A Novel Functional Artificial Ion Channel

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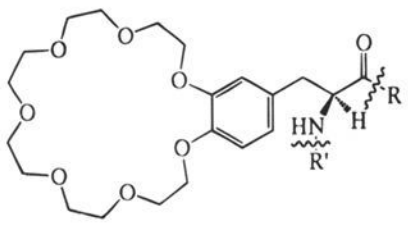
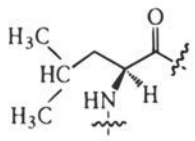
Ion channel proteins are complex membrane proteins that control and regulate the transport of ions across cell membranes. Because of their involvement in several diseases,[1] their potential use in biosensors,[2] and their inherent difficulty of isolation, intense efforts have been devoted to the preparation of artificial ion channels.[3] Here, we report a very simple, rapid, and efficient strategy for the preparation of such functional channel molecules. The strategy 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.

N-t-Boc-(Leu-**3**-Leu-Leu-Leu-**3**-Leu)$_3$-OMe **1**

N-t-Boc-Leu-**3**-Leu-Leu-Leu-**3**-Leu-OMe **2**

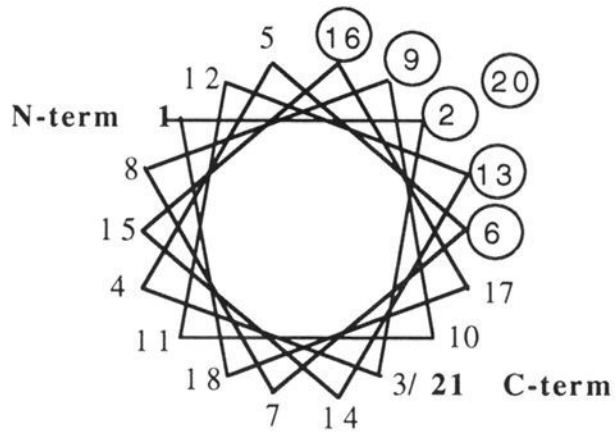


3 (a: R=OH; R'= COO-tBu)
(b: R=OMe; R'= COO-tBu)



= Leu = L-Leucine

The designed molecule **1** is a 21 amino acid peptide composed of 15 L-leucines and six 21-crown-7 L-phenylalanines. These amino acids were chosen because they are hydrophobic and a peptide composed of these amino acids should be lipophilic enough to incorporate in a lipid bilayer membrane. Also, these amino acids have a high propensity to favor the α-helix conformation;[4] therefore peptide **1** should adopt that conformation in solution. If the crown ether residues are incorporated judiciously at positions 2, 6, 9, 13, 16, and 20 of the sequence



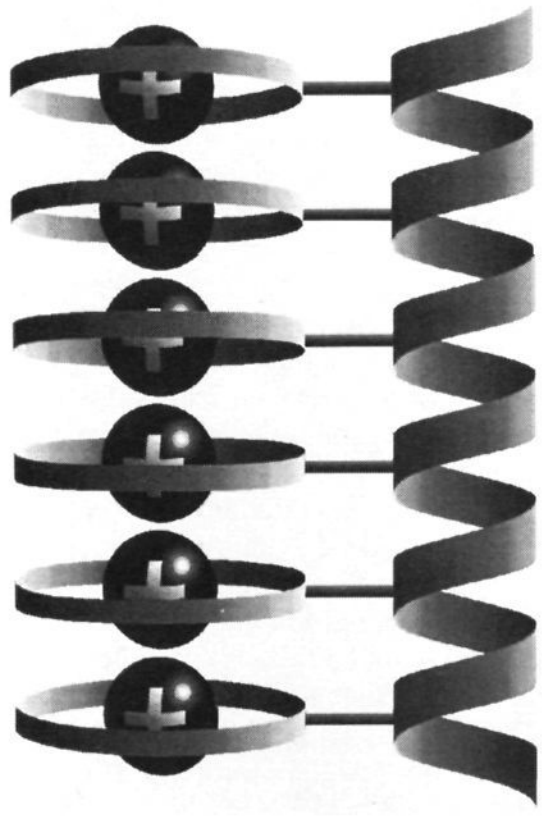


Figure 1. (a, top) Axial projection of the helical structure of **1** showing the positions of the crown ether amino acid **3** (circled positions). (b, bottom) Schematic representation of the proposed active form of the artificial ion channel **1**.

