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Water-Soluble Calix[4]arene Derivatives: Binding Stoichiometry and Spectroscopic Evaluation of the Host-Guest Recognition Mechanism

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1. Introduction

If hydrophobic molecules are inserted into an aqueous medium, the water molecules order around the hydrophobic ones to build a quasi crystalline surface. In this way, the hydrogen bonding of the water molecules around a hydrophobic surface is maximized. If two hydrophobic molecules meet they will associate with their hydrophobic surfaces towards each other. The water molecules, previously attached to these surfaces, will be distributed back into the bulk solvent resulting in favourable entropy. The entropic gain is responsible for almost all associations in the medium water and hence extremely important for life (e.g. formation of membranes, micelles, and for protein folding where folding starts often with tryptophan residues forming a hydrophobic core). The hydrophobic effect is shown below (**Figure 1**).

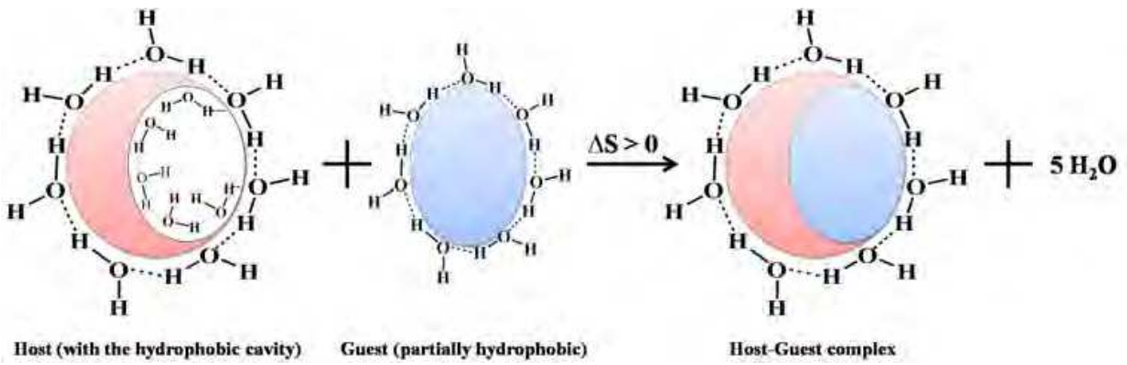

Fig. 1. Host-guest binding mechanism in aqueous medium.

Similarly, in most cases the protein-substrate binding is a result of the hydrophobic effect. However, there are evidences suggesting that the water molecules play an important role in the protein-substrate binding. Water molecules could participate in hydrogen bonding networks that link side chain and main chain atoms with the functional groups on the bases,

and the phosphodiester backbone anionic oxygens.[1] Macromolecular crystallography provided the necessary supportive view, that water molecules act as major contributors to stability and specificity.[2,3,4]

Thermodynamic analyses of protein-DNA binding suggest that water released from protein-DNA interfaces is favourable to binding. Structural analyses of the remaining water at the interface in protein-DNA complexes indicate that a majority of these water molecules promote binding by screening protein and DNA electrostatic repulsions between electronegative atoms/like charges. A small fraction of the observed interfacial waters act as linkers to form extended hydrogen bonds between the protein and the DNA, compensating for the lack of a direct hydrogen bond.[5]

Is it by design or by default that water molecules are observed at the interfaces of some protein-DNA complexes? Both experimental and theoretical studies on the thermodynamics of protein-DNA binding overwhelmingly support the extended hydrophobic view that water release from interfaces supports binding. Structural and energy analyses indicate that the remaining waters at the protein-DNA complexes interfaces ensure liquid-state packing densities, screen the electrostatic repulsions between like charges (which seems to be by design), and in a few cases act as linkers between complementary charges on the biomolecules (which may well be by default). Protein-drug binding and DNA-small molecule binding also revealed the possibility of the role played by the water molecules in the receptors binding pockets. The binding of the cardiac toponin-I (cTnI) with the small molecule (Fluorescent probe) revealed the enzyme hydrophobic binding region as shown in **Figure 2**.[6]

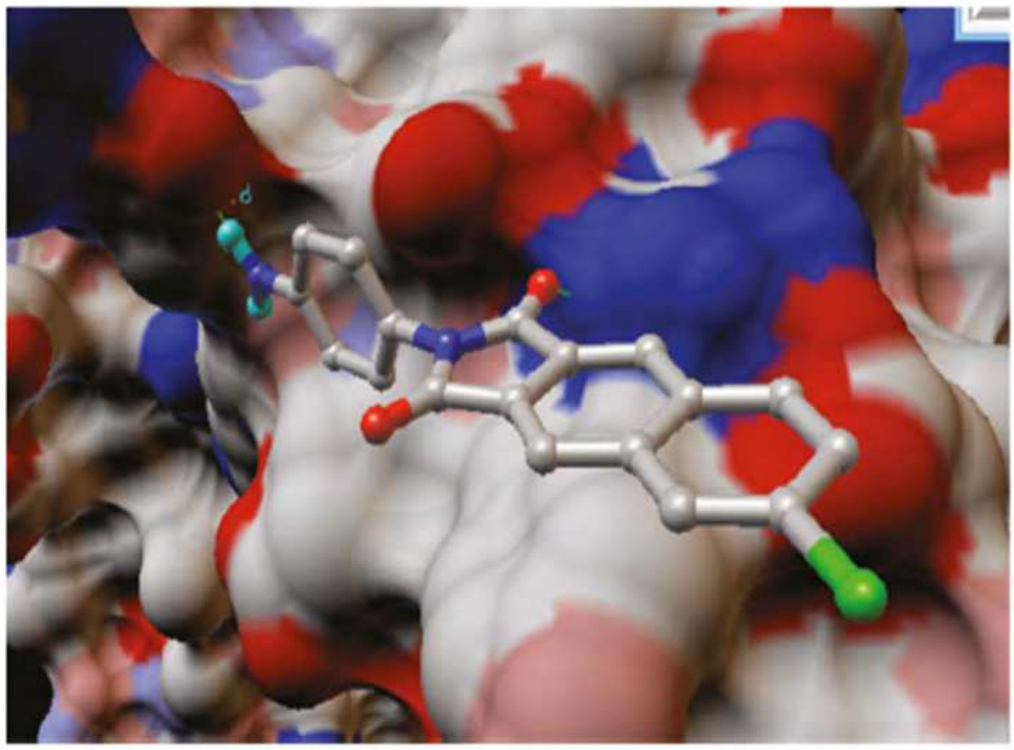

Fig. 2. Binding mode of cardiac toponin-I (cTnI) with the fluorescent probe. (*Reproduced from J. Am. Chem Soc.*133(38):14972-14974).

The protein enzymatic activity, being a surface function, depends on the recognition efficiency of negatively charged and polar amino acids of the substrate peptide.[7] The bulk-like environment in the close vicinity of the surface would enhance the interaction with the substrate (**Figure 3**). On the other hand, the structured water molecules are needed around the protein surface to be part of an efficient chemistry and possibly maintain a three dimensional structure.[8] From these observations, it is clear that the water molecules play a crucial role in the receptor-substrate binding, probably be due to the hydration effect or hydrophobic effect. Scientist has always tried to find the answers related to the biological receptor-substrate interactions using the host-guest chemistry of synthetic counterparts.

