

## **Chapter 3**

**Towards the understanding of structural factors inducing cell transfection properties in arginino-calix[4]arenes**

## 3.1 Introduction

The results discussed in Chapter 2 indicated that compound **3** (Fig 3.1) is the best calix[4]arene based vector for cell transfection synthesized so far.

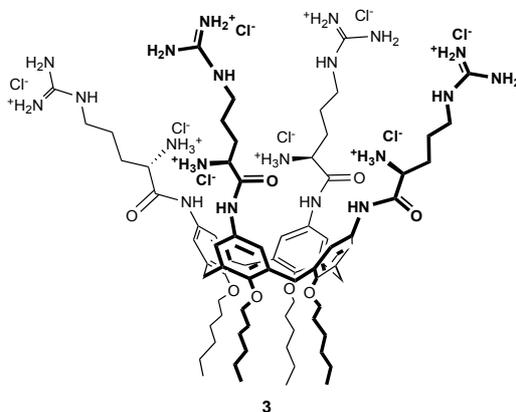


Fig 3.1 Structural formula of compound 3.

After the evaluation of the importance of the macrocyclic structure in relation to the complexation and delivery capabilities through the comparison of **3** with the acyclic Gemini analogue **8**, in order to better understand the other factors affecting the biological properties of the argininocalixarene, several structural modifications respect to **3** were explored. Synthesis and properties of the compounds obtained in this way are described in this chapter.

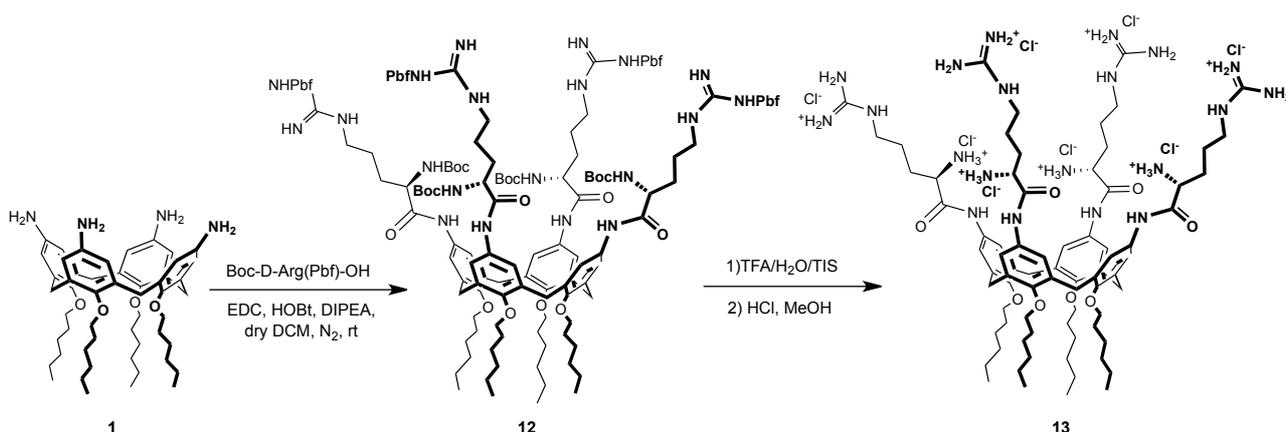
## 3.2 Results and discussion

### 3.2.1 Synthesis

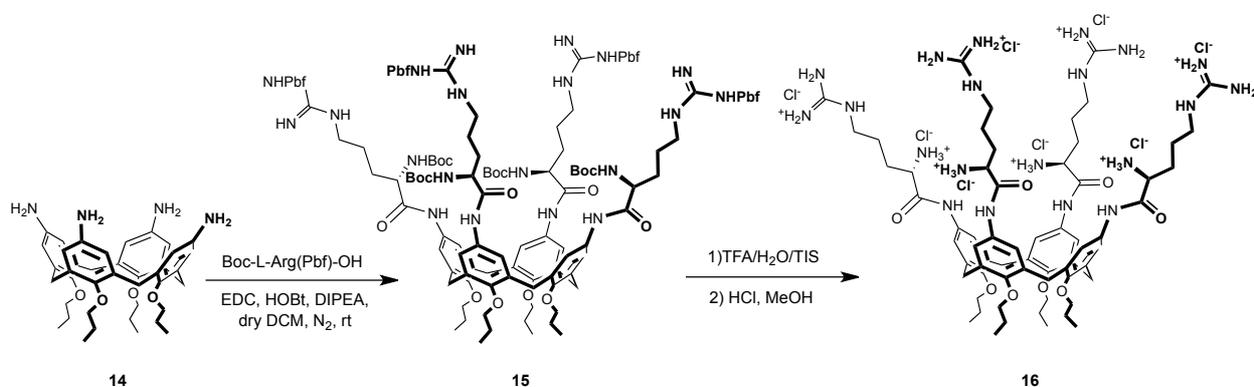
It is known that sequences of arginine-rich peptide comprising L-amino acids are susceptible to proteolytic digestion and metabolic decomposition. We have actually no data about the stability of L-argininocalixarene **3** in biological environment and if the amide bond linking the L-amino acid to the calixarene scaffold can undergo hydrolysis by peptidases. First of all, then, calix[4]arene **13**, containing D- instead of L-arginine units, was prepared through the same synthetic pathway used for the synthesis of **3**, using Boc-D-Arg(Pbf)-OH (Scheme 1), and investigated as new potential non-viral vector. Possible differences in properties between **3** and **13** could be ascribed to different stability and/or

different efficiency in steps of the gene delivery process where the stereochemistry of the vector plays a key role.

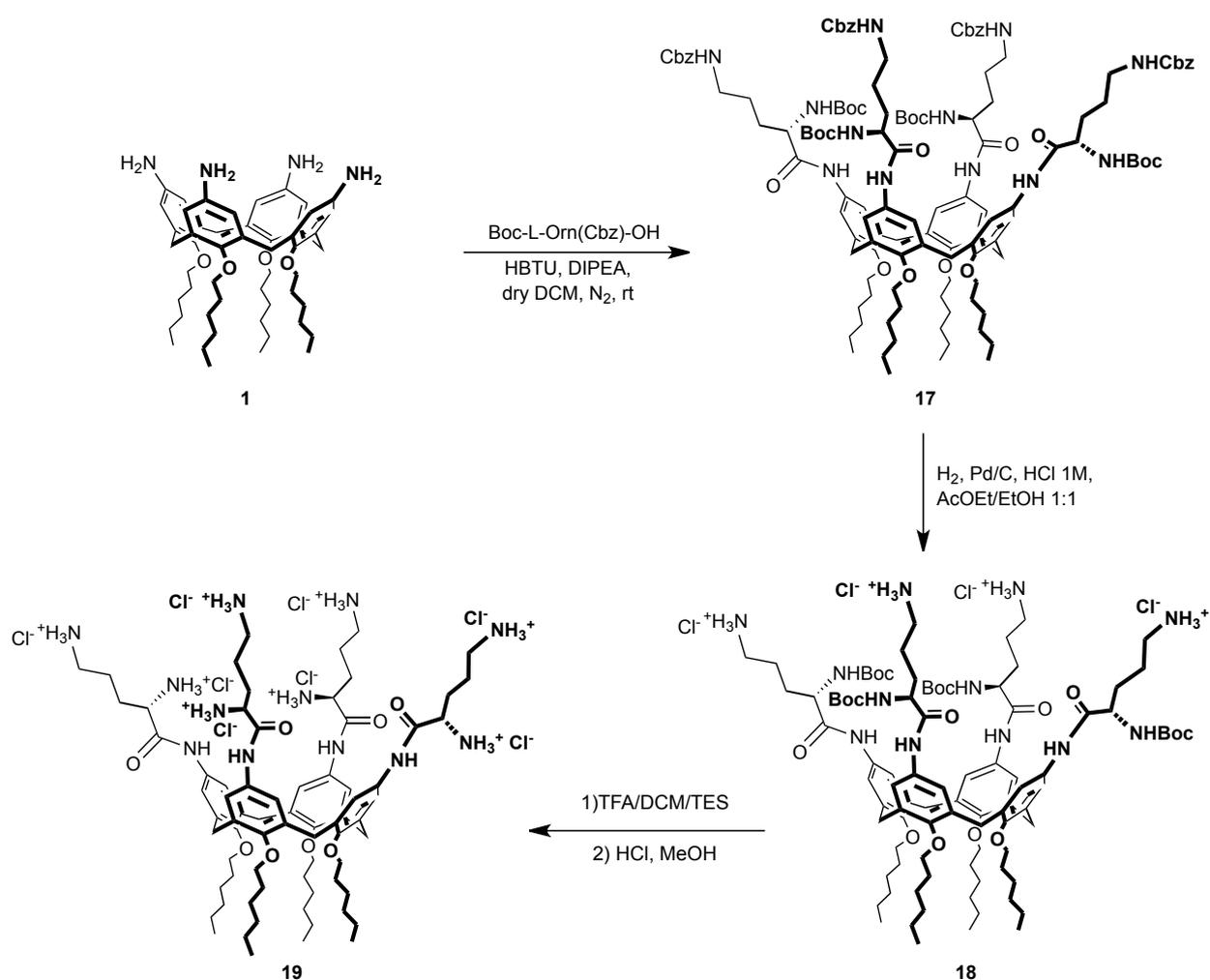
It is to this end reported<sup>1</sup> that D-arginine containing delivery systems resulted in some uptake experiments more efficient than the corresponding L-arginine based analogues, even if control experiments showed that the quantitative difference in uptake could not be attributed to increased decomposition of and L- versus a D-peptide by cellular or serum proteases.



As second variation, compound **16**, having shorter lipophilic tails compared to **3**, was synthesized to study the influence of the lower rim substituents on establishing hydrophobic interactions. This derivative was obtained starting from the amino precursor **14**<sup>2</sup> functionalized at the lower rim with propyl chains and following the coupling/deprotection sequence (**Scheme 2**) already adopted for **3**.



In addition, it was decided to modify the positively charged groups on the amino acid side chain by the synthesis of **19** bearing at the upper rim L-ornithine moieties (**Scheme 3**). The introduction of this non-natural amino acid led to a compound that presents on one side the polar heads displayed by lysino-calixarene **5** for DNA complexation, and on the other side the same distance between the apolar cavity and the charged groups offered by arginine unit in lead compound **3**. Respect to **5**, this derivative should verify the effects of guanidinium replacement with ammonium taking strictly fixed distances and all other structural parameters. The availability Boc-L-Orn(Cbz)-OH determined the need of two different deprotection steps in sequence, the first by catalytic hydrogenation, the second by treatment with TFA, to obtain the desired product **19**. It was decided to avoid the use of too strong acidic conditions, such as the use of HBr, that could remove simultaneously the two protecting groups but also damage the final compound.

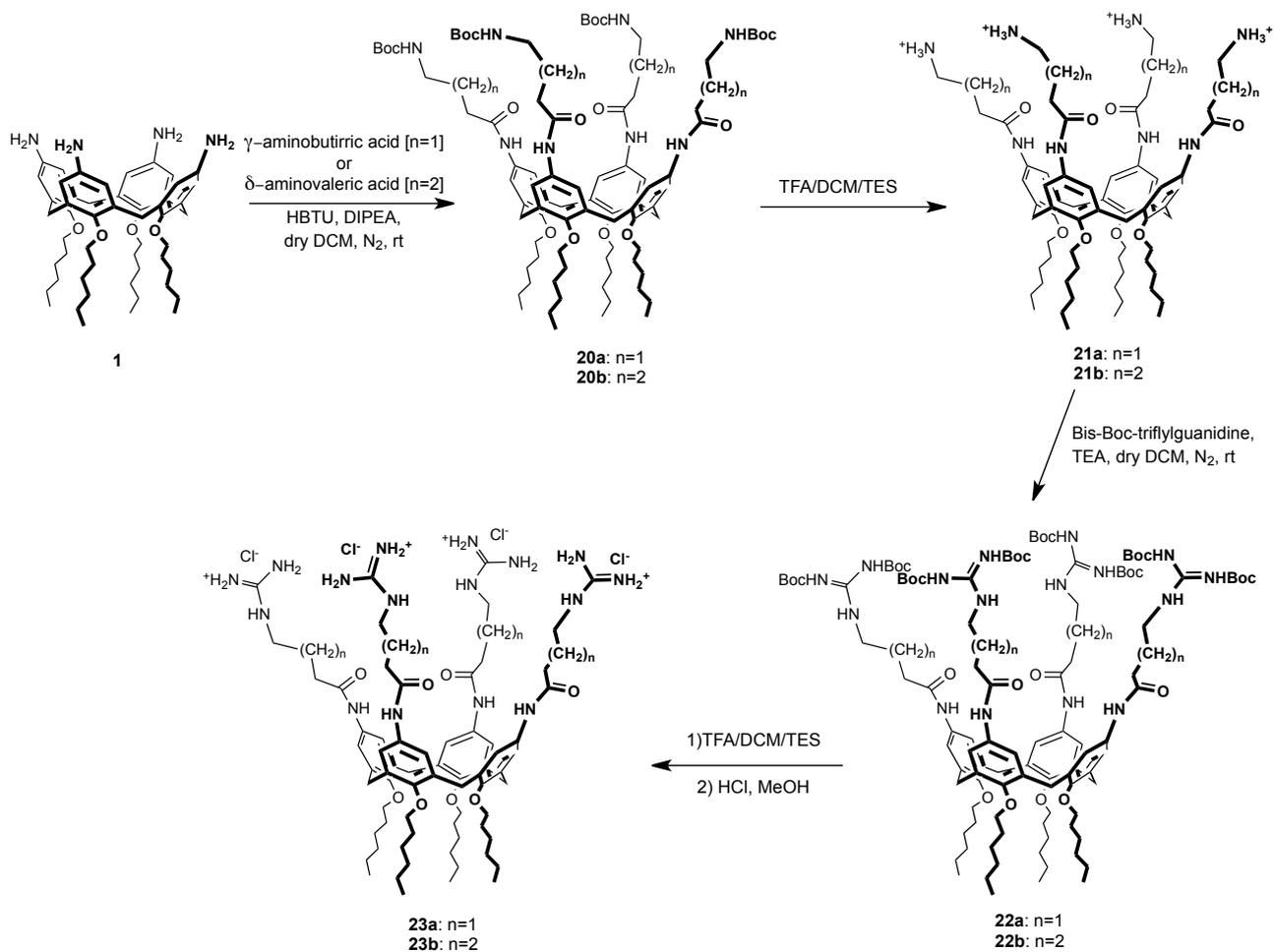


**Scheme 3:** synthesis of calixarene **19**.

Further modifications were finalised to disclose the importance of arginine  $\alpha$ -amino group in giving to compound **3** its excellent transfection abilities. For this purpose, four new calix[4]arenes (**23a** and **b**, **31** and **33**) were synthesized where the amino group was absent or involved in an amide bond. The lower rim was always functionalized with hexyl chains.

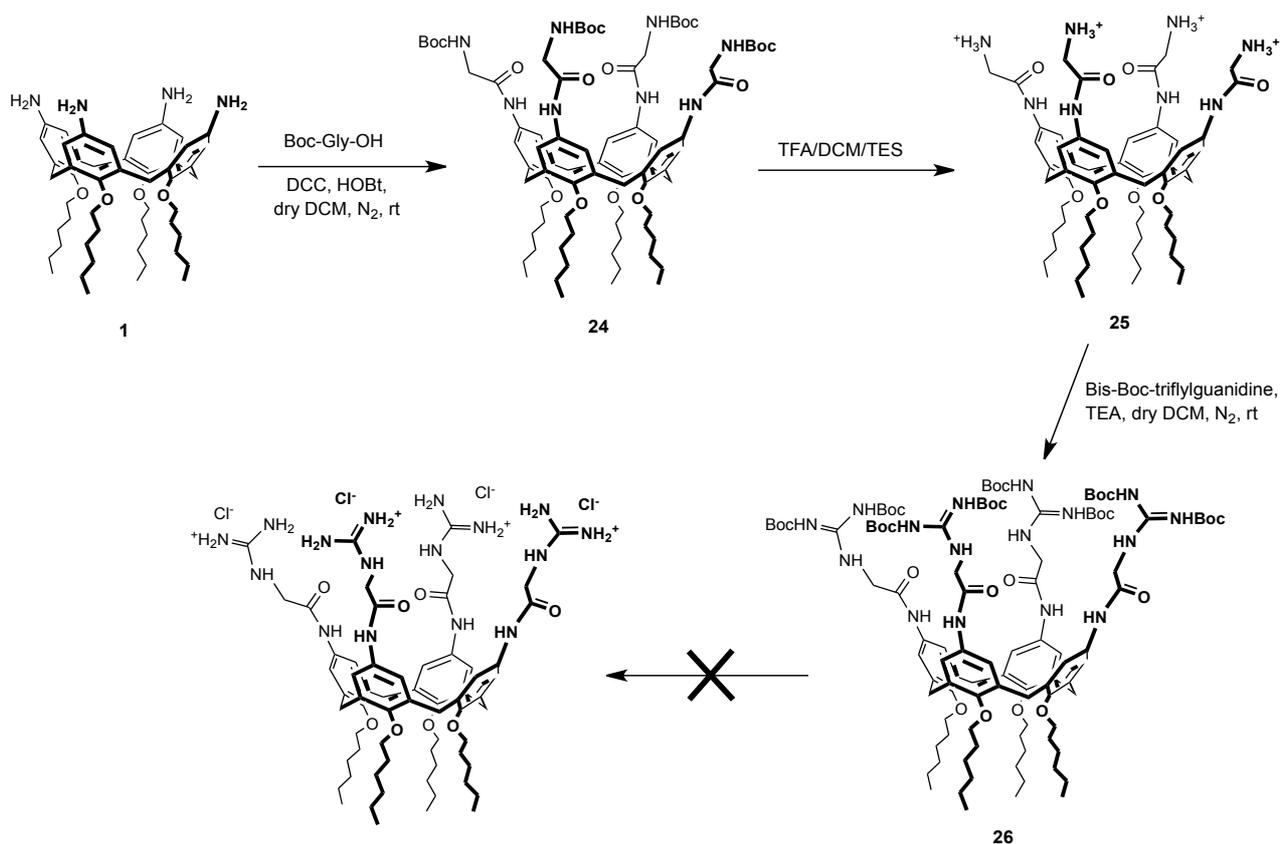
The first synthetic pathway (**Scheme 4**) was addressed to the introduction of only the guanidinium moieties at different distances from the macrocyclic cavity exploiting the amino terminal group of amino acids not belonging to the series of the  $\alpha$ -amino acids and lacking a side chain.

The functionalization was performed by coupling reactions between the upper rim tetraamino-calix[4]arene **1** and Boc protected  $\gamma$ -amino-butiric and  $\delta$ -amino-valeric acids to give intermediates **20a** and **20b**, respectively. In both cases HBTU (O-benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate) was used as coupling agent. Subsequent removal of the amino protecting groups (compounds **21a,b**), reaction with bis-boc-triflylguanidine (compounds **22a,b**) in presence of triethylamine, and final deprotection, again from Boc groups by reaction with TFA and TES (Triethylsilane), gave the guanidinylated compounds **23a** and **23b** (**Scheme 4**).



**Scheme 4:** synthesis of guanidinylated-calixarenes **23a** and **23b**.

We tried to carry out the same procedure also to build the guanidinium group on the natural amino acid glycine (**Scheme 5**), in order to place the cationic head closer to the cavity and investigate the importance of the spacer in this class of ligands.

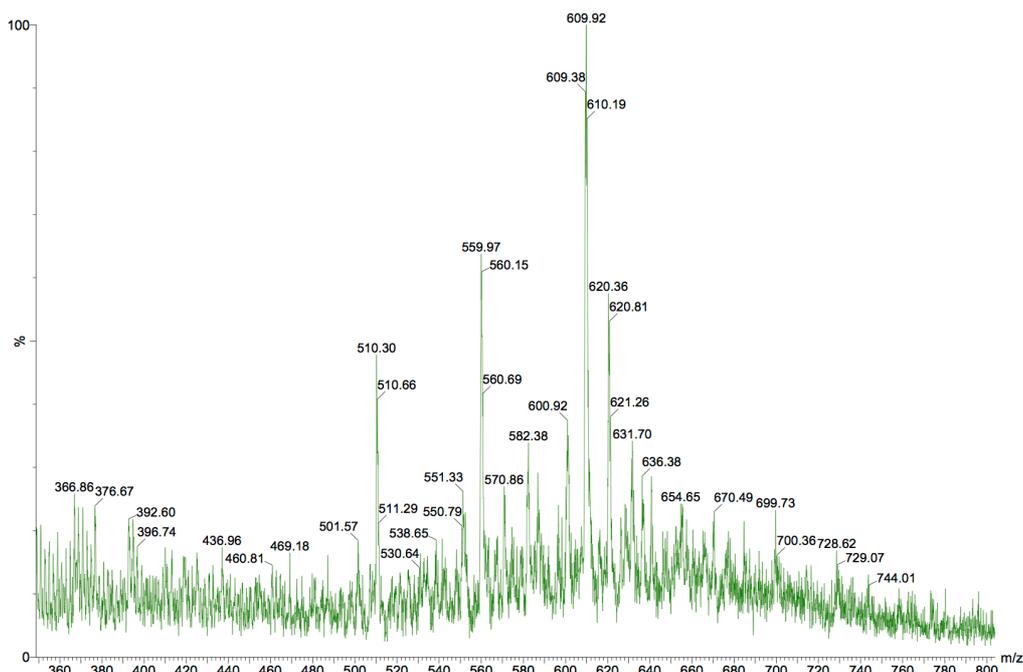


**Scheme 5:** guanidinio-construction on glycine units.

Unfortunately, during the final deprotection step, although attempted in many different conditions, such as TFA 5% in DCM at 0°C or HCl 10% in dioxane, the cleavage of the amide bond between the amino acid and the calixarene occurred as evidenced following the reaction progress by ESI-MS. In the spectra (**Fig 3.2**) in fact it is possible to observe, besides the peak at  $m/z$  609.9, relative to the desired product as  $[M + 2H]^{2+}$ , other ions at  $m/z$  560.0 and 510.3 are present, corresponding to the derivatives lacking one and two guanidylated side chains, respectively.

