Zinc(II) as an Allosteric Regulator of Liposomal Membrane Permeability Induced by Synthetic Template-Assembled Tripodal Polypeptides

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Abstract: Three copies of peptide sequences from the peptaibol family, known to affect the permeability of the lipid bilayer of membranes, were connected to tris(2-aminoethyl)amine (TREN), a tripodal metal ion ligand, to prepare functional peptides capable of modifying the permeability of liposomal membranes. Some of the resulting tripodal polypeptide derivatives are very effective in promoting carboxyfluorescein (CF) leakage from CF-loaded unilamellar vesicles composed of a 70:30 phosphatidylcholine/cholesterol blend. The activity of these novel compounds was shown to be tunable upon metal ion coordination of the TREN subunit; the tripodal apopeptide was far more effective than its Zn^{II} complex. Leakage experiments showed that a minimum number of five amino acids per peptide chain is required to form active systems. A mechanism is proposed in which the Zn^{II} ion changes the conformation of the template from extended to globular and thus acts as an allosteric regulator of the activity of the systems. Molecular modeling studies indicate that when the three peptide chains are connected to the template in the extended conformation, the resulting tripodal polypeptide is able to span across the membrane, thus allowing the formation of permeable channels made of a cluster of molecules. The same change of conformation induces, to some extent, fusion of the membranes of different liposomes.

Keywords: allosterism · liposomes · membrane permeability · peptides · zinc

Introduction

Biological membranes are organized assemblies of lipids and proteins whose primary functions are containing, compartmentalizing, and regulating the transfer of metabolites and macromolecules in a living organism.[1] Their permeability is highly selective because of the occurrence of specific carriers or channel-formers for ions or organic molecules.[2] Several physiologically important channel proteins have been isolated and sequenced,[3] and the preparation of artificial ion channels is an ongoing ambitious goal attracting considerable efforts.[4] To this aim, different strategies have been used. Artificial proteins, based on specifically tailored peptides with predictable structural features and properties have been designed and synthesized.[5] The so-called "bouchet approach" has been exploited to prepare ion channels based on membrane spanning monomers attached to a pre-organized macrocyclic central core.[6] Very efficient transmembrane channels have recently been prepared by designing macrocyclic peptides that can adopt flat-ring conformations and stack one upon the other to form self-assembling nanotubes.[7]

A common feature of many biological systems is the presence of regulatory elements that allow modulation of the permeation processes across the membranes.[1] A reversible modulation of any given physicochemical property is the basic feature of any molecular or supramolecular device.[8] Artificial ionophores which can reversibly modulate membrane permeability in response to a chemical or electrochemical input are examples of such artificial devices.[9] Such a regulation may be achieved by taking advantage of allosteric effects, such as those resulting from a change of conformation owing to interaction with an effector in a remote subsite of the molecule which is not directly involved in the process. Examples of systems whose functions have been regulated by means of allosteric interactions have been reported.[10] They include hydrolytically active catalysts,[11] and enzyme inhibitors.[12]

Peptaibols[13] constitute an interesting class of natural antibiotics produced by soil fungi. It is widely accepted that

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these linear peptides act on cell membranes through channel formation involving leakage of the cytoplasmic material and, eventually, lead to cell death. They are characterized by closely related sequences of linear peptides, ranging from 19 amino acids (alamethicin)[14] to 11 amino acids (trichogin)[15], and even to as low as six amino acids (trichodecenins).[16] Peptaibols are known to contain a large portion of α-aminoisobutyric acid (Aib), which strongly promotes helix formation.[17] While the helix formed by the 19-amino acid sequence is long enough to span across the double layer of membranes, shorter peptides such as trichogin require the presence of a fatty acid chain at the N-terminus to elicit membrane activity (hence the term *lipo*peptides).[18] A mixed $\alpha/3_{10}$ helix was proposed for natural trichogin GA IV from extensive solution[15a,c] and crystal-state studies.[15b]

In 1996, we reported[19] the synthesis and the metal ion modulation of membrane permeability properties induced by a polypeptide template.[20] This was designed by connecting together three copies of a decapeptide analogue of trichogin GA IV through an appropriate spacer to the known metal ion ligand tris(2-aminoethyl)amine (TREN).[21] Membrane activity was tested by monitoring the leakage of trapped carboxyfluorescein (CF) from small unilamellar vesicles.[15a,c, 18, 22] Here, we report a comparative study of a wide class of compounds obtained by functionalizing the same ligand with peptides of different main chain length aimed at defining the minimal requirements for affecting membrane permeability as well as identifying the source of the activity observed.

Results and Discussion

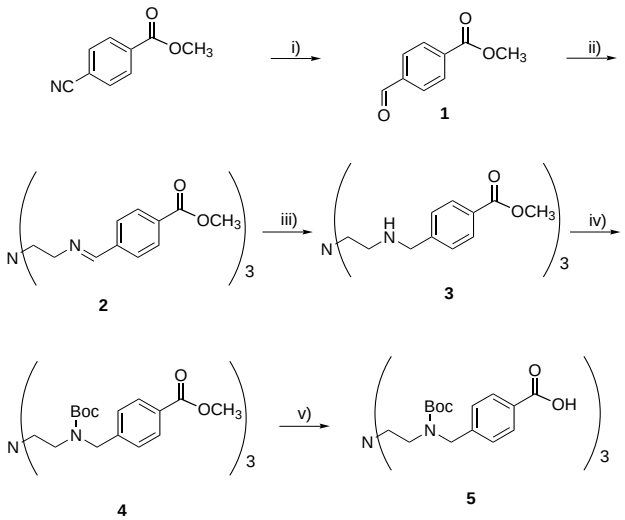
Peptide design and conformation: The peptides used for the construction of our systems are made of sequences ranging from three to ten amino acids; all of them are portions of the naturally occurring antibiotic trichogin. The constituent amino acids and the acronyms used for their identification are given below. Peptides **P4**, **P5**, **P6**, and **P10** correspond to the C-terminal sequence of trichogin, except for the C-terminal Lol which was replaced by Leu–OMe (the non-reduced amino acid ester). According to previous studies, this modification has little influence on the activity of the antibiotic.[15c, 23] Furthermore, all sequences contain at least one helicogenic Aib residue. The synthesis and characterization of peptides **P3**–**P6** and **P10** have been reported elsewhere.[23]

Trichogin GA IV	*n*-C_7H_{15}CO-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Lol
C_8P_{11}	*n*-C_7H_{15}CO-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-LeuOMe
P10	Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-LeuOMe
P6	Gly-Leu-Aib-Gly-Ile-LeuOMe
P5	Leu-Aib-Gly-Ile-LeuOMe
P4	Aib-Gly-Ile-LeuOMe
P3	Gly-Leu-Aib-NH*i*Pr
Ac(P11)	CH_3CO-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-LeuOMe
Z(P10)	$PhCH_2OCO$-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-LeuOMe

A conformational investigation carried out by FT-IR absorption spectroscopy in deuterochloroform on the Z-protected peptides used for the preparation of template-assembled **T(P4)$_3$**, **T(P6)$_3$** and **T(P10)$_3$** reveals the following features: 1) the presence of free (stretching band at $\tilde{\nu} > 3400\ cm^{-1}$) and hydrogen-bonded (stretching band in the $3356-3319\ cm^{-1}$ range) NH groups, 2) an increase in the intensity of the hydrogen-bonded NHs as the number of amino acids in the peptide chain increases. This behavior strongly suggests formation of a folded structure even with the short, four-residue peptides. Upon increasing the length of the peptide, the content of the folded conformation tends to increase. The crystal structures of the Z-protected tetrapeptide **P4** and pentapeptide **P5** confirm the spectroscopic evidence, although they show that in the crystals, the molecules assume conformations not amenable to a regular 3_{10} or α helix.[24] For the purpose of our investigation, which was carried out in a lipid bilayer, it is conceivable that the environment experienced by the peptide in the membrane would be similar to that in the chloroform solution used for the spectroscopic investigations. Hence, the folded conformation of the peptides is probably maintained when bound to the liposomes. This is also supported by our recent observation that peptides rich in C^{α}-tetrasubstituted amino acids assume a prevailing helical conformation even in aqueous solutions.[25]

Synthesis and conformation of the template-assembled peptides: Peptides **P3**–**P6** and **P10** were used for the functionalization of TREN. It is known that coordination of N-functionalized TREN to Zn^{II} involves, typically, formation of pentacoordinate trigonal bipyramidal complexes with four amino groups and an extra apical ligand.[21] A 4-carboxy-1-methylbenzene spacer was introduced between the TREN molecule and the peptide sequences to avoid undesired interactions between the vacant coordination site of the metal and the peptide chains, which, inter alia, could result in the modification of their secondary structures.

The series of five related compounds was prepared as indicated in Scheme 2, while the templating platform was prepared according to Scheme 1. Thus, using $SnCl_2$ and HCl,



Scheme 1. Synthesis of the TREN-based template. i) $SnCl_2$, HCl, Et_2O; ii) TREN, CH_3CN; iii) $NaBH_4$, EtOH; iv) $(Boc)_2O$; v) NaOH, EtOH, then diluted HCl.

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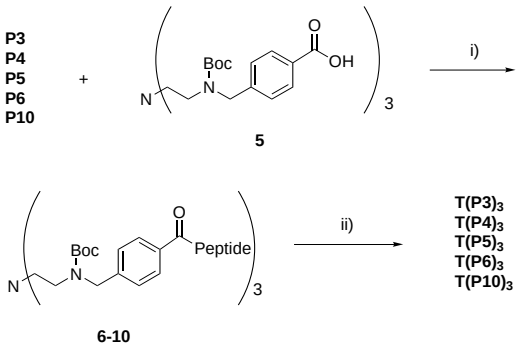
4-cyanomethylbenzoate was converted into the corresponding aldehyde, which was then treated with TREN, and the resulting tris-imine was reduced with $NaBH_4$. This TREN derivative was subsequently protected with Boc and the methyl ester was hydrolyzed to give the free carboxylic acid. All peptide sequences were prepared in solution by stepwise elongation of the peptide chain starting from the C-terminal leucine methyl ester.[23] N^{α}-Benzyloxycarbonyl amino acid derivatives were incorporated in the peptide chain either by the mixed anhydride or by the symmetrical anhydride coupling method. Docking between the core unit and the peptide sequences was performed by coupling the free benzoyl groups of the ligand with the N-terminus of each different peptide using 1-hydroxybenzotriazole (HOBt) and *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC) as coupling reagents. Removal of the Boc protecting groups and semi-preparative reversed-phase HPLC purification gave the final products.