of **1**, the crown ethers are all located on the same side of the helix and form a channel for ions, as illustrated schematically in Figure 1, long enough to span a bilayer membrane. One additional reason for the use of crown ethers as the pore-forming moieties is the fact that their binding ability can be engineered to specific needs. In this case, the 21-crown-7 ligand was chosen because it binds alkali metal ions rather poorly, a necessary requirement to obtain ion channel activity. The 21 amino acid peptide **1** was efficiently prepared in a 10% pure isolated overall yield following a segment condensation strategy we have reported[5] using the oxime resin.[6] The purification of **1** was easily achieved by reverse phase HPLC, and it was characterized by ^{1}H NMR and FAB mass spectrometry.[7]

The ion transport ability of **1** as well as its shorter heptapeptide analogue **2**, the *N*-BOC-21-crown-7 L-phenylalanine methyl ester **3b**, and the natural ion channel gramicidin A was

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(2) (a) Cammann, K.; Lemke, U.; Rohen, A.; Sander, J.; Wilken, H.; Winter, B. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 516−39. (b) Cass, A. E. G., Ed. *Biosensors, A Practical Approach*; Oxford University Press: Oxford, U.K., 1990. (c) Buch, R. M.; Rechnitz, G. A. *Anal. Chem.* **1989**, *61*, 533a−42a.

(3) For reports on unimolecular channels, see: (a) Cross, G. G.; Fyles, T. M.; James, T. D.; Zojaji, M. *Synlett* **1993**, 449−60 and references cited therein; (b) Nakano, A.; Xie, Q.; Mallen, J. V.; Echegoyen, L.; Gokel, G. W. *J. Am. Chem. Soc.* **1990**, *112*, 1287−9; (c) Julien, L. Lazrak, T.; Canceill, J.; Lacombe, L.; Lehn, J.-M. *J. Chem. Soc. Perkin Trans. II* **1993**, 1011−20; (d) Kragton, U. F.; Roks, M. F.; Nolte, R. J. M. *J. Chem. Soc. Chem. Commun.* **1985**, 1275−6; (e) Stankovic, C. J.; Heinemann, S. H.; Schreiber, S. L. *J. Am. Chem. Soc.* **1990**, *112*, 3702−4. For reports on self-assembling channels, see: (f) Åkerfeldt, K. S.; Lear, J. D.; Wasserman, Z. R.; Chung, L.; DeGrado, W. F. *Acc. Chem. Res.* **1993**, *26*, 191−7 and references cited therein. (g) Montal, M.; Montal, M. S.; Tomich, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 6929. (h) Ghadhiri, M. R.; Granja, J. R.; Buehler, L. K. *Nature* **1994**, *369*, 301−4. (i) Tabushi, I.; Kuroda, Y.; Yokota, Y. *Tetrahedron Lett.* **1982**, *23*, 4601−4. (j) Kobuke, Y.; Ueda, K.; Sokabe, M. *J. Am. Chem. Soc.* **1992**, *114*, 7618−22. (k) Menger, F. M.; Davis, D. S.; Persichetti, R. A.; Lee, J. J. *Ibid.* **1990**, *112*, 2451−2.

(4) (a) Schultz, G. E.; Schirmer, R. H. *Principles of Protein Structure*; Springer Verlag: New York, 1979; Chapter 6. (b) Yamamoto, H.; Hayakama, T. *Polymer* **1979**, *18*, 979−83.