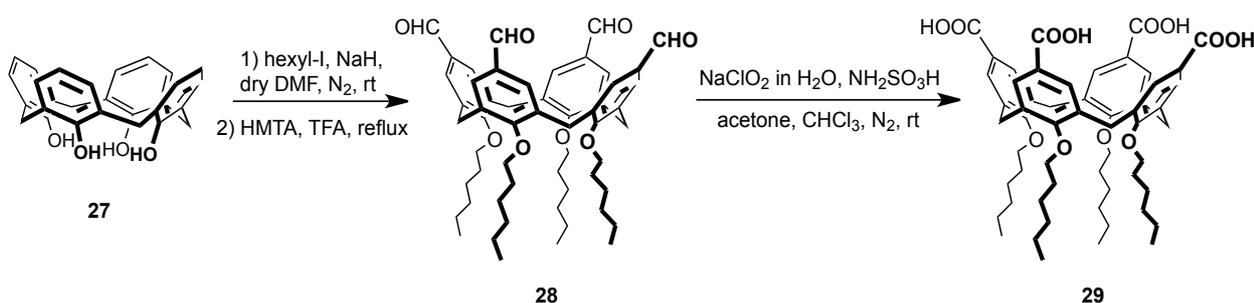
Probably a “creatine to creatinine”-type cyclization takes place leading to the amide cleavage, perhaps also favoured by the good “leaving group” nature of the aniline (calixarene) moiety.

Unfortunately this cleavage takes place already before than the Boc-removal was completed, thus preventing the possibility to obtain the desired product even shortening the reaction times.



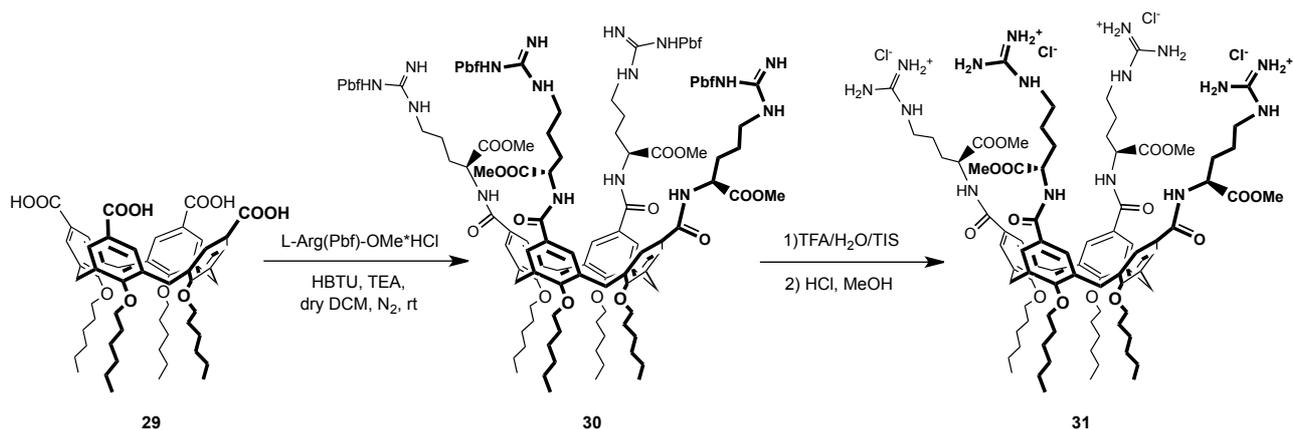
**Fig 3.2** ESI-MS spectrum of the products obtained from the deprotection step of **26**.

As mentioned above, an alternative strategy to overcome the basicity of the  $\alpha$ -amino groups in the vector structure has been to involve them in amide bonds. Then we initially decided to link the arginine units through their amine units to a tetracarboxylic acid derivative of calix[4]arene (**29**). This was obtained (**Scheme 6**) starting from the native tetrahydroxycalix[4]arene **27** subsequently alkylated and formylated to **28**. This was then oxidized to the corresponding tetracarboxylic acid **29**.



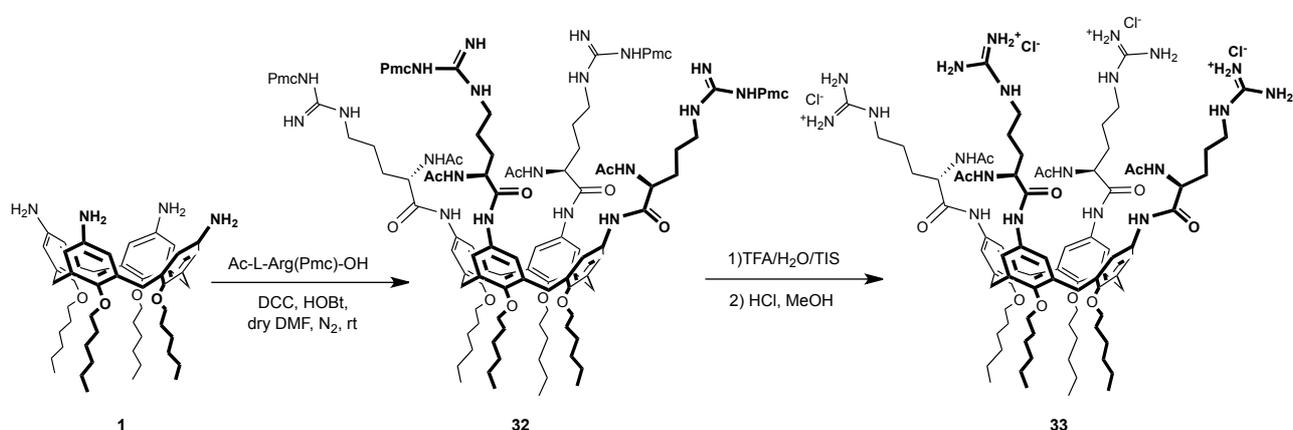
**Scheme 6:** synthesis of calixarene **29**.

The coupling reaction (**Scheme 7**) was carried out with L-Arg(Pbf)-OMe-HCl, in presence of HBTU as coupling agent and an excess of TEA (triethylamine). The final deprotection step gave the desired product **31** in quantitative yield.



**Scheme 7:** synthesis of the N-linked calixarene **31**.

In alternative, maintaining the same connection used in **3** between amino acid and calixarene scaffold, we transformed the  $\alpha$ -amino groups in N-acetyl-amides. The designed compound **33** was synthesized through the reaction sequence described in **Scheme 8**. We directly used Ac-L-Arg(Pmc)-OH after some unsuccessful attempts to introduce the acetyl group on arginine units already linked to the calixarene scaffold and still protected on guanidine group.

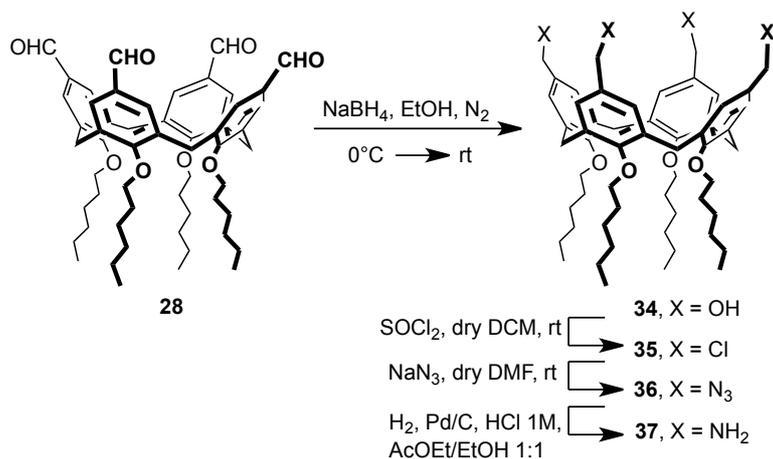


**Scheme 8:** synthesis of calixarene **33**.

As described in Chapter 2, the upper rim arginino-calix[4]arene **3** resulted a very efficient gene delivery system, but its synthesis involves a rather inefficient coupling step that reduces the overall yield and makes preparation of **3** significantly more costly. Therefore, we decided to explore the possibility to introduce small spacers between the cavity and the

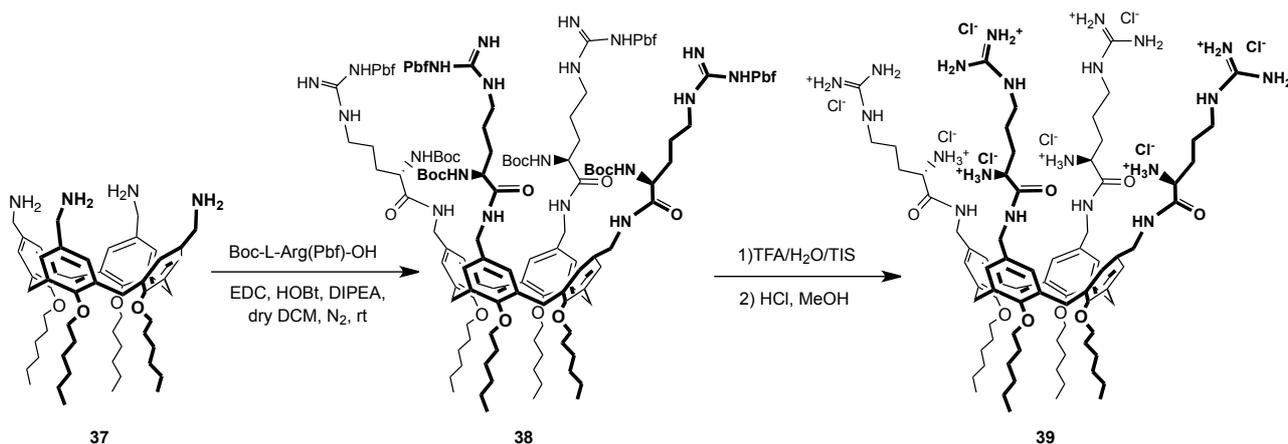
arginine units, with the aim of improving the synthesis but maintaining the very good biological properties.

First, we tried to increase the reactivity of the calixarene in the conjugation reaction replacing the aromatic amines of derivative **1** (Cap. 2) with more nucleophilic benzyl amines and therefore prepared the tetraaminomethyl calixarene **37** (Scheme 9).



**Scheme 9:** synthesis of calixarene **37** with aminomethyl groups.

The synthetic pathway included the reduction of the previously obtained tetraformyl-calixarene **28** in presence of  $\text{NaBH}_4$ , two subsequent reactions with  $\text{SOCl}_2$  and  $\text{NaN}_3$ , respectively, followed by the final hydrogenation of azide groups to afford the desired product **37**.

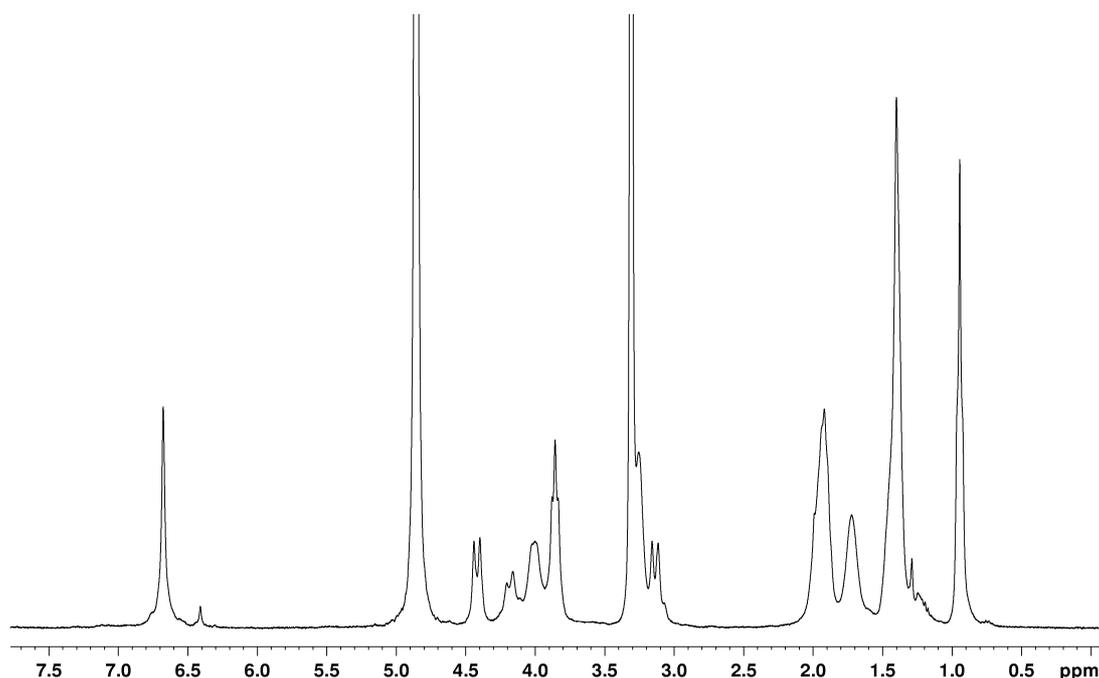


**Scheme 10:** synthesis of calixarene **39**.

The coupling reaction between **37** and Boc-L-Arg(Pbf)-OH was carried out in presence of EDC and HOBt. Unexpectedly, this step was slow and needed several subsequent additions of amino acid and coupling reagents. Moreover the purification by chromatography of the resulting crude was difficult and some solubility problems affected the yield (25%) that at the end resulted lower than that obtained for the synthesis of **3**.

Then deprotection step proceeded in TFA solution (95%), in presence of TIS (triisopropylsilane) as carbocation scavenger, and after the treatment with diluted HCl the final pure product **39** was obtained in quantitative yield.

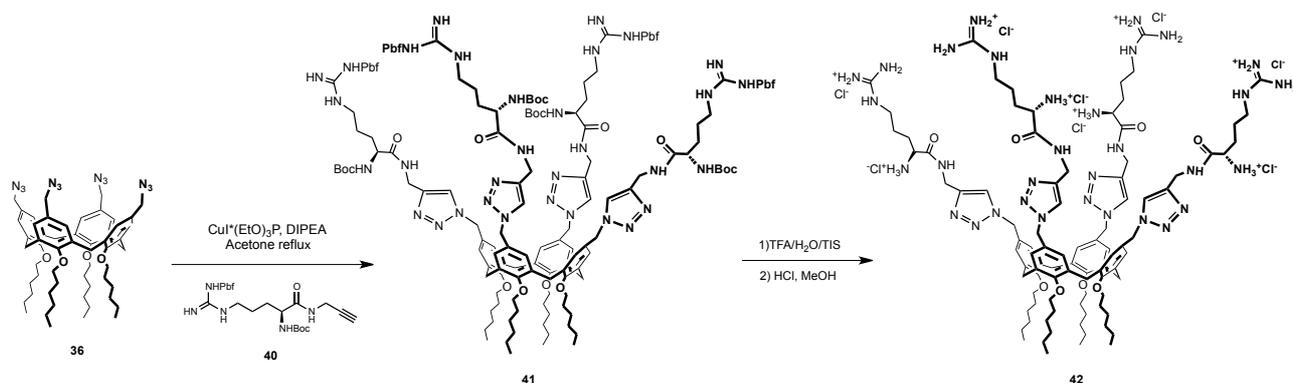
In the NMR spectrum of this compound (**Fig. 3.3**), it can be noted that, differently from **3** (e.g **Fig 2.11** in chapter 2), all the aromatic protons resonate at the same frequency giving rise to a single signal. The presence in fact of methylene spacers between the amide units and the calixarene scaffold exclude the possibility of resonance between the nitrogen atom and aromatic ring and, moreover, also increases the distance between the aromatic protons and the chiral center of the arginine, being both cause of the splitting in two signals for the aromatic protons of **3**



**Fig 3.3**  $^1\text{H-NMR}$  spectrum (MeOD, 400 MHz) of **39**.

The second synthetic pathway was, on the contrary, based on the use of a completely different mode of linkage. We decided in fact to exploit one of the most widely used click

chemistry reaction,<sup>3</sup> the Copper(I)-catalyzed Azide-Alkyne Cycloaddition (CuAAC).<sup>4</sup> We identified as useful reagent the tetraazido intermediate **36** of the previous procedure and prepared the propargylamide of Boc-L-Arg(Pbf)-OH (**40**) that, incorporating a terminal carbon-carbon triple bond, behaves as ideal partner for the CuAAC. The reaction between the two compounds **36** and **40** (**Scheme 11**) successfully produced derivative **41** with triazole rings as spacers.



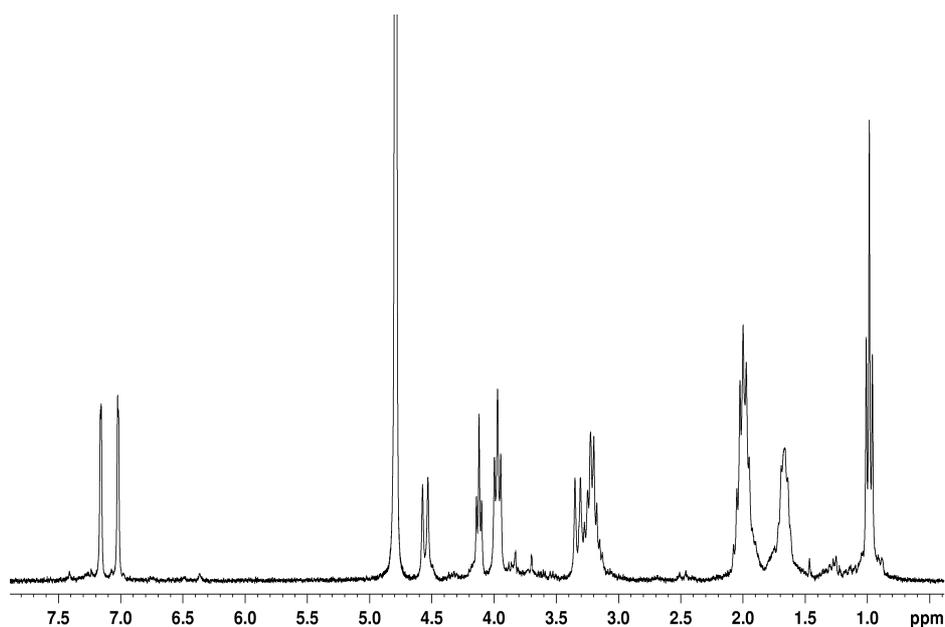
**Scheme 11:** synthesis of calixarene **42**.

Deprotection step was carried out in standard conditions (TFA/TIS/ $\text{H}_2\text{O}$ ) and furnished the target calixarene **42** in quantitative yield.

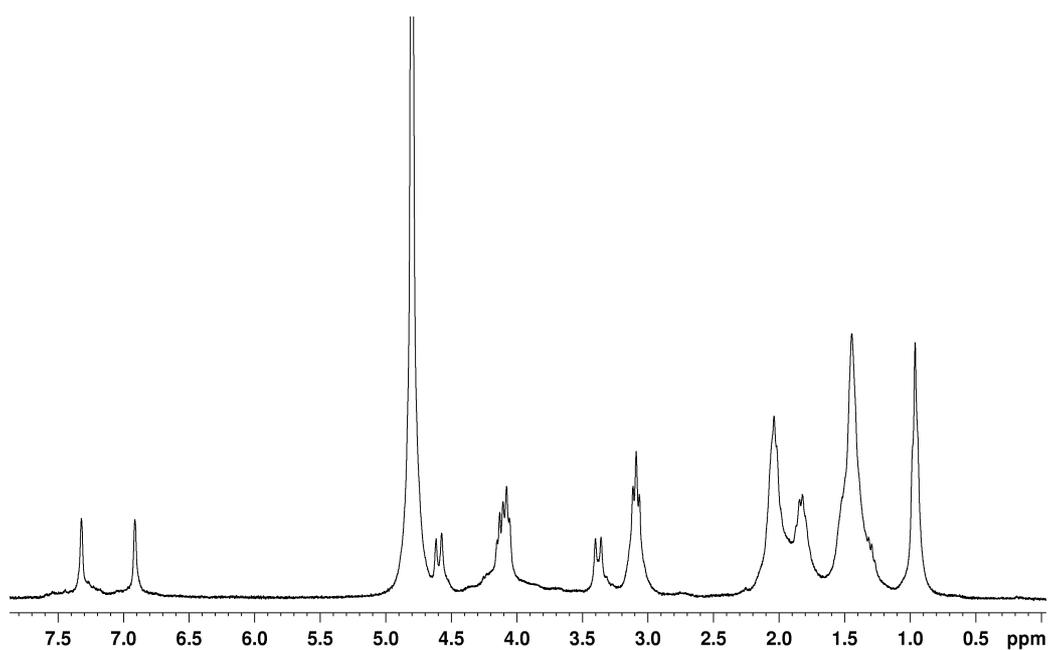
### 3.2.2 Aggregation properties in water

Most of the synthesized compounds were found to be soluble in water at 1mM concentration, except in case of the D-arginine containing calixarene **13**, which resulted poorly soluble, like the reference macrocycle **3** and the modified derivatives **31** and **39** having the  $\alpha$ -amine involved in the amide bond and the methylene spacer between arginine and calixarene scaffold, respectively.