Scheme 2. Synthesis of the tripodal polypeptide templates. i) HOBt, EDC, CH_2Cl_2; ii) TFA, CH_2Cl_2.

The purity and the identity of these compounds were assessed by analytical HPLC, ^{1}H NMR and mass spectrometry (MALDI). Final compounds where isolated as trifluoroacetate salts; in this form they are all soluble in methanol, insoluble in chloroform and dichloromethane. Template-assembled derivatives **T(P5)$_3$**, **T(P6)$_3$**, and **T(P10)$_3$** are soluble in water up to about 5×10^{-5} M at pH 7.3, while **T(P3)$_3$** and **T(P4)$_3$** exhibit a slightly higher solubility in this solvent.

The uncomplexed ligands have a flexible structure with no geometrical constraint. Previous work conducted in our laboratory[10d] has shown that on binding to Zn^{II} ions, a benzyl-functionalized TREN derivative forms a pentacoordinate complex with the three phenyl groups pointing in the same direction, defining a basket-like pseudocavity. Thus, in principle, the tertiary structure of the template-assembled polypeptides can be controlled by metal ion complexation. The binding constant of the TREN platform for the 1:1 complex with Zn^{II} ions is estimated from literature data to be about 10^{10} M^{-1} at pH 7.[26] This means that a very strong complex can be formed, and also that it is reasonable to assume that a negligible amount of free metal ion is present when a 1:1 solution of ligand and metal ion is prepared and diluted to a concentration of 10^{-7} M.

The FT-IR absorption spectra of apopeptides **T(P5)$_3$**, **T(P6)$_3$**, and **T(P10)$_3$** in deuterochloroform solution (Figure 1)

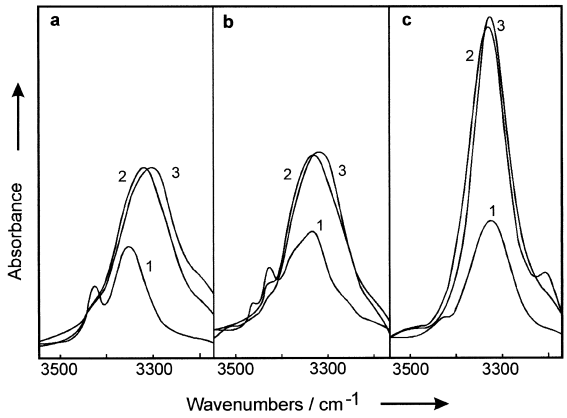


Figure 1. FT-IR absorption spectra of the Z-protected peptides (1), the tripodal polypeptides (2), and their Zn^{II} complexes (3) recorded in $CDCl_3$ (10% CD_3CN added for **T(P10)$_3$**). a) **P5** and **T(P5)$_3$**; b) **P6** and **T(P6)$_3$**; c) **P10** and **T(P10)$_3$**.

indicate an increase of the hydrogen-bonded versus free NH groups when compared to those of the corresponding single, Z-protected peptides. This behavior suggests that, within the template-assembled tripodal peptides, the peptide chains tend to interact reciprocally, and this interaction induces formation of additional hydrogen bonds. Although it is not possible to know from this behavior whether this extra hydrogen bonding occurs within the same peptide chain or between different chains, it clearly suggests some sort of interaction between the peptides in the template leading to an overall increase of hydrogen bonding. We note, however, that the main amide I band occurs at 1662 cm^{-1}, in full accord with a 3_{10} helical conformation.[27] Induced helicity by the clustering of several peptide molecules is well documented in the literature.[20a, 28] Upon formation of the Zn^{II} complexes, the only change observed is a shift (10–15 cm^{-1}) of the absorption maximum towards lower wavenumbers in accordance with a further strengthening of the hydrogen bonds, probably owing to a stronger interaction between the peptide chains. This could imply a Zn^{II} template conformation that can induce the alignment of the three peptide chains.

Further support for a change of conformation in the presence of Zn^{II} is obtained by dynamic light scattering experiments. Figure 2 shows the amount of scattered intensity of aqueous solutions of **T(P10)$_3$** as a function of its concentration as a free ligand and as a Zn^{II} complex. Since the intensity of scattered light depends not only on the concentration but also on the size of the system, a steeper gradient in Figure 2 indicates a larger object in solution. Hence, the data suggest a more compact (probably globular, see above) conformation for the Zn^{II} complex. From the relatively high intensity of the scattered light, it appears that both systems form aggregates under the conditions employed. The size of the aggregates does not change in the concentration interval explored, as there is no change of gradient and both lines show the same intercept that represents the background intensity in the absence of added peptide. Hence, aggregates are already present at the lowest concentration explored. As mentioned above, the onset of aggregation or a change in the size of the aggregates would result in a net change of gradient. However,

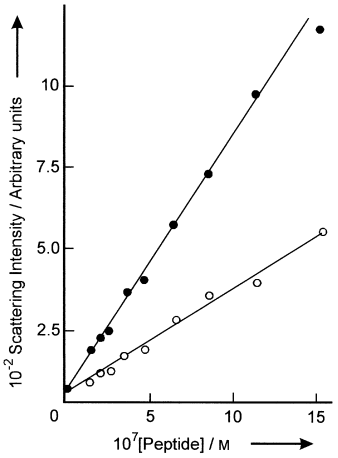


Figure 2. Dependence of the scattering intensity of aqueous solutions of **T(P10)$_3$** (●) and its Zn^{II} complex (○) as a function of the concentration of tripodal peptide at 25 °C.

we cannot rule out that the difference in gradient is related, at least in part, to a different number of monomers constituting the two different aggregates.

These observations lend support to the hypothesis that complexation with Zn^{II} induces a change of conformation of the polypeptide template with the formation of a three-helix bundle, similar to that depicted in Figure 3b, while the polypeptide template devoid of the metal ion assumes a more open conformation like, for instance, that illustrated in Figure 3a.

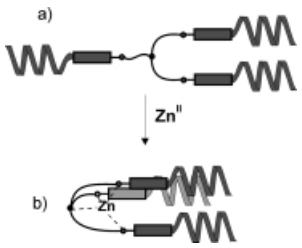


Figure 3. Suggested conformational change induced by the addition of Zn^{II} ions to the tripodal polypeptides.

Trapped-dye release from liposomes: As stated in the Introduction, our basic idea was to verify if the template-assembled polypeptides would be able to alter the permeability of a liposomal membrane, and if the complexation of a Zn^{II} ion could modulate this effect. Furthermore, we wanted to know the influence of the length of each peptide chain on the efficiency of the modification of membrane permeability. The CF-leakage technique[15a,c, 18, 22] takes advantage of the increase in fluorescence of the dye when released into the surrounding medium from a condition of self-quenching due to its high concentration when trapped inside the liposome. Membrane-modifying properties of the new compounds were thus examined by following their effect on CF leakage from small unilamellar vesicles prepared from a 70:30 blend of egg L-α-phosphatidylcholine and cholesterol loaded with a high concentration (5×10^{-2} M) of the fluorescent dye. Vesicles were prepared by sonication, and unencapsulated carboxyfluorescein was separated by passing the resulting dispersion through a Sephadex column. Light scattering experiments on the vesicle solutions devoid of the trapped dye gave a hydrodynamic diameter of 63 ± 5 nm. This fact and the method of preparation of the aggregates (sonication) are consistent with the formation of unilamellar aggregates. The time course of the fluorescence emission is very similar to that reported for analogous experiments conducted on antibiotics of the peptaibol family.[29] A typical profile is shown in Figure 4 for **T(P6)$_3$** and **T(P10)$_3$**. An initial burst of fluorescence emission, the amount of which was found to be

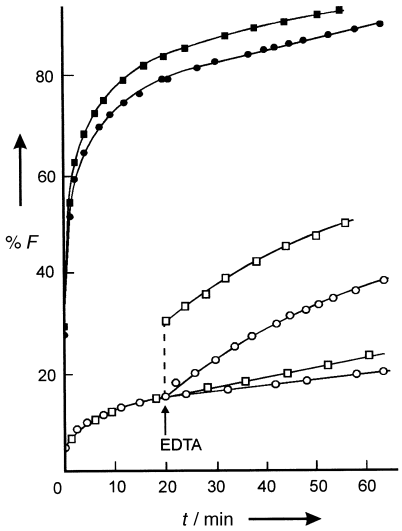


Figure 4. Time course of the release of trapped CF from CF-loaded liposomes at 25 °C upon addition of apopeptides (filled symbols) and Zn^{II}-peptides (open symbols). Squares: **T(P10)$_3$**; circles: **T(P6)$_3$**. The arrow indicates the time of addition of excess EDTA to a cuvette containing the Zn^{II} peptides while a second cuvette was left unchanged for reference. Conditions: [tripodal polypeptide] = $[Zn^{II}] = 5 \times 10^{-7}$ M, pH 7.4.

dependent on the amount of peptide added, is immediately observed, followed by a slower increase until the final fluorescence intensity is eventually reached. Because of the kinetic complexity of the fluorescence versus time profiles we found it convenient to evaluate the efficiency of the systems by determining the percentage of fluorescence increase observed after 20 min, a time that was chosen after a preliminary screening of the different peptide templates.

The results obtained for the different systems and their Zn^{II} complexes are shown in Figure 5 as a function of their concentration. Table 1 and Figure 6 report the percentage of fluorescence increase after 20 min at a fixed concentration of 5×10^{-7} M polypeptide. Table 1 also reports the amount of CF leaked spontaneously by the vesicles, and the effect due to the single peptide models **Ac(P11)** and **Z(P10)** at the same concentration.

