Journal of Inclusion Phenomena and Macrocyclic Chemistry **43:** 305–310, 2002.

Determination of the Stability Constants of Inclusion Complexes of *p*-H-37-(2-carboxy-methyloxy)-calix-[6]-arene and *p*-sulphonato-37-(2-carboxy-methyloxy)-calix-[6]-arene with 15 Amino acids by RP-HPLC

O. I. KALCHENKO[1], E. DA SILVA[2] and A. W. COLEMAN[2]*
[1]*Institute of Organic Chemistry, National Academy of Sciences of Ukraine, Murmanskaya, 5, 02094, Kiev-94, Ukraine;*
[2]*IBCP, CNRS UMR 5086, 7, Passage du Vercors, 69367 Lyon Cedex 07, France*

(Received: 18 January 2002; in final form: 24 May 2002)

Key words: inclusion complexes, amino acids, water-soluble calixarenes, reversed-phase high-performance liquid chromatography (RP-HPLC)

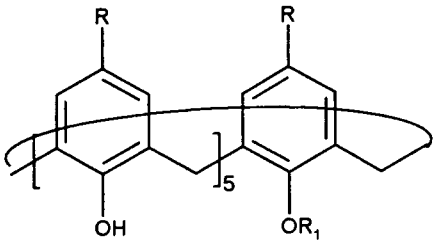
Abstract

Reversed-phase high performance liquid chromatography (Separon SGX CN, UV detection at 254 nm and water as mobile phase) was applied to the study of the host–guest complexation of *p*-H-37-(2-carboxy-methyloxy)-calix-[6]-arene (**1**) and *p*-sulphonato-37-(2-carboxy-methyloxy)-calix-[6]-arene (**2**) with 15 amino acids in the mobile phase. It was established that the formation of the inclusion complexes results in changes in the retention times and capacity factors of amino acids. Stability constants of the complexes were determined. The variations in stability constant values may be explained in terms of the different interactions, which may occur between amino acids and **1** and **2**.

Introduction

The calix-[6]-arenes, composed of six phenolic units linked *via* methylene groups, have been studied as cavity-shaped host molecules able to recognize a range of guest molecules [1] but have received less attention than the calix-[4]-arenes [2, 3]. The binding properties of these compounds have been examined in solution [4, 5] and in the crystalline state [6]. In order to increase the binding properties of calixarenes in aqueous solutions, compounds **1** and **2** were synthesized as given in the literature [7]. In view of the activity of *p*-sulphonato-calix-[6]-arene (**3**) towards different proteins, including chloride-ion channels [8], lysyl oxidase [9] and its anti-thrombotic activity [10], knowledge of the nature and strength of the interactions of **3** and its derivatives with amino acids should provide important information on the mechanism of the binding of *p*-sulphonato-calix-[6]-arenes to complex bio-macromolecules. The solid-state structure of **1** shows a bridge shaped double cavity [7]. Interestingly in the structure only one guest site was occupied, suggesting that a preferential binding site may exist.

Previous NMR [11] and micro-calorimetric [12] studies on the interactions of **3** with Lys and Arg have shown the presence of both 1:1 and 1:2 complexes. Previous RP-HPLC work on the binding on the interaction of *p*-sulphonato-calix-[4]-arene with 10 amino acids was carried out, by ourselves, in aqueous/organic mixtures, and showed strong binding by Arg (K_{ass} = 2587 M^{-1}), Lys (K_{ass} = 1221 M^{-1} and Trp (K_{ass} = 1518 M^{-1}) [13]. Arena *et al.* have reported on the NMR complexation studies of this compound with a number of amino acids [14]. We have recently described the binding of the *p*-sulphonato-calix-[n]-arenes to Bovine Serum Albumin (BSA) by electrospray mass spectrometry, the observed binding order was *p*-sulphonato-calix-[4]-arene ≫ *p*-sulphonato-calix-[6]-arene > *p*-sulphonato-calix-[8]-arene [15], interestingly the inverse order is observed for the anti-thrombotic activity of the *p*-sulphonato-calix-[n]-arenes [16].



