# Chapter 3

# Towards the understanding of the factors affecting cell transfection by calix[4]arene guanidinium derivatives

# 3.1 Results

## 3.1.1 Synthesis and properties of structural variants of 5b

The results described in **Chapter 2** clearly indicate that compound **5b** (**Fig. 3.1**) is the best calix[4]arene based vector for cell transfection synthesized so far.

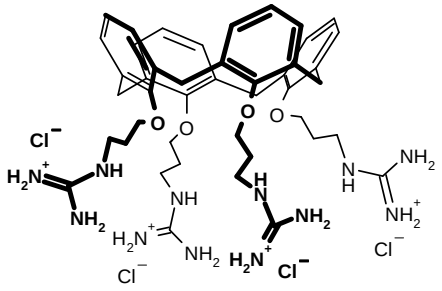


**Fig. 3.1**. Structural formula of compound **5b**.

In order to better understand the factors affecting DNA condensation and cell transfection in this series of ligands, several structural variations were performed, by synthesizing several new ligands (**Fig. 3.2**), whose properties are described in this chapter. First, compound **32** having longer alkyl chains compared to **5b** was synthesized, to study the influence of the distance between the macrocyclic cavity and the positively charged head groups. The synthetic strategy is similar to that followed for **5b** but the alkylation was carried out with N-(6-bromohexyl)phthalimide.

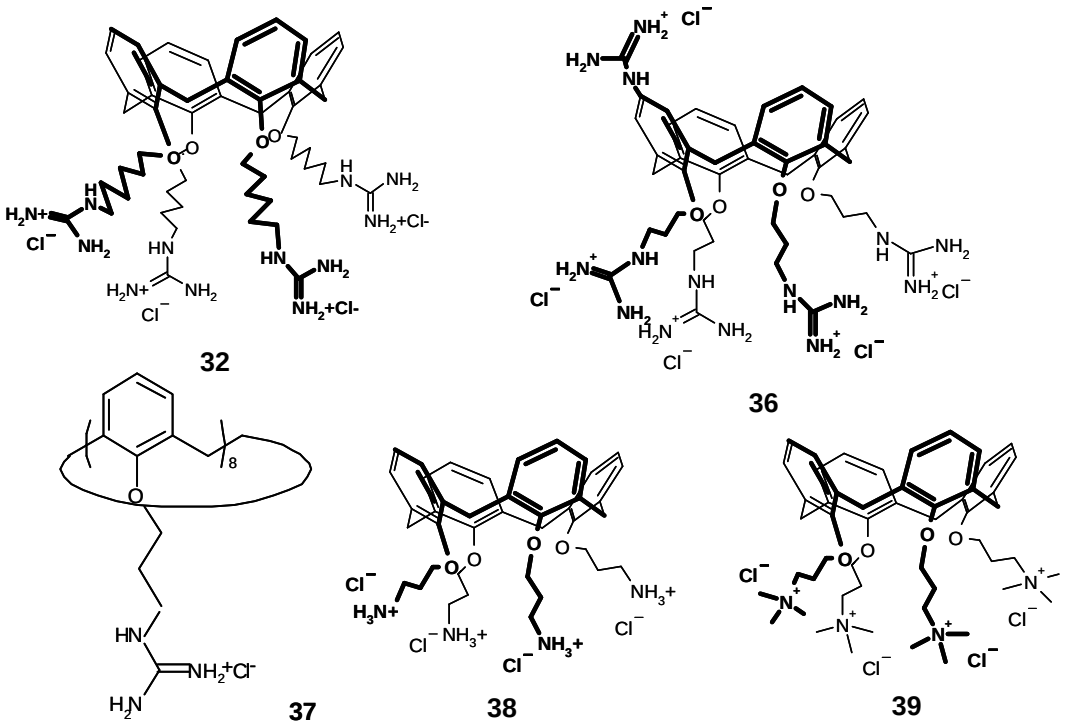
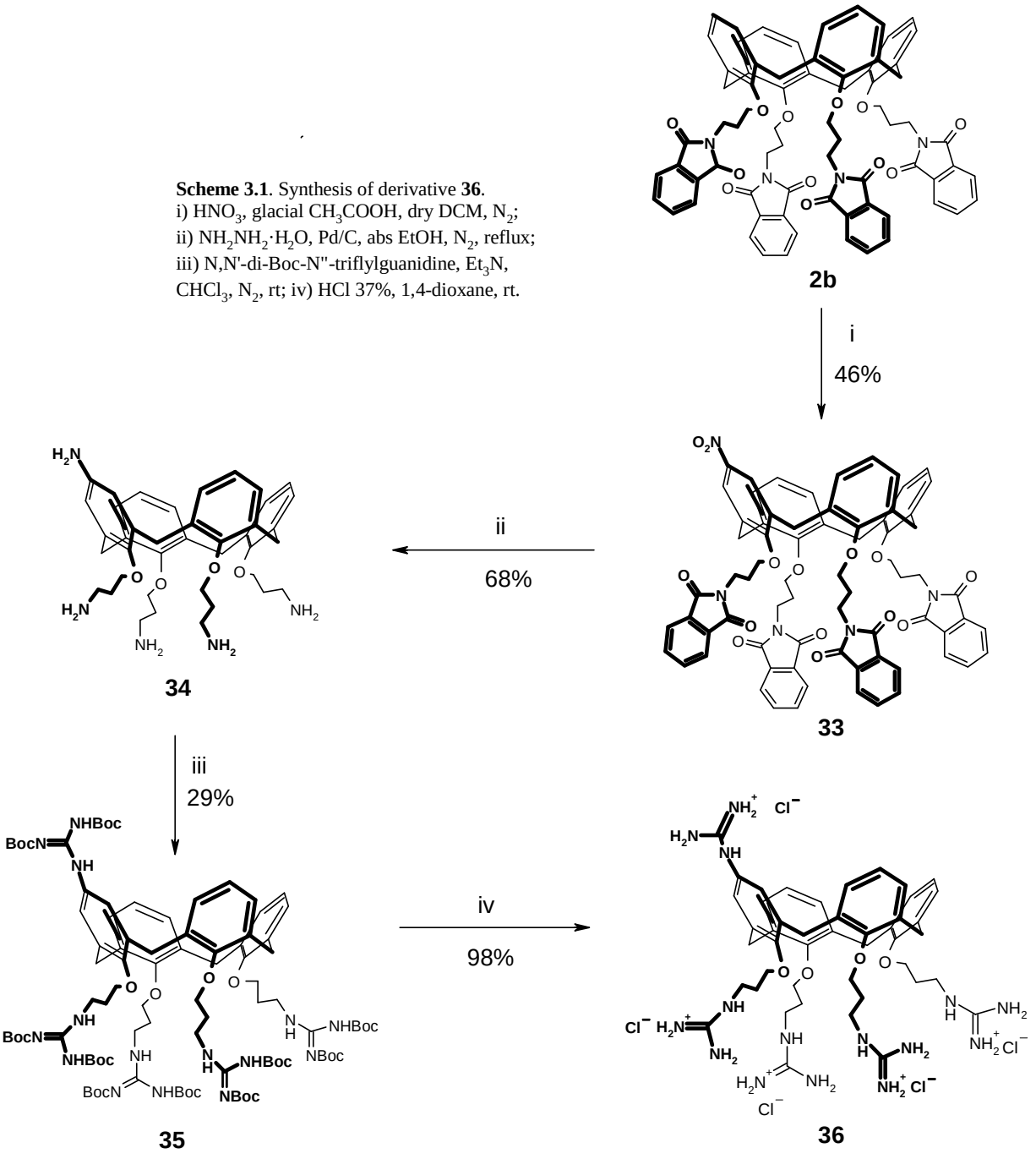



**Fig. 3.2**. Structural formulas of compounds **32**, and **36**-**39**.

As further modification, it was decided to introduce an additional positive charge at the upper rim (see compound **36**), maintaining constant all the other structural characteristics of **5b**.




**Scheme 3.1**. Synthesis of derivative **36**.
i) $HNO_3$, glacial $CH_3COOH$, dry DCM, $N_2$;
ii) $NH_2NH_2 \cdot H_2O$, Pd/C, abs EtOH, $N_2$, reflux;
iii) N,N'-di-Boc-N''-triflylguanidine, $Et_3N$, $CHCl_3$, $N_2$, rt; iv) HCl 37%, 1,4-dioxane, rt.

For this purpose, after the introduction of the phthalimido-propyl terminating chains, a mono-nitration[1] reaction was carried out (**Scheme 3.1**), using $HNO_3$ (65%) and glacial $CH_3COOH$ in $CH_2Cl_2$. The reaction is known not to be particularly selective since all the calixarene aromatic rings have the same reactivity, but, at least, the phthalimide groups are stable under these conditions. Thus, in order to isolate a satisfying amount of the mononitro derivative **33**,

the reaction was carefully followed by TLC and stopped when all the starting calixarene **2b** disappeared and before a significant amount of polinitration products formed, especially the dinitro derivative. Anyway, a mixture of products was obtained, which was purified by flash chromatography. In the following step the phthalimido groups were removed and simultaneously the nitro group at the upper rim was reduced to amine. In fact, using $NH_2NH_2 \cdot H_2O$ in abs EtOH in presence of Pd/C 10% as catalyst, it was possible to remove the phthalimido group and reduce the nitro group, in a single step. Even in this case, however, the work-up had to be done very quickly to avoid carbonatation of the amino group at the lower rim of **34**. The reaction with hydrazine occurred in 12 h and its yield was not quantitative probably because some of the product was lost into water. The following reaction was the functionalisation of all the amino groups with Boc protected guanidine, using N,N'-di-Boc-N"-triflylguanidine in $CHCl_3$ and in presence of $Et_3N$, in order to allow also the reaction of the weakly nucleophilic aromatic amino group. The progression of the reaction was monitored by TLC, using ninhydrin, to verify the presence of compounds having free amino groups. After 48 h the reaction was complete and the pure compound **35** was isolated by flash chromatography. The last step to obtain **36** was the deprotection of guanidines from Boc groups, always carried out with HCl 37% in dioxane.

Using similar synthetic methodologies developed for the cone conformer **5b**, we also synthesized the larger and conformationally mobile calix[8]arene derivative **37** (**Fig. 3.2**), starting from native calix[8]arene. In the last step an HPLC purification was necessary, because a by-product originated after the alkylation reaction was impossible to be removed before.


**Scheme 3.2**. Synthesis of compound **39**.

Moving further towards a better understanding of the contribution of each structural element to the transfection efficiency of **5b**, the cationic part was modified to see if it must have some

specific characteristics. For this purpose, the aminocalixarene **3b** was fully protonated by 2 M HCl to give **38** (**Fig. 3.2**), which has positive charges dependent on the pH. On the other hand, permethylation of **3b** (**Scheme 3.2**) gave compound **39** having four permanently charged tetraalkyl ammonium head groups.

The permethylation reaction was initially performed in the presence of an organic base ($But_3N$) as reported in literature,[2] but it was not complete even warming at 40 °C for several days. More successful was the reaction performed in the presence of a relatively weaker base ($KHCO_3$) as reported by Chen and Benoiton.[3] After several days of stirring at room temperature, following the reaction by ESI-MS and performing the final $I^-/Cl^-$ exchange using a DOWEX 11 resin (Cl form), it was possible to isolate **39** in almost quantitative yield.

The $^1H$ NMR spectra in $D_2O$ of compounds **32**, **36**, **37**, **38**, **39** (**Fig. 3.3**, **3.4**, **3.5**, **3.6** and **3.7** respectively) at room temperature are sharp (**32** and **36** at least up to 100 μM, **38** and **39** at least up to 1 mM). For calix[8]arene **37** broadening occurred around 5 mM, due to aggregation phenomena and not to conformational problems since by dilution to 1 mM the peaks become sharp (**Fig. 3.5**). So, also for these compounds at the concentration ($10^{-5}$ M) used in the following studies, we did not consider any aggregation of our ligands in water solution.

