Inclusion of naturally occurring amino acids in water soluble calix[4]arenes: a microcalorimetric and ^{1}H NMR investigation supported by molecular modeling

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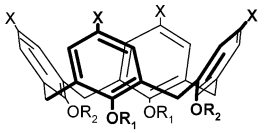
The thermodynamic parameters for the inclusion of some naturally occurring amino acids into a series of *p*-sulfonated calix[4]arenes, were determined *via* both ^{1}H NMR and calorimetric titrations in buffered aqueous solution at 25 °C. The calorimetric data show that inclusion is enthalpically driven in all cases, regardless of flexibility of the *host* and the nature of the *guest*. The most efficient receptor is the calix[4]arene tetrasulfonate **1**, which exists in solution at pH 7 in a cone conformation, stiffened by H-bonding at the lower rim.
Molecular mechanics data help in the understanding of why some hosts do not form inclusion complexes at all. The comparison of our data with literature reports shows that there are dramatic buffer-dependent changes in the binding affinities.

Introduction

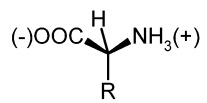
Molecular recognition is one of the key processes in living systems.[1] Mimicking this process in water, where the vast majority of biological processes takes place, using simplified synthetic models, is of paramount importance for the understanding of the forces involved in the recognition process. Among these studies, the inclusion of amino acids and peptides in appropriate cavities assumes a pivotal role. Whereas the inclusion of these small guests in natural cyclodextrins has been extensively studied,[2] very few studies have been reported on the inclusion of amino acids and peptides in synthetic receptors. On the other hand, quite recently several examples of biologically active, water soluble calixarenes[3] have been reported in the literature.

Pinhal *et al.*[4] found that *p*-sulfonatocalix[8]arene stimulates the synthesis of heparan sulfate proteoglycan secreted by endothelial cells from rabbit aortae and human umbilical veins in culture. Coleman *et al.*[5] reported that water soluble calixarenes are excellent candidates as heparin mimics with respect to peptide folding and even protein–protein interactions. The considerable interest in water-soluble calixarenes for biological and medical applications has resulted in a few patents; in fact, they have been registered as antiviral agents,[6] blockers of chloride ion channels,[7] antithrombotic agents[8] as well as inhibitors of lysyl oxidase activity.[9]

Due to this wide interest, we studied the complexation of several guests of biological relevance in water soluble calix[4]arenes and were the first to report quantitative values on the inclusion of naturally occurring amino acids into a series of calixarenes (Scheme 1) in physiological conditions[10] whereas other authors had investigated the inclusion of arginine and lysine into some *p*-sulfonato-calix[*n*]arenes ($n = 4, 6, 8$) exploring the acidic region only.[5]



1 X = SO_3H, $R_1 = R_2 = H$
2 X = SO_3H, $R_1 = R_2 = CH_2COOH$
3 X = H, $R_1 = R_2 = CH_2COOH$
4 X = SO_3H, $R_1 = H$, $R_2 = CH_2CH_2OCH_2CH_3$
5 X = SO_3H, $R_1 = R_2 = CH_2CH_2OCH_2CH_3$



R (aliphatic):
L-Ala, L-Val, L-Leu.
R (aromatic):
L-Phe, L-Tyr, L-His, L-Trp

Scheme 1

Following our earlier preliminary studies,[10,11] we now report a full thermodynamic (^{1}H NMR and calorimetric) characterization of the inclusion of some naturally occurring amino acids into a series of water soluble calix[4]arenes (Scheme 1). This allows better identification of the factors that control this binding process, as knowledge of the nature and strength of the interactions may provide important information on the mechanism of the binding[12] of *p*-sulfonated calixarenes to complex bio-macromolecules. As shown in the scheme, we have included both sulfonated (**1**, **2**, **4** and **5**) and non-sulfonated (**3**) calixarenes, to evaluate the role played by the presence of negative charges ($-SO_3^-$); we have also varied the nature, the length and the number (*viz.* **4** and **5**) of the groups attached at the lower rim. Both the calorimetric and ^{1}H NMR experiments were carried out at pH 7 (pD 7.3) and in 0.1 mol dm^{-3} phosphate buffer to reproduce the physiological conditions as much as possible: moreover, using two different techniques allows the stability constants to be cross checked. The molecular mechanics optimization of the systems

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shown in Scheme 1 provides additional information that helps in understanding the binding mechanism.