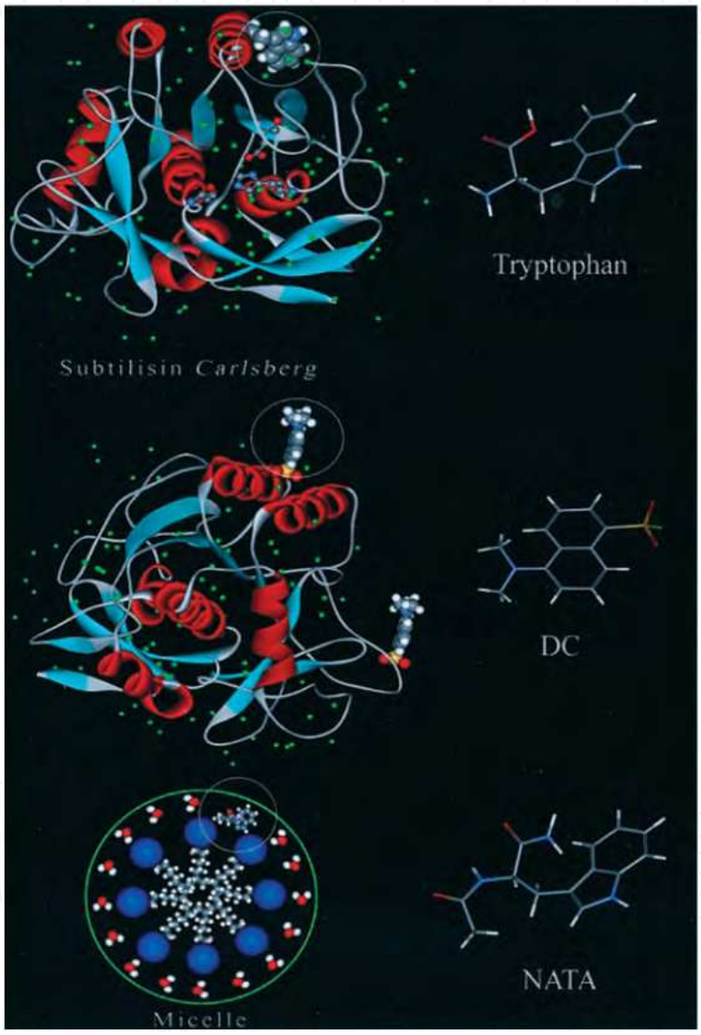

Fig. 3. High-resolution X-ray structure of the Subtilisin *Carlsberg* (SC) protein. This structure was downloaded from the Protein Data Bank and processed with WEBLAB-VIEWERLITE, Accelrys, San Diego, CA. (*Top*) Position of the protein single Trp residue. Note the bound water molecules around this residue. (*Middle*) Two of the nine potential binding sites for DC labeling are shown. (*Bottom*) Illustration of a micelle with a NATA molecule included. Molecular structures of the probes are presented on the right of each illustration. (*Reproduced from Proc. Nat. Acad. Sci.* 2002, 99(4): 1763–1768)

This chapter presents a survey of the current literature on water-soluble calix[4]arenes (hosts) complexes with various substrates (guests), elaborating the water-soluble calix[4]arenes applications. Moreover, a critique of various data interpretations, in the context of the water molecules role in host-guest binding and in general of the water-soluble calix[4]arene guest recognition principles, is also provided.

2. Stoichiometry of the water-soluble calix[4]arene complexes: The methods

2.1 Binding constant

The thermodynamic stability of a host-guest (e.g. metal–macrocycle) complex, in a given solvent (often water or methanol) at a given temperature, is gauged by the binding constant, *K*, measurement. The binding constant is the most widely used method for host-guest affinity assessment in solution, and it is of fundamental importance in supramolecular chemistry. The binding constant is merely the equilibrium constant for the reaction between a Host, H, and Guest, G, in water, described in the following equation:

$$H(H_2O)n + G(H_2O)n \rightleftharpoons [H.G] + n(H_2O)$$

$$K = \frac{[H.G]}{[H(H_2O)n][G(H_2O)n]}$$

Thus a large binding constant corresponds to a high equilibrium concentration of bound guest, and hence to a more stable host–guest complex.

If a sequential process of more than one guest is involved in the binding process, then two *K* values may be measured for the 1:1 and 1:2 complexes, respectively: K_1 and K_2.

$$H(H_2O)n + G(H_2O)n \overset{K_1}{\rightleftharpoons} [H.G] + n(H_2O)$$

$$[H.G](H_2O)n + G(H_2O)n \overset{K_2}{\rightleftharpoons} [H.G_2] + n(H_2O)$$

$$K_2 = \frac{[H.G_2]}{[[H.G](H_2O)n][G(H_2O)n]}$$

In these circumstances, an overall binding constant, β, may be defined for the complete process, with the individual *K* values known as the stepwise binding constants:

$$\beta = K_1 \, X \, K_2$$

Magnitudes of binding constants can widely change, so they are often reported as log *K*, hence:

$$\log\beta = \log(K_1 \, X \, K_2) = \log K_1 + \log K_2)$$

The host-guest complex binding constant depends on the complex stoichiometry. As shown in the equations above, a key aspect of such calculations is the use of the correct stoichiometry model (i.e. the ratio of host to guest, which must be assumed or determined), so it is worthy spending some time in understanding the method to determine it.

2.2 Job plot (method of continuous variation)

There are different methods to determine the stoichiometry, e.g. Continuous Variation Methods (**Figure 4**),[9] the Slope Ratio Method,[10] the Mole Ratio Method,[11] and others. Being the Continuous Variation Method the most popular among these, this method has been adopted here to determine the stoichiometry.

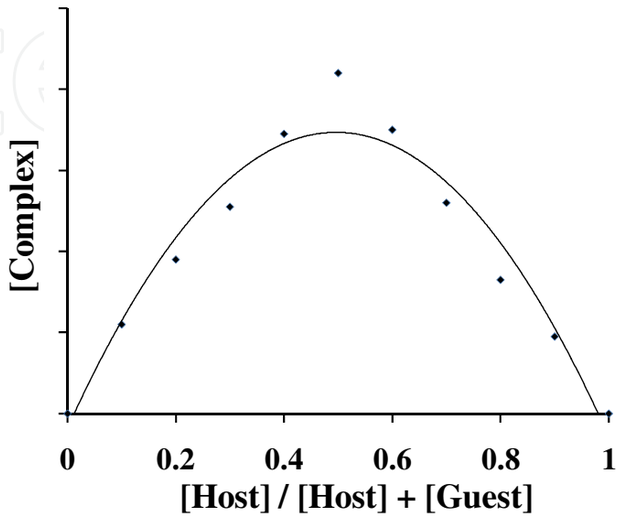

Fig. 4. Job plot for a 1:1 host–guest complex.