Calixarenes **16** and **19**, with propyl chains at the lower rim and ornithine units at the upper, respectively, showed spectra in  $\text{D}_2\text{O}$  with sharp signals at room temperature (**Figures 3.4 and 3.5**).

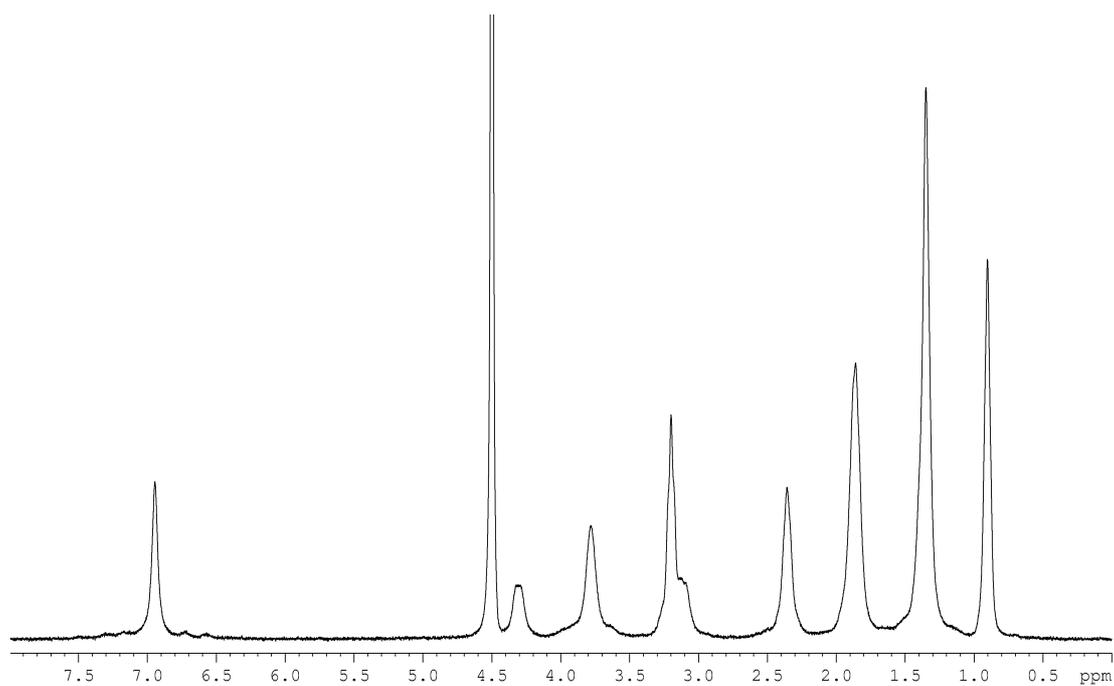


**Fig 3.4**  $^1\text{H}$  NMR spectrum ( $\text{D}_2\text{O}$ , 300 MHz, 298 K) of compound **16**.

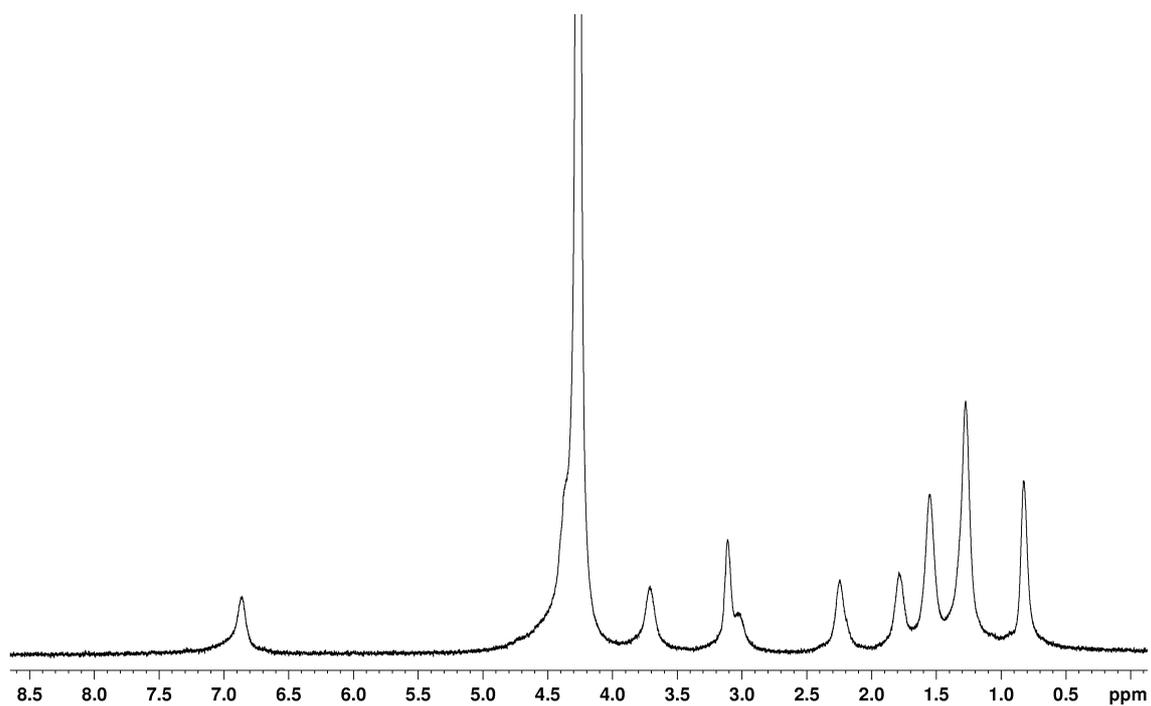


**Fig 3.5**  $^1\text{H}$  NMR spectrum ( $\text{D}_2\text{O}$ , 300 MHz, 298 K) of compound **19**.

Different behaviour was observed for the two derivatives without  $\alpha$ -amine groups **23a** and **23b** (Figures 3.6 and 3.7), for which, although well soluble, the NMR signals remained rather broad even at 70 °C, suggesting aggregation phenomena.



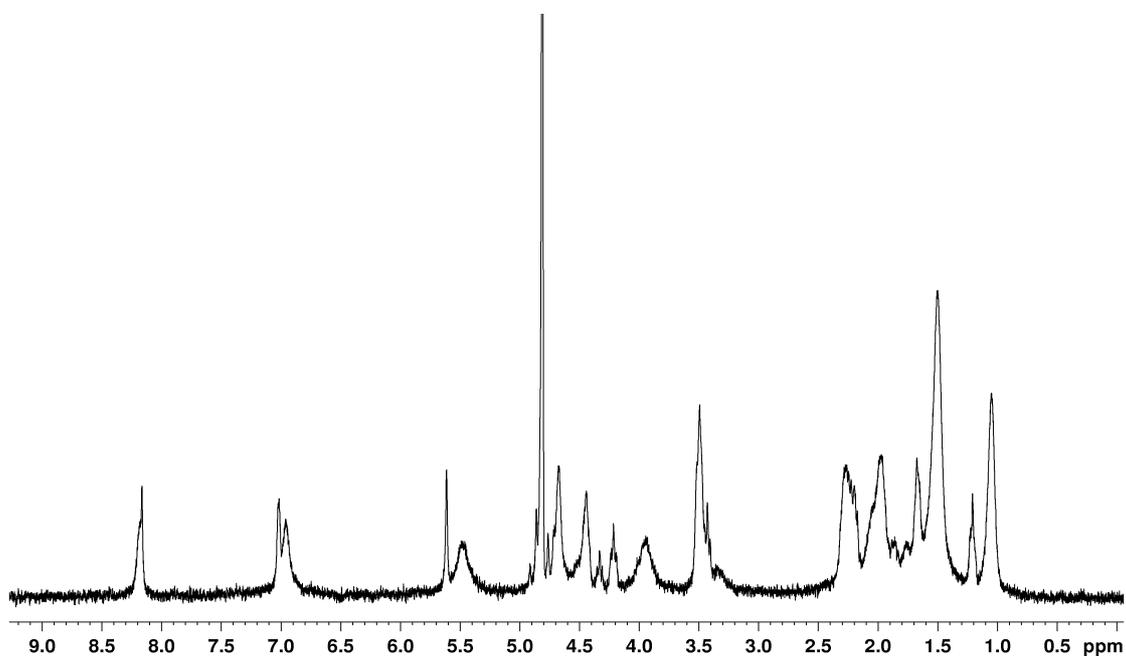
**Fig 3.6**  $^1\text{H}$  NMR spectrum ( $\text{D}_2\text{O}$ , 300 MHz, 323 K) of compound **23a**.



**Fig 3.7**  $^1\text{H}$  NMR spectrum ( $\text{D}_2\text{O}$ , 300 MHz, 353 K) of compound **23b**.

For calixarene **42** with triazole rings as spacers, the  $^1\text{H}$  NMR signals at room temperature were substantially undetectable from the baseline of the spectrum. At 50 °C on the

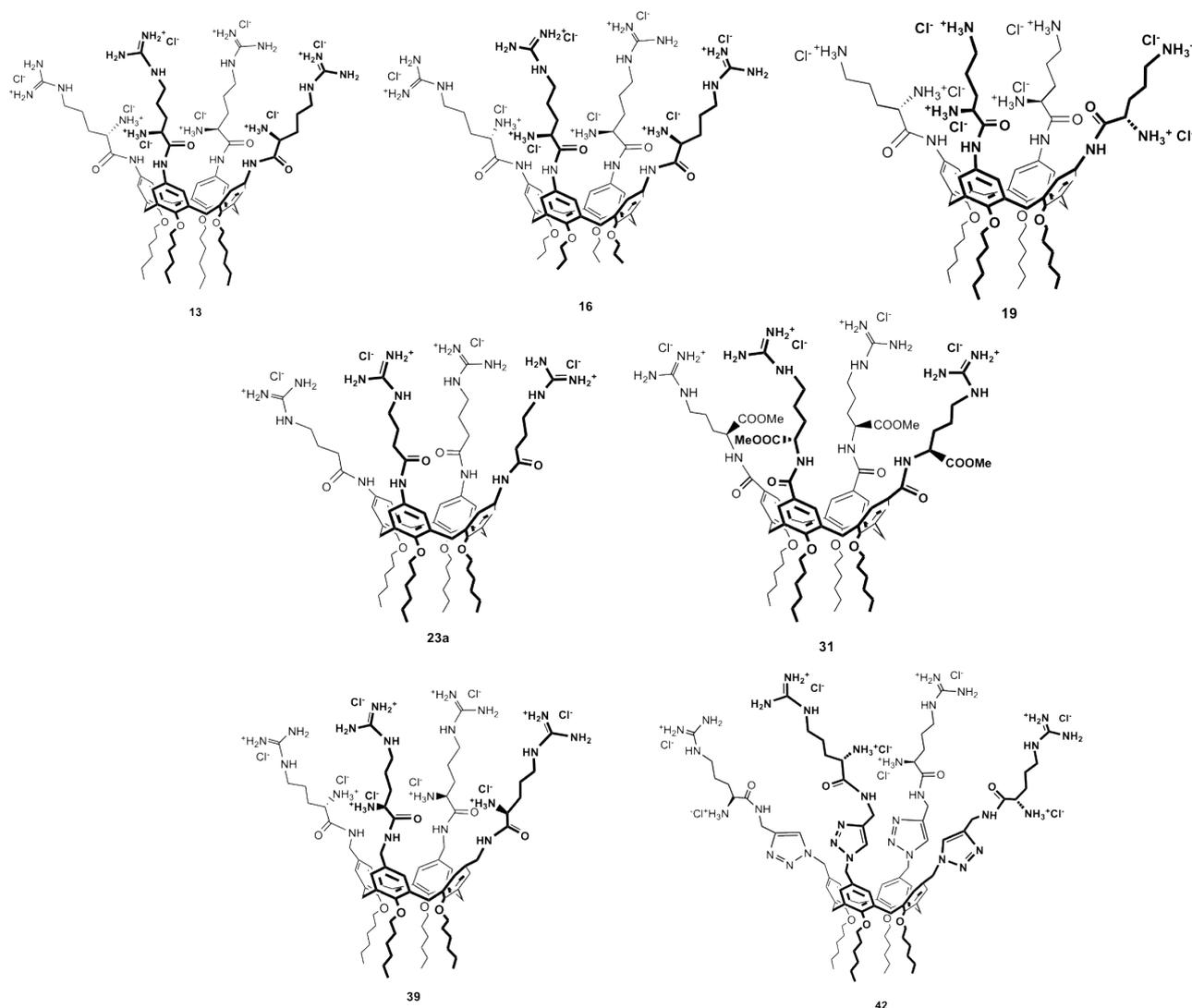
contrary it showed a spectrum with rather sharp signals (**Fig 3.8**), but also more complicate than the expected for this symmetrically functionalized molecule. It is possible that more species are present in solution, in equilibrium and slow exchange on the NMR time scale.



**Fig 3.8**  $^1\text{H}$  NMR spectrum ( $\text{D}_2\text{O}$ , 300 MHz, 323 K) of compound **42**.

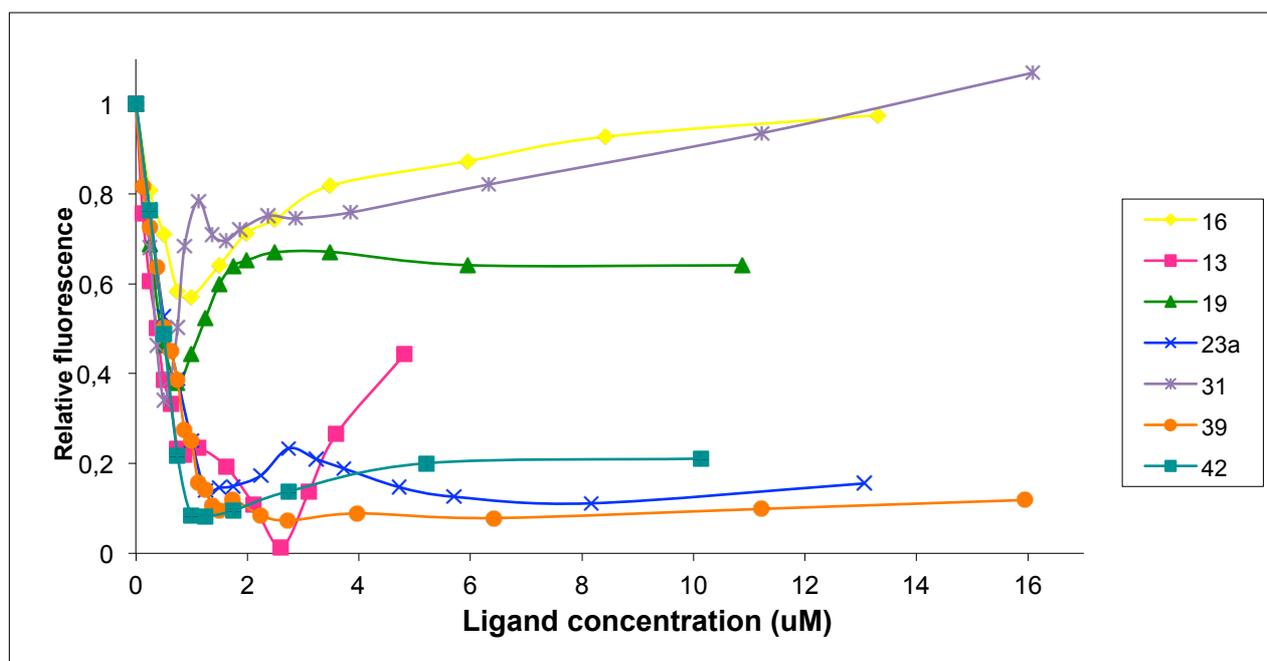
### 3.2.3 DNA binding and cell transfection studies

The ability of these new ligands (**Fig 3.9**) to bind plasmid pEGFP-c1 was preliminarily studied through Ethidium Bromide Displacement assays.



**Fig 3.9** Some structural formulas of some calix[4]arenes studied in this chapter.

All titrations showed an evident interaction between calixarenes and DNA (**Fig 3.10**). After the first additions to the EB/DNA mixture, a drastic fluorescence quenching occurred for all ligands. However, the curve trend was not the same for each of them. While for derivatives **23a**, **39** and **42** there was a similar rapid decrease of the relative fluorescence almost till to zero with a subsequent almost asymptotic trend, with ligands **16**, **19** and **31** the inversion of curve slope was observed as previously seen for the upper rim arginino-calixarene **3** and lysino-calixarene **5**.

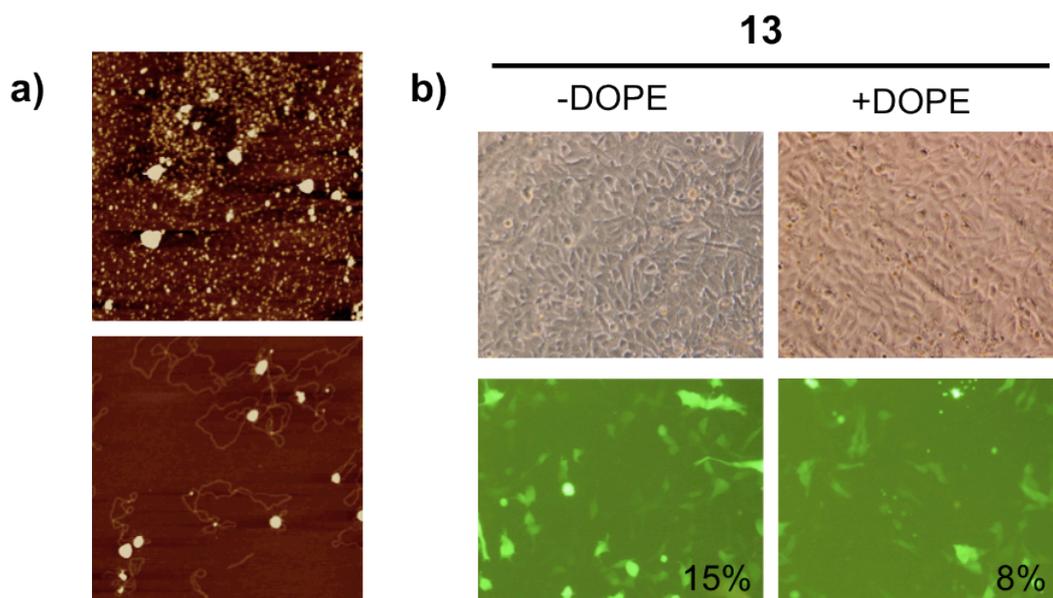


**Fig 3.10** Ethidium Bromide Displacement Assays. Relative fluorescence vs ligand concentration. Fluorescence studies (excitation at 530 nm, emission at 600 nm) were performed collecting the emission spectra of buffer solutions (4 mM HEPES, 10 mM NaCl) of 50 mM ethidium bromide (relative fluorescence = 0), mixture of 0.5 nM plasmid DNA (pEGFP-C1) and 50 mM ethidium bromide (relative fluorescence = 1) and after addition of increasing amounts of ligand to the DNA/ethidium bromide mixture.

The curve of compound **13**, ligand functionalized with D-arginine, is the only one that regularly reaches the value of zero at a concentration of ligand around 2.5  $\mu\text{M}$  with a complete displacement of EB, before a marked increasing trend occurred. On the whole, however, we were not able to find correlations between the results of these titration experiments and the structural features of the studied molecules.

The interaction with DNA was then studied by AFM.

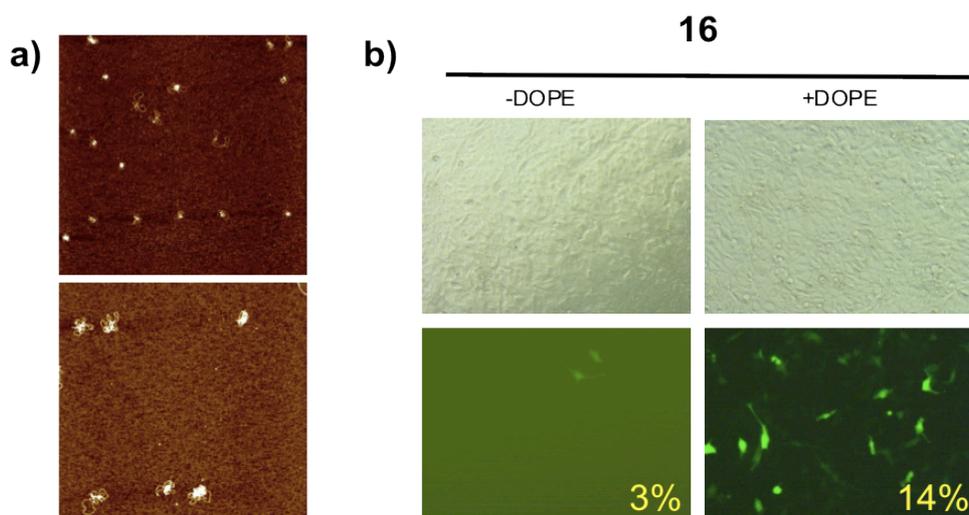
For D-argininocalixarene **13** at 2 and 1  $\mu\text{M}$ , analogously to its enantiomer **3**, nanometric condensates and partially relaxed aggregates, respectively, were observed (**Fig 3.11 a**).



