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Tuning Chloride Binding, Encapsulation, and Transport by Peripheral Substitution of Pseudopeptidic Tripodal Small Cages

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Abstract: A highly efficient synthesis of small pseudopeptidic cages from simple precursors has been achieved by the triple S_N2 reaction between tripodal tris(amido amines) and several 1,3,5-tris(bromomethyl)benzene electrophiles. The success of the macrobicyclization strongly depends on the central triamine scaffold, which dictates the correct preorganization of the intermediates. The chloride binding properties of the protonated pseudopeptidic cages have been studied in the solid state (by X-ray diffraction) as well as in solution (by NMR spectroscopy and ESI-MS) and in the gas phase (by collision-induced dissociation (CID)-MS). The crystal structure of the HCl salts of several cages show a chloride partially or completely caged within the cavity of the macrobicycle. Both the amino acid side chain and the substitution at the aromatic tripodal ring have an effect on the chloride binding ability. The cages derived from the 1,3,5-benzene moiety show low affinity, whereas the triple substitution in the ring (either with Me or Et) increases the chloride binding by one order of magnitude. Besides, the cages derived from aliphatic amino acids display a stronger interaction than those derived from phenylalanine. The basis for the different mode of binding depending on the receptor structure is proposed according to the structural data (X-ray and NMR spectroscopy). Finally, the transport of the chloride anion through lipid bilayers has been studied for selected cages. Despite the important differences in the chloride binding, the transport properties are better correlated with the lipophilicity of the molecules. Therefore, the pseudopeptidic cages sharing the same binding motif for chloride rendered very different interaction and transport properties depending on the peripheral substitution.

Keywords: cage compounds · chloride binding · pseudopeptides · supramolecular chemistry · transport

Introduction

The study of macrocycles and cage-like structures derived from amino acids (peptides and pseudopeptides) is an emerging research topic, closely connected with synthetic chemistry,[1] natural products,[2] material science,[3] and biological chemistry.[4] The geometrical constraints produced by the cyclic frame are extremely useful to implement a rigid conformation within a small peptidic sequence.[5] This has allowed the chemists to design structural scaffolds with interesting biological activities.[6] Moreover, this type of compounds has been used as receptors for some species of interest, either ions (cations[7] and anions[8]) or small neutral molecules.[9] Among these structures, those defining a three-dimensional inner space are especially attractive for recognition purposes, because they usually lead to stronger interaction and higher selectivity towards a given complementary guest. Thus, the study of three-dimensional cavities as molecular containers is a hot topic nowadays.[10] In this context, macrobicyclic pseudopeptides defining a cage-like architecture[11] have become very appealing as synthetic targets for molecular recognition purposes[12] and biomimetic studies.[13] However, their preparation is very often challenging, requiring multi-step synthetic pathways with low yields and tedious purification steps. In this regard, the key step is usually the reaction leading to the macrobicyclic structure,

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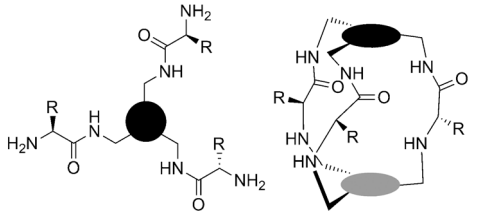
which very often leads to an untreatable mixture of cyclic and open-chain oligomers.[14] Some interesting approaches have been developed to improve the efficiency of the cyclization processes, mainly based on the implementation of a defined preorganization, which would stabilize the correct pre-cyclic conformation in the transition state of the reaction.[15] Then, a faster and a more selective formation of the intended cyclic structure can be achieved. This preorganization can be obtained by using geometrically rigid building blocks[16] or after tuning the protecting groups[17] or the solvent,[18] by using some additional non-covalent interactions[19] or by taking advantage of designed templates, either kinetically or thermodynamically.[20]

On the other hand, chloride is the most abundant anion in biological systems; therefore, it is an interesting target for synthetic receptors. Despite the structural simplicity of chloride anion, its physicochemical properties make its selective and efficient molecular recognition very challenging, especially in highly competitive media, like aqueous solution.[21] The control of chloride transport and maintenance of appropriate chloride concentrations is fundamental for the function of living organisms. Defective or altered functioning of the natural transport mechanism is associated with serious diseases termed channelopathies, such as cystic fibrosis.[22] Recently, the development of synthetic small molecules designed for the transport of chloride through lipid bilayers has received an increasing attention.[23] These compounds can facilitate chloride transport in living cells and display biological activity.[24] A number of these molecules have been shown to promote apoptosis in cancer cells as a result of their anionophoric activity.[25] On the other hand, the design and preparation of new transporters displaying low toxicity is also needed. In this regard, pseudopeptidic cages are very appealing targets, because it is relatively simple to modulate their physicochemical properties by the variation of the side chains from different amino acids. Moreover, the preparation of simple transporters with systematic structural changes would allow the understanding of the transport process at the fundamental level in order to propose some structure–activity relationships.

Within our ongoing research project devoted to the synthesis and the study of the properties of new pseudopeptidic compounds,[26] we envisioned the preparation of three-dimensional cage-like structures (Scheme 1). A simple and reasonable proposal to reach this aim should be the preparation of pseudopeptidic tris(amido amines) based on a tripodal central scaffold (black circle in Scheme 1) and three amino acidic units. Compounds of this type have recently shown interesting properties in nanotechnology and for the preparation of new biomaterials.[27] These tripodal pseudopeptides can be used to synthesize cage-like structures by the reaction with a suitable tripodal complementary moiety (grey circle in Scheme 1). Molecular models (CPK) show that the inner cavity of these cages is suitable for hosting a chloride anion.

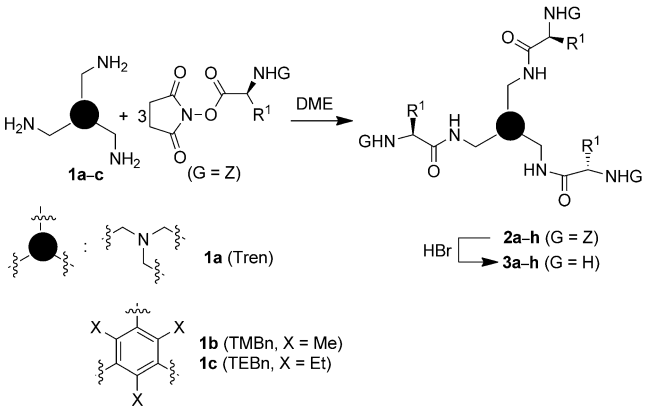


Scheme 1. Tripodal pseudopeptides.

Here we report on the simple preparation of tripodal pseudopeptides and on their macrobicyclization reaction toward the corresponding pseudopeptidic cages. Our studies allowed us to understand the structural factors affecting the macrobicyclization reaction and to define the scope and limitations of the proposed synthetic procedure. Moreover, the ability of the cages to bind chloride has been studied in gas, solution and solid states. Finally, the chloride transport through lipid bilayers as a model for cell membranes was also studied for selected cages.

Results and Discussion

Design and synthesis of the tripodal cages: Our design of the corresponding tripodal pseudopeptides is quite simple and straightforward. As the central scaffold, we used three different triamines, having three-fold symmetry (Scheme 2),



Scheme 2. Synthesis of the tripodal pseudopeptidic tris(amido amines). DME = dimethoxyethane.

which are either commercially available (Tren, **1a**) or easily accessible from simple starting materials[28] (TMBn and TEBn, **1b** and **1c**, respectively). These have been described forming part of different receptors, where a preorganized concave conformation is proposed.[29] This geometrical disposition would favor the use of the corresponding tripodal tris(amido amines) for the closure of a macrobicyclic architecture, leading to the preparation of small pseudopeptidic molecular cages. The coupling of the triamines (**1a–c**) with the Z-protected (Z = benzyloxycarbonyl) *N*-hydroxysuccinimide esters of the amino acids led to the Z-protected tripodal pseudopeptides **2a–h** (Scheme 2, Table 1). Further con-

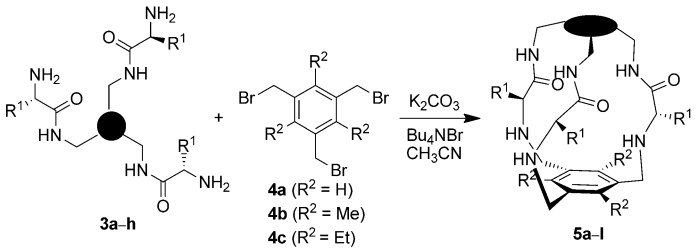
Table 1. Synthesis of the tripodal pseudopeptides **3a–h**.