There is a strong bias in the host-guest chemistry literature towards the fitting of data to 1:1 stoichiometries, and it is a common mistake to neglect higher complexes. Binding stoichiometry may be confirmed in most kinds of titration experiments, allowing the complex concentration to be determined by making up a series of solutions with varying host-guest ratios such that the total concentration of host and guest remains constant. Monitoring the changing concentration of the host–guest complex in these samples allows a plot of [Complex] against ([Host]/([Host] + [Guest])) to be constructed (**Figure 4**). For a 1:1 complex, this kind of representation (referred to as a Job plot) should give a peak at 0.5 (**Figure 4**), a peak at 0.66 would correspond to a 2:1 stoichiometry and so on. The complex concentration is generally taken to be related to an observable quantity such as $\Delta\delta$ according to following equation

$$[\text{Complex}] \; \alpha \; \Delta\delta \; \text{X mole fraction of host}$$

In a spectrophotometric experiment, absorbance, at a properly chosen wavelength, is usually directly proportional to the complex concentration.

2.3 Methods for association constant measurements

Generally, the complex formation mechanism between a host and a guest is a basic and important process in supramolecular chemistry. Selectivity in the complexation is a crucial property in determining the molecular recognition ability of the host molecule, which discriminates among different guest species. The association constant's ratio of the corresponding complexation is usually treated as a measure of the selectivity. However,

theoretically the association constants can be measured by any experimental technique yielding information about the concentration of a complex, [Host-Guest], as a function of the host or guest concentration changes. In practice, the methods described below are of common use. In every case a concentration range must be chosen to have equilibrium between significant amounts of complexed and free host and guest, limiting the range of binding constants that can be measured by a particular technique. If the binding by the target host is too strong, then a competing host is sometimes added, in order to reduce the apparent (measured) association constant according to the difference in guest affinity between the two hosts. The true affinity can then be extrapolated from the knowledge of the binding constant of the guest for the host with the lower affinity.

2.3.1 Potentiometric titrations

If the host molecules are susceptible to protonation (e.g. the aminocalix[4]arenes with their basic tertiary amine nitrogen), the protonation constants, and consequently the p*K*a values, may be readily determined using pH electrodes to monitor a simple acid–base titration. Initially, this will give the acid dissociation constant (p*K*a) of the hosts conjugate acid, $H.H^+$.[12] Addition of a guest cation will perturb the hosts basicity by competition with H^+ ions for the ligand lone pair(s) and hence will affect the titration curves shape. Analysis of the various equilibrium by a curve-fitting computer program (such as *sigmaplot* or *Hyperquad*), along with knowledge of the hosts p*K*a, allows the determination of the amount of uncomplexed host and subsequently the concentration of the complex and the stability constants for the host-guest complexation reaction, as shown in the following equation,

$$K = \frac{[\mathrm{H}^+][H]}{[G.H^+]}$$

2.3.2 Nuclear magnetic resonance titration

If the exchange of complexed and un-complexed guest is slow on the nuclear magnetic resonance (NMR) time scale, then the association constant may be approximately evaluated under the prevailing conditions of concentration, temperature solvent *etc.* by simple integration of the NMR signals for complexed and un-complexed host or guest. However, most host–guest equilibrium are fast on the (relatively slow) NMR spectroscopic time scale, and the chemical shift observed for a particular resonance (that is sensitive to the complexation reaction) is a weighted average between the chemical shift of the free and bound species.

In a typical NMR titration experiment, small aliquots of guest are added to a host solution of known concentration in a deuterated solvent, and the NMR spectrum of the sample monitored as a function of guest concentration, or host:guest ratio. Commonly, changes in chemical shift ($\Delta\delta$) are noted for various atomic nuclei present (e.g. ^{1}H in ^{1}H NMR) as a function of the guest binding influence on their magnetic environment. As a result, two kinds of information are gained. Firstly, the location of the most affected nuclei may give qualitative information about the guest binding regioselectivity (e.g. is the guest inside the host cavity?). More importantly, the treatment of the titration curve data (a plot of $\Delta\delta$ against added guest concentration, e.g. Figure 1.4) by different methods such as the Benesi-Hildebrand (Hanna-Ashbaugh) treatment,[13] the Rose-Drago,[14] the Scatchard (Foster-Fyfe)

method,[15,16] and the non-linear curve fitting analysis (few examples of the software programs are EQNMR, AGRNMRL, GRAFIT, and GRAPHPAD PRISM)[17], give quantitative information about the association constant. NMR spectroscopic methods are useful for binding constants in the range 10–10^4 M^{-1}. Recently, the diffusion NMR spectroscopy has become popular in supramolecular chemistry, due to its application in determining association constants in many systems.[18,19]

2.3.3 Fluorescence titration

Fluorescence titration measurements are based on the proportion of fluorescence intensity to fluorophore concentration (i.e. concentration of fluorescent species in solution; this is often a fluorescent guest, G). For a 1:1 complex with host, H, with stability constant *Ks*= [HG]/[H][G] the fluorescence intensity *F* is given by the following equation:

$$F = k\mathrm{G}\,[\mathrm{G}] + k\mathrm{s}\,[\mathrm{HG}]$$

Where, the *k*G and *k*s represent proportionality constants for the guest and the 1:1 host–guest complex respectively. In the absence of host the fluorescence intensity, *F*o, is given by:

$$F_0 = K \circ \mathrm{G}_{\mathrm{total}}$$

$$\text{Where } \mathrm{G}_{\mathrm{total}} = [\mathrm{G}] + [\mathrm{HG}].$$

Combining these two relationships gives the following equation, which provides the basis for almost all the fluorimetric methods for stability constant (*K*11) measurements:

$$\frac{F}{F_0} = \frac{(K_G/K_G^0) + (K_s/K_G^0)K_sH}{1 + K_sH}$$

This equation is greatly simplified when either the guest or host–guest complex are non-fluorescent (i.e. the fluorescence is 'turned on' by complexation, or, in the case of quenching, by the host), in which case either K_G or K_s become zero. For example, for $K_G = K^0_G$ 0 and $K_s = 0$, we obtain:

$$\frac{F_0}{F} = 1 + K_sH$$

A simple plot of F_o/F against [H] from the quenching host titration into a guest solution should yield a straight line of slope K_s.

2.3.4 UV-Vis Spectrophotometric titration

UV-Vis spectroscopic titration (or Spectrophotometric titration) involves monitoring the intensity of an electronic absorption band at a particular wavelength, characteristic of either the complex or free host or guest, and it is closely related to the fluorescence titration method. An absorbance intensity *vs*. concentration plot is generated by adding a guest to a solution of constant host concentration.[10,11,12,13,14] Software such as *Specfit*® can then be used, in association with an appropriate stoichiometry model, to evaluate the association constant. Both fluorescent and UV-Vis spectroscopic methods have the advantage over NMR methods of being more sensitive, hence lower concentrations of host and guest can be used. Unlike

fluorescence methods, the observation of one or more clear isosbestic points is common in absorption spectroscopic titrations. An isosbestic point is reached when the observed absorption intensity remains constant throughout the titration. Furthermore, the observation of an isosbestic point is a good evidence for the free host conversion into a complex without any other significant intermediate species involved. The understanding of the statistical treatment of the obtained data to determine the association constants with the knowledge of primary statistics is the main feature of this method. When the complex stoichiometry is not 1 to 1, or when other premises are not satisfied, the data treatment should be changed or modified. Nonlinear least square data manipulation is one of the best approximations.