**Fig 3.11 a)** AFM image showing the effects induced on plasmid pEGFP-C1 incubated with **13** 1  $\mu\text{M}$  ( $2 \times 2 \mu\text{m}$ ); **b)** Transfection experiments performed with 1nM plasmid DNA and calixarene **13** on RD-4 cells: phase contrast images and fluorescence microscopy images of the transfected cells.

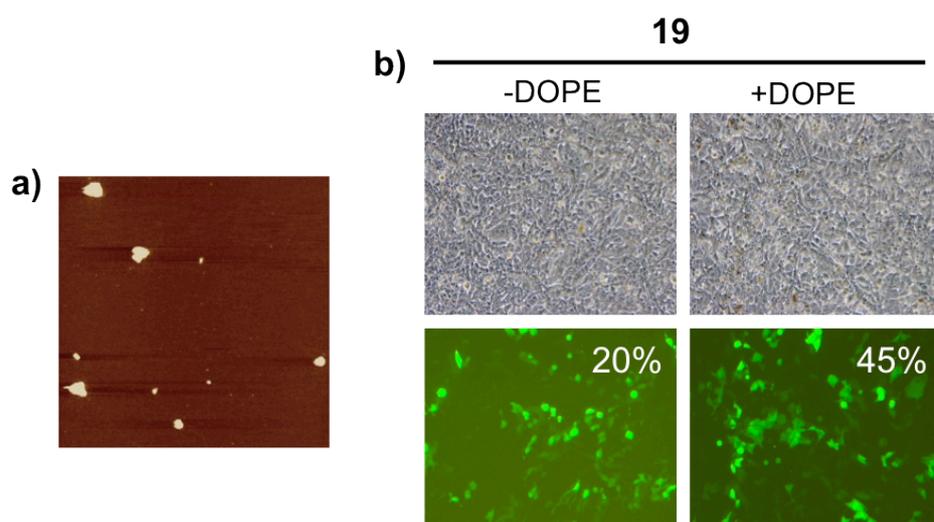
Surprisingly, when tested as gene delivery vector for pEGFP-C1 on RD-4 cells, this ligand indeed resulted much less efficient than **3** (**Fig 3.11 b**). It was able to give only 8% of cell transfection in presence of DOPE, and 15% in its absence, suggesting that stereochemistry of the amino acid units plays an important role in one or more steps of the transfection process.

Compound **16** compacted almost completely pDNA in globular aggregates at concentration 2  $\mu\text{M}$ , but did not give alone cell transfection (**Fig 3.12**). Only in presence of DOPE it reached 14% of transfection. In this case, presumably, the propyl chains do not bring an appropriate apolar contribution to molecule **16**. The consequence is that the ligand would not be amphiphilic enough to originate the hydrophobic interactions needed for an efficient delivery through cell membranes. Accordingly, the  $^1\text{H-NMR}$  experiments showed that this compound has not self-aggregation propensity in water. The helper lipid, instead, seemed to overcompensate this lack of lipophilicity allowing a little transfection activity.



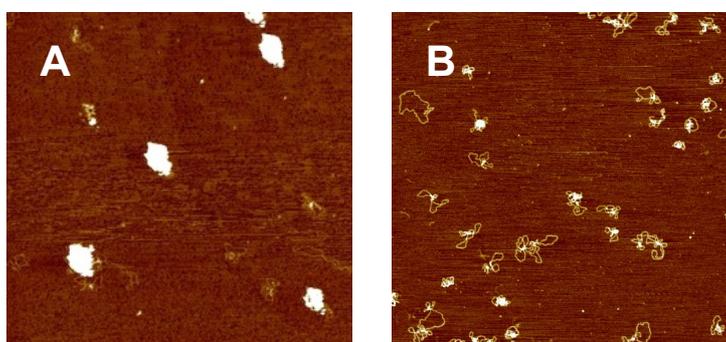
**Fig 3.12 a)** AFM images showing the effects induced on plasmid pEGFP-C1 incubated with **16** 2  $\mu\text{M}$  (top: 4 $\times$ 4  $\mu\text{m}$ ; bottom: 2 $\times$ 2  $\mu\text{m}$ ); **b)** Transfection experiments performed with 1nM plasmid DNA and calixarene **16** on RD-4 cells: phase contrast images and fluorescence microscopy images of the transfected cells.

The behavior of calixarene **19** containing the ornithine units was very similar to the lysine-based vector **5**, both in DNA binding and transfection properties (**Fig 3.13**). Although by AFM big, tight condensates were visualized, some others appeared characterized by proper dimensions for the cell membrane crossing. These could in fact justify the observed 20% of transfection efficiency. The formulation of the complex between DNA and **19** with DOPE, like in the case of **5**, increased transfection efficiency up to 45%; this percentage is higher than that of lipofectamine LTX.



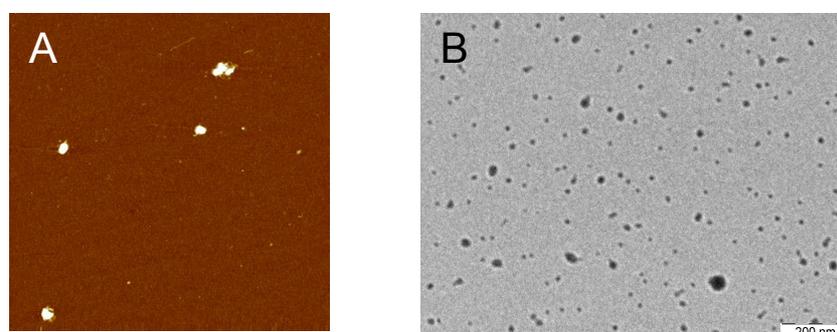
**Fig 3.13 a)** AFM image showing the effects induced on plasmid pEGFP-C1 incubated with **19** 2  $\mu\text{M}$  (5 $\times$ 5  $\mu\text{m}$ ); **b)** Transfection experiments performed with 1nM plasmid DNA and calixarene **19** on RD-4 cells: phase contrast images and fluorescence microscopy images of the transfected cells.

The three derivatives **23a**, **23b** and **33**, all lacking free amine groups corresponding to the  $\alpha$ -amine of the arginine in **3**, were unable to transfect Rhabdomyosarcoma RD-4 cells; only calixarene **31**, where the amino groups of the arginines at the upper rim are involved in the amide bond with the calixarene scaffold, showed a maximum of 7% of transfection with adjuvant. These results could be related to their observed behavior in the interaction with pDNA that, although different, can justify poor or completely absent transfection abilities. For example, compound **23b** formed very large aggregates (**Fig 3.14 A**), whereas for compound **31** a tendency to give species involving only a single plasmid was shown but the condensation ability was scarce (**Fig 3.14 B**).



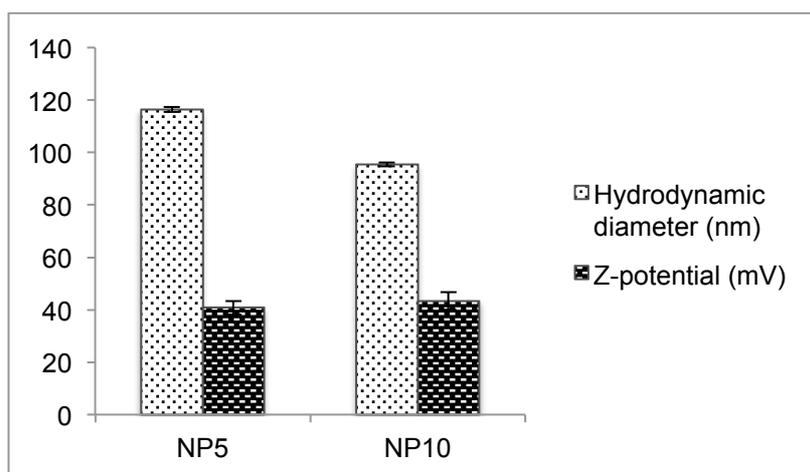
**Fig 3.14** AFM images (4×4  $\mu\text{m}$ ) showing the effects induced on plasmid pEGFP-C1 by A) ligand **23a** 1  $\mu\text{M}$  and B) ligand **31** 2  $\mu\text{M}$ .

The two structural variations at level of the linker did not affect significantly the mode of interaction with pDNA. In both cases microscopy techniques (AFM and TEM) evidenced the formation of globular tight condensates, with a diameter smaller than 100 nm and each consisting of a single filament of ds-pDNA (**Fig 3.15**).



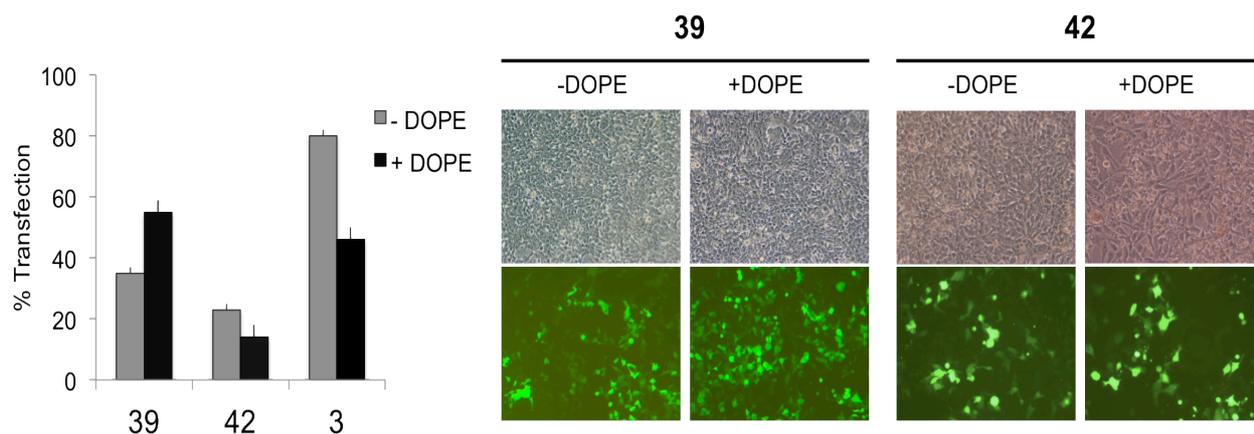
**Fig 3.15** A) AFM image (2×2  $\mu\text{m}$ ) showing the effects induced on plasmid pEGFP-C1 by ligand **39** 2  $\mu\text{M}$  and B) TEM image of calixarene **42**:pDNA.

Unfortunately, for compound **42**, analysed by TEM, it was not possible to observe the internal microstructure of lipoplexes, as on the contrary done with **3**. By DLS, their hydrodynamic diameters and positive Z-potentials were determined for NP $\geq$ 5 used for transfection experiments (**Fig 3.16**). Calixarene **42**:pDNA complexes exhibited slightly larger diameter as compared with those of vector **3**. Additionally, values of Z-potential were found at about 40 mV, lower than those obtained previously for **3**. All these results suggest that these complexes could not have well-defined compact packing.



**Fig 3.16** Values of hydrodynamic diameter and Z-potential for compound **42** at two different N/P.

Experiments, performed using plasmid pEGFP-C1 in RD-4 cells, demonstrated the superior abilities of the vector **3** as transfection system also versus these two L-arginine containing ligands (**Fig 3.17**). Even a little modification, like the introduction of a methylene bridge between the macrocyclic cavity and the arginine units in the case of **39**, significantly affected the transfection capabilities reducing the percentage of the transfected cells from 80% found for **3** to 35%. Calixarene **39** however showed good delivery properties in presence of DOPE transfecting 55% of treated cells. This percentage was even higher than that achieved by **3** in the presence of the helper lipid, as well as of that of LTX and PEI.



**Fig 3.17** Transfection experiments performed with 1nM pEGFP-C1 plasmid, compounds **39** and **42**, alone and in presence of DOPE (1/2 molar ratio, 10/20  $\mu$ M), and lipofectamine LTX to RD-4 cells. Left image: in vitro transfection efficiency as percentage of transfected cells upon treatment with the two ligands compared to **3**, alone and in presence of DOPE (1/2 molar ratio, 10/20  $\mu$ M). Right images: phase contrast images and fluorescence microscopy images of the transfected cells.

### 3.3 Conclusions

Various structural modifications of **3** were carried out and studied. All the synthesized compounds resulted worse vectors compared to the lead compound **3**; despite so, important informations to understand the structural requirements necessary to assure transfection efficiency to this class of new macrocyclic amphiphilic vectors were obtained.

The stereochemistry change of arginine units (calixarene **13**) reduced drastically the delivery properties, although the toxicity did not increase compared to **3**. We could hypothesize, from the collected data, that one or more events during the whole transfection process are strongly influenced by stereochemical requirements with an undoubted preference for the L-enantiomer of arginine. These results also would support the idea that specific recognition processes take place involving the amino acid units present in the ligand structure.

Other modifications resulting in compounds **16**, **39** and **42** probably determined notable changes in the lipophilicity-hydrophilicity ratio respect to vector **3**. In these derivatives the lack of a properly balanced amphiphilic character caused worse biological properties, even if apparently did not generate marked differences in DNA binding, all giving rise to small condensates of pDNA. Therefore, this fact evidenced that condensation of plasmid in nanometric globular aggregates is a necessary but not sufficient preliminary condition for efficient gene delivery.

All compounds without free  $\alpha$ -amines showed an almost total inefficiency of transfection. These data evidenced the great importance of this moiety in determining the transfection properties, that in compound **3** could be related at least in part to the proton sponge effect to which these basic centers can contribute. However, we could not draw conclusions on this aspects but only make hypotheses because the compounds at issue demonstrated inability in DNA condensation, then evidencing inefficiency also in steps preceding cell entry and in particular endosomal escape where indeed the proton sponge is involved.

The results obtained with the ornithine-bearing vector confirmed what we already found with the lysinocalixarene **5**. The replacement of the guanidinium group with ammonium determines a drastic decrease of transfection efficiency with the need of using DOPE to reach transfection percentages better or at least comparable with one among LTX and PEI, but always lower than those of **3** alone. This decreased translocation ability of lysine and ornithine compared to arginine also supports the observation that, while charge is necessary, it is not sufficient, as both the guanidinium and the ammonium group have a single positive charge. Obviously, the degree of protonation, the presence of protonable groups with different pKa and the phosphate binding ability are other important factors in conferring to the arginine cluster better transfection efficiency than the lysine/ornithine analogs.

In conclusion, in this small calixarene-based library of vectors, if the lack of amines as supporting groups to the guanidinium moieties is highly detrimental, the presence of ammonium instead of guanidinium is definitely unfavourable when the ligand is used alone but in the presence of DOPE a remarkable transfection activity is in any case observed making the vectors able to compete in *in vitro* experiments with commercially available and largely used transfectants such as LTX lipofectamine and PEI.

### 3.4 Experimental section

**General Information.** All moisture sensitive reactions were carried out under nitrogen atmosphere, using previously oven-dried glassware. All dry solvents were prepared according to standard procedures, distilled before use and stored over 3 or 4 Å molecular sieves. Most of the solvents and reagents were obtained from commercial sources and used without further purification. Analytical TLC were performed using prepared plates of silica gel (Merck 60 F-254 on aluminum) and then, according to the functional groups

present on the molecules, revealed with UV light or using staining reagents: FeCl<sub>3</sub> (1% in H<sub>2</sub>O/CH<sub>3</sub>OH 1:1), ninhydrin (5% in EtOH), basic solution of KMnO<sub>4</sub> (0.75% in H<sub>2</sub>O). Reverse phase TLC were performed by using silica gel 60 RP-18 F-254 on aluminum sheets. Merck silica gel 60 (70-230 mesh) was used for flash chromatography and for preparative TLC plates. <sup>1</sup>H NMR and <sup>13</sup>C-NMR spectra were recorded on Bruker AV300 and Bruker AV400 spectrometers (observation of <sup>1</sup>H nucleus at 300 MHz and 400 MHz respectively, and of <sup>13</sup>C nucleus at 75 MHz and 100 MHz respectively). All chemical shifts are reported in part per million (ppm) using the residual peak of the deuterated solvent, which values are referred to tetramethylsilane (TMS, δ<sub>TMS</sub> = 0), as internal standard. All <sup>13</sup>C NMR spectra were performed with proton decoupling. For <sup>1</sup>H NMR spectra recorded in D<sub>2</sub>O at values higher than the room temperature, the correction of chemical shifts was performed using the expression δ = 5.060 - 0.0122 × T (°C) + (2.11 × 10<sup>-5</sup>) × T<sup>2</sup> (°C) to determine the resonance frequency of water protons (Gottlieb, H. E., Kotlyar, V., and Nudelman, A. *J. Org. Chem.* **1997**, *62*, 7512–7515). Electrospray ionization (ESI) mass analyses were performed with a Waters spectrometer. Melting points were determined on an Electrothermal apparatus in closed capillaries. Optical rotations were measured at 20°C in 1-dm tubes on a Perkin-Elmer 341 polarimeter.

### **Synthesis of 5,11,17,23-Tetrakis[(Boc-D-Arg(Pbf))amino]-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (12)**

The compound **12** was synthesized according to the procedure described in Chapter 2 for compound **2**. The pure product was isolated by flash column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 96:4) as white solid in 35% yield.

[α]<sub>D</sub>: +45° (c = 7.36 · 10<sup>-4</sup>, CDCl<sub>3</sub>/CH<sub>3</sub>OH: 1/1).

<sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>/MeOD: 1/1) δ 7.10 (bs, 4H, ArH), 6.62 (bs, 4H, ArH), 4.45 (d, J = 12.8 Hz, 4H, ArCH<sub>ax</sub>Ar), 4.12 (bs, 4H, COCHNH), 3.88 (bs, 8H, OCH<sub>2</sub>), 3.14-3.11 (m, 12H, CH<sub>2</sub>NH and ArCH<sub>eq</sub>Ar), 2.97 (s, 8H, CH<sub>2</sub> Pbf), 2.57 (s, 12H, CH<sub>3</sub> Pbf), 2.51 (s, 12H, CH<sub>3</sub> Pbf), 2.07 (s, 12H, CH<sub>3</sub> Pbf), 1.93 (bs, 8H, OCH<sub>2</sub>CH<sub>2</sub>), 1.73 (bs, 8H, COCHCH<sub>2</sub>), 1.60 (bs, 8H, COCHCH<sub>2</sub>CH<sub>2</sub>), 1.45-1.27 (m, 84H, C(CH<sub>3</sub>)<sub>3</sub> Boc, C(CH<sub>3</sub>)<sub>2</sub> Pbf, O(CH<sub>2</sub>)<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 0.95 (m, 12H, CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (m/z): [M + 2Na]<sup>2+</sup> calcd for C<sub>148</sub>H<sub>220</sub>N<sub>20</sub>O<sub>28</sub>S<sub>4</sub> 1450.2, found 1451.0.

### **Synthesis of 5,11,17,23-Tetra(D-Arg-amino)-25,26,27,28-tetrakis(*n*-**

### hexyloxy)calix[4]arene, octahydrochloride (13)

The compound **12** was deprotected according to the general procedure described in chapter 2. The pure product was isolated as white solid in quantitative yield.

$[\alpha]_D$ :  $-7^\circ$  ( $c=0.001$ ,  $\text{CH}_3\text{OH}$ ).

$^1\text{H-NMR}$  (300 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  7.17 (s, 4H, ArH), 6.97 (s, 4H, ArH), 4.49 (d,  $J = 12.3$  Hz, 4H,  $\text{ArCH}_{\text{ax}}\text{Ar}$ ), 4.08 (bs, 4H,  $\text{COCHNH}$ ), 3.93 (t,  $J = 7.2$  Hz, 8H,  $\text{OCH}_2$ ), 3.21-3.17 (m, 12H,  $\text{ArCH}_{\text{eq}}\text{Ar}$  e  $\text{CH}_2\text{NH}$ ), 2.02 (bs, 16H,  $\text{OCH}_2\text{CH}_2$  and  $\text{COCHCH}_2$ ), 1.78 (bs, 8H,  $\text{COCHCH}_2\text{CH}_2$ ), 1.45 (m, 24H,  $\text{O}(\text{CH}_2)_2\text{CH}_2\text{CH}_2\text{CH}_2$ ), 0.99 (t,  $J = 6.3$  Hz, 12H,  $\text{CH}_2\text{CH}_3$ ).