Entry	Triamine	X	R^1 (Aaa)	**2** ([%])[a]	**3** ([%])[a]
1	**1a** (Tren)	–	Bn[b] (Phe)	**2a** (85)	**3a** (74)
2	**1a** (Tren)	–	*i*Pr (Val)	**2b** (87)	**3b** (72)
3	**1a** (Tren)	–	*i*Bu (Leu)	**2c** (95)	**3c** (84)
4	**1a** (Tren)	–	*s*Bu (Ile)	**2d** (96)	**3d** (62)
5	**1b** (TMBn)	Me	Bn (Phe)	**2e** (94)	**3e** (71)
6	**1b** (TMBn)	Me	*i*Pr (Val)	**2f** (71)	**3f** (76)
7	**1c** (TEBn)	Et	Bn (Phe)	**2g** (81)	**3g** (76)
8	**1c** (TEBn)	Et	*i*Pr (Val)	**2h** (82)	**3h** (71)

[a] Yields of the isolated products are given in parenthesis. [b] Bn = benzyl.

ventional deprotection of the carbamates **2a–h** in acidic medium produced the intended tripodal pseudopeptides **3a–h** in good overall yields. The synthetic procedures can be easily carried out in relatively large scale, because none of the steps required a chromatographic purification, and the final products can be isolated as analytically pure solids by precipitation and washing procedures. No significant differences in the reactivity of the systems due to the central scaffold or to the amino acid side chains were observed, being the different final yields ascribed to a slight difference in the solubility of the compounds.

We also used these tripodal pseudopeptides for the construction of small pseudopeptidic cages. To this aim, we performed the triple S_N2 reaction between the corresponding tris(amido amine) and the symmetric triply electrophilic aromatic bromides **4a–c** (Scheme 3). Different substitution



Scheme 3. Macrobicyclization reaction for the synthesis of the pseudopeptidic cages **5a–l**.

patterns in the electrophiles were assayed (R^2 = H, Me, Et) for studying the steric effect on the formation of the cages. The reaction was performed in acetonitrile, which was heated to reflux, by using an excess of anhydrous potassium carbonate as a base. Moreover, we used tetrabutylammonium bromide as an additive (0.5 mol with respect to compounds **3a–h**). This highly soluble salt can act as a phase-transfer catalyst and the anionic bromide also serves as a stabilizing species of the transition states, as we have recently observed in the formation of pseudopeptidic macrocycles.[30] The obtained results are displayed in Table 2.

For the Tren derivatives, very good yields of the isolated products were obtained (Table 2, entries 1–12), considering that three substitution reactions are performed in one pot and that a careful chromatographic purification process is

Table 2. Synthesis of the pseudopeptidic tripodal cages.

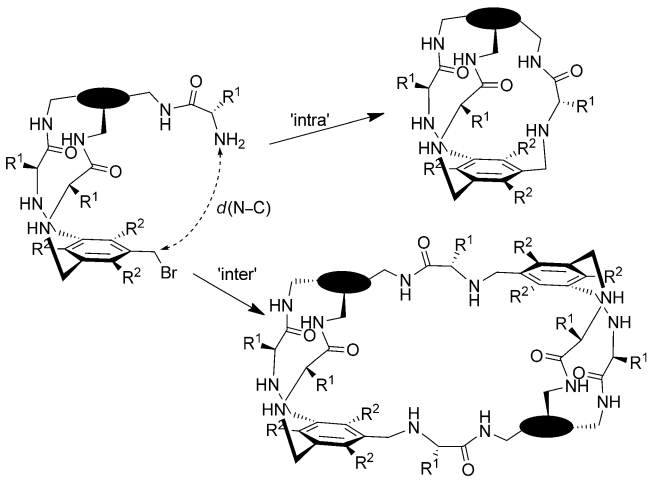
Entry	**3**	Scaffold	R^1	Aaa	**4** (R^2)	Cage ([%])[a]
1	**3a**	Tren	Bn	Phe	**4a** (H)	**5a** (38)
2	**3b**	Tren	*i*Pr	Val	**4a** (H)	**5b** (43)
3	**3c**	Tren	*i*Bu	Leu	**4a** (H)	**5c** (20)
4	**3d**	Tren	*s*Bu	Ile	**4a** (H)	**5d** (34)
5	**3a**	Tren	Bn	Phe	**4b** (Me)	**5e** (39)
6	**3b**	Tren	*i*Pr	Val	**4b** (Me)	**5f** (47)
7	**3c**	Tren	*i*Bu	Leu	**4b** (Me)	**5g** (25)
8	**3d**	Tren	*s*Bu	Ile	**4b** (Me)	**5h** (53)
9	**3a**	Tren	Bn	Phe	**4c** (Et)	**5i** (33)
10	**3b**	Tren	*i*Pr	Val	**4c** (Et)	**5j** (49)
11	**3c**	Tren	*i*Bu	Leu	**4c** (Et)	**5k** (37)
12	**3d**	Tren	*s*Bu	Ile	**4c** (Et)	**5l** (42)
13	**3e**	TMBn	Bn	Phe	**4b** (Me)	–[b]
14	**3f**	TMBn	*i*Pr	Val	**4b** (Me)	–[b]
15	**3g**	TEBn	Bn	Phe	**4b** (Me)	–[b]
16	**3h**	TEBn	*i*Pr	Val	**4b** (Me)	–[b]

[a] Yields of the isolated products after chromatographic purification. [b] A complicated mixture was obtained as observed by ^{1}H NMR spectroscopy and ultra-performance liquid chromatography (UPLC)-MS analysis of the crude reaction mixture.

involved, which is necessary for the isolation of analytically pure materials. This purification process is usually the limiting step for the preparation of pseudopeptidic-related compounds. Anyway, ^{1}H NMR spectroscopy and UPLC-MS analysis of the crude reaction mixture suggested that the reaction is very selective towards the formation of the cage (see the Supporting Information). Within the Tren derivatives, the yields were better for the pseudopeptides derived from Val and Ile although, once again, the differences are more related to the purification process than to the reactivity of the compounds. No important differences were found for the substitution pattern in compounds **4a–c**, suggesting a low effect of the steric hindrance in the reaction.

Unfortunately, the reactions performed with the tripodal amido amines bearing an aromatic central scaffold (**3e–h**, Table 2, entries 13–16) led, in all cases, to a more complex mixture of compounds (as observed by TLC, ^{1}H NMR spectroscopy, and UPLC-MS). The accurate UPLC-MS analysis of the crude reaction mixtures allowed us to identify the intended [1+1] cage, accompanied with a large amount of the bigger [2+2] cage, along with other open-chain oligomeric minor products (see the Supporting Information). In most of the examples, these two cages were the major products and in a very similar proportion as inferred from the UPLC traces. After many attempts, we were unable to isolate both cage compounds as pure products, even by using careful and exhaustive preparative HPLC techniques. Thus, we concluded that the macrobicyclization reaction is much more efficient in the case of the Tren derivatives **3a–d** than for the compounds having a central aromatic scaffold (TMBn or TEBn, **3e–h**). These results seemed quite intriguing, because similar aromatic scaffolds have been used to preorganize tripodal moieties for the preparation of concave receptors. Thus, a more in-depth study was performed in order to find a reasonable explanation for the different behavior. First of all, ^{1}H NMR spectra (500 MHz, CD_3CN) obtained for repre-

sentative precursors (**3b**, **3f**, and **3h**, $R^1 = i$Pr) showed that the starting tris(amido amine) is able to weakly interact with the bromide anion present in the reaction mixture (added as a catalyst and also formed during the reaction process). The tripodal pseudopeptides set hydrogen bonding between the amide and amino NH hydrogen atoms and the Br anion, and molecular modeling suggested that these interactions would produce a concave conformation with the three amino nitrogen atoms in a similarly close proximity (see Figures S1–S4 in the Supporting Information). Therefore, we concluded that no important differences in the preorganization of the precursors should be expected. However, we must consider that the key step to render either the [1+1] or the [2+2] cage is the third S_N2 reaction in a hypothetical macrocyclic intermediate, as depicted in Scheme 4. From