2.3.5 Calorimetric titration

Calorimetric titration, also known as isothermal titration calorimetry (ITC), involves accurate measurement of the heat (enthalpy) evolved from a carefully insulated sample as a function of added guest or host concentration.[20,21,22] The gradient of the ITC curve can be fitted to determine the binding constant and $\Delta G_{complex}$. Integration of the total area under the ITC plot gives the complexation enthalpy ($\Delta H_{complex}$)which allows for all the system thermodynamic parameters evaluation, being $\Delta G_{complex} = \Delta H_{complex} - T\Delta S_{complex}$. ITC is useful for determination of binding constants in range from *ca.*10^2 - 10^7 M^{-1}.

2.3.6 Mass spectrometry

Several electrospray-mass spectrometry (ESI-MS)-based methods are available for association constants (K_S) determination between a protein and a small substrate. Electrospray ionization is today the most widely used ionization technique in chemical and biochemical analysis. Interfaced with a mass spectrometer, it allows the investigation of the molecular composition of liquid samples. A large variety of chemical substances can be ionized with electrospray. Moreover, there is no limitation in mass which thus enables even the investigation of large non-covalent protein complexes. Its high ionization efficiency profoundly changed biomolecular sciences because proteins can be identified and quantified on trace amounts in a high throughput fashion.[23,24,25]

3. Molecular recognition by water-soluble calix[4]arenes

3.1 Cation recognition

Non-covalent interactions play a dominant role in many forefront areas of modern chemistry, from materials design to molecular biology. A detailed understanding of the physical origin and scope of such interactions has become a major goal of physical organic chemistry. The cation-π interaction is an important non-covalent kind of interaction, including hydrogen bonds, ion pairs (salt bridges), and the hydrophobic interaction.

3.1.1 Metal ion recognition

The first patent explicitly describing a calixarene for a practical application of p-tert-butylcalix[8]arene for the recovery of cesium from nuclear wastes, came in 1984. Numerous papers relating to the complexation of cesium by modified calixarenes have appeared since then.

Several other calixarenes for complexation of nuclides have also garnered attention (**Figure 5**), including a water-soluble calix[4]arene-bis-benzocrown-6 (**1)** for selective Cs^+ complexation (1:1) in moderately salted media.[26] The water-soluble calix[4]arene-bis-benzocrown-6 (**1)** derivatives are also reported to separate the caesium–sodium by nanofiltration–complexation.[27] The voltammetric study on a water-soluble calix[4]arene (calix[4]arene-triacid-monoquinone (**2)**, CTA, and calix[4]arene-triacid-diquinone (**3)**), which bind with the Ca^{2+}, Sr^{2+}, Ba^{2+} in basic aqueous solution, provided important information about the unique electrochemical behaviour of Ca^{2+}–CTA 1:1 complex at pH= 8.2.[28,29]

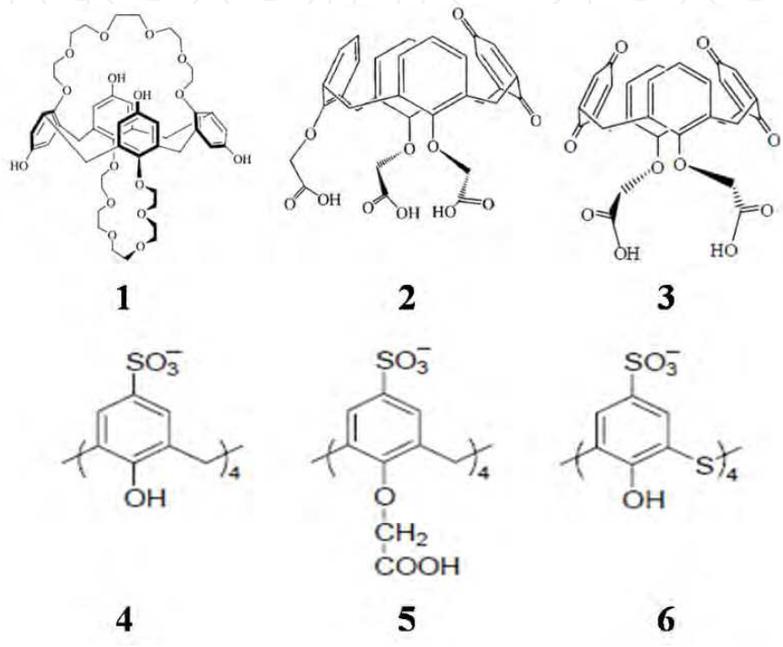

Fig. 5. water-soluble calix[4]arene derivatives **1 - 6**

The 1:1 stoichiometric complexation of lanthanoid(III) nitrates (La-Gd, Tb) with water-soluble calix[4]arenesulfonate (**4)**, and its structurally similar derivatives (**5)** and (**6)** is reported (**Table 1**).[30] The water-soluble calix[4]arenesulfonates (**5)** possessing four carboxylic groups at the lower rim of parent calix[4]arenesulfonate (**4)**, displayed the enhanced binding abilities for Sm^{3+}. As compared with (**4)** and (**5)**, *p*-sulfonatothiacalix[4]arene (**6)** gives not only the lower binding constants for all of lanthanoid(III) ions but also lower cations selectivity. Thermodynamically, the resulting complexes of lanthanoid(III) ions with (**4)** and its derivatives (**5)** and (**6)** are entirely entropy-driven in aqueous solution, typically showing larger positive entropy changes. These changes ($T\Delta S^\circ$), and somewhat smaller positive enthalpy changes (ΔH°), are directly contributed to the stability of the complexes as a compensative consequence.

It is interesting to notice that in all cases the solvated metal ions are the guests which form respective complexes with the water-soluble calix[4]arene derivatives (hosts) in the aqueous medium. Therefore, further studies are needed to evaluate the cations hydration shell effect on the complexation with water-soluble hosts.