R= -H	R1= -CH_2COOH	**1**
R= -SO_3H	R1= -CH_2COOH	**2**
R= -SO_3H	R1= -H	**3**

Scheme 1. Structure of compounds **1**, **2** and **3**.

In this paper, we report on the host–guest interaction of **1** and **2** (Scheme 1) with 15 amino acids (Scheme 2) using HPLC, in purely aqueous conditions. The use of a Separon SGX CN column was essential to the study, allowing high

* Author for correspondence. E-mail: aw.coleman@ibcp.fr

reproducibility in the results. The column retained its selective properties even during the long initial runs with the pure amino acids, and no artefact phenomena were observed. In contrast to the separon SGX C18 column long duration studies at a reasonable temperature (36 °C) and with high flow rates are possible with the SGX CN Column. Moreover, the column can be easily cleaned and regenerated.

Experimental

Reagents. Distilled water was used as mobile phase. Compounds **1** and **2** were synthesised by the published method [7]. Amino acids were purchased from Sigma and used without further purification.

Apparatus. HPLC analysis conditions were as follows:

The LC system consisted of a high-pressure pump HPP 4001 (Laboratorni Pristroje, Praha, Czechia) connected to a Rheodyne Model sample 7120 injector (20 μl, Rheodyne Inc., Berkeley, CA) and an ultraviolet-visible (UV-vis) detector LCD 2563 (Laboratorni Pristroje, Praha, Czechia). The column (150 × 3 mm i.d.) was packed with Separon SGX CN (5 μm) (Lachema, Czechia).

HPLC analysis

The solutions were unbuffered to prevent interference from buffer-calix interactions, solution pHs are in the range missing value. The mobile phase aqueous solutions containing **1** and **2** at concentrations of 0.035, 0.07, 0.14, 0.28 × 10^{-4} M and 0.208, 0.416, 0.832 × 10^{-4} M, respectively were prepared by dissolving the corresponding calixarenes in the mobile phase at ambient temperature (20 °C). Each concentration was analysed five times. The concentrations of amino acids in the injected solutions (identical to the mobile phase) were 10^{-4} M. The amount of the sample injected was 10 μl. Each sample was analysed five times. All chromatograms were obtained at 36 °C. The flow rate was 0.8 mL/min, and the UV detector operated at a wavelength of 254 nm. The dead time (t_0) was measured with the disodium salt of EDTA. Mobile phases with **1** and **2** as additive were equilibrated for 1.5 h before analysis.

Results and discussion

The influence of **1** *and* **2** *in the mobile phase on retention of amino acids*

The effects of adding **1** and **2** to the mobile phase on the retention time, t_R, and the capacity factor, k', of the amino acids were studied. Capacity factors, k', and retention times, t_R of amino acids were determined and are presented in Tables 1 and 2.

Determination of stability constants

Stability constants of calixarene complexes with organic molecules in solution have usually been determined by NMR spectroscopy [17–18]. However, RP-HPLC studies provide an alternate method for determining K_{ass} values, as with recently reported work on calix-[4]-resorcinarene [4], calix-[4]-arene [19], calix-[8]-arene [5] and *p*-sulphonato-calix-[4]-arene [13], the introduction of **1** and **2** into the mobile phase results in a decrease in the retention times and the capacity factors of the amino acids, indicating formation of a host–guest inclusion complex. As illustrated in Figures 1–3, the dependences are essentially linear for all amino acids implying the formation of complexes with a 1:1 apparent stoichiometry [17–18]. From the dependence obtained for $1/k'$ with the concentration of the calixarene additive, the

Table 1. $1/k'$ values and 1:1 stability constants, K_{ass} (M^{-1}, for complexes of **1** with amino acids. Column Separon SGX CN, mobile phase water, 0.8 mL/min, 36 °C, 254 nm