Scheme 4. Key step for the competition between the inter-/intramolecular S_N2 reactions.

this intermediate, an effective intramolecular reaction would exclusively produce the intended [1+1] cage, whereas the competing intermolecular reaction would give access to the larger [2+2] cage. According to the difference observed in the outcome of the reactions depending on the central scaffold of the tripodal pseudopeptide, we hypothesized that a rational explanation for our observations could be obtained by studying the corresponding macrocyclic intermediate in selected representative cases. Because the nucleophilic substitution is an irreversible reaction, the spatial proximity between the reacting centers in the intermediate (namely the distance between the amino nitrogen atom and the methylene bromide (d(N−C) in Scheme 4) must be fundamental regarding the competition between the intra- and intermolecular reactions. The closer are the two reacting atoms, the more efficient should be the intramolecular reaction, whereas a long distance would increase the feasibility of the intermolecular process. Theoretical calculations on representative intermediates (see Figure S7 in the Supporting Information) showed that d(N−C) was significantly larger for the tripodal aromatic spacer, thus explaining the observed differences in the corresponding reactivity.

All the isolated pure cages were unambiguously characterized by spectroscopic techniques, showing the expected C_3 symmetry by NMR spectroscopy and the formation of the [1+1] macrobicycle by mass spectrometry. Besides, a definitive proof for the cage structure was obtained by X-ray diffraction analysis (see below).

Crystal structures of the chloride complexes: We obtained crystals suitable for X-ray diffraction with several cages as the corresponding tetrahydrochloric acid salts, obtained by slow evaporation of a methanolic solution of the given compound in the presence of a slight excess of concentrated aqueous hydrochloric acid. Thus, we were able to get suitable crystals for all the Phe derivatives, that is, **5a** ($R^2 = $H), **5e** ($R^2 = $Me), and **5i** ($R^2 = $Et); and for two of the Val derivatives, that is, **5f** ($R^2 = $Me) and **5j** ($R^2 = $Et). The comparison between the observed structures illustrates the effect of the amino acid nature (R^1) and of the substitution at the tripodal aromatic ring (R^2). All the crystals showed the presence of four chloride anions per cage unit, confirming the protonation of the four amino groups of the receptors. The cages crystallized with solvent (water or methanol) molecules, being in most cases disordered. For the crystals showing two geometries per asymmetric unit (**5a**, **5f**, and **5j**), very little differences (mainly in the disposition of the amino acid side chains) were observed by the corresponding superposition of the structures (see Figures S32, S36, and S38 in the Supporting Information). Thus, a unique geometry per crystal structure will be discussed. Remarkable differences between different receptors were observed attending to the cage–chloride contacts and to the cage geometry (see Figures 1 and 2). By comparing the structures obtained for the Phe cages, a very intriguing trend was observed (Figure 1). The cages bearing a Me (**5e**) or Et (**5i**) residue at R^2 showed a tight inclusion complex with chloride (Figure 1B and C). Thus, one chloride anion settles in the center of the inner cavity of the cages, stabilized by four electrostatic hydrogen-bonding interactions with all the ammonium groups of the cages (at a Cl···HN distance of 2.1 Å for the tertiary nitrogen atom and 2.6–2.9 Å for the secondary nitrogen atoms), defining the anion coordination with tetrahedral geometry. Besides, the included chloride is positioned on top of the centroid of the tripodal aromatic ring (at a distance of 3.09 Å) suggesting the participation of an anion–π interaction.[31]

Interestingly, the amide NH groups of **5e** and **5i** are pointing outwards and are hydrogen bonded to crystal water molecules, and do not interact with any of the outer chloride ions. Thus, the main interaction of the included chloride is through electrostatic hydrogen bonds with the ammonium nitrogen atoms. However, a very different situation was obtained for compound **5a**, lacking substitution at the tripodal aromatic ring ($R^2 = $H, Figure 1A). For this molecule, the crystal structure showed a less symmetric cage without any encapsulated chloride (Figure 1A). The inner cavity of the

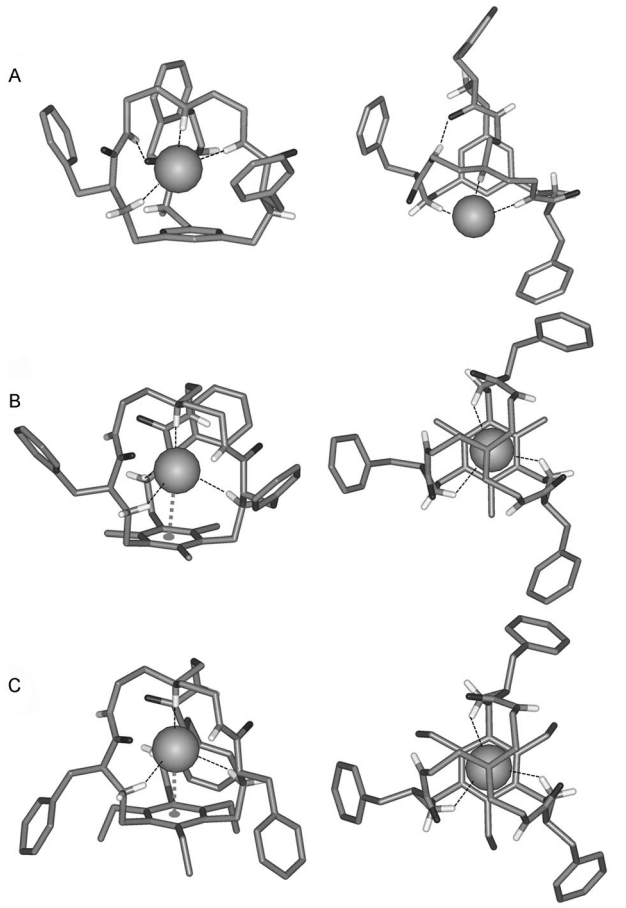


Figure 1. Side and upper view of the crystal structures of the corresponding tetrahydrochloric acid salts of A) **5a**, B) **5e**, and C) **5i**. Only the closest chloride is shown as a CPK model (30% of van der Waals radius), whereas the additional chloride atoms, solvent molecules, and non-polar hydrogen atoms have been omitted for clarity. Hydrogen bonds are shown as dashed black lines, whereas possible anion–π contacts are shown as dashed gray lines.

cage is highly reduced by the collapse of one of the arms, which sets an intramolecular hydrogen bond between an amide carbonyl group and the amide NH group of the closer arm (see Figure 1 A). As a consequence, the closest chloride is bound out of the cavity, interacting through both ammonium and amide NH···Cl hydrogen bonds (Cl···HN distances of 2.2–2.3 Å, Figure 1 A). The other three chloride ions are found to interact with ammonium groups and water molecules, one of them being also located close to one of the cage windows (see Figure S31 in the Supporting Information). Thus, surprisingly, the substitution on the aromatic tripodal ring improves the ability of the cages for including the chloride within the inner cavity, at least as observed in the solid state.