Results and discussion

Binding constants

The results obtained by us *via* ^{1}H NMR in buffered aqueous solutions (pD = 7.3; pH = 7.0) are reported in Table 1, together with some literature data obtained under comparable conditions. All the amino acids are in their zwitterionic form in the experimental conditions studied by us. The data reported by Coleman *et al.*[13] for **1** have not been included in Table 1 since they were obtained under conditions that differ significantly from those employed in the present study as far both the pH (pH = 2) and the solvent (acetonitrile–water–trifluoroacetic acid or methanol–water–trifluoroacetic acid) are concerned. The log*K* values obtained by us *via* ^{1}H NMR and calorimetry under homogeneous conditions are close to one another and are well within the experimental error. Obtaining consistently the same results by probing two different parameters (chemical shifts and heat) makes us confident of the accuracy of the data reported in the present investigation.

The changes in chemical shift resulting from complexation ($\Delta\delta_{obs}$) for **2**-L-Leu are shown Fig. 1 as an example. They show that upon inclusion, the aliphatic protons of the guest undergo a significant upfield shift, the methyl protons being the most shifted ones; the same pattern is obtained for the inclusion of L-Val. This indicates that these aliphatic amino acids form inclusion complexes by inserting their apolar tail into the calixarene cavity; the same occurs with the aromatic amino acids. Our experiments also show no detectable complex formation of any of the

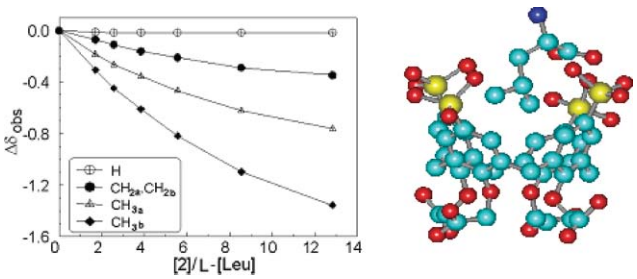

Fig. 1 Plots of $\Delta\delta_{obs}$ (ppm) *versus* [**2**]/[L-Leu], in D_2O, 25 °C, [L-Leu] = 1×10^{-3} mol dm^{-3}, pD 7.3 (0.1 mol dm^{-3} phosphate buffer).

Table 1 Stability constants for the complexes of L-aminoacids with hosts **1–5**

Complex[a]	log *K*	pH/pD[b]	Buffer	Medium/Conc.	Technique	Reference
1-L-Ala	ND[c]	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
	1.89	8[b]	No/adj[d]	?	^{1}H NMR	16
1-L-Val	1.2 (1)	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
	1.1 (2)	7.0	PBS	PBS/0.1 mol dm^{-3}	Calorimetry	This work
	3.20	NS[e]	No	?	Calorimetry	17
1-L-Leu	1.7 (1)	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
	1.8 (1)	7.0	PBS	PBS/0.1 mol dm^{-3}	Calorimetry	This work
	3.08	NS[e]	No	?	Calorimetry	17
	2.89	8[b]	No/adj[d]	?	^{1}H NMR	16
1-L-Phe	1.8 (1)	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
	1.7 (1)	7.0	PBS	PBS/0.1 mol dm^{-3}	Calorimetry	This work
	3.14	NS[e]	No	?	Calorimetry	17
	2.91	8[b]	No/adj[d]	?	^{1}H NMR	16
1-L-His	1.3 (1)	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
	2.70	8[b]	No/adj[d]	?	^{1}H NMR	16
1-L-Trp	1.4	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
2-L-Ala	ND[c]	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
2-L-Val	1.0 (1)	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
	1.1 (4)	7.0	PBS	PBS/0.1 mol dm^{-3}	Calorimetry	This work
2-L-Leu	1.9 (1)	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
	1.9 (1)	7.0	PBS	PBS/0.1 mol dm^{-3}	Calorimetry	This work
2-L-Phe	1.7 (1)	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
	1.5 (1)	7.0	PBS	PBS/0.1 mol dm^{-3}	Calorimetry	This work
2-L-His	ND[c]	—	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
2-L-Trp	1.3 (1)	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
3-L-AA	NCF[f]					This work
4-L-Ala	ND[c]	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
4-L-Phe	1.4 (1)	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
4-L-Val	ND[c]	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
4-L-Leu	0.9 (1)	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
4-L-His	ND[c]	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
4-L-Trp	1.0 (1)	7.3[b]	PBS	PBS/0.1 mol dm^{-3}	^{1}H NMR	This work
5-L-AA	NCF[f]					This work