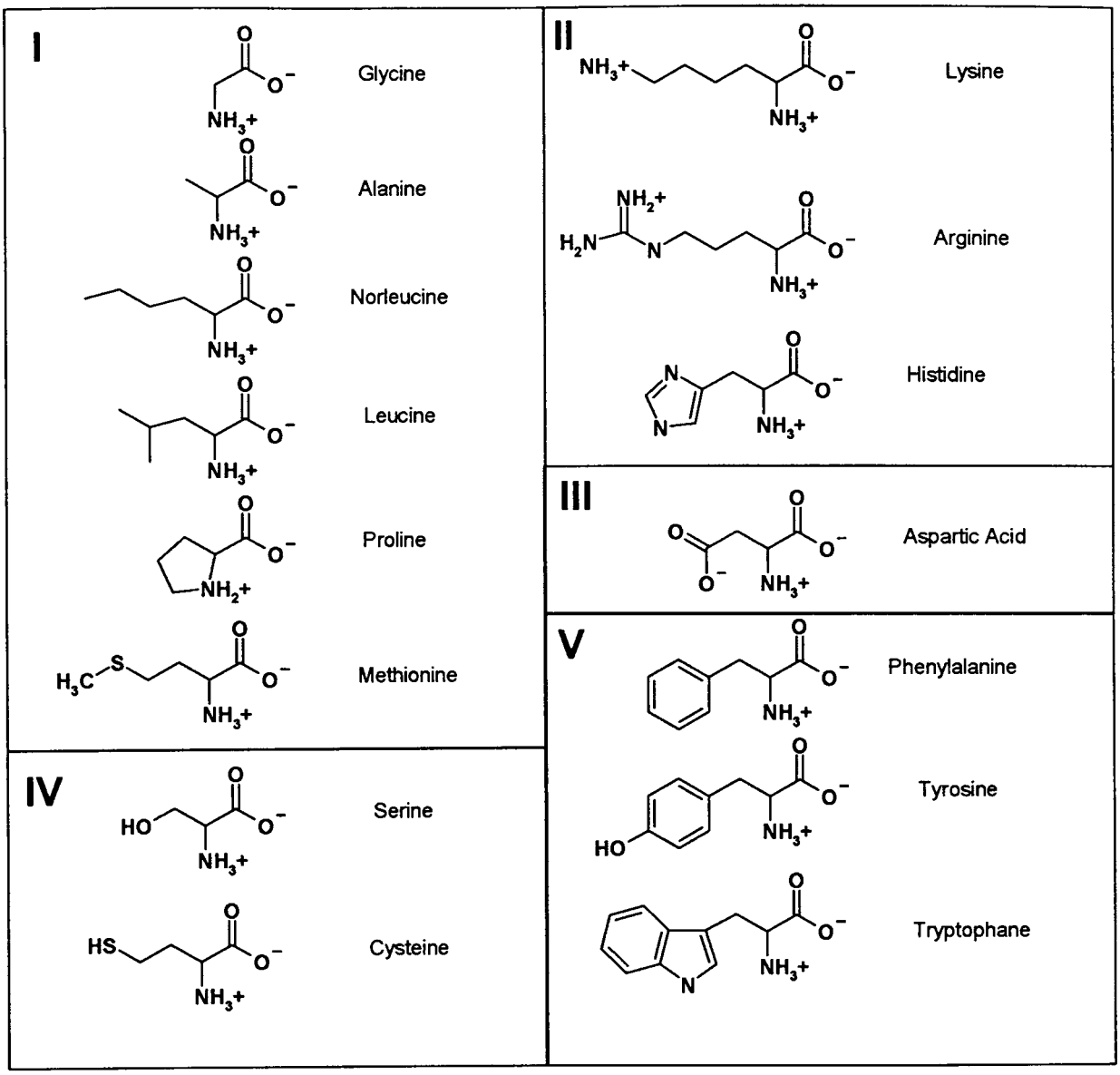
Class	Amino acids	[calixarene] in mobile phase × 10^{-4} M					K_{ass}/M^{-1} (RSD, %)
		0	0.035	0.07	0.14	0.28	
I	Gly	0.472	0.475	0.479	0.484	0.499	1949 ± 7
	Ala	0.462	0.465	0.468	0.475	0.482	1805 ± 9
	Met	0.401	0.402	0.404	0.407	0.409	758 ± 7
	Nor	0.398	0.4	0.403	0.408	0.412	1365 ± 21
	Leu	0.376	0.378	0.38	0.383	0.386	1164 ± 15
	Pro	0.394	0.396	0.399	0.403	0.407	1518 ± 15
II	His	0.51	0.514	0.522	0.543	0.598	2241 ± 25
	Lys	2.02	2.04	2.067	2.099	2.141	3134 ± 15
	Arg	0.596	0.602	0.611	0.623	0.635	3031 ± 13
III	Asp	1.866	1.909	1.972	2.04	2.242	6585 ± 9
IV	Ser	0.475	0.477	0.481	0.486	0.49	1447 ± 20
	Cys	0.478	0.481	0.483	0.487	0.494	1307 ± 9
V	Phe	0.305	0.309	0.311	0.315	0.322	4157 ± 20
	Tyr	0.42	0.423	0.426	0.432	0.438	2041 ± 19
	Trp	0.204	0.206	0.21	0.214	0.219	2801 ± 24