Analysis of Figures 5, 6, and Table 1 allows one to make the following observations: 1) Some of the systems synthesized are very effective in promoting CF leakage from the vesicles. In the best cases, the efficiency is higher than that of the trichogin analogue **C$_8$(P11)**. 2) The Zn^{II} complexes are consistently much less effective than the apopeptides in promot-

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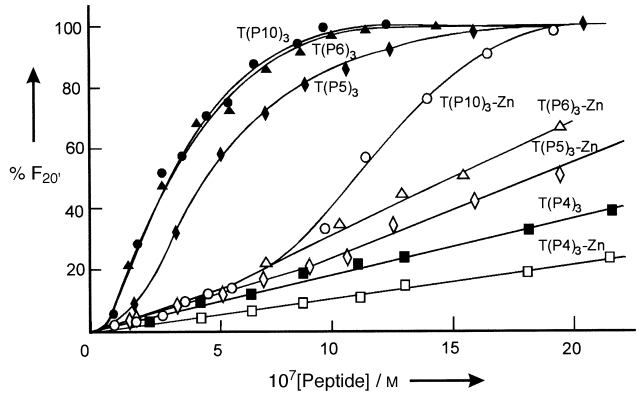

Figure 5. Percentage of CF released after 20 min at 25 °C and pH 7.4 from CF-loaded liposomes upon addition of increasing amounts of the different tripodal polypeptides and their Zn^{II} complexes.

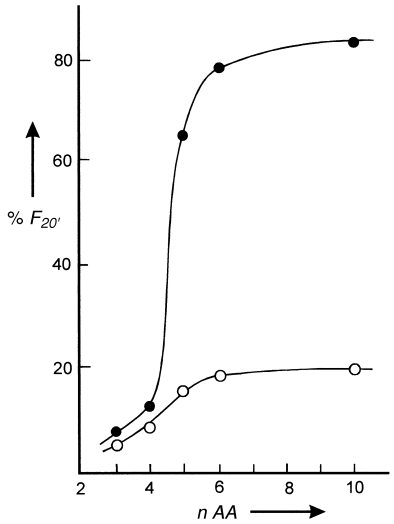

Figure 6. Percentage of CF released after 20 min at 25 °C from CF-loaded liposomes as a function of the number of amino acids present in each peptide chain. [Tripodal polypeptide] = 5×10^{-7} M; filled symbols refer to apopeptides while open symbols refer to the Zn^{II} complexes.

Table 1. Percentage increase of fluorescence intensity at 520 nm after 20 min upon addition of 5×10^{-7} M polypeptide (free or as a 1:1 Zn^{II} complex) or the same concentration of other additives to CF-loaded liposomes at 25 °C.

Additive	F_{20} [%]	
	free	**Zn^{II}**
$T(P10)_3$	83	18
$T(P6)_3$	79	18
$T(P5)_3$	65	15
$T(P4)_3$	12	8
$T(P3)_3$	7	5
C_8P_{11}	55	
Ac(P11)	3	
Z(P10)	5	
TOMe[a]	3	
none	3	

[a] The methyl ester of the TREN platform devoid of peptide chains.

ing CF release from the vesicles. 3) The six residue per chain tripodal polypeptide (**$T(P6)_3$**) is as effective as the longer homologue **$T(P10)_3$**. 4) As the number of amino acids in each peptide chain decreases, the efficiency decreases as well, and eventually vanishes completely with **$T(P3)_3$**. 5) The presence of the template is critical for the obtainment of an active system. Single peptide systems **Ac(P11)** and **Z(P10)**, although made of ten amino acids, are essentially inactive. 6) The TREN platform, which is devoid of peptide functionalization, is also inactive.

For **$T(P6)_3$** and **$T(P10)_3$**, the permeability can, at least in part, be reversibly controlled (Figure 4). When we start the experiment with the Zn^{II} complexes, we observe a very slow increase of fluorescence, in accord with the behavior described above. However, the rate of CF release becomes much faster when an excess of ethylendiaminetetraacetic acid (EDTA) is added to the solution. EDTA ($\log K_b = 16.7$ for Zn^{II})[30] removes the metal ion from the TREN platform, thereby transforming the tripodal polypeptide into a more efficient system for promoting CF release from vesicles. The time course of an experiment which starts with the free ligand is less amenable to unravel such a reversible control of permeation, as the very fast initial CF release is followed by a slower process (see above) which is difficult to differentiate from that observed in the presence of the Zn^{II} complex.

"Snapshots" taken after 20 min for different vesicular solutions exposed to 2.5×10^{-7} M **$T(P6)_3$** in the presence of an increasing concentration of metal ion clearly indicate that one equivalent of Zn^{II} required to elicit the maximum inhibition of the effect of the free tripodal polypeptide (Figure 7). This finding rules out any perturbation of permeability associated with an interaction of Zn^{II} with the lipid rather than with the TREN template. Indeed, it has been reported that divalent ions (including Zn^{II}) decrease membrane permeability,[31] although this effect shows up at concentrations far higher than those employed in our experiments.

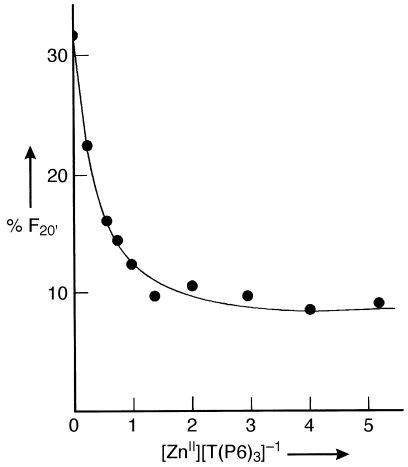

Figure 7. Percentage of CF released after 20 min at 25 °C and pH 7.4 from CF-loaded liposomes as a function of the $[Zn^{II}]/[\mathbf{T(P6)_3}]$ ratio. $[\mathbf{T(P6)_3}] = 2.5 \times 10^{-7}$ M.

Membrane fusion experiments: The kinetic experiments illustrated in Figure 4 indicate that the process of dye release is by no means unimodal. What was particularly intriguing was the very fast increase of fluorescence at the very early stages

of the experiments. It occurred to us that the amphiphilic nature of the active templates could be responsible for a fusion process between the membranes of different liposomes. A fast fusion process could be associated with a rapid release of part of the trapped dye. For this purpose, we have run lipid mixing experiments using vesicle preparations containing 1% of hexadecylphosphatidylethanolamine functionalized at the amine of the head group with the 7-nitrobenzen-2-oxo-1,3-diazol-4-yl group (PE-NBD) and lissamine-rhodamine (PE-LRB), respectively. Excitation of PE-NBD (450 nm) leads to an emission band at 530 nm, which is very close to the absorption band of PE-LRB. When the two dyes are on the membrane of the same liposome, their proximity leads, upon excitation of PE-NBD to the emission of PE-LRB and a concomitant decrease of the emission of PE-NBD because of energy transfer between the two systems.[32]

Accordingly, when two liposome preparations containing PE-NBD and PE-LRB, respectively, are mixed in the presence of a fusogenic agent, the mixing of their lipids leads to the phenomenon described above. Figure 8 shows the effect

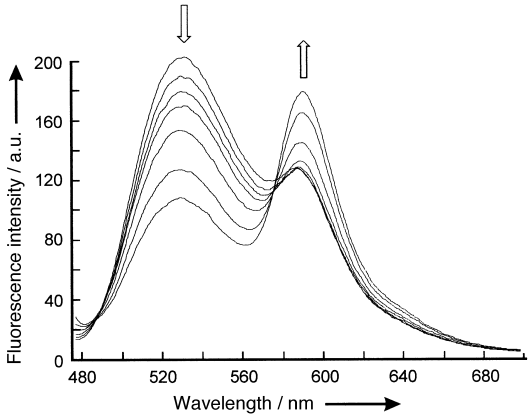

Figure 8. Fluorescence emission spectra (λ_{ex} 450 nm) of an equimolar mixture of PE-NBD and PE-LRB containing vesicles (1 mol% fluorescent probe/total lipid; [total lipid] = 1.14×10^{-4} M) and increasing concentration of **T(P10)$_3$**. The different curves are relative to the following percentage of [**T(P10)$_3$**] respect to the total lipid concentration = 0, 0.5, 0.9, 1.4, 1.8, 2.3, and 2.7%.

on the fluorescent emission of a solution of two liposome preparations, each containing one of the two fluorescent lipids, upon addition of increasing amounts of **T(P10)$_3$**. The fluorescent emission of PE-NBD decreases, while that of PE-LRB increases; this behavior suggests that the template induces considerable fusion between the two liposome preparations. The phenomenon is instantaneous and occurs immediately after the addition of the peptide template with no further time-dependent change of fluorescence. When the same experiment is carried out with **T(P10)$_3$**-Zn^{II} the effect is almost negligible (data not shown), in line with the lack of the burst of fluorescence in the kinetic experiments in the presence of the complex (see Figure 4). We suggest that the fast fusion process induced by **T(P10)$_3$** is also responsible for the burst in the release of trapped CF in the experiments described in the previous paragraph.

Effects of the polypeptides on the liposomal membranes: The experiments reported above point to at least two different effects exerted by the polypeptide templates on the liposomal membrane: 1) fusion, and 2) altered permeability.

The former is a fast process occurring immediately after the addition of the templates (devoid of Zn^{II}) to the liposomes. During fusion some of the trapped dye is released and this accounts for the instantaneous burst of fluorescence in Figure 4. Interestingly enough, a similar kinetic behavior has been observed with other antibiotics of the peptaibol family and we speculate that in those cases also, induced fusion may be the explanation. In the present case, it is conceivable that several polypeptide templates in their extended conformation (see Figure 3a) bind to two different liposomes, inserting one peptide chain into the membrane of one liposome and the other two into the membrane of the second liposome. This should allow the interaction of the constituent lipids that eventually leads to fusion. In the presence of Zn^{II}, the system assumes a conformation that does not allow this interaction (see Figure 3b) and, consequently, fusion is negligible.