Host	Guest (Cation)	Log Ks	-ΔG◦	ΔH◦	TΔS◦
4	La^{3+}	4.23	24.1 ± 0.3	9.2 ± 0.1	33.3 ± 0.4
	Nd^{3+}	4.08	23.3 ± 0.3	9.5 ± 0.2	32.8 ± 0.5
	Sm^{3+}	3.82	21.8 ± 0.2	10.4 ± 0.2	32.2 ± 0.4
	Eu^{3+}	3.83	21.9 ± 0.2	12.5 ± 0.2	34.4 ± 0.4
	Gd^{3+}	3.94	22.5 ± 0.3	9.8 ± 0.3	32.2 ± 0.6
5	La^{3+}	3.73 ± 0.03	21.3 ± 0.4	5.1 ± 0.5	26.5 ± 0.3
	Ce^{3+}	3.82 ± 0.01	21.8 ± 0.1	5.1 ± 0.3	26.9 ± 0.4
	Pr^{3+}	3.97 ± 0.04	22.7 ± 0.3	4.5 ± 0.4	27.2 ± 0.1
	Nd^{3+}	4.09 ± 0.03	23.4 ± 0.6	4.0 ± 0.1	27.4 ± 0.2
	Sm^{3+}	4.08 ± 0.02	23.3 ± 0.4	3.9 ± 0.1	27.2 ± 0.8
	Eu^{3+}	3.51 ± 0.04	20.1 ± 0.1	7.3 ± 0.3	27.4 ± 0.1
	Gd^{3+}	3.86 ± 0.05	22.0 ± 0.3	5.5 ± 0.2	27.5 ± 0.3
	Tb^{3+}	3.63 ± 0.01	20.9 ± 0.2	6.8 ± 0.7	27.7 ± 0.5
6	La^{3+}	3.45 ± 0.02	19.7 ± 0.1	7.2 ± 0.2	26.8 ± 0.3
	Ce^{3+}	3.41 ± 0.02	19.4 ± 0.2	7.0 ± 0.1	26.5 ± 0.2
	Pr^{3+}	3.42 ± 0.03	19.6 ± 0.3	6.9 ± 0.1	26.5 ± 0.3
	Nd^{3+}	3.40 ± 0.01	19.4 ± 0.1	6.8 ± 0.3	26.2 ± 0.1
	Sm^{3+}	3.37 ± 0.04	19.2 ± 0.2	7.2 ± 0.2	26.4 ± 0.4
	Eu^{3+}	3.26 ± 0.03	18.6 ± 0.4	7.5 ± 0.3	26.0 ± 0.3
	Gd^{3+}	3.30 ± 0.02	17.7 ± 0.6	9.0 ± 0.1	26.6 ± 0.1
	Tb^{3+}	3.33 ± 0.02	19.0 ± 0.1	7.7 ± 0.1	26.7 ± 0.5

Values are the averages of more than three independent measurements in pH = 2 acidic aqueous solution

Table 1. Complex stability constants (log *Ka*) and thermodynamic parameters (kJ mol^{-1}) for complexation of lanthanoid(III) nitrates with 4, 5, and 6 in acidic aqueous solution (pH = 2) at 25 ◦C.

3.1.2 Molecular cation recognition and hydrophobic cavity depth of water-soluble calix[4]arenes

The complexation of molecular cations by the water-soluble calix[4]arenes is widely studied. Shinkai and coworkers[31] were the first to investigate molecular cation complexation with p-sulfonatocalix[4]arenes (**7)** (**Figure 6**) as hosts and trimethylanilinium as a guest.

By measuring the ^1H NMR shift values over a temperature range of 0–80^0C, they calculated ΔG^o, ΔH^o and ΔS^o values and concluded that complexation with the cyclic tetramer (7.8, n=4) was driven by a favourable enthalpy change (stronger electrostatic interaction). It was emphasized that in studies with water soluble calixarenes an important feature that had to be taken into consideration was their aggregation properties.[32] A calix[4]arene with anionic groups (SO_3^- and CO_2^-) on both exo and endo rims, forms fairly strong complexes with cations such as $PhCH_2NMe_3^+$ (Ks = 2500M^{-1}) (**15)**.[33] For cations derived from amines, the organic moiety introduces a significant steric factor, with the ammonium cation included in the host cavity.[34,35,36] A closely related

study[37] involving a variety of ammonium guests, including acetylcholine and N-methylquinuclidinium, reached the same conclusion that the guest is in the cavity and that its ammonium portion is closely associated with the calixarene aromatic rings. This interaction was discussed as a π-cation interaction.[38,39,40] The N,N,N,-trimethylanilinium (TMA) cation (**14)** orientation was reported to have a dual binding mode (charged group vs. aromatic moiety inclusion) which occurs in a nonselective fashion with flexible water-soluble calix[4]arene hosts (**7a**, n=4). The binding mode can, however, be effectively controlled and turned into a selective process by preorganization of the calixarene cavity into the cone structure (**7b**, n=4). The presence of sulfonate groups at the upper rim provides, anchoring points for the positively charged guests, the sulfonate groups significantly deepening the cavity of host (**7)**, thus improving its inclusion capability.

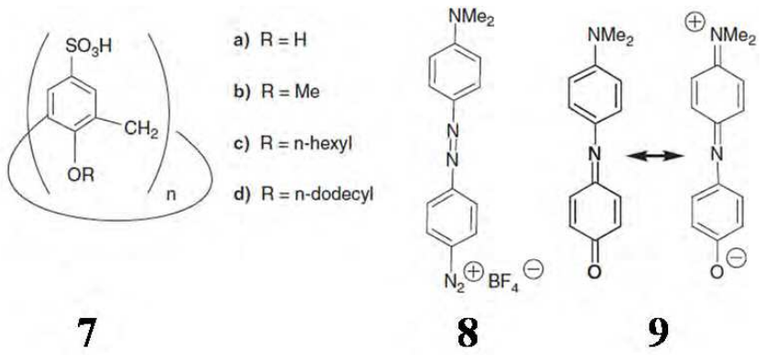

Fig. 6. Water-soluble calix[4]arene host(s) **7** and guests **8, 9.**

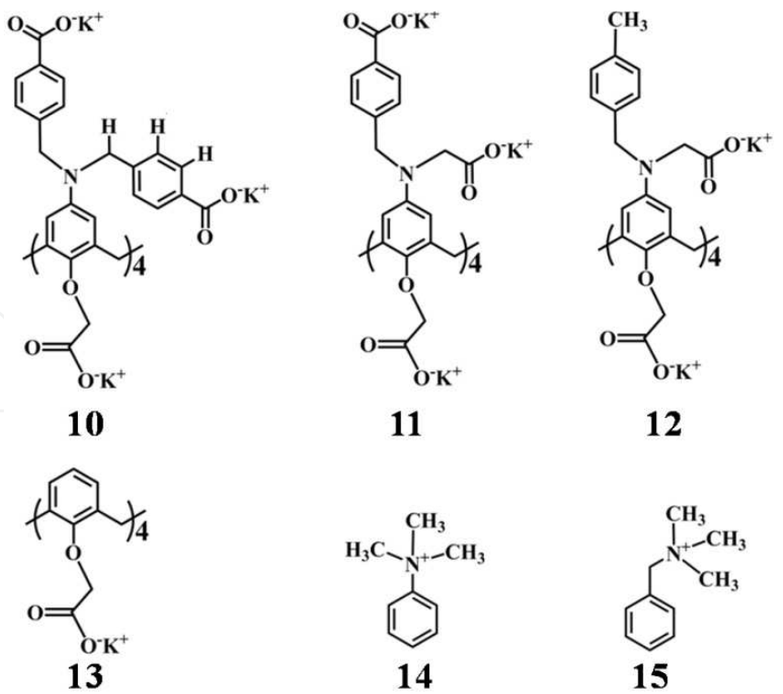

Fig. 7. Hosts with deep hydrophobic cavities (**10 - 12**), and guests **14** (Phenyltrimethylammonium chloride), **15** (Benzyltrimethylammonium chloride).

