Glucosylthioureidocalix[4]arenes: Synthesis, conformations and gas phase recognition of amino acids†

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The gas-phase recognition of native amino acids and the conformational properties of three glucosylthioureidocalix[4]arenes (**1-3**) were studied theoretically and experimentally using *ab initio* calculations, ESI-FTICR, ^{1}H and ^{13}C NMR MS. The conformational and complexation properties of the glucocalixarenes were dependent on the number of glucose units at the upper rim and the length of the alkyl chains at the lower rim of the calixarene skeleton. ESI-MS experiments showed the compounds to form 1 : 1 complexes with the amino acids, with a marked preference for amino acids containing an aromatic nucleus and an additional H-bonding group in their side chain (Trp, Tyr, Phe ≫ Ser, Leu and Asp). The experimental data were rationalized by the results of *ab initio* calculations. ESI-MS competitions carried out with enantiomeric-labelled (EL) amino acids showed enantiomeric selectivities ranging from 0.61 (Phe(D)/Phe(L) with ligand **3**) to 2.58 (Tyr(D)/Tyr(L) with ligand **2**). In gas-phase hydrogen–deuterium (H/D) exchange reactions, diglucosylcalix[4]arene **2** exhibited extremely slow exchange rates, which were attributed to the close proximity and strong hydrogen bonding between the facing glucosylthioureido groups. H/D exchange rates were much higher for the tetraglucosylcalix[4]arenes **1** and **3** and their amino acid complexes, and the more rigid tetrapropoxy derivative **3** showed more selective H/D exchange reactions than the calixarene **1**. Bi- or trimodal H/D exchange distribution was observed for the tetraglucosyl derivatives indicating that these ligands exist in multiple isomeric forms in gas phase.

Introduction

The recognition of amino acids and peptides by synthetic receptors is a topic of great interest in supramolecular and bioorganic chemistry.[1] Over past years a variety of chiral hosts have been synthesised, with the purpose of obtaining enantioselective recognition of α-amino acids or their derivatives.[2] Some of these hosts are based on calixarenes.[3] Amines, amides, ureas, thioureas and guanidiums are commonly used as chiral units,[4] but with the exception of cyclodextrins,[5] only few studies have looked at the recognition of simple amino acids by carbohydrate containing ligands.[6] Even fewer studies, experimentally and theoretically, have been performed in the gas phase,[7] although such studies could provide fundamental insight in this area and guide the design of new efficient and selective chiral receptors.

Mass spectrometric investigations of noncovalent recognition became possible along with the availability of soft ionization techniques, such as electrospray ionization (ESI).[8] With sensitive ionization techniques, the relatively weak inter- and intramolecular noncovalent interactions of biomolecules and synthetic ligands can be preserved intact, and detailed information on their complexation and gas-phase properties can be obtained.[9] Besides the fast and simple screening of potential substrates with minimal sample consumption made possible by modern and sophisticated MS techniques (such as FTICR MS), different experiments can be carried out to acquire information in solvent free conditions.[10] During the past decade, hydrogen–deuterium (H/D) exchange has increasingly been used to clarify the conformational properties of biomolecules both in solution and in gas phase.[11] However, gas-phase H/D exchange has been used rarely for exploring the conformational properties of synthetic supramolecular receptors and the interactions in their complexes.[12] Gas-phase techniques can provide a useful complement to solution phase techniques and enable evaluation of the role of solvent in conformational properties of the receptors.[13] Increasingly, mass spectrometry is also being used for the chiral discrimination between enantiomers, an important but still difficult area of analysis.[14]

We have previously synthesised a series of thiourea-linked calixarene glycoclusters,[15,16] and studied their interactions with proteins[17-20] and anionic species.[19] In the latter case, evidence was obtained of a possible cooperation between the aromatic cavity of the calix[4]arene, the thiourea linker and the sugar units in anion binding. Here we report the synthesis of a conformationally mobile glucosylthioureido calix[4]arene (**1**) and the results of an extensive investigation of the gas-phase recognition of α-amino acids (Scheme 2) by a small collection of glucosylthioureido

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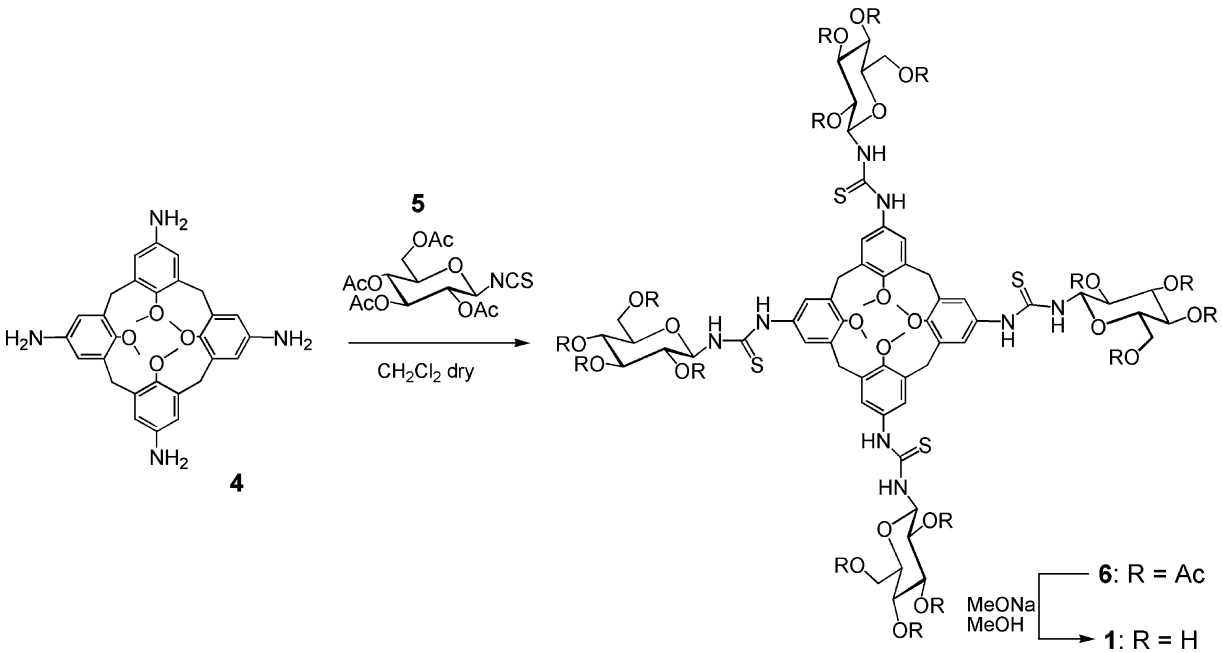
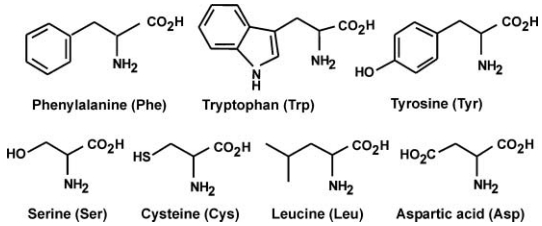
† Electronic supplementary information (ESI) available: ^{1}H NMR spectra of **6**, VT-NMR, ^{13}C NMR and HR-MS spectra of **1**, ESI-MS profile spectra, competitive complexation of amino acids in the presence of **1** and **3**, *semi-empirical* modelling of **1**, CID-MS spectra of [**2** + Tyr–H]$^{-}$ and [**3** + Tyr–2H]$^{2-}$, and *ab initio* optimized structure of **3**, **2** and complexes **2** × **Phe**, **2** × **Tyr** and **2** × **Trp**. See DOI: 10.1039/b916268b



Scheme 1 Synthesis of tetraglucosylthioureido-tetramethoxycalix[4]arene **1**.

calix[4]arenes. These are the conformationally mobile tetraglucosyl derivative **1** (Scheme 1) and the di- and tetrafunctionalized receptors **2** and **3** (Fig. 1), locked in the cone conformation. Gas-phase studies were carried out by electrospray ionization mass spectrometry (ESI-MS) in negative ion mode and the recognition studies were complemented by *ab initio* optimizations.



Scheme 2 Amino acids used in this study.

Results and discussion

Synthesis of ligands and their conformational properties in solution

The tetragluco-tetramethoxycalix[4]arene **1** was synthesised (Scheme 1) by coupling the tetra-amino-tetramethoxy-calix[4]arene **4**[21] with tetracetyl-β-D-glucopyranosyl isothiocyanate (**5**) in dry dichloromethane. After purification by flash chromatography, compound **6** was deprotected from acetyl groups to compound **1** by the Zemplen method. The *cone* compounds **2** and **3** were prepared as previously described.[19]

The glucocalix[4]arene **6** is conformationally mobile in solution. As can be deduced from the analysis of the ^{1}H (see ESI†) and ^{13}C NMR spectra, a mixture of the *cone*, *partial cone* and *1,3-alternate* conformers[22] exist in $CDCl_3$. This is due to the strong intramolecular H-bonding between NH thioureido protons of one arm and the S or O atoms of another arm. In DMSO-d_6 at 363 K, on the other hand, single sets of sharp signals are seen indicating a breaking of the intramolecular H-bonds and fast exchange between the different conformers (see ESI†).

The deacetylated tetraglucocalix[4]arene **1** is soluble in water up to 8×10^{-4} M and its NMR spectra are also strongly dependent on the type of solvent. Its ^{1}H NMR spectrum in water (Fig. 2) shows sharp peaks, excluding the presence of aggregates. The presence of a singlet at 3.99 ppm in the ^{1}H NMR spectrum and of a signal at 35.9 ppm in the ^{13}C NMR spectrum, related to the bridging methylene groups ($ArCH_2Ar$), clearly suggests a *1,3-alternate* conformation in D_2O. As was observed for other water-soluble tetramethoxycalix[4]arenes,[17] this conformation is highly preferred since it allows minimization of the contacts between the apolar aromatic nuclei of the macrocycle and the bulk water molecules. The absence of two distinct parts for this conformation, one lipophilic and one hydrophilic, prevents the aggregation previously observed for the tetrapropoxy analogue **3**, which is fixed in the *cone* structure.[19]

The situation is rather different in CD_3OD, where there is clear NMR evidence for the presence of two conformers, the *partial cone* and the *cone* in about 2 : 1 ratio, in slow exchange (see VT-NMR in ESI†). However, a conformational search carried out for **1** by *semi*-empirical methods at PM3 level (see ESI†) shows that *in vacuo* the *cone* structure is highly preferred over the *partial cone* (ΔE = 123 kJ mol^{-1}). Probably this is also the most stable conformation in the gas phase because of the more extensive intramolecular H-bonding between the saccharide units.

In CD_3OD both tetrapropoxy derivatives, **2** and **3**, are present in *cone* conformation owing to the synthetic procedure followed and to the bulkier propyl groups which prevent interconversion to the *partial cone* or *1,3-alternate* structures. However, while the ^{1}H NMR spectrum of compound **2** in CD_3OD indicates the presence of a *closed flattened cone* conformation (Fig. 1),

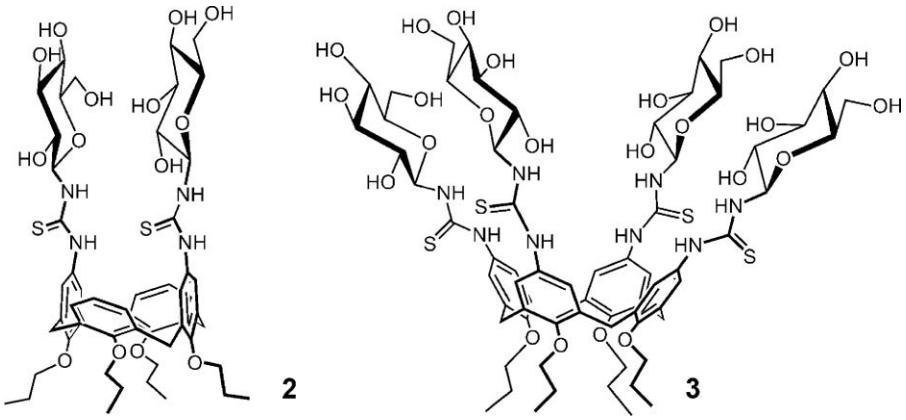


Fig. 1 The tetrapropoxy diglucosyl- (**2**) and tetraglucosyl- (**3**) thioureidocalix[4]arenes in the *closed flattened cone* and C_4 symmetric *cone* conformation, respectively.

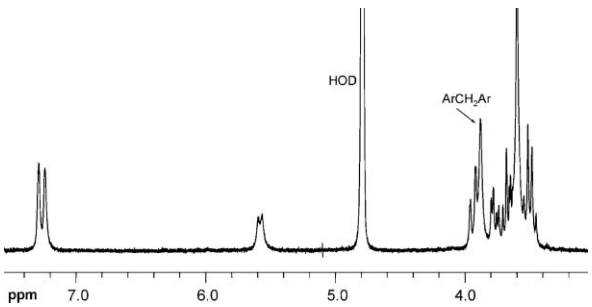


Fig. 2 ^{1}H NMR (D_2O, 300 MHz, 298 K) of **1**.

probably arising from the H-bonding interactions between the thiourea groups and sugar oxygen atoms, the ^{1}H NMR spectrum of tetraglucocalixarene **3** is consistent with a more regular C_4 symmetric *cone* structure (Fig. 1), at least on average. For the purpose of the present study, detailed VT-NMR experiments of compounds **2** and **3** in CD_3OD[19] do not add too much to the conformational features of these receptors also because the conformations in the gas phase are better described by *in vacuo* calculations (*vide infra*).

Complex formation with amino acids

According to negative ion ESI-MS spectra, the diglucocalixarene **2** formed only singly charged $[M + Guest–H]^-$ complex ions with the amino acids (Fig. 3a). The tetraglucocalixarenes **1** and **3** instead formed mainly doubly charged $[M + Guest–2H]^{2-}$ complex ions. In general, the doubly charged ions formed by **3** and especially by **1** were more abundant than the corresponding singly charged ions indicating higher stability of doubly charged ions (Fig. 3b). Profile spectra revealed that glucocalix[4]arenes form complexes with tryptophan (Trp), tyrosine (Tyr), phenylalanine (Phe), serine (Ser), leucine (Leu) and aspartic acid (Asp) (ESI†). However, in the case of Asp the complexation was observed only with glucocalix[4]arenes **1** and **2**. Mass peaks corresponding to complexes that contained cysteine (Cys) were not observed under any conditions, which most likely results from the weak hydrogen bonding donor nature of SH group.[23] Structures of amino acids studied are presented in Scheme 2.

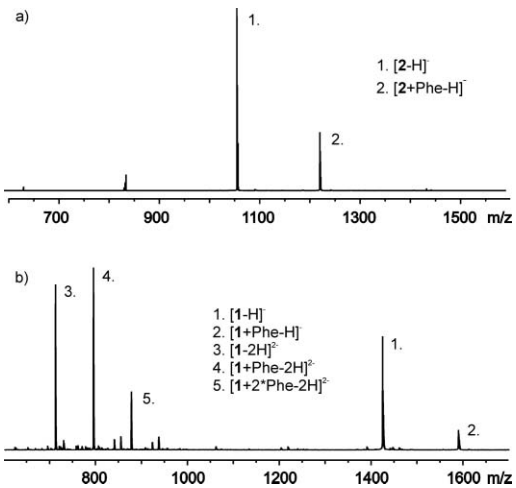


Fig. 3 Profile spectra measured from samples containing Phe in the presence of a) **2** and b) **1** (2 μM, calixarene/phenylalanine 1 : 3).

To put this selectivity order on a more quantitative basis, we evaluated the relative affinities of glucocalixarenes towards the amino acids in competition experiments[24] with amino acid pairs Trp *vs.* Tyr, Tyr *vs.* Phe, Phe *vs.* Ser and Ser *vs.* Leu. Competition between Ser and Leu was measured only with **1** and **3** owing to the low abundance of $[2+ Leu–H]^-$ ion. Within the limits of accuracy, the affinity of **2** towards amino acids followed the order Trp > Tyr > Phe > Ser (Fig. 4a). This is in line with the NMR data obtained in DMSO-d_6 solution,[19] which show a clear selectivity for guests containing aromatic nuclei (benzylphosphonate *vs.* dihydrogenphosphate and β-D-glucose-6-phosphate; benzoate *vs.* acetate).

The affinities of **3** and **1** followed the order Trp > Tyr > Phe > Leu > Ser and Trp > Tyr > Phe > Ser > Leu, respectively (see ESI†).

The comparison between the amino acid complexation reveals that side-chain H-bonding donor group containing Tyr is clearly favored over Phe, which does not have this capability. Tyr is also favored over Ser in complexation, which indicates that the affinity of glucocalixarenes towards the amino acids is influenced by the aromatic nature of the complexing amino acid. The comparison also reveals that the strength of the H-bonding donor group has

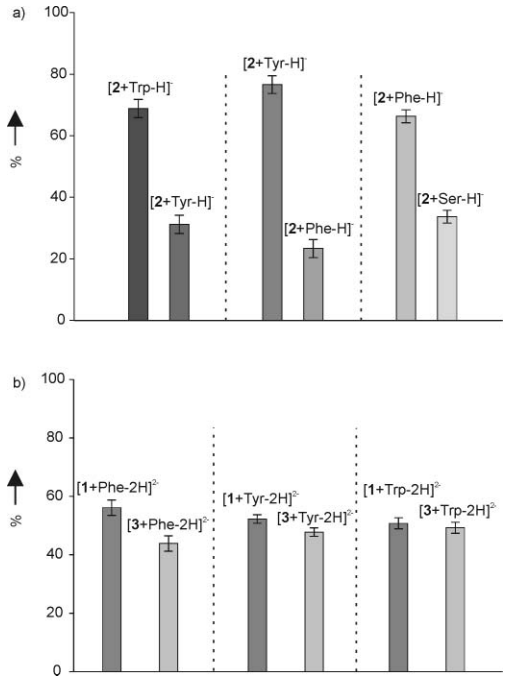


Fig. 4 Competitive complexation of a) amino acids in the presence of **2** and b) hosts **1** and **3** in the presence of amino acids (relative intensities: $\% = ([\mathbf{M} + \text{Guest}_1\text{–H}]^-/([\mathbf{M} + \text{Guest}_1\text{–H}]^- + [\mathbf{M} + \text{Guest}_2\text{–H}]^-)) \times 100$).

effect on the complexation since Ser (hydroxyl group in the side-chain) was observed to form complexes with all glucocalixarenes studied and Cys (thiol group in the side-chain) did not form any complexes at all. Altogether, these observations suggest that the overall affinity of glucocalixarenes towards the amino acids decreases with decrease in the number of H-bonding groups, the strength of H-bonding donor group and the aromatic nature (or size) of the amino acid.

Enantiomeric selectivity of glucocalixarenes towards the aromatic amino acids was studied by competition measurements between D-amino acids Phe, Tyr and Trp and their multiply deuterated L-amino acids. All glucocalixarenes favoured the L-enantiomer in complexation with Phe and Trp and D-enantiomer in complexation with Tyr (Table 1). The inversion of the enantioselectivity observed for Tyr relative to Phe and Trp is not surprising: inversion has often been reported in the literature and especially where the type of residue and number of H-bonding groups on the guest deviate noticeably.[25] Here also the inversion of enantioselectivity was observed with an amino acid (Tyr), which has a fundamentally different H-bonding ability than especially Phe, or Trp. The inversion of enantioselectivity for Tyr implies that the introduction of a polar H-bonding group onto the aromatic ring alters the H-bonding ability of amino acid and plays a major role in enantioselective complexation. Even though the enantioselectivity values obtained are moderate they can be considered highly reliable.[13] The present values ranging from 0.61 to 2.58 suggest that this type of glycosylcalixarene based receptors could have potential in applications relevant to chiral recognition and/or differentiation.

Competition experiments between the two tetraglucocalixarenes **1** and **3** with a single amino acid (Phe, Tyr or Trp) indicate that the conformationally mobile compound **1** is slightly preferred in amino acid complexation over derivative **3**, which is fixed in the *cone* conformation (Fig. 4b). This preference decreases as the steric hindrance of the lateral chain increases and is almost absent for the bulkiest amino acid (Trp).

Table 1 D/L ratios determined in competition experiments between amino acid enantiomers of Phe, Tyr and Trp

Competition	D/L[a]
1 + L-Phe(d-8) + D-Phe	0.66 ± 0.04
2 + L-Phe(d-8) + D-Phe	0.78 ± 0.16
3 + L-Phe(d-8) + D-Phe	0.61 ± 0.06
1 + L-Tyr(d-7) + D-Tyr	1.34 ± 0.11
2 + L-Tyr(d-7) + D-Tyr	2.58 ± 0.71
3 + L-Tyr(d-7) + D-Tyr	1.17 ± 0.12
1 + L-Trp(d-5) + D-Trp	0.62 ± 0.04
2 + L-Trp(d-5) + D-Trp	0.85 ± 0.05
3 + L-Trp(d-5) + D-Trp	0.70 ± 0.03

[a] Ratio from the absolute mass peak intensities (I_D/I_L).

Dissociation and kinetic stability of the complexes

In the collision induced dissociation (CID) experiments, the ion of interest is isolated, excited and allowed to collide with inert collision gas. This leads to competitive dissociation of the ions and fragment ions are observed in resulting spectra. With CID it is possible to get valuable gas phase information about structures and the relative kinetic stabilities of complexes.

The deprotonated complexes of **2** dissociate and produce [**2**-H]$^-$ ion and a neutral amino acid in CID experiments. As the energy is increased the [**2**-H]$^-$ ion dissociates into its fragments (see ESI†). The doubly deprotonated complexes of **1** and **3** dissociate and produce deprotonated amino acids and doubly deprotonated [M–2H]$^{2-}$ ion as CID fragments (ESI†). Interestingly, [M–H]$^-$ ions are not observed as fragments even though the complexes dissociate leaving one of the charges to amino acids. Probably this results from the short lifetime and instability of the [M–H]$^-$ ions formed in the gas phase during CID.

The kinetic stabilities of the complexes were compared by carrying out CID experiments as a function of energy, plotting the dissociation curves and calculating $E^{0.5}_{com}$ values (Table 2). The deprotonated complexes of **2** dissociate with low energy and there are no significant differences between the $E^{0.5}_{com}$ values. The same is observed with the deprotonated complexes of **1** and **3**.

Table 2 $E^{0.5}_{com}$ values of the complexes of glucocalixarenes with amino acids

Ion	$E^{0.5}_{com}$/eV	R^{2a}
[**1** + Phe–2H]$^{2-}$	4.679	0.995
[**1** + Trp–2H]$^{2-}$	3.694	0.985
[**1** + Tyr–2H]$^{2-}$	3.318	0.981
[**2** + Tyr–H]$^-$	1.152	0.970
[**2** + Trp–H]$^-$	1.132	0.938
[**2** + Ser–H]$^-$	0.930	0.977
[**2** + Phe–H]$^-$	0.877	0.989
[**3** + Phe–2H]$^{2-}$	4.076	0.996
[**3** + Tyr–2H]$^{2-}$	3.921	0.983
[**3** + Trp–2H]$^{2-}$	3.576	0.984
[**3** + Leu–2H]$^{2-}$	2.725	0.991

[a] Correlation for curve fitting.

Although the competition studies showed clear differences in the affinities of the glucocalixarenes towards amino acids, the CID studies revealed only minor to moderate differences in the kinetic stabilities (Table 2). Comparison of the $E_{com}^{0.5}$ values of the doubly charged complexes of **1** and **3** revealed mostly relatively minor differences between the $E_{com}^{0.5}$ values of complexes formed with aromatic amino acids; exceptionally, in the case of **1**, the kinetic stability was noticeably higher for the Phe complex than for complexes with the other amino acids. More significant differences were observed between the complexes formed with aromatic and aliphatic amino acids (Leu) indicating higher kinetic stability of the complexes with aromatic amino acids as compared to complexes with aliphatic amino acids (Fig. 5 and Table 2).

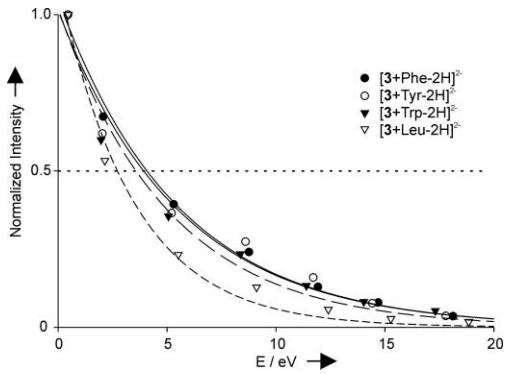


Fig. 5 Dissociation curves for doubly charged complexes of **3**.

Computational calculations

The optimizations on *ab initio* level were performed only for neutral **3**, for neutral **2** and its complexes with L-Tyr, L-Phe and L-Trp. Earlier it has been stated that amino acids exist mainly in non-zwitterionic form in the gas phase and therefore the complexes with non-zwitterionic amino acids were calculated.[26]

The optimization of neutral **2** resulted in a lowest energy structure in which the two glycosyl substituents stand almost upright above the cavity and are in close contact with each other (Fig. 6a). Overall, there are six possible intramolecular H-bonds and one of them is located between the facing glucose units. This conformation is compact and the space between the substituents is so small that it is virtually impossible for any amino acid to locate between the glycosyl groups or in the cavity. This means that major rearrangement of the calixarene structure is likely to occur during complex formation. The structure of **3** is also fairly compact, and seven of the total fourteen intramolecular H-bonds are formed between glucose units in proximal or distal position (ESI†). This structure is consistent with that obtained by *semi*-empirical methods, which was used as an initial structure (ESI†). Although calixarenes **2** and **3** both have significant intramolecular interactions between the substituents, only **2** appears to adopt a *closed flattened cone* conformation. The same was indicated in previous NMR experiments.[19]

In complexation with amino acids, calixarene **2** adopts a much wider conformation (Fig. 6b–d). In the Phe complex the amino acid has two H-bonds with one of the glycosyl substituents (Fig. 6b). In the complex of **2** with Tyr, the amino acid is able to form a total of three H-bonds with the glucose substituents due

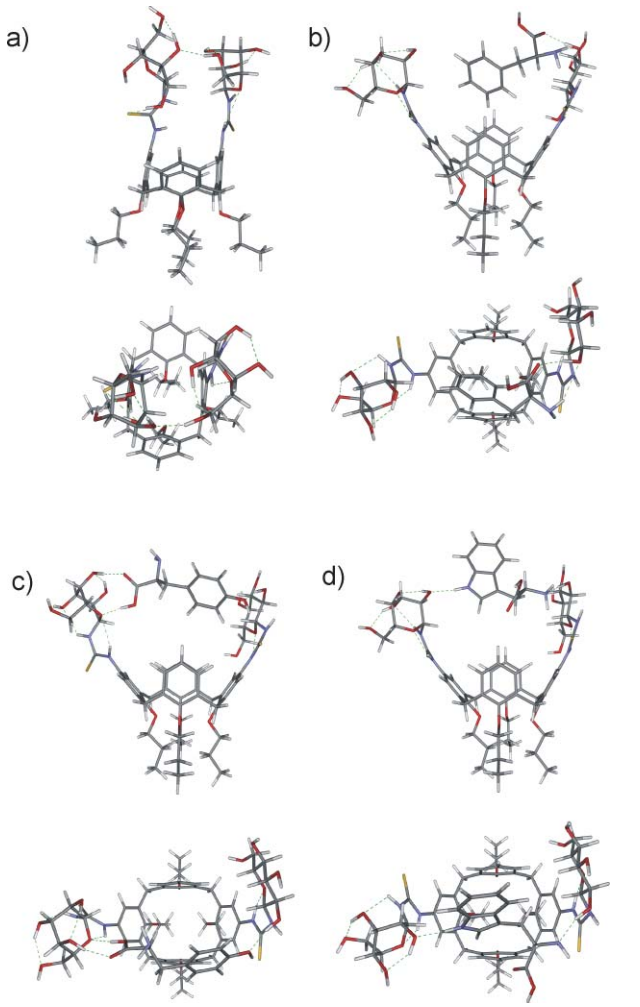


Fig. 6 Optimized structures (density functional BP86) for a) **2**, b) **2** with Phe, c) **2** with Tyr and d) **2** with Trp (lateral and apical views, possible H-bonds marked with dotted lines).

to the phenol hydroxy group. The position of Trp in the complex is similar to that of Phe and the number of hydrogen bonding is the same even if the nitrogen atom of the indole ring allows the second H-bond and the benzene nucleus is now pointing out with respect to the calixarene cavity. Interestingly, both the phenol hydroxy group of Tyr and the indole NH of Trp are H-bonded with a sugar unit affording one of the three contact points necessary for the chiral recognition.

According to the interaction energies, the stability of the complexes increases in the order [**2** + Phe] ($\Delta E = -59.95$ kJ mol^{-1}, two intermolecular H-bonds with one glycosyl substituent) < [**2** + Trp] ($\Delta E = -88.37$ kJ mol^{-1}, two intermolecular H-bonds with two glycosyl substituents) < [**2** + Tyr] ($\Delta E = -132.8$ kJ mol^{-1}, three intermolecular H-bonds with two glycosyl substituents). It seems therefore that both the number of formed H-bonds and the involvement of both glucosyl groups in the interaction are important for the formation of a stable complex. The competition experiments showed a stronger complexation of Trp as compared to Tyr. According to calculations, however, L-Tyr complex seems more stable than the L-Trp complex. It has to be notified that interactions with solvent molecules play a role in mass spectrometric competition experiments in a form of ESI† response

factor, whereas the environment in calculations is solvent-free. It is therefore likely that there are some minor inconsistencies in results obtained by the theoretical and experimental methods. However, both theoretical and experimental results clearly evidenced the preference in complexation for aromatic amino acids having a polar group capable of additional H-bonding involving the second glucose unit.

In each of the modelled complexes, despite of the initial structure used in the calculations, the amino acid moved away from the calixarene cavity towards the glycosyl substituents where H-bonding takes place. Only in the structure of the Phe complex of **2** (Fig. 6b) there is a possibility of a weak π–π interaction between the aromatic rings of Phe and the calixarene skeleton. However, the complexes of ligand **2** with these aromatic amino acids seem mainly stabilized by H-bonds. Also carbohydrate-arene interactions (CH-π) which were often indicated to take place between the aromatic moieties and CH-hydrogens of a carbohydrate, especially in water solution,[27] were not pointed out by our calculations. In any case, we cannot exclude that these type of interactions play an important role especially in water solution and in the case of tetrasubstituted calixarenes, where the two additional glycosyl units should stand close to the aromatic residues of the amino acid.

MS conformational studies

H/D exchange reactions were performed to obtain information about the conformations of glucocalixarenes and their amino acid complexes in the gas phase. According to current knowledge, steric factors, proton affinities and hydrogen bonding of the labile hydrogens have a considerable effect on the rates of the gas-phase H/D exchange.[29] Moreover, gas-phase H/D exchange reactions are more selective than the corresponding reactions in solution, which means that the gas-phase H/D exchange can be exploited to study conformational as well as intrinsic properties of ions.

Three hydrogens of the [**2**–H]$^-$ ion were observed to be replaced by deuterium during the longest reaction delay of 540 s. The slow reaction rate could result from the close proximity and strong hydrogen bonds between the two glucosylthioureido groups, which prevent their co-operation in H/D exchange and also decrease the exchange rate. Moreover, due to the instability (see CID results) of the [**2**–H]$^-$ ion, the collisions with the reagent might cause dissociation of the ion rather than exchange. For complexes of **2**, H/D exchanges were not observed but dissociation to [**2**–H]$^-$ ions occurred. The singly charged complexes of **1** and **3** showed similar behaviour to that of **2**: dissociation of complexes to [**1**–H]$^-$ and [**3**–H]$^-$ ions. In contrast, the deprotonated **1** and **3** ions ([M – H]$^-$) exchanged 22 out of 23 labile hydrogens, and interesting bimodal exchange distribution was observed. In doubly deprotonated **1** and **3** and their complexes, all labile hydrogens were replaced by deuterium, and bi- or trimodal exchange distribution was observed as shown for compound **3** in Fig. 7. The trimodal exchange distribution was observed only for [**3** + Trp–2H]$^{2-}$ and [**3** + Tyr–2H]$^{2-}$ complex ions.

The bimodal (or trimodal) exchange distribution implies that, in the gas phase, there are at least two (or three) populations of ions that react with MeOD with clearly different rate. Multimodal distributions in H/D exchange spectra have usually been associated with the existence of multiple isomeric forms.[29]

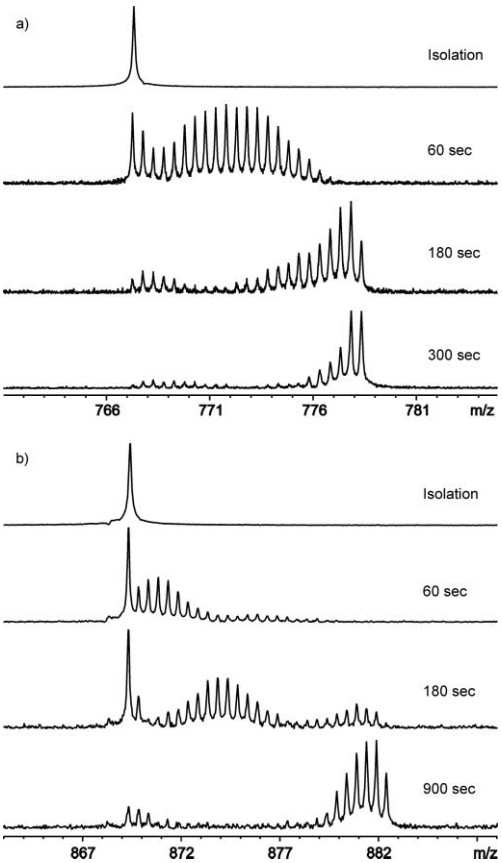


Fig. 7 H/D exchange spectra of a) [**3**–2H]$^{2-}$ and b) [**3** + Trp–2H]$^{2-}$.

It must be emphasized, however, that the bimodal distribution does not limit the number of possible isomeric structures to two, since two or more structures may have closely similar H/D exchange rates. In addition, whether the behaviour observed in H/D exchange reactions originate from clearly distinct structures or from structures able to interconvert to each other cannot be determined on the basis of the recorded spectra alone and activated H/D exchange experiments are required.[30]

Activated H/D exchange experiments were performed for ions [**1**–2H]$^{2-}$, [**3**–2H]$^{2-}$ and [**3** + Trp–2H]$^{2-}$ at reaction delays in which the bimodal (or trimodal) distribution was clearly visible. However, the activation was not observed to affect the H/D exchange distributions of the ions, and we concluded that the energy barrier for interconversion of these structures was not exceeded.

Since the glucocalixarenes have relatively flexible substituents, it is likely that different isomeric forms are represented in the isolated ion population. The observation of a bimodal distribution is reasonable therefore. Nevertheless, there are at least two aspects of the results deserving closer attention. First of all