(5) Voyer, N. *Ibid.* **1991**, *113*, 1818−21. Briefly, the key heptapeptide *N*-BOC-Leu-**3**-Leu$_3$-**3**-Leu-OMe was prepared on the oxime resin.[6] Coupling reactions were performed using *N*-BOC amino acids and DIC/HOBT as reagent in CH_2Cl_2/DMF (1:1). The *N*-BOC group was deprotected by a 30 min treatment with a 50% CF_3COOH solution in CH_2Cl_2. The completion of coupling reactions was monitored by the ninhydrin test. Cleavage of the peptide from the resin was realized using a 0.5 M solution of CH_3COOH·Leu-OMe in $CHCl_3$.[6] The key peptide was then selectively deprotected with CF_3COOH or 1 M NaOH in MeOH and dimerized (DIC/HOBT) to yield the 14-residue intermediate. The latter was deprotected with CF_3COOH and coupled again with the *N*-BOC heptapeptide acid to give crude **1**.

(6) (a) DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1982**, *47*, 3258−61; (b) **1980**, *45*, 1295−300.

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investigated using vesicles by the pH stat method.[8] Briefly, a sample of 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.[8] Then, ions and FCCP,[9] a proton carrier, are added. In the absence of a transporter, no proton leakage is observed. Upon addition of a functional transporter, its transport ability is monitored by the release of protons required to maintain the electroneutrality across the vesicle wall. The protons are neutralized continuously to maintain a constant solution pH of 7.6. The graph of the volume of base added vs time allows the determination of the transport mode.[8,10] Typical results are illustrated in Figure 2 for the case of Cs^+. As it can be seen, the monomeric crown ether **3b** and the heptapeptide **2**, too short to span the membrane, act as typical carriers by slowly and constantly transporting Cs^+ at a similar rate. However, addition of the hexa-crown peptide **1** as well as gramicidin A led to a very rapid release of protons that reached saturation after less than 2 min. These results suggest that **1** functions as an artificial ion channel which is as efficient as gramicidin A. Indeed, the obtention of a plateau at around 80% of the total value of protons entrapped is typical of channel molecules that do not migrate between vesicles contrarily to carriers like valinomycin.[8,11] In addition, the turbidity of the vesicle solution is constant throughout the experiments, but the addition after 1 h of a surfactant, Triton X-100, results almost instantly in a clear solution by lysing all vesicles. This proved that peptide **1** does not act like a simple surfactant. This is further proved by the observation that no proton release was observed when **1** was added to the vesicle solution in the absence of ions (Figure 2b).[12] Furthermore, the hexa-crown peptide **1** exhibited the same channel activity with Li^+, Na^+, K^+, and Rb^+. On the other hand, circular dichroism studies in trifluoroethanol demonstrated that **1** adopts a stable α-helix conformation, which suggests that this conformation is the most stable for **1** in a low-polarity environment like a bilayer membrane.