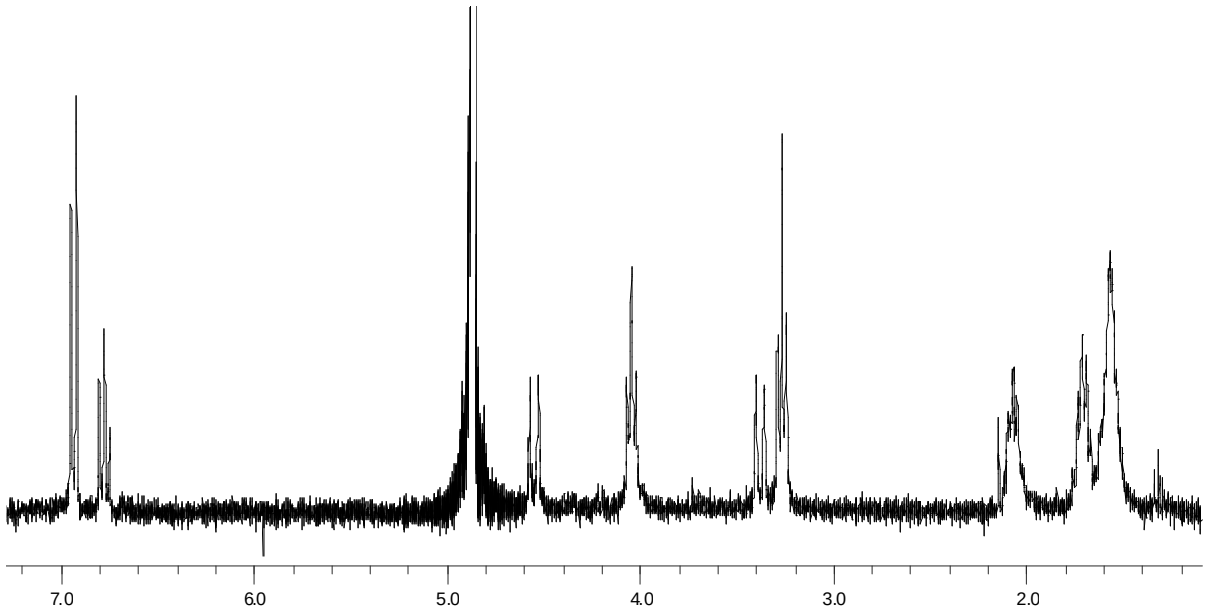



**Fig. 3.3**. $^1H$ NMR spectrum ($D_2O$, 300 MHz, 298 K) of **32**.

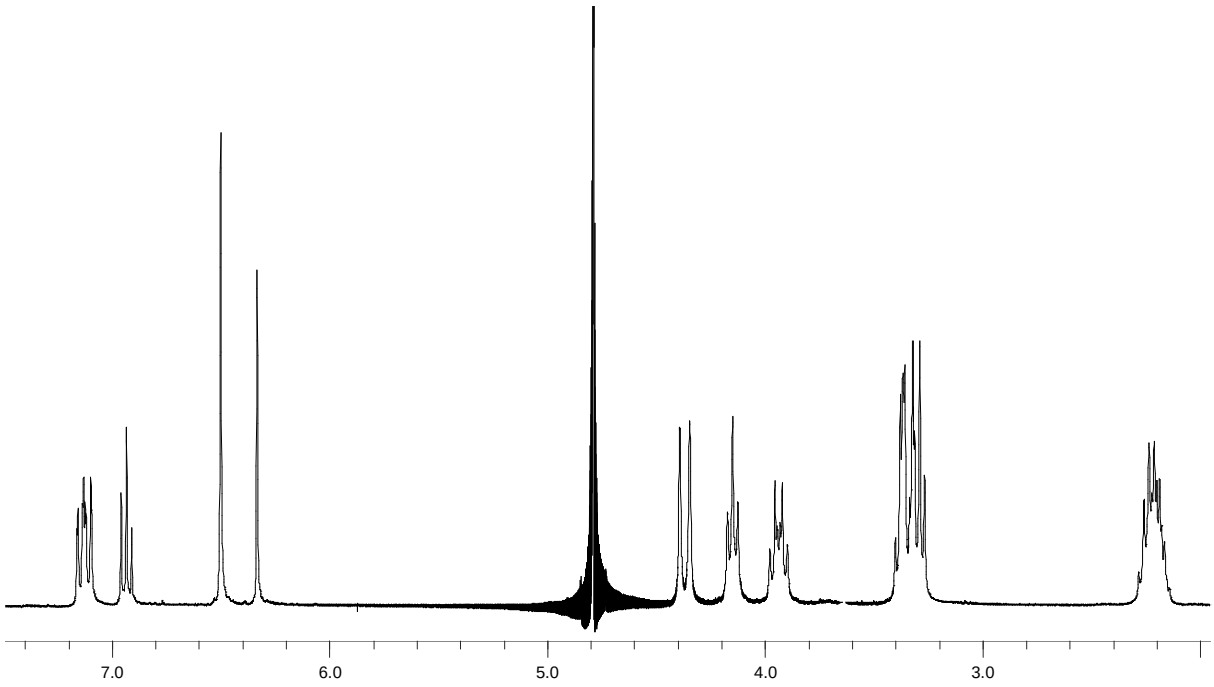



**Fig. 3.4**. $^{1}$H NMR spectrum ($D_2O$, 300 MHz, 298 K) of **36** 100 μM.

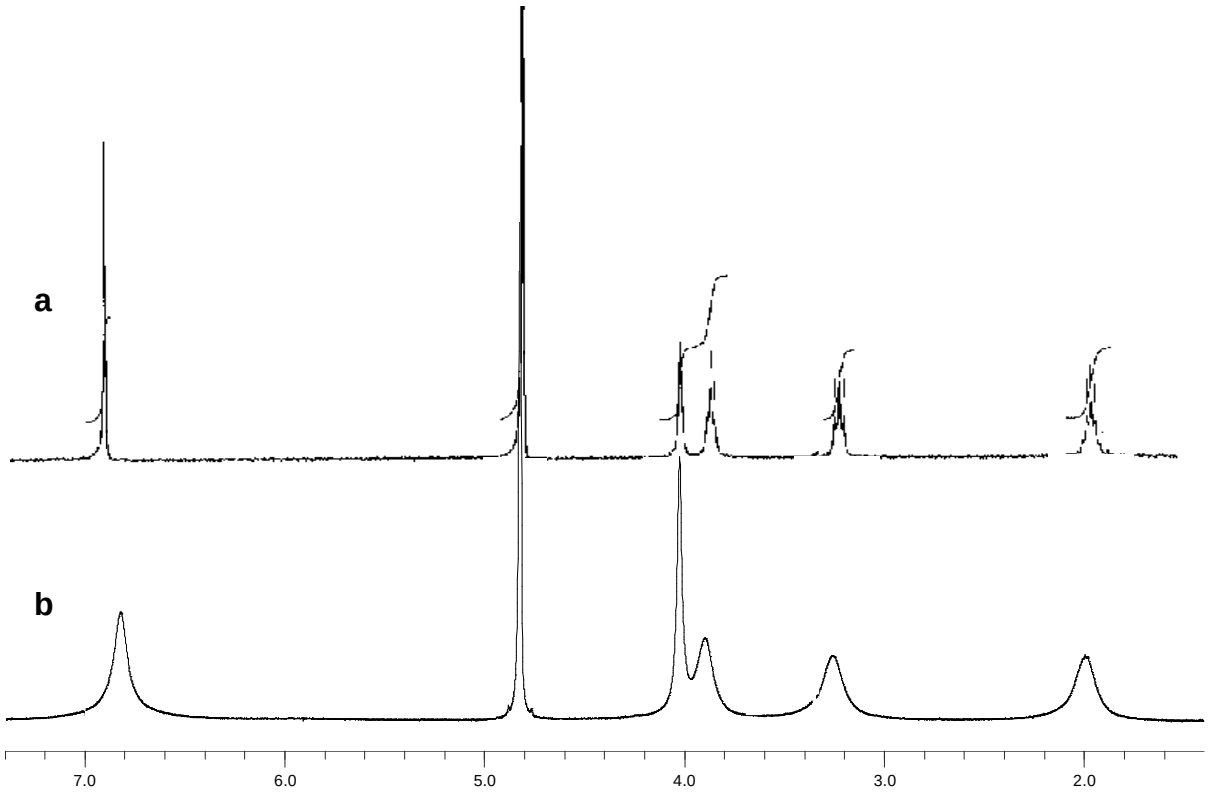



**Fig. 3.5**. $^{1}$H NMR spectrum ($D_2O$, 300 MHz, 298 K) of **37** a) 0.8 mM and b) 7.8 mM.

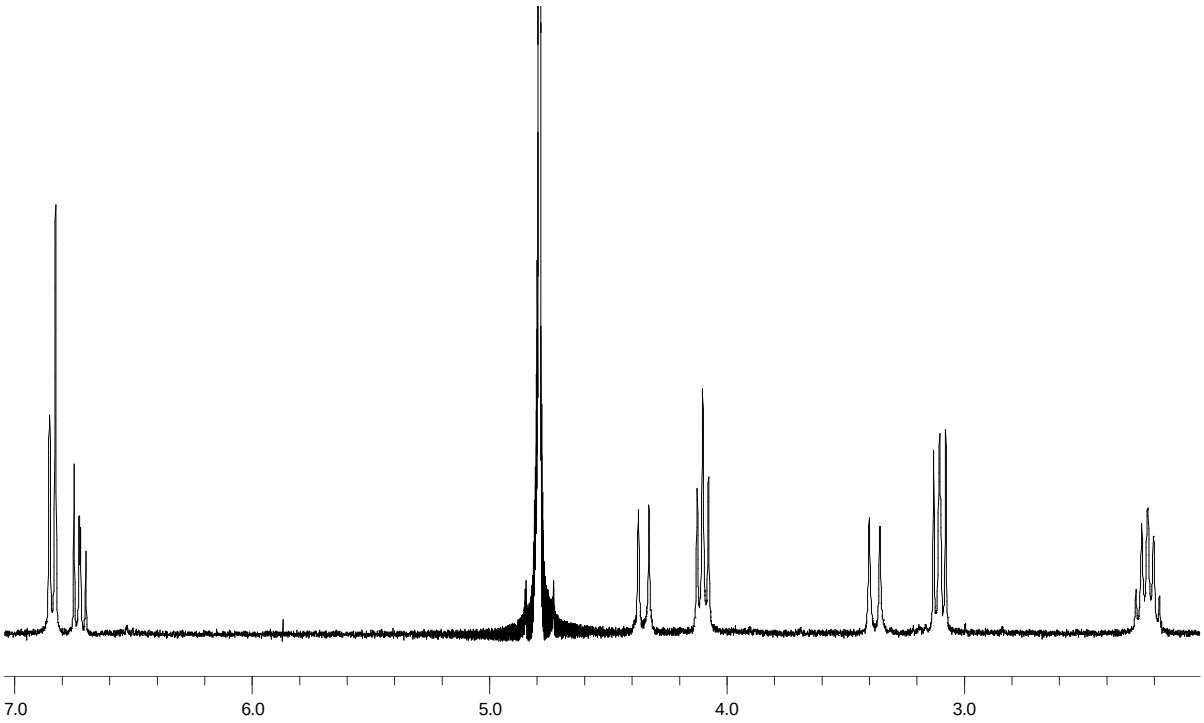



**Fig. 3.6**. $^{1}$H NMR spectrum ($D_2O$, 300 MHz, 298 K) of **38** 1 mM.

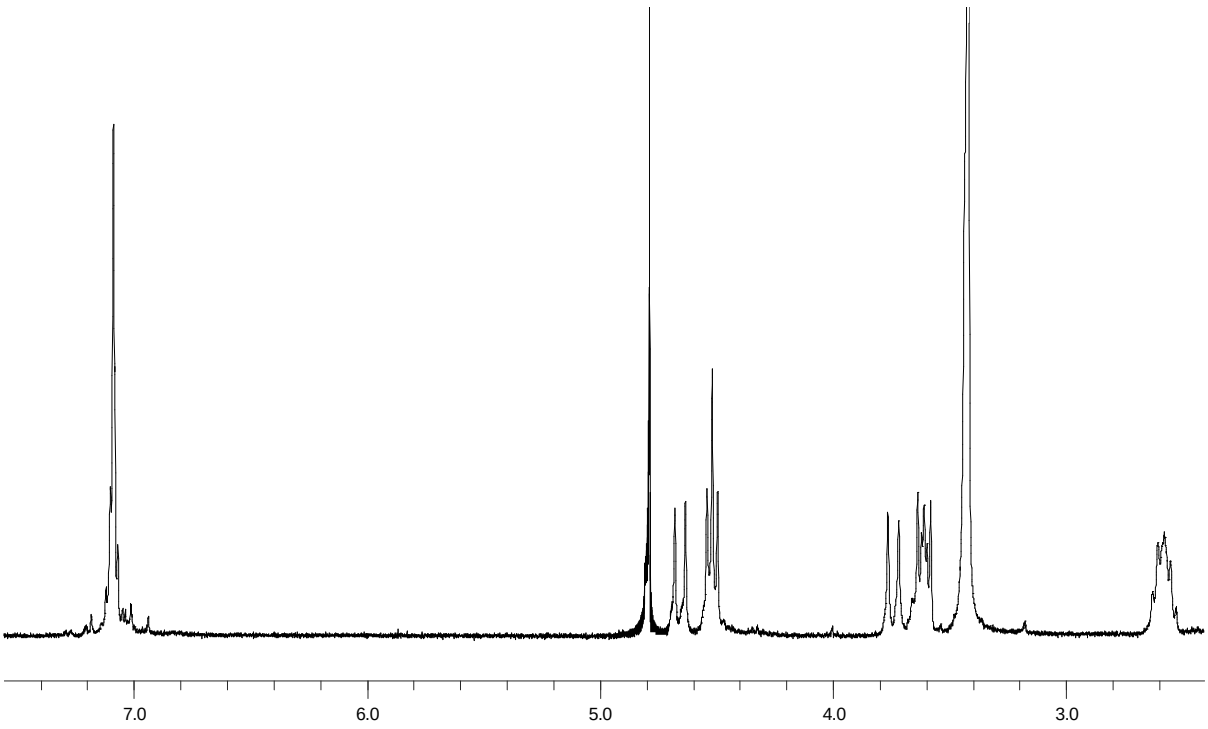



**Fig. 3.7**. $^{1}$H NMR spectrum ($D_2O$, 300 MHz, 323 K) of **39** 1 mM.

## 3.1.2 Fluorescent guanidinium calix[4]arenes

Another important structural modification was the introduction of a fluorophore at the upper rim of the calix[4]arene **5b** to follow this vector into the cell by fluorescence or confocal microscopy and better understand its fate and the mechanism determining the transfection efficiency, even after the DNA release inside the cell. The compounds **48** and **50** were therefore synthesized. The coumarin was chosen as fluorophore (displayed in blue) because the wavelength of its emission should not interfere with the absorbance and emission of the EGFP (displayed in green), expressed in our cells after the transfection experiments.

With the same purpose also ligand **55** was synthesized, as a fluorescent analogue of the previously reported upper rim guanidinium calixarenes which also showed transfection activity.[4]

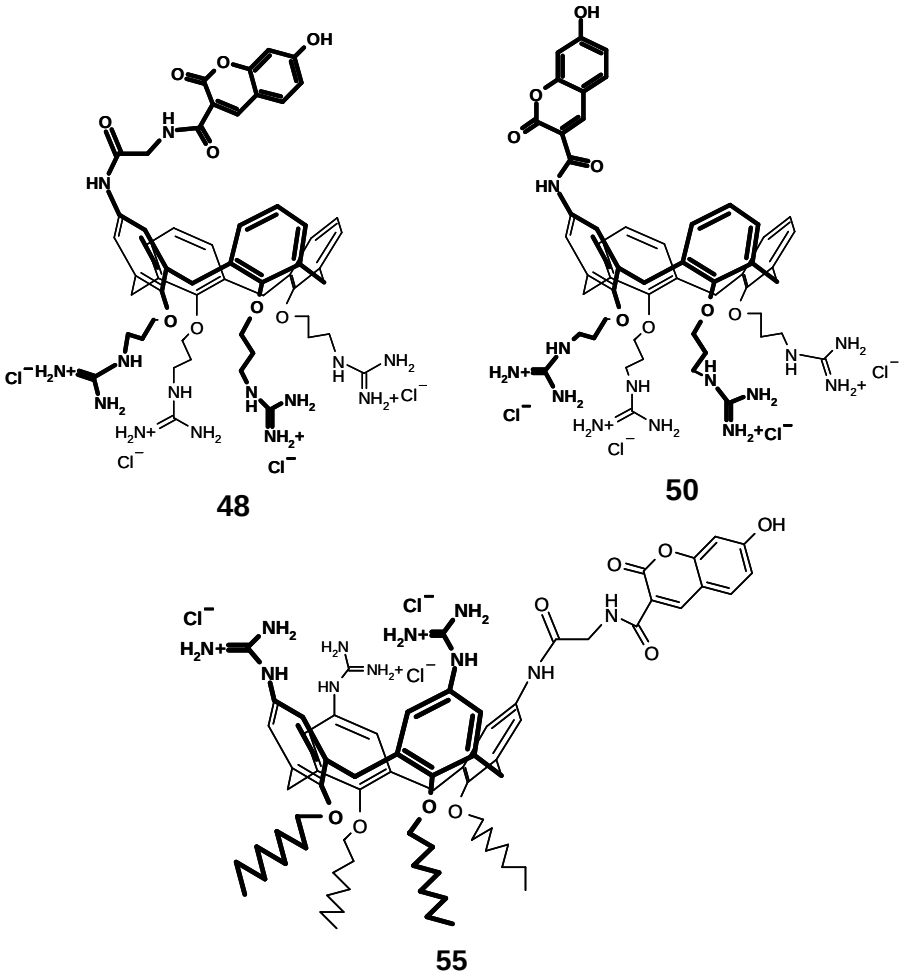
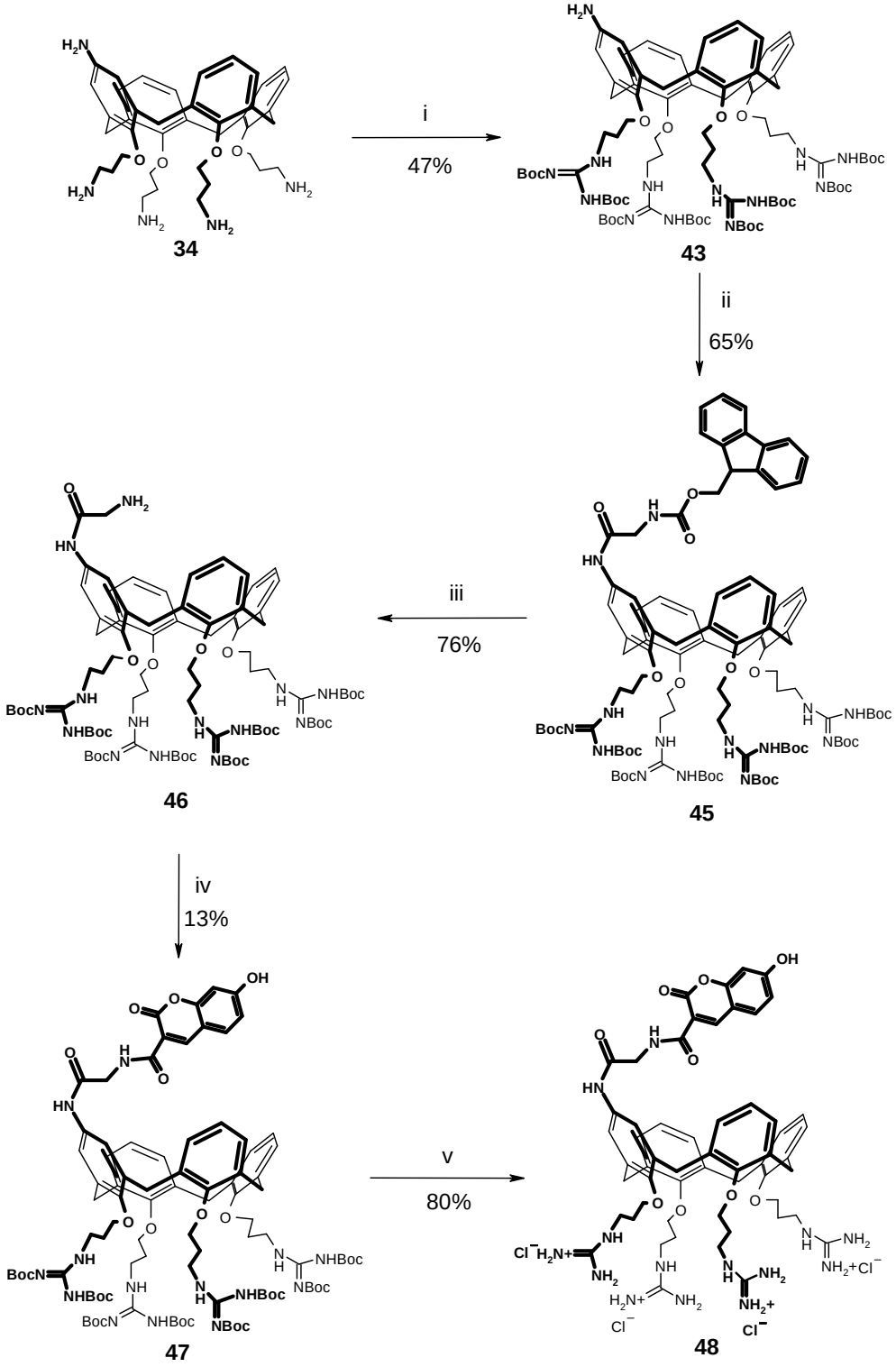



**Fig. 3.8**. Structural formulas of the fluorescent-calix[4]arenes **48**, **50**, **55**.

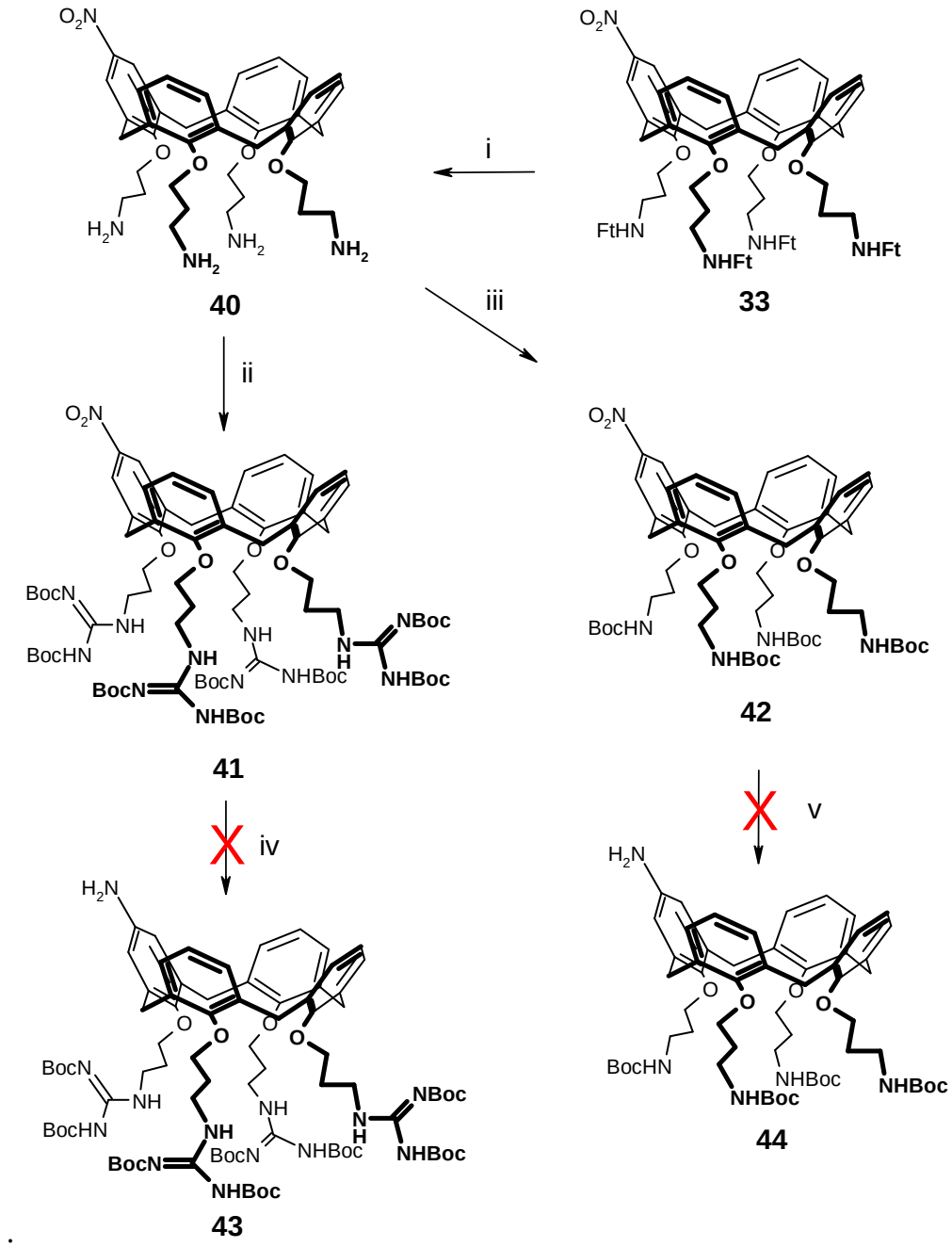
The lower rim guanidinium compounds **48** and **50** differ only for a glycine unit, used in **48** as a spacer between the cavity and the fluorophore to modify its mobility and verify a possible influence of the distance from the cavity in the photophysical properties.

To obtain compound **48** the pentamine **34** was used as starting material, synthesized through the reaction sequence outlined in **Scheme 3.1**.




**Scheme 3.3**. Synthesis of **48**. i) N,N'-di-Boc-N''-triflylguanidine, $CHCl_3$, $N_2$, rt; ii) Fmoc-Gly-OH, HOBt, DCC, dry DMF, $N_2$, rt; iii) piperidine 20% V/V, dry $CH_2Cl_2$, $N_2$, rt; iv) 7-hydroxycoumarin-3-carboxylic acid, HOBt, DCC, dry DMF, $N_2$, rt; v) HCl 37%, 1,4-dioxane, rt.