As for the alteration of permeability, this may be attained by the following mechanisms: 1) formation of pores or channels that span across the membrane,[4] 2) formation of lipophilic complexes with the trapped dye (carrier mechanism),[33] or, 3) formation of defects or "soft" patches in the bilayer with enhanced permeability.[34] This last mechanism occurs more frequently when large amounts of additives (typically amphiphilic molecules) are added to the vesicular preparation. The very low concentration at which the present tripodal polypeptides exert their effects ($\approx$1% with respect to the lipid concentration) allows us to dismiss the possibility that this is actually the mechanism occurring. The channel or carrier mechanisms appear more likely. With the data obtained from the present investigation we favor the aforementioned mechanism 1) on the basis of the following arguments:

1) A concentration lag is observed before the onset of activity of the tripodal polypeptide. All profiles of Figure 5 are sigmoidal as typically expected for a cooperative process. In the present case, this finding could be related to the obtainment of the minimum concentration required for the formation of a channel by a cluster of tripodal polypeptides within the vesicular membrane. In the case of the carrier mechanism, a lower efficiency and a linear dependence on tripodal peptide concentration would be expected.[35]
2) It has been suggested[6a, 35] and experimentally verified[6a] with naturally occurring ionophores operating with channel or carrier mechanism, that the carrier mechanism becomes much less effective in membranes in their gel state, that is, below their phase transition temperature (T_c). For this purpose we have tested the tripodal polypeptide **T(P10)$_3$** with CF-loaded liposomes made of diphosphatidylcholine (DPPC), a lipid characterized by a T_c of 41 °C and, hence, in the gel state at 25 °C. By contrast, the membrane of the liposomes of egg phosphatidylcholine used in the leakage experiments described above are in their fluid state at 25 °C (T_c in the range -15 to -7 °C). As can be seen in Figure 9, the amount of released CF is

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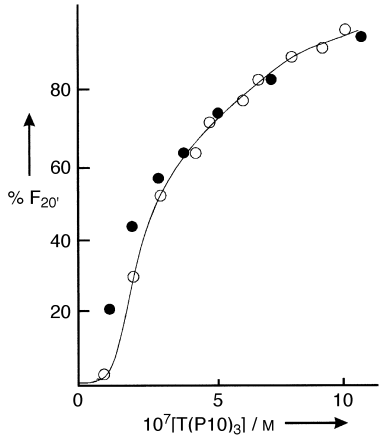

Figure 9. Percentage of CF released after 20 min at 25 °C from CF-loaded liposomes (open circles, egg phosphatidylcholine, filled circles DPPC) upon addition of increasing amounts of **T(P10)$_3$**.

practically the same irrespective of the fluidity of the membrane of the liposomes, lending strong support to the channel mechanism.

3) Molecular models of the Zn^{II}-ligated polypeptide template (Figure 10) obtained by manually docking three copies of the decapeptide (in the conformation obtained from the crystallographic data of trichogin GA IV)[15b] to the TREN platform[10d] and subsequently minimizing the energy of the structure for the new bonds introduced, have an estimated distance between the termini of the helices and the CH_2 groups of TREN close to the pivotal nitrogen of approximately 18 Å, that is, half the length of the phospholipid bilayer (ca. 36 Å). However, in the absence of the metal ion and, hence, in the extended conformation, the polypeptide template is able to span the full length of the bilayer.

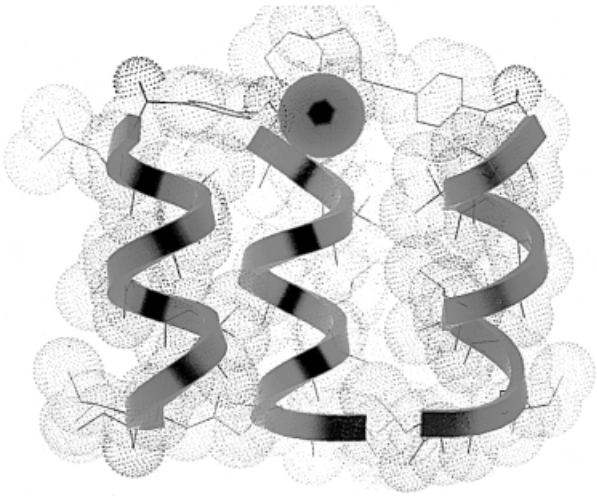

Figure 10. Computer graphic picture of the Zn^{II} complex of tripodal polypeptide **T(P10)$_3$**.

On the basis of the above considerations, we propose the following mechanism to explain the alteration of liposomal membrane permeability induced by the tripodal polypeptides. The tripodal polypeptide binds to a liposome mostly in its extended conformation.[36] When a critical concentration is reached within a liposome, a cluster of tripodal polypeptides is formed. They may interact with another liposome to give membrane fusion or, by inserting more deeply into the membrane, they get aligned and oriented in such a way as to expose the hydrophobic surface of each helix to the hydrocarbon chains of the surrounding lipids, while the more polar surface defines a channel suitable for the permeation of polar species. As the number of amino acids in each peptide decreases below six, the length of the tripodal polypeptide is too short to span across the membrane and, furthermore, its helical conformation becomes less stable. Consequently, the activity decreases. Activity is also much lower if it binds to the membrane in its globular conformation as in the Zn^{II} complex. In this case again, the system is too short to allow for the fusion process or to ensure the formation of a channel spanning the membrane, and the residual activity observed could be due to the occasional alignment of two clusters residing on different leaflets of the membrane.[37]

Conclusion

By connecting three copies of a series of peptides, with amino acid sequences representing portions of the peptaibol trichogin GA IV, to a modified TREN platform, we have obtained template-assembled polypeptides that are very effective in promoting CF leakage from unilamellar vesicles. A minimum number of five amino acids per peptide chain are required to elicit activity in the polypeptide template. The rate of CF release can be controlled by formation of a Zn^{II} complex with the TREN subunit in a reversible process. The apopeptides are far more effective than the Zn^{II} polypeptides in inducing CF leakage. This behavior is probably associated with a change of conformation of the polypeptide template from an extended one, able to span across the vesicular membrane, to a globular one, too short for this purpose. We note, finally, that this conformational change may regulate the activity of a system according to its functions. In the present case, Zn^{II} binding leads to inhibition of activity. In a recent example from our own laboratory based on the same templating unit,[12] Zn^{II} complexation leads, in contrast, to enhancement of the efficiency of a HIV-1 protease inhibitor. In this latter case, the conformational change induced by metal ion binding ensures a tighter binding with the dimerization interface of the protein.

Experimental Section

Proton NMR spectra were recorded at 200 MHz on a Bruker AC200F spectrometer, at 250 MHz on a Bruker AC250F spectrometer, or at 400 MHz on a Bruker AM400 spectrometer. All chemical shifts are reported in parts per million (ppm) as downfield shifts from Me_4Si. Light scattering experiments were performed with a Spectra Physics 2016 argon laser operating at 488 nm, interfaced with a Nicomp 370 Model particle sizing autocorrelator. The experiments were carried out at 25 °C on previously centrifuged samples. Positive Maldi-MS measurements were performed at the CNR Research Area, Camin, Padova.

HPLC purification: All peptide derivatives were purified by high-performance liquid chromatography. HPLC was performed using a Pharmacia LKB-LCC2252 liquid chromatograph equipped with a UV Uvicord SV detector operating at 226 nm. All separations were accomplished on a reversed-phase semi-preparative C_{18} column (Vydac model218TP1010) using a solvent gradient (solvent A: 0.05% TFA in H_2O; solvent B: 0.05% TFA in 9:1 CH_3CN/H_2O). Purity was confirmed to be at least 95% by analytical HPLC performed using a Gilson liquid chromatograph equipped with a model306 double pump, a manometric device model306, a Rainin UV detector (DINAMAX operating at 226 nm), and a reversed-phase C_{18} column (Vydac model218TP54) using the same solvent mixture.

Membrane permeability measurements: Leakage from vesicles was evaluated using the CF-entrapped vesicle technique.[15a,c, 18, 22] CF-encapsulated vesicles were prepared according to the following procedure: egg phosphatidylcholine (20.5 mg) and cholesterol (4.5 mg) were dissolved in freshly distilled CH_2Cl_2 (5 mL). The resulting mixture was stirred for 5 min under nitrogen and then evaporated under a steady nitrogen stream. The residue was dried under vacuum and then allowed to swell for one night at room temperature under nitrogen in a pH 7.4 HEPES solution (0.05 M, 5 mL) containing CF (50 mM). The slurry was sonicated (Braunsonic immersion probe sonicator, 40% duty cycle, output control set to five) at 0 °C for 45 min, and the solution was filtered through a 0.45 μm Millipore filter. Unencapsulated CF was removed by passing the solution through a Sephadex G-75 column (0.1 M NaCl, 0.01 M HEPES, pH 7.4). The fractions containing the liposomes were collected, and the total lipid concentration was determined by phosphate analysis.[38] The liposomes were then diluted with buffer to a 4×10^{-5} M for the leakage experiments. Three mL of this solution were used for each experiment. The phospholipid concentration was kept constant, and increasing [peptide]/[lipid] molar ratios were obtained by adding aliquots of the peptide dissolved in a 1:1 methanol/water mixture (pH 7.4, HEPES 0.01 M; the pH refers to the aqueous phase before mixing). Methanol concentration was kept below 2% in all the experiments. After peptide addition the mixture was vigorously stirred, and the time course of the fluorescence change corresponding to CF efflux was recorded at 25 °C on a Perkin–Elmer LS50B instrument (λ_{em} 520 nm, λ_{ex} 488 nm). The percentage of released CF was determined according to Equation (1):

$$\% \ CF = (F_t - F_0)/(F_T - F_0) \times 100 \quad (1)$$

where F_0 is the fluorescence intensity of the vesicles suspension in the absence of peptide, F_t is the fluorescence intensity at time t in the presence of peptide, and F_T is the total fluorescence determined by disrupting the vesicles by addition of 30 μL of a 10% Triton X-100 solution.