The correlation coefficients for all plots were greater than 0.97.

Table 2. $1/k'$ values and 1:1 stability constants, K_{ass} (M^{-1}, for complexes of **2** with amino acids. Column Separon SGX CN, mobile phase water, 0.8 mL/min, 36 °C, 254 nm

Class	Amino acids	[calixarene] in mobile phase × 10^{-4} M				K_{ass}/M^{-1} (RSD, %)
		0	0.208	0.416	0.832	
I	Gly	0.416	0.421	0.427	0.431	578 ± 16
	Ala	0.357	0.362	0.369	0.376	673 ± 11
	Met	0.323	0.328	0.334	0.341	744 ± 8
	Nor	0.191	0.194	0.198	0.203	839 ± 7
	Leu	0.379	0.387	0.392	0.4	941 ± 8
	Pro	358	0.365	0.374	0.386	985 ± 6
II	His	2.236	2.349	2.471	2.584	475 ± 6
	Lys	3.597	3.861	4.117	4.535	2310 ± 5
	Arg	3.03	3.257	4.265	5.569	3601 ± 14
III	Asp	3.984	4.176	4.356	4.663	4091 ± 5
IV	Ser	0.426	0.431	0.44	0.447	564 ± 15
	Cys	0.433	0.441	0.45	0.459	888 ± 14
V	Phe	0.206	0.21	0.215	0.221	933 ± 6
	Tyr	0.327	0.34	0.358	0.376	1923 ± 6
	Trp	0.158	0.166	0.176	0.187	2460 ± 9

The correlation coefficients for all plots were greater than 0.97.



Scheme 2. List of amino acids and ionisation states at pH 5.

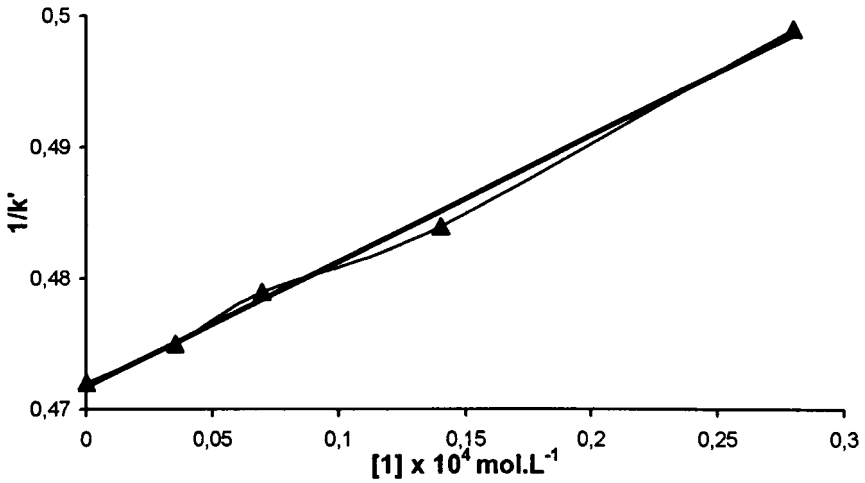


Figure 1. Plots for $1/k'$ for Gly (▲) vs concentration of **1**.