On the other hand, the compounds from aliphatic amino acids showed a remarkably different structure in the solid state, though the conformation was very similar between the Val cages bearing either Me (**5f**) or Et (**5j**) at R^2 (Figure 2, for a superposition of both cages, see Figure S39 in the Supporting Information). Two chloride atoms were found close

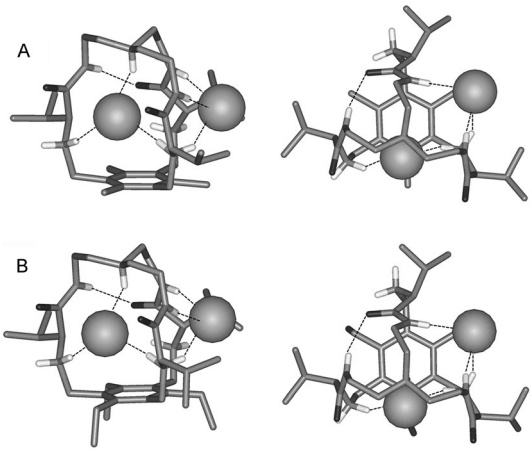


Figure 2. Side and upper view of the crystal structures of the corresponding tetrahydrochloric acid salts of A) **5f** and B) **5j**. Only the closest chloride ions are shown as a CPK model (30% of van der Waals radius), whereas the additional chloride ions, solvent molecules, and non-polar hydrogen atoms have been omitted for clarity. Hydrogen bonds are shown as dashed black lines.

to the cavity of the receptors. One of these chloride ions is partially included in the inner cavity of the molecule, namely at the entrance of the cage. This Cl(1) interacts through three ammonium NH···Cl hydrogen bonds with two secondary nitrogen atoms and the tertiary N atom (at Cl···HN distances of 2.2–2.4 Å). The second chloride Cl(2) is hydrogen bonded through one ammonium (Cl···HN distances ca. 2.4–2.5 Å) and two amide NH groups (Cl···HN distances ca. 2.3–2.4 Å), being at the outer face of the cage. Also in these Val cages, an intramolecular weak hydrogen bond was observed between the C=O group and the amide NH group of two arms of the capsule. These results underscore remarkable differences in the mode of binding of the chloride anions due to the aliphatic/aromatic side chain. The Phe cages (with R^2 = Me or Et) showed the inclusion of one chloride ion mainly interacting through ammonium NH bonds, whereas the corresponding Val cages (with R^2 = Me or Et) displayed a more complex binding implicating ammonium and amide groups and two chloride ions per cage. Thus, the nature of the side chain could change the mode of chloride recognition, at least from the information gathered in their corresponding crystal structures.

Chloride binding in solution and in the gas phase

ESI-MS experiments: The chloride recognition abilities of selected hosts were also investigated by ESI-MS-based competition experiments.[32] To this aim, we used the cages derived from Phe (**5a**, **5e**, and **5i**) and Val (**5b**, **5f**, and **5j**) in order to screen the effect of the substitution at both R^1 (aromatic vs. aliphatic) and R^2 (H, Me, Et). A compound derived from Leu (**5k**) was also added to the study for the comparison of different aliphatic side chains. The ESI mass spectra of solutions of an equimolar mixture of the studied receptors in the absence of HCl and in the presence of a 30-

fold excess of HCl in CH_2Cl_2/CH_3OH (7:3) showed important differences (Figure S8 in the Supporting Information). In the absence of added HCl, the ESI mass spectrum revealed the dominant presence of a singly charged $[M+H]^+$ species and a minor doubly charged $[M+2H]^{2+}$ peak (where M stands for the mass of the corresponding cages). When a 30-fold excess of HCl was added, the doubly charged species $[M+2H]^{2+}$ became dominant with respect to the singly charged peaks, indicating that the protonation of the amine groups has occurred as expected after the addition of HCl. Moreover, the corresponding 1:1 host–chloride adducts of the general formula $[M+2H+Cl]^+$ were clearly identified on the basis of both accurate m/z determinations as well as their characteristic isotopic pattern, and remarkably their intensity was strongly dependent on the identity of the host. This is illustrated in Figure 3 where the expanded region in the $m/z = 500$–850 range of the ESI mass spectrum of the equimolar mixture of the cages in the presence of a 30-fold excess of HCl is presented.

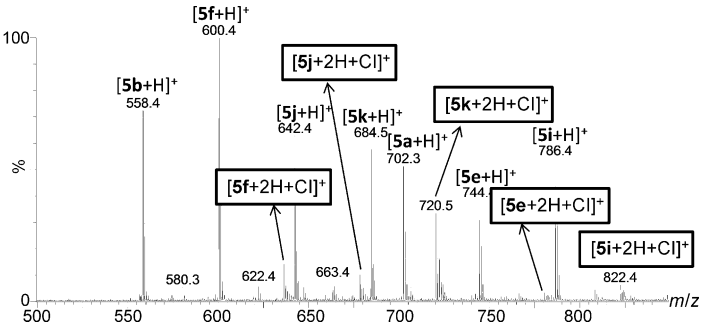


Figure 3. ESI mass spectrum in the $m/z = 500$–850 range of an equimolar mixture of the selected cages (**5a**, **5b**, **5e**, **5f**, **5i**, **5j**, and **5k**) in the presence of a 30-fold excess of HCl.

Two clear trends in the chloride selectivity can be seen along the series, as judged by ESI mass spectrometry. First of all, except for $M =$ **5a** and **5b**, the species $[M+2H+Cl]^+$ are detected. We reasonably take this as an experimental evidence for a weaker binding of chloride by the hosts **5a** and **5b**, thus anticipating the importance of the alkyl substituents in the tripodal aromatic ring to promote chloride fixation in the capsule. We hypothesize that this observed selectivity does not rely on electronic anion–π interactions, because an enhanced stability of the Cl–π interaction is expected for the more electron-poor non-substituted phenyl groups present in **5a** and **5b**.[33] However, this observation in the ESI-MS competition experiments parallels the behavior observed in solid state for the Phe derivatives, where **5a** ($R^2 = H$) showed a less efficient geometry for the interaction with the closest chloride within the crystal cell. Another interesting trend is observed when comparing the corresponding cages bearing aliphatic versus aromatic side chains. Thus, whereas for $M =$ **5e** and **5i**, that is, cages that feature Phe residues, $[M+2H+Cl]^+$ cations are barely detected in the ESI mass spectrum, for $M =$ **5f**, **5j**, and **5k**, that is, cages displaying Val and Leu residues, more intense signals are observed. This suggests that the receptors from aliphatic amino acids seem to bind the anion more efficiently. We have already observed that the Phe pseudopeptidic macrocycles usually displayed a reduced ability to participate in non-covalent interactions with a given substrate or upon self-assembly,[34] and we reasoned this trend as a shielding effect of the aromatic ring of the side chain, which precludes the close amide and ammonium groups from establishing hydrogen-bonding interactions. On the basis of the ESI-MS competition results we propose host **5k** (Leu derivative with $R^2 = Et$) as the most efficient chloride receptor.

Energy-resolved collision-induced dissociation (CID) experiments were also carried out on the formed $[M+2H+Cl]^+$ cations. In general, the expelled fragment in all cases is HCl in the collision energy range investigated, to yield the $[M+H]^+$ singly charged free receptor. Breakdown profiles for each host–guest couple are represented in Figure 4A and illustrative CID spectra at increasing collision energy (CE) for the $[\mathbf{5k}+2H+Cl]^+$ cation in Figure 4B. The differences between the gas dissociation of the complexes were not very large, although a higher stability of those formed with the cages bearing Et at R^2 was apparent. Also in this case, the best receptor was **5k**, like in the competition experiments.

NMR experiments: The interaction between the pseudopeptidic cages and chloride anions in solution was also studied by NMR spectroscopic titration. To this aim, the addition of chloride (as the tetrabutylammonium salt, TBACl) to a solution of the cages was monitored by ^1H NMR (500 MHz, 303 K) spectroscopy. Interestingly, when the experiments were performed with the receptors as the free amine, a very weak interaction was observed. However, when performing the experiment with the protonated cages (either as the $HClO_4$ or the trifluoroacetic acid (TFA) salts) a strong binding was observed by the perturbation of the chemical shifts of several ^1H NMR signals upon the addition of TBACl. After trying different experimental conditions, we obtained suitable NMR spectra (reasonably well-defined signals for the accurate quantitative analysis) by titrating the corresponding tetra-TFA salts of the cages in aqueous acetonitrile solution (CD_3CN/H_2O 95:5) with increasing amounts of TBACl (see the Supporting Information for details). Several signals of the cages were affected by the presence of the chloride anion, mainly those flanking the secondary amino nitrogen atoms corresponding to the benzylic positions of the tripodal aromatic ring (labeled as HB/HB′) and the proton at the chiral center (the one at the α carbon of the amino acid, labeled as HA), as well as the amide NH proton signal (which was visible because we used non-deuterated water). Other representative signals from the Tren moiety also changed upon chloride addition, though severe broadening and important overlapping precluded their accurate analysis. A single set of signals was observable during the titration experiments, implying fast exchange on the ^1H NMR timescale. We performed the titrations with all the Phe de-

