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ORIGINAL ARTICLE

Mass spectrometric behavior of functionalized calix[4]arenes: the screening ability of host–guest complex formation with amino acid methyl esters

Andrei Medvedovici · Florin Albu · Abdelwaheb Hamdi · Rachid Souane · Lidia Kim · Lucia Mutihac · Jacques Vicens

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Abstract The ESI–MS and MS/MS behavior of functionalized calix[4]arenes (**1**–**5**) has been studied in both positive and negative-ion mode. Liquid chromatography coupled to ESI–MS has been successfully used for separation of the byproducts issuing from the functionalization pathways, through the application of a simple reversed phase mechanism. The ability of (**1**–**5**) to host methyl esters of amino acids, tyrosine, tryptophan, phenylalanine, cysteine, valine, serine, leucine, isoleucine, and threonine has been evaluated by means of MS identification of the host–guest resulting in protonated molecular ions. The direct infusion within the ESI source of the solutions containing the two partners (i.e., calixarene and amino acid derivative) could act as a fast screening means for the evaluation of hosting capability. Only positive ionization may offer information about the host–guest complexes being formed. The influence of the excess of a partner in the infused solution strongly alters ionization yields, making quantitative approaches meaningless. Attempts to chromatographically isolate the host–guest complexes failed, probably due to the fact that interactions of the partners with the mobile and stationary phases are higher than the inclusion interactions. Structures consisting of combined fragments of the host–guest partners resulting from the collisional induced dissociation have not been observed.

Keywords Functionalized calix[4]arenes · Amino acids methyl esters · Host–guest interaction · ESI–MS · MS/MS

A. Medvedovici · L. Kim · L. Mutihac (✉)
Department of Analytical Chemistry,Faculty of Chemistry, University of Bucharest, 1-4, Blvd. Regina Elisabeta, 030018 Bucharest, Romania
e-mail: mutihac@astralnet.ro

F. Albu
Bioanalytical Laboratory, S.C. Labormed S.A, Th. Pallady Blvd. no. 44B, 032266 Bucharest, Romania

A. Hamdi · R. Souane · J. Vicens
IPHC-UdS-ECPM-CNRS, 25, rue Becquerel, 67087 Strasbourg, France

Introduction

Advances in the construction of novel hosts with applications in supramolecular chemistry have generated an increasing demand of new analytical techniques for studying their binding properties with minimal sample consumption. The binding properties of a host and the characterisation and evidence of the host–guest formation in solution have been obtained from many conventional methods including nuclear magnetic resonance, UV-Vis and fluorescence spectrophotometry, circular dichroism, X-ray structures determination, light scattering, calorimetry, conductometry, scanning tunnelling, atomic force microscopy, and computer simulations [1, 2]. Mass spectrometry is an additional quite recent tool for studying non-covalent interactions in the gas phase and probing molecular recognition. Recent reviews have been published on such topics [3–9].

Although the amount of published data on complexation of amino acids by calixarenes and their derivatives, relatively few studies have been devoted to the investigation of their complex formation by mass spectrometry techniques [9].

In 2002, Stone et al. published matrix-assisted laser desorption ionization (MALDI) mass spectrometric studies on non-covalent complexes formed between twenty amino acids and methyl, ethyl, and propyl ester derivatives of calix[6]arenes. Amino acid complexes were observed for

nearly all the amino acids in time-of-flight (TOF) analysis. In Fourier transform mass spectrometry (FTMS) analysis, only the most basic amino acids arginine, histidine, and lysine formed stable adducts. The complexes were abundant under conditions suggesting favourable interactions between host and guest [10].

By the very same year, Perret et al. published the results of their research based on electrospray mass spectrophotometry (ESI/MS) of the formation of supramolecular complexes between a diphosphoryloxycalix[4]arene (DPhC) and a series of amino acids (Ala, Asp, Arg, His, Ser, and Cys) alone and in the presence of metal cations (Na^+, K^+, Mg^{2+}, Ca^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}) [11]. When alone, the calixarene derivative showed the best selectivity for histidine. In the presence of monovalent cations, the decomplexation of amino acids was observed. With divalent cations, ternary complexes were observed DPhC–Cu^{2+}-Arg, DPhC–Cu^{2+}-Lys, and DPhC–Ni^{2+}-Arg, DPhC–Ni^{2+}-Lys. For Zn^{2+}, stronger complexes were observed with Cys and Ser, the highest complex being obtained with His. ^{1}H NMR spectroscopy showed that His is probably attached to Zn^{2+}, which is chelated to the oxygen atoms of the phosphoryloxy groups [11].

In 2010, Torvinen et al. [12] reported the gas phase recognition of native amino acids (Phe, Trp, Tyr, Ser, Cys, Leu, and Asp) by three glucosylthioureidocalix[4]arenes. The glucocalixarenes exhibited clear selectivity towards aromatic amino acids, the complex formation being enhanced by the introduction of a polar, H-bonding group, to the side chain of the amino acid [12]. ESI–MS enantiomeric-labelling studies used to access the enantioselectivity for the recognition of D- versus L-amino acids showed selectivity ranging from 0.61 to 2.58 [12].

Moreover, Bew et al. [13] reported a mass spectrometry investigation of the complexation of four *N*-protected α-amino acids by a series of variously substituted bis-1,3-*N*-benzylureas calix[4]arenes. Best complexation was observed for those calixarenes derivatives having methylene groups between the calix platform and the urea function. Interestingly, it was shown that selectivity can be found during the submission of mixtures of *N*-Fmoc-α-amino acids to a single bis-1,3-*N*-benzylureas calix[4]arene [13].