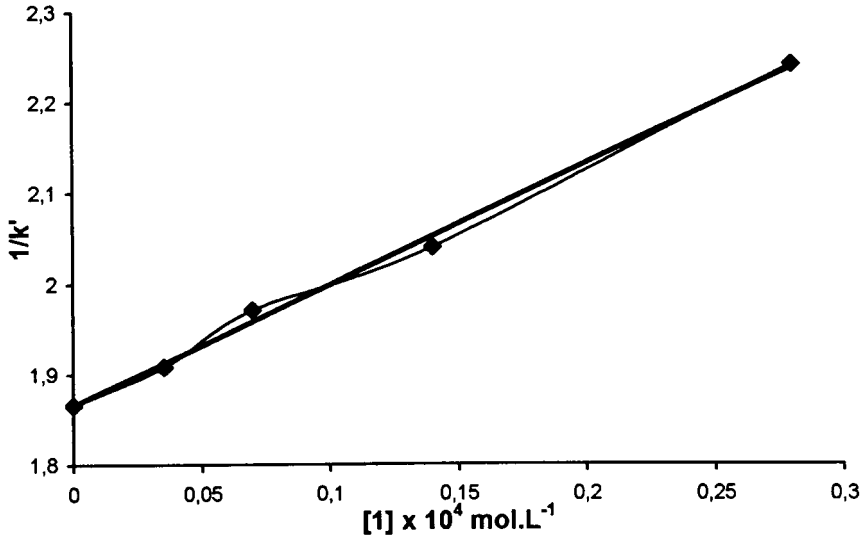


Figure 2. Plots for $1/k'$ for Asp (♦) vs concentration of **1**.

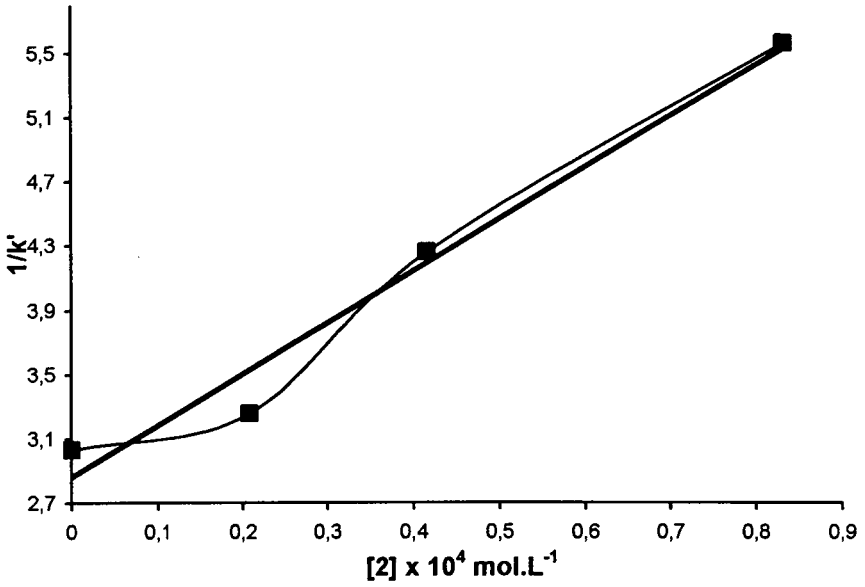


Figure 3. Plots for $1/k'$ for Arg (■) vs concentration of **2**.

stability constants of the complexes were calculated from Equation (1).

$$1/k' = 1/k'_0 + [\text{Host}]/K_D \times k'_0, \qquad (1)$$

where k'_0 is the capacity factor in the absence of a host, [Host] is the concentration of **1** or **2** in the mobile phase and, K_D is the dissociation constant of the complex [20].

It is known that the equation of the liquid chromatographic retention can be used for the calculation of binding constants for a guest-amino acid to a host-calixarene molecule [19]. For all complexes, the possible stoichiometries of the complexes formed, 2:1, 1:1 and 1:2 were verified using the equations proposed by Armstrong *et al.* [20]. For all the complexes the correlation constants were significantly higher (>0.97) for 1:1 complexes than those calculated for either 2:1 or 1:2 complexes. We, thus, postulate that only 1:1 binding is significant for the interaction between **1** and **2** and the amino acids studied here.