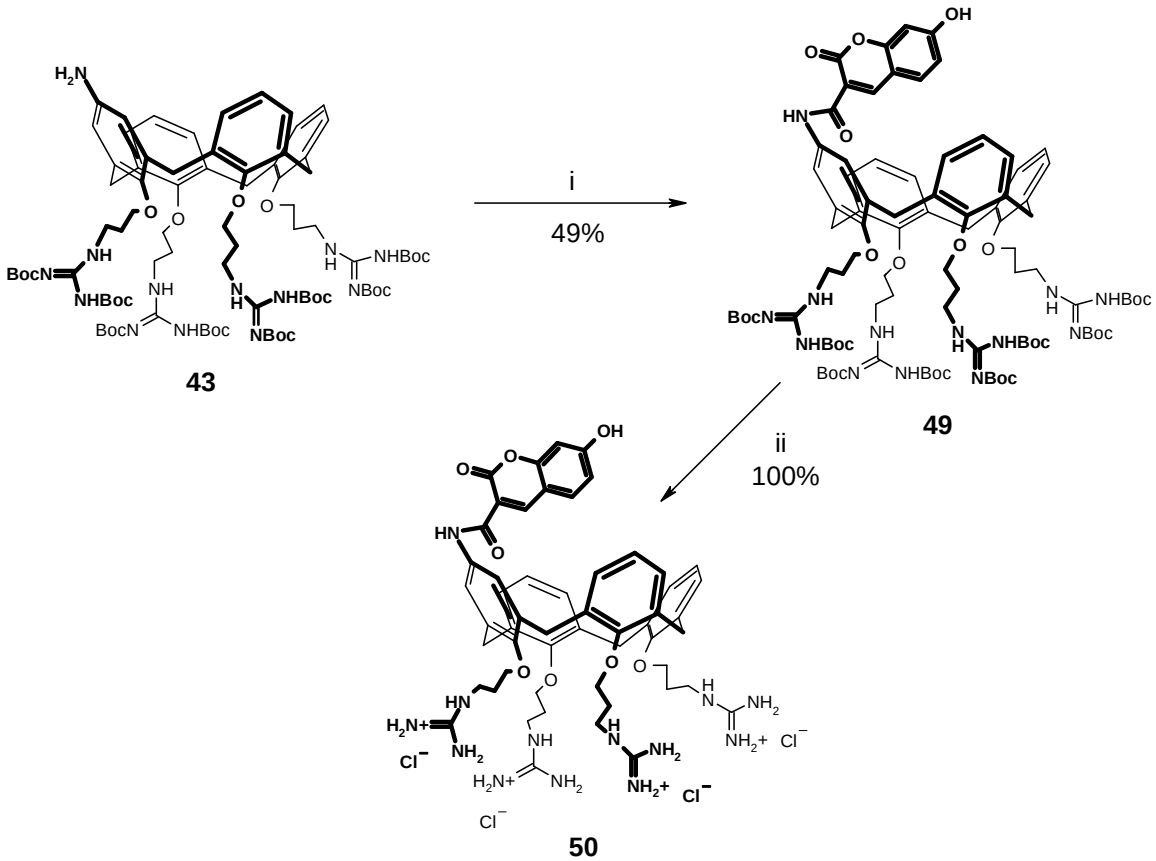
By exploiting the higher reactivity of aliphatic *vs* aromatic $NH_2$ groups (**Scheme 3.3**), a reasonable yield (47%) of compound **41**, having a free amino group at the upper rim was obtained when reacting **34** with N,N'-di-Boc-N"-triflylguanidine. The yield was decreased since the formation of small amount of the completely guanidilated compound (**35**) could not be avoided.A previously attempted synthetic pathway to obtain **43**, starting from **33** and described in **Scheme 3.4**, failed.




**Scheme 3.4**. i) $NH_2NH_2 \cdot H_2O$, abs EtOH, reflux, $N_2$; ii) N,N'-di-Boc-N"-triflylguanidine, $CHCl_3$, $N_2$; iii) Boc anhydride, dry DCM, $N_2$, rt; iv) Pd/C, abs EtOH, $H_2$ 1.1 bar, rt and Pd/C, $NH_2NH_2 \cdot H_2O$, abs EtOH, reflux, $N_2$; v) Pd/C, abs EtOH, $H_2$ 1.1 bar, rt.

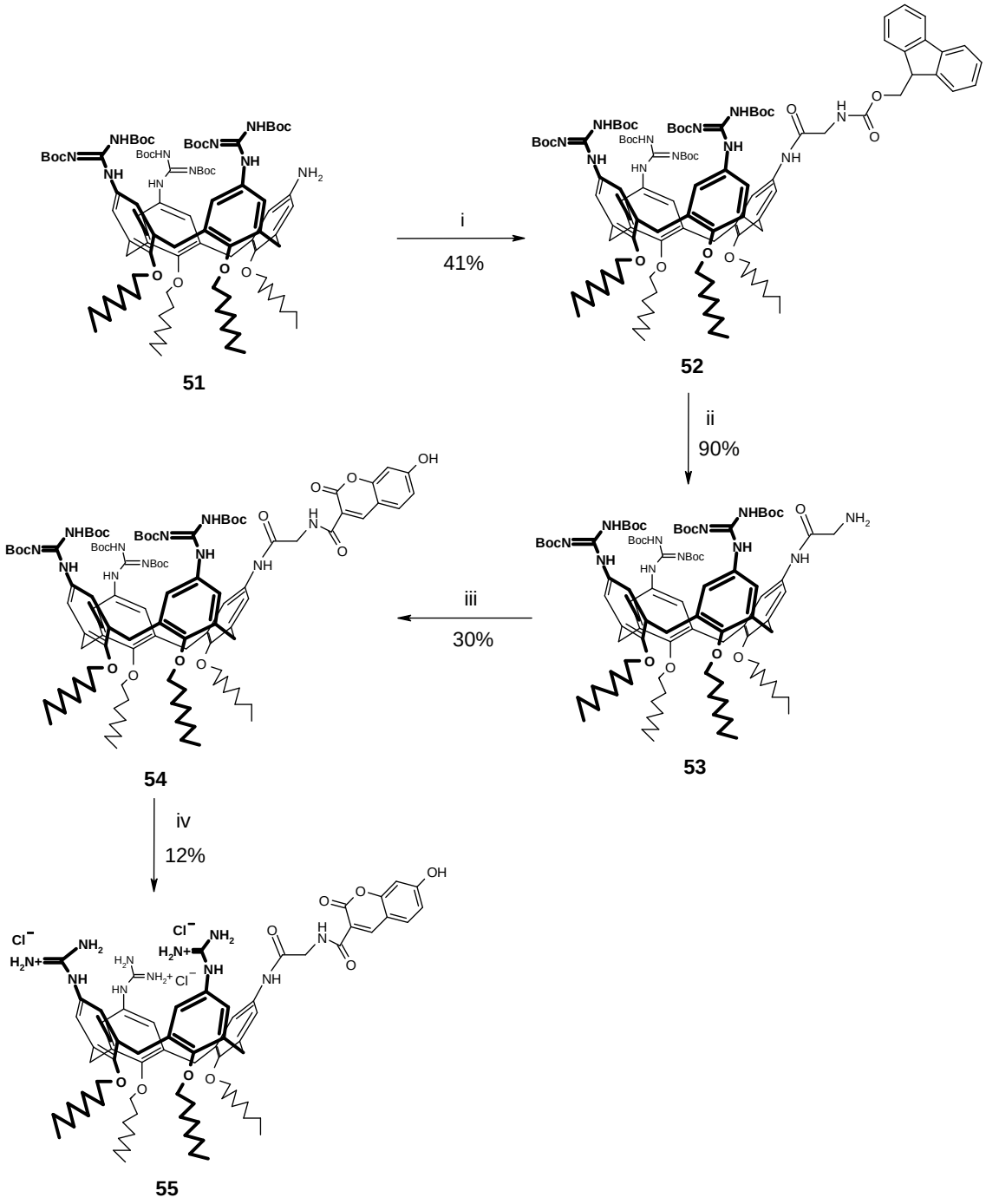
To obtain compound **40**, the phthalimido groups were removed with $NH_2NH_2 \cdot H_2O$ and the resulting amino groups were derivatized by reaction either with N,N'-di-Boc-N''-triflylguanidine or Boc anhydride giving **41** or **42**, respectively. Then, the attempts to reduce both intermediates to the corresponding amino derivatives **43** and **44** with Pd/C and $H_2$ or $NH_2NH_2 \cdot H_2O$ were unsuccessful due to the unexpted loss of Boc protecting groups and formation of several by-products as detected by ESI-MS. Likely the Boc groups present on the C=N moiety are rather unstable under these reductive conditions.

Back to **Scheme 3.3**, the following step was a coupling reaction with the Fmoc protected glycine, in presence of DCC and HOBt, in dry DMF, to give compound **45**, followed by the removal of the protecting group with piperidine which gave **46**. The coupling with 7-hydroxycoumarin-3-carboxylic acid followed, obtaining **47**. After the purification from DCU, the coumarin in excess and DCU-N-acylisourea, the guanidinium groups were deprotected to obtain the final compound **48**. No degradation of the coumarin lactone was observed. For the synthesis of **50** the guanidilated compound **43** was immediately reacted with 7-hydroxycoumarin-3-carboxylic acid (**Scheme 3.5**) getting **49**, that was deprotected and transformed in the guanidinium salt **50**.




**Scheme 3.5**. Synthesis of **50**. i) 7-hydroxycoumarin-3-carboxylic acid, HOBt, DCC, dry DMF, $N_2$, rt; ii) HCl 37%, 1,4-dioxane, rt.

The synthesis of the upper rim guanidinium-coumarin calixarene **55** was in some way suggested by the availability of the protected triguanidine **51** (**Scheme 3.6**). This was obtained as unexpected by-product of the guanidilation reaction of tetraamino-tetraoctiloxy calix[4]arene performed with N,N'-di-Boc-N''-triflylguanidine, not sufficiently reactive to give the fully functionalized derivative.




**Scheme 3.6**. Synthesis of derivative **55**. i) Fmoc-Gly-OH, HOBt, DCC, dry DMF, $N_2$, rt; ii) piperidine 20% V/V, dry $CH_2Cl_2$, $N_2$, rt; iii) 7-hydroxycoumarin-3-carboxylic acid, HOBt, DCC, dry DMF, $N_2$, rt; iv) HCl 37%, 1,4-dioxane, rt.

The presence of glycine as spacer was planned also in this case in order to minimize steric repulsion during the condensation of coumarin with the scaffold and to avoid too close proximity of the fluorophore and guanidinium charged groups in the final product.

Then, starting from compound **51** a synthetic procedure based on the same reactions described in **Scheme 3.3** from **43** to **48** was followed and compound **55** was obtained pure after HPLC purification.

Compounds **48** and **50** are soluble in water, while **55** is soluble in methanol but not in water, because of the long alkyl chains and a lower number of charged groups compared to other similar compounds prepared so far. $^1$H NMR spectra of **48** in $D_2O$ at two different concentrations suggest a possible aggregation phenomenon involving the inclusion of coumarin of a calixarene into the cavity of another one. A broadening of the signals is in fact observable at 1 mM, which are on the contrary sharp at 0.1 mM. Moreover signals relative to the fluorophore undergo a downfield shift by dilution towards resonance values detectable in the spectrum of **55** in $CD_3OD$, where intermolecular inclusion can be ruled out. A $^1$H NMR in $D_2O$ of **50** at 100 μM (**Fig. 3.9**)appears as sharp as for **48**.

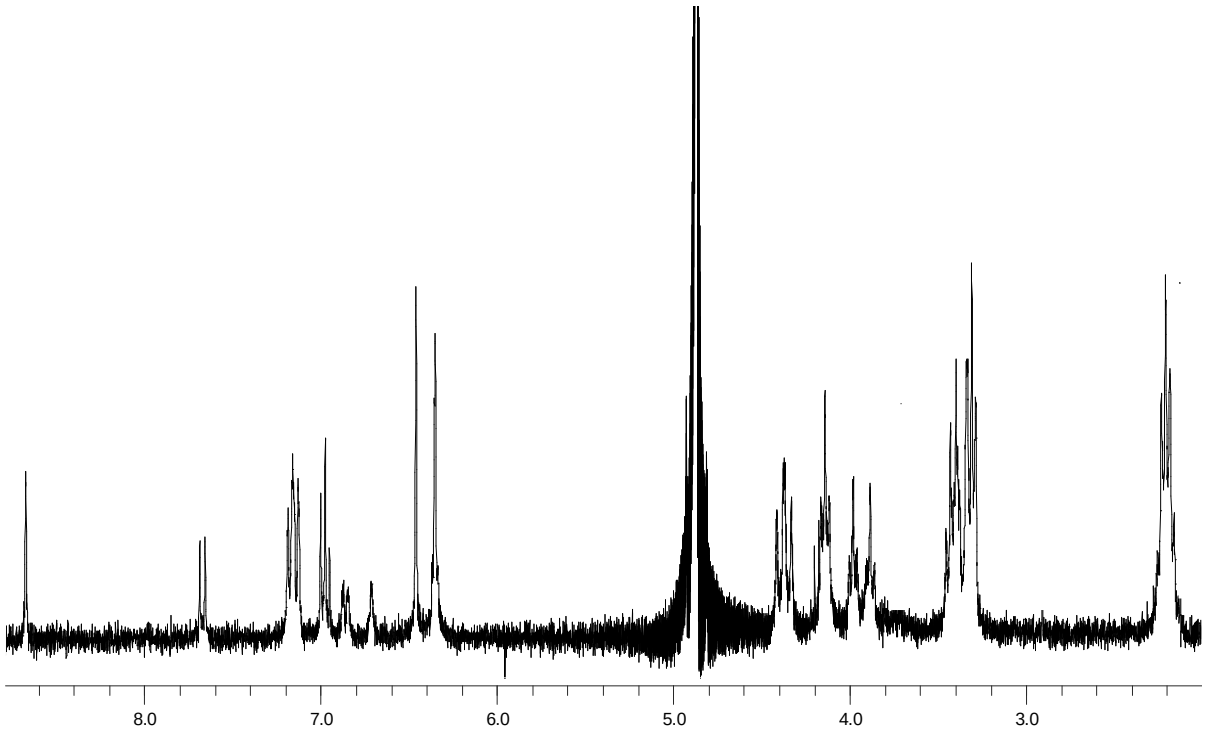



**Fig. 3.9**. $^1$H NMR spectrum ($D_2O$, 300 MHz, 298K) of **50** 100 μM.

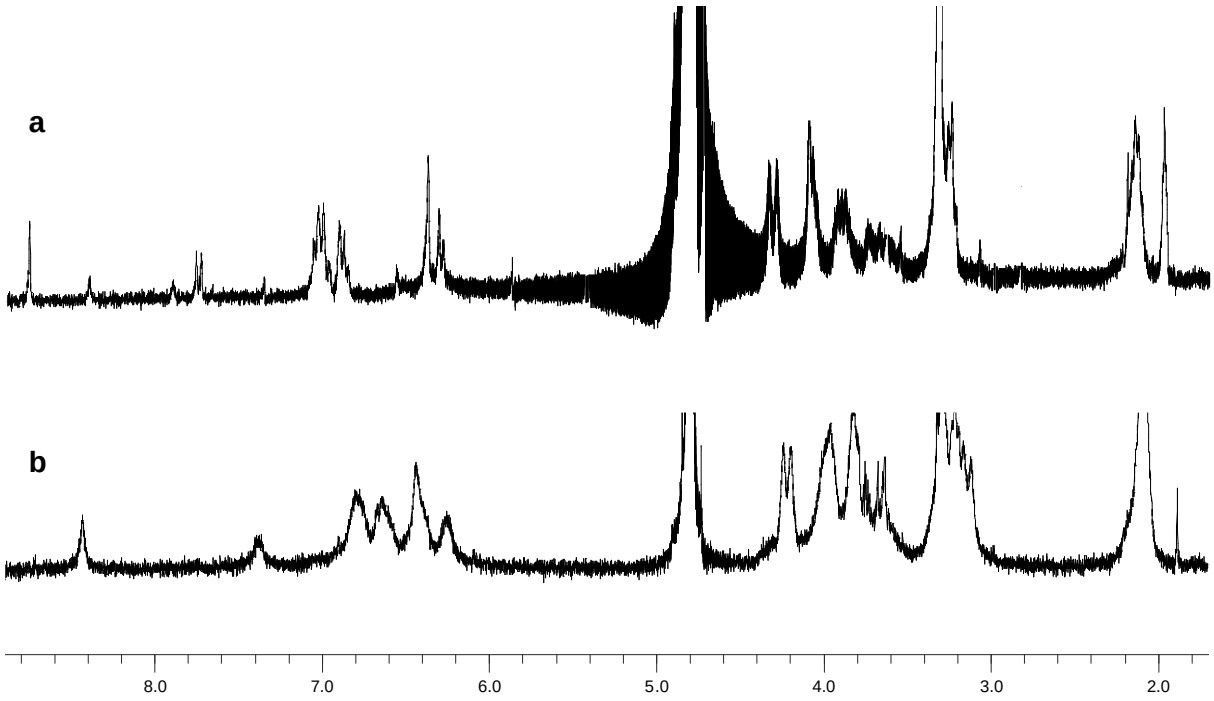



**Fig. 3.10**. $^{1}$H NMR spectra ($D_2O$, 300 MHz, 298K) of **48** a) 90 μM and b) 1 mM.

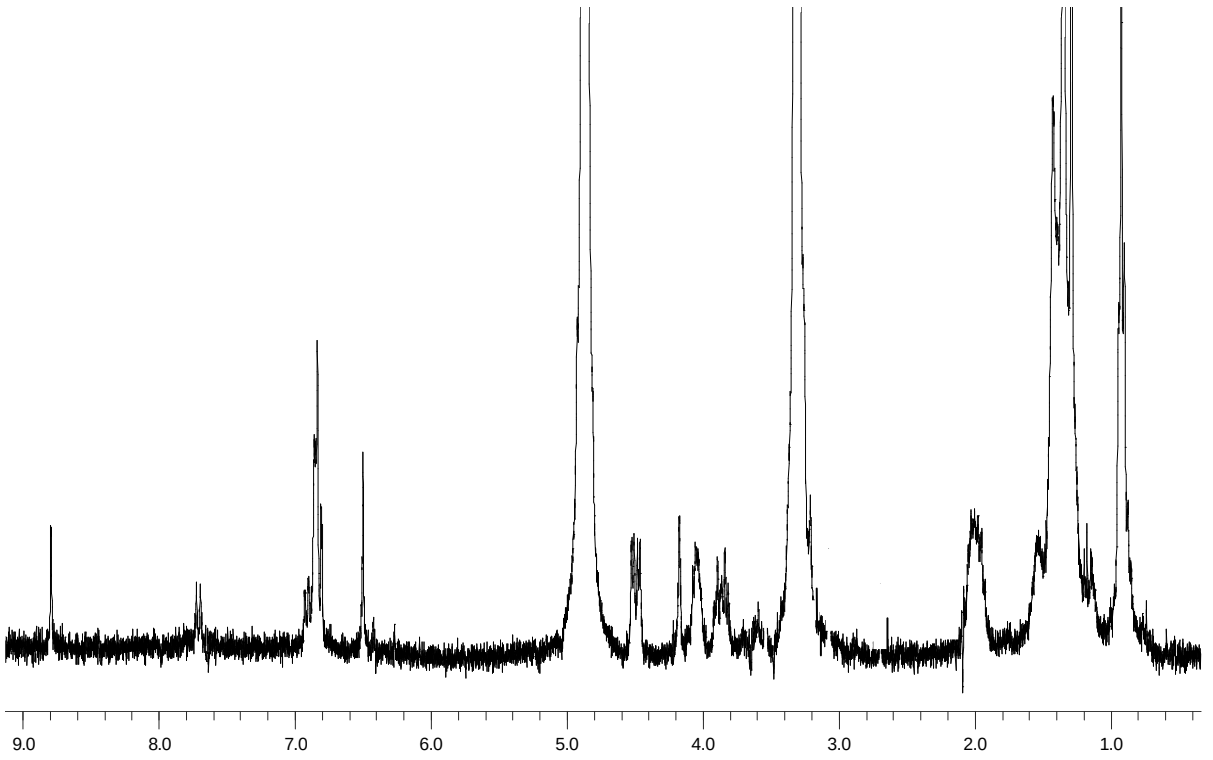
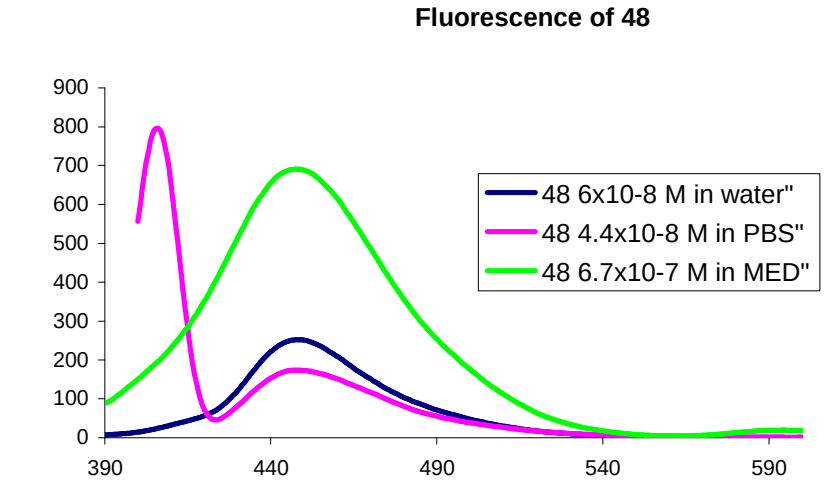
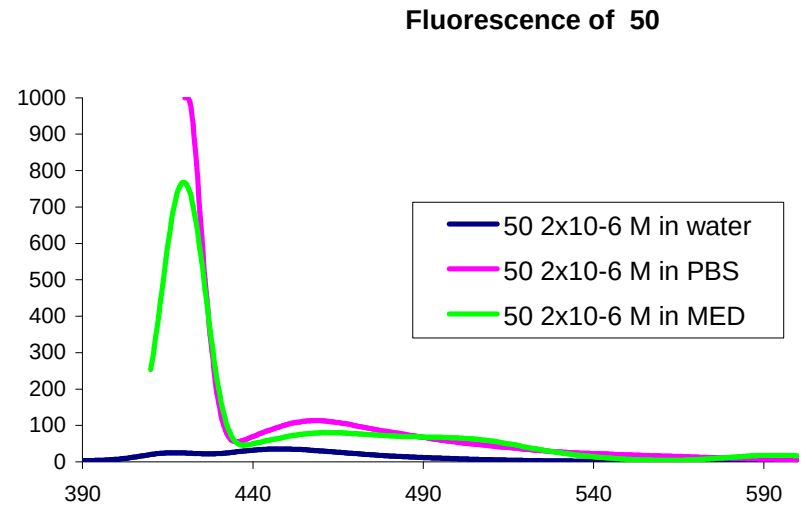