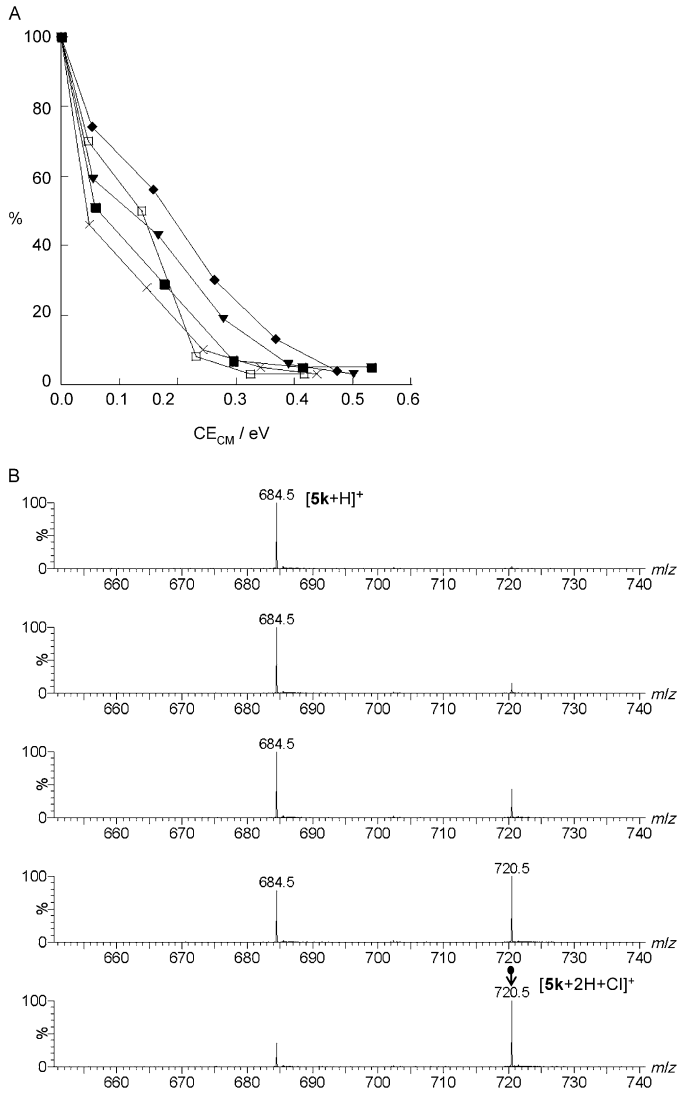


Figure 4. A) Breakdown profiles of mass-selected $[M+2H+Cl]^+$ complexes at increasing center of the mass collision energies (× = **5e** (Phe, Me), □ = **5i** (Phe, Et), ■ = **5f** (Val, Me), ▼ = **5j** (Val, Et), and ◆ = **5k** (Leu, Et)) and B) illustrative CID spectra of mass-selected $[\mathbf{5k}+2H+Cl]^+$ at $m/z = 720.5$ at increasing CE_{lab} from 1, 3, 6, 9, and 12 eV (from the bottom to the top).

rivatives, that is, **5a**, **5e**, and **5i**, to show the effect of different substitution at the tripodal aromatic ring (R^2). Additionally, the effect of the amino acid side chain (R^1) was studied by measuring all the cages with $R^2 = Et$, that is, **5i** (Phe), **5j** (Val), **5k** (Leu), and **5l** (Ile). As a general trend, most of the signals move downfield during the first points of the titration, up to the addition of one to two equivalents of chloride. Then, some signals continue moving downfield upon further addition, whereas others started to move upfield. This behavior suggests the formation of a strong 1:1 cage–chloride complex accompanied with complexes with higher chloride stoichiometry (see below). The methylene protons at the benzylic position of the tripodal aromatic ring (HB/HB′) showed a very interesting trend. These protons appeared as an AB quartet in all the cases and their corresponding anisochrony increased in the presence of chloride. This suggests that the formation of chloride complexes produces a more rigid cage, with a more different chemical environment for these protons. For the illustration of the effect of the substitution at the tripodal aromatic ring, the chloride-induced chemical shifts for **5a** ($R^2 = H$) and **5e** ($R^2 = Me$) are shown in Figure 5. For a fair comparison, the

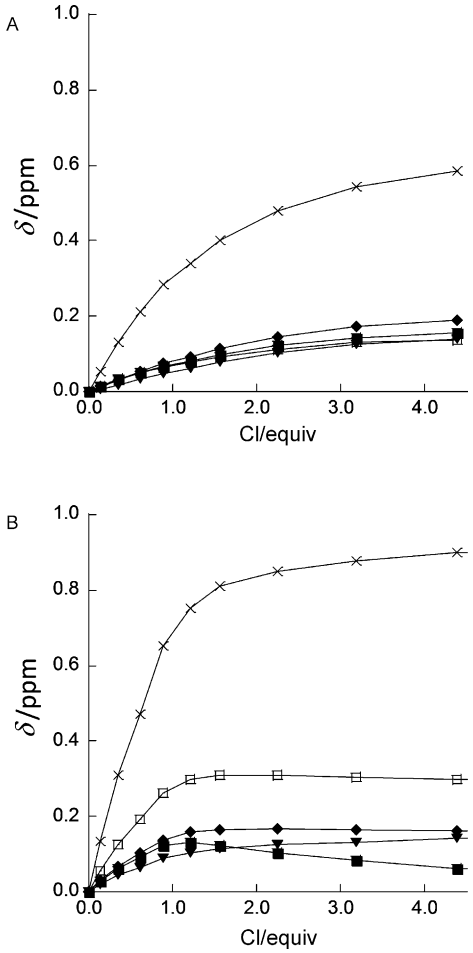


Figure 5. Effect of the substitution on R^2 on the chloride binding properties illustrated by the plot of the chloride-induced chemical shifts of several signals of cages A) **5a** ($R^2 = H$, ◆) and B) **5e** ($R^2 = Me$, ◆). NMR titrations were performed at 500 MHz, 303 K, and in CD_3CN/H_2O 95:5 (× = NH, □ = HB, ■ = HB′, ▼ = HA).

same scales were used for both representations. The plots clearly show a stronger interaction of chloride with the Me derivative (see below for a quantitative analysis). Thus, no signs of saturation were observed for **5a** after four equivalents of the anion were added, whereas most of the signals of **5e** clearly showed saturation after addition of less than two equivalents of chloride. Noteworthy, both the signals from the benzylic protons (HB/HB′) and the signal for the methyl groups directly attached to the aromatic ring in **5e** reached saturation at approximately 1.2 equivalents of chloride, suggesting that these groups are directly involved in the