On one hand, calixarenes, with their unique three-dimensional surface and conformational rigid structure, are one of the best known host molecules along with cyclodextrins, cucurbiturils, cryptands, and crown ethers. By their availability and easy functionalization at either the upper and/or lower rim of the molecular skeleton among potential building blocks, calixarenes have become important receptors in synthesis and applications as supramolecular receptors for molecular recognition, sensing, catalysis, nanoscience, drug delivery, and separation science [14–20]. The ability of calixarenes and their derivatives to form stable complexes with a wide range of guest molecules renders them attractive receptors with large applications in host–guest chemistry [14–20].

On the other hand, amino acids, as fundamental constituents of a wide variety of biological macromolecules, are interesting targets in host–guest chemistry or supramolecular chemistry. Understanding molecular recognition properties of amino acids by receptors in aqueous solutions, gas phase, or solid state, is important for developing procedures of synthesis, purification, and separation of amino acids, as well as in elucidation of the principle of their transport through biological membranes. A limited number of reviews has been published so far on the complexation of amino acids by calixarenes derivatives [21, 22].

We have previously presented studies on the ability of functionalized calix[4]arenes (**1**–**5**) (see Chart 1) variously substituted by acid or amido functions, glycolic chains, and hydroxyl groups as extractants [23] and carriers [24] towards amino acids (L-Try, L-Phe, and L-Tyr) and their methyl ester derivatives (see Chart 2). The extraction results suggest that these aromatic amino acid methyl esters are extracted to a larger extent from an aqueous to an organic phase as compared with the native ones [23]. The presence of OH groups, glycolic chains and amido functions able to form hydrogen bonds and oxygen-cation interactions and electrostatic interactions showed that the amino acids esters are likely to be maintained at the lower rim of the calixarenes [23]. In a subsequent paper, calixarenes (**1**–**5**) were employed for the selective transport of tryptophan, phenylalanine, and tyrosine methyl esters through a liquid membrane [24]. All receptors were found to transport the aromatic amino acids methyl esters from an aqueous source phase to an aqueous receiving phase. The better results were obtained in the transport of tryptophan and phenylalanine by **1** and **5**. The results pointed out that the structure of calix[4]arenes is one of the most important parameter for the recognition of tryptophan and phenylalanine. As in the case of calix[4]arene (**1**), the tetraamido calix[4]arene (**5**) exhibited lower transport through membrane towards tyrosine methyl ester. The receptors diamido calix[4]arene (**3**) and diamido calix[4]arene (**2**) also showed an efficient transport of tryptophan methyl ester and phenylalanine methyl ester. Under our experimental conditions, it was noticed that receptor **1** is selective for tryptophan and phenylalanine methyl esters over tyrosine methyl ester and a similar behaviour was observed for receptor **2** concerning the same amino acid derivatives [24].

In the present paper, we report our recent studies by ESI–MS and MS–MS on the complexes formed between functionalized calixarenes and aromatic and aliphatic amino acid derivatives. These investigations were

performed in order to compare the formation of complexes in solution and in vacuum, as well as to show some possible solvating effects due to the solvents in extraction and transport.

Experimental

Reagents

Acetonitrile was HPLC grade from Merck (Darmstadt, Germany). Water for chromatography (resistivity minimum 18.2 MΩ and TOC maximum 30 ppb) was produced within the laboratory by means of a TKA Lab HP 6UV/UF device and used throughout experiments. Functionalized calixarenes (see Chart 1) were in-house synthesized according to the procedures described elsewhere [23, 25]. Methyl esters of the L-amino acids (phenylalanine, tryptophan, tyrosine, leucine, serine, iso-leucine, valine, cysteine and threonine) were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) as hydrochlorides and are of 98% purity or higher (see Chart 2). Formic acid was extra pure grade obtained from Merck.

Equipments

Experiments were performed on a system built up from Agilent series 1200 modules (Agilent Technology, Waldbronn, Germany) as following: degasser (G1322 A); binary pump SL (G1312 B); thermostated autosampler (G1367 C); column thermostat (G1330 B). Detection was made through a MS/MS triple quadrupole detector (G2571 A) using an atmospheric pressure electrospray ion source (ESI) operated under negative and positive modes. System control, data acquisition, and interpretation were carried out by using the Agilent Mass Hunter software version B 01.00 (B48).

Chromatographic method

Chromatographic separation was applied to verify purity of the in-house synthesized functionalized calixarenes. The chromatographic separation was performed on a Zorbax SB-C18 (Rapid resolution Cartridge) 2.1 × 30 mm, 3.5 μm, thermostated at 25 °C. The column was operated under isocratic conditions, the mobile phase consisting in 3/7 (v/v) aqueous 0.2% formic acid and acetonitrile, at a flow rate of 0.5 mL/min. A standard chromatographic run of 15 min was applied. The injection volume was 0.1 μL from solutions having a concentration of 1 mg/mL for each of the functionalized calixarenes. For MS and MS^2 studies of calixarenes (host analytes), amino acid methyl esters (guest analytes) and the host–guest resulting products, direct 1 μL injections of the respective solutions were made in a 0.5 mL/min flow carrier containing 50% (v/v) aqueous 0.2% formic acid and 50% acetonitrile passing through a capillary tube of 2 m length and 0.12 mm i.d. Solutions of analytes were made in acetonitrile, at a nominal concentration of 0.1 mg/mL. The solutions of the host–guest products were obtained under a 1/9 (w/w) excess of the guest amino acid derivative with respect to the host functionalized calixarene (kept at 0.1 mg/mL concentration level).

MS detection

The operational parameters of the ESI source were the following: vaporizing temperature 350 °C; pressure of the nebulising gas (nitrogen) 60 psi; flow of the drying gas (nitrogen) 13 L/min; capillary potential 4,000 V. The fragmentor energy was set to 140 V for both positive and negative ionization conditions. The collision energy for MS^2 experiments (nitrogen as collision gas) was 20 V under negative polarity and 25 V for the positive counterpart. The electron multiplier was set to 600 V. Scans were made on the mass to charge ratio interval ranging from 50 to 1,500 a.m.u. with a scan time of 500 ms.