Vesicle fusion assay: Peptide-induced vesicle fusion was monitored using resonance energy transfer (RET) between PE-NBD and PE-LRB probes.[31] PE-NBD and PE-LRB-loaded vesicles were prepared as following: egg phosphatidylcholine (11.0 mg) and cholesterol (2.4 mg) were dissolved in freshly distilled CH_2Cl_2 (2 mL). This solution was split in two aliquots (each 1 mL), to which were added PE-NBD (230 μL of a 3.12×10^{-4} M solution in CH_2Cl_2) and PE-LRB (100 μL of a 7.14×10^{-4} M solution in CH_2Cl_2) to obtain a lipid/probe ratio of 100:1. The organic solvent was evaporated under a steady nitrogen stream followed by high vacuum for two hours. The two lipid preparations were dispersed in 10 mL of a pH 7.4, 0.01 M HEPES/0.1 M NaCl buffer solution, sonicated separately (Braunsonic immersion probe sonicator, 40% duty cycle, output control set to five) at 0 °C for 45 min, and the solutions obtained were filtered through a 0.45 μm Millipore filter. The fluorophore-labeled liposomes (160 μL of each) were diluted with buffer in the same cuvette to a final volume of 2 mL. Fluorescence emission spectra (475–700 nm, λ_{ex} 450 nm) were recorded at 25 °C on a Perkin–Elmer LS-50B instrument before and after the addition of increasing amounts of the peptide dissolved in a 1:1 methanol/water mixture (pH 7.4, HEPES 0.01 M; the pH refers to the aqueous phase before mixing).

Syntheses: The synthesis of Z-protected peptides **P3**, **P4**, **P5**, **P6**, and **P10** has already been reported.[23] Deprotection was performed by hydrogenation on Pd/C following standard procedures. Analytical HPLC profiles of compounds **T(P4)$_3$**–**T(P6)$_3$** and **T(P10)$_3$**, their ^{1}H NMR spectra and MALDI-MS spectra are reported in the Supporting Information.

4-Formylmethylbenzoate (1): Stannous chloride (33.33 g, 175.7 mmol) was suspended in diethyl ether (100 mL) and HCl gas was bubbled into the mixture until two liquid layers separated. A solution of methyl-4-cyanobenzoate (10 g, 62.05 mmol), dissolved in diethyl ether (50 mL), was added and the mixture was vigorously stirred for two days. A white precipitate formed, the mixture was decanted overnight, and the solid collected by filtration. A 10% $NaHCO_3$ solution was then added to the residue until all the acid was neutralized. The mixture was extracted three times with dichloromethane, the organic phase was then dried over Na_2SO_4, and the solvent was evaporated to dryness. Purification on silica gel chromatography ($CHCl_3$) afforded the desired product **1** (5.18 g, 51%). M.p. 62 °C (lit. 61–62 °C)[39]; ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 3.95$ (s, 3H; OCH_3), 7.92, 7.95, 8.15, 8.18, (AA′BB′, 4H; Ph), 10.07 (s, 1H; CHO).

***N,N,N*-Tris{2-[(4-methoxycarbonylphenyl)methylenimino]ethyl}amine (2)**: TREN (1.54 g, 10.5 mmol) was dissolved in dry CH_3CN (40 mL) and then added dropwise to a magnetically stirred solution of **1** (5.18 g, 31.5 mmol) in the same solvent (100 mL). After 4 h, a white precipitate formed. The solid was collected by filtration and then washed with additional solvent. The organic solution was evaporated to dryness, and the oily residue was dissolved in toluene (10 mL) and then evaporated to dryness again. After repeating this procedure three times, a white solid compound was recovered. This was washed with CH_3CN and combined to the previous fraction to obtain compound **2** (5.2 g, 85%). The product was used directly in the following step. ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 2.95$ (t, $J = 6.25$ Hz, 6H; NCH_2CH_2), 3.70 (t, $J = 6.25$ Hz, 6H; NCH_2CH_2), 3.94 (s, 9H; OCH_3), 7.55, 7.59, 7.97, 8.01 (AA′BB′, 12H; Ph), 8.13 (s, 3H; NC*H*Ph).

***N,N,N*-Tris{2-[(4-methoxycarbonylphenyl)methylamino]ethyl}amine (3)**: A solution of **2** (5.1 g, 87.2 mmol) dissolved in the minimum amount of methanol was carefully added to a suspension of $NaBH_4$ (1.5 g, 39.7 mmol) in the same solvent (100 mL) which was stirred under nitrogen at 0 °C in an ice bath. The ice bath was removed, and the mixture was stirred at room temperature overnight. Water (3 mL) was added, and the mixture was stirred for 30 min. The solvent was removed under vacuum, and H_2O (50 mL) was added to the resulting oily residue. The aqueous phase was extracted with CH_2Cl_2 (3×50 mL), the combined organic phases were dried over Na_2SO_4, and the solvent was removed under vacuum to afford compound **3** (5.15 g, 99%) as a yellow oil. ^{1}H NMR (250 MHz, $CDCl_3$): $\delta =$ 1.78 (br s, 3H; NH), 2.57–2.59 (m, 6H; NCH_2CH_2), 2.63–2.65 (m, 6H; NCH_2CH_2), 3.78 (s, 9H; OCH_3), 3.90 (s, 6H; CH_2Ph), 7.29, 7.32, 7.91, 7.94 (AA′BB′, 12H; Ph).

***N,N,N*-Tris{2-[(4-methoxycarbonylphenyl)methyl(*tert*-butoxycarbonyl)-amino]ethyl}amine (4)**: A mixture of di-*tert*-butyldicarbonate (2.97 g, 13.6 mmol) and compound **3** (2.41 g, 4.08 mmol) in freshly distilled CH_2Cl_2 (100 mL) was stirred overnight at room temperature with protection by a $CaCl_2$ drying tube. $NaHCO_3$ (5%, 30 mL) was added and the mixture was stirred for 30 min. The aqueous phase was extracted with CH_2Cl_2 ($3 \times$ 10 mL). The organic layers were combined, dried over Na_2SO_4, and the solvent was evaporated. Purification on silica gel ($CHCl_3$/ethyl acetate 6:1) afforded the desired product **4** (2.5 g, 69%) as a yellow oil. ^{1}H NMR (250 MHz, $CDCl_3$, 328 K): $\delta = 1.40$ (s, 27H; Boc), 2.55 (br t, 6H; NCH_2CH_2), 3.18 (br t, 6H; NCH_2CH_2), 3.91 (s, 9H; OCH_3), 4.43 (s, 6H; CH_2Ph), 7.23, 7.26, 7.96, 7.99 (AA′BB′, 12H; Ph).

***N,N,N*-Tris{2-[(4-carboxyphenyl)methyl(*tert*-butoxycarbonyl)amino]-ethyl}amine (5)**: NaOH (1 M, 20 mL) was added to a solution of **4** (2.5 g, 2.81 mmol) in EtOH (140 mL). After stirring the mixture at room temperature for 24 h, no more starting material was detected by TLC ($CHCl_3$/MeOH 9:1). The solvent was evaporated leaving a white residue that was collected and dissolved in H_2O (30 mL). After adjusting to pH 4 with 1 M HCl, a white solid precipitated. This was separated by centrifugation, washed several times with neutral water, and then dried under vacuum in the presence of anhydrous $CaCl_2$ to obtain compound **5** (1.7 g, 71%). M.p. 153–155 °C; ^{1}H NMR (250 MHz, CD_3OD, 328 K): $\delta = 1.44$ (s, 27H; Boc), 2.62 (br m, 6H; NCH_2CH_2), 3.26 (br m, 6H; NCH_2CH_2), 4.51 (s, 6H; CH_2Ph), 7.32, 7.36, 8.00, 8.03 (AA′BB′, 12H; Ph).

***N,N,N*-Tris{2-[(4-(isopropyl-NH-Aib-Leu-Gly)carboxyphenyl)methyl-(*tert*-butoxycarbonyl)amino]ethyl}amine (6)**: EDC (80 mg, 0.42 mmol) was added to a solution of compound **5** (95 mg, 0.112 mmol) and HOBt (50 mg, 0.37 mmol) in freshly distilled CH_2Cl_2 (2 mL) which was kept at 0 °C. The mixture was stirred for 15 min at the same temperature and then mixed with a second solution prepared by dissolving tripeptide **P3** (200 mg, 0.64 mmol) in the same solvent (30 mL). The mixture was left stirring at room temperature overnight. Further EDC (20 mg) was added, and the

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mixture was stirred for an additional 3 h. The organic solution was washed with H_3BO_3 (0.5 M, 3 × 10 mL), $NaHCO_3$ (10%, 3 × 10 mL), and H_2O (3 × 10 mL), dried over Na_2SO_4, and then concentrated under reduced pressure. The oily residue was precipitated from CH_2Cl_2/light petroleum and purified by flash chromatography ($CHCl_3$/EtOH 15:1 to 7:1) to afford compound **6** (131 mg, 71%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.88$ (dd, 18H; LeuδCH_3), 1.02 (dd, 18H; NHCH(CH_3)$_2$), 1.40 (s, 27H; Boc), 1.45 (s, 18H; AibβCH_3), 1.65 (m, 9H; LeuβCH_2, γCH), 2.46 (br m, 6H; NCH_2CH$_2$), 3.10 (br m, 6H; NCH$_2$CH_2), 3.85–4.00 (m, 6H; GlyαCH_2), 4.00–4.20 (m, 3H; NHCH(CH$_3$)$_2$), 4.20–4.30 (m, 3H; LeuαCH), 4.30 (br s, 6H; CH_2Ph), 6.40 (br d, 3H; NHCH(CH$_3$)$_2$), 6.88 (s, 3H; AibαNH), 7.15, 7.17 (AA′BB′, 6H; Ph), 7.29 (br s, 3H; LeuαNH), 7.67 (br s, 3H; GlyαNH), 7.72, 7.75 (AA′BB′, 6H; Ph).