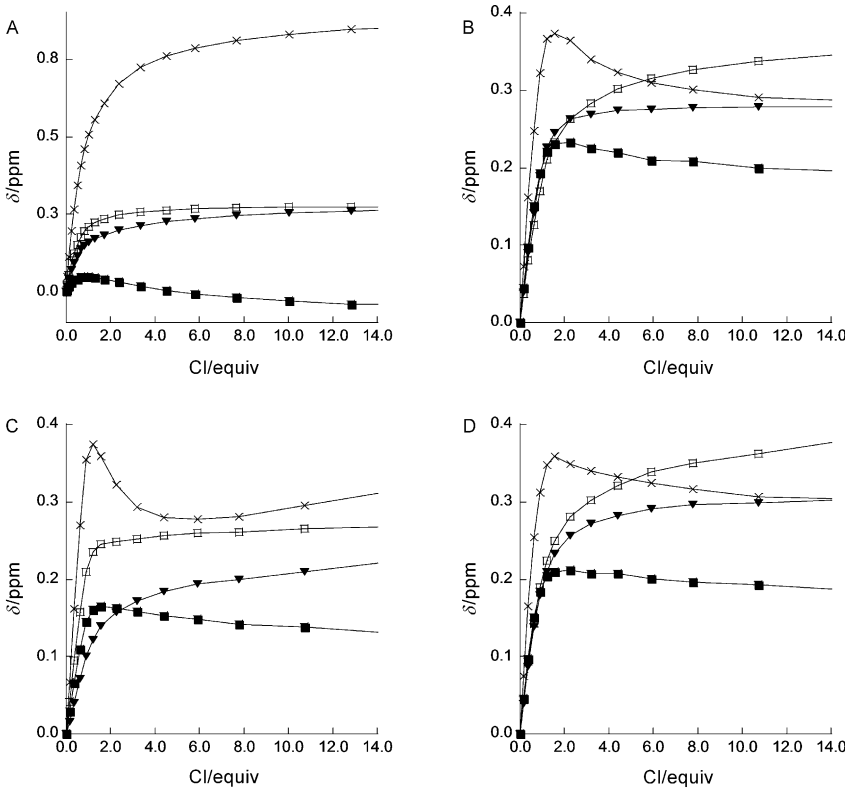


Figure 6. Effect of the nature of the amino acid side chain on the binding of chloride, illustrated by the plot of the chloride-induced chemical shifts of several signals of A) **5i** (Phe), B) **5j** (Val), C) **5k** (Leu), and D) **5l** (Ile). NMR titrations were performed at 500 MHz, 303 K, and in CD_3CN/H_2O 95:5 (× = NH, □ = HB, ■ = HB′, ▼ = HA).

binding of the first chloride anion. These data are in perfect agreement with the results observed in the solid state, that is, the formation of the cage–chloride 1:1 supramolecular complex.

For the study of the effect of the nature of the amino acid side chain, similar plots of the chloride-induced chemical shifts are shown in Figure 6 for the cages bearing an ethyl moiety at R^2. A remarkable difference between the aromatic and aliphatic amino acids was observed. The Leu cage seems to be the one forming the most stable complexes with chloride. In the aliphatic cages, a very interesting behavior was observed for the chemical shift of the amide NH proton. In all cases, the signal moves downfield until reaching a maximum at approximately 1.2 equivalents of chloride and, after that, a marked upfield shift is evident. This suggests that in the case of the aliphatic cages, the amide NH proton is directly implicated in the binding of the first chloride anion and that the formation of the complexes of higher stoichiometry further affects the chemical environment of the amide group, probably due to a conformational change. These experimental observations in solution are in a very good agreement with the data obtained in the solid state by X-ray diffraction of the corresponding chloride complexes of Phe and Val cages (see above).

For the quantitative analysis of the data, we followed the fitting procedure proposed by Roelens and co-workers.[35] To that, the simultaneous non-linear regression fit of all the available signals was performed (see the Supporting Information for details). Complexes with different stoichiometry were detected by fitting the titration data. However, in all cases the 1:1 complex was more stable, suggesting the preference for the interaction with one chloride ion. For a suitable comparison of supramolecular complexes of different stoichiometry, we used the BC_{50}^0 parameter that gives an idea of the intrinsic chloride affinity, independently on the stoichiometry of the considered complexes.[36] Because we observed multinuclear complexes in the titration agent (chloride), the BC_{50}^0 parameter is here defined as the concentration of chloride necessary to bind 50% of the corresponding cage, starting from the free species and considering all the possible complexes formed. For simple 1:1 complexes, the BC_{50}^0 parameter is equal to the dissociation constant of the corresponding supramolecular complex and, therefore, the lower is the BC_{50}^0 value, the stronger is the host–guest interaction. The obtained results are shown in Table 3, whereas the titration experiments and

Table 3. Chloride-binding data in solution, obtained by ^{1}H NMR spectroscopic titration (500 MHz, 5% aqueous CD_3CN, 303 K) of the corresponding TFA salts of the cages with TBACl.

Entry	Cage	R^1 (Aaa)	R^2	Logβ[a] (cage/Cl)	BC_{50}^0 [μM]
1	**5a**	Bn (Phe)	H	(2.35±0.03) (1:1)[b]	(4467±309)
2	**5e**	Bn (Phe)	Me	(4.01±0.08) (1:1)[b] (6.2±0.1) (1:2)[c]	(96±17)
3	**5i**	Bn (Phe)	Et	(3.37±0.09) (1:1)[b] (5.1±0.1) (1:2)[c]	(417±83)
4	**5j**	*i*Pr (Val)	Et	(3.68±0.07) (1:1)[b] (5.86±0.09) (1:2)[c]	(202±31)
5	**5k**	*i*Bu (Leu)	Et	(4.35±0.11) (1:1)[b] (6.9±0.1) (1:2)[c] (7.9±0.3) (1:3)[d]	(44±11)
8	**5l**	*s*Bu (Ile)	Et	(3.87±0.08) (1:1)[b] (5.9±0.1) (1:2)[c]	(133±24)

[a] Obtained by simultaneous non-linear regression fitting of all the available ^{1}H NMR signals. [b] We defined β (1:1) as the formation constant for the 1:1 cage/Cl complex in M^{-1}. [c] In this case β (1:2) is the cumulative formation constant for the corresponding 1:2 cage/Cl complex in M^{-2}. [d] In this case β (1:3) is the cumulative formation constant for the 1:3 cage/Cl complex in M^{-3}. Despite the weak binding of the third chloride ion, it was necessary for an accurate fitting of the data.

the corresponding fittings are given in the Supporting Information. Interestingly, the substitution at the tripodal aromatic ring produced an increase in the strength of the chloride binding by more than one order of magnitude from $BC_{50}^{0}=4$ to 0.1 mM for $R^2=H$ or Me, respectively. The large increase of the chloride binding, which is influenced by the R^2 substituent, is in very good agreement with the observations in the solid state by single-crystal X-ray diffraction and in solution by ESI-MS competition experiments. As previously anticipated, this difference cannot be ascribed to enhanced anion–π interactions. We reasoned that the substitution at R^2 could produce an efficient desolvation of the nearby benzylic ammonium groups, thus leaving the corresponding NH_2^+ sites more accessible for the binding to the anion. A slight decrease in the chloride binding was observed by going from Me to Et substitution among the Phe cages, which can be ascribed to an increased steric hindrance in the Et derivative. Stronger interactions are obtained by changing from an aromatic to an aliphatic amino acid side chain, as also observed in the competition ESI-MS experiments and for other pseudopeptidic macrocyclic hosts.[34] Besides, we consistently observed the formation of complexes of 1:2 stoichiometry, as well as chloride binding through the participation of the amide NH protons, which is also in agreement with the mode of binding observed in the solid state by X-ray diffraction for the Val cages. The cage showing the strongest interaction (with $BC_{50}^{0}=44$ μM) was **5k**, which is in perfect accordance with the competition ESI-MS and CID experiments. Among the cages bearing aliphatic side chains, the one from Leu lacks substitution at the β carbon atom of the amino acid, which could decrease the steric hindrance for the binding, thus explaining the improved chloride recognition of **5k** versus **5l** and **5j**.

The chloride recognition ability of cage **5i** was also studied by ^{35}Cl NMR spectroscopy, in order to know whether the inclusion complex observed in the solid state was retained in solution.[37] Thus, the ^{35}Cl NMR spectrum of **5i**·4HCl was acquired in a highly competitive solvent (CD_3OD/D_2O 1:1), in addition to the ^{35}Cl NMR spectrum of HCl in the same medium as a reference (Figure 7). The spectrum of **5i**·4HCl showed an almost undetectable very broad signal at room temperature (Figure 7B), which slightly sharpened at higher temperature (333 K, Figure 7C). The very sharp peak at $\delta=3.29$ ppm observed for HCl in this medium (Figure 7A) suggested that the Cl nuclei in the sample of **5i**·4HCl are involved in a dynamic exchange process within a non-symmetrical environment.[38] Interestingly, the signal observed at 333 K was non-symmetrical, and the accurate de-convolution by line-shape fitting rendered two broad ^{35}Cl NMR peaks at $\delta=17.83$ and 10.86 ppm in a relative intensity of approximately 1:3, respectively. These data suggest that the 1:1 chloride inclusion complex is also present in solution and that the chloride in/out exchange occurs at an intermediate rate on the ^{35}Cl NMR timescale, but fast on the ^{1}H NMR timescale as observed in the titration experiments. Considering the resonance frequencies of both nuclei and our experimental observations, we estimated the chloride in/out exchange within the low milliseconds scale.