Results and discussions

MS and MS^2 data obtained through direct infusion of the solutions of the functionalized calixarenes in the ESI source are given in Table 1. Electrospray ionization produces, under positive polarity, the protonated molecular species. Adducts with sodium, ammonium, and potassium are always formed with different intensities compared to the protonated molecular ion. For instance, signals corresponding to $[MH]^+$ and $[MNH_4]^+$ are comparable and have increased intensities with respect to the $[MNa]^+$ adduct in the case of receptor **1**. A reversed situation is obtained for receptor **4**, where the intensity of the sodium adduct is much higher than the intensity of the protonated molecular ion, while the ammonium adduct is not formed. Calixarene **5** produces a double protonated molecular ion $[M+2H]^{2+}$ as major ion, while the single charged protonated ion and the sodium adduct have similar intensities. A mixed double charged species $[MHNa]^{2+}$ is obtained with an increased probability. This ion is able to host small neutral molecules, such as CO_2 or HCN, produced within the source. For the receptor **3**, the sodium adduct has much lower intensity compared to the protonated molecular ion, while the major ion is produced through dehydration. Calixarene **2** produces the protonated molecular ion as the major signal. The signal of the sodium adduct is also considerable, being much larger than the potassium one. In-source

Table 1 MS and MS^2 data for functionalized calixarenes (direct infusion in the ESI source)

Calixarene	Molecular formula	M_w	Exact Mass	Polarity	*m/z* in MS spectra/attribution	Precursor ion	*m/z* of product ions/attribution
1	$C_{48}H_{60}O_8$	765.01	764	(+)	765/$[MH]^+$	765	709/$[MH–(H_2C=C(CH_3)_2)]^+$
					782/$[MNH_4]^+$		653/$[MH–2 \times (H_2C=C(CH_3)_2)]^+$
					787/$[MNa]^+$		597/$[MH–3 \times (H_2C=C(CH_3)_2)]^+$
					803/$[MK]^+$		541/$[MH–4 \times (H_2C=C(CH_3)_2)]^+$
					709/$[MH–(H_2C=C(CH_3)_2)]^+$		495/$[MH–4 \times (H_2C=C(CH_3)_2)–HCOOH]^+$
				(−)	763/$[M–H]^-$	763	705/$[M–H–CH_3COOH]^-$
							647/$[M–H–2 \times CH_3COOH]^-$
2	$C_{54}H_{74}N_2O_{10}$	911.2	910	(+)	911/$[MH]^+$	911	893/$[MH–H_2O]^+$
					933/$[MNa]^+$		
					949/$[MK]^+$		
					965/$[MNa+MeOH]^+$		
					893/$[MH–H_2O]^+$		
					483/$[MHNa+MeOH]^{2+}$		
				(−)	909/$[M–H]^-$	909	No CID
3	$C_{56}H_{78}N_2O_{12}$	971.25	970	(+)	971/$[MH]^+$	971	953/$[MH–H_2O]^+$
					993/$[MNa]^+$		
					953/$[MH–H_2O]^+$		
					810/$[MH–(CHCONHC(CH_2OH)_3)]+$		
				(−)	969/$[M–H]^-$	969	No CID
4	$C_{52}H_{64}O_{12}$	881.08	880	(+)	881/$[MH]^+$	881	777/$[MH–CO_2–HCOOH]^+$
					903/$[MNa]^+$		721/$[777–(H_2C=C(CH_3)_2)]^+$
					835/$[MH–HCOOH]^+$		665/$[777–2 \times (H_2C=C(CH_3)_2)]^+$
					825/$[MH–(H_2C=C(CH_3)_2)]^+$		609/$[777–3 \times (H_2C=C(CH_3)_2)]^+$
					777/$[MH–CO_2–HCOOH]^+$		
				(−)	879/$[M–H]^-$	879	No CID
5	$C_{68}H_{100}N_4O_{16}$	1229.57	1228	(+)	1229/$[MH]^+$	1229	1167/$[MH–HO(CH_2)_2OH]^+$
					1251/$[MNa]^+$		1022/$[MH–HO(CH_2)_2OH–(HO(CH_2)_2O(CH_2)_2NHCO=CH_2)]^+$
					615/$[M+2 \times H]^{2+}$	615	553/$[M–2 \times HO(CH_2)_2OH]^{2+}$
					626/$[MHNa]^{2+}$		525/$[M–2 \times HO(CH_2)_2OC_2H_5]^{2+}$
					637/$[M+2\times H+CO_2]^{2+}$		494/$[M–2 \times HO(CH_2)_2OC_2H_5–N_2–2 \times H_2O]^{2+}$
					642/$[M+2\times H+2\times HCN]^{2+}$		406/$[494–2 \times HO(CH_2)_2OCH=CH_2]^{2+}$
							246/$[(CH_3)_3C\text{-}C_6H_3(CH_3)(0CH_2CONHCH=CH_2)]^+$
						626	595/$[MHNa–HO(CH_2)_2OH]^{2+}$
							546/$[MHNa–(O=CH\text{-}CO–NH(CH_2)_2O(CH_2)_2OH)]^{2+}$
						637	564/$[M+2H–(CH_3CONHCH_2CH_2OCH_2CH=O)]^{2+}$
				(−)	1227/$[M–H]^-$	1227	1082/$[M–H–(CH_3CONHCH_2CH_2OCH_2CH=O)]^-$
					1263/$[M+Cl]^-$		937/$[M–H–2 \times (CH_3CONHCH_2CH_2OCH_2CH=O)]^-$

dehydration produces a signal comparable to the sodium adduct, while the latter may also trap a methanol molecule.