***N,N,N*-Tris{2-[(4-(isopropyl-NH-Aib-Leu-Gly)carboxyphenyl)methylamino]ethyl}amine (T(P3)$_3$)**: Compound **6** was dissolved in a 3:1 CH_2Cl_2/CF_3COOH solution (20 mL) and the mixture was stirred at room temperature for 1 h. The solvent was then evaporated under vacuum to afford the title compound as a white solid. M.p. 130–132 °C; $[\alpha]_D^{20} = -18.4$ ($c = 0.5$ in MeOH); ^{1}H NMR (250 MHz, CD_3OD): $\delta = 0.99$ (dd, 18H; LeuδCH_3), 1.04 (dd, 18H; NHCH(CH_3)$_2$), 1.49 (s, 18H; AibβCH_3), 1.60 (m, 6H; LeuβCH_2), 1.64 (m, 3H; LeuγCH), 2.99 (br t, 6H, NCH_2CH$_2$), 3.26 (br t, 6H; NCH$_2$CH_2), 3.94 (m, 3H; NHCH(CH$_3$)$_2$), 4.11 (dd, 6H; GlyαCH_2), 4.23 (m, 3H; LeuαCH), 4.34 (s, 6H; CH_2Ph), 7.62, 7.65, 7.95, 7.99 (AA′BB′, 12H; Ph); elemental analysis calcd (%) for $C_{75}H_{120}O_{12}N_{16} \cdot 8\,CF_3COOH \cdot H_2O$: C 46.16, H 5.53, N 9.46; found: C 46.03, H 5.49, N 9.00.

***N,N,N*-Tris{2-[(4-(CH$_3$O-Leu-Ile-Gly-Aib)carboxyphenyl)methyl(*tert*-butoxycarbonyl)amino]ethyl}amine (7)**: EDC (25.1 mg, 0.13 mmol) was added to a solution of compound **5** (33.1 mg, 0.04 mmol) and HOBt (15.2 mg, 0.11 mmol) in freshly distilled CH_2Cl_2 (1 mL) at 0 °C. The mixture was stirred for 15 min at this temperature and then mixed with a second solution prepared by dissolving tetrapeptide **P4** (89.9 mg, 0.22 mmol) in the same solvent (3 mL). After 2 h, EDC (15 mg) was added, and the mixture stirred for an additional 24 h. The organic solution was then washed with H_3BO_3 (0.5 M, 3 × 10 mL), $NaHCO_3$ (10%, 3 × 10 mL), and H_2O (3 × 10 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residual crude oily product was purified by silica column chromatography ($CHCl_3$/EtOH 9:1 → 8:2). Concentration of the proper fractions gave compound **7** (58.2 mg, 74%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.80$–1.10 (m, 42H; LeuδCH_3, IleγCH_2, δ-, $\gamma' CH_3$), 1.10–1.35 (m, 3H; LeuγCH), 1.39–1.75 (m, 51H; Boc, AibβCH_3, LeuβCH_2), 1.95–2.13 (m, 3H; IleβCH), 2.46 (br m, 6H; NCH_2CH$_2$), 3.10 (br m, 6H; NCH$_2$CH_2), 3.67 (s, 9H; OCH$_3$), 3.80–4.10 (m, 6H; GlyαCH_2), 4.35 (d, $J = 6.25$ Hz, 3H; IleαCH), 4.49–4.62 (m, 3H; LeuαCH), 4.90 (br s, 6H; CH_2Ph), 6.75 (br m, 6H; NH), 7.10 (br m, 3H; NH), 7.25 (br m, 3H; NH), 7.30, 7.35, 7.72, 7.76 (AA′BB′, 12H; Ph).

***N,N,N*-Tris{2-[(4-(CH$_3$O-Leu-Ile-Gly-Aib)carboxyphenyl)methylamino]-ethyl}amine (T(P4)$_3$)**: Compound **7** (58.2 g, 0.03 mmol) was dissolved in a 3:1 CH_2Cl_2/CF_3COOH solution (4 mL) and the mixture was stirred at room temperature for 1 h. The solvent was then evaporated under vacuum to leave a white solid that was purified by semi-preparative reversed-phase HPLC (isocratic conditions, 47% solvent B, flow rate 2 mL min^{-1}, 11.57 min retention time). The fractions containing the pure product were combined and lyophilized to obtain the title compound (12.3 mg, 21%) as a white solid; the purity was ascertained by analytical reversed-phase HPLC (linear gradient from 40% to 59% solvent B over 36 min, flow rate 1 mL min^{-1}, retention time 12.04 min). M.p. 142–144 °C; $[\alpha]_D^{20} = -24.0$ ($c = 0.1$ in MeOH); ^{1}H NMR (400 MHz, CD_3OD): $\delta = 0.80$–1.10 (m, 42H; LeuδCH_3, IleγCH_2, $\gamma' CH_3$), 1.20–1.40 (m, 3H; LeuγCH), 1.50–1.80 (m, 27H; AibβCH_3, LeuβCH_2), 2.00–2.20 (m, 3H; IleβCH), 2.92 (br m, 6H; CH_2CH$_2$N), 3.23 (br m, 6H; CH$_2$CH_2N), 3.73 (s, 9H; OCH$_3$), 3.74–4.00 (m; GlyαCH_2), 4.29 (d, $J = 6.25$ Hz, 3H; IleαCH), 4.38 (s, 6H; CH_2Ph), 4.40–4.50 (m, 3H; LeuαCH), 7.67, 7.69, 8.07, 8.09 (AA′BB′, 12H; Ph); MALDI-MS: m/z: 1732.1 $[M+K]^+$, 1716.0 $[M+Na]^+$, 1694.4 $[M]^+$.

***N,N,N*-Tris{2-[(4-(CH$_3$O-Leu-Ile-Gly-Aib-Leu)carboxyphenyl)methyl-(*tert*-butoxycarbonyl)amino]ethyl}amine (8)**: A procedure similar to that described for the synthesis of compound **7** was followed using EDC (34.4 mg, 0.18 mmol), compound **5** (45.4 mg, 0.05 mmol), HOBt (20.8 mg, 0.15 mmol), and pentapeptide **P5** (158 mg, 0.31 mmol). The crude oily material obtained was purified by column chromatography (silica gel, $CHCl_3$/EtOH 8.5:1.5) to give compound **8** (85 mg, 69%). ^{1}H NMR (250 MHz, CD_3OD): $\delta = 0.80$–1.05 (m, 42H; LeuδCH_3, IleδCH_3, γCH_2 and $\gamma' CH_3$), 1.30–1.90 (m, 54H; LeuγCH, Boc, AibβCH_3, LeuβCH_2), 1.95–2.15 (m, 3H; IleβCH), 2.62 (br m, 6H; NCH_2CH$_2$), 3.25 (br m, 6H; NCH$_2$CH_2), 3.70 (s, 9H; OCH$_3$), 3.71–4.00 (m, 6H; GlyαCH_2), 4.21–4.41 (m, 6H; IleαCH, LeuαCH), 4.50 (br s, 6H; CH_2Ph), 4.55–4.70 (m, 3H; LeuαCH), 7.25–7.45 (br AA′, 6H; Ph), 7.87, 7.9 (BB′, 6H; Ph).

***N,N,N*-Tris{2-[(4-(CH$_3$O-Leu-Ile-Gly-Aib-Leu)carboxyphenyl)methyl-amino]ethyl}amine (T(P5)$_3$)**: Compound **8** (85 mg, 0.04 mmol) was dissolved in 3:1 CH_2Cl_2/CF_3COOH (16 mL), and the mixture was stirred at room temperature for 1 h. The solvent was evaporated under vacuum to give a residue that was purified by semi-preparative reversed-phase HPLC. (gradient from 50% to 62% solvent B over 25 min, flow rate 2.5 mL min^{-1}, retention time 16.7 min). The fractions containing the pure product were combined and lyophilized to give the title compound (44 mg, 51%) as a white solid; the purity was ascertained by analytical reversed-phase HPLC (linear gradient from 50% to 68% solvent B over 36 min, flow rate 1 mL min^{-1}, retention time 18.81 min). M.p. 155–158 °C; $[\alpha]_D^{20} = -35.0$ ($c = 0.1$ in MeOH); ^{1}H NMR (400 MHz, CD_3OD): $\delta = 0.75$–1.10 (m, 42H; LeuδCH_3, IleδCH_3, γCH_2, $\gamma' CH_3$), 1.20–1.90 (m, 36H; LeuγCH, AibβCH_3, LeuβCH_2), 1.90–2.10 (m, 3H; IleβCH), 2.93 (br m, 6H; NCH_2CH$_2$), 3.23 (br m, 6H; NCH$_2$CH_2), 3.72 (s, 9H; OCH$_3$), 3.72, 3.78, 3.88, 3.94 (AB, 6H; GlyαCH_2), 4.20–4.40 (m, 12H; LeuαCH, IleαCH, GlyαCH_2, CH_2Ph), 4.60–4.70 (m, 3H; LeuαCH), 7.62, 7.64, 7.97, 8.00 (AA′BB′, 12H; Ph); MALDI-MS: m/z: 2059 $[M+Na]^+$, 2037 $[M]^+$.

***N,N,N*-Tris{2-[(4-(CH$_3$O-Leu-Ile-Gly-Aib-Leu-Gly)carboxyphenyl)methyl(*tert*-butoxycarbonyl)amino]ethyl}amine (9)**: A procedure similar to that described for the synthesis of compound **7** was followed using EDC (37.3 mg, 0.195 mmol), compound **5** (47.2 mg, 0.055 mmol), HOBt (24 mg, 0.18 mmol), and hexapeptide **P6** (174 mg, 0.305 mmol). The crude oily material was purified by column chromatography (silica gel, $CHCl_3$/EtOH from 9:1 to 7:3) to give compound **9** (55 mg, 39%). ^{1}H NMR (250 MHz, CD_3OD): $\delta = 0.80$–1.10 (m, 60H; LeuδCH_3, IleδCH_3, γCH_2, $\gamma' CH_3$), 1.20–1.80 (m, 57H; AibβCH_3, Boc, LeuβCH_2), 1.97–2.20 (m, 3H; IleβCH), 2.63 (br m, 6H; NCH_2CH$_2$), 3.25 (br m, 6H; NCH$_2$CH_2), 3.68 (s, 9H; OCH$_3$), 3.90–4.60 (m, 27H; GlyαCH_2, LeuαCH, IleαCH, CH_2Ph), 7.35–7.45 (br AA′, 6H; Ph), 7.83, 7.89, (BB′, 6H; Ph).