[a] The complex results from the reaction: C + L-AA ⇌ C–L-AA where C and L-AA are the appropriate calixarene (**1**, **2**, **3**, **4**,or **5**) and amino acid, respectively. [b] pD values. [c] ND indicates not detected in our experimental conditions, [d] No/adj. indicates that no buffer is used. The pD of the titrate is brought up to 8 by simply adding the appropriate amount of NaOH. [e] NS indicates not specified. [f] NCF indicates no complex formed with any of the amino acids investigated.

investigated ligands with alanine in the experimental conditions used. This is not surprising; Kazakova *et al.*[14] investigating the inclusion of amino acids into calixresorcinarenes in 0.1 mol dm^{-3} phosphate buffer, concluded that only the amino acids possessing hydrophobic moieties or a long hydrophobic chain form inclusion complexes.

It is very likely that the alanine side chain (*i.e.* the $-CH_3$ group) is too short to be inserted into the cavity while the charged residue of the amino acid simultaneously interacts with the sulfonato groups and the water molecules.

The data confirm the importance of the sulfonato groups at the upper rim.[15] Host **3**, which lacks these negatively charged residues, forms no complexes with any of the investigated amino acids, showing that the docking provided by the interaction between the sulfonato groups and the protonated amino residue helps somehow the insertion of the aliphatic (L-Val, L-Leu) or aromatic (L-Phe, L-His or L-Trp) apolar tail in the calixarene cavity. It is the combination of these two factors that determines the inclusion of the amino acid. Tyrosine does not form any adduct probably because its polar OH group cannot be suitably accommodated by the apolar cavity of the receptor. In this context, we were rather surprised that host **5**, possessing the sulfonato anchoring groups, forms no adducts with any of the amino acids. The molecular modeling, reported below (see Molecular Modeling section), helps in the understanding of why this host forms no adducts with any of the amino acids investigated.

However, our data are sizably lower than those reported by both Coleman *et al.*[16] and Buschmann *et al.*[17] and this deserves some comments. Both our ^{1}H NMR and calorimetric data were obtained in a phosphate buffer (pD = 7.3; pH = 7). This medium has been extensively used although there are conflicting reports, even by the same authors, on the use of this medium.[13,16,18] In fact, this buffer is sometimes used without any comment[13] or is said to have no apparent influence on complexation[18] or is said not to be totally neutral.[16]

We are firmly convinced that these experiments must be carried out in buffered solutions for the following reasons: (*i*) the ionic medium (0.1 mol dm^{-3} in our case) ensures small changes of the activity coefficients from the beginning to the end of the titration; (*ii*) the species distribution remains virtually unchanged in the course of the experiments and (*iii*) the inclusion of the amino acids, mainly investigated for its biomedical potential,[5,10] is studied under pH conditions that reproduce the physiological value, at least to a certain extent.

As to the first point, obviously, the values derived at different ionic strength are apparent constants and can be compared only with values obtained under the same conditions; therefore, caution must be used when comparing values obtained at different ionic strength (and or temperature). However, a simple calculation shows that the ionic medium term may account for some 0.7 log units.[19] At higher buffer concentrations, the interaction between the sulfonato groups and the positive tail of the amino acid, that is responsible of the anchoring of the guest, decreases, thus rendering the inclusion of the amino acid weaker. It is important to emphasize that the values obtained by Coleman *et al.*[16] and by Buschmann *et al.*[17] for **1**-L-Leu and **1**-L-Phe (Table 1) differ from one another by some 0.3 and 0.2 log units, respectively. The only difference in the experimental set up is that Coleman *et al.* adjust the initial pH of the titrate to 8 whilst Buschmann *et al.* simply titrate an unbuffered aqueous solution of the appropriate amino acid with an unbuffered solution of **1**. This shows that even seemingly negligible differences in the procedure adopted, leading to different ionic media and speciation, may result in non-negligible differences.