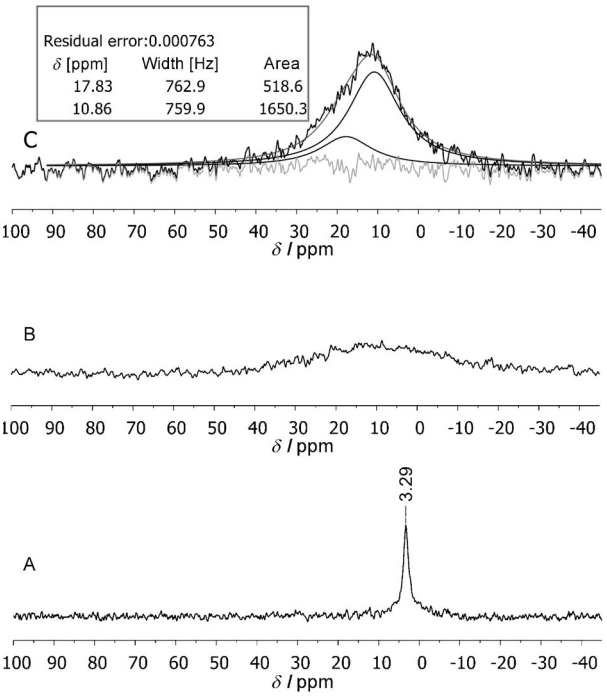


Figure 7. ^{35}Cl NMR spectra (48.97 MHz, CD_3OD/D_2O 1:1) of A) HCl at 303 K, B) **5i**·4HCl at 297 K, and C) **5i**·4HCl at 333 K. In C) the experimental spectrum was de-convoluted into two bands (black traces), being the corresponding addition simulated spectrum (gray trace) and the difference between the simulated and the experimental spectra (light gray trace) also shown. The numerical results of the fitting are shown in the inset.

Chloride transport through lipid bilayers: The potential of these compounds to facilitate the transmembrane transport of chloride in model phospholipid bilayers was explored by using 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) liposomes. Chloride efflux from chloride-loaded vesicles was monitored over time by using a chloride-selective electrode. The liposome suspension was placed in an isotonic, chloride-free medium and the compounds studied were added as a solution in DMSO. Addition of a detergent at the end of the experiment lysed the vesicles and provided a final chloride concentration value corresponding to 100% chloride release. The results obtained are shown in Figure 8. Compound **5i** was found to be the most active chloride carrier in these assays, promoting around 50% chloride release. Compounds **5e**, **5k**, and **5l** promoted similar chloride efflux accounting for approximately 35%, and limited chloride efflux was detected in the case of compound **5a**. These results showed that in compounds **5a**, **5e**, and **5i**, which are derived from Phe, the transport efficiency increased significantly as the substitution of the tripodal aromatic ring changed from hydrogen (**5a**) to methyl (**5e**) and ethyl (**5i**) groups. The fact that **5a** was found to be the less active derivative of these compounds is in good agreement with the

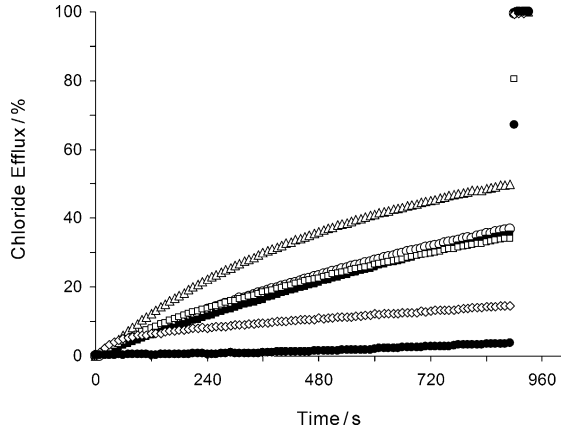


Figure 8. Chloride efflux promoted by 25 μM (5 mol% carrier-to-lipid concentration) of the indicated compounds in unilamellar POPC vesicles. The vesicles were loaded with 489 mM NaCl buffered at pH 7.0 with 10 mM phosphate dispersed in 489 mM $NaNO_3$ buffered at pH 7.0. Each trace represents the average of three trials (△=**5i**, ○=**5k**, ■=**5l**, □=**5e**, ◇=**5a**, ●=DMSO).

results observed in both the solid state and in solution. Thus, compound **5a** did not encapsulate chloride in the solid state and the chloride binding affinity was an order of magnitude lower than that of the other derivatives. In order to further rationalize these results we also studied the lipophilicity of these compounds. This parameter has been shown to greatly influence the transmembrane transport activity of other ionophores.[39] Average log *P* values were calculated by using computational methods.[40] Moreover, an experimental measure of the lipophilicity was obtained by reverse-phase HPLC.[41] The results indicated that an increase in the lipophilicity of the compounds resulted in an increase of the transport activity (Table S1 in the Supporting Information), with the most lipophilic derivative (**5i**) being the most active transporter and the most hydrophilic derivative (**5a**) only displaying limited activity as chloride transporter. The similar values of both log *P* and the retention time for compound **5e**, **5k**, and **5l** were in agreement with the results observed in the liposome assays.

Conclusion

We described herein the efficient and selective synthesis of a new family of small pseudopeptidic cages, through a triple nucleophilic substitution and from simple tripodal precursors. The success of the reaction highly depends on the structure of the central scaffold of the tris(amido amine) precursor. The pseudopeptides derived from tris(2-aminoethyl)amine (Tren) led to the [1+1] cages in very good yields and complete selectivity, whereas those bearing a central aromatic scaffold produced complex mixtures, mainly containing the [1+1] and the [2+2] cages. The obtained results have been rationalized in terms of the preorganization of the corresponding monomacrocyclic intermediate, previous to the closure of the macrobicycle. The binding of chloride ions by the protonated forms of the pseudopeptidic cages has been studied in the solid state (by X-ray diffraction), in solution (by NMR spectroscopy and competition ESI-MS) and in the gas phase (by tandem MS CID), showing trends, which are in very good agreement. Despite the fact that the binding pockets of the different cages are essentially identical (same size, shape, and potential binding sites), the chloride recognition abilities are strongly affected by the peripheral substitution, such as the residues at the aromatic tripodal ring (R^2) and the nature of the amino acid side chain (R^1). The substitution at R^2 is favoring the inclusion of the chloride ion (as observed by X-ray diffraction) and is improving the binding in solution (as observed by competition ESI-MS and NMR spectroscopy) by more than one order of magnitude. This is very surprising because the more substituted aromatic ring should display reduced anion–π interactions, but can be explained with a less efficient solvation of the ammonium groups in the more substituted cages. On the other hand, the cages built from aliphatic amino acids interact more strongly with the chloride than the cages derived from Phe. Interestingly, the aliphatic/aromatic substitution seems to change the mode of the binding to chloride, as inferred from the NMR spectroscopic and X-ray data. Thus, the Phe cages are more prone to completely encapsulate the chloride ion by electrostatic ammonium NH···Cl^- hydrogen bonds, whereas the aliphatic cages bind two chloride ions through ammonium and amide NH groups, leading to a partial encapsulation. An important counterintuitive conclusion can be extracted by comparing the results of the Phe and Val cages bearing an Et group at R^2: the chloride inclusion within the cage structure does not necessarily imply a more efficient binding. Thus, the Phe cages (with Me or Et at R^2) fully encapsulate the anion but the interaction is stronger with the receptors built from aliphatic amino acids. The ability of some of these cages to transport chloride through lipid bilayers has also been demonstrated. Once again, remarkable differences were observed in the chloride transport rates of the different cages, which are better correlated to the corresponding lipophilicity than to the chloride binding. Therefore, we have demonstrated that slight changes in the structure of these pseudopeptidic cages produce important differences in the corresponding chloride affinity and transport ability, even if they have apparently the same binding pocket. The behavior of these minimalistic systems parallels the situation in Nature, where far structural mutations in natural receptors can enormously affect their function, and even produce pathological disorders. Our deep study by using small and simple molecules could serve for the understanding of more complicated systems, as well as a platform for the further design of improved chloride receptors and transporters based on pseudopeptidic molecules.