***N,N,N*-Tris{2-[(4-(CH$_3$O-Leu-Ile-Gly-Aib-Leu-Gly)carboxyphenyl)methylamino]ethyl}amine (T(P6)$_3$)**: Compound **9** (55 mg, 0.02 mmol) was dissolved in 3:1 CH_2Cl_2/CF_3COOH (14 mL) and the mixture was stirred at room temperature for 1 h. The solvent was evaporated under vacuum to give a residue that was purified by semi-preparative reversed-phase HPLC (linear gradient from 48 to 60% solvent B over 22 min, flow rate 2.5 mL min^{-1}). The fractions containing the pure product were combined and lyophilized to obtain the title compound (18 mg, 32%) as a white solid whose purity was ascertained by analytical reversed-phase HPLC (linear gradient from 50 to 68% solvent B over 36 min, flow rate 1 mL min^{-1}, retention time 20.96 min). M.p. 183–185 °C; $[\alpha]_D^{20} = -43.6$ ($c = 0.5$ in MeOH); ^{1}H NMR (400 MHz, CD_3OD): $\delta = 0.80$–1.10 (m, 60H; LeuδCH_3, IleγCH_2, $\gamma' CH_3$, δCH_3), 1.20–1.80 (m, 36H; LeuγCH, LeuβCH_2, AibβCH_3), 2.00–2.20 (m, 3H; IleβCH), 2.93 (br m, 6H; NCH_2CH$_2$), 3.23 (br m, 6H; NCH$_2$CH_2), 3.51, 3.56, 3.66, 3.90 (AB, 6H; GlyαCH_2), 3.69 (s, 9H; OCH$_3$), 4.10–4.50 (m, 21H; GlyαCH_2, LeuαCH, IleαCH, CH$_2$Ph), 7.62, 7.64, 7.95, 7.97 (AA′BB′, 12H; Ph); MALDI-MS: m/z: 2225 $[M+Na]^+$.

***N,N,N*-Tris{2-[(4-(CH$_3$O-Leu-Ile-Gly-Aib-Leu-Gly-Gly-Aib-Leu-Gly)carboxyphenyl)methyl(*tert*-butoxycarbonyl)amino]ethyl}amine (10)**: A procedure similar to that described for the synthesis of compound **7** was employed using EDC (14.7 mg, 0.08 mmol), compound **5** (18.6 mg, 0.02 mmol), HOBt (8.9 mg, 0.065 mmol), and decapeptide **P10** (96.2 mg, 0.11 mmol). The resulting oily material was purified by column chromatography (silica gel, $CHCl_3$/MeOH 9:1) to give compound **10** (66.6 mg, 88%). ^{1}H NMR (250 MHz, CD_3OD, 398 K): $\delta = 0.85$–1.15 (m, 78H; LeuδCH_3, IleδCH_3, γCH_2, $\gamma' CH_3$), 1.20–1.85 (m, 90H; AibβCH_3, Boc, LeuβCH_2, LeuγCH), 2.00–2.20 (m, 3H; IleβCH), 2.65 (br t, 6H; NCH_2CH$_2$), 3.30 (br t, 6H, NCH$_2$CH_2), 3.67–3.83 (m, 15H; GlyαCH_2, OCH$_3$), 3.89 (d, $J = 10.5$ Hz, 12H; GlyαCH_2), 4.08 (d, $J = 8.7$ Hz, 6H; GlyαCH_2), 4.33 (t, $J = 7.5$ Hz, 12H; LeuαCH, IleαCH), 7.34, 7.37, 7.86, 7.89 (AA′BB′, 12H; Ph).

***N,N,N*-Tris{2-[(4-(CH$_3$O-Leu-Ile-Gly-Aib-Leu-Gly-Gly-Aib-Leu-Gly)carboxyphenyl)methylamino]ethyl}amine (T(P10)$_3$)**: Compound **10** (66.6 mg, 0.02 mmol) was dissolved in 3:1 CH_2Cl_2/CF_3COOH (10 mL) and the mixture was stirred at room temperature for 1 h. The solvent was

evaporated under vacuum to leave a residue that was purified by semi-preparative reversed-phase HPLC (isocratic conditions 63% solvent B, flow rate 2 mL min^{-1}, retention time 22.2 min). The fractions containing the pure product were combined and lyophilized to obtain the title compound (11 mg, 16%) as a white solid; the purity was ascertained by analytical reversed-phase HPLC (linear gradient from 60% to 88% solvent B over 30 min, retention time 20.96 min). M.p. 197–199 °C; $[\alpha]_D^{20} = -31.6$ ($c = 0.5$ in MeOH); ^{1}H NMR (400 MHz, CD_3OD): $\delta = 0.85-1.15$ (m, 78H; LeuδCH_3, IleδCH_3, γCH_2, $\gamma' CH_3$), 1.30–1.90 (m, 65H; AibαCH_3, LeuβCH_2, LeuγCH), 1.90–2.20 (m, 3H; IleβCH), 2.95 (br m, 6H; NCH_2CH_2), 3.25 (br m, 6H; NCH_2CH_2), 3.71 (s, 9H; OCH_3), 3.70–4.50 (m, 36H; GlyαCH_2, LeuαCH, IleαCH), 7.62, 7.64, 7.95, 7.97 (AA′BB′, 12H; Ph); MALDI-MS: m/z: 3183 $[M+K]^+$.