Moreover, a change of the species distribution may have significant implications for the calculation of the heat assumed to be associated with the specific species and will be discussed below.

Thermodynamic characterization

The enthalpy and entropy values derived from our calorimetric measurements are shown in Table 2 together with the values determined by Buschman *et al.*[17] L-Trp was not soluble enough to give a satisfactory heat–volume curve in our experimental conditions. We were not able to obtain a satisfactory heat–volume

Table 2 Thermodynamic parameters for the inclusion of L-Val, L-Leu, L-Phe, L-Trp and L-His into hosts **1**, **2** and **4**

Reaction	log K^a	ΔG/kJ mol^{-1}	ΔH/kJ mol^{-1}	ΔS/J mol^{-1} deg^{-1}
1 + L-Val ⇌ **1**-L-Val	1.2	−6.7(5)	−9.6(7)[b]	−9(2)
	3.20[c]		−46.7[c]	−96[c]
1 + L-Leu ⇌ **1**-L-Leu	1.7	−9.7(5)	−15.5(8)	−19(3)
	3.08[c]		−51.7[c]	−115[c]
1 + L-Phe ⇌ **1**-L-Phe	1.8	−10.3(5)	−17.5(8)	−24(3)
	3.14[c]		−36.0[c]	−61[c]
1 + L-Trp ⇌ **1**-L-Trp	1.4	−8.0(5)	—[d]	—
1 + L-His ⇌ **1** L-His	1.3	−7.4(5)	−15(1)	−26(4)
2 + L-Val ⇌ **2**-L-Val	1.0	−5.7(5)	−9.5(9)	−13(3)
2 + L-Leu ⇌ **2**-L-Leu	1.9	−10.8(5)	−21.6(8)	−36(3)
2 + L-Phe ⇌ **2**-L-Phe	1.7	−9.7(5)	−17.1(9)	−25(3)
2 + L-Trp ⇌ **2**-L-Trp	1.3	—[d]		
4 + L-Leu ⇌ **4**-L-Leu	0.9	−5.1(5)	—[e]	—
4 + L-Phe ⇌ **4**-L-Phe	1.4	−8.0(5)	−18(2)	−33(5)
4 + L-Trp ⇌ **4**-L-Trp	1.0	−5.9(5)	—[d]	—

[a] All the values have been determined in 0.1 mol dm^{-3} phosphate buffer at 25 °C. [b] σ in parentheses. [c] Ref. 17: ΔS values derived from the $T\Delta S$ figures given in this reference by using a value of 298.16 K for T. [d] Too poorly soluble to obtain a reliable/reproducible calorimetric measurement. [e] Too little heat to obtain a reliable/reproducible ΔH.

curve for the adduct of **4** with L-Leu either; this is probably due to relatively low stability constants of this adduct, that is the smallest of all the systems investigated. The heat–volume curve was satisfactorily fitted by assuming the presence of 1 : 1 species only. Table 2 shows that the ΔH values reported by Buschmann *et al.*[17] are larger than those determined in the present investigation. It has to be underlined that our values were determined under constant ionic strength whereas Buschmann's values were obtained under "non-buffered conditions". In addition to the difference resulting from the different ionic strength (see the Binding constants section), working in "non-buffered conditions" may affect also the heat assumed to be associated to the specific species, which in turn affects the log$K$ determined *via* calorimetric experiments. The heat produced during the titration should be corrected not only for all non-chemical energy terms but also for the chemical heat effects due to the formation (or disappearance) of species other than the 1 : 1 assumed to be the only species being formed. Whilst this assumption holds in buffered conditions where the pH change is negligible, a simple calculation[20] shows that the addition of the *p*-sulfonatocalix[4]arene to a solution of the amino acid (*e.g.* alanine) causes a pH change that, in turn, causes a change of the species distribution. Neglecting the heat associated with the species being either formed or destroyed and attributing all the heat produced to the only species assumed to form introduces an error in both the log$K$ and the ΔH value calculated for the 1 : 1 species.