The complexation of the water-soluble aminocalix[4]arenes containing deep hydrophobic cavities with cations have been reported.[41] However, the guest recognition and the orientation in the cavity of the host were reported to be dependent on the depth of the host hydrophobic cavity. The host (**11**, **12)** interacts with both the cationic function and the aromatic moiety in the guests (**14**, **15**), but with a slight preference for the cationic functions. The host (**13)** selectively recognizes the trimethylammonium functions of the guests (**14** and **15)**.

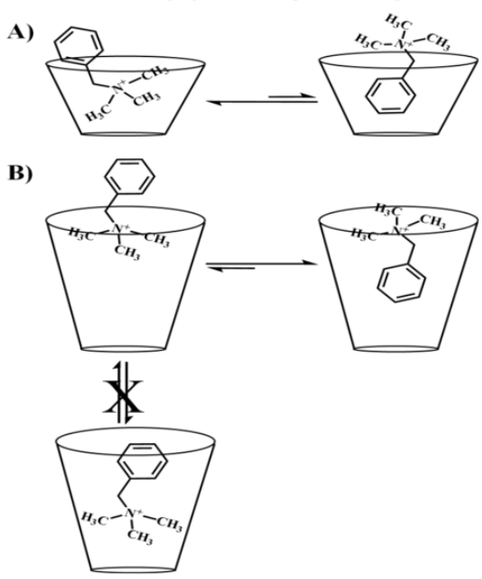

Fig. 8. A) Inclusion mode of the guest **15** by hosts **11-13**; B) Inclusion mode of the guest **15** by host **10**

However, the host (**10)** selectively recognizes the aromatic moiety of the ditopic trimethylammonium guests (**14** and **15)**. These results suggest that the water molecules around the calix[4]arene nucleus in the hosts (**11 – 13)** may assist the hydrophilic trimethylammonium function in entering the cavity. Furthermore, in case of the host (**10),** possessing a deep hydrophobic cavity, the trimethylammonium function cannot deeply enter into the calix[4]arene nucleus, being solvated by the water. As the guest molecules trimethylammonium function is engaged on the mouth of the host **(10)** deep hydrophobic cavity, the guest aromatic moiety is selected by the host (**10)** to form the inclusion complexes. These results suggest that the guest recognition and orientation in the cavity of the host are directly dependent on the host hydrophobic cavity depth.

The water-soluble iminecalix[4]arene (**16, Figure 9**) with deep hydrophobic cavity was also recognized for its selective recognition of the guest.[42,43] The negatively charged four carboxylate functions on the top of the deep hydrophobic cavity play a major role in the recognition of charged molecular species. The ^{1}H NMR titration experiments revealed that host (**16)** binds with cationic (**15**, **21, 22**) and neutral guests (**17-20**) in water, with high binding constants in order of 10^{4}-10^{5} M^{-1}. Cationic guest (**15)** showed the highest binding constant of 2.81×10^{5} M^{-1} . These studies revealed that except for the -CH···π and π-π stacking interactions, the hydrophobic interactions proved to be crucial in the molecular recognition process in aqueous medium.

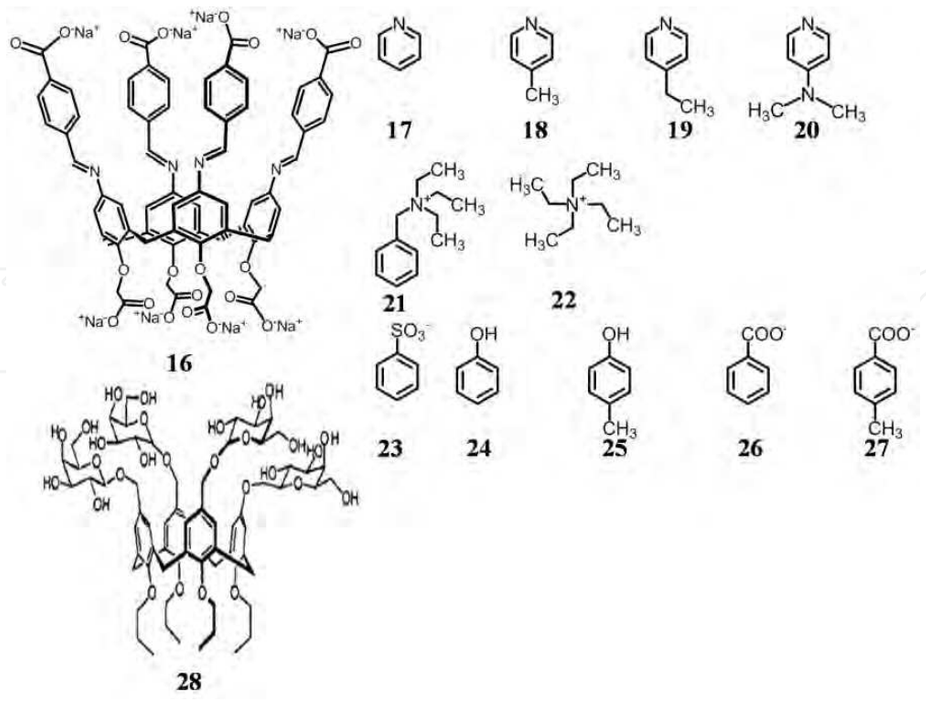

Fig. 9. Water-soluble iminecalix[4]arene **16**, **28** and guests **17-27.**

3.2 Anion recognition

Anion recognition (binding) plays an important role in a variety of chemical reactions and biochemical events as outlined in various reports.[44] This molecular recognition process has been the subject of numerous experimental and theoretical studies in recent years.[45,46]

3.2.1 Inorganic anions

The hydrogen-bond dynamics of water molecules solvating a Cl^-, Br^-, or I^- anion is slow compared with neat liquid water, indicating that the aqueous solvation shells of these ions are rigid. This rigidity can play an important role in the overall dynamics of chemical reactions in aqueous solution.[47]

Furthermore, the anions complexation can be more difficult than that of cations, and a variety of considerations come into play, including (a) the charge, (b) the size, which is often larger than the metal cation one, (c) the shape; whereas the metal cations are spherical, the anions frequently are not, (d) pH dependence, often more critical than in the case of metal cations and (e) solvation, which has a strong influence on the binding strength. There are enormous reports on the recognition of various anions (inorganic) by the calix[4]arene derivatives in the organic solvents but there are only few reports on the anion complexation by the water-soluble calix[4]arenes in the aqueous medium,[48] which opens a new direction for such studies.