ESI-MS ( $m/z$ ):  $[\text{M} + 3\text{H-8HCl}]^{3+}$  calcd for  $\text{C}_{76}\text{H}_{132}\text{N}_{20}\text{O}_8\text{Cl}_8$  482.7, found 482.8,  $[\text{M} + 2\text{H-8HCl}]^{2+}$  calcd 723.5, found 723.5,

The product shows the same spectroscopic properties reported in Ref. <sup>5</sup>

### Synthesis of 5,11,17,23-Tetramino-25,26,27,28-tetrakis(*n*-propyloxy)calix[4]arene (14)

It was synthesized according to a literature procedure.<sup>2</sup>

### Synthesis of 5,11,17,23-Tetrakis[(Boc-L-Arg(Pbf))amino]-25,26,27,28-tetrakis(*n*-propyloxy)calix[4]arene (15)

The compound **15** was synthesized according to the procedure described in chapter 2 for compound **3**. The pure product was isolated as white solid in 39% yield.

Mp: 189.0-192.3 °C.  $^1\text{H NMR}$  (300 MHz, MeOD)  $\delta$  7.06 (bs, 4H, ArH), 6.64 (bs, 4H, ArH), 4.46 (d,  $J = 13.2$  Hz, 4H,  $\text{ArCH}_{\text{ax}}\text{Ar}$ ), 4.08 (bs, 4H,  $\text{COCHNH}$ ), 3.86 (bs, 8H,  $\text{OCH}_2$ ), 3.23-3.05 (m, 12H,  $\text{CH}_2\text{NH}$  and  $\text{ArCH}_{\text{eq}}\text{Ar}$ ), 2.97 (s, 8H,  $\text{CH}_2$  Pbf), 2.55 (s, 12H,  $\text{CH}_3$  Pbf), 2.50 (s, 12H,  $\text{CH}_3$  Pbf), 2.04 (s, 12H,  $\text{CH}_3$  Pbf), 2.05-1.85 (m, 8H,  $\text{OCH}_2\text{CH}_2$ ), 1.80-1.26 (m, 76H,  $\text{C}(\text{CH}_3)_3$  Boc,  $\text{COCH}(\text{CH}_2)_2$  and  $\text{C}(\text{CH}_3)_2$  Pbf), 1.01 (bs, 12H,  $\text{CH}_2\text{CH}_3$ ).

$^{13}\text{C NMR}$  (100 MHz, MeOD)  $\delta$  172.8 (C=O), 159.8 (C Ar Pbf), 158.1 (C=N), 157.9 (C=O), 154.7 (C Ar calix), 139.4 (C Ar Pbf), 136.3 (C Ar calix), 134.3 and 133.6 (C Ar Pbf), 133.1 (C Ar calix), 126.0 (C Ar Pbf), 122.3 and 121.7 (C Ar calix), 118.5 (C Ar Pbf), 87.7 ( $\text{C}(\text{CH}_3)_2$  Pbf), 80.8 ( $\text{C}(\text{CH}_3)_3$  Boc), 78.1 ( $\text{OCH}_2$ ), 56.1 ( $\text{COCHNHBOC}$ ), 44.0 ( $\text{CH}_2$  Pbf), 41.6 ( $\text{N}=\text{CNHCH}_2$ ), 32.2 ( $\text{COCHCH}_2$ ), 31.1 ( $\text{ArCH}_2\text{Ar}$ ), 29.0 and 28.8 ( $\text{C}(\text{CH}_3)_2$  and  $\text{C}(\text{CH}_3)_3$ ), 27.0 ( $\text{N}=\text{CNHCH}_2\text{CH}_2$ ), 24.4 ( $\text{OCH}_2\text{CH}_2$ ), 19.7 and 18.5 ( $\text{CH}_3$  Pbf), 12.6 ( $\text{CH}_2\text{CH}_3$ ), 10.8 ( $\text{CH}_3$  Pbf).

ESI-MS ( $m/z$ ):  $[\text{M} + 2\text{H}]^{2+}$  calcd for  $\text{C}_{136}\text{H}_{196}\text{N}_{20}\text{O}_{28}\text{S}_4$  1365.7, found 1366.3.

## Synthesis of 5,11,17,23-Tetra(L-Arg-amino)-25,26,27,28-tetrakis(*n*-propyloxy)calix[4]arene, octahydrochloride (16)

The compound **15** was deprotected according to the general procedure described in chapter 2. The pure product **16** was isolated as white solid in quantitative yield.

Mp: 224-225 °C dec. <sup>1</sup>H NMR (400 MHz, MeOD) δ 7.17 (s, 4H, ArH), 6.96 (s, 4H, ArH), 4.50 (d, *J* = 12.8 Hz, 4H, ArCH<sub>ax</sub>Ar), 4.08 (bs, 4H, COCHNH<sub>3</sub><sup>+</sup>), 3.89 (t, *J* = 7.6 Hz, 8H, OCH<sub>2</sub>), 3.31 (8H, N=CNHCH<sub>2</sub>, determined by COSY), 3.19 (d, *J* = 12.8 Hz, 4H, ArCH<sub>eq</sub>Ar), 2.15-1.88 (m, 16H, OCH<sub>2</sub>CH<sub>2</sub> and COCHCH<sub>2</sub>), 1.78 (bs, 8H, N=CNHCH<sub>2</sub>CH<sub>2</sub>), 1.01 (bs, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>1</sup>H NMR (300 MHz, D<sub>2</sub>O, c = 1 mM) 7.16 (d, *J* = 2.1 Hz, 4H, ArH), 7.02 (d, *J* = 2.1 Hz, 4H, ArH), 4.55 (d, *J* = 13.2 Hz, 4H, ArCH<sub>ax</sub>Ar), 4.12 (t, *J* = 6 Hz, 4H, COCHNH), 3.97 (t, *J* = 7.8 Hz, 8H, OCH<sub>2</sub>), 3.33 (d, *J* = 13.2 Hz, 4H, ArCH<sub>eq</sub>Ar), 3.28-3.11 (m, 8H, N=CNHCH<sub>2</sub>), 2.09-1.82 (m, 16H, OCH<sub>2</sub>CH<sub>2</sub> and COCHCH<sub>2</sub>), 1.80-1.26 (m, 8H, COCHCH<sub>2</sub>CH<sub>2</sub>), 0.98 (t, *J* = 7.5 Hz, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, MeOD) δ 167.8 (C=O), 158.6 (C=N), 154.8, 136.3, 132.9, 122.5 and 122.1 (C Ar), 78.3 (OCH<sub>2</sub>), 54.6 (COCHNH<sub>3</sub><sup>+</sup>), 41.9 (N=CNHCH<sub>2</sub>), 32.0 (COCHCH<sub>2</sub>), 29.8 (ArCH<sub>2</sub>Ar), 25.5 (N=CNHCH<sub>2</sub>CH<sub>2</sub>), 24.5 (OCH<sub>2</sub>CH<sub>2</sub>), 10.7 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (*m/z*): [M + 2H-8HCl]<sup>2+</sup> calcd for C<sub>64</sub>H<sub>108</sub>N<sub>20</sub>O<sub>8</sub>Cl<sub>8</sub> 639.4, found 639.2.

## Synthesis of 5,11,17,23-Tetrakis[(Boc-L-Orn(Cbz)amino)-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (17)

To a solution in dry DCM of Boc-L-Orn(Cbz)-OH and DIPEA (1.5 equiv and 1.7 equiv for each calixarene NH<sub>2</sub> group, respectively), HBTU [O-Benzotriazole-N,N,N',N'-tetramethyluronium-hexafluoro-phosphate] (1.5 equiv for each calixarene NH<sub>2</sub> group) was added. Then aminocalixarene **1** (100 mg, 0.122 mmol) was added. The mixture was stirred at room temperature for 24 h. Then the reaction was quenched with water; the organic layer was washed with brine, and then dried over anhydrous MgSO<sub>4</sub>. The solvent was removed under reduced pressure and the pure products were isolated by flash column chromatography (CHCl<sub>3</sub>/MeOH 98:2). The pure product was isolated as white solid in 35% yield (96 mg).

Mp: 178.9-179.7 °C. <sup>1</sup>H NMR (300 MHz, DMSO-d<sub>6</sub>) δ 9.24 (bs, 2H, NH), 7.42-7.10 (m, 20H, ArH<sub>Cbz</sub>), 6.95-6.62 (m, 8H, ArH<sub>calix</sub>), 4.99 (s, 8H, CH<sub>2</sub><sub>Cbz</sub>), 4.33 (d, *J* = 12.6 Hz, 4H, ArCH<sub>ax</sub>Ar), 4.15-3.62 (m, 12H, COCHNH and OCH<sub>2</sub>), 3.19-2.77 (m, 12H, ArCH<sub>eq</sub>Ar and

CH<sub>2</sub>NHCbz), 2.02-1.75 (m, 8H, OCH<sub>2</sub>CH<sub>2</sub>), 1.62-1.08 (m, 76H, (CH<sub>3</sub>)<sub>3</sub> Boc, COCH(CH<sub>2</sub>)<sub>2</sub> and O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 0.90 (bs, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>) δ 170.9, 156.5, 155.6 (C=O), 152.4 (C Ar<sub>calix</sub>), 137.7 (C Ar<sub>Cbz</sub>), 134.7, 133.3 (C Ar<sub>calix</sub>), 128.8, 128.1 (C Ar<sub>Cbz</sub>), 120.5, 119.9 (C Ar<sub>calix</sub>), 78.4 (C(CH<sub>3</sub>)<sub>3</sub>), 75.6 (OCH<sub>2</sub>), 65.5 (CH<sub>2</sub><sub>Cbz</sub>), 54.9 (COCHNHBoc), 39.5 (CH<sub>2</sub>NHCbz, determined by HSCQ), 32.1 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 31.3 (ArCH<sub>2</sub>Ar), 30.2 (COCHCH<sub>2</sub>), 30.1 (OCH<sub>2</sub>CH<sub>2</sub>), 28.7 (C(CH<sub>3</sub>)<sub>3</sub>), 26.6 (COCHCH<sub>2</sub>CH<sub>2</sub>), 25.9 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 22.8 (CH<sub>2</sub>CH<sub>3</sub>), 14.3 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (m/z): [M + 2H]<sup>2+</sup> calcd for C<sub>124</sub>H<sub>172</sub>N<sub>12</sub>O<sub>24</sub> 1129.6, found 1129.9.

### Synthesis of 5,11,17,23-Tetrakis[(Boc-L-Orn-amino)-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene, tetrahydrochloride (18)

Calix[4]arene **17** (80 mg, 3.6×10<sup>-2</sup> mmol) was dissolved in EtOH/AcOEt (1:1, 10 mL), and a catalytic amount of Pd/C (10%) and HCl 1M (100μL) were added. Hydrogenation for Cbz group removal was carried out at 2 atm in a Parr reactor for 27 h. The reaction was stopped by catalyst filtration and the solvent removed under reduced pressure. The pure product **18** was isolated as white solid in quantitative yield (60.2 mg).

Mp: 168°C dec. <sup>1</sup>H NMR (300 MHz, MeOD) δ 7.12 (bs, 4H, ArH), 6.67 (bs, 4H, ArH), 4.46 (d, *J* = 13.2 Hz, 4H, ArCH<sub>ax</sub>Ar), 4.15 (bs, 4H, COCHNH), 3.89 (bs, 8H, OCH<sub>2</sub>), 3.22-2.77 (m, 12H, ArCH<sub>eq</sub>Ar and CH<sub>2</sub>NH<sub>3</sub><sup>+</sup>), 2.07-1.62 (m, 24H, OCH<sub>2</sub>CH<sub>2</sub> and COCH(CH<sub>2</sub>)<sub>2</sub>), 1.58-1.30 (m, 60H, (CH<sub>3</sub>)<sub>3</sub> Boc and O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 0.95 (bs, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, MeOD) δ 172.3, 157.8, (C=O), 154.7, 136.2, 133.1, 122.6, 121.7 (C Ar<sub>calix</sub>), 80.9 (C(CH<sub>3</sub>)<sub>3</sub>), 76.5 (OCH<sub>2</sub>), 55.7 (COCHNH), 40.4 (CH<sub>2</sub>NH<sub>3</sub><sup>+</sup>), 33.4 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 32.2 (ArCH<sub>2</sub>Ar), 31.4 (OCH<sub>2</sub>CH<sub>2</sub>), 30.7 (COCHCH<sub>2</sub>), 28.9 (C(CH<sub>3</sub>)<sub>3</sub>), 27.3 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 25.0 (COCHCH<sub>2</sub>CH<sub>2</sub>), 24.0 (CH<sub>2</sub>CH<sub>3</sub>), 14.0 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (m/z): [M + 2H-4HCl]<sup>2+</sup> calcd for C<sub>92</sub>H<sub>152</sub>N<sub>12</sub>O<sub>16</sub>Cl<sub>4</sub> 839.6, found 839.9.

### Synthesis of 5,11,17,23-Tetra[L-Orn-amino]-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene, octahydrochloride (19)

The compound **18** was deprotected according to the general procedure described in chapter 2. The pure product **19** was isolated as white solid in quantitative yield.

Mp: >198°C dec. <sup>1</sup>H NMR (400 MHz, MeOD) δ 7.18 (d, *J* = 2.4 Hz, 4H, ArH), 6.94 (d, *J* = 2.4 Hz, 4H, ArH), 4.49 (d, *J* = 13.0 Hz, 4H, ArCH<sub>ax</sub>Ar), 4.13 (t, *J* = 6.4 Hz, 4H,

COCHNH<sub>3</sub><sup>+</sup>), 3.92 (t, *J* = 7.2 Hz, 8H, OCH<sub>2</sub>), 3.22-2.95 (m, 12H, ArCH<sub>eq</sub>Ar and CH<sub>2</sub>NH<sub>3</sub><sup>+</sup>), 2.18-1.77 (m, 24H, OCH<sub>2</sub>CH<sub>2</sub> and COCH(CH<sub>2</sub>)<sub>2</sub>), 1.58-1.32 (m, 24H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 0.98 (t, *J* = 7.4 Hz 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, MeOD) δ 167.5 (C=O), 154.8, 136.1 132.8, 122.4, 122.0 (C Ar), 76.7 (OCH<sub>2</sub>), 54.2 (COCHNH<sub>3</sub><sup>+</sup>), 40.1 (CH<sub>2</sub>NH<sub>3</sub><sup>+</sup>), 33.4 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 32.0 (ArCH<sub>2</sub>Ar), 31.5 (OCH<sub>2</sub>CH<sub>2</sub>), 29.7 (COCHCH<sub>2</sub>), 27.2 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 24.1 (COCHCH<sub>2</sub>CH<sub>2</sub>), 24.0 (CH<sub>2</sub>CH<sub>3</sub>), 14.5 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (*m/z*): [M + 2H-8HCl]<sup>2+</sup> calcd for C<sub>72</sub>H<sub>124</sub>N<sub>12</sub>O<sub>8</sub>Cl<sub>8</sub> 639.4, found 639.5.

### Synthesis of 4-(Boc-amino)butyric acid

It was synthesized according to a literature procedure<sup>6</sup>

### Synthesis of 5,11,17,23-Tetrakis[4-(Boc-amino)butyramino]-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (20a)

To a solution in dry DCM of 4-(Boc-amino)butyric acid and DIPEA (1.5 equiv and 1.7 equiv for each calixarene NH<sub>2</sub> group, respectively), HBTU (1.5 equiv for each calixarene NH<sub>2</sub> group) was added. Then aminocalixarene **1** (100 mg, 0.122 mmol) was added. The mixture was stirred at room temperature for 24 h. Then the reaction was quenched with water; the organic layer was washed with brine, and then dried over anhydrous MgSO<sub>4</sub>. The solvent was removed under reduced pressure and the pure product was isolated by flash column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 95:5) and centrifugation with hexane as white solid in 44% yield (83 mg).

Mp: 101.8-102.3°C. <sup>1</sup>H-NMR (300 MHz, CD<sub>3</sub>OD) δ 6.89 (s, 8H, ArH), 4.45 (d, *J* = 12.9 Hz, 4H, ArCH<sub>ax</sub>Ar), 3.89 (t, *J* = 7.2 Hz, 8H, OCH<sub>2</sub>), 3.15-3.05 (m, 12H, ArCH<sub>eq</sub>Ar and CH<sub>2</sub>NHBoc), 2.26 (t, *J* = 7.5 Hz, 8H, NHCOCH<sub>2</sub>), 2.05-1.90 (m, 8H, NHCOCH<sub>2</sub>CH<sub>2</sub>), 1.85-1.72 (m, 8H, OCH<sub>2</sub>CH<sub>2</sub>) 1.55-1.30 (m, 60H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub> and C(CH<sub>3</sub>)<sub>3</sub>), 1.00- 0.90 (m, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C-NMR (100 MHz, CD<sub>3</sub>OD) δ 173.5 and 158.7 (C=O), 154.5, 136.3, 133.8 and 122.0 (C Ar), 80.1 (C(CH<sub>3</sub>)<sub>3</sub>), 76.7 (OCH<sub>2</sub>), 41.1 (CH<sub>2</sub>NHBoc), 35.3 (COCH<sub>2</sub>), 33.6, (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 32.3 (ArCH<sub>2</sub>Ar), 31.7 (OCH<sub>2</sub>CH<sub>2</sub>), 29.0 (C(CH<sub>3</sub>)<sub>3</sub>), 27.5 and 27.4 (COCH<sub>2</sub>CH<sub>2</sub> and CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 24.2 (CH<sub>2</sub>CH<sub>3</sub>), 14.7 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (*m/z*): [M + Na]<sup>+</sup> calcd for C<sub>88</sub>H<sub>136</sub>O<sub>16</sub>N<sub>8</sub> 1584.6, found 1584.8, [M + 2Na]<sup>2+</sup> calcd 803.5, found 803.7.

### Synthesis of 5,11,17,23-Tetrakis[5-(Boc-amino)valeriamo]-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (20b)

To a solution in dry DCM of 5-(Boc-amino)valeric acid and DIPEA (1.5 equiv and 1.7 equiv for each calixarene NH<sub>2</sub> group, respectively), HBTU (1.5 equiv for each calixarene NH<sub>2</sub> group) was added. Then aminocalixarene **1** (100 mg, 0.122 mmol) was added. The mixture was stirred at room temperature for 24 h. Then the reaction was quenched with water; the organic layer was washed with brine, and then dried over anhydrous MgSO<sub>4</sub>. The solvent was removed under reduced pressure and the pure product was isolated by flash column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 96:4) and centrifugation with hexane as white solid in 50% yield (98.5 mg).

Mp: 118.5-120.2°C. <sup>1</sup>H-NMR (300 MHz, CD<sub>3</sub>OD) δ 6.89 (s, 8H, ArH), 6.61 (bt, CH<sub>2</sub>NH), 4.44 (d, *J* = 12.9 Hz, 4H, ArCH<sub>ax</sub>Ar), 3.88 (t, *J* = 7.2 Hz, 8H, OCH<sub>2</sub>), 3.17-2.96 (m, 12H, ArCH<sub>eq</sub>Ar and CH<sub>2</sub>NHBoc), 2.26 (t, *J* = 7.2 Hz, 8H, NHCOCH<sub>2</sub>), 2.06-1.88 (m, 8H, OCH<sub>2</sub>CH<sub>2</sub>), 1.72-1.57 (m, 8H, CH<sub>2</sub>CH<sub>2</sub>NHBoc) 1.55-1.28 (m, 68H, COCH<sub>2</sub>CH<sub>2</sub>, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub> and C(CH<sub>3</sub>)<sub>3</sub>), 0.95 (t, *J* = 6.6 Hz, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C-NMR (100 MHz, CD<sub>3</sub>OD) δ 173.3 and 158.1 (C=O), 154.0, 135.8, 133.3 and 121.5 (C Ar), 79.4 (C(CH<sub>3</sub>)<sub>3</sub>), 76.1 (OCH<sub>2</sub>), 40.5 (CH<sub>2</sub>NHBoc), 37.0 (COCH<sub>2</sub>), 33.0 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 31.7 (ArCH<sub>2</sub>Ar), 31.1 (OCH<sub>2</sub>CH<sub>2</sub>), 30.2 (CH<sub>2</sub>CH<sub>2</sub>NHBoc), 28.5 (C(CH<sub>3</sub>)<sub>3</sub>) and CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 26.5 (COCH<sub>2</sub>CH<sub>2</sub>), 23.6 (CH<sub>2</sub>CH<sub>3</sub>), 14.1 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (*m/z*): [M + Na]<sup>+</sup> calcd for C<sub>92</sub>H<sub>144</sub>O<sub>16</sub>N<sub>8</sub> 1640.1, found 1640.9.

### Synthesis of 5,11,17,23-Tetrakis[(4-aminobutiramino)-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene, tetrahydrotrifluoroacetate (21a)

The compound **20a** was deprotected according to the general procedure described in Chapter 2. The product **21a** has not been treated with HCl and was isolated as white solid in quantitative yield.