Fig. 2 gives a straightforward idea of the parameters driving the inclusion of the amino acids and shows that the full thermodynamic characterization of the binding process is one of the key elements to understand how and why the units assemble and what are the stabilizing factors.[12] The inclusion of all the investigated amino acids is enthalpically favoured and entropically unfavoured regardless of the nature of the side chain; the driving force for inclusion will be a CH-π interaction of the alkyl chain for L-Val and L-Leu or a π–π interaction for L-Phe and L-His. The ΔH value overwhelms the TΔS contribution, thus showing that it is the interaction between the side chain and the calixarene cavity, rather than the desolvation of the interacting particles, that mainly determines the ΔG value.[15] Compared to L-Val, the inclusion of the longer L-Leu chain produces a larger ΔH value with both **1** and **2**.[16]

Perhaps the L-Leu side chain has the optimum length to simultaneously permit a good interaction of the hydrophobic portion with the cavity while still leaving the polar residues exposed to both the sulfonato groups and the solvent. The ΔH (and ΔS values) associated with the inclusion of L-Phe with **1**, **2** and **4** are essentially the same and suggest that the inclusion of this amino acid is independent of the more or less pronounced pre-organization of the calixarene host. Imidazole has less pronounced affinity for the phenolic rings of the host, compared to that shown by the benzene residues[6] of L-Phe, and this is reflected by the ΔH difference found for **1**–L-His and **1**–L-Phe.

Molecular modeling

The optimization of both the free ligands and their complexes by molecular mechanics calculations provides further support to the description based on ^{1}H NMR and calorimetric data. The structures and the distances of interest are reported in Fig. 3 and Table 3, respectively. Free hosts **1**, **4** and **5** have a C_{2v} elongated shape, as already shown for hosts **2** and **3**,[15] with the C_2 and C_4 benzene rings that move outward and the C_1 and C_3 benzene rings that tilt inward. Molecular dynamics[21] and NMR[22, 23] studies, performed on other similar cone calix[4]arenes, demonstrate that each host undergoes a rapid C_{2v}–C_{2v} interconversion, leading on the average to a C_{4v} *cone*.

Host **1** has the most symmetrical cavity among all the investigated receptors (Table 3). It is likely that this is due to hydrogen bonding between the deprotonated phenolate oxygen and the neighbouring OH groups that leads to the fixation of the calixarene into the cone conformation[24] as demonstrated by ^{1}H NMR data at neutral pH.

The C_1–C_3 distance decreases from host **1** to host **5** paralleling the capability of each calix to include the amino acids. The C_1–C_3 distance is only 4.40 Å for host **5** that does not include any amino acid. The steric hindrance of the lower rim substituents causes them to move away from one another (see Fig. 3d), which, in turn, results in a very elongated cavity

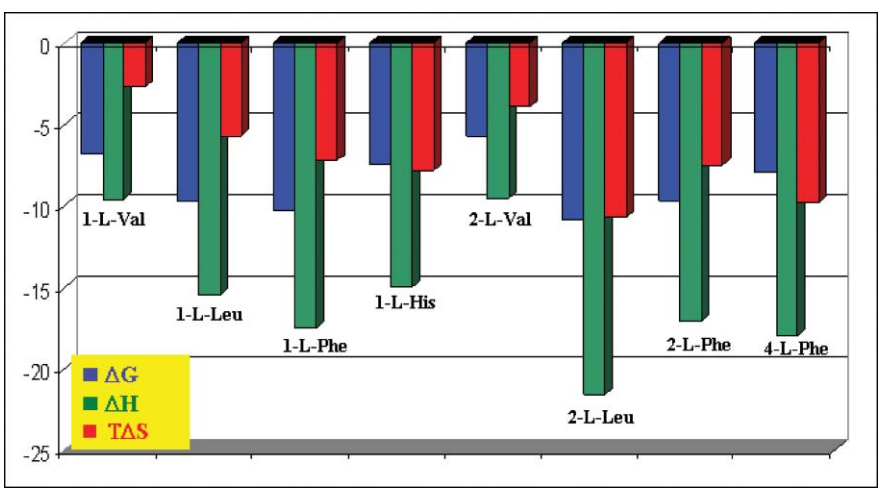

Fig. 2 ΔG, ΔH and $T\Delta S$ values for the inclusion complexes. ΔG, ΔH and $T\Delta S$ are expressed in kJ mol^{-1}.