Protonated molecular ions isolated as precursors for collisional induced dissociation (CID) are cleaved to product ions, while sodium adducts are not producing CID. Mixed $[MHNa]^{2+}$ species also dissociate. This actually remains valid for other double charged precursors. Generally, the breakdowns arise to the moieties functionalized to the phenol groups of the calixarenes. Exception is made by receptor **1**, where dissociation is produced to the *t*-butyl substituents of the aromatic rings.

Proton elimination under negative ionization conditions represents the general pattern for all tested compounds. Additionally, receptor **5** produces the capture of a chloride ion, with almost similar probability compared to $[M\text{-}H]^-$ formation. The negative molecular ions are resistant to CID in the cases of receptors **2**–**4**. When dissociation occurs under negative MS^2 conditions, breakdowns arise to the functionalized phenol moieties.

Calixarenes **1** and **3** produce complicated ionization patterns under positive electrospray ionization. Attribution of other signals than those enlisted in Table 1 was almost impossible, leading to the conclusion that substances contain other byproducts issuing from the synthesis pathways. Consequently, we attempted the chromatographic separations under conditions given in the Experimental section. Indeed, our assumptions were confirmed, as illustrated in Fig. 1.

For receptor **1**, an additional compound was found to elute later in the chromatogram. Ions with *m/z* of 779, 801, and 796 a.m.u. found in the MS spectrum of the infused solution characterize the second peak in the chromatogram and are attributed to $[MH]^+$, $[MNH4]^+$ and $[MNa]^+$ structures corresponding to a compound having a molecular weight of 778. This might be corresponding to a calixarene having a methyl esterified carboxyl group. The ammonium adduct ion is the most intense signal, followed by the protonated one and by the sodium adduct, which illustrate a similar behavior to the parent receptor **1**. The 777 a.m.u. ion signal under negative ionization conditions was also confirmed. To illustrate our findings, (+)/(−) MS, and (+)/(−) MS^2 spectra for receptor **1** are given in Fig. 2 (A–C).

Two additional peaks are observed in the chromatogram of the **3** compound. The **3**/A byproduct should have a molecular mass of 881 u, as it produces ions characterized by *m/z* values of 882 a.m.u. $[MH]^+$, 904 a.m.u. $[MNa]^+$, and 864 a.m.u. $[MH\text{-}H_2O]^+$, having intensities decreasing from the sodium adduct to the protonated molecular ion and the dehydrated one. A mixed $[MHNa]^{2+}$ double charged ion at *m/z* of 452.5 could also be observed with a lower intensity. The signal with m/z of 880 a.m.u. in the negative ion spectrum confirms our findings. In the MS^2 spectrum, dissociation is achieved through a water molecule loss. No CID was observed under negative ionization conditions. The structure of the byproduct may be tentatively attributed to a **3** analogue having one phenolic moiety functionalized by means of the *N*-methylacetamide group ($–CH_2CONHCH_3$). The advanced structure confirms also the elution order, the corresponding product being more hydrophobic compared to the parent one.

The **3**/B byproduct has a molecular mass of 849 a.m.u., as it generates the protonated molecular ion with *m/z* of 850 a.m.u. and the sodium adduct of 872 a.m.u., the intensity of the molecular ion being higher than the intensity of the sodium adduct. The mixed double charged $[MHNa]^{2+}$ adduct is observable at a lower intensity (about 20% from the major signal). The formation of the ion with *m/z* at 848 a.m.u. under negative polarity confirms the above mentioned data. No CID was observed for the negative precursor ion. As a tentative attribution of a structure for **3**/B byproduct we may advance a symmetrical analogue of receptor **3**, both phenolic groups being functionalized with *N*-hydroxyethylacetamide groups ($–CH_2CONHCH_2CH_2OH$).

MS characterization of the two peaks in the chromatogram of the **3** product could not explain additional signals at *m/z* of 793, 815, and 831 a.m.u. observed in the spectrum of the infused solution. Finally, we considered that the signals are produced by another compound with a molecular mass of 792 a.m.u., having a retention time higher than

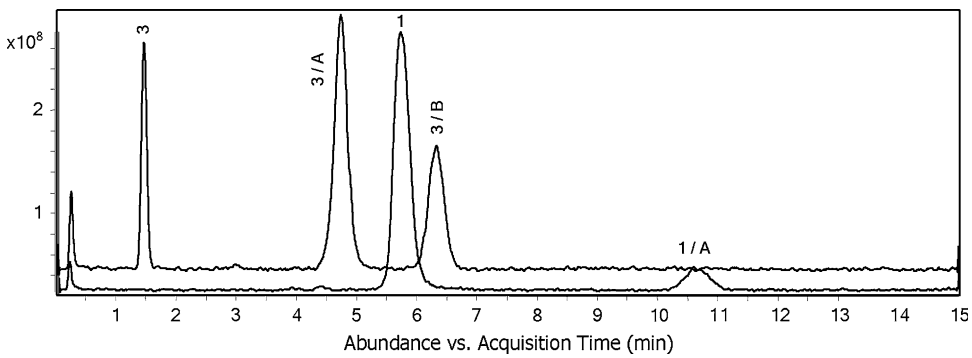

Fig. 1 Liquid chromatographic separation under RP elution mechanism of byproducts in the tested calixarenes 1 and 3 (conditions are given in the "Experimental")