**Fig. 3.11**. $^{1}$H NMR spectrum ($CD_3OD$, 300 MHz, 298K) of **55**.

For these molecules bearing fluorescent groups, the wavelength and intensity of the fluorescence emission were determined.



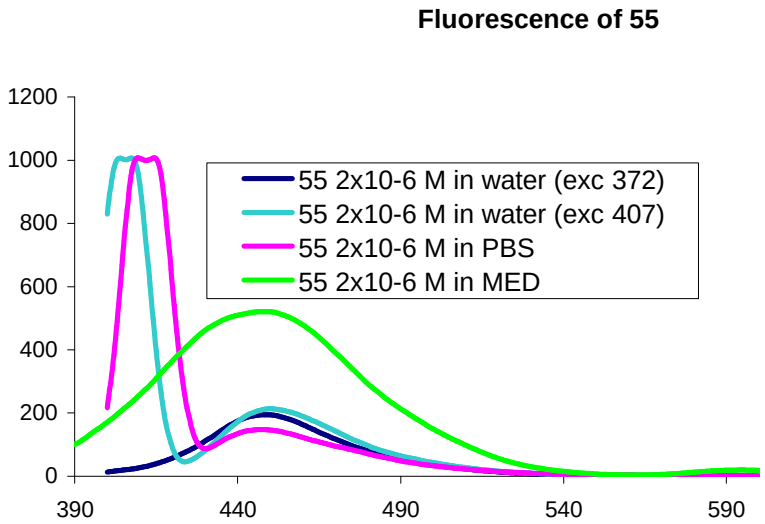




**Fig. 3.12**. Fluorescence spectra of **48** (excitation at 354 nm in $H_2O$, 407 nm in PBS, 358 nm in MED), **50** (excitation at 365 nm in $H_2O$, 417 nm in PBS, 420 nm in MED) and of **55** (excitation at 372 and 407 nm in $H_2O$, 413 nm in PBS, 358 nm in MED).

First the UV spectra were registered to know the wavelength of their absorption maxima in three different media: $H_2O$, phosphate buffer (PBS) and in the medium where the cells grow and used for transfection (MED). The values were then used to select the excitation wavelengths for recording the molecules for determining the fluorescence spectra (**Fig. 3.12**).
On the basis of these spectra, **48** emits with a maximum at 449 nm (blue), with a very high intensity especially in the medium of cell growth. The concentrations at which the measurements were performed were much lower ($6\times10^{-8}$ M), compared to those used for the other two compounds ($2\times10^{-6}$ M). In principle it could be possible to study the fate of this calixarene inside the cell. The same possibility could be considered for **55**, that shows a quite high fluorescence emission intensity with a maximum at 448.5 nm (blue), although at $2\times10^{-6}$ M, much higher than that used for **48**. On the other hand, compound **50** showed a different behaviour, because it has a very low emission even at $2\times10^{-6}$ M, perhaps because the proximity of coumarin to the cavity causes a significant fluorescence quenching.
All these preliminary studies on fluorescent calix[4]arene vectors are important for exploring the possibility of tracking the cellular uptake of these macrocycles and their DNA complexes. Such studies are currently in progress and some preliminary results will be mentioned in the next section, devoted to DNA binding and cell transfection.

### 3.1.3 DNA binding and cell transfection studies

Compound **32** showed characteristics similar to compound **5b** in the capacity of binding DNA although AFM studies revealed some peculiarities.

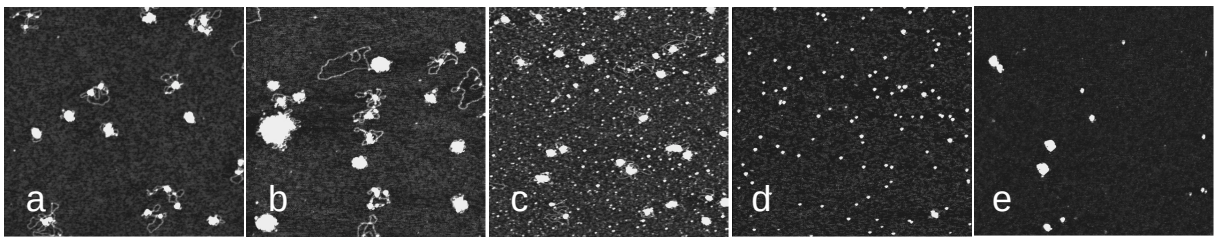



**Fig. 3.13**. AFM images (2×2 μm) of plasmid 0.5 nM in presence of **32** a) 1 μM, b) 2 μM, c) 2 μM + 10% EtOH, d) 2 μM, no DNA + 10% EtOH, e) 2 μM, no DNA.

At 1 μM concentration it forms DNA condensates of simple filaments (**Fig. 3.13a**), probably even a bit more efficiently than **5b**. Their size is increased when the ligand concentration is increased (**Fig. 3.13b**), but they again appear as single-filament condensates when a 10% of ethanol is present in the incubation solution (**Fig. 3.13c**). In these conditions, also very small

aggregates of few nm are present on the mica surface which are lacking DNA filaments and can then be related to calixarene aggregates. In fact, the AFM analysis of calixarene **32** alone at the concentration 2 μM, left in the deposition buffer in presence of 10% ethanol for the usual incubation time, reveals the presence of the same type of aggregates (**Fig. 3.13d**), which are bigger in absence of ethanol (**Fig. 3.13e**).

Rather surprisingly **32**, immediately tested as gene delivery vector for pEGFP-C1 plasmid DNA, appeared less efficient than **5b**, both in the absence and in the presence of DOPE (**Fig. 3.14**) and overall, it resulted strongly cytotoxic.

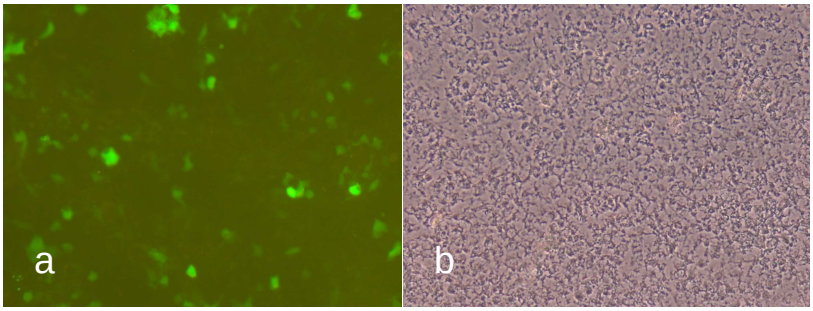



**Fig. 3.14**. Images obtained in presence of a) ligand **32**/DOPE (5/10 μM), visualized with fluorescence microscopy and b) **32**/DOPE (10/20 μM), visualized with phase contrast microscopy, in which nearly all the cells are dead.

For a further elaboration of these results, the data of **32** and **5b** obtained were compared also by luciferase assay experiment (**Fig. 3.15**). In this experiment, the vector containing the gene reporter is used to transfect cells. After a while the cells are lysed, and luciferine (luciferase substrate), Mg and an ATP excess are introduced in the cell extract. The possibly expressed luciferase enzyme catalyses the oxidative carboxylation of luciferin causing luminescence, which can be quantified by a luminometer or by a scintillation counter.

In fact as an alternative to EGFP expression, also another type of plasmid can be used that encodes for the luciferase protein; in this case the transfection efficiency is defined in terms of fluorescence intensity, of a product of the enzymatic activity of the luciferase itself, measured after cell lysis.

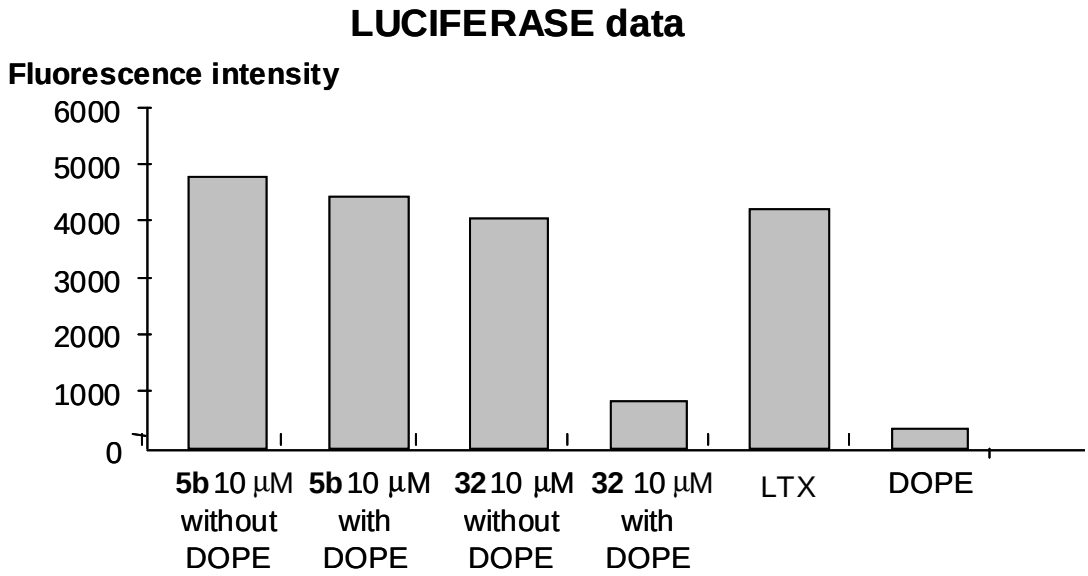



**Fig. 3.15**. Analysis of data for compounds **5b** and **32**obtained with luciferase.

From this kind of experiments with luciferase, **35** and **5b**, in absence of DOPE, do not have a very different transfection efficiency, while, in presence of helper, **5b** confirmed to be much more efficient even if the difference between the two is not so large as found with the previous method.

AFM images (**Fig. 3.16**) relative to pentaguanidinium compound **36** evidenced the expected strong interaction with plasmids which, however, leads to large aggregates (200-300 nm).

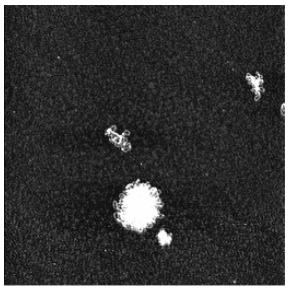


**Fig. 3.16**. AFM image (2×2 μm) of plasmid 0.5 nM in presence of **36**, 1 μM.

EMSA and AFM experiments were done also with the conformationally mobile calix[8]arene derivative **37**. In the gel plates (**Fig. 3.17**) **37** blocks all the DNA filaments in the well at 50 μM, as observed for **5b**.

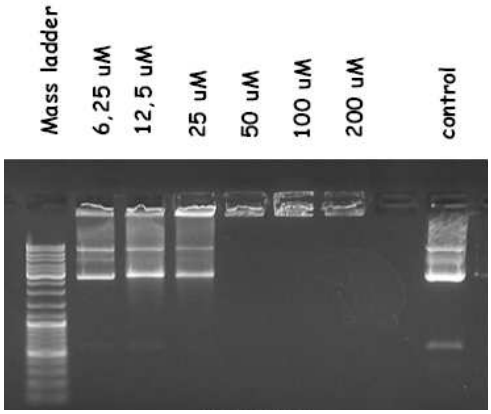



**Fig. 3.17**. EMSA conducted with pEGFP-C1 plasmid and the ligand **37**.

In AFM experiments **37** showed a behaviour very similar to **32**, giving rise to condensates of different sizes with DNA filaments (**Fig. 3.18a**,**b**) which partially relax by addition of EtOH (**Fig. 3.18c**). The presence of the alcohol, as for **32**, determines also the formation of small regular aggregates of calixarene both in presence and in absence of DNA.

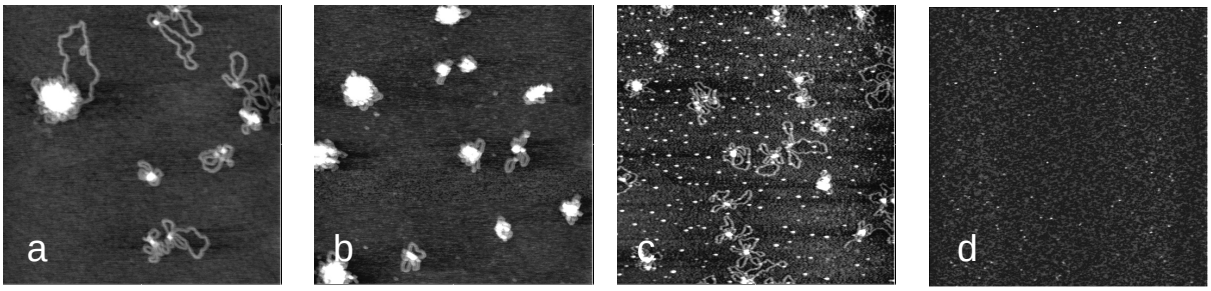



**Fig. 3.18**. AFM image (2×2 μm) of plasmid 0.5 nM in presence of **37** a) 0.5 μM, b) 0.7 μM, c) 0.5 μM + 10% EtOH, d) 0.5 μM, no DNA + 10% EtOH.

The substitution of guanidinium units at the lower rim with primary and quaternary ammonium groups determined a dramatic loss of efficiency in DNA binding and condensation. As reported for **39** in **Fig. 3.19**, even at a concentration of ligand 200 μM in EMSA, DNA moves in the gel as the control without ligand.

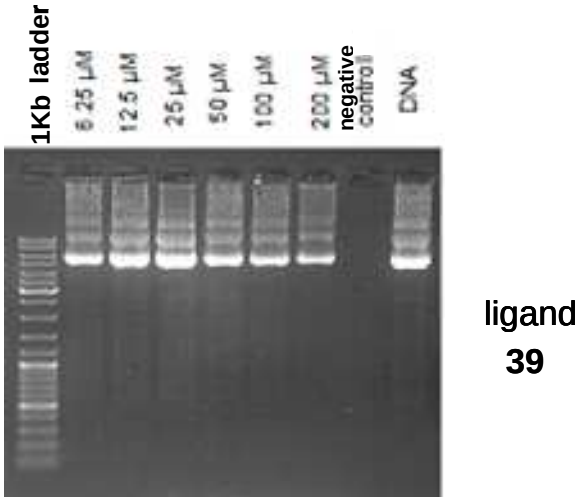



**Fig. 3.19**. EMSA experiments conducted with pEGFP-C1 plasmid and the ligand **39**.

Therefore the AFM experiments revealed that both these compounds interact with the DNA filaments, but have a low efficiency in their condensation. For **38**, bearing primary ammonium groups, different images were taken, modifying the concentration (from $10^{-6}$ to $5\times10^{-6}$ M), the pH of the solution used for the calixarene-DNA incubation and for the deposition (5.9 and 7.4), eventually adding EtOH (10% v/v), but without getting in any case significant improvements in the capacity of generating condensates (**Fig**. **3.20a**).

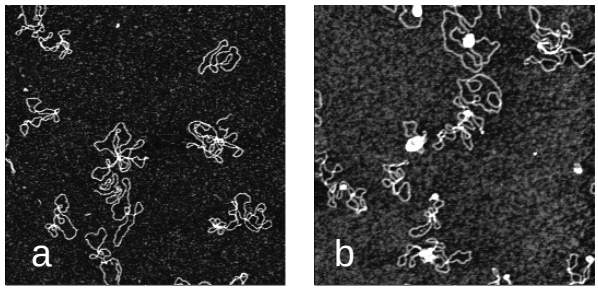



**Fig. 3.20**. AFM images (2×2 μm) of plasmid 0.5 nM in presence of a) **38** $2.5\times10^{-6}$ M + 10% EtOH at pH= 5.9, b) **39** $5\times10^{-6}$ M + 10% EtOH.

Also for **39** some experimental conditions were modified every time, in particular the concentration (from $10^{-6}$ to $5\times10^{-6}$ M), the incubation time and the presence or not of EtOH. But even for this compound no significant results in terms of DNA condensation were obtained, although at least at $5\times10^{-6}$ M of cationic ligand, the interaction capacity clearly modifies the filaments conformation (**Fig. 3.20b**) compared to the typical relaxed situation of DNA alone deposited onto mica.

All these four derivatives **36-39** resulted unable to transfect rhabdomyosarcoma cells. From the toxicity point of view **37** resulted very cytotoxic. Considering the conformational mobility of this ligand, a modification in the preparation of the usual formulation with DOPE was made. While the incubation is always performed after mixing the components in the order ligand, DNA and then DOPE, for **37** it was also tried to mix before ligand and DOPE, and then adding DNA. The idea was to allow DOPE to interact first with the ligand, blocking it in a defined conformation thus creating a "supramolecular" framework and allowing this assembly to interact with DNA in a way more suitable for transfection. However, even after this new protocol, no gene delivery was observed, although a drastic decrease of cytotoxicity resulted, probably because **37** is sequestered by the helper lipid and is less available in the interaction with cell.