Table 3 Relevant distances for compounds **1**, **2**, **4** and **5** and their complexes with L-Val, L-Leu, L-Phe, L-His, L-Trp

Free host					Complex			Complex			Complex			Complex		
Distance	C_1–C_3	C_2–C_4	S_1–S_3	S_2–S_4		C_1–C_3	C_2–C_4		C_1–C_3	C_2–C_4		C_1–C_3	C_2–C_4		C_1–C_3	C_2–C_4
1	5.78	9.60	6.09	12.24	**1**-L-Val	7.61	8.62	**1**-L-Leu	8.07	8.10	**1**-L-Phe	6.73	9.49	**1**-L-Trp	6.75	9.29	**1**-L-His	7.40	8.63
2	4.91	10.01	4.81	12.87	**2**-L-Val	7.60	8.64	**2**-L-Leu	7.54	8.80	**2**-L-Phe	6.77	9.28	**2**-L-Trp	6.71	9.36			
4	4.80	9.87	4.61	12.50				**4**-L-Leu	7.36	8.85	**4**-L-Phe	6.89	9.29	**4**-L-Trp	6.74	9.17			
5	4.73	9.75	4.40	12.36															

Distances are given in Å. C1, C2, C3 and C4 are the calixarene aromatic carbon atoms bearing the S1, S2, S3 and S4 sulfonato groups, respectively (see Fig. 3).

that does not leave enough room for the amino acid to be included. This picture is in good agreement with the stability constants derived from both the ^{1}H NMR and the calorimetric data: the inclusion of the investigated amino acids (Scheme 1) depends on the cavity dimension and accessibility, which, in turn, depends on the residues attached both to the upper and lower rim.

The cavity of the hosts becomes more symmetrical upon complexation (Table 3). This effect is particularly pronounced for the aliphatic amino acids (L-Val and L-Leu), which are included into the cavity *via* their aliphatic residues, thanks to CH_3–π type interactions.[25] For the complexes with L-Phe and L-Trp, instead, the cavity maintains a more C_{2v} boat-like shape. In this case, in fact, the guests enter the cavity of the hosts *via* their aromatic residues (Figs. 3h, 3m and 3p (Trp), 3g, 3l and 3o (Phe)), as also shown by the ^{1}H NMR upfield shifts observed for the aromatic protons of both L-Trp and L-Phe. In the L-Phe and L-Trp complexes, the retention of the cofacial orientation of two aromatic rings of the free hosts permits the aromatic residue of the guests to be accommodated in a sandwich type arrangement, which maximizes the π–π interaction. This is not the case for the **1**-L-His complex; for this complex, in fact, the C_1–C_3, C_2–C_4 distances are more similar to those obtained for the complexes of the aliphatic amino acids. The imidazole residue does not lie in the C_2–C_4 symmetry plane (Fig. 3i), the cavity has a more symmetrical shape, which determines a somehow less efficient π–π interaction with the aromatic moieties of the host. This effect is probably due to the different nature of the aromatic residue of His (*viz.* imidazole); in fact, a larger stabilization is observed for benzene and substituted benzenes than for imidazole in parallel stacking.[26] It has also to be taken into account that the imidazole residue of L-His ($pK_{a2} = 6.04$)[27] is partially protonated (*ca* 10%) in our conditions and this reduces the apolar character of its aromatic portion. Hence, L-His, among the amino acids reported here, has a low affinity for the hydrophobic cavity of the investigated hosts. These findings corroborate NMR data: only host **1**, the most efficient receptor, is able to complex histidine.