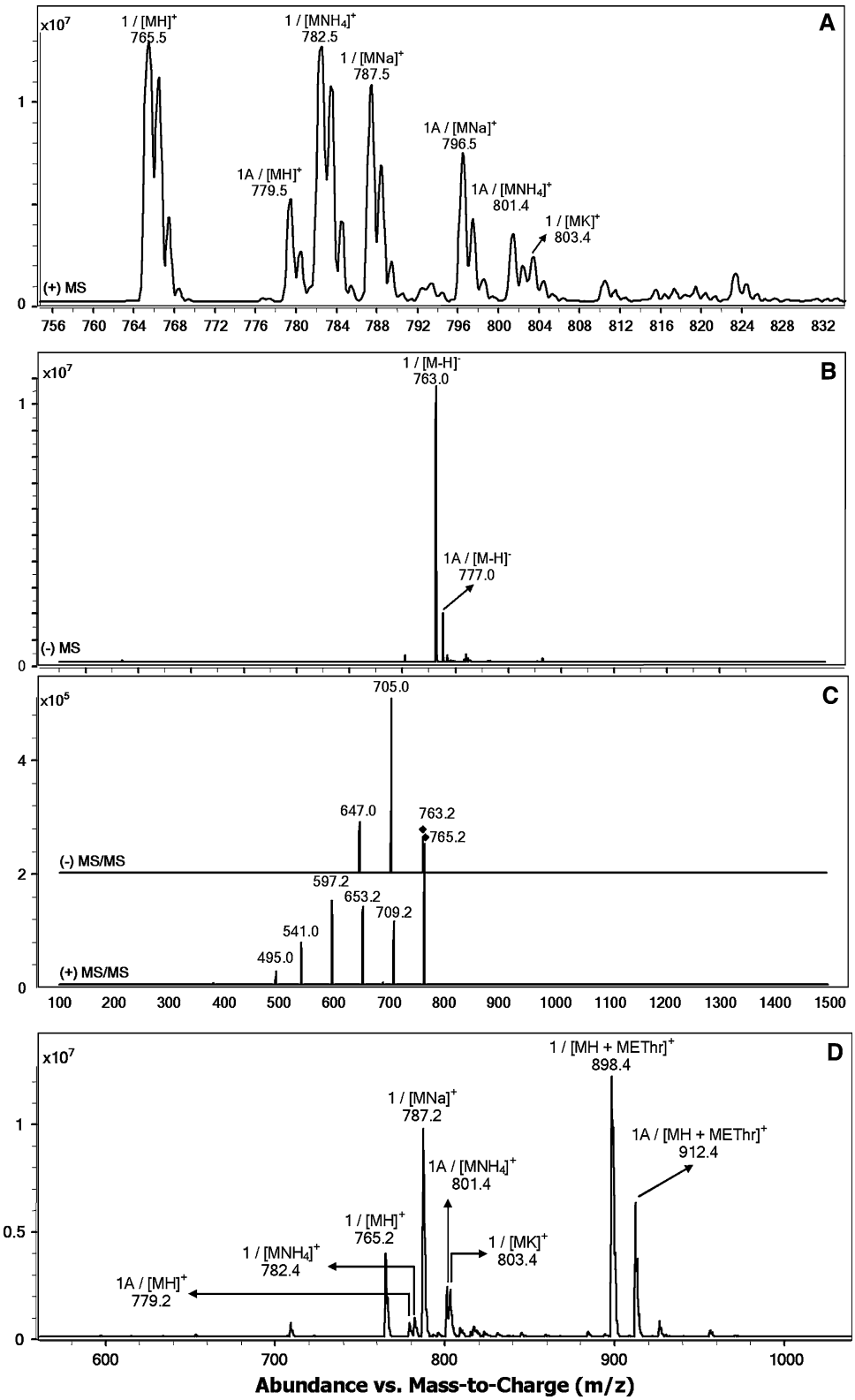

Fig. 2 MS and MS^2 spectra of receptor **1** (**a**, **b**, **c**) and of the host–guest complex made between receptor **1** and METhr (**d**)

15 min., and producing the protonated molecular ion at *m/z* 793 a.m.u., a sodium adduct at *m/z* 815 a.m.u., and a potassium adduct at *m/z* of 831 a.m.u., the intensity of the sodium adduct being much higher than intensities of the other two species. Such a structure may correspond to a symmetrical analogue of the **3**/A compound, with two phenolic groups substituted by the *N*-methylacetamide moiety. However, confirmation of these assumptions