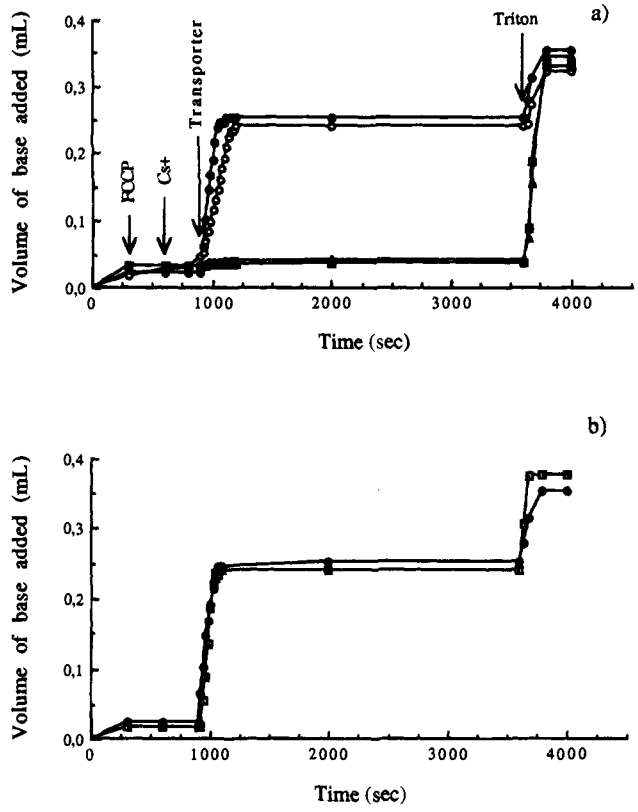

Figure 2. (a) Cs^+ transport ability of the monomeric crown ether **3b** (△), the heptapeptide **2** (■), the 21-residue peptide **1** (●), and the natural ion channel gramicidin A (○) in vesicles using the pH state technique.[8] The transport ability is monitored by the release of protons from the vesicles as a function of time. (b) Control experiment showing that the transport results are not dependent on the order of addition of the components: (⊡) FCCP, **1**, then Cs^+; (●) FCCP, Cs^+, then **1**.

In conclusion, we have developed a novel type of functional artificial ion channel and demonstrated its effectiveness for the transport of ions. The synthetic strategy to prepare **1** is simple and rapid and allows further molecular engineering of this type of compound. Although the results described support the proposed working model in Figure 1b, the precise mode of action of **1** has yet to be established. Work is currently under way to prepare analogues of **1** and to elucidate their transport mechanism.

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Supplementary Material Available: Circular dichroism spectra of **1** in trifluoroethanol (1 page). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(7) Salient data for **1**: FAB MS (thioglycerol) $C_{222}H_{363}N_{21}O_{66}$, 4404, M + Na^+; ^1H NMR (δ in ppm, DMSO-d_6) 0.72−0.90 (m, 90H, *i*-Bu CH_3), 1.20−1.65 (m, 45H, *i*-Bu CH_2CH), 1.35 (s, 9H, BOC *t*-Bu), 2.65−2.98 (m, 12H, β-CH_2 of **3**), 3.45−3.61 (m, 96H, crown CH_2), 3.62 (s, 3H, OMe), 3.65−3.78 (m, 24H, crown CH_2), 3.95−4.09 (m, 24H, crown CH_2), 4.15−4.39 (m, 15H, Leu α-CH), 4.45−4.56 (m, 6H, α-CH of **3**), 6.60−6.80 (m, 18H, arom H), 6.95 (d, 1H, BOC-NH), 7.67−8.25 (m, 20H, amides H).

(8) The pH stat method and the preparation of vesicles have been described in detail: Fyles, T. M.; James, T. D.; Kaye, K. C. *J. Am. Chem. Soc.* **1993**, *115*, 12315−21. See also ref 3a. 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. After the evaporation of ether, the vesicles were suspended in an unbuffered solution and purified by filtration through a 0.45 μm syringe filter to remove the large aggregates and by gel filtration on Sephadex G-25. The 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 sulfate and 0.093 M D-mannitol in doubly distilled deionized 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,[9] 0.5 mL of Cs_2SO_4 (0.5 M), and 10 μL of a 0.275 mM MeOH solution (2.75 nmol) of the transporter. The release of protons starts at this point, and the pH is maintained at 7.5 by addition of a choline hydroxide solution. After 1 h the remaining entrapped protons are liberated by the lysis of the vesicles by addition of Triton X-100. The experiments were performed under a stream of N_2.

(9) FCCP = 1,3-dinitrilo-2-propanone (4-(trifluoromethoxy)phenyl)-hydrazone is a proton carrier used to ensure that proton transport can occur rapidly: Herve, M.; Cybulska, B.; Gary-Bobo, C. M. *Eur. J. Biophys.* **1985**, *12*, 121−8. See also ref 8.

(10) Fyles, T. M.; James, T. D.; Kaye, K. C. *Can. J. Chem.* **1990**, *68*, 976−8.

(11) It is also possible that **1** does not empty all the vesicles because of the presence of some multilamellar liposomes whose interiors remain inaccessible to **1**.

(12) This experiment also demonstrates that choline cations from the buffer solution are not transported by **1**.