In conclusion, we have shown that the inclusion of amino acids into a series of *p*-sulfonatocalix[4]arenes is enthalpically driven and that the entity of the interaction depends on the length or nature of the side chain. Receptor **1** exists at pH > 5 as pentanion held in a cone structure by *intra*molecular hydrogen bonding between the phenolate anion and two adjacent OH groups at the lower rim, as previously shown.[24] It turns out that **1** is more efficient than receptor **4** and **5** which exist in the cone structure thanks to the four alkyl groups at the lower rim, but experience a high degree of conformational mobility between two symmetrical C_{2v} conformations.[23] Here we find another piece of evidence on how very small changes of the substitution pattern at the lower rim profoundly influence the binding properties of the apolar cavity of calix[4]arenes both in organic media[28] and in water solution.[29] We have also shown that working in unbuffered conditions may have significant implications resulting both from the different ionic strength determined by the presence of the medium and to the different speciation arising from pH changes during the titration as this results in both an ionic strength difference and in a different speciation arising from pH changes during the titration.

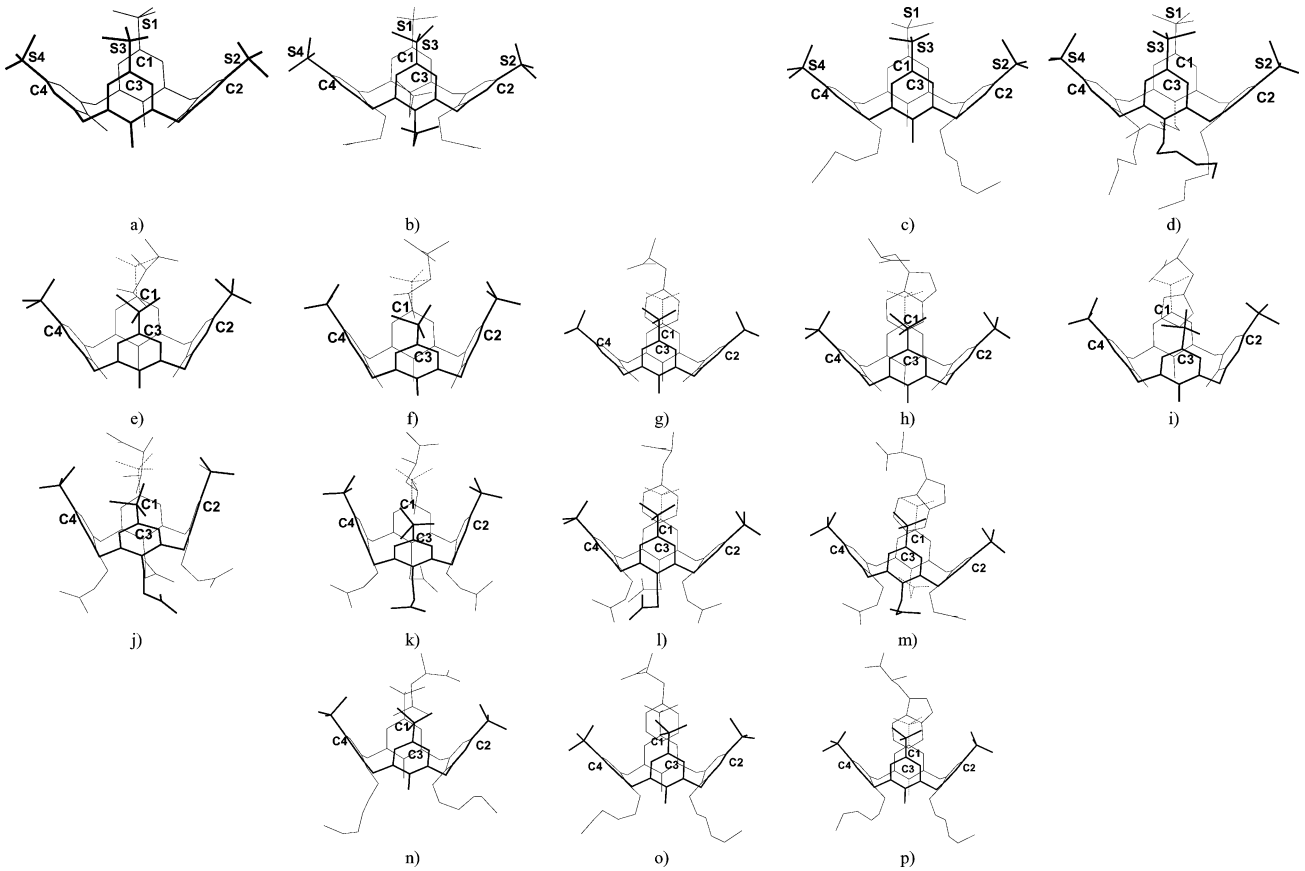

Fig. 3 Optimized structures (molecular mechanics) in water generated for (a) host**1**; (b) host **2**; (c) host **4**; (d) host **5**; (e) **1**-L-Val; (f) **1**-L-Leu; (g) **1**-L-Phe; (h) **1**-L-Trp; (i) **1**-L-His; (j) **2**-L-Val; (k) **2**-L-Leu; (l) **2**-L-Phe; (m) **2**-L-Trp; (n) **4**-L-Leu; (o) **4**-L-Phe; (p) **4**-L-Trp.

Experimental

Materials

Amino acids were purchased from Sigma (purity >99.5%) and were used without further purification. Sodium dihydrogen phosphate and disodium hydrogen phosphate used to prepare buffer solution were obtained from Carlo Erba (purity >99%). NaD_2PO_4 and Na_2DPO_4 were prepared by deuteration of the above commercial products. Doubly distilled water and Grade A glassware used throughout.

Synthesis

1 was synthesized according to Shinkai *et al.*;[30] **2**, **3** and **5** were synthesized according to the procedure described by Casnati *et al.*[31] **4** was synthesized as described previously.[32] All hosts were checked thermogravimetrically as described by Arena *et al.* in ref. 15 and in ref. 24.

NMR Spectra