Experimental Section

General: Reagents and solvents were purchased from commercial suppliers (Aldrich, Fluka, or Merck) and were used without further purification.

NMR spectroscopy: The NMR experiments were carried out on a Varian INOVA 500 spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C, and 49 MHz for ^{35}Cl). Chemical shifts are reported in [ppm] by using TMS as a reference. Titrations experiments followed by ^{1}H NMR spectroscopy were performed in CD_3CN/H_2O 95:5 at 303 K and data fitting was carried out with HypNmr 2008 version 4.0.71 software (see the Supporting Information for details).

Mass spectrometry: A Q-TOF Premier mass spectrometer with an electrospray source (Waters, Manchester) operating in the V-mode was used. The drying gas as well as the cone gas was nitrogen at a flow of 400 L h^{-1} and 60 L h^{-1}, respectively. The temperature of the source block was set to 120 °C and the desolvation temperature was set to 150 °C. A capillary voltage of 3.5 kV was used in the positive scan mode. For characterization purposes, methanol sample solutions were infused through a syringe pump directly connected to the ESI source at a flow rate of 10 μL min^{-1} and the cone voltage was set to 20 V. Mass calibration was performed by using a mixture of 0.05 M NaOH and 10 % formic acid (50:50) from m/z = 50–1250. For the accurate mass measurements, a solution of leucine enkephalin (m/z = 556.2771) was introduced through the lock spray needle at a flow rate of 30 μL min^{-1}. For competition experiments, to an equimolar mixture of the receptors (1×10^{-3} M in CH_2Cl_2/CH_3OH 7:3) a 30-fold excess of HCl was added. The resulting mixture was diluted to a final concentration of 1×10^{-5} M and the ESI mass spectra were recorded. Collision-induced dissociation (CID) experiments were performed by mass-selecting the monoisotopic peak of interest with Q1 (isolation width 1 Da), interacted with argon in the T-wave collision cell while analyzing the ionic fragments with the TOF analyzer. The collision energy was systematically stepped in the E_{lab} = 1–12 eV range. For a qualitative analysis of the energy-dependent CID experiments, the laboratory collision energies were converted to the center-of-mass frame, $E_{CM} = m/(m+M)E_{lab}$, where m and M stand for the masses of the collision gas and the ionic species, respectively. For the breakdown profile representations, the signal intensities were obtained from the average of 80 scans and measuring of the area of the fragmentation peaks. These graphs were represented by taking into account the relative abundance of the precursor and the product peaks of each compound ($I_{\text{precursor ion}}/[I_{\text{precursor ion}}+I_{\text{product ion}}]$) against E_{CM}. We selected the value of the collision energy required for 50 % reduction of the precursor ion ($E_{1/2}$) as a semi-quantitative measure of the intrinsic gas-phase stability of the studied non-covalent complexes.

Infrared spectroscopy: FTIR spectra were acquired in a JASCO 6200 equipment having a MIRacle Single Reflection ATR Diamond/ZnSe accessory.

X-ray crystallographic analysis: Data were collected on a STOE IPDS II two-circle diffractometer with graphite-monochromated $Mo_{K\alpha}$ radiation. Empirical absorption corrections were performed by using the MULABS[42] option in PLATON.[43] The structures were solved by direct methods by using the program SHELXS[44] and refined against F^2 with full-matrix least-squares techniques by using the program SHELXL-97.[44] Hydrogen atoms were geometrically positioned and refined by using a riding model. Hydrogen atoms of water and methanol molecules could not be unequivocally located and were omitted from the refinement. The absolute configuration of all molecules was determined by refining the Flack parameter. In compound **5f**, three isopropyl groups are disordered over two positions. CCDC-883493 (**5f**), 883494 (**5a**), 883495 (**5j**), 883496 (**5i**), and 883497 (**5e**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Molecular modeling: Theoretical conformational studies were performed by Monte Carlo searches and MMFF minimizations with the Spartan 06 program. Thus, over 10000 geometries were stochastically generated and minimized with the MMFF force field. The 100 most stable local minima were analyzed and ordered attending to their relative energy. Their population was also calculated by using a Boltzmann distribution with the same software.

General procedure for the preparation of the cages

Synthesis of ***5a***: *N*-*N*′-*N*″-tris(*N*-L-phenylalanine)-Tren (**3a**) (761 mg, 1.295 mmol), anhydrous K_2CO_3 (1.789 g, 12.95 mmol), tetrabutylammonium bromide (208.64 mg, 0.648 mmol), and 1,3,5-tris(bromomethyl)-benzene (476.39 mg, 1.295 mmol) were placed in a flask containing dry CH_3CN (500 mL) and the mixture was heated to reflux for 12 h under an argon atmosphere. The reaction mixture was filtered and the solvent evaporated under reduced pressure. The crude product was dissolved in $CHCl_3$ (50 mL) and extracted with aqueous NaOH 0.01 M (3×50 mL). The organic phase was dried over anhydrous $MgSO_4$ and the solvent was evaporated under reduced pressure. The product was purified by silica flash chromatography by using $MeOH/CH_2Cl_2$ (1:40) as eluent. Compound **5a** was obtained in 38 % (345.34 mg, 0.492 mmol) yield. M.p. 94–96 °C; $[\alpha]_D^{25} = -78.5$ (c = 0.01, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.83 (s, 1H), 2.24 (t, J = 6.2 Hz, 2H), 2.79 (dd, J = 9.9, 14.0 Hz, 1H), 2.99 (q, J = 6.0 Hz, 2H), 3.33 (dd, J = 4.0, 14.1 Hz, 1H), 3.43 (d, J = 14.5 Hz, 1H), 3.50 (dd, J = 4.1, 9.9 Hz, 1H), 3.76 (d, J = 14.5 Hz, 1H), 7.01 (s, 1H), 7.07 (t, J = 5.6 Hz, 1H), 7.22–7.25 (m, 1H), 7.28–7.38 ppm (m, 4H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 37.9, 38.8, 51.9, 54.6, 64.5, 125.5, 126.8, 128.8, 129.1, 138.00, 141.0, 173.8 ppm; IR (ATR): 3318, 2930, 2830, 1653, 1509, 1451 cm^{-1}; HRMS (ESI-TOF)$^+$ calcd for $C_{42}H_{51}N_7O_3$ $[M+H]^+$: 702.4132; found 702.4134; elemental anal. calcd for $C_{42}H_{51}N_7O_3{\cdot}H_2O$: C 70.07, H 7.42, N 13.62; found: C 69.7, H 7.1, N 13.3.

Preparation of the phospholipid vesicles: A solution of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (Sigma–Aldrich) (chloroform, 20 mg mL^{-1}) was evaporated and the obtained lipid film was dried under high vacuum for at least 2 h. The lipid film was rehydrated by addition of a solution of sodium chloride (489 mM, 10 mM phosphate buffer, pH 7.0) followed by careful vortexing. The obtained lipid suspension was then subjected to nine freeze-thaw cycles and twenty-nine extrusions through a 200 nm polycarbonate Nucleopore membrane by using a LiposoFast Basic extruder (Avestin, Inc.). The resulting unilamellar vesicles were dialyzed against a solution of $NaNO_3$ (489 mM, and 10 mM phosphate buffer, pH 7.0) to remove unencapsulated chloride.

Ion selective electrode (ISE) transport assays: Unilamellar POPC vesicles (200 nm mean diameter) containing an encapsulated solution of NaCl (489 mM) and phosphate buffer (10 mM, pH 7.0) were suspended in a solution containing $NaNO_3$ (489 mM) and phosphate buffer (10 mM, pH 7.0) for a final lipid concentration of 0.5 mM and a total volume of 5 mL. A solution of the carrier molecule in DMSO (12.5 μL, 10 mM) was added and the chloride release from the vesicles was monitored by using a sympHony chloride-selective electrode. At the end of the experiment the vesicles were lysed with a detergent (triton-X 10 % dispersion in water, 60 μL) to release all chloride ions. This final reading value was considered to represent 100 % release and used as such.

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