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[1] M. Jain, *Introduction to Biological Membranes*, 2nd ed., Wiley, New York, **1988**, Chapter 9.
[2] D. W. Urry, *Top. Curr. Chem*. **1985**, *128*, 175.
[3] a) M. Montal, *Curr. Opin. Struct. Biol.* **1995**, *5*, 501; b) F. Hucho, C. Weise, *Angew. Chem*. **2001**, *113*, 3194; *Angew. Chem. Int. Ed*. **2001**, *40*, 3101 and references therein.
[4] a) T. M. Fyles, T. D. James, K. C. Kaye, *J. Am. Chem. Soc.* **1993**, *115*, 12315; b) M. F. M. Rocks, R. J. M. Nolte, *Macromolecules* **1992**, *25*, 5398; c) H. Wagner, K. Harms, U. Koert, S. Meder, G. Boheim, *Angew. Chem*. **1996**, *108*, 2836; *Angew. Chem. Int. Ed. Engl*. **1996**, *35*, 2643; d) Y. Tanaka, Y. Kobuke, M. Sokabe, *Angew. Chem*. **1995**, *107*, 717; *Angew. Chem. Int. Ed. Engl*. **1995**, *34*, 693; e) G. W. Gokel, O. Murillo, *Acc. Chem. Res.* **1996**, *29*, 425; f) F. M. Menger, D. S. Davis, R. A. Persichetti, J. J. Lee, *J. Am. Chem. Soc*. **1990**, *112*, 2451; g) F. M. Menger, P. Aikens, *Angew. Chem*. **1992**, *104*, 919; *Angew. Chem. Int. Ed. Engl*. **1992**, *31*, 898; h) U. F. Kragten, M. F. M. Roks, R. J. M. Nolte, *J. Chem. Soc. Chem. Commun*. **1985**, 1275; i) N. Voyer, M. Robitaille, *J. Am. Chem. Soc*. **1995**, *117*, 6599; j) T. M. Fyles, D. Loock, X. W. F. van Straaten-Nijenhuis, X. Zhou, *J. Org. Chem*. **1996**, *61*, 8866; k) O. Murillo, I. Suzuki, E. Abel, G. W. Gokel, *J. Am. Chem. Soc*. **1996**, *118*, 7628; l) N. Sakai, K. C. Brennan, L. A. Weiss, S. Matile, *J. Am. Chem. Soc*. **1997**, *119*, 8726; m) N. Sakai, S. Matile, *Chem. Eur. J.* **2000**, *6*, 1731; n) A. F. DiGiorgio, S. Otto, P. Bandyopadhyay, S. L. Regen, *J. Am. Chem. Soc.* **2000**, *122*, 11029; o) G. W. Gokel, *Chem. Commun.* **2000**, 1; p) N. Yoshino, A. Satake, Y. Kobuke, *Angew. Chem*. **2001**, *113*, 471; *Angew. Chem. Int. Ed*. **2001**, *40*, 457; q) G. W. Gokel, A. Mukhopadhyay, *Chem. Soc. Rev*. **2001**, *30*, 274.
[5] a) A. Grove, M. Mutter, J. E. Rivier, M. Montal, *J. Am. Chem. Soc.* **1993**, *115*, 5919; b) K. S. Åkerfeldt, J. D.Lear, Z. R. Wasserman, L. A. Chung, W. F. DeGrado, *Acc. Chem. Res.* **1993**, *26*, 191; M. S. P. Sansom, *Prog. Biophys. Mol. Biol*. **1991**, *55*, 139.
[6] a) M. J. Pregel, L. Jullien, J. Canceill, L. Lacombe, J. M. Lehn, *J. Chem. Soc. Perkin Trans. 2* **1995**, 417; b) L. Jullien, T. Lazrak, J. Canceill, L. Lacombe, J. M. Lehn, *J. Chem. Soc. Perkin Trans. 2* **1993**, 1011.
[7] a) R. M. Ghadiri, J. R. Granja, L. K. Buehler, *Nature* **1994**, *26*, 301; b) N. Khazanovich, J. R. Granja, D. E. McRee, R. A. Milligan, M. R. Ghadiri, *J. Am. Chem. Soc*. **1994**, *116*, 6011; c) M. R. Ghadiri, J. R. Granja, R. A. Milligan, D. E. McRee, N. Khazanovich, *Nature* **1993**, *366*, 324; d) H. S. Kim, J. D. Hartgerink, M. R. Ghadiri, *J. Am. Chem. Soc*. **1998**, *120*, 4417; e) S. Fernandez-Lopez, H.-S. Kim, E. C. Choi, M. Delgado, J. R. Granja, A. Khasanov, K. Kraehenbuehl, G. Long, D. A. Weinberger, K. M. Wilcoxen, M. R. Ghadiri, *Nature* **2001**, *412*, 452.
[8] J. M. Lehn, *Angew. Chem*. **1990**, *102*, 1347; *Angew. Chem. Int. Ed. Engl.* **1990**, *29*, 1304.
[9] a) G. A. Woolley, A. S. I. Jaikaran, Z. Zhang, S. Peng, *J. Am. Chem. Soc.* **1995**, *117*, 4448; b) T. M. Fyles, B. Zeng, *Chem. Commun.* **1996**, 2295; c) T. M. Fyles, D. Loock, X. Zhou, *J. Am. Chem. Soc.* **1998**, *120*, 2297; d) M. M. Tedesco, B. Ghebremariam, N. Sakai, S. Matile, *Angew. Chem*. **1999**, *111*, 523; *Angew. Chem. Int. Ed. Engl.* **1999**, *38*, 541; e) N. Sakai, B. Baumeister, S. Matile, *ChemBioChem* **2000**, *2*, 123; f) C. D. Hall, G. J. Kirkovits, A. C. Hall, *Chem. Commun.* **1999**, 1897; g) D. Wang, L. Guo, J. Zhang, L. R. Jones, Z. Chen, C. Pritchard, R. W. Roeske, *J. Pept. Res.* **2001**, *57*, 301.
[10] For selected examples see: a) S. Shinkai, M. Ikeda, A. Sugasaki, M. Takeuchi, *Acc. Chem. Res.* **2001**, *34*, 494; b) F. Wang, A. W. Schwabacher, *J. Org. Chem*. **1999**, *64*, 8922; c) P. Tecilla, U. Tonellato, A. Veronese, F. Felluga, P. Scrimin, *J. Org. Chem*. **1997**, *62*, 7621; d) P. Scrimin, P. Tecilla, U. Tonellato, G. Valle, A. Veronese, *J. Chem. Soc. Chem. Commun*. **1995**, 1163; e) R. Baldes, H.-J. Schneider, *Angew. Chem*. **1995**, *107*, 380; *Angew. Chem. Int. Ed. Eng*. **1995**, *34*, 321.
[11] a) I. O. Fritsky, R. Ott, R. Krämer, *Angew. Chem*. **2000**, *112*, 3403; *Angew. Chem. Int. Ed*. **2000**, *39*, 3255; b) I. O. Fritsky, R. Ott, H. Pritzkow, R. Krämer, *Chem. Eur. J.* **2001**, *7*, 1221.
[12] S. Valente, M. Gobbo, G. Licini, A. Scarso, P. Scrimin, *Angew. Chem*. **2001**, *113*, 4017; *Angew. Chem. Int. Ed.* **2001**, *40*, 3899.
[13] a) E. Benedetti, A. Bavoso, B. Di Blasio, V. Pavone, C. Pedone, C. Toniolo, G. M. Bonora, *Proc. Natl. Acad. Sci USA* **1982**, *79*, 7951; b) M. S. P. Sansom, *Q. Rev. Biophys.* **1993**, *26*, 365.
[14] a) R. C. Pandey, J. C. Cook, Jr., K. L. Rinehart, *J. Am. Chem. Soc.* **1977**, *99*, 8469; b) R. O. Fox, F. M. Richards, *Nature* **1982**, *300*, 325.
[15] a) C. Auvin-Guette, S. Rebuffat, Y. Prigent, B. Bodo, *J. Am. Chem. Soc.* **1992**, *114*, 2170; b) C. Toniolo, C. Peggion, M. Crisma, F. Formaggio, X. Shui, D. S. Eggleston, *Nat. Struct. Biol*. **1994**, *1*, 908; c) V. Monaco, E. Locardi, F. Formaggio, M. Crisma, S. Mammi, E. Peggion, C. Toniolo, S. Rebuffat, B. Bodo, *J. Pept. Res.* **1998**, *52*, 261.
[16] a) T. Fujita, S. Wada, A. Iida, T. Nishimura, M. Kanai, N. Toyama, *Chem. Pharm. Bull.* **1994**, *42*, 489; b) V. Monaco, F. Formaggio, M. Crisma, C. Toniolo, X. Shui, D. S. Eggleston, *Biopolymers* **1996**, *39*, 31; c) R. Gurunath, P. Balaram, *Biopolymers* **1995**, *35*, 21.
[17] a) C. Toniolo, E. Benedetti, *Trends Biochem. Sci*. **1991**, *16*, 350; b) C. Toniolo, E. Benedetti, *Macromolecules* **1991**, *24*, 4004; c) I. L. Karle, P. Balaram, *Biochemistry* **1990**, *29*, 6747.
[18] T. Le Doan, M. El Hajji, S. Rebuffat, M. R. Rajesvari, B. Bodo, *Biochim. Biophys. Acta* **1986**, *858*, 1.
[19] P. Scrimin, A. Veronese, P. Tecilla, P. Tonellato, V. Monaco, F. Formaggio, M. Crisma, C. Toniolo, *J. Am. Chem. Soc*. **1996**, *118*, 2505.
[20] For template-assembled synthetic peptides see: a) M. Mutter, S. Vuilleumier, *Angew. Chem*. **1989**, *101*, 551; *Angew. Chem. Int. Ed. Engl*. **1989**, *28*, 535; b) M. Mutter, P. Dumy, P. Garrouste, C. Lehmann, M. Mathieu, C. Peggion, S. Peluso, A. Razaname, G. Tuchscherer, *Angew. Chem*. **1996**, *108*, 1587; *Angew. Chem. Int. Ed. Engl*. **1996**, *35*, 1482. For peptide bundles affecting membrane permeability see: c) K. S. Åkerfeldt, R. M. Kim, D. Camac, J. T. Groves, J. D. Lear, W. F. DeGrado, *J. Am. Chem. Soc*. **1992**, *114*, 9656; d) A. Matsubara, K. Asami, A. Akagi, N. Nishino, *J. Chem. Soc. Chem. Commun*. **1996**, 2069.
[21] R. H. Prince in *Comprehensive Coordination Chemistry* (Eds.: G. Wilkinson, R. D. Gillard, A. McCleverty), Pergamon Press, London, **1987**, pp. 937–938.
[22] J. N. Winstein, S. Yoshikami, P. Henkari, R. Blumenthal, W. H. Hagins, *Science* **1977**, *195*, 489.
[23] C. Toniolo, M. Crisma, F. Formaggio, C. Peggion, V. Monaco, C. Goulard, S. Rebuffat, B. Bodo, *J. Am. Chem. Soc*. **1996**, *118*, 4952.
[24] a) M. Crisma, G. Valle, V. Monaco, F. Formaggio, C. Toniolo, *Acta Crystallogr. Sect. C* **1994**, *50*, 563; b) M. Crisma, G. Valle, F. Formaggio, V. Monaco, C. Toniolo, *Lett. Pept. Sci.* **1996**, *3*, 121.
[25] F. Formaggio, M. Crisma, P. Rossi, P. Scrimin, B. Kaptein, Q. B. Broxterman, J. Kamphuis, C. Toniolo, *Chem. Eur. J.* **2000**, *6*, 4498.
[26] a) G. Anderegg, V. Gramlich, *Helv. Chim. Acta* **1994**, *77*, 685; b) J. W. Canary, J. Xu, J. M. Castagnetto, D. Rentzeperis, L. A. Marky, *J. Am. Chem. Soc*. **1995**, *117*, 11545.
[27] D. F. Kennedy, M. Crisma, C. Toniolo, D. Chapman, *Biochemistry* **1991**, *30*, 6541.
[28] For selected examples see: a) M. R. Ghadiri, C. Soares, C. Choi, *J. Am. Chem. Soc*. **1992**, *114*, 4000; b) T. Sasaki, S. T. Kaiser, *J. Am. Chem. Soc*. **1989**, *111*, 380.
[29] G. Schwarz, C. H. Robert, *Biophys. J.* **1990**, *58*, 577.
[30] D. D. Perrin, B. Dempsey, *Buffers for pH and Metal Ion Control*, Chapman and Hall, London, **1974**.

  0947-6539/02/0812-2762 $ 17.50+.50/0

[31] M. Kaszuba, G. R. Hunt, *J. Inorg. Biochem*. **1990**, *40*, 217.
[32] a) C. Puyal, L. Maurin, G. Miquel, A. Bienvenüe, J. Philippot, *Biochim. Biophys. Acta* **1994**, *1195*, 259; b) D. Hoekstra, *Biochemistry* **1982**, *21*, 2833; c) D. K. Struck, D. Hoekstra, R. E. Pagano, D. Hoekstra, *Biochemistry* **1981**, *20*, 4093.
[33] P. Lauger, *Science* **1972**, *178*, 24.
[34] a) Y. Liu, S. L. Regen, *J. Am. Chem. Soc*. **1993**, *115*, 708; b) P. Scrimin, P. Tecilla, R. A. Moss, K. Bracken, *J. Am. Chem. Soc*. **1998**, *120*, 1179.
[35] Recently Regen has reported compelling evidence that ionophores may act with a dominant carrier mechanism at low concentrations and with a channel mechanism at high concentrations (see: G. Deng, M. Merritt, K. Yamashita, V. Janout, A. Sadownik, S. L. Regen, *J. Am. Chem. Soc*. **1996**, *118*, 3307.). We cannot rule out that this is the case with the present tripodal peptides and that the lower part of each sigmoidal curve represents the carrier-mechanism contribution to the transport process.
[36] A similar extended geometry of complexation to a lipid bilayer by a tetrasteroid-functionalized porphyrin has been reported: J. Lahiri, G. D. Fate, S. B. Ungashe, J. T. Groves, *J. Am. Chem. Soc*. **1996**, *118*, 2347.
[37] A reviewer pointed out that the role of Zn^{II} could be to decrease the partition of the template into the membrane as a consequence of a lower lipophilicity of the metal complex. However, we note that at pH 7.4 the TREN platform is triprotonated ($pK_a = 10.1$; 9.5; 8.4 see: R. M. Smith, A. E. Martell, *Critical Stability Constants*, *Vol. 6*, Plenum Press, New York, **1989**). In our experience the Zn^{II} complexes are slightly less water soluble than the metal free (protonated) templates.
[38] P. S. Chen, T. Y. Toribara, H. Warner, *Anal. Chem*. **1956**, *28*, 1756.
[39] K. Hass, M. Bender, *J. Am. Chem. Soc*. **1949**, *71*, 1767.

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