Mp: 202° C dec. <sup>1</sup>H-NMR (300 MHz, CD<sub>3</sub>OD) δ 6.88 (s, 8H, ArH), 4.46 (d, *J* = 13.2 Hz, 4H, ArCH<sub>ax</sub>Ar), 3.89 (t, *J* = 7.2 Hz, 8H, OCH<sub>2</sub>), 3.12 (d, *J* = 13.2 Hz, 4H, ArCH<sub>eq</sub>Ar), 2.98 (t, *J* = 7.2 Hz, 8H, CH<sub>2</sub>NH<sub>3</sub><sup>+</sup>), 2.42 (t, *J* = 6.9 Hz, 8H, NHCOCH<sub>2</sub>), 2.05-1.87 (m, 16H, OCH<sub>2</sub>CH<sub>2</sub> and COCH<sub>2</sub>CH<sub>2</sub>), 1.55-1.32 (m, 24H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 1.02- 0.89 (m, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C-NMR (100 MHz, CD<sub>3</sub>OD) δ 172.4 (C=O), 154.7, 136.4, 133.6 and 122.2 (C Ar), 76.7 (OCH<sub>2</sub>), 40.5 (CH<sub>2</sub>NH<sub>3</sub><sup>+</sup>), 34.5 (COCH<sub>2</sub>), 33.5 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 32.3 (ArCH<sub>2</sub>Ar), 31.6 (OCH<sub>2</sub>CH<sub>2</sub>), 27.5 (COCH<sub>2</sub>CH<sub>2</sub>), 24.5 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 24.2 (CH<sub>2</sub>CH<sub>3</sub>), 14.7 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (m/z):  $[M + Na - 4CF_3COOH]^+$  calcd for  $C_{76}H_{108}N_8O_{16}F_{12}$  1183.3, found 1183.6.

### Synthesis of 5,11,17,23-Tetrakis(5-aminovaleriamino)-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene, tetrahydrotrifluoroacetate (21b)

The compound **20b** was deprotected according to the general procedure described in Chapter 2. The product **21b** has not been treated with HCl and was isolated as white solid in quantitative yield.

Mp: 225°C dec.  $^1H$ -NMR (300 MHz,  $CD_3OD$ )  $\delta$  6.89 (s, 8H, ArH), 4.46 (d,  $J = 13.0$  Hz, 4H, ArCH<sub>ax</sub>Ar), 3.89 (t,  $J = 7.2$  Hz, 8H, OCH<sub>2</sub>), 3.12 (d,  $J = 13.0$  Hz, 4H, ArCH<sub>eq</sub>Ar), 2.93 (t,  $J = 6.9$  Hz, 8H, CH<sub>2</sub>NH<sub>3</sub><sup>+</sup>), 2.33 (t,  $J = 6.3$  Hz, 8H, NHCOCH<sub>2</sub>), 2.03-1.88 (m, 8H, OCH<sub>2</sub>CH<sub>2</sub>), 1.80-1.61 (m, 16H, CH<sub>2</sub>CH<sub>2</sub>NHBoc and COCH<sub>2</sub>CH<sub>2</sub>), 1.55-1.30 (m, 24H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 1.02- 0.89 (m, 12H, CH<sub>2</sub>CH<sub>3</sub>).

$^{13}C$ -NMR (75 MHz,  $CD_3OD$ )  $\delta$  173.1 (C=O), 154.6, 136.2, 133.4 and 121.9 (C Ar), 76.7 (OCH<sub>2</sub>), 40.4 (CH<sub>2</sub>NH<sub>3</sub><sup>+</sup>), 36.8 (COCH<sub>2</sub>), 33.4 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 32.2 (ArCH<sub>2</sub>Ar), 31.5 (OCH<sub>2</sub>CH<sub>2</sub>), 28.1(CH<sub>2</sub>CH<sub>2</sub>NHBoc), 27.4 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 24.0 (COCH<sub>2</sub>CH<sub>2</sub>), 23.4 (CH<sub>2</sub>CH<sub>3</sub>), 14.7 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (m/z):  $[M + 2H - 4CF_3COOH]^{2+}$  calcd for  $C_{76}H_{108}N_8O_{16}F_{12}$  609.4, found 609.3.

### Synthesis of 5,11,17,23-Tetrakis[(Boc-Gly-amino)]-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (24)

To a solution in dry DMF of Boc-Gly-OH and HOBt (1.5 equiv and 1.7 equiv for each NH<sub>2</sub> group, respectively), DDC (1.5 equiv for each NH<sub>2</sub> group) was added. After 10-15 min aminocalixarene **1** (100 mg, 0.12 mmol) in 2 mL of DMF was added. The mixture was stirred at room temperature for 24 h. Ethyl acetate was added (10 mL), DCU was filtered off by gravity on a PTFE filter, and the solvent was removed under reduced pressure. The crude was dissolved in ethyl acetate (10 mL) and washed with a saturated NaHCO<sub>3</sub> aqueous solution (10 mL), brine (10 mL) and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solvent was removed under reduced pressure giving a crude material that was purified by flash column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 95:5) to obtain the pure product as a white solid in 38% yield (67 mg).

Mp: 110-112°C dec.  $^1H$  NMR (300 MHz, MeOD)  $\delta$  6.87 (s, 8H, ArH), 4.45 (d,  $J = 13.2$  Hz, 4H, ArCH<sub>ax</sub>Ar), 3.89 (t,  $J = 7.2$  Hz, 8H, OCH<sub>2</sub>), 3.74 (s, 8H, COCH<sub>2</sub>NHBoc), 3.11 (d,  $J =$

13.2 Hz, 4H, ArCH<sub>eq</sub>Ar), 2.04-1.88 (m, 8H, OCH<sub>2</sub>CH<sub>2</sub>), 1.55-1.31 (m, 60H, (CH<sub>3</sub>)<sub>3</sub> Boc, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 0.95 (t, *J* = 6.9 Hz 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C NMR (75 MHz, MeOD) δ 168.7 and 157.3 (C=O), 153.1, 134.8 131.9, 120.4 (C Ar), 79.3 (C(CH<sub>3</sub>)<sub>3</sub>), 75.1 (OCH<sub>2</sub>), 43.5 (COCH<sub>2</sub>NHBoc), 32.0 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 30.7 (ArCH<sub>2</sub>Ar), 30.1 (OCH<sub>2</sub>CH<sub>2</sub>), 27.4 (C(CH<sub>3</sub>)<sub>3</sub>), 25.9 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 22.6 (CH<sub>2</sub>CH<sub>3</sub>), 13.1 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (*m/z*): [M + Na]<sup>+</sup> calcd for C<sub>80</sub>H<sub>120</sub>N<sub>8</sub>O<sub>16</sub> 1471.9, found 1472.2.

### Synthesis of 5,11,17,23-Tetrakis(Gly-amino)-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene, tetrahydrochloride (25)

The compound **24** was deprotected according to the general procedure described in Chapter 2. The pure product was isolated as white solid in quantitative yield.

Mp: >210°C dec. <sup>1</sup>H NMR (300 MHz, MeOD) δ 6.92 (s, 8H, ArH), 4.47 (d, *J* = 13.0 Hz, 4H, ArCH<sub>ax</sub>Ar), 3.91 (t, *J* = 7.3 Hz, 8H, OCH<sub>2</sub>), 3.78 (s, 8H, COCH<sub>2</sub>NH<sub>3</sub><sup>+</sup>), 3.14 (d, *J* = 13.0 Hz, 4H, ArCH<sub>eq</sub>Ar), 2.07-1.82 (m, 8H, OCH<sub>2</sub>CH<sub>2</sub>), 1.62-1.26 (m, 24H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 0.95 (t, *J* = 6.4 Hz 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C NMR (75 MHz, MeOD) δ 165.0 (C=O), 154.8, 136.5 133.0, 121.9 (C Ar), 76.7 (OCH<sub>2</sub>), 42.2 (COCH<sub>2</sub>NH<sub>3</sub><sup>+</sup>), 33.5 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 32.2 (ArCH<sub>2</sub>Ar), 31.6 (OCH<sub>2</sub>CH<sub>2</sub>), 27.4 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 24.1 (CH<sub>2</sub>CH<sub>3</sub>), 14.6 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (*m/z*): [M + Na]<sup>+</sup> calcd for C<sub>60</sub>H<sub>88</sub>N<sub>8</sub>O<sub>8</sub> 1071.6, found 1071.7.

### General procedure for guanidylation:

To a solution of calix[4]arenes **21a**, **21b** and **25** (0.063 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (10 mL) and Et<sub>3</sub>N (4 eq. for NH<sub>2</sub> group), *N,N'*-Bis(tert-butoxycarbonyl)-*N''*-triflylguanidine (2 eq. for NH<sub>2</sub> group) was added and the mixture was stirred for 16 h. The mixture was transferred to a separatory funnel and washed with 2 M aqueous sodium bisulfate (10 mL) and with saturated sodium bicarbonate (10 mL). Each aqueous layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (2×5 mL). The combined organic phases were washed with brine (15 mL), dried over anhydrous MgSO<sub>4</sub> and concentrated under reduced pressure. Pure products were isolated by flash column chromatography (gradient from CH<sub>2</sub>Cl<sub>2</sub> to CH<sub>2</sub>Cl<sub>2</sub>/MeOH 95:5).

### Synthesis of 5,11,17,23-Tetrakis[(γ-(*N,N'*-di-Boc-guanidyl)butyramino]-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (22a)

The pure product **22a** was isolated as yellowish solid in 56% yield (74.3 mg).

Mp: >130°C dec. <sup>1</sup>H-NMR (300 MHz, CD<sub>3</sub>OD) δ 6.90 (s, 8H, ArH), 4.44 (d, *J* = 13.1 Hz, 4H, ArCH<sub>ax</sub>Ar), 3.88 (t, *J* = 7.2 Hz, 8H, OCH<sub>2</sub>), 3.40 (t, *J* = 7.2 Hz, 8H, CH<sub>2</sub>NH), 3.10 (d, *J* = 13.1 Hz, ArCH<sub>eq</sub>Ar), 2.30 (t, *J* = 7.2 Hz, 8H, COCH<sub>2</sub>), 2.03-1.82 (m, 16H, OCH<sub>2</sub>CH<sub>2</sub> and COCH<sub>2</sub>CH<sub>2</sub>), 1.55-1.34 (m, 96H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub> and C(CH<sub>3</sub>)<sub>3</sub>), 1.00- 0.87 (m, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C-NMR (100 MHz, CD<sub>3</sub>OD) δ 173.1 (C=O), 164.7 (C=N), 157.9 and 154.5 (C=O), 154.3, 136.3, 133.9 and 122.1 (C Ar), 84.6 and 80.5 (C(CH<sub>3</sub>)<sub>3</sub>), 76.7 (OCH<sub>2</sub>), 41.4 (CH<sub>2</sub>NH), 35.2 (COCH<sub>2</sub>), 33.6 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 32.4 (ArCH<sub>2</sub>Ar), 31.7 (OCH<sub>2</sub>CH<sub>2</sub>), 28.8 and 28.5 (C(CH<sub>3</sub>)<sub>3</sub>), 27.5 (COCH<sub>2</sub>CH<sub>2</sub>), 26.6 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 24.2 (CH<sub>2</sub>CH<sub>3</sub>), 14.7 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (m/z): [M + 2Na]<sup>2+</sup> calcd for C<sub>112</sub>H<sub>176</sub>N<sub>16</sub>O<sub>24</sub> 1087.9, found 1088.0.

### Synthesis of 5,11,17,23-Tetrakis[(δ-(N,N'-di-Boc-guanidyl)valeramino]-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (22b)

The pure product **22b** was isolated as yellowish solid in 41% yield (56 mg).

Mp:135.1-137 °C. <sup>1</sup>H-NMR (300 MHz, CD<sub>3</sub>OD) δ 6.91 (s, 8H, ArH), 4.45 (d, *J* = 12.9 Hz, 4H, ArCH<sub>ax</sub>Ar), 3.89 (t, *J* = 6.9 Hz, 8H, OCH<sub>2</sub>), 3.38 (t, *J* = 6.6 Hz, 8H, CH<sub>2</sub>NH), 3.12 (d, *J* = 12.9 Hz, 4H, ArCH<sub>eq</sub>Ar), 2.29 (bt, 8H, COCH<sub>2</sub>), 2.07-1.86 (m, 8H, OCH<sub>2</sub>CH<sub>2</sub>), 1.55-1.34 (m, 112H, COCH<sub>2</sub> CH<sub>2</sub>CH<sub>2</sub>, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub> and C(CH<sub>3</sub>)<sub>3</sub>), 0.97 (t, *J* = 6.9 Hz, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C-NMR (75 MHz, CD<sub>3</sub>OD) δ 173.5 (C=O), 164.6 (C=N), 157.6 and 154.4 (C=O), 154.2, 136.2, 133.8 and 121.0 (C Ar), 84.5 and 80.4 (C(CH<sub>3</sub>)<sub>3</sub>), 76.6 (OCH<sub>2</sub>), 41.5 (CH<sub>2</sub>NH), 37.3 (COCH<sub>2</sub>), 33.5 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 32.2 (ArCH<sub>2</sub>Ar), 31.6 (OCH<sub>2</sub>CH<sub>2</sub>), 29.8 (CH<sub>2</sub>CH<sub>2</sub>NH), 28.8 and 28.4 (C(CH<sub>3</sub>)<sub>3</sub>), 27.5 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 24.1 (COCH<sub>2</sub>CH<sub>2</sub> and CH<sub>2</sub>CH<sub>3</sub>, determined by HSCQ), 14.7 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (m/z): [M + 2Na]<sup>2+</sup> calcd for C<sub>116</sub>H<sub>184</sub>N<sub>16</sub>O<sub>24</sub> 1116.0, found 1116.1.

### Synthesis of 5,11,17,23-Tetrakis[α-(N,N'-di-Boc-guanidyl)acetamino]-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (26)

Pure product was isolated as yellowish solid in 33% yield (42 mg).

Mp: 159-162°C. <sup>1</sup>H-NMR (300 MHz, CD<sub>3</sub>OD) δ 6.89 (s, 8H, ArH), 4.36 (d, *J* = 13.2 Hz, 4H, ArCH<sub>ax</sub>Ar), 4.03 (s, 8H, COCH<sub>2</sub>), 3.89 (t, *J* = 7.2 Hz, 8H, OCH<sub>2</sub>), 3.13 (d, *J* = 13.2 Hz, ArCH<sub>eq</sub>Ar), 2.02-1.83 (m, 8H, OCH<sub>2</sub>CH<sub>2</sub>), 1.61-1.29 (m, 96H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub> and C(CH<sub>3</sub>)<sub>3</sub>), 1.00-0.89 (m, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD, 19/1) δ 169.7 (C=O), 166.7 (C=N), 159.7 and 157.3 (C=O), 156.4, 139.1, 135.5 and 124.3 (C Ar), 87.3 and 83.5 (C(CH<sub>3</sub>)<sub>3</sub>), 79.2 (OCH<sub>2</sub>), 48.1 (COCH<sub>2</sub>), 35.9 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 35.0 (ArCH<sub>2</sub>Ar), 34.0 (OCH<sub>2</sub>CH<sub>2</sub>), 32.1 and 31.8 (C(CH<sub>3</sub>)<sub>3</sub>), 29.8 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 26.7 (CH<sub>2</sub>CH<sub>3</sub>), 17.7 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (m/z): [M + 2Na]<sup>2+</sup> calcd for C<sub>104</sub>H<sub>160</sub>N<sub>16</sub>O<sub>24</sub> 1031.6, found 1032.0.

#### **General procedure for Boc deprotection in case of guanidinylated derivatives:**

A solution of calix[4]arene (10 mmol) in DCM/TFA/TES (92.5/5/2.5, 10 mL) was stirred at 0°C. The progression of the reaction was followed using mass spectroscopy. After completion (24-48 h), the volatiles were removed under reduced pressure. The crude material was precipitated, washed and centrifuged with anhydrous diethyl ether (3×5 mL). The trifluoroacetate anion of the resulting TFA salts was exchanged by adding 10 mM HCl solution (3×3 mL) followed by evaporation under reduced pressure

#### **Synthesis of 5,11,17,23-Tetrakis[γ-guanidinobutiramino]- 25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene, tetrahydrochloride (23a)**

The pure product **23a** was isolated as white solid in 80% yield.

Mp: >210°C dec. <sup>1</sup>H-NMR (400 MHz, CD<sub>3</sub>OD) δ 9.48 (bs, NHCO), 7.53 (bt, CH<sub>2</sub>NH), 6.90 (s, 8H, ArH), 4.46 (d, *J* = 13.0 Hz, 4H, ArCH<sub>ax</sub>Ar), 3.89 (t, *J* = 7.2 Hz, 8H, OCH<sub>2</sub>), 3.25 (bt, 8H, CH<sub>2</sub>NH), 3.12 (d, *J* = 13.0 Hz, 4H, ArCH<sub>eq</sub>Ar), 2.39 (t, *J* = 6.4 Hz, 8H, COCH<sub>2</sub>), 2.05-1.80 (m, 16H, OCH<sub>2</sub>CH<sub>2</sub> and COCH<sub>2</sub>CH<sub>2</sub>), 1.55-1.30 (m, 24H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 1.05-0.90 (m, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C-NMR (100 MHz, CD<sub>3</sub>OD) δ 172.8 (C=O), 158.6 (C=N), 154.4, 136.1, 133.4 and 121.9 (C Ar), 76.4 (OCH<sub>2</sub>), 41.8 (CH<sub>2</sub>NH), 34.0 (COCH<sub>2</sub>), 33.2 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 32.0 (ArCH<sub>2</sub>Ar), 31.3 (OCH<sub>2</sub>CH<sub>2</sub>), 27.2 (COCH<sub>2</sub>CH<sub>2</sub>), 25.7 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 23.9 (CH<sub>2</sub>CH<sub>3</sub>), 14.4 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (m/z): [M + 2H - 4HCl]<sup>2+</sup> calcd for C<sub>72</sub>H<sub>116</sub>N<sub>16</sub>O<sub>8</sub>Cl<sub>4</sub> 665.4, found 665.4.

#### **Synthesis of 5,11,17,23-Tetrakis(δ-guanidinovaleramino)-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene, tetrahydrochloride (23b)**

The pure product **23b** was isolated as white solid in quantitative yield.

Mp: >190°C dec. <sup>1</sup>H-NMR (400 MHz, CD<sub>3</sub>OD) δ 6.91 (s, 8H, ArH), 4.48 (d, *J* = 13.2 Hz, 4H, ArCH<sub>ax</sub>Ar), 3.91 (t, *J* = 7.6 Hz, 8H, OCH<sub>2</sub>), 3.21 (t, *J* = 6.8 Hz, 8H, CH<sub>2</sub>NH), 3.13 (d, *J* = 13.2 Hz, 4H, ArCH<sub>eq</sub>Ar), 2.33 (t, *J* = 6.8 Hz, 8H, COCH<sub>2</sub>), 2.04-1.91 (m, 8H, OCH<sub>2</sub>CH<sub>2</sub>)

1.78-1.57 (m, 16H COCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 1.55-1.32 (m, 24H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 0.97 (t, *J* = 6.8 Hz, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C-NMR (100 MHz, CD<sub>3</sub>OD) δ 173.6 (C=O), 158.6 (C=N), 154.5, 136.2, 133.6 and 122.1 (C Ar), 76.6 (OCH<sub>2</sub>), 42.3 (CH<sub>2</sub>NH), 37.1 (COCH<sub>2</sub>), 33.4 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 32.2 (ArCH<sub>2</sub>Ar), 31.5 (OCH<sub>2</sub>CH<sub>2</sub>), 29.4 (CH<sub>2</sub>CH<sub>2</sub>NH), 27.4 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 24.0 (CH<sub>2</sub>CH<sub>3</sub>), 23.8 (COCH<sub>2</sub>CH<sub>2</sub>), 14.5 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (*m/z*): [M + 2H – 4HCl]<sup>2+</sup> calcd for C<sub>76</sub>H<sub>124</sub>N<sub>16</sub>O<sub>8</sub>Cl<sub>4</sub> 692.8, found 693.0.

### Synthesis of 5,11,17,23-Tetraformyl-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (28)

In a two neck round bottom flask HMTA [hexamethylenetetramine] (16.57 g, 118.3 mmol) was dissolved in TFA (150 mL); this solution was stirred at 100 °C for 10 min. 25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (synthesized starting from calixarene **27** according to a literature procedure<sup>7</sup>) (2.50 g, 3.29 mmol) was added and the mixture was stirred at reflux for 4 days. Then the reaction was quenched by addition of HCl 1 M (400 mL) and stirred for 3h. The aqueous layer was extracted with DCM (2x250 mL); the combined organic phases were washed with sat. Na<sub>2</sub>CO<sub>3</sub> (200 mL) and brine (200 mL) till neutral pH, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtrated and the solvent was removed under reduced pressure. The product (1.78g, yield 62%) was purified by trituration in hexane (50mL).

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 9.58 (s, 4H, ArCHO), 7.15 (s, 8H, ArH), 4.49 (d, *J* = 13.8 Hz, 4H, ArCH<sub>ax</sub>Ar), 3.96 (t, *J* = 7.5 Hz, 8H, OCH<sub>2</sub>), 3.34 (d, *J* = 13.8 Hz, 4H, ArCH<sub>eq</sub>Ar), 2.00-1.80 (m, 8H, OCH<sub>2</sub>CH<sub>2</sub>), 1.50-1.20 (m, 24H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 1.00-0.80 (m, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 191.3 (ArCHO), 162.0, 135.6, 131.4 and 130.2 (C Ar), 75.8 (OCH<sub>2</sub>), 31.9 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 30.9 (ArCH<sub>2</sub>Ar), 30.3 (OCH<sub>2</sub>CH<sub>2</sub>), 25.8 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 22.8 (CH<sub>2</sub>CH<sub>3</sub>), 14.0 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (*m/z*): [M + Na]<sup>+</sup> calcd for C<sub>56</sub>H<sub>72</sub>O<sub>8</sub> 895.5, found 895.9.