The amino acids studied can be divided into 5 classes, depending on the nature of the side chain and the likely interaction with **1** and **2**. For class I, Gly, Ala, Pro, Met, Leu and Nor, the lateral functions are non-polar and will probe the effect of hydrophobic alkyl chain length on the interactions. Class II consists of the positively charged amino acids, Lys, Arg and His, here favourable electrostatic interactions should exist with the sulphonate groups present in **2** but will be absent with **1**. Asp is the negatively charged amino acid of class III; here unfavourable electrostatic interactions will exist with **2**, but the formation of a doubly bridged hydrogen bonded carboxylic acid–carboxylic acid complex is possible with the pendant carboxylic acid function on **1** and **2**. In class IV, the lateral chains of Ser and Cys are polar and hydrogen bonding may occur between the phenolic groups of **1** and **2** and the guest molecules. For class V, Phe, Tyr and Trp, aromatic groups on the lateral chains may undertake aromatic–aromatic interactions with **1** and **2**.

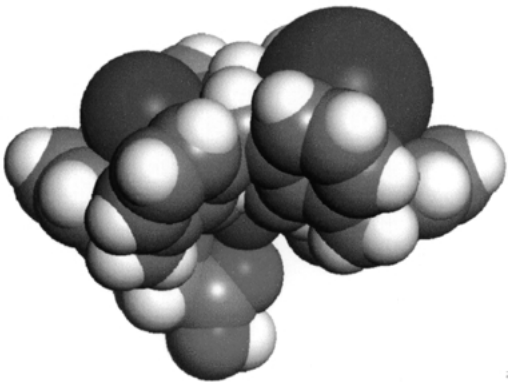
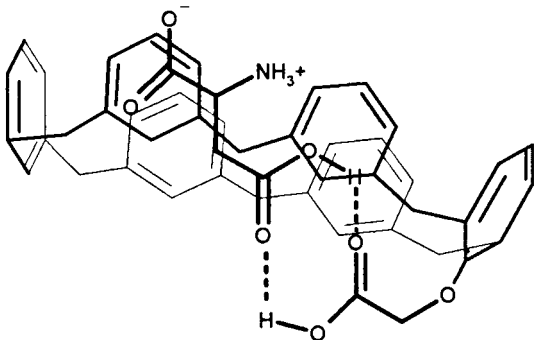


Figure 4. Space filling view of **1** derived from crystallographic data and showing the three possible binding pockets of 85 Å^3, 62 Å^3 and 39 Å^3 volume.



Scheme 3. Proposed H bonding pocket between **1** and Asp. The hydroxyl groups of calixarene are omitted for clarity.

Table 3. The volume (Å^3) of the cavities of the host molecule **1** and the volume (Å^3) of amino acids

Amino acid	Surface (Å^2)	Volume (Å^3)
Gly	75	60.1
Ala	115	88.6
Nor	–	-
Leu	170	166.7
Pro	145	112.7
Met	185	162.9
Lys	200	168.6
Arg	225	173.4
His	195	153.2
Asp	150	111.1
Phe	210	189.9
Tyr	230	193.6
Trp	255	227.8
Ser	115	89
Cys	135	108.5
Compound **1**	Upper rim	
	Cavity 1	85
	Cavity 2	62
	Lower rim	39

Figure 4 shows the available binding sites in **1**, based on crystallographic data [7]. At the upper rim two distinct sites are available having van der Waals volumes of 85 Å^3 and 62 Å^3. Comparing these to known volumes of the amino acids (Table 3), it can be seen that the volume of the second cavity is too small for any strong inclusion with all the amino acids other than glycine. This effectively explains the 1:1 complexation observed although some caution must be taken due to the higher molecular flexibility which will exist in solution. The pendant carboxylic acid group is positioned so that interactions between this function and suitable functions on the lateral chain of various amino acids may occur.

In the case of the interactions between **1** and the various amino acids of class I, having only aliphatic groups on the side chain, the association constant ranges from 758 M^{-1} for Met to 1949 M^{-1} for Gly. While in general there seems to be a decrease in the association constant with increasing lateral chain length, the added steric bulk of the function in Leu reduces the K_{ass}. The order of the observed association constants is Gly > Ala > Nor > Leu.

