*H*$_2$–CH_2–CO), 1.24 (m, 2H, C*H*$_2$–CH_2–CH_2–NH), 0.86 (s, 27H, *t*-Bu), 0.00 (s, 18H, Si–CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 172.4, 156.6, 136.8–128.3, 66.8, 61.8, 60.7, 41.1, 37.5, 29.9, 26.6, 26.1, 25.5, 18.4, −5.3. ESMS *m*/*z* ($M + H^+$) 711.

[Tris(*tert*-butyldimethylsilyloxymethyl)methyl]6-aminohexanamide 9. The protected amine **8** (66 mg, 0.093 mmol) was deprotected in MeOH with H_2/Pd on activated carbon for 2 h at room temperature. The mixture was filtered on Celite, solvent was evaporated and the amine **9** was used in next step without further purification.

Typical procedure for amino acids coupling on solid support. The amino acid (5 equiv.) was activated with DIC/HOBt during 30 min at 0 °C in CH_2Cl_2/DMF (1 : 1), then added to the resin swollen in DMF and 1.5 equiv. of DIEA were added. 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 × 50 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.

***N-tert*-BOC-(L-(21-C-7)-L-L-L-(21-C-7)-L)$_3$-OMe 1.** The peptide on oxime resin *N-tert*-BOC-(L-(21-C-7)-L-L-L-(21-C-7)-L)$_3$-resin was synthesized in the same manner as described previously.[5] Cleavage of the 21mer peptide from 500 mg of this resin was performed in MeOH using 2 equiv. of DBU.[20] Purification was done by reverse 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 a fluffy white solid. ^{1}H NMR (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, 12 CH_2–OAr), 3.75–3.60 (m, 24H, 12 C*H*$_2$–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, 6 βCH_2 21-C-7), 1.65–1.35 (m, 54H, 15 Leu βCH_2 + 15 Leu γCH + 9 H *t*-Bu), 0.95–0.65 (m, 90H, Leu CH_3). MALDI TOF-MS *m*/*z* ($M + Na)^+$ 4406.

6-(L-(21-C-7)-L-L-L-(21-C-7)-L)$_3$-resin 10. *N-tert*-BOC-(L-(21-C-7)-L-L-L-(21-C-7)-L)$_3$-resin was deprotected by a 30 min treatment with a 50% CF_3COOH solution in DCM. The acid **6** (0.25 g, 0.45 mmol) was activated with DIC/HOBt (5 equiv.) during 30 min at 0 °C in CH_2Cl_2/DMF (1 : 1), and then added, with 1.5 equiv. of DIEA, to the deprotected 21mer on resin (0.21 g, 0.089 mmol) swollen with CH_2Cl_2. The mixture was shaken mechanically for 2 h at room temperature. The resin was drained and washed thoroughly with DMF (3 × 50 mL), MeOH (3 × 50 mL), DMF (3 × 50 mL), MeOH (3 × 50 mL) and then dried *in vacuo*. The completion of coupling reaction was monitored by the ninhydrin test.

6-(L-(21-C-7)-L-L-L-(21-C-7)-L)3-OMe 2. Cleavage of the 21mer peptide from 100 mg of **10** resin was performed in MeOH (10%)/THF using 2 equiv. of DBU. Deprotection was completed in acetic acid after 90 h. Purification was done on Sephadex LH-20 in MeOH. Solvents were removed *in vacuo* to give a colorless oil that was dissolved in acetic acid, and lyophilized to obtain **2** as a fluffy white solid. Purity was verified by reverse-phase HPLC (0–100% B in 45 min). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.45–7.80 (m, 22H, NH), 6.85–6.55 (m, 18H, H Ar), 4.30–3.80 (m, 54H, 6 αCH 21-C-7 + 12 CH_2–Oar + 12 C*H*$_2$–CH_2–OAr), 3.80–3.40 (m, 120H, 51 CH_2–O +15 Leu αCH + OCH_3), 3.35–2.90 (m, 16H, 6 βCH_2 21-C-7 + CH_2–CO), 1.90–1.20 (m, 45H, 15 Leu βCH_2 +15 Leu γCH), 0.95–0.60 (m, 90H, Leu CH_3). MALDI TOF-MS *m*/*z* ($M + Na)^+$ 4504.

***N-tert*-BOC-(L-(21-C-7)-L-L-L-(21-C-7)-L)$_3$-9 3.** Cleavage of the peptide *N-tert*-BOC-(L-(21-C-7)-L-L-L-(21-C-7)-L)$_3$-resin from the oxime resin was performed in CH_2Cl_2 using 1 equiv. of **9** and acetic acid in catalytic amount. Deprotection was effected with 2.5 equiv. of TBAF in THF. Purification was done on Sephadex LH-20 in MeOH. Solvents were removed

in vacuo to give a colorless oil that was dissolved in acetic acid, and lyophilized to obtain **3** as a fluffy white solid. Purity was verified by reverse-phase HPLC (0–100% B in 45 min). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.50–7.70 (m, 23H, NH), 6.85–6.50 (m, 18H, H Ar), 4.30–3.80 (m, 54H, 6 αCH 21-C-7 + 12 CH_2–OAr + 12 CH_2–CH_2–OAr), 3.80–3.40 (m, 117H, 51 CH_2–O + 15 Leu αCH), 3.35–2.85 (m, 16H, 6 βCH_2 21-C-7 + CH_2–NH + CH_2–CO), 2.00–1.30 (m, 60H, 15 Leu βCH_2 + 15 Leu γCH + 3 CH_2–(CH_2)$_3$–CH_2 + 9 H *t*-Bu), 1.00–0.65 (m, 90H, Leu CH_3). MALDI TOF-MS *m*/*z* $(M + Na)^+$ 4603.

6-(L-(21-C-7)-L-L-L-(21-C-7)-L)$_3$-9 4. Cleavage of the 21mer peptide from 500 mg of **10** resin was performed as described for **3** using 1 equiv. of **9**. Deprotection was done with 2.5 equiv. TBAF in THF. Purification was done on Sephadex LH-20 in MeOH. Solvents were removed *in vacuo* to give a colorless oil that was dissolved in acetic acid, and lyophilized to obtain **4** as a fluffy white solid. Purity was checked by reverse-phase HPLC (0–100% B in 45 min). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.40–7.60 (m, 24H, NH), 6.85–6.55 (m, 18H, H Ar), 4.30–3.80 (m, 54H, 6 αCH 21-C-7 + 12 CH_2–OAr + 12 CH_2–CH_2–OAr), 3.80–3.40 (m, 123H, 54 CH_2–O + 15 Leu αCH), 3.35–2.80 (m, 20H, 6 βCH_2 21-C-7 + CH_2–NH + 3 CH_2–CO), 1.90–1.20 (m, 51H, 15 Leu βCH_2 + 15 Leu γCH + 3 CH_2–(CH_2)$_3$–CH_2), 0.95–0.65 (m, 90H, Leu CH_3). MALDI TOF-MS *m*/*z* $(M + Na)^+$ 4707.

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