Functionalisation of calix[4]arenes with carbohydrate moieties results in receptors which show considerable water solubility. A number of calixsugars have been developed[49] and their binding characteristics studied. Neutral guests such as carbohydrates and N-protected

amino acids failed to bind. However, 1:1 complexation of dihydrogen phosphate was seen for (**28),** offering opportunities for the binding of larger, phosphate containing biological substrates.

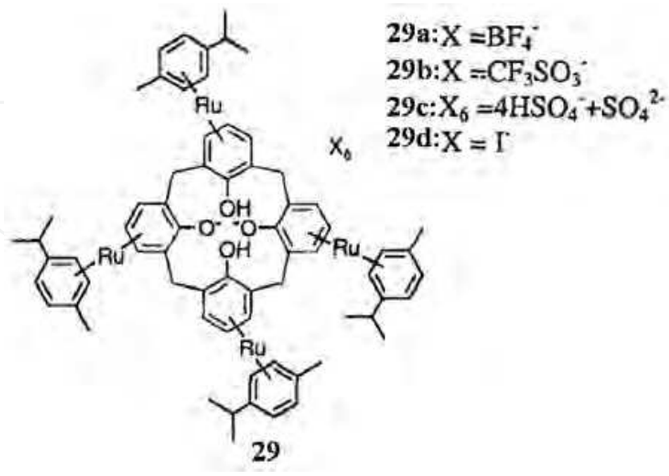

Fig. 10. Water-soluble π-metalated calix[n]arene **29** and complexes

A series of π-metalated calix[n]arenes were synthesised, among which compounds **29** are water-soluble due to the presence of six positive charges.[50] The calixarenes cavities are therefore electron-poor and able to complex anions both in the solid state and in water. The X-ray crystal structures of compounds **29a, 29c** and **29d** showed that a BF_4^-, SO_4^{2-}, and I^- anion is complexed in the calixarene cavity, respectively, tetrafluoroborate being the most deeply included one. Acetate, phosphate and sulfate anions are not bound by host (**29b),** due to their high hydrophilicity. An interesting inversion of the expected selectivity, on the basis of the free hydration energy order (Hofmeister series), is observed for halide ions due to size complementarity between the guest and the calixarene cavity.[51]

3.2.2 Molecular anion recognition and depth of hydrophobic cavity of water-soluble calix[4]arenes

Very few examples of anion complexation by water-soluble calixarenes have been reported so far. This is probably due to the fact that anion recognition is a rather new field in supramolecular chemistry and that anions are more highly hydrated than cations of comparable size and, therefore, their complexation in water is a remarkably difficult task. In the case of l-anilino-8-naphthalenesulfonate (ANS)[52] and 2-*p*-toluidino-6-naphthalenesulfonate (TNS) [53] the lipophilic residue of the guest is included inside the calixarene cavity.[54]

A cationic calix[4]arene derivative (**30)** binds both aliphatic (**31**, **32)** and aromatic, sulfonate (**23)** and carboxylate (**26)** anions in aqueous solution with a Log *K* of 1.50, 1.48, 2.44, 2.32, respectively, as a result of concerted electrostatic and hydrophobic interactions. The sulfonate ion in guest **23** may show good electrostatic interaction with the cations on the top of the cavity. However, the sulfonate guest inclusion is affected by the host different mobility caused by the pH change. An interesting example of the anionic host (**10)** complexation with the anionic sulfonate (**23)** (Log *K*= 4.3, pH=7.3; Log *K*= 0, pH=5.8) has

been recently reported.[55] The pH of the solution shows a significant effect on the dynamics of the gate (formed by eight benzylic functions) and portal on the hydrophobic cavity of the water-soluble aminocalix[4]arene host (**10)**. At pH 5.8 the gate closes and prevents the entry of anionic guests. However, at pH 7.3 the gate opens and allows the entry of anionic guests (**23**, **24)** to the hydrophobic cavity. Host **10** not only shows a similar behaviour towards guests **23** and **24** but also shows a preference for sulfonate derivatives. This preference can be assigned to the tripodal symmetry of sulfonate function, instead of dipodal in carboxylate, and its electron withdrawing effect. The tripodal symmetry gives extra room for negative charges of guest molecules on the cavity of host **10** reducing the electrostatic repulsion. The electron withdrawing effect prevails and increases the π-π stacking interactions between the guest (**23)** and the host (**10)**. The deep hydrophobic cavity of the water-soluble aminocalix[4]arene role in the recognition of anionic guests cannot be neglected, despite the absence of favourable electrostatic interaction shown by host **30** towards guests **23** and **24**, host **10** showing strong binding with them**.**

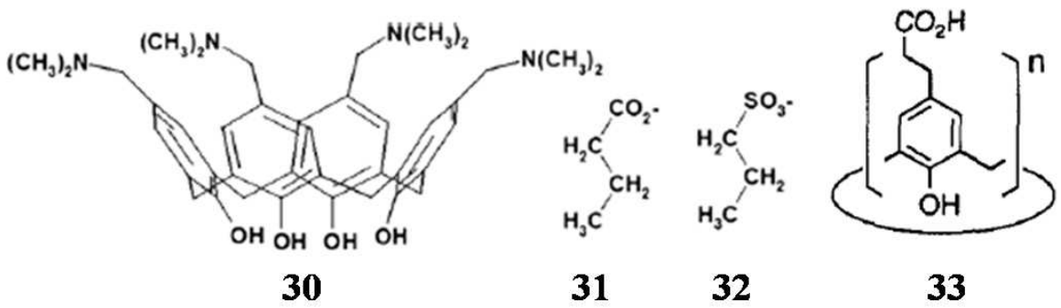

Fig. 11. Water-soluble calix[4]arene derivatives **30** (cationic), **33.**

3.3 Recognition of neutral molecules

The complexation of neutral molecules by water-soluble calixarenes was carried out in the eighties and has been already critically reviewed.[56,57,58] The pioneering work[59] on the complexation of aromatic hydrocarbons by hosts **33** (n=4) has however to be mentioned, since it disclosed a rough correlation between binding constants and host-guest complementarily. Calix[4]arenes are too small to host durene or naphthalene, calix[5]- or -[6]arenes preferring naphthalene, anthracene and phenanthrene.

Sciotto et al. have studied the interactions between alcohols, ketones, nitriles and p-sulfonatocalixarenes (**4)** and its derivatives by 1H NMR spectroscopy,[60,61] proving that the apolar aliphatic portions of the guests were included into the host hydrophobic cavity with the terminal polar groups directed towards the polar sulfonate groups of the host and to the solvent. The two most important factors for the complexation of the investigated hosts and guests are conformational properties of the receptors and electrostatic effects. Methanol is not included by p-sulfonatocalixarenes at all, probably due to the fact that the small methyl group inclusion inside the hydrophobic cavity would lead to a partial inclusion of polar OH group, causing the polar hydroxyl group to be less exposed to polar solvent.