The lower binding efficiency of both these ligands was confirmed by EMSA (**Fig. 3.21**) even if a further demonstration of the behaviour analogy of both compared to **5b** was obtained because already at 50 μM there is a significant percentage of plasmid that is kept in the well, that reaches 100% when the calixarene ligand is 100 μM. For **50** a fluorescent spot was observed at 200 μM that, respect to the starting line, moved to the opposite direction of the usual DNA shift. This spot could be the fluorescent ligand in excess and not bound to the plasmids or also the DNA-ligand lipoplex in which the total net charge becomes positive, then moving towards the negative pole.

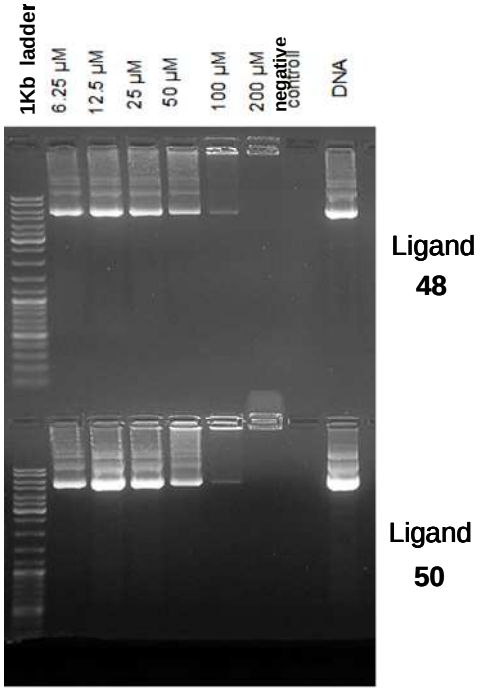



**Fig. 3.21**. EMSA experiments conducted with pEGFP-C1 plasmid and ligand **48** (upper row) and ligand **50** (lower row).

Concerning the two coumarin containing compounds **48** and **50**, the analysis made by AFM and EMSA evidenced they have DNA complexation properties similar to the ligand **5b**.
Noticeable from the AFM images collected for example with **50** (**Fig. 3.22**), the DNA condensation is evident, like for **5b**, if in the incubation solution is present the EtOH. From a more quantitative point of view, the concentration at which **50** is able to generate condensates ($5\times10^{-6}$ M) is 2.5 times that necessary for **5b**, showing a lower efficiency.

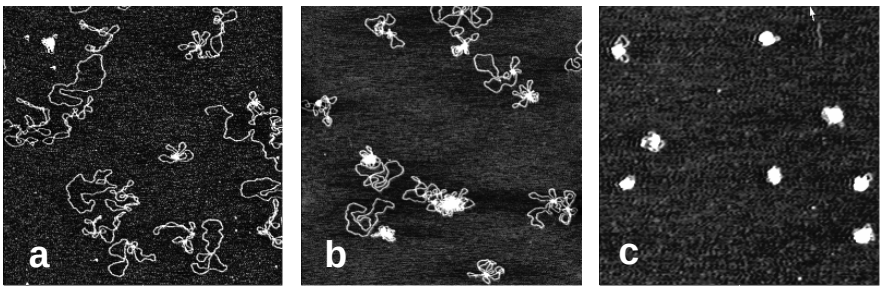



**Fig. 3.22**. AFM images (2×2 μm) of plasmid 0.5 nM in presence of ligand **50** a) $10^{-6}$ M, b) $5\times10^{-6}$ M, c) $5\times10^{-6}$ M + 10% EtOH.

After these preliminary binding experiments, **48** and **50** were obviously tested for transfection but we were strongly disappointed because no delivery at all was observed.
Moreover, during transfection attempts, both **48** and **50** were not emitting once incubated with the cells. Though being not able to transfect, the calixarene was expected to emit in the blue, while nothing was visible. Actually, from fluorescence studies described before, this result could be somehow understandable for **50**, which gave a very low emission intensity, while it is unexpected for **48**, that is the one with the highest emission efficiency between the three derivatives bearing coumarin.
A test to reveal the fluorescence of the two compounds with the microscopy used to follow the transfection evolution confirmed that nothing was visible with sample **50**. With **48** instead, dissolved in the medium for the cell growth, in presence of DNA and DOPE, little fluorescent blue spots were observed. Unfortunately this emission was not detectable anymore in presence of the cells.
An experiment was performed with a mixture of **5b** and **48**, in the order to exploit the former as vector and the latter as probe, hoping its involvement in the formation of condensates, but no blue fluorescence was observed.

The AFM image of the upper rim guanidinium calixarene **55** evidenced a significant interaction capacity with the plasmid already at $10^{-6}$ M (**Fig. 3.22**) and the encouraging presence of some condensates.

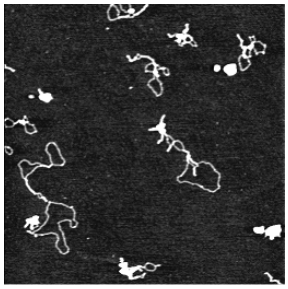


**Fig. 3.23**. AFM image (2×2 μm) of plasmid 0.5 nM in presence of the ligand **55** $10^{-6}$ M.

Nevertheless, as the other two fluorescent guanidinium calix[4]arenes, **55** did not result able to transfect cells neither in absence nor in presence of the helper DOPE. In this case some aggregates were observed that stay outside the cells and emit, as foreseen, in the blue (**Fig. 3.24**), but inactivity as vector cancels for the moment any possibility to use it as probe to follow the delivery process.

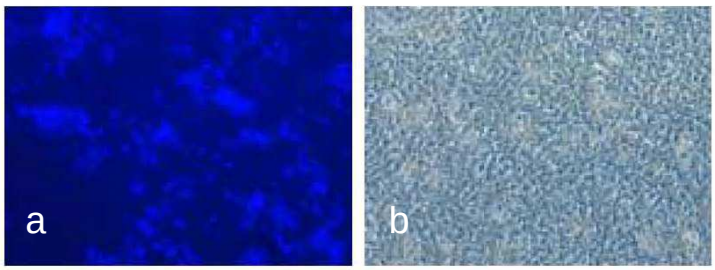



**Fig. 3.24**. Images obtained in presence of a) **55**/DOPE (10 μM/20 μM), visualized with fluorescence microscopy and b) **55**/DOPE (10 μM/20 μM) visualized with phase contrast microscopy.

## 3.2 Discussion

Different modifications of the structure of **5b** were carried out and studied. When an additional positive charge was introduced at the upper rim (compound **36**) it resulted not useful in terms of transfection, although the toxicity decreased compared to **5b**. An explanation of this behaviour can be found in the AFM experiments, which show that **36** forms large aggregates probably not able to cross the cell membrane. The additional charge at the upper rim changes the polarity of this side of the molecule which could be important for an efficient interaction with the helper lipid DOPE, essential for the activity of **5b**. For other reasons, in particular the conformational freedom, also calix[8]arene probably does not own a defined apolar cavity, and also this ligand does not show transfection activity although, as **32**,

shows ability in giving small condensates of DNA. These two compounds, with respect to this property, evidence moreover that condensation in nanometric “blobs” of nucleic acid is a necessary but not sufficient preliminary condition for gene delivery. Reduction of lipophilicity and availability of calixarene cavity look as important drawbacks, and in line with this, the fluorescent calix[4]arenes **48** and **50**, having a polar and hindering fluorophore at the upper rim, do not transfect as well. Additional interesting information was obtained studying the properties of **38** and **39**, which have four positive charges at the lower rim, different from guanidinium groups. The EMSA experiments done with these two derivatives confirmed that their capacity of binding DNA is lower compared to **5b**. While in fact this last compound showed to hold a large amount of DNA in the well already at 50 μM, for compound **39**, even at 200 μM, the DNA continues to move on the gel like in absence of ligand.

Because of their inability of compacting DNA, the two compounds showed a total inefficiency of transfection even if they are not cytotoxic. In summary the presence of positive charges is not sufficient to make the calixarene a good non viral vector for transfection, but it is necessary to have a functional group with specific characteristics like the guanidinium cation, which stays protonated in a larger pH range than primary ammonium groups and that has a geometry clearly more suitable to a proper interaction with DNA. For these non viral vectors based on the calix[4]arene scaffold the guanidinium group revealed to be fundamental for the DNA binding and the transfection.

Since the fluorescent calix[4]arenes **48**, **50** and **55** so far were not of help in finding the fate and mechanism that determines the transfection efficiency, we decided to acquire, in collaboration with the group of Prof. Piero Baglioni of the University of Florence, some additional chemico-physical data on the guanidinium calixarenes and their complexes with DNA in the absence and in the presence of DOPE. Meanwhile, Prof. Gaetano Donofrio in Parma, will perform biological tests aimed at the same goal.

## 3.3 Conclusions

Starting from the reference compound **5b** which revealed to be the best non viral vector based on the calixarene scaffold obtained so far, new cationic calix[4]arenes were synthesized modifying, stepwise, different structural features, such as i) increasing the distance between positive charges and calixarene cavity; ii) adding a positive charge at upper rim; iii) increasing the macrocycle size; iv) substituting the guanidinium group with a different

cationic group; v) linking to the ligand a fluorophore group to use the product as probe in the cellular tests.

The products obtained were all characterized by ESI-MS and $^{1}H$ NMR and $^{13}C$ NMR spectroscopy and their ability to interact with DNA through AFM and EMSA experiments were studied. Then, through transfection tests monitored by fluorescence and contrast phase microscopy, their capacity to act as non-viral vectors was evaluated. It resulted clear that all these more or less subtle structural changes make these compounds worse vectors compared to **5b**, but important information was obtained about the structural requirements, necessary to assure transfection efficiency to this class of new cationic lipids.

Unfortunately, the fluorescent products **48**, **50** and **55** do not act as probes because not able to deliver DNA to cells and/or not fluorescent when used in transfection experiments.

## 3.4 Experimental section

All the reactions were carried out under a nitrogen atmosphere. All dry solvents were prepared according to standard procedures and stored over molecular sieves. Melting points were determined on an Electrothermal apparatus in capillaries sealed under nitrogen. $^{1}H$ and $^{13}C$ NMR spectra were recorded on Bruker AV300 and AC300 spectrometers (partially deuterated solvents were used as internal standards). Mass spectra were recorded in ESI mode on a single quadrupole instrument SQ Detector, Waters (capillary voltage 3.8 kV, cone voltage 30-160 eV, extractor voltage 3 eV, source block temperature 80 °C, desolvation temperature 150 °C, cone and desolvation gas ($N_2$) flow rates 1.6 and 8 L/min, respectively). UV and Fluorimetric experiments were performed on Perkin Elmer UV-Vis Lambda BIO 20 spectrophotometer and LS55 Perkin Elmer fluorimeter, respectively. TLC was performed on Merck 60 F254 silica gel and flash column chromatography on 230-240 mesh Merck 60 silica gel.

N-(6-Bromohexyl)phthalimide (97%) was purchased from Alfa Aesar.

**General procedure for the alkylation of calix[4]arene 1b with *N*-(ω-bromohexyl)phthalimide and calix[8]arene with N-(3-bromopropyl)phthalimide** (see **Experimental section** in **Chapter 2**).

**25,26,27,28-Tetrakis(6-phthalimidohexyloxy)calix[4]arene (2d).**

The crude compound was purified by preparative TLC (eluent: hexane/ethyl acetate= 1:1) to obtain the pure product as a light yellow oil in 77% yield.

$^1$H NMR (300 MHz, $CDCl_3$) δ 7.81-7.75 (m, 8H, Pht), 7.68-7.64 (m, 8H, Pht), 6.59-6.50 (m, 12H, Ar*H*), 4.38 (d, *J* = 13.3 Hz, 4H, ArC*H*$_2$Ar), 3.85 (t, *J* = 7.4 Hz, 8H, OC*H*$_2$), 3.66 (t, *J* = 7.1 Hz, 8H, C*H*$_2$N), 3.11 (d, *J* = 13.3 Hz, 4H, ArC*H*$_2$Ar), 1.95-1.80 (m, 8H, $OCH_2CH_2$), 1.75-1.64 (m, 8H, $CH_2CH_2N$), 1.50-1.30 (m, 16H, $OCH_2CH_2CH_2CH_2$). $^{13}$C NMR (75 MHz, $CDCl_3$) δ 168.2, 156.4, 135.0, 133.7, 132.1, 130.9, 128.0, 123.0, 121.8, 74.7, 37.9, 31.0, 30.0, 28.7, 26.9, 25.7. MS (ESI): calculated for $[M + Na]^+$ *m*/*z* = 1363.6, found *m*/*z* = 1364.4.

**49,50,51,52,53,54,55,56-Octakis(3-phthalimidopropoxy)calix[8]arene (2e).**

It was synthesized according to a literature procedure.[5]

**Synthesis of 5-Nitro-25,26,27,28-tetrakis(3-phthalimidopropoxy)calix[4]arene (33).**

To a solution of calix[4]arene **2b** (1 g, 0.85 mmol) in dry $CH_2Cl_2$ (20 mL) and glacial acetic acid (1.52 mL, 37.94 mmol), 65% $HNO_3$ (0.52 mL, 7.47 mmol) was added. The reaction mixture turned pink and it was stirred for 2 h at room temperature. The black solution was quenched by addition of distilled water (30 mL) and vigorously stirred for 30 min. The organic orange layer was separated and washed with distilled water (50 mL) and the separated aqueous layer was extracted with $CH_2Cl_2$ (50 mL). The combined organic phases were dried over anhydrous $Na_2SO_4$ and the solvent distilled off under reduced pressure. The crude was purified by flash chromatography (eluent: cyclohexane/ethyl acetate= 5:4.8) to obtain the product as a pale yellow foam in 46% yield.

Mp: 107-110 °C. $^1$H NMR (300 MHz, $CDCl_3$) δ 7.73-7.52 (m, 16H, Pht), 7.08 (s, 2H, Ar*H* in ortho to $NO_2$), 6.92-6.78 (m, 6H, Ar*H*), 6.20 (s, 3H, Ar*H* opposite to $ArNO_2$), 4.53 (d, 2H, *J* = 14.4 Hz, ArC*H*$_2$Ar), 4.44 (d, 2H, *J* = 13.9 Hz, ArC*H*$_2$Ar), 4.27-3.83 (m, 16H, $OCH_2CH_2CH_2$), 3.23 (d, 2H, *J* = 14.4 Hz, ArC*H*$_2$Ar), 3.18 (d, 2H, *J* = 13.9 Hz, ArC*H*$_2$Ar), 2.36-2.23 (m, 8H, $OCH_2CH_2$). $^{13}$C NMR (75 MHz, $CDCl_3$) δ 168.1, 161.1, 156.9, 155.6, 142.6, 136.4, 135.8, 134.7, 133.7, 133.6, 133.5, 132.1, 132.0, 129.6, 128.7, 127.8, 123.3, 123.1, 122.9, 122.7, 121.9, 73.0, 72.6, 35.5, 35.3, 35.2, 31.2, 31.0, 29.7, 29.6. MS (ESI): calculated for $[M + Na]^+$ *m*/*z* = 1240.4, found *m*/*z* = 1241.0.

**General procedure for the removal of the phthaloyl protecting groups** (see **Experimental section** in **Chapter 2**).

**25,26,27,28-Tetrakis(6-aminohexyloxy)calix[4]arene (3d).**

The pure compound was obtained as a white solid in 94% yield.

Mp: 140-143 °C. $^{1}$H NMR (300 MHz, $CDCl_3$) δ 6.61-6.52 (m, 12H, Ar*H*), 4.41 (d, *J* = 13.3 Hz, 4H, ArC*H*$_2$Ar), 3.87 (t, *J* = 7.4 Hz, 8H, OC*H*$_2$), 3.13 (d, *J* = 13.3 Hz, 4H, ArC*H*$_2$Ar), 2.70 (t, *J* = 6.6 Hz, 8H, C*H*$_2$NH$_2$), 1.96 (bs, 8H, N*H*$_2$), 1.97-1.85 (m, 8H, OCH$_2$C*H*$_2$), 1.58-1.35 (m, 24H, OCH$_2$CH$_2$C*H*$_2$C*H*$_2$C*H*$_2$). $^{13}$C NMR (75 MHz, $CDCl_3$) δ 156.4, 135.0, 128.1, 121.9, 74.8, 42.1, 33.7, 31.0, 30.2, 27.1, 26.1. MS (ESI): calculated for $[M + Na]^+$ *m*/*z* = 843.6, found *m*/*z* = 843.9.

**49,50,51,52,53,54,55,56-Octakis(3-aminopropoxy)calix[8]arene (3e).**

It was synthesized according to literature procedure.[5]

**5-Nitro-25,26,27,28-tetrakis[3-aminopropoxy]calix[4]arene (40).**

The pure compound was obtained as a yellow oil in 91% yield.

$^{1}$H NMR (300 MHz, $CDCl_3$): δ 7.09 (s, 2H, Ar*H*), 6.98-6.88 (m, 4H, Ar*H*), 6.88-6.81 (m, 2H, Ar*H*), 6.21 (s, 3H, Ar*H*), 4.43 (d, *J* = 13.4 Hz, 2H, ArC*H*$_2$Ar), 4.38 (d, *J* = 13.4 Hz, 2H, ArC*H*$_2$Ar), 4.15-3.93 (m, 6H, OC*H*$_2$), 3.83 (t, *J* = 6.7 Hz, 2H, OC*H*$_2$), 3.22 (d, *J* = 13.4 Hz, 2H, ArC*H*$_2$Ar), 3.18 (d, *J* = 13.4 Hz, 2H, ArC*H*$_2$Ar), 2.94 (q, *J* = 6.9 Hz, 4H, OCH$_2$CH$_2$C*H*$_2$), 2.79 (t, *J* = 7.0 Hz, 4H, OCH$_2$CH$_2$C*H*$_2$), 2.12-1.92 (m, 8H, OCH$_2$C*H*$_2$), 1.73 (bs, 2H, N*H*$_2$). $^{13}$C NMR (75 MHz, $CDCl_3$): δ 161.0, 156.8, 155.4, 142.6, 136.4, 135.7, 134.7, 133.6, 129.5, 128.6, 127.8, 123.2, 122.6, 121.8, 73.2, 72.8, 72.6, 39.4, 39.2, 34.1, 33.9, 31.0, 30.9. MS (ESI): calculated for $[M + H]^+$ *m*/*z* = 698.4, found *m*/*z* = 698.6.

**General procedure for the guanidilation at the lower and upper rim,** see the **Experimental Section** in **Chapter 2**.

**25,26,27,28-Tetrakis[6-(bis-Boc-guanidine)hexyloxy]calix[4]arene (4d).**

The residue was purified by flash column chromatography on silica gel (eluent: from $CH_2Cl_2$ to $CH_2Cl_2$/ethyl acetate= 98:2) to obtain the pure product as a colourless oil in 63% yield.

$^{1}$H NMR (300 MHz, $CDCl_3$) δ 11.50 (s, 4H, BocN*H*), 8.30 (bs, 4H, CH$_2$N*H*), 6.61-6.52 (m, 12H, Ar*H*), 4.40 (d, *J* = 13.3 Hz, 4H, ArC*H*$_2$Ar), 3.87 (t, *J* = 7.3 Hz, 8H, OC*H*$_2$), 3.44-3.38 (m, 8H, C*H*$_2$NH$_2$), 3.14 (d, *J* = 13.3 Hz, 4H, ArC*H*$_2$Ar), 1.95-1.80 (m, 8H, OCH$_2$C*H*$_2$), 1.65-

1.50 (m, 8H, C*H*$_2$CH$_2$NH$_2$), 1.49 (s, 36H, *t*Bu), 1.48 (s, 36H, *t*Bu), 1.48-1.30 (m, 16H, OCH$_2$CH$_2$C*H*$_2$C*H*$_2$). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 163.5, 156.4, 156.0, 153.2, 135.0, 128.1, 121.9, 82.9, 79.1, 74.6, 40.8, 31.0, 29.9, 29.1, 28.2, 28.0, 26.9, 25.7. MS (ESI): calculated for [M + Na]$^+$ *m*/*z* = 1812.1, found *m*/*z* = 1812.7.