For the three positively charged amino acids, the association constants are considerably higher in the range 2241 M^{-1} for His to 3134 M^{-1} for Lys. At pH 5, the measured pH value of the solutions, the pendant function of **1** will be present in an equilibrium of the charged carboxylate and neutral carboxylic acid, the stabilisation of the complexes with Lys, Arg and His can arise from either hydrogen bonding or favourable electrostatic interactions.

For Asp, the only amino acid having a carboxylic acid function present on the lateral chain, the highest 1:1 K_{ass} of 6585 M^{-1} is observed. Given that the pKa of the lateral chain is 3.65, the formation of a doubly hydrogen bonded carboxylic acid dimer (Scheme 3) may strongly stabilise the structure of the complex.

The two polar amino acids Ser and Cys of class IV show 1:1 K_{ass} values similar to those observed for the aliphatic amino acids. This would suggest that hydrogen bonding between the alcohol and thiol present on the lateral chains and the carboxylic acid of **1** does not play a significant role.

The 1:1 K_{ass} values for the amino acids of class V, having aromatic functions on the lateral chain are higher (2041–4157 M^{-1}) than those observed for class I and in the range of those observed for the positively charged amino acids. This is probably due to the presence of Π–Π aromatic-aromatic interactions between these molecules and **1**.

In the case of **2**, the addition of six sulphonate groups at the upper rim of the calixarene skeleton will, evidently, greatly increase the polarity of the host. It is thus unsurprising that with regard to the non-polar amino acids where the main interactions with the calixarene should be simple hydrophobic interactions, that there is large decrease in the 1:1 association constants which now range from 578 M^{-1} for Gly to 985 M^{-1} for Pro. The value of K_{ass} for Met undergoes the smallest decrease between the two hosts, being 758 M^{-1} with **1** and 744 M^{-1} for **2**. Interestingly the order of the 1:1 K_{ass} is now Gly < Ala < Nor < Leu for the molecules having simple alkyl chains as the lateral functions. Thus in contrast to **1** the association constant increases with increasing chain length.

For the positively charged amino acids Lys and Arg, the 1:1 K_{ass} vary only slightly between **1** and **2**, with that of Lys decreasing slightly and that for Arg increasing by about 20%. One would expect that the electrostatic interactions with the sulphonate groups would dominate for **2**, as observed in the tight binding of Lys to the sulphonate functions observed in the solid state structure of the 2:1 Lys: *p*-sulphonato-calix-[4]-arene complex [21]. Apparently the energetics of the electrostatic interactions between the positive charge of the amino acids and the carboxylate in **1** and the sulphonate/carboxylate functions in **2** are roughly equivalent. The small variations may arise from the difference in polarity between the alkyl chain present in Lys and the guanidinium present in Arg and the increased polarity of the host in going from **1** to **2**.

As with **1**, the K_{ass} for the complex between **2** and Asp is the highest observed, 4091 M^{-1}. This value is lower than that observed between Asp and **1** (6585 M^{-1}), showing that while some unfavourable electrostatic interactions may have been introduced, the proposed existence of the doubly hydrogen bonded carboxylic acid – carboxylic acid system between the function on the pendant arm of the calixarene and the lateral chain function of Asp dominates energetically in the complexation process. For the polar amino acids the 1:1 K_{ass} values decrease strongly between **1** and **2**, this adds weight to the hypothesis that for Ser and Cys the forces driving inclusion complex formation are hydrophobic and not hydrogen bonding.

Finally for the aromatic amino acids of class V, the 1:1 K_{ass} values for Tyr and Trp remain effectively constant between **1** and **2** being respectively 2041 M^{-1}, 1923 M^{-1} and 2801 M^{-1}, 2460 M^{-1}. For the strictly non-polar Phe again a large decrease in 1:1 K_{ass} is observed decreasing from 4157 M^{-1} to 933 M^{-1} between **1** and **2**. It is possible that for Tyr and Trp there may be secondary interactions involving the polar functions and the hydroxyl or sulphonate groups present in **1** and **2**. This difference in the K_{ass} values between these amino acids and Ser and Cys may arise from a deeper interaction into the cavity bringing the polar functions into closer contact.