### Synthesis of 5,11,17,23-Tetrahydroxycarbonyl-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (29)

A solution of compound **28** (1.5 g, 1.72 mmol) in CHCl<sub>3</sub>/acetone (100mL, 1/1 v/v) was cooled to 0°C and treated with an aqueous solution (12mL) of NaClO<sub>2</sub> (2.91 g, 25.77 mmol) and H<sub>2</sub>NSO<sub>3</sub>H (3.00 g, 30.92 mmol). The mixture was vigorously at rt for 30h and,

then, evaporated to dryness at reduced pressure. HCl 2M (20 mL) was added to give **29** as white solid that was crystallized from MeOH (1.064 g, yield 66%).

Mp: > 300 °C dec. <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD/CDCl<sub>3</sub> 9/1) δ 7.37 (s, 8H, ArH), 4.49 (d, *J* = 13.5 Hz, 4H, ArCH<sub>ax</sub>Ar), 3.98 (t, *J* = 7.2 Hz, 8H, OCH<sub>2</sub>), 3.31 (under the CD<sub>3</sub>OD signal, 4H, ArCH<sub>eq</sub>Ar), 2.00-1.85 (m, 8H, OCH<sub>2</sub>CH<sub>2</sub>), 1.50-1.35 (m, 24H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 0.93 (t, *J* = 6.6 Hz, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD 19:1) δ 168.9 (ArCOOH), 160.7, 134.7, 130.2 and 123.8 (C Ar), 75.3 (OCH<sub>2</sub>), 31.8 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 30.8 (ArCH<sub>2</sub>Ar), 30.0 (OCH<sub>2</sub>CH<sub>2</sub>), 25.7 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 22.6 (CH<sub>2</sub>CH<sub>3</sub>), 13.8 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (*m/z*): [M - H]<sup>-</sup> calcd for C<sub>56</sub>H<sub>72</sub>O<sub>12</sub> 936.1, found 936.1.

### **Synthesis of 5,11,17,23-Tetrakis{carbonyl-N-[L-Arg(Pbf) methyl ester]}-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (**30**)**

L-Arg(Pbf)-OMe·HCl (300 mg, 0.64 mmol) and TEA (100 μL, 0.72mmol) are added to a suspension of **29** (100 mg, 0.11 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (9 ml). The solution is stirred at room temp until the complete solubilization of the amino acid, then HBTU (240 mg, 0.64 mmol) and triethylamine (100 μL, 0.72mmol) are added. During the first 30 min pH is frequently checked and triethylamine is added to maintain it at ca. 8.5. After 4 h the reaction is quenched by addition of 1M HCl (10 ml), then the organic layer is separated, washed with 5% NaHCO<sub>3</sub> (10 ml) and with water (10 ml), dried over anhydrous MgSO<sub>4</sub>. The solvent was removed under reduced pressure giving a crude material that was purified by preparative TLC (eluent: AcOEt/hexane= 94:6) to obtain the pure product as a white solid in 64% yield (185 mg).

Mp: 148-149 °C; <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD/CDCl<sub>3</sub> 19 /1) δ 7.24 (bs, 8H, ArH), 4.49 (d, *J* = 13.5 Hz, 4H, ArCH<sub>ax</sub>Ar), 4.38 (bs, 4H, NHCHCOOCH<sub>3</sub>), 3.98 (bs, 8H, OCH<sub>2</sub>), 3.60 (s, 12H, COOCH<sub>3</sub>), 3.31 (under the CD<sub>3</sub>OD signal, 4H, ArCH<sub>eq</sub>Ar), 3.18 (bs, 8H, CH<sub>2</sub>NH), 2.96 (s, 8H, CH<sub>2</sub> Pbf), 2.54 (s, 12H, CH<sub>3</sub> Pbf), 2.49 (s, 12H, CH<sub>3</sub> Pbf), 2.10-1.90 (m, 20H, CH<sub>3</sub> Pbf and OCH<sub>2</sub>CH<sub>2</sub>), 1.81 (bs, 8H, CONHCHCH<sub>2</sub>), 1.65-1.30 (m, 56H, CONHCHCH<sub>2</sub>CH<sub>2</sub>, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub> and C(CH<sub>3</sub>)<sub>2</sub> Pbf), 1.00-0.90 (m, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD/CDCl<sub>3</sub> 19/1) δ 174.6 (C=O), 169.7 (C=N), 160.9 (C=O), 159.9 (C Ar Pbf), 158.1 (C Ar calix), 139.4 (C Ar Pbf), 136.1 (C Ar calix), 134.4, 133.6, 129.7, 128.9 and 128.7 (C Ar calix), 126.2 (C Ar Pbf), 118.4 (C Ar Pbf), 87.7 (C(CH<sub>3</sub>)<sub>2</sub> Pbf), 76.8 (OCH<sub>2</sub>), 54.4 (NHCHCOOCH<sub>3</sub>), 52.8 (COOCH<sub>3</sub>), 44.0 (CH<sub>2</sub> Pbf), 41.5 (N=CNHCH<sub>2</sub>), 33.4

(OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 31.9 (ArCH<sub>2</sub>Ar), 31.6 (OCH<sub>2</sub>CH<sub>2</sub>), 29.4 (NHCHCH<sub>2</sub>), 28.8 (C(CH<sub>3</sub>)<sub>2</sub>), 27.3 (N=CNHCH<sub>2</sub>CH<sub>2</sub>), 27.2 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 24.0 (CH<sub>2</sub>CH<sub>3</sub>), 19.7 (CH<sub>3</sub> Pbf), 18.5 (CH<sub>3</sub> Pbf), 14.6 (CH<sub>2</sub>CH<sub>3</sub>), 12.6 (CH<sub>3</sub> Pbf).

ESI-MS (m/z): [M + 2Na]<sup>2+</sup> calcd for C<sub>136</sub>H<sub>192</sub>O<sub>28</sub>N<sub>16</sub>S<sub>4</sub> 1335.9, found 1336.1.

### **Synthesis of 5,11,17,23-Tetrakis{carbonyl-N-[L-Arg methyl ester]}-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene, tetrahydrochloride (31)**

The compound **30** was deprotected according to the general procedure described in Chapter 2. The pure product **31** was isolated as white solid in quantitative yield.

Mp: > 210 °C dec. <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD) δ 7.31 (bs, 4H, ArH), 7.26 (bs, 4H, ArH), 4.52 (d, *J* = 13.8 Hz, 4H, ArCH<sub>ax</sub>Ar), 4.46 (t, *J* = 7.2 Hz, 4H, NHCHCOOCH<sub>3</sub>), 4.05-3.95 (m, 8H, OCH<sub>2</sub>), 3.73 (s, 12H, COOCH<sub>3</sub>), 3.40 (d, *J* = 13.8 Hz, 4H, ArCH<sub>eq</sub>Ar), 3.31 (under the CD<sub>3</sub>OD signal, 8H, CH<sub>2</sub>NH), 2.05-1.93 (m, 16H, OCH<sub>2</sub>CH<sub>2</sub> e CONHCHCH<sub>2</sub>), 1.85-1.70 (m, 8H, CONHCHCH<sub>2</sub>CH<sub>2</sub>), 1.55-1.40 (m, 24H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 0.96 (t, *J* = 6.8 Hz, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD) δ 174.9 (C=O), 170.2 (C=N), 161.3 (C=O), 158.6, 136.4, 129.5 and 128.4 (C Ar), 76.8 (OCH<sub>2</sub>), 55.3 (NHCHCOOCH<sub>3</sub>), 52.9 (COOCH<sub>3</sub>), 42.0 (N=CNHCH<sub>2</sub>), 33.4 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 31.9 (ArCH<sub>2</sub>Ar), 31.7 (OCH<sub>2</sub>CH<sub>2</sub>), 29.1 (NHCHCH<sub>2</sub>), 27.4 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 27.0 (N=CNHCH<sub>2</sub>CH<sub>2</sub>), 24.1 (CH<sub>2</sub>CH<sub>3</sub>), 14.6 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (m/z): [M + 3H – 4HCl]<sup>3+</sup> calcd for C<sub>84</sub>H<sub>132</sub>O<sub>16</sub>N<sub>16</sub>Cl<sub>4</sub> 540.4, found 540.5.

### **Synthesis of 5,11,17,23-Tetrakis{[N-Ac-L-Arg(Pmc)]amino}--25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (32)**

To a solution in dry DMF of Ac-L-Arg(Pmc)-OH and HOBt (1.5 equiv and 1.7 equiv for each NH<sub>2</sub> group, respectively), DCC (1.5 equiv for each NH<sub>2</sub> group) was added. After 10-15 min aminocalixarene **1** (100 mg, 0.12 mmol) in 2 mL of DMF was added. The mixture was stirred at room temperature for 24 h. Ethyl acetate was added (10 mL), DCU was filtered off by gravity on a PTFE filter, and the solvent was removed under reduced pressure. The crude was dissolved in ethyl acetate (10 mL) and washed with a saturated NaHCO<sub>3</sub> aqueous solution (10 mL), brine (10 mL) and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solvent was removed under reduced pressure giving a crude material that was purified by flash column chromatography (gradient CH<sub>2</sub>Cl<sub>2</sub>/MeOH from 94:6 to 92:8) to obtain the pure product as a white solid in 10% yield (32 mg).

Mp: 163-165 °C.  $^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{OD}/\text{CDCl}_3$  19:1)  $\delta$  6.99 (bs, 4H, ArH), 6.73 (bs, 4H, ArH), 4.52-4.23 (m, 8H,  $\text{ArCH}_{\text{ax}}\text{Ar}$  and  $\text{COCHNHAc}$ ), 3.87 (bs, 8H,  $\text{OCH}_2$ ), 3.30-2.95 (m, 12H,  $\text{CH}_2\text{NH}$  e  $\text{ArCH}_{\text{eq}}\text{Ar}$ ), 2.70-2.40 (m, 32H,  $\text{CH}_2_{\text{Pmc}}$ ,  $\text{CH}_3_{\text{Pmc}}$  and  $\text{CH}_3_{\text{Pmc}}$ ), 2.06 (s, 12H,  $\text{CH}_3_{\text{Pmc}}$ ), 2.05-1.85 (m, 20H,  $\text{CH}_3\text{CO}$  and  $\text{OCH}_2\text{CH}_2$ ), 1.78 (t,  $J = 6.3$  Hz, 8H,  $\text{CH}_2_{\text{Pmc}}$ ), 1.85-1.10 (m, 64H,  $\text{O}(\text{CH}_2)_2\text{CH}_2\text{CH}_2\text{CH}_2$ ,  $\text{COCHCH}_2\text{CH}_2$  and  $\text{C}(\text{CH}_3)_2_{\text{Pmc}}$ ), 1.00-0.83 (m, 12H,  $\text{CH}_2\text{CH}_3$ ).

$^{13}\text{C}$  NMR (400 MHz,  $\text{CD}_3\text{OD}/\text{CDCl}_3$  19:1)  $\delta$  173.3 and 172.3 (C=O), 157.9 (C=N), 157.8 (C Ar  $_{\text{Pmc}}$ ), 154.8 (C Ar  $_{\text{calix}}$ ), 136.6 and 136.2 (C Ar  $_{\text{Pmc}}$ ), 134.4 and 133.0 (C Ar  $_{\text{calix}}$ ), 125.0 and 122.8 (C Ar  $_{\text{Pmc}}$ ), 122.3 (C Ar  $_{\text{calix}}$ ), 119.4 (C Ar  $_{\text{Pmc}}$ ), 76.7 ( $\text{C}(\text{CH}_3)_2_{\text{Pbf}}$ ), 74.9 ( $\text{OCH}_2$ ), 55.0 ( $\text{COCHNHAc}$ ), 41.5 ( $\text{N}=\text{CNHCH}_2$ ), 33.8 ( $\text{CH}_2_{\text{Pmc}}$ ), 33.4 ( $\text{OCH}_2\text{CH}_2\text{CH}_2$ ), 32.1 ( $\text{ArCH}_2\text{Ar}$ ), 31.5 ( $\text{OCH}_2\text{CH}_2$ ), 30.5 ( $\text{COCHCH}_2$ ), 27.3 ( $\text{N}=\text{CNHCH}_2\text{CH}_2$  and  $\text{CH}_2\text{CH}_2\text{CH}_3$ ), 27.1 ( $\text{C}(\text{CH}_3)_2$ ), 24.0 ( $\text{CH}_2\text{CH}_3$ ), 22.8 ( $\text{CH}_3\text{CO}$ ), 22.4 ( $\text{CH}_2_{\text{Pmc}}$ ), 19.1 and 18.0 ( $\text{CH}_3_{\text{Pmc}}$ ), 14.6 ( $\text{CH}_2\text{CH}_3$ ), 12.4 ( $\text{CH}_3_{\text{Pmc}}$ ).

ESI-MS (m/z):  $[\text{M} + 2\text{Na}]^{2+}$  calcd for  $\text{C}_{140}\text{H}_{204}\text{N}_{20}\text{O}_{24}\text{S}_4$  1362.0, found 1363.1.

### Synthesis of 5,11,17,23-Tetrakis[(N-Ac-L-Arg)amino]-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene, tetrahydrochloride (33)

The compound **32** was deprotected according to the general procedure described in Chapter 2 for protecting group Pbf.

The crude (26 mg) was dissolved in MeOH (5mL) and formic acid (10  $\mu\text{L}$ ), and purified by semi-preparative RP-HPLC using a  $\text{C}_{12}$  column (column: Jupiter 4u Proteo 90A, C-12, 90A, 10 mm  $\times$  250 mm, elution conditions: eluent A 100%  $\text{H}_2\text{O}$  + 0.1% formic acid; eluent B: 100% MeOH + 0.1% formic acid; 100% A over 5 min, 100% A to 31/69 A/B over 5 min, 31/69 to 21/79 A/B over 20 min, at 4 mL/min; retention time: 13.1 min). The fractions containing the pure product were collected and evaporated under reduced pressure. The formate anion of the resulting salt was exchanged by adding 10 mM HCl solution in MeOH (3 $\times$ 5 mL) followed by evaporation under reduced pressure, to give **33** as white powder (1,1mg, 42% yield).

$^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  6.98 (bs, 4H, ArH), 6.62 (bs, 4H, ArH), 4.46 (d,  $J = 13.2$  Hz, 4H,  $\text{ArCH}_{\text{ax}}\text{Ar}$ ), 4.45-4.30 (m, 4H,  $\text{COCHNHAc}$ ), 4.00-3.80 (m, 8H,  $\text{OCH}_2$ ), 3.26-3.08 (m, 12H,  $\text{CH}_2\text{NH}$  and  $\text{ArCH}_{\text{eq}}\text{Ar}$ ), 2.03 (s, 12H,  $\text{CH}_3\text{CO}$ ), 2.02-1.20 (m, 48H,  $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2$  and  $\text{COCHCH}_2\text{CH}_2$ ), 0.95 (t,  $J = 6.0$  Hz, 12H,  $\text{CH}_2\text{CH}_3$ ).

ESI-MS (m/z):  $[\text{M} + 3\text{H} \cdot 4\text{HCl}]^{3+}$  calcd for  $\text{C}_{140}\text{H}_{208}\text{N}_{20}\text{O}_{24}\text{Cl}_4$  538.7, found 539.2.

**Synthesis of 5,11,17,23-Tetrahydroxymethyl-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (34)**

A suspension of **28** (0.91 g, 1.04 mmol) in EtOH abs. (50 mL) was stirred with NaBH<sub>4</sub> (0.24 g, 6.25 mmol) at room temperature for 18 h. The solution was treated with HCl 1 M (20 mL) and then concentrated. The residue was extracted with EtOAc (50 mL). The organic layer was washed with H<sub>2</sub>O (50 mL) and sat. NaHCO<sub>3</sub> (50 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated to give **34** as white solid (642 mg, yield 70%).

Mp: 218.3 – 219.7 °C. <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD) δ 6.66 (s, 8H, ArH), 4.46 (d, *J* = 13.2 Hz, 4H, ArCH<sub>ax</sub>Ar), 4.24 (s, 8H, CH<sub>2</sub>OH), 3.90 (t, *J* = 7.2 Hz, 8H, OCH<sub>2</sub>), 3.15 (d, *J* = 13.2 Hz, 4H, ArCH<sub>eq</sub>Ar), 2.00-1.88 (m, 8H, OCH<sub>2</sub>CH<sub>2</sub>), 1.52-1.35 (m, 24H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 0.95 (t, *J* = 6.6 Hz, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD) δ 156.8, 135.7, 135.4 and 127.9 (C Ar), 76.0 (OCH<sub>2</sub>), 64.8 (CH<sub>2</sub>OH), 33.0(OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 31.7 (ArCH<sub>2</sub>Ar), 31.2 (OCH<sub>2</sub>CH<sub>2</sub>), 27.0 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 23.6 (CH<sub>2</sub>CH<sub>3</sub>), 14.2 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (*m/z*): [M + Na]<sup>+</sup> calcd for C<sub>56</sub>H<sub>80</sub>O<sub>8</sub> 904.1, found 904.1.

**Synthesis of 5,11,17,23-Tetrachloromethyl-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (35)**

To a solution of calixarene **34** (600 mg, 0.68 mmol) in dry DCM (10 mL) SOCl<sub>2</sub> (0.99 mL, 13.60 mmol) was added dropwise and the mixture was stirred for 4 h at room temperature. The progress of reaction was monitored via TLC (eluent DCM/MeOH 9/1). The solvent was evaporated under reduced pressure and the pure product was isolated as yellow solid (622 mg, yield 95%).

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 6.64 (s, 8H, ArH), 4.39 (d, *J* = 13.2 Hz, 4H, ArCH<sub>ax</sub>Ar), 4.29 (s, 8H, CH<sub>2</sub>Cl), 3.86 (t, *J* = 7.6 Hz, 8H, OCH<sub>2</sub>), 3.13 (d, *J* = 13.2 Hz, 4H, ArCH<sub>eq</sub>Ar), 1.95-1.80 (m, 8H, OCH<sub>2</sub>CH<sub>2</sub>), 1.45-1.25 (m, 24H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 1.00-0.85 (m, 12H, CH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 157.0, 135.2, 130.9 and 128.6 (C Ar), 75.4 (OCH<sub>2</sub>), 46.6 (CH<sub>2</sub>Cl), 32.0 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 30.9 (ArCH<sub>2</sub>Ar), 30.2 (OCH<sub>2</sub>CH<sub>2</sub>), 25.9 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 22.8 (CH<sub>2</sub>CH<sub>3</sub>), 14.1(CH<sub>2</sub>CH<sub>3</sub>).

**Synthesis of 5,11,17,23-Tetraazidomethyl-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (36)**

Calix[4]arene **35** (290 mg, 0.30 mmol) was dissolved in dry DMF (15mL) and then NaN<sub>3</sub> (120 mg, 1.82 mmol) was added. The mixture was stirred at room temperature for 20 h. the reaction was quenched with with HCl 1 M (50 mL); AcOEt was added and the organic layer separated, washed with with H<sub>2</sub>O (2×50 mL) and dried over Na<sub>2</sub>SO<sub>4</sub>. Evaporation of the solvent yielded quantitatively pure product **36** as yellowish solid.

Mp: 105.0-105.8 °C; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 6.62 (s, 8H, ArH), 4.45 (d, *J* = 13.2 Hz, 4H, ArCH<sub>ax</sub>Ar), 3.96 (s, 8H, CH<sub>2</sub>N<sub>3</sub>), 3.89 (t, *J* = 7.2 Hz, 8H, OCH<sub>2</sub>), 3.17 (d, *J* = 13.2 Hz, 4H, ArCH<sub>eq</sub>Ar), 2.00-1.85 (m, 8H, OCH<sub>2</sub>CH<sub>2</sub>), 1.48-1.30 (m, 24H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 0.93 (t, *J* = 6.9 Hz, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 156.4, 135.0, 128.5 and 128.4 (C Ar), 75.2 (OCH<sub>2</sub>), 54.0 (CH<sub>2</sub>N<sub>3</sub>), 31.1 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 30.7 (ArCH<sub>2</sub>Ar), 30.1 OCH<sub>2</sub>CH<sub>2</sub>), 25.8 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 22.6 (CH<sub>2</sub>CH<sub>3</sub>), 13.9 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (*m/z*): [M + Na]<sup>+</sup> calcd for C<sub>56</sub>H<sub>76</sub>O<sub>4</sub>Cl<sub>4</sub> 1003.6, found 1004.1.

**Synthesis of 5,11,17,23-Tetraaminomethyl-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene, tetrahydrochloride (37)**

Calix[4]arene **36** (300 mg, 3.1×10<sup>-1</sup> mmol) was dissolved in EtOH/AcOEt (1:1, 50 mL), and a catalytic amount of Pd/C (10%) and HCl 1M (4mL) were added. Hydrogenation was carried out at 2 atm in a Parr reactor for 44 h. The reaction was stopped by catalyst filtration and the solvent removed under reduced pressure. The pure product **37** was isolated as white solid in quantitative yield (307 mg).