^{1}H NMR spectra were obtained at 25 °C with a Varian Inova 500 MHz spectrometer and with a Bruker AC-200 MHz spectrometer. Chemical shifts (δ, ppm) in water were externally referenced to DSS in order to avoid any possible interaction with the calix[4]arene derivatives as well as with the guest molecule; all experiments were performed in deuterated phosphate buffer (0.1 mol dm^{-3}) to have a pD value of 7.3. ^{1}H NMR titrations were carried out keeping the guest concentration fixed (1×10^{-3} mol dm^{-3}) and varying the host concentration to obtain the desired host/guest ratio. Chemical shifts of each titration were refined to obtain the final log K value.[33]

Calorimetric measurements and calculations.

The calorimetric measurements were performed at 25.000 ± 0.001 °C using a Tronac 450 isoperibolic calorimeter equipped with a 4 ml titration dewar. To minimize heat leakage the total volume of the solution (titrant + titrate) never exceeded 3 ml. However, the small volume cell was chosen to reduce the amount of host employed for the titrations that were run at least in triplicate. The use of the small volume cell requires an accurate determination of the heat leaking out of the cell into the surrounding bath.[34] This was accomplished thanks to a software program set up for this purpose.[35] The heat produced during the titration was corrected for all non-chemical energy terms. The corrected Q values, that practically refer to one single species, as the pH did not practically change over the entire titration run, were refined by using DOEC,[20] that minimizes the function $U = \Sigma(Q_{i,\text{calcd}} - Q_{i,\text{exptl}})$,[2] where Q_i is the corrected heat of reaction of the i-th titration point to obtain $\Delta H°$ and $\Delta S°$ values; where feasible, the corrected Q values were also refined by using EQDH to double check log$K$ values.[36]

Molecular modeling

Molecular modeling calculations were performed in two steps.[37] In the first step, the atomic charges of each single molecule were calculated by the MNDO method.[38] In the second step, each calix as well as the amino acid were inserted in a box containing 350 water molecules and were minimized. Finally, amino acids were included into the calixarenes and the resulting complexes were inserted in a box containing 350 water molecules. The calculations were performed by MM + force field, using the standard parameters of the package. The minimization was performed by using the conjugate gradient method (Fletcher–Reeves) and was carried out until the RMS gradient reached a value of 0.1 kcal $Å^{-1}$ mol^{-1} (average number of iterations equal to 400). The species minimized for each amino acid as well as for each calixarene was the one really existing in the working conditions (*i.e.* pH = 7).

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