Chart 1 Structures of the functionalized calix[4]arenes

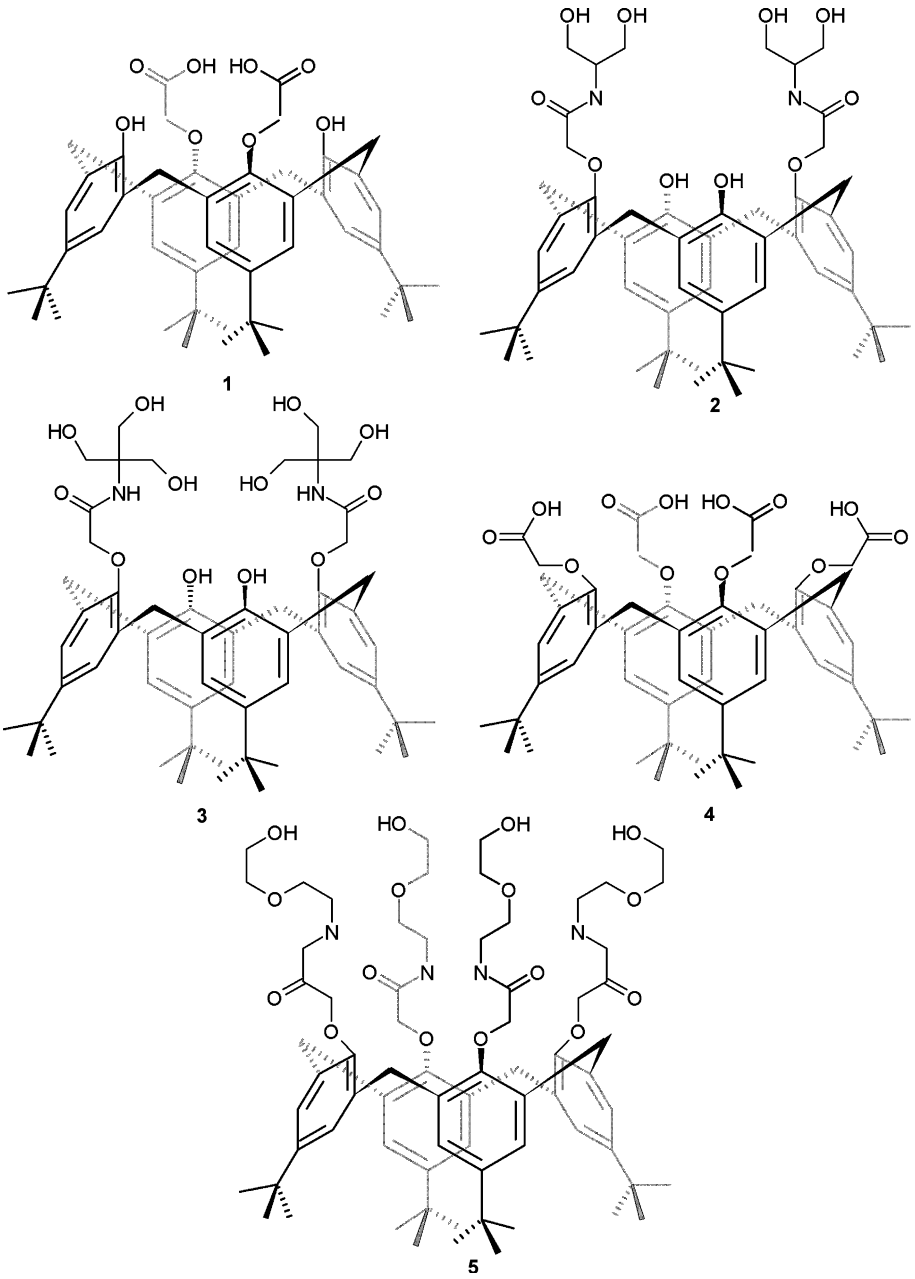

Chart 2 Structures of the tested amino acid methyl esters

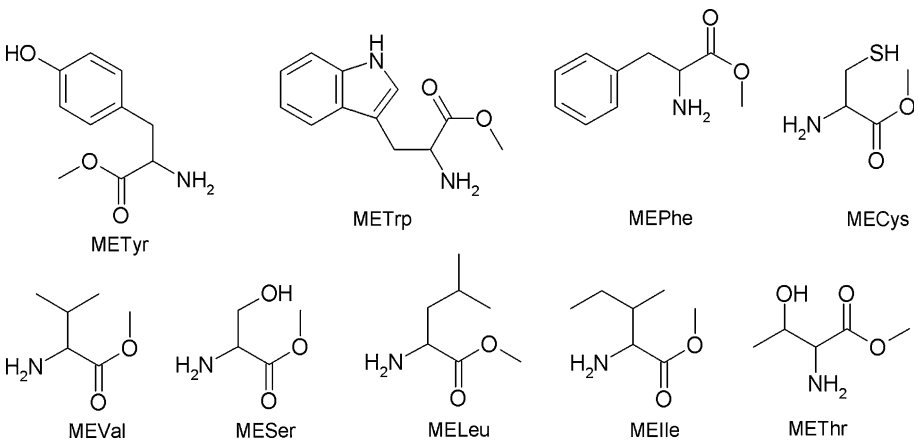

Table 2 ESI ionization of the amino acids methyl esters (*m/z* values in bold indicate the major ions)

#	Amino acid derivative (acronym)	Exact mass	Polarity	MS behavior *m/z*/attribution
1	METyr	195	(+)	119/$[MH–NH_3–CH_3OH–CO]^+$
				136/$[MH–CH_3OCHO]^+$
				147/$[MH–NH_3–CH_3OH]^+$
				179/$[MH–NH_3]^+$
				196/$[MH]^+$
				218/$[MNa]^+$
			(−)	155/$[M+OH^-–CO–CHO]^-$
				194/$[M–H]^-$
2	METrp	218	(+)	132/$[MH–CO–CH_2O–CH2=NH]^+$
				143/$[MH–OH^•–CH_3^•–CO\text{-}NH_2^•]^+$
				159/$[MH–CH_3OH–CO]^+$
				170/$[MH–OH^•–CH_3OH]^+$
				202/$[MH–OH^•]^+$
				219/$[MH]^+$
				241/$[MNa]^+$
3	MEPhe	179	(+)	77/$[MH–CH_3OH–CO–NH_3–C_2H_2]^+$
				103/$[MH–CH_3OH–CO–NH_3]^+$
				120/$[MH–CH_3OH–CO]^+$
				131/$[MH–OH^•–CH_3OH]^+$
				163/$[MH–OH^•]^+$
				180/$[MH]^+$
				202/$[MNa]^+$
4	MECys	135	(+)	87/$[MH–NH_3–CH_3OH]^+$
				102/$[MH–NH_3–OH^•]^+$
				119/$[MH–NH_3]^+$
				136/$[MH]^+$
				158/$[MNa]^+$
				269/$[M_2–H_2+H]^+$
				291/$[M_2–H_2+Na]^+$
5	MEVal	131	(+)	**132**/$[MH]^+$
6	MESer	119	(+)	**102**/$[MH–H_2O]^+$
				120/$[MH]^+$
7	MELeu	145	(+)	**146**/$[MH]^+$
8	MEIle	145	(+)	**146**/$[MH]^+$
9	METhr	133	(+)	102/$[MH–CH_3OH]^+$
				116/$[MH–H_2O]^+$
				134/$[MH]^+$

through the *m/z* signal at 791 u in the negative MS spectrum has not been obtained.

The methyl esters of the amino acids are generally intensively cleaved within the ESI source. It is the case of methyl esters of threonine, serine, tyrosine, tryptophan, phenylalanine and cysteine). The ionization patterns are illustrated in Table 2. Only for the methyl esters of isoleucine, leucine, and valine, the protonated molecular ions represent the major ones too. Adducts with sodium are observed only for tyrosine, tryptophan, phenylalanine, and cysteine derivatives. Cysteine dimerises to the thioether within the source producing its protonated form and the sodium adduct. Negative ionization is produced by the methyl ester of tyrosine only, due to its phenolic moiety.

In order to evaluate the ability of functionalized calixarenes for hosting a methyl ester of an amino acid, the two compounds were mixed together. The mixing ratio was 1/9 (w/w) between the calixarene and the amino acid

Table 3 Formation of the host–guest complexes between the functionalized calixarene and the methyl esters of the amino acids, as it results from the (+) mass spectra obtained from infused solutions containing the calixarene and the amino acid derivative in a mass ratio of 1:9

Amino acid methyl ester	Calixarene																	
	1		1/A		2		3		3/A		3/B		3/C		4		5	
	m/z[a]	Int.[b]	*m/z*	Int.	*m/z*	Int.	*m/z*	Int.	*m/z*	Int.	*m/z*	Int.	*m/z*	Int.	*m/z*	Int.	*m/z*	Int.
METyr	960	M	975	H	1106	L	1166	N.F.	1077	N.F.	1045	N.F.	988	N.F.	1076	M	1424	N.F.
METrp	983	M	998	H	1129	M	1189	L	1100	H	1068	L	1011	H	1099	M	1447	N.F.
MEPhe	944	M	959	H	1090	L	1150	N.F.	1061	N.F.	1029	N.F.	972	N.F.	1060	M	1408	N.F.
MECys	900	M	915	H	1046	N.F.	1106	N.F.	1017	N.F.	985	N.F.	928	N.F.	1016	H	1364	N.F.
MEVal	896	M	911	H	1042	L	1102	N.F.	1013	N.F.	981	N.F.	924	N.F.	1012	M	1360	N.F.
MESer	884	M	899	H	1030	N.F.	1090	N.F.	1001	N.F.	969	N.F.	912	N.F.	1000	H	1348	N.F.
MELeu	910	M	925	H	1056	L	1116	N.F.	1027	N.F.	995	N.F.	938	N.F.	1026	M	1374	N.F.
MEIle	910	M	925	H	1056	L	1116	N.F.	1027	N.F.	995	N.F.	938	N.F.	1026	M	1374	N.F.
METhr	898	M	913	H	1044	N.F.	1104	N.F.	1015	N.F.	983	N.F.	926	N.F.	1014	H	1362	N.F.

M major ion, *H* high intensity, *L* low intensity, *N.F.* not formed

[a] The mass to charge ratio characterizing the host–guest complex between the functionalized calixarene and the methyl ester of the amino acid

[b] The relative intensity of the signal assigned to the protonated host–guest complex with respect to the signal of the protonated calixarene

methyl ester. The excess of the amino acid derivative was used to force inclusion within the calixarene cavity. The concentration of the calixarene in the solution was identical (0.1 mg/mL) to the concentration of the solution used to asses its MS spectral behavior, in view of some quantitative comparison (e.g., signal area for an extracted ion). The solutions containing the host–guest pair were directly infused within the ESI source. Attempts to chromatographically separate the host–guest complex failed, probably due to stronger interactions of the amino acid derivative with the mobile phase, and the calixarene with the stationary phase compared to the host–guest interaction. Experimental results are illustrated in Table 3.

One easily observe that receptors **1** and **4** are hosting the amino acids methyl esters, as the signals of the protonated complexes are major or intense within the mass spectra acquired in the positive polarity. To illustrate MS behavior corresponding to formed host–guest complexes, in Fig. 1**d** the spectra corresponding to receptor **1** and METhr is given. The impurity found in receptor **1** is readily functioning similarly to its congener, exhibiting an evident affinity for all amino acid derivatives. No host–guest complexes are formed with receptor **5** (possibly because it is some sterical hindrance of the moieties functionalizing the phenol groups responsible for the lack of inclusion). Receptor **2** poorly interacts with the amino acids methyl esters, with the exception of the tryptophan derivative. No interactions are evidenced by the MS behavior in the case of cysteine, serine, and threonine methyl esters. Receptor **3** poorly interacts only with the tryptophan derivative only. However, the related compounds of **3**, specifically congeners **3**/A and **3**/C, are better hosting the amino acid derivatives. Receptor **3**/B behaves similarly to its parent product. In all cases, the mass signals of the free calixarene are observable, meaning that the host–guest complex was not quantitatively formed or it may be cleaved within the ion source. None of the signals corresponding to fragments of the calixarene hosting amino acid derivatives or fragments of amino acids hosted by calixarene, or both, are observed.

Negative ionization is not producing any signal evidencing the formation of the host–guest complexes.

In order to get some quantitative insights about the processes, ion extraction was made according to the *m/z* corresponding to the protonated ion of the free calixarene, either in positive or negative polarity, from traces obtained after infusion of solutions containing the calixarene and the mixture calixarene/amino acid derivative, respectively. The signal areas were measured and compared. In all cases sustaining formation of the host–guest complex, the consumption of the free calixarene was around 80%. Surprisingly, for cases not evidencing formation of the complexes, apparent consumption of the free calixarene was around 50%. Monitoring of the negative molecular ion of the calixarene in different solutions (alone and together with the amino acid derivative) leads to reduction with around 50% of the recorded signal intensities. As such, it comes out that the presence of the excess of the free amino acid derivative within the source affects ionization yields both in positive and negative polarity modes, making quantitative interpretation meaningless.

When applying CID to precursors consisting in protonated host–guest complex ions, the fragmentation patterns illustrated in Table 4 are observed.

Table 4 Product ions resulting from CID of the precursors representing the complexes formed between calixarenes and amino acid methyl esters

Calixarene/amino acid methyl ester complex	Precursor ion(*m/z*)	Product ions (*m/z*)						
1-METyr	960	196	–	–	597	653	709	***765***
1-METrp	983	219	202	–	597	653	***709***	765
1-MEPhe	944	180	120	541	597	653	***709***	765
1-MECys	900	136	–	–	597	653	709	***765***
1-MEVal	896	132	–	541	597	653	***709***	765
1-MESer	884	120	–	541	597	653	***709***	765
1-MELeu	910	146	–	541	597	653	***709***	765
1-MEIle	910	146	–	541	597	653	***709***	765
1-METhr	898	134	–	541	597	653	***709***	765
Attributions	$[CalAAH]^+$	$[AAH]^+$	AA fragm	$[CalH\text{-}4 \times Fr]^+$	$[CalH\text{-}3 \times Fr]^+$	$[CalH\text{-}2 \times Fr]^+$	$[CalH\text{-}Fr]^+$	$[CalH]^+$
2-METyr	1189	–	–	893	*911*	–	–	–
2-METrp	1056	–	–	***893***	911	–	–	–
2-MEPhe	1129	–	–	893	***911***	–	–	–
2-MEVal	1106	–	–		911	–	–	–
2-MELeu	1042	–	–	893	***911***	–	–	–
2-MEIle	1056	–	–	***893***	911	–	–	–
Attributions	$[CalAAH]^+$	–	–	$[MH\text{–}H_2O]^+$	$[CalH]^+$	–	–	–
3-METrp	1189	–	–	*953*	971	–	–	–
Attributions	$[CalAAH]^+$	–	–	$[CalH\text{-}H_2O]^+$	$[CalH]^+$	–	–	–
4-METyr	1076	196	–	***777***	835	881	–	–
4-METrp	1099	219	–	***777***	835	881	–	–
4-MEPhe	1060	180	–	***777***	835	881	–	–
4-MECys	1016	–	–	***777***	835	881	–	–
4-MEVal	1012	132	–	***777***	835	881	–	–
4-MESer	1000	120	–	***777***	835	881	–	–
4-MELeu	1026	146	–	***777***	835	881	–	–
4-MEIle	1026	146	–	***777***	835	881	–	–
4-METhr	1014	134	–	***777***	835	881	–	–
Attributions	$[CalAAH]^+$	$[AAH]^+$	–	$[MH\text{-}CO_2\text{-}CH_3COOH]^+$	$[MH\text{-}HCOOH]^+$	$[CalH]^+$	–	–

Data in *bold* and *italics* represents the major product ions being formed

CalAA calixarene/amino acid derivative complex, *Cal* Calixarene, *AA* Amino acid methyl ester, *H* proton, *Fr* $[H_2C{=}C(CH_3)_2]$

Notice that no fragmentation may occur at the two partners (calixarene and/or amino acid methyl ester) with conservation of the inclusion complex. The hosted amino acid derivative is not influencing the CID pathways occurring to the calixarene cavity, fragmentation being made according to the principal patterns identified for the standalone compound. Usually, the amino acid derivative is excluded from the cavity under CID conditions as a protonated molecular ion. In some cases (e.g., **2** host–guest complexes) amino acid elimination is accomplished as a neutral form, and consequently does not yield any associated product ions.

The comparison of the results presented in Tables 3 and 4 with previous data [23] obtained for the extraction of methyl esters METyr, METrp, and MEPhe by calixarenes **1–5** (see Table 5) indicates that no rational correlations can could be rendered indicative of solvent positive or negative effects in the complex formation process.

Table 5 Extraction (%) of aromatic amino acid derivatives by functionalized calix[4]arenes [23]

Calix[4]arenes	MeTrp (%)	MePhe (%)	MeTyr (%)
1	50	12	23
2	41	11	3
3	61	21	16
4	5	8	<3
5	65	13	6

$c_{amino\ acid} = 2.5 \times 10^{-4}$–$1.0 \times 10^{-3}$ M; $c_{calix[4]arene} = 1 \times 10^{-4}$–$5.0 \times 10^{-4}$M; pH 5.5 (MES/NaOH buffer), $T = 298.15$ K

Conclusions

Functionalized calix[4]arenes are generating positive and negative ions within the ESI source of a mass spectrometer. Adduct formation with sodium, potassium, and ammonium ions are more often observed under positive ionization. Fragmentation may occur, generally taking place at the functionalized phenol groups, although cleavage of the *t*-butyl moiety was also observed. Negative molecular ions are obtained through proton elimination. Further, CID of the isolated molecular precursor under negative polarity has not generally been observed. If happening, the fragmentation patterns are simpler under negative polarity compared to positive MS^2 ionization. The ability of the calixarene to trap an amino acid methyl ester can be easily screened, though the observation of the formation of the protonated molecular ion of the host–guest complex, under positive ionization is highlighted. Inclusion of an amino acid derivative stabilizes calixarene towards negative ionization. Quantitative approaches in the studies of the host–guest complex formation are difficult, since the presence of an excess of a partner (usually the amino acid derivative) strongly influences the ionization yields of the host. Although the separation and characterization of the calixarene synthesis byproducts is possible by means of HPLC-ESI/MS, all attempts to perform chromatographic isolation of an inclusion complex have failed so far. Isolation of the molecular ions corresponding to the host–guest complexes and their further CID has lead to the conclusion that fragmentation can not occur at the level of the two partners with a simultaneous conservation of the inclusion aggregate.

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