Mp: 230 °C dec.; <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD) δ 6.87 (s, 8H, ArH), 4.49 (d, *J* = 13.2 Hz, 4H, ArCH<sub>ax</sub>Ar), 3.92 (t, *J* = 7.2 Hz, 8H, OCH<sub>2</sub>), 3.84 (s, 8H, CH<sub>2</sub>NH<sub>3</sub><sup>+</sup>), 3.31 (under the CD<sub>3</sub>OD signal, 4H, ArCH<sub>eq</sub>Ar) 2.00-1.85 (m, 8H, OCH<sub>2</sub>CH<sub>2</sub>), 1.55-1.30 (m, 24H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 1.00-0.85 (m, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD) δ 157.0, 135.4, 129.2 and 126.7 (C Ar), 75.2 (OCH<sub>2</sub>), 42.6 (CH<sub>2</sub>NH<sub>2</sub>), 31.9 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 30.3 (ArCH<sub>2</sub>Ar), 30.1 (OCH<sub>2</sub>CH<sub>2</sub>), 25.8 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 22.5 (CH<sub>2</sub>CH<sub>3</sub>), 13.1 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (*m/z*): [M +Na-4HCl]<sup>+</sup> calcd for C<sub>56</sub>H<sub>88</sub>N<sub>4</sub>O<sub>4</sub>Cl<sub>4</sub> 899.6, found 899.8.

### Synthesis of 5,11,17,23-Tetrakis([Boc-L-Arg(Pbf)]aminomethyl)-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (38)

The compound **38** was synthesized according to the procedure described in Chapter 2 for compound **3**. The pure product was isolated as white solid in 25% yield.

Mp: 160.0-161.3 °C; <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD) δ 6.53 (bs, 8H, ArH), 4.37 (d, *J* = 11.4 Hz, 4H, ArCH<sub>ax</sub>Ar), 4.15-3.80 (m, 20H, OCH<sub>2</sub>, ArCH<sub>2</sub>NH and NHCOCH), 3.25-2.90 (m, 20H, CH<sub>2</sub>NHCNH, ArCH<sub>eq</sub>Ar and CH<sub>2</sub> Pbf), 2.56 (s, 12H, CH<sub>3</sub> Pbf), 2.50 (s, 12H, CH<sub>3</sub> Pbf), 2.07 (s, 12H, CH<sub>3</sub> Pbf), 1.92 (bs, 8H, OCH<sub>2</sub>CH<sub>2</sub>), 1.80-1.20 (m, 100H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>, COCH(CH<sub>2</sub>)<sub>2</sub>, (CH<sub>3</sub>)<sub>3</sub> Boc and C(CH<sub>3</sub>)<sub>2</sub> Pbf), 1.00-0.80 (m, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD) δ 174.8 and 160.0 (C=O), 158.2 (C=N), 157.9 (C Ar Pbf), 157.1 (C Ar calix), 139.6 (C Ar Pbf), 136.4 (C Ar calix), 134.5 and 133.7 (C Ar Pbf), 133.0 and 129.1 (C Ar calix), 126.2 and 118.6 (C Ar Pbf), 87.8 (C(CH<sub>3</sub>)<sub>2</sub> Pbf), 80.7 (C(CH<sub>3</sub>)<sub>3</sub>), 76.6 (OCH<sub>2</sub>), 55.9 (COCHNHBoc), 44.2 and 44.1 (CH<sub>2</sub> Pbf and ArCH<sub>2</sub>), 41.5 (N=CNHCH<sub>2</sub>), 33.6 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 32.1 (ArCH<sub>2</sub>Ar), 31.7 (OCH<sub>2</sub>CH<sub>2</sub>), 30.9 (COCHCH<sub>2</sub>), 29.0 and 28.9 (C(CH<sub>3</sub>)<sub>2</sub> and C(CH<sub>3</sub>)<sub>3</sub>), 27.5 (COCHCH<sub>2</sub>CH<sub>2</sub>), 27.4 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 24.2 (CH<sub>2</sub>CH<sub>3</sub>), 19.9 and 18.7 (CH<sub>3</sub> Pbf), 14.7 (CH<sub>2</sub>CH<sub>3</sub>), 12.8 (CH<sub>3</sub> Pbf).

ESI-MS (*m/z*): [M + 2Na]<sup>2+</sup> calcd for C<sub>152</sub>H<sub>228</sub>O<sub>28</sub>N<sub>20</sub>S<sub>4</sub> 1477.9, found 1478.4, [M + 3Na]<sup>3+</sup> calcd 993.0, found 993.3.

### Synthesis of 5,11,17,23-Tetrakis([L-Arg]aminomethyl)-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (39)

The compound **38** was deprotected according to the general procedure described in Chapter 2. The pure product **39** was isolated as white solid in quantitative yield.

Mp: >187 °C dec; <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD) δ 6.68 (bs, 8H, ArH), 4.42 (d, *J* = 13.0 Hz, 4H, ArCH<sub>ax</sub>Ar), 4.18 (d, *J* = 13.6 Hz, 4H, ArCHNH), 4.08-3.90 (m, 8H, ArCHNH and COCHNH<sub>3</sub><sup>+</sup>), 3.87 (t, *J* = 7.4 Hz, 8H, OCH<sub>2</sub>), 3.26 (bs, 8H, CH<sub>2</sub>NHCNH), 3.14 (d, *J* = 13.0 Hz, 4H, ArCH<sub>eq</sub>Ar), 2.05-1.80 (bs, 16H, OCH<sub>2</sub>CH<sub>2</sub> and COCHCH<sub>2</sub>), 1.74 (bs, 8H, COCHCH<sub>2</sub>CH<sub>2</sub>), 1.50-1.25 (m, 24H, O(CH<sub>2</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>), 1.00-0.85 (m, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD) δ 169.6 (C=O), 158.7 (C=N), 157.3, 136.4, 132.6 and 129.4 (C Ar), 76.5 (OCH<sub>2</sub>), 54.2 (COCHNH<sub>3</sub><sup>+</sup>), 44.3 (ArCH<sub>2</sub>), 41.9 (N=CNHCH<sub>2</sub>), 33.4 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 31.9 (ArCH<sub>2</sub>Ar), 31.5 (OCH<sub>2</sub>CH<sub>2</sub>), 29.8 (COCHCH<sub>2</sub>), 27.4 4 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 25.4 (COCHCH<sub>2</sub>CH<sub>2</sub>), 24.0 (CH<sub>2</sub>CH<sub>3</sub>), 14.5 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (*m/z*): [M + 3H – 8HCl]<sup>3+</sup> calcd for C<sub>80</sub>H<sub>140</sub>N<sub>20</sub>O<sub>8</sub>Cl<sub>8</sub> 501.1, found 501.4.

**Synthesis of  $N_{\alpha}$ -Boc- $N_{\omega}$ -Pbf-L-arginine- $N$ -propargylamide (40)** (procedure similar to ref. <sup>8</sup>)

$N_{\alpha}$ -Boc- $N_{\omega}$ -Pbf-L-arginine (1g, 1.9 mmol), DIPEA (0.78 mL, 2.28 mmol) and HBTU (865 mg, 2.28 mmol) were dissolved in dry DMF (15 mL) under Ar atmosphere. After stirring for 30 min at room temperature, propargylamine (0.146 mL, 2.28 mmol) was added and the mixture was stirred for 15 h. The solvent was removed under reduced pressure; ethyl acetate was added to the residue and washed with water and NaHCO<sub>3</sub> sat. The organic layer was dried over MgSO<sub>4</sub>, evaporated and purified through flash column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 97:3), then a white solid (751 mg, yield 70%) was isolated.

M.p.: 125.5-125.9°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 7.34 (bs, 1H, NHCO), 6.27 (m, 3H, NH-guanidine), 5.59 (bs, 1H, NHBoc), 4.20 (bs, 1H, COCHNH), 3.99 (m, 2H, CH<sub>2</sub>CCH), 3.27 (bs, 2H, N=CNHCH<sub>2</sub>), 2.96 (s, 2H, CH<sub>2</sub> Pbf), 2.51 (s, 3H, CH<sub>3</sub> Pbf), 2.50 (s, 3H, CH<sub>3</sub> Pbf), 2.18 (t,  $J = 2.5$  Hz, CH<sub>2</sub>CCH), 2.09 (s, 3H, CH<sub>3</sub> Pbf), 1.81 (bs, 2H, COCHCH<sub>2</sub>), 1.60 (bs, 2H, COCHCH<sub>2</sub>CH<sub>2</sub>), 1.46 (s, 6H, C(CH<sub>3</sub>)<sub>2</sub> Pbf), 1.41 (s, 9H, C(CH<sub>3</sub>)<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 165.9 (C=O), 159.0 (C Ar), 156.8 (C=N), 156.1 (C=O), 138.3 - 117.6 (C Ar), 86.4 (C(CH<sub>3</sub>)<sub>2</sub>), 80.2 (C(CH<sub>3</sub>)<sub>3</sub>), 79.6 (C alkyne), 71.4 (CH alkyne), 60.4 (COCHNHBoc), 43.2 (CH<sub>2</sub> Pbf and N=CNHCH<sub>2</sub>), 29.9 (COCHCH<sub>2</sub>), 29.1 (COCHCH<sub>2</sub>CH<sub>2</sub>), 28.6 (C(CH<sub>3</sub>)<sub>2</sub>), 28.3 (C(CH<sub>3</sub>)<sub>3</sub>), 19.3, 17.9 and 12.5 (CH<sub>3</sub> Pbf).

ESI-MS (m/z): [M + Na]<sup>+</sup> calcd for C<sub>27</sub>H<sub>41</sub>N<sub>5</sub>O<sub>6</sub>S 586.3, found 586.3.

### Synthesis of 41

To a solution of **36** (35mg, 35.7 μmol) in acetone (5 mL), compound  $N_{\alpha}$ -Boc- $N_{\omega}$ -Pbf-L-arginine- $N$ -propargylamide (104.5 mg, 0.185 mmol), DIPEA (25 μL, 0.143 mmol) and CuI\*P(EtO)<sub>3</sub> (5.1 mg, 14.3 μmol) were added and the reaction mixture was refluxed for 24 h. The solvent was removed under reduced pressure and the residue was purified by flash chromatography (gradient CH<sub>2</sub>Cl<sub>2</sub>/MeOH from 99:1 to 9:1); then a white solid (82 mg, 71%) was isolated.

<sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD) δ 7.69 (bs, 4H, CH triazole), 6.49 (bs, 8H, ArH), 5.16 (bs, 8H, triazol-CH<sub>2</sub>-Arg), 4.42-4.20 (m, 12H, ArCH<sub>ax</sub>Ar and ArCH<sub>2</sub>), 3.91 (bs, 4H, COCHNHBoc), 3.72 (t,  $J=7.4$  Hz, 8H, OCH<sub>2</sub>), 3.11-2.92 (m, 12H, ArCH<sub>eq</sub>Ar and N=CNHCH<sub>2</sub>), 2.85 (s, 8H, CH<sub>2</sub> Pbf), 2.44 (s, 12H, CH<sub>3</sub> Pbf), 2.39 (s, 12H, CH<sub>3</sub> Pbf), 1.94 (s, 6H, CH<sub>3</sub> Pbf), 1.86-1.72 (m, 8H, OCH<sub>2</sub>CH<sub>2</sub>), 1.68-1.35 (m, 16H, COCHCH<sub>2</sub>CH<sub>2</sub>), 1.35-1.12 (m, 84H,

O(CH<sub>2</sub>)<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>, C(CH<sub>3</sub>)<sub>3</sub> and C(CH<sub>3</sub>)<sub>2</sub> Pbf), 0.90-0.74 (m, 12H, CH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD) δ 173.5 (C=O), 158.4 (C=N), 156.4 (C Ar), 156.2 (C=O), 145.0 (C triazole), 138.0 (C Ar Pbf), 135.1 (C Ar calix), 132.1 (C Ar Pbf), 128.8 (C Ar Pbf), 128.0, 127.9 (C Ar Pbf), 124.6 (C Ar calix), 122.7 (C Ar Pbf), 122.6 (CH triazole), 117.0 (C Ar calix), 86.2 (C(CH<sub>3</sub>)<sub>2</sub> Pbf), 79.1 (C(CH<sub>3</sub>)<sub>3</sub>), 75.1 (OCH<sub>2</sub>), 54.2 (COCHNHBoc), 53.3 (triazol-CH<sub>2</sub>-arginine), 42.4 (CH<sub>2</sub> Pbf), 39.8 (N=CNHCH<sub>2</sub>), 34.3 (ArCH<sub>2</sub>), 31.8 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 30.1 (ArCH<sub>2</sub>Ar), 30.0 (OCH<sub>2</sub>CH<sub>2</sub>), 29.0 (COCHCH<sub>2</sub>), 27.2 (C(CH<sub>3</sub>)<sub>2</sub>, C(CH<sub>3</sub>)<sub>3</sub>), 25.8 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 25.5 (COCHCH<sub>2</sub>CH<sub>2</sub>), 22.5 (CH<sub>2</sub>CH<sub>3</sub>), 18.2, 17.0 (CH<sub>3</sub> Pbf), 13.0 (CH<sub>2</sub>CH<sub>3</sub>), 11.1 (CH<sub>3</sub> Pbf).

ESI-MS (m/z): [M + 2Na]<sup>2+</sup> calcd for C<sub>164</sub>H<sub>240</sub>N<sub>32</sub>O<sub>28</sub>S<sub>4</sub> 1639.9, found 1640.4.

## Synthesis of 42

The compound **41** was deprotected according to the general procedure described in Chapter 2. The pure product **42** was isolated as white solid in quantitative yield.

<sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD) δ 8.21 (bs, 4H, CH triazole), 6.71 (bs, 8H, ArH), 5.39 (bs, 8H, triazol-CH<sub>2</sub>-Arg), 4.72-4.33 (m, 12H, ArCH<sub>ax</sub>Ar and ArCH<sub>2</sub>), 4.00 (bs, 4H, COCHNH), 3.85 (t, J=7.1 Hz, 8H, OCH<sub>2</sub>), 3.27-3.08 (m, 12H, ArCH<sub>eq</sub>Ar and N=CNHCH<sub>2</sub>), 2.08-1.82 (m, 16H, OCH<sub>2</sub>CH<sub>2</sub> and COCHCH<sub>2</sub>), 1.78-1.61 (m, 8H, COCHCH<sub>2</sub>CH<sub>2</sub>), 1.52-1.25 (m, 24H, O(CH<sub>2</sub>)<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 1.03-0.84 (m, 12H, CH<sub>2</sub>CH<sub>3</sub>).

<sup>13</sup>C NMR (75 MHz, CD<sub>3</sub>OD) δ 170.1 (C=O), 158.6 (C=N), 158.2 and 137.0 (C Ar), 136.8 (C triazole), 130.0 and 129.8 (C Ar), 129.7 (CH triazole), 76.6 (OCH<sub>2</sub>), 55.6 (COCHNH<sub>3</sub><sup>+</sup>), 54.1 (triazol-CH<sub>2</sub>-arginine), 41.8 (N=CNHCH<sub>2</sub>), 35.5 (ArCH<sub>2</sub>), 33.3 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 31.6 (ArCH<sub>2</sub>Ar), 31.5 (OCH<sub>2</sub>CH<sub>2</sub>), 29.6 (COCHCH<sub>2</sub>), 27.3 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 25.4 (COCHCH<sub>2</sub>CH<sub>2</sub>), 24.0 (CH<sub>2</sub>CH<sub>3</sub>), 14.5 (CH<sub>2</sub>CH<sub>3</sub>).

ESI-MS (m/z): [M + Na – 8HCl]<sup>+</sup> calcd for C<sub>92</sub>H<sub>160</sub>N<sub>32</sub>O<sub>12</sub>Cl<sub>8</sub> 1841.3, found 1841.4.

**DNA preparation and storage.** Plasmid DNA (pEGFP-C1) was purified through cesium chloride gradient centrifugation (Maniatis, T., Fritsch, E. F., and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Laboratory, New York). A stock solution of the plasmid 0.35 mM in milliQ water (Millipore Corp., Burlington, MA) was stored at -20 °C.

**Fluorescence studies.** Ethidium Bromide Displacement Assays (excitation at 530 nm, emission at 600 nm) were performed collecting on a PerkinElmer LS 55 the emission spectra of buffer solutions (4 mM Hepes, 10 mM NaCl, pH 7.4) of 50 mM ethidium bromide (relative fluorescence = 0), mixture of 0.5 nM plasmid DNA (pEGFP-C1) and 50 mM ethidium bromide (relative fluorescence = 1) and after addition of increasing amounts of ligand. Experiments with Nile Red were performed on the same instrument (excitation at 530 nm) in buffer solutions (4 mM Hepes, 10 mM NaCl, 2 mM MgCl<sub>2</sub>, pH = 7.4) of the dye (0.2 mM) and mixture of the dye with ligand (2 mM) and pEGFP-C1 DNA (0.5 nM).

**Sample preparation and AFM imaging.** DNA samples were prepared by diluting the plasmid DNA to a final concentration of 0.5 nM in deposition buffer (4 mM Hepes, 10 mM NaCl, 2 mM MgCl<sub>2</sub>, pH = 7.4) either in the presence or absence of ligands. When needed, ethanol at a defined concentration was added to the deposition buffer prior to addition of DNA and calixarenes. The mixture was incubated for 5 min at room temperature, then a 20 mL droplet was deposited onto freshly-cleaved ruby mica (Ted Pella, Redding, CA) for 1.5 min. The mica disk was rinsed with milliQ water and dried with a weak nitrogen stream. AFM imaging was performed on the dried sample with a Nanoscope IIIA Microscope (Digital Instruments Inc. Santa Barbara, CA) operating in tapping mode. Commercial diving board silicon cantilevers (NSC-15 Micromash Corp., Estonia) were used. Images of 512×512 pixels were collected with a scan size of 2 mm at a scan rate of 3-4 lines per second and were flattened after recording using Nanoscope software.

**Measurement of the Size of the Complexes by Dynamic Light Scattering (DLS) and of the  $\zeta$ -Potential.** The average sizes of the calix-pDNA complexes were measured using a Zetasizer nano with the following specification: sampling time, automatic; number of measurements, 3 per sample; medium viscosity, 1.054 cP; refractive index, 1.33; scattering angle, 173°;  $\lambda$  = 633 nm; temperature, 25°C. Data were analyzed using the multimodal number distribution software included in the instrument. Results are given as volume distribution of the major population by the mean diameter with its standard deviation. Zeta-potential measurements were made using the same apparatus with “mixed-mode measurement” phase analysis light scattering (M3-PALS). M3-PALS consists of both slow field reversal and fast field reversal measurements, hence the name “mixed-mode measurement”; it improves accuracy and resolution. The following specifications

were applied: sampling time, automatic; number of measurements, 3 per sample; medium viscosity, 1.054 cP; medium dielectric constant, 80; temperature, 25°C. Before each series of experiments, the performance of the instruments was checked with either a 90 nm monodisperse latex beads (Coulter) for DLS or with DTS 50 standard solution (Malvern) for  $\zeta$  potentials.

**Transmission Electron Microscopy (TEM).** Formvar-carboncoated grids were placed on top of small drops of the calix-pDNA complex (HEPES 20 mM, pH 7.4, DNA 60  $\mu$ M phosphate). After 1-3 min of contact, grids were negatively stained with a few drops of 1% aqueous solution of uranyl acetate. The grids were then dried and observed using an electron microscope working under standard conditions.

**Cell culture and transient transfection assay.** RD-4 [human Rhabdomyosarcoma cell line (obtained from David Derse, National Cancer Institute, Frederick, Maryland)], was grown in EMEM medium containing NEAA, 10% FBS, 2 mM L-glutamine, 100 IU/mL penicillin and 100 mg/mL streptomycin. All cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were subcultured to a fresh culture vessel when growth reached 70-90% confluence (i.e. every 3-5 days) and incubated at 37 °C in a humidified atmosphere of 95% air-5% CO<sub>2</sub>. Transfections were performed in 24 well plates, when cells were 80% confluent (approximately  $5 \times 10^4$  cells) on the day of transfection. 2.5 mg of plasmid and different concentrations of ligands were added to 1 mL of serum-free medium (DMEM, 2 mM L-glutamine and 50  $\mu$ g/ml), mixed rapidly and incubated at room temperature for 20 min. When used, serum was added at this point to the transfection solution. Following the removal of the culture medium from the cells, 0.5 mL of transfection mixture were carefully added to every well. Lipoplex formulations with helper lipid were prepared adding a 2 mM ethanol solution of DOPE to plasmid-ligand mixture at 1:2 ligand:DOPE molar ratio, where ligand concentration was kept to 10  $\mu$ M. These solutions administered to the cells were completely clear and homogeneous. LTX™ transfection reagent was used according to manufacturer's protocol as positive transfection control. The mixture and cells were incubated at 37 °C in a humidified atmosphere of 95% air-5% CO<sub>2</sub> for 5 h. Finally, transfection mixture was removed and 1 mL of growth medium added to each transfected well and left to incubate for 72 h. Five fields were randomly selected from each well without viewing the cells (one in the centre

and one for each quadrant of the well) and examined. The transfected cells were observed under fluorescence microscope for EGFP expression. Each experiment was done three times. Statistical differences between treatments were calculated with Student's test and multifactorial ANOVA.

### 3.5 References

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