**49,50,51,52,53,54,55,56-Octakis[3-(bis-Boc-guanidine)propoxy]calix[8]arene (4e).**

The residue was purified by flash column chromatography on silica gel (eluent: from $CH_2Cl_2$ to $CH_2Cl_2$/ethyl acetate= 92:8) to obtain the pure product as a colourless oil in 20% yield.

$^1$H NMR (300 MHz, CDCl$_3$) δ 11.50 (s, 8H, BocN*H*), 8.59 (t, *J* = 5.2 Hz, 8H, CH$_2$N*H*), 6.98-6.65 (m, 24H, Ar*H*), 4.12 (s, 16H, ArC*H*$_2$Ar), 3.95-3.45 (m, 32H, OC*H*$_2$CH$_2$C*H*$_2$), 2.17-1.80 (m, 16H, OCH$_2$C*H*$_2$), 1.46 (s, 72H, *t*Bu), 1.43 (s, 72H, *t*Bu). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 163.6, 156.1, 154.5, 153.0, 134.3, 134.1, 128.9, 124.3, 82.7, 78.9, 71.3, 38.3, 31.2, 29.7, 28.2, 28.0. MS (ESI): calculated for [M + 2H]$^{++}$ *m*/*z* = 1621.9, found *m*/*z* = 1622.4.

**5-(Bis-Boc-guanidine)-25,26,27,28-tetrakis[3-(bis-Boc-guanidine)propoxy]calix[4]arene (35).**

The residue was purified by flash column chromatography on silica gel (eluent: from $CH_2Cl_2$ to $CH_2Cl_2$/ethyl acetate= 99:1) to obtain the pure product as a colourless oil in 34% yield.

$^1$H NMR (300 MHz, CDCl$_3$) δ 11.53, 11.51, 11.48 (3×s, 5H, N*H*Boc), 9.76 (s, 1H, ArN*H*), 8.45-8.30 (m, 4H, CH$_2$N*H*), 6.74 (s, 2H, Ar*H*CNH), 6.77-6.48 (m, 9H, Ar*H*), 4.37 (d, *J* = 13.5 Hz, 2H, ArC*H*$_2$Ar), 4.33 (d, *J* = 13.5 Hz, 2H, ArC*H*$_2$Ar), 4.03-3.86 (m, 8H, OC*H*$_2$), 3.73-3.42 (m, 8H, C*H*$_2$N), 3.18 (d, *J* = 13.5 Hz, 2H, ArC*H*$_2$Ar), 3.17 (d, *J* = 13.5 Hz, 2H, ArC*H*$_2$Ar), 2.26-2.10 (m, 8H, OCH$_2$C*H*$_2$), 1.51, 1.47, 1.46 (3×s, 90H, *t*Bu). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 163.5, 156.1, 156.0, 153.3, 153.1, 135.2, 134.9, 134.5, 130.6, 128.4, 128.2, 122.6, 122.3, 122.0, 83.2, 82.8, 79.1, 78.9, 72.1, 72.0, 37.8, 31.0, 29.8, 29.6, 28.2, 28.0. MS (ESI): calculated for [M + H + Na]$^{++}$ *m/z* 951.0, found *m/z* 951.6, calculated for [M + 2Na]$^{++}$ *m/z* 962.0, found *m/z* 962.6.

**5-Nitro-25,26,27,28-tetrakis[3-(bis-Boc-guanidine)propoxy]calix[4]arene (41).**

The crude was purified by flash column chromatography on silica gel (eluent: $CH_2Cl_2$, $CH_2Cl_2$/ethyl acetate = 99:1) to obtain the pure product as a colourless oil in 55% yield.

$^1$H NMR (300 MHz, CDCl$_3$): δ 11.52-11.42 (m, 4H, N*H*Boc), 8.40 (t, *J* = 5.2 Hz, 2H, N*H*C=N), 8.35 (t, *J* = 5.2 Hz, 2H, N*H*C=N), 7.09 (s, 2H, Ar*H*CNO$_2$), 6.95-6.85 (m, 4H, Ar*H*),

6.82 (t, *J* = 7.3 Hz, 2H, Ar*H*), 6.21 (s, 3H, Ar*H*), 4.42 (d, *J* = 14.0 Hz, 2H, ArC*H*$_2$Ar), 4.35 (d, *J* = 13.8 Hz, 2H, ArC*H*$_2$Ar), 4.14-3.95 (m, 6H, OC*H*$_2$), 3.83 (t, *J* = 6.7 Hz, 2H, OC*H*$_2$), 3.68-3.42 (m, 8H, OCH$_2$CH$_2$C*H*$_2$), 3.24 (d, *J* = 14.0 Hz, 2H, ArC*H*$_2$Ar), 3.18 (d, *J* = 13.8 Hz, 2H, ArC*H*$_2$Ar), 2.25-2.05 (m, 8H, OCH$_2$C*H*$_2$), 1.47, 1.45, 1.43 (3×s, 72H, *t*Bu). $^{13}$C NMR (75 MHz, $CDCl_3$): δ 163.5, 160.8, 156.6, 156.1, 156.0, 155.2, 153.1, 142.6, 136.2, 135.7, 134.6, 133.7, 129.6, 128.7, 127.8, 123.3, 122.8, 121.9, 83.0, 82.9, 79.0, 78.9, 72.6, 72.1, 72.0, 37.9, 37.6, 37.4, 31.1, 31.0, 29.9, 29.7, 28.2, 28.0. MS (ESI): calculated for $[M + Na]^+$ *m*/*z* = 1688.9, found *m*/*z* = 1689.6.

**5-Amino-25,26,27,28-tetrakis[3-(bis-Boc-guanidine)propoxy]calix[4]arene (43).**

The crude was purified by flash column chromatography on silica gel (eluent: $CH_2Cl_2$, $CH_2Cl_2$/ethyl acetate = 98:2) to obtain the pure product as a colourless oil in 66% yield.

$^1$H NMR (300 MHz, $CDCl_3$ ): δ 11.48 (bs, 4H, N*H*Boc), 8.38 (bs, 4H, N*H*C=N), 6.70-6.50 (m, 9H, Ar*H*), 5.92 (s, 2H, Ar*H*CNH$_2$), 4.38 (d, *J* = 13.6 Hz, 2H, ArC*H*$_2$Ar), 4.29 (d, *J* = 13.3 Hz, 2H, ArC*H*$_2$Ar), 3.95 (bt, 6H, OC*H*$_2$), 3.87 (t, *J* = 6.9 Hz, 2H, OC*H*$_2$), 3.68-3.50 (m, 8H, NC*H*$_2$), 3.19 (d, *J* = 13.6 Hz, 2H, ArC*H*$_2$Ar), 3.05 (d, *J* = 13.3 Hz, 2H, ArC*H*$_2$Ar), 2.27-2.07 (m, 8H, OCH$_2$C*H*$_2$), 1.47 (s, 36H, *t*Bu), 1.46 (s, 36H, *t*Bu). $^{13}$C NMR (75 MHz, $CDCl_3$): δ 163.5, 156.1, 156.0, 153.1, 149.0, 140.6, 135.3, 134.8, 128.2, 122.1, 121.9, 115.4, 82.8, 78.9, 72.0, 39.8, 31.0, 29.8, 29.7, 28.6, 28.2, 28.0. MS (ESI): calculated for $[M + H]^+$ *m*/*z* = 1636.9, found *m*/*z* = 1637.1, calculated for $[M + Na]^+$ *m*/*z* = 1658.9, found *m*/*z* = 1659.5

**5,11,17-Tris(bis-Boc-guanidine)-23-amino-25,26,27,28-tetraoctyloxycalix[4]arene (51).**

The residue was purified by flash column chromatography on silica gel (eluent: from $CH_2Cl_2$ to $CH_2Cl_2$/ethyl acetate = 98:2) to obtain the pure product as a light orange solid in 70% yield. Mp: >250 °C dec. $^1$H NMR (300 MHz, $CDCl_3$) δ 11.71 (s, 2H, BocN*H*), 11.52 (bs, 1H, BocN*H*), 10.28 (s, 2H, ArN*H*), 9.36 (s, 1H, ArN*H*), 7.30 (s, 4H, Ar*H*), 6.19 (s, 4H, Ar*H*), 5.42 (s, 4H, Ar*H*), 4.37 (d, *J* = 13.6 Hz, 2H, ArC*H*$_2$Ar), 4.33 (d, *J* = 13.9 Hz, 2H, ArC*H*$_2$Ar), 4.05-3.90 (m, 4H, OC*H*$_2$), 3.65-3.50 (m, 4H, OC*H*$_2$), 3.10 (d, *J* = 13.6 Hz, 2H, ArC*H*$_2$Ar), 3.02 (d, *J* = 13.9 Hz, 2H, ArC*H*$_2$Ar), 2.90 (bs, 2H, ArN*H*$_2$), 1.95-1.70 (m, 8H, OCH$_2$C*H*$_2$), 1.55-1.41 (4×s, 54H, *t*Bu), 1.35-1.10 (m, 40H, O(CH$_2$)$_2$(C*H*$_2$)$_5$), 0.95-0.80 (m, 12H, C*H*$_3$). $^{13}$C NMR (75 MHz, $CDCl_3$) δ 163.6, 155.2, 153.8, 153.6, 153.4, 153.2, 148.5, 140.7, 137.4, 136.8, 133.4, 133.3, 130.2, 130.1, 123.5, 122.6, 122.3, 114.1, 83.4, 82.9, 79.3, 79.0, 75.2,

75.0, 31.9, 31.2, 31.1, 30.5, 30.4, 30.0, 29.9, 29.7, 29.4, 28.2, 28.1, 26.7, 26.6, 25.9, 22.7, 22.6, 14.0. MS (ESI): calculated for $[M + Na]^+$ *m*/*z* = 1659.1, found *m*/*z* = 1660.9.

**Synthesis of 25,26,27,28-Tetrakis(3-aminopropoxy)calix[4]arene, tetrachloride (38).**

A solution of calix[4]arene **2b** (0.23 g, 0.2 mmol) and hydrazine monohydrate (1.99 mL, 40 mmol) in absolute ethanol (6.2 mL) was refluxed overnight. The solvent was removed under reduced pressure and the residue was dissolved in $CH_2Cl_2$ (10 mL) and distilled water (10 mL). The organic layer was separated and the water phase was extracted with $CH_2Cl_2$ (3×10 mL). The combined organic layers were dried over anhydrous $Na_2SO_4$ and the residue dissolved in 2 M HCl (2 mL). The pure compound was obtained after evaporation of the solvent under reduced pressure to obtain a white solid in 83% yield.

Mp: >250 °C dec. $^1$H NMR (300 MHz, $CD_3OD$): δ 6.65-6.55 (m, 12H, Ar*H*), 4.38 (d, *J* = 13.4 Hz, 4H, ArC*H*$_2$Ar), 4.04 (t, *J* = 7.2 Hz, 8H, OC*H*$_2$), 3.23 (d, *J* = 13.4 Hz, 4H, ArC*H*$_2$Ar), 3.17 (t, *J* = 8.1 Hz, 8H, C*H*$_2$N$^+$), 2.33 (quint, *J* = 7.5 Hz, 8H, OCH$_2$C*H*$_2$). $^1$H NMR (300 MHz, $D_2O$) δ 6.88 (d, 8H, *J* = 7.3 Hz, Ar*H*), 6.79-6.74 (m, 4H, Ar*H*), 4.39 (d, *J* = 13.3 Hz, 4H, ArC*H*$_2$Ar), 4.14 (t, *J* = 7.2 Hz, 8H, OC*H*$_2$), 3.23 (d, *J* = 13.3 Hz, 4H, ArC*H*$_2$Ar), 3.17 (t, *J* = 7.4 Hz, 8H, C*H*$_2$N$^+$), 2.26 (quint, *J* = 7.8 Hz, 8H, OCH$_2$C*H*$_2$). $^{13}$C NMR (75 MHz, $CD_3OD$) δ 157.2, 136.3, 129.9, 124.0, 73.4, 38.8, 32.3, 29.6. MS (ESI): calculated for $[M + H - 4HCl]^+$ *m*/*z* = 653.4, found *m*/*z* = 653.6, calculated for $[M + Na - 4HCl]^+$ *m*/*z* = 675.4, found *m*/*z* = 675.5.

**Synthesis of 25,26,27,28-Tetrakis[3-(trimethylammonium)propoxy]calix[4]arene, tetrachloride (39).**

The tetraminocalix[4]arene **3b** (0.26 g, 0.4 mmol) was dissolved in MeOH (8 mL), $KHCO_3$ (0.41 g, 4.1 mmol) and MeI (0.8 mL, 12.7 mmol) were added and the mixture was stirred at room temperature. The reaction was followed by mass spectroscopy and stopped after 7 days. MeOH/$CH_2Cl_2$ = 9:1 (10 mL) was added, the unsoluble inorganic salts were filtered off and the tetralkylammonium salt was obtained after evaporation of the solvent under reduced pressure. The residue was dissolved (0.53 g, 0.4 mmol) in $H_2O$ (5 mL), and 5 mL of a $Cl^-$ DOWEX11 resin were added and stirred for 30 min. After removal of the resin, the pure compound **39** was obtained by evaporation of the solvent under reduced pressure. A white solid can be obtained, in 98% yield, liophilizing this residue.

$^{1}$H NMR (400 MHz, $CD_3OD$, 298 K): δ 6.70-6.60 (m, 12H, Ar*H*), 4.43 (d, *J* = 13.8 Hz, 4H, ArC*H*$_2$Ar), 4.19 (t, *J* = 7.0 Hz, 8H, OC*H*$_2$), 3.70-3.60 (m, 8H, $OCH_2CH_2CH_2$), 3.35 (d, *J* = 13.8 Hz, 4H, ArC*H*$_2$Ar), 3.26 (s, 36H, C*H*$_3$), 2.39 (quint, *J* = 7.0 Hz, 8H, $OCH_2CH_2$). $^{1}$H NMR (300 MHz, $D_2O$, 323K): δ 6.84-6.79 (m, 12H, Ar*H*), 4.38 (d, *J* = 13.6 Hz, 4H, ArC*H*$_2$Ar), 4.24 (t, *J* = 6.8 Hz, 8H, OC*H*$_2$), 3.46 (d, *J* = 13.6 Hz, 4H, ArC*H*$_2$Ar), 3.42-3.28 (m, 8H, $OCH_2CH_2CH_2$), 3.14 (s, 36H, C*H*$_3$), 2.40-2.24 (m, 8H, $OCH_2CH_2$). $^{13}$C NMR (75 MHz, $CD_3OD$) δ 155.0, 134.8, 128.4, 122.5, 70.6, 63.7, 52.5, 31.1, 23.7. MS (ESI): calculated for $[M + 4H - 4Cl]^{4+}$ *m*/*z* = 207.2, found *m*/*z* = 206.3.

**Synthesis of 5-Nitro-25,26,27,28-tetrakis[3-(Boc-amino)propoxy]calix[4]arene (42).**

To a solution of calixarene **40** (87 mg, 0.125 mmol) in dry $CH_2Cl_2$ (8 mL), Boc anhydride (0.12 mL, 0.5 mmol) was added. The mixture was stirred for 24 h and the solvent removed under reduced pressure. The crude was purified by flash column chromatography on silica gel (eluent: $CH_2Cl_2$, $CH_2Cl_2$/EtOAc= 95:5) to obtain the pure product as a white solid in 52% yield.

Mp: 172-174 °C. $^{1}$H NMR (300 MHz, $CDCl_3$): δ 7.09 (s, 2H, Ar*H*$CNO_2$), 7.02-6.75 (m, 6H, Ar*H*), 6.21 (s, 3H, Ar*H*), 5.71 (bs, 2H, N*H*Boc), 5.62 (bs, 1H, N*H*Boc), 5.49 (bs, 1H, N*H*Boc), 4.37 (d, *J* = 14.0 Hz, 2H, ArC*H*$_2$Ar), 4.32 (d, *J* = 14.3 Hz, 2H, ArC*H*$_2$Ar), 3.94 (q, *J* = 7.5 Hz, 4H, OC*H*$_2$), 3.85 (bt, 2H, OC*H*$_2$), 3.74 (t, *J* = 5.7 Hz, 2H, OC*H*$_2$), 3.42 (bs, 4H, $OCH_2CH_2CH_2$), 3.27-3.06 (m, 4H, $CH_2CH_2CH_2$), 3.19 (d, *J* = 14.0 Hz, 2H, ArC*H*$_2$Ar), 3.14 (d, *J* = 14.3 Hz, 2H, ArC*H*$_2$Ar), 2.20-1.95 (m, 8H, $OCH_2CH_2$), 1.44 (s, 36H, *t*Bu). $^{13}$C NMR (75 MHz, $CDCl_3$): δ 161.0, 156.8, 155.4, 142.6, 136.4, 135.7, 134.7, 133.6, 129.5, 128.6, 127.8, 123.2, 122.6, 121.8, 73.2, 72.8, 72.6, 39.4, 39.2, 34.1, 33.9, 31.0, 30.9. MS (ESI): calculated for $[M + H]^{+}$ *m/z* = 698.4, found *m/z* = 698.6.

**General procedure for the coupling reaction (**see the **Experimental Section** in **Chapter 4**).

**Synthesis of 5-[(N-Fmoc-Gly)amino]-25,26,27,28-tetrakis[3-(bis-Boc-guanidine)propoxy]calix[4]arene (45).**

The crude was purified by preparative TLC (eluent: hexane/ethyl acetate = 3:2) to obtain the pure product as a colourless oil in 65% yield.

$^1$H NMR (300 MHz, $CDCl_3$): δ 11.49 (s, 4H, N*H*Boc), 8.56-8.26 (m, 4H, N=CN*H*), 7.76 (d, *J* = 7.4 Hz, 2H, Ar*H* Fmoc), 7.59 (d, *J* = 7.2 Hz, 2H, Ar*H* Fmoc), 7.39 (t, *J* = 7.3 Hz, 4H, Ar*H* Fmoc), 7.35-7.15 (m, 5H, Ar*H* Fmoc and ArN*H*), 6.80-6.48 (m, 9H, Ar*H*), 6.49 (s, 2H, Ar*H*NH), 5.47 (bs, 1H, N*H*COO), 4.44 (d, *J* = 6.9 Hz, 2H, COOC$H_2$), 4.36 (d, 2H, *J* = 13.4 Hz, ArC$H_2$Ar), 4.35 (d, 2H, *J* = 13.4 Hz, ArC$H_2$Ar), 4.22 (t, 1H, *J* = 6.9 Hz, $COOCH_2CH$), 4.15-3.80 (m, 10H, OC$H_2$ and COC$H_2$), 3.70-3.38 (m, 8H, $OCH_2CH_2CH_2$), 3.17 (d, 2H, *J* = 13.4 Hz, ArC$H_2$Ar), 3.16 (d, 2H, *J* = 13.4 Hz, ArC$H_2$Ar), 2.30-2.05 (m, 8H, $OCH_2CH_2$), 1.47 (s, 36H, *t*Bu), 1.46 (s, 36H, *t*Bu). $^{13}$C NMR (75 MHz, $CDCl_3$): δ 166.1, 163.5, 156.6, 156.1, 155.9, 153.2, 153.0, 143.6, 141.2, 135.3, 135.1, 134.6, 131.1, 128.6, 128.4, 128.2, 127.7, 127.1, 125.0, 122.4, 121.8, 120.4, 120.0, 82.9, 79.0, 72.1, 67.3, 47.0, 45.3, 37.9, 31.0, 29.9, 29.7, 28.2, 28.0. MS (ESI): calculated for $[M + H]^+$ *m*/*z* = 1616.0, found *m*/*z* = 1616.8, calculated for $[M + Na]^+$ *m*/*z* = 1938.0, found *m*/*z* = 1938.8.