Conclusion

In conclusion we have shown that RP-HPLC analysis of the interactions between calix-[6]-arene derivatives and amino acid is a valid tool even for quite insoluble host molecules. The variations in the observed association constants can be explained in terms of the various possible interactions between the groups on the lateral chains of the amino acids and the host. The variation in the polarity between the two hosts considered can modulate strongly the interactions for hydrophobic lateral chains, but has much smaller effects for the charged amino acids. It should be noted that as one aim of this study is to prepare biosensors, the strong interactions between Asp and the hosts will be removed if the pendant carboxylic group on the calix-[6]-arenes is used in the anchoring function to surfaces. However, the possibility to modulate the interactions, quite selectivity, as a function of the para substituent on the calixarene opens up possibilities for the use of these molecules as modulators in proteomic separation experiments.

Acknowledgment

One of us, O. I. K., acknowledges the support of the IBCP (CNRS) in carrying on this work.

References

1. V. Böhmer: *Angew. Chem. Int. Ed. Engl.* **34**, 713 (1995).
2. G. Arena, A. Contino, F.G. Gulino, A. Magrì, D. Sciotto and R. Ungaro: *Tetrahedron Lett.* **41**, 9327 (2000).
3. S. Shinkai, K. Araki, T. Matsuda, N. Nishiyasu, H. Ikeda, L. Takasu and M. Iwamoto: *J. Am. Chem. Soc.* **112**, 9053 (1990).
4. J. Lipkowski, O.I. Kalchenko, J. Slowikowska, V.I. Kalchenko, O.V. Lukin, L.N. Markovsky and R. Nowakowski: *J. Phys. Org. Chem.* **11**, 426 (1998).
5. O.I. Kalchenko, J. Lipkowski, V.I. Kalchenko, M.A. Vysotsky and L.N. Markovsky: *J. Chromatogr. Sci.* **36**, 269 (1998).
6. A.V. Nabok, A.K. Hassan, A.K. Ray, O. Omar and V.I. Kalchenko: *Sens. Actuators, B.* **45**, 115 (1997).
7. E. Da Silva, L. Memmi, A.W. Coleman, B. Rather and M.J. Zaworotko: *J. Supramol. Chem.*, in press (2002).
8. J.L. Atwood, R.J. Bridges, R.K. Juneja and A.K. Singh: U.S. Patent 5,489,612 (1996).
9. E. Aubert-Foucher, A.W. Coleman and D.J.S. Hulmes: French Patent FR 2782007 (1998).
10. K.M. Hwang, Y.M. Qi, S.Y. Liu, T.C. Lee, W. Choy and J. Chen: U.S. Patent 5,409,959 (1995).
11. N. Douteau-Guevel, A.W. Coleman, J.-P. and N. Morel-Desrosiers: *J. Phys. Org. Chem.* **11**, 693 (1998).
12. N. Douteau-Guevel, A.W. Coleman, J.-P. Morel and N. Morel-Desrosiers: *J. Chem. Soc. Perkin Trans. 2*, 629, (1998).
13. O.I. Kalchenko, F. Perret and A.W. Coleman: *J. Chem. Soc. Perkin Trans. 2*, 258 (2001).
14. G. Arena, A. Contino, F.G. Gulino, A. Magri, F. Sansone, D. Sciotto and R. Ungaro: *Tetrahedron Lett.* **40**, 1597 (1999).
15. L. Memmi, A. Lazar, A. Brioude, V. Ball and A.W. Coleman: *J. Chem. Soc. Chem. Commun.* 2474 (2001).
16. A.W. Coleman, unpublished results.
17. G. Arena, A. Contino, G.G. Lombardo and D. Sciotto: *Thermochim. Acta* **254**, 1 (1995).
18. C.D. Gutsche: Calixarenes (Royal Society of Chemistry), Cambridge (1989).
19. O.I. Kalchenko, A.V. Solovyov, J. Lipkowski and V.I. Kalchenko: *J. Chem. Res., S.*, 60 (1999).
20. D.W. Armstrong, F. Nome, L.A. Spino and T. D. Golden, *J. Am. Chem. Soc.* **108**, 1481 (1986).
21. M. Selkti, A.W. Coleman, I. Nicolis, N. Douteau-Guevel, F. Villain, A. Tomas and C. de Rango: *J. Chem. Soc. Chem. Commun.*, 161 (2000).