The interactions of aromatic substrates (**34, 35, 36,** and **37**) (**Figure 12**) with **4** were studied by Schatz and co-workers via ^{1}H NMR titration experiments and molecular modelling

studies combined with abinitio NMR shift calculation at neutral aqueous solutions.[62] All the guests are included into the hosts cavities, with a mechanism which is mainly driven by enthalpy term. In most cases, the five aromatic protons are pointing inside and the guest functional group is located outside the hosts cavities due to hydrophobic and π-π interactions. For (**34)**, the complex binding mode is different, probably because the methyl group is included into the host cavity, contributing to the favorable C–H-π interactions and hydrophobic interactions.[63]

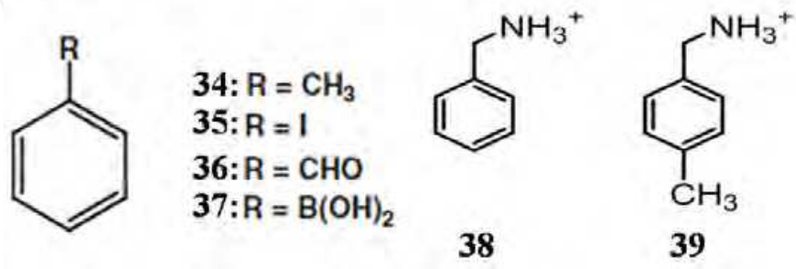

Fig. 12. Aromatic neutral guests (**34**-**37**), and cationic guests **38**, **39.**

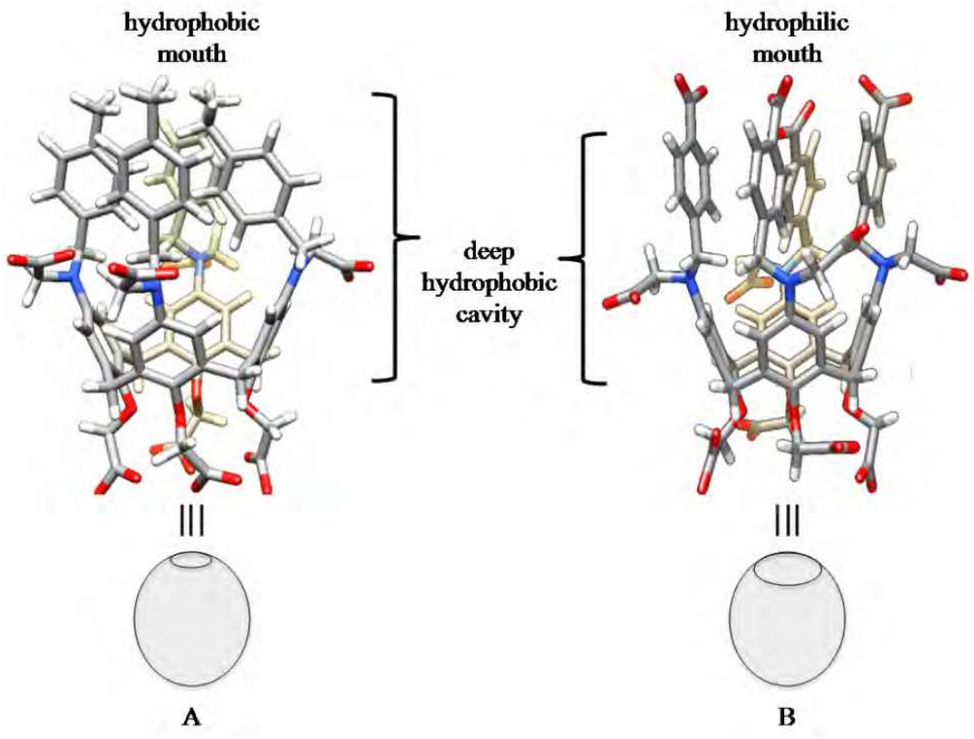

Fig. 13. Hosts **11** and **12,** A) host **12** (side view), B) host **11** (side view).

The new water-soluble aminocalix[4]arene hosts **11** and **12** with deep hydrophobic cavity facilitate hydrophilic mouth and hydrophobic mouth, respectively.[64] The ^{1}H NMR titrations revealed that host **12** shows high selectivity for neutral guests (**18** and **19)**, with log *K* of 4.2 and 4.6, respectively. The host **11** shows log *K* of 4.9 for binding with guest **39**. Moreover, the host **11** binding ability for guest **38** is stronger by a factor of 1000 than that of the host **12**.

The NMR investigations indicate that host **11** and **12** can form 1:1 host–guest inclusion complexes with aromatic cationic guests and pyridine derivatives with high binding constants. Both hosts refused to recognize the hydrophilic anionic guests, possibly due to the electrostatic repulsion arising from carboxylate functions on the cavity of the host. The host **12**, with hydrophobic mouth, showed high binding constant for 4-methylbenzylammonium, as the carboxylate functions of the mouth showed strong electrostatic interactions with the ammonium function. However, the hydrophilic mouth of host **11** enhances the binding of 4-ethylpyridine. It is clear from the data that the cavity of both hosts has a preference for structurally flat guests containing methyl groups (either a CH_3 in *para* position of an aromatic ring or a presence of trimethylammonium group) and a very poor one for smaller but more hydrophilic primary ammonium groups, which indeed do not enter the hydrophobic cavity.

4. Conclusion

Mimicry of the molecular recognition features of naturally occurring proteins by synthetic receptors is one of the challenging research topics of supramolecular chemistry. The substrates and enzymes (host-guest) features can be studied by Potentiometry, NMR Spectroscopy, UV-Visible Spectroscopy, Fluorescence Spectroscopy, and Calorimetry. In some cases the ESI-MS can be employed to study the protein-protein, or protein-small molecule interactions. It is quite obvious that the exact host-guest complex stoichiometry is the most critical parameter in the evaluation of the host-guest interactions. The molecular recognition properties of the water-soluble calix[4]arene derivatives revealed that the hydrophobic cavity of these hosts play an important role in the guests recognition. Increasing the hydrophobic cavity depth, like in the water-soluble aminocalix[4]arene hosts, results in an increased binding of the guest into the hosts deep hydrophobic pockets. Synthetically tailored hosts based on the calix[4]arene framework can be used to probe the naturally occurring biomolecular reactions based on the non-covalent interactions.

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Stoichiometry and Research - The Importance of Quantity in Biomedicine

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The aim of this book is to provide an overview of the importance of stoichiometry in the biomedical field. It proposes a collection of selected research articles and reviews which provide up-to-date information related to stoichiometry at various levels. The first section deals with host-guest chemistry, focusing on selected calixarenes, cyclodextrins and crown ethers derivatives. In the second and third sections the book presents some issues concerning stoichiometry of metal complexes and lipids and polymers architecture. The fourth section aims to clarify the role of stoichiometry in the determination of protein interactions, while in the fifth section some selected experimental techniques applied to specific systems are introduced. The last section of the book is an attempt at showing some interesting connections between biomedicine and the environment, introducing the concept of biological stoichiometry. On this basis, the present volume would definitely be an ideal source of scientific information to researchers and scientists involved in biomedicine, biochemistry and other areas involving stoichiometry evaluation.

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