**Synthesis of 5,11,17-Tris(bis-Boc-guanidine)-23-[(*N*-Fmoc-Gly)amino]-25,26,27,28-tetraoctyloxycalix[4]arene (52).**

The residue was purified by preparative TLC (eluent: ethyl acetate/hexane= 1:3) to obtain the pure product as a colourless oil in 41% yield.

$^1$H NMR (300 MHz, $CDCl_3$) δ 11.71 (s, 2H, BocN*H*), 11.15 (bs, 1H, BocN*H*), 10.34 (s, 2H, ArN*H*CN), 9.60 (bs, 1H, ArN*H*CN), 8.30 (s, 1H, ArN*H*CO), 7.73 (d, *J* = 7.5 Hz, 2H, ArFmoc), 7.56 (d, *J* = 7.6 Hz, 2H, ArFmoc), 7.45 (d, *J* = 2.1 Hz, 2H, ArH), 7.37 (t, *J* = 7.5 Hz, 2H, ArFmoc), 7.28-7.25 (m, 4H, ArFmoc and ArH), 6.38 (s, 2H, Ar*H*), 6.00 (s, 2H, Ar*H*), 5.51 (bs, 1H, N*H*COO), 4.41 (d, *J* = 13.6 Hz, 2H, ArC$H_2$Ar), 4.37 (d, *J* = 13.1 Hz, 2H, ArC$H_2$Ar), 4.28 (d, *J* = 6.9 Hz, 2H, C$H_2$Fmoc), 4.20-4.10 (m, 1H, C*H*Fmoc), 4.10-3.90 (m, 4H, OC$H_2$), 3.76-3-50 (m, 6H, OC$H_2$ and C$H_2$Gly), 3.17 (d, *J* = 13.6 Hz, 2H, ArC$H_2$Ar), 3.06 (d, *J* = 13.1 Hz, 2H, ArC$H_2$Ar), 2.00-1.70 (m, 8H, $OCH_2CH_2$), 1.65-1.10 (94H, *t*Bu and $O(CH_2)_2(CH_2)_5$), 0.90-0.88 (m, 12H, C$H_3$). $^{13}$C NMR (75 MHz, $CDCl_3$) δ 165.8, 163.5, 154.9, 153.3, 153.0, 143.9, 141.1, 137.3, 136.2, 133.5, 133.4, 130.5, 130.3, 127.5, 127.0, 125.2, 122.7, 122.4, 122.1, 121.8, 119.8, 83.4, 79.3, 75.7, 75.0, 66.8, 47.1, 43.2, 31.9, 31.3, 30.4, 30.0, 29.8, 29.7, 29.4, 28.2, 28.0, 27.9, 26.6, 26.5, 25.9, 22.7, 22.6, 14.1. MS (ESI): calculated for $[M + Na]^+$ *m*/*z* = 1961.2, found *m*/*z* = 1962.1.

**General procedure for the removal of the Fmoc protecting groups (**see the **Experimental Section** in **Chapter 4).**

**5-Glicinylamino-25,26,27,28-tetrakis[3-(bis-Boc-guanidine)propoxy]calix[4]arene (46)**.

The product is used without further purification and the identity of the product was checked by ESI-MS.

MS (ESI): calculated for $[M + H]^+$ *m/z* = 1693.9, found *m/z* = 1694.6, calculated for $[M + Na]^+$ *m/z* = 1715.9, found *m/z* = 1715.8.

**5,11,17-Tris(bis-Boc-guanidine)-23-glicinylamino-25,26,27,28-tetraoctyloxycalix[4]arene (53).**

The product is used without further purification and the identity of the product was checked by ESI-MS.

MS (ESI): calculated for $[M + Na]^+$ *m/z* = 1739.1, found *m/z* = 1739.7.

**General procedure for the coupling reaction (**see the **Experimental Section** in **Chapter 4**).

**5-[(7-hydroxycoumarin-3-carbonyl)glicinylamino]-25,26,27,28-tetrakis[3-(bis-Boc-guanidine)propoxy]calix[4]arene (47).**

The crude was purified by preparative TLC (eluent: hexane/ethyl acetate= 4:7) to obtain the pure product as a colourless oil in 13% yield.

$^1$H NMR (300 MHz, $CDCl_3$): δ 11.51 (s, 4H, N*H*Boc), 9.49 (t, 1H, *J* = 5.4 Hz, $COCH_2NH$), 8.94 (s, 1H, C*H*CCOCou), 8.60-8.30 (m, 5H, N=CN*H* and ArN*H*), 7.49 (d, 1H, *J* = 8.6 Hz, Ar*H*Cou), 6.99 (s, 2H, Ar*H*CNH), 6.94 (dd, *J* = 8.7, 2.1 Hz, 1H, CHC*H*COHCou), 6.86-6.68 (m, 3H, Ar*H*), 6.83 (d, *J* = 1.8 Hz, 1H, C*H*COCCou), 6.41-6.23 (m, 6H, Ar*H*), 4.42 (d, 2H, *J* = 4.8 Hz, NHCOC$H_2$), 4.31 (d, 2H, *J* = 13.6 Hz, ArC$H_2$Ar), 4.17 (d, 2H, *J* = 13.5 Hz, ArC$H_2$Ar), 4.18-3.85 (m, 6H, OC$H_2$), ), 4.18-3.85 (t, *J* = 6.9 Hz, 2H, OC$H_2$), 3.63-3.39 (m, 8H, $OCH_2CH_2CH_2$), 3.13 (d, 2H, *J* = 13.6 Hz, ArC$H_2$Ar), 2.90 (d, 2H, *J* = 13.5 Hz, ArC$H_2$Ar), 2.30-2.02 (m, 8H, $OCH_2CH_2$), 1.48, 1.47 (2×s, 72H, *t*Bu). $^{13}$C NMR (75 MHz, $CDCl_3$): δ 166.6, 164.3, 163.6, 163.4, 163.3, 163.2, 161.0, 156.9, 156.7, 156.2, 156.1, 156.0, 155.0, 153.2, 153.1, 136.0, 135.7, 133.7, 133.3, 132.1, 131.3, 128.7, 127.9, 122.4, 122.0, 120.2, 115.0, 112.8, 111.3, 103.1, 83.1, 82.9, 79.3, 79.0, 78.4, 72.2, 71.8, 44.7, 38.0, 37.9, 30.9, 30.8, 29.8, 29.6, 28.2, 28.0. MS (ESI): calculated for $[M + 2Na]^{++}$ *m/z* = 963.5, found *m/z* = 964.0.

**Synthesis of 5-[(7-hydroxycoumarin-3-carbonyl)amino]-25,26,27,28-tetrakis[3-(bis-Boc-guanidine)propoxy]calix[4]arene (49).**

The crude was purified by preparative TLC (eluent: hexane/ethyl acetate = 1:1) to obtain the pure product as a light green oil in 49% yield.

$^1$H NMR (300 MHz, $CDCl_3$): δ 11.62, 11.55, 11.50 (3×bs, 4H, N*H*Boc), 10.48 (s, 1H, ArN*H*), 8.90 (s, 1H, C*H*CCOCou), 8.68 (t, *J* = 5.7 Hz, 1H, $CH_2$N*H*), 8.49 (t, *J* = 5.1 Hz, 2H, $CH_2$N*H*), 8.40 (t, *J* = 5.4 Hz, 1H, $CH_2$N*H*), 7.53 (d, *J* = 8.8 Hz, 1H, Ar*H*Cou), 7.00 (s, 2H, Ar*H*NH), 6.88 (dd, *J* = 8.7, 2.1 Hz, 1H, CHC*H*COHCou), 6.85 (d, *J* = 8.0 Hz, 2H, Ar*H*), 6.74 (t, *J* = 7.5 Hz, 1H, Ar*H*), 6.70 (d, *J* = 2.0 Hz, 1H, C*H*COCCou), 6.40-6.22 (m, 6H, Ar*H*), 4.34 (d, *J* = 13.4 Hz, 2H, ArC$H_2$Ar), 4.22 (d, *J* = 13.5 Hz, 2H, ArC$H_2$Ar), 4.15-3.92 (m, 4H, OC$H_2$), 3.84 (t, *J* = 6.5 Hz, 4H, OC$H_2$), 4.73-3.42 (m, 8H, $OCH_2CH_2CH_2$), 3.14 (d, *J* = 13.4 Hz, 2H, ArC$H_2$Ar), 3.00 (d, *J* = 13.5 Hz, 2H, ArC$H_2$Ar), 2.35-2.12 (m, 8H, $OCH_2CH_2$), 1.50, 1.48, 1.46, 1.44 (4×s, 72H, *t*Bu). $^{13}$C NMR (75 MHz, $CDCl_3$): δ 163.6, 163.4, 163.0, 159.6, 156.7, 156.5, 156.3, 156.1, 155.0, 153.2, 153.1, 148.8, 135.9, 135.8, 133.5, 133.3, 132.1, 131.2, 128.7, 127.8, 122.4, 122.2, 120.5, 115.0, 113.9, 112.0, 103.1, 83.2, 83.0, 82.9, 79.5, 79.1, 79.0, 72.2, 71.9, 38.0, 30.9, 29.9, 29.6, 28.2, 28.0. MS (ESI): calculated for $[M + H]^+$ *m*/*z* = 1824.9, found *m*/*z* = 1825.5, calculated for $[M + Na]^+$ *m*/*z* = 1846.9, found *m*/*z* = 1847.9.

**5,11,17-Tris(bis-Boc-guanidine)-23-{[(7-hydroxycoumarin-3-carbonyl)glicinyl]amino}-25,26,27,28-tetraoctyloxycalix[4]arene (54).**

The residue was purified by preparative TLC. (eluent: $CH_2Cl_2$/MeOH= 40:1) to obtain the pure product as a yellowish oil in 37% yield.

$^1$H NMR (300 MHz, $CDCl_3$) δ 11.70 (bs, 3H, N*H*Boc), 11.70 (bs, 2H, N*H*Boc), 11.10 (bs, 1H N*H*Boc), 10.38 (s, 3H, O*H*Cou and ArN*H*), 9.70 (s, 1H, ArN*H*), 8.86 (bt, 1H, CON*H*$CH_2$), 8.59 (bs, 1H, ArN*H*CO), 8.23 (s, 1H, C*H*CCOCou), 7.45 (d, *J* = 2.3 Hz, 2H, Ar*H*), 7.30 (d, *J* = 2.2 Hz, 2H, Ar*H*), 7.10 (d, *J* = 8.7 Hz, 1H, C*H*CHCOHCou), 6.75 (dd, *J* = 8.6, 2.2 Hz, 1H, CHC*H*COHCou), 6.61 (d, *J* = 2.1 Hz, 1H, C*H*COCCou), 6.38 (s, 2H, Ar*H*), 6.03 (s, 2H, Ar*H*), 4.41 (d, *J* = 12.6 Hz, 2H, ArC$H_2$Ar), 4.37 (d, *J* = 13.1 Hz, 2H, ArC$H_2$Ar), 4.15-4.02 (m, 4H, OC$H_2$), 3.86 (d, *J* = 5.5 Hz, 2H, C$H_2$Gly), 3.77-3.75 (m, 4H, OC$H_2$), 3.18 (d, 2H, *J* = 13.1 Hz, ArC$H_2$Ar), 3.06 (d, 2H, *J* = 12.6 Hz, ArC$H_2$Ar), 2.05-1.20 (m, 102H, $OCH_2CH_2CH_2CH_2CH_2CH_2CH_2$ and *t*Bu), 1.07-0.82 (m, 12H, C$H_3$). $^{13}$C NMR (75 MHz, $CDCl_3$) δ 168.0, 164.2, 163.5, 163.1, 162.5, 161.5, 156.1, 154.9, 153.6, 153.5, 153.3, 153.1, 152.8, 147.7, 137.2, 136.9, 136.5, 136.2, 134.7, 133.6, 130.6, 130.5, 130.0, 122.7, 122.5,

122.1, 121.9, 115.2, 112.6, 110.8, 103.3, 83.9, 83.5, 79.8, 79.4, 75.7, 75.1, 41.8, 31.9, 31.3, 30.4, 30.0, 29.8, 29.7, 29.4, 28.1, 26.6, 26.5, 26.1, 22.7, 22.6, 14.1. MS (ESI): calculated for $[M + Na]^+$ *m*/*z* = 1927.1, found *m*/*z* = 1927.8.

**General procedure for the removal of the Boc protecting groups (**see the **Experimental Section** in **Chapter 2).**

**25,26,27,28-Tetrakis(6-guanidiniumhexyloxy)calix[4]arene, tetrachloride (32).**

The pure compound was obtained as a white powder in 87% yield.

Mp >250 °C dec. $^1$H NMR (300 MHz, $CD_3OD$) δ 6.62-6.46 (m, 12H, Ar*H*), 4.51 (d, *J* = 13.2 Hz, 4H, ArC$H_2$Ar), 3.92 (t, *J* = 7.3 Hz, 8H, OC$H_2$), 3.19 (t, *J* = 7.0 Hz, 8H, C$H_2$N), 3.13 (d, *J* = 13.2 Hz, 4H, ArC$H_2$Ar), 2.03-1.87 (m, 8H, $OCH_2CH_2$), 1.72-1.38 (m, 8H, C$H_2CH_2$N), 1.57-1.41 (m, 16H, $OCH_2CH_2CH_2CH_2$). $^1$H NMR (300 MHz, $D_2O$) δ 6.89 (d, *J* = 7.6 Hz, 8H, Ar*H*), 6.73 (t, *J* = 7.6 Hz, 4H, Ar*H*), 4.44 (d, *J* = 13.1 Hz, 4H, ArC$H_2$Ar), 4.00 (t, *J* = 6.8 Hz, 8H, OC$H_2$), 3.22 (t, *J* = 6.3 Hz, 8H, C$H_2$N), 3.33 (d, *J* = 13.1 Hz, 4H, ArC$H_2$Ar), 2.06-1.95 (m, 8H, $OCH_2CH_2$), 1.71-1.57 (m, 8H, C$H_2CH_2$N), 1.57-1.41 (m, 16H, $OCH_2CH_2CH_2CH_2$). $^{13}$C NMR (75 MHz, $CD_3OD$) δ 158.9, 158.0, 136.5, 129.6, 123.4, 76.2, 42.9, 32.3, 31.6, 30.4, 28.2, 27.5. MS (ESI): calculated for $[M + H - 4\ HCl]^+$ *m*/*z* = 989.7, found *m*/*z* = 989.8, $[M + Na - 4\ HCl]^+$ *m*/*z* = 1011.7, found *m*/*z* = 1011.8.

**5-guanidinium-25,26,27,28-Tetrakis(3-guanidiniumpropoxy)calix[4]arene, pentachloride (36).**

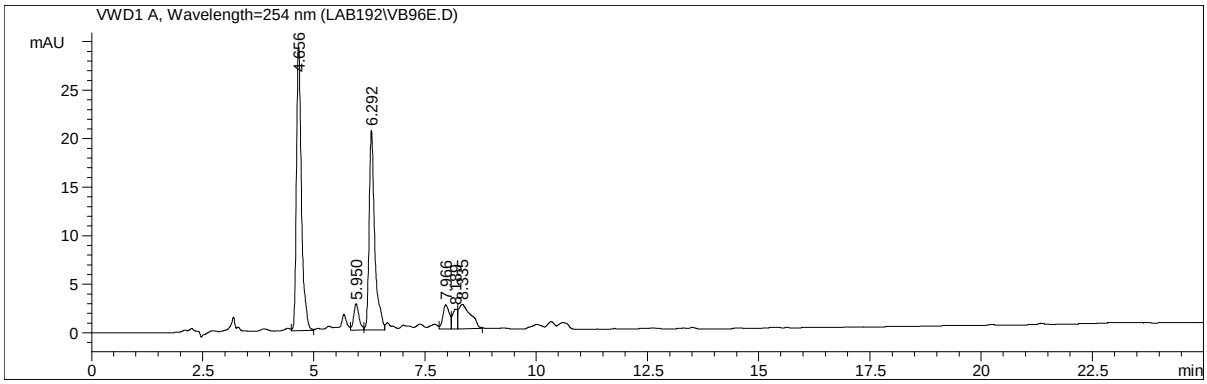
The pure compound was obtained as a light yellow powder in quantitative yield. Hygroscopic. $^1$H NMR (300 MHz, $CD_3OD$) δ 8.80 (s, 1H, ArN*H*), 7.85-7.70 (m, $CH_2$N*H*), 7.10-6.98 (m, 4H, Ar*H*), 6.86 (t, *J* = 7.5 Hz, 2H, Ar*H*), 6.46-6.32 (m, 3H, Ar*H*), 6.23 (s, 2H, Ar*H*CNH), 4.44 (d, *J* = 13.2 Hz, 4H, ArC$H_2$Ar), 4.17 (t, *J* = 7.2 Hz, 4H, OC$H_2$), 4.03-3.87 (m, 4H, OC$H_2$), 3.50-3.32 (m, 8H, C$H_2$N), 3.33-3.20 (m, 4H, ArC$H_2$Ar), 2.35-2.12 (m, 8H, $OCH_2CH_2$). $^1$H NMR (300 MHz, $D_2O$): δ 7.22-7.12 (m, 4H, Ar*H*), 6.97 (t, 2H, *J* = 7.5 Hz, Ar*H*), 6.54 (s, 3H, Ar*H*), 6.37 (s, 2H, Ar*H*), 4.40 (d, 4H, *J* = 13.4 Hz, ArC$H_2$Ar), 4.18 (t, 4H, *J* = 7.1 Hz, $OCH_2$), 3.99 (t, 2H, *J* = 6.9 Hz, OC$H_2$), 3.96 (t, 2H, *J* = 6.9 Hz, OC$H_2$), 3.48-3.28 (m, 12H, NHC$H_2$ and ArC$H_2$Ar), 2.35-2.16 (m, 8H, $OCH_2CH_2$). $^{13}$C NMR (75 MHz, $CD_3OD$): δ 159.0, 158.3, 157.7, 156.8, 156.1, 137.9, 137.6, 137.1, 135.4, 130.8, 130.4, 130.0,

129.3, 126.0, 124.3, 123.8, 74.1, 73.9, 73.5, 40.3, 40.2, 32.3, 31.2, 31.0. MS (ESI): calculated $[M + H - 5HCl]^+$ *m/z* 878.5, found *m/z* 878.7.

**49,50,51,52,53,54,55,56-Octakis(3-guanidiniumpropoxy)calix[8]arene, octachloride (37).** The crude (36 mg, $1.9\times10^{-2}$ mmol) was dissolved in water (9 mL) and purified by semi-preparative RP-HPLC, with a $C_{12}$ column, after having found the correct gradient with an analytical $C_{12}$.

- **Column:** analytical $C_{12}$ "Jupiter 4u Proteo 90A"
- **Particles diameter:** 4 μm
- **Porosity:** 90 Å
- **Dimensions:** 250×4.6 mm
- **Detector:** UV-visible at λ = 254 nm
- **Eluents mixture:** A: 100% $H_2O$ + 0.05% TFA
  B: 100% $CH_3CN$ + 0.05% TFA
- **Injection Volume:** 5 μL
- **Gradient:**

| Time (min) | Flow | %A | %B |
|---|---|---|---|
| - | 4.00 mL/min | 70% | 30% |
| 20 | 4.00 mL/min | 40% | 60% |
| 25 | 4.00 mL/min | 40% | 60% |

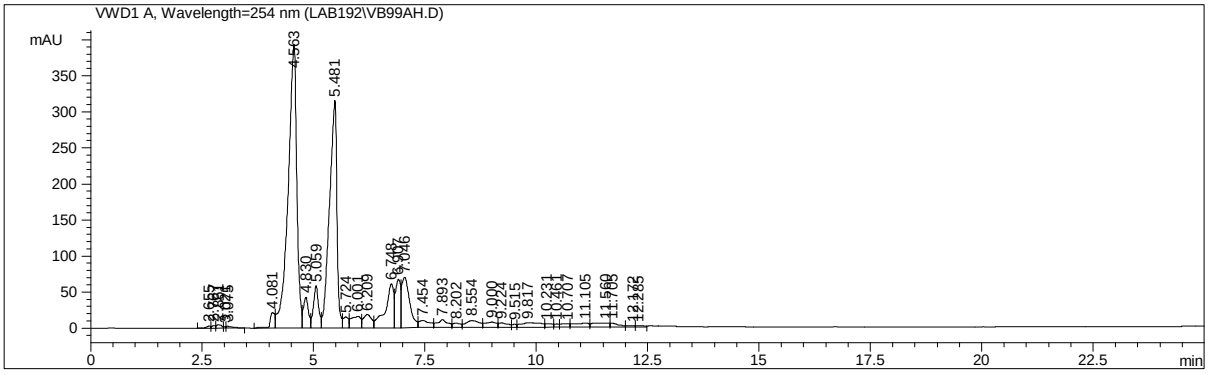



- **Retention time:** 4.656 min
- **Column:** semi-preparative "Jupiter 4u Proteo 90A" $C_{12}$

- **Particles diameter:** 4 μm
- **Porosity:** 90Å
- **Dimensions:** 250×10 mm
- **Detector:** UV-visible at λ = 254 nm
- **Eluents mixture:** A: 100% $H_2O$ + 0.05% TFA
  B: 100% $CH_3CN$ + 0.05% TFA
- **Injection volume:** 300 μL
- **Gradient:**

| Time (min) | Flow | %A | %B |
|---|---|---|---|
| - | 4.00 mL/min | 70% | 30% |
| 20 | 4.00 mL/min | 40% | 60% |
| 25 | 4.00 mL/min | 40% | 60% |

- **Retention time:** 5.563 min




The fractions corresponding to pure product were collected and the solvent was evaporated under reduced pressure. The residue was taken three times with 10 mM HCl (3×5 mL) and each time the volatiles were removed at the rotavapor.

The pure compound was obtained as a white powder in 27% yield.

Mp: >250 °C dec. $^1$H NMR (300 MHz, $CD_3OD$) δ 6.85 (s, 24H, Ar*H*), 4.06 (s, 16H, ArC$H_2$Ar), 3.88 (bt, 16H, OC$H_2$), 3.36 (bt, 16H, $OCH_2CH_2CH_2$), 2.03 (bs, *J* = 6.2 Hz, 16H, $OCH_2CH_2$). $^1$H NMR (300 MHz, $D_2O$) δ 6.91 (s, 24H, Ar*H*), 4.04 (s, 16H, ArC$H_2$Ar), 3.88 (t, *J* = 5.9 Hz, 16H, OC$H_2$), 3.24 (t, *J* = 6.7 Hz, 16H, $OCH_2CH_2CH_2$), 1.98 (quint, *J* = 6.2 Hz,

16H, $OCH_2CH_2$). $^{13}C$ NMR (75 MHz, $CD_3OD$) δ 158.9, 156.4, 135.8, 130.5, 125.8, 71.9, 40.1, 31.2, 30.8. MS (ESI): calculated for $[M + 2H - 8HCl]^{++}$ *m*/*z* = 821.5, found *m*/*z* = 822.0

**5-[(7-hydroxycoumarin-3-carbonyl)glicinylamino]-25,26,27,28-tetrakis[3-(bis-Boc-guanidine)propoxy]calix[4]arene, tetrachloride (48).**

The pure compound was obtained as pale yellow oil in 80% yield.

$^1H$ NMR (300 MHz, $CD_3OD$): δ 8.73 (s, 1H, C*H*CCOCou), 7.61 (d, 1H, *J* = 8.4 Hz, Ar*H*Cou), 6.82 (dd, *J* = 9.0, 2.1 Hz, 1H, CHC*H*COHCou), 6.87-6.68 (m, 8H, Ar*H*), 6.70 (d, *J* = 3.3 Hz, 1H, C*H*COCCou), 6.51 (s, 3H, Ar*H*), 4.41 (d, 2H, *J* = 13.5 Hz, ArC$H_2$Ar), 4.40 (d, 2H, *J* = 13.5 Hz, ArC$H_2$Ar), 4.13 (s, 2H, NHCOC$H_2$), 4.06 (t, *J* = 7.5 Hz, 4H, OC$H_2$), 4.00 (t, *J* = 6.9 Hz, 4H, OC$H_2$), 3.45-3.35 (m, 8H, $OCH_2CH_2CH_2$), 3.23 (d, 2H, *J* = 13.5 Hz, ArC$H_2$Ar), 3.21 (d, 2H, *J* = 13.5 Hz, ArC$H_2$Ar), 2.31-2.14 (m, 8H, $OCH_2CH_2$). $^1H$ NMR (300 MHz, $D_2O$): δ 8.79 (s, 1H, C*H*CCOCou), 7.61 (d, 1H, *J* = 8.4 Hz, Ar*H*Cou), 6.82 (dd, *J* = 9.0, 2.1 Hz, 1H, CHC*H*COHCou), 6.87-6.68 (m, 8H, Ar*H*), 6.70 (d, *J* = 3.3 Hz, 1H, C*H*COCCou), 6.51 (s, 3H, Ar*H*), 4.41 (d, 2H, *J* = 13.5 Hz, ArC$H_2$Ar), 4.40 (d, 2H, *J* = 13.5 Hz, ArC$H_2$Ar), 4.13 (s, 2H, NHCOC$H_2$), 4.06 (t, *J* = 7.5 Hz, 4H, OC$H_2$), 4.00 (t, *J* = 6.9 Hz, 4H, OC$H_2$), 3.45-3.35 (m, 8H, $OCH_2CH_2CH_2$), 3.23 (d, 2H, *J* = 13.5 Hz, ArC$H_2$Ar), 3.21 (d, 2H, *J* = 13.5 Hz, ArC$H_2$Ar), 2.31-2.14 (m, 8H, $OCH_2CH_2$). $^{13}C$ NMR (75 MHz, $CD_3OD$): δ 168.7, 165.9, 164.9, 163.0, 158.7, 158.4, 157.6, 157.0, 153.7, 149.9, 136.5, 136.1, 135.7, 133.8, 133.0, 129.9, 129.8, 129.5, 123.7, 123.5, 121.7, 115.7, 114.3, 112.8, 103.1, 73.2, 44.2, 40.0, 32.1, 30.9, 30.8. MS (ESI): calculated for $[M + H - 4HCl]^+$ *m*/*z* =1081.5, found *m*/*z* = 1081.5.

**5-[(7-hydroxycoumarin-3-carbonyl)amino]-25,26,27,28-tetrakis[3-guanidinylpropoxy]calix[4]arene, tetrachloride (50).**

The pure compound was obtained as a white powder in quantitative yield.

$^1H$ NMR (300 MHz, $CD_3OD$): δ 10.36 (s, 1H, ArN*H*), 8.79 (s, 1H, C*H*CCOCou), 7.72 (bs, N=CN*H*), 7.69 (d, *J* = 8.7 Hz, 1H, Ar*H*Cou), 6.90 (dd, *J* = 8.7, 2.3 Hz, 1H, CHC*H*COHCou), 6.88-6.67 (m, 8H, Ar*H*), 6.70 (d, *J* = 2.4 Hz, 1H, Ar*H*Cou), 6.46 (s, 3H, Ar*H*), 4.43 (d, 2H, *J* = 13.4 Hz, ArC$H_2$Ar), 4.42 (d, 2H, *J* = 13.3 Hz, ArC$H_2$Ar), 4.20-3.92 (m, 8H, OC$H_2$), 3.55-3.30 (m, 8H, $OCH_2CH_2CH_2$), 3.25 (d, 4H, *J* = 13.3 Hz, ArC$H_2$Ar), 3.23 (d, 4H, *J* = 13.4 Hz, ArC$H_2$Ar), 2.32-2.15 (m, 8H, $OCH_2CH_2$). $^1H$ NMR (300 MHz, $D_2O$): δ 8.63 (s, 1H, C*H*CCOCou), 7.62 (d, *J* = 8.8 Hz, 1H, Ar*H*Cou), 7.11 (t, *J* = 8.0 Hz, 4H, Ar*H*), 6.93 (t, *J* =

7.4 Hz, 2H, Ar*H*), 6.87 (d, *J* = 8.1 Hz, 1H, CHC*H*COHCou), 6.72 (s, 1H, Ar*H*Cou), 6.41 (s, 2H, Ar*H*), 6.35-6.26 (m, 3H, Ar*H*), 4.34 (d, 2H, *J* = 13.8 Hz, ArC$H_2$Ar), 4.30 (d, 2H, *J* = 13.2 Hz, ArC$H_2$Ar), 4.15-4.02 (m, 4H, OC$H_2$), 3.93 (t, *J* = 6.5 Hz, 2H, OC$H_2$), 3.83 (t, *J* = 6.4 Hz, 2H, OC$H_2$), 3.43-3.18 (m, 12H, $OCH_2CH_2CH_2$ and ArC$H_2$Ar), 2.25-2.16 (m, 8H, $OCH_2CH_2$). $^{13}$C NMR (75 MHz, $CD_3OD$): δ 166.2, 163.9, 162.0, 159.0, 158.6, 158.0, 157.2, 154.5, 150.3, 137.0, 136.7, 136.5, 135.9, 133.4, 133.3, 130.3, 130.2, 129.6, 124.0, 123.7, 122.7, 122.6, 116.2, 114.9, 113.3, 103.4, 73.5, 40.3, 32.5, 32.4, 31.2, 31.1. MS (ESI): calculated for $[M + H - 4HCl]^+$ *m/z* = 1824.5, found *m/z* = 1824.8, calculated for $[M + Na]^+$ *m/z* = 1046.5, found *m/z* = 1047.8.

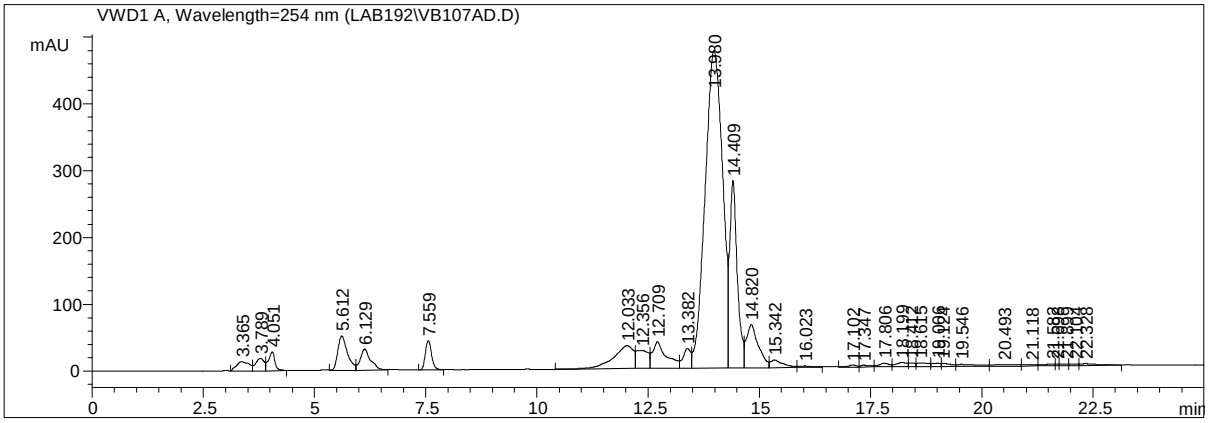
**5,11,17-Trisguanidinium-23-{[(7-hydroxycoumarin-3-carbonyl)glicinyl]amino}-25,26,27,28-tetraoctyloxycalix[4]arene, trichloride (55).**

The crude (18 mg, $1.3\times10^{-2}$ mmol) was dissolved in methanol (6 mL) and purified by semi-preparative RP-HPLC, with a $C_{12}$ column, after having found the correct gradient conditions with an analytical $C_{12}$ column.

- **Column:** semi-preparative "Jupiter 4u Proteo 90A" $C_{12}$
- **Particles diameter:** 4 μm
- **Porosity:** 90Å
- **Dimensions:** 250×10 mm
- **Detector:** UV-visible at λ = 254 nm
- **Eluents mixture:** A: 100% $H_2O$ + 0.05% TFA
  B: 100% MeOH + 0.05% TFA
- **Injection volume:** 300 μL
- **Gradient:**

| Time (min) | Flow | %A | %B |
|---|---|---|---|
| - | 4.00 mL/min | 20% | 80% |
| 20 | 4.00 mL/min | 0% | 100% |
| 25 | 4.00 mL/min | 0% | 100% |

- **Retention time:** 13.98 min




$^{1}$H NMR (300 MHz, $CD_3OD$) δ 8.80 (s, 1H, C*H*CCOCou), 7.71 (d, *J* = 8.4 Hz, 1H, C*H*CHCOHCou), 6.91 (dd, *J* = 8.4, 2.7 Hz, 1H, CHC*H*COHCou), 6.89-6.82 (m, 6H, Ar*H*), 6.81 (d, *J* = 2.4 Hz, 1H, C*H*COCCou), 6.50 (s, 2H, Ar*H*), 4.50 (d, *J* = 12.6 Hz, 2H, ArC$H_2$Ar), 4.37 (d, *J* = 13.5 Hz, 2H, ArC$H_2$Ar), 4.17 (s, 2H, C$H_2$Gly), 4.09-3.98 (m, 4H, OC$H_2$), 3.90 (t, *J* = 6.9 Hz, 2H, OC$H_2$), 3.84 (t, *J* = 6.0 Hz, 2H, OC$H_2$), ArC$H_2$Ar under the $CD_3OD$, 2.13-1.88 (m, 8H, $OCH_2CH_2$), 1.65-1.10 (m, 40H, $OCH_2CH_2CH_2CH_2CH_2CH_2CH_2$), 0.97-0.89 (m, 12H, C$H_3$). $^{13}$C NMR (75 MHz, $CD_3OD$): δ 169.2, 166.3, 165.5, 163.3, 158.7, 158.2, 157.9, 157.7, 157.5, 156.5, 154.5, 150.3, 138.9, 138.4, 137.6, 135.9, 133.9, 133.4, 130.1, 130.0, 127.2, 127.1, 126.6, 121.9, 116.1, 114.4, 113.0, 103.4, 77.3, 77.1, 44.7, 33.6, 32.0, 31.9, 31.6, 31.3, 31.2, 28.1, 27.9, 24.2, 14.8. MS (ESI): calculated for $[M -3 HCl + 2H]^{++}$ *m*/*z* = 652.9, found *m*/*z* = 653.0.

**DNA preparation and storage.** Plasmid DNA was purified through cesium chloride gradient centrifugation (T. Maniatis, E.F. Fritsch, J. Sambrook, in *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory: New York, 1989). A stock solution of the plasmid 0.350 μM in milliQ water (Millipore Corp., Burlington, MA) was stored at -20 °C.

**Electrophoresis mobility shift assay (EMSA)**

Binding reactions were performed in a final volume of 14 μL with 10 μL of 20 mM Tris/HCl pH 8, 1 μL of plasmid (1 μg of pEGFP-C1) and 3 μL of compound at different final concentrations, ranging from 25 to 200 μM. Binding reaction was left to take place at room temperature for 1 h; 5 μL of 1 g/mL in $H_2O$ of glycerol was added to each reaction mixture and loaded on a TA (40 mM Tris–Acetate) 1% agarose gel. At the end of the binding reaction 1 μL (0.01 mg) of ethidium bromide solution is added. The gel was run for 2.5 h in TA buffer

at 10 V/cm. EDTA was omitted from the buffers because it competes with DNA in the reaction.

**Ethidium Bromide Displacement Assays.** 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 guanidinium ligand.

**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 $MgCl_2$, 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 minutes at room temperature, then a 20 μL 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 μm at a scan rate of 3-4 lines per second and were flattened after recording using Nanoscope software.

**Cell culture and transient transfection assay**. The human rhabdomyosarcoma cell line RD-4, obtained from David Derse, National Cancer Institute, Frederick, Maryland, was maintained as a monolayer using growth medium containing 90% DMEM, 10% FBS, 2 mM l-glutamine, and 100 IU/mL penicillin, 10 μg/mL streptomycin. 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% $CO_2$. Transfections were performed in 6 well plates, when cells were 80% confluent (approximately 3×105 cells) on the day of transfection. 3 μg of plasmid, and different concentration of ligands were added to 1 mL of serum-free medium, mixed rapidly and incubated at room temperature for 20 min. Each mixture was carefully added to the cells following the removal of the culture medium from the cells. Lipoplex formulations were performed adding DOPE to plasmid-ligand

mixture at 1: 2 ligand: DOPE molar ratio, where ligand concentration was kept to 10 μM. 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% $CO_2$ for 5 h. Finally, transfection mixture was removed and 3 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.

Vero (African green monkey, ATCC CRL-1586), BoMac (bovine macrophage, obtained from J. Stabel, National Animal Disease Center, Ames, IA, USA), N2a (Mouse neuroblastoma, ATCC CCL-131), AUBEK (bovine foetal kidney cell line, ATCC CCL 163) and hMSC (human mesenchymal stem cells, obtained from R. Sala, University of Parma, Italy) were grown in EMEM medium containing NEAA, 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. All cultures were incubated at 37 °C in a humidified atmosphere containing 5% $CO_2$. Transfection were performed as described for RD-4 cells.

**MTT survival assay for cell viability determination.** Following transfection, complete medium (90% DMEM, 10% FBS, 2 mM l-glutamine, and 100 IU/mL penicillin, 10 μg/mL streptomycin) containing MTT (5 mg/mL) was added to the culture for 4 h. Then, after the addition of an equal volume of solubilisation solution (10% SDS in HCl 0.01 M) cells were incubated at 37 °C overnight. Specific optical density was measured at 540 nm, using 690 nm as reference wavelength in an SLT-Lab microreader (Salzburg, Austria). Each experiment was done three times and each treatment was performed with eight replicates. Statistical differences among treatments were calculated with Student's test and multifactorial ANOVA.

**Luciferase reporter assay**

Luciferase reporter assay was performed with a Dual Luciferase Reporter Assay System kit (Promega) with minor modifications. Following treatments, cells were washed with PBS, lysed with 100 μl of lysis passive buffer by freeze-thawing at −80 °C. 10 μl of the cell lysate was added to 50 μl of LAR and Luciferase activity were determined with a PerkinElmer Victor Multilabel Counter, according to the manufacturer's specifications. Individual assays

were normalized for *Renilla* Luciferase activity with a second reading, adding 50 µl of Stop & Glo substrate.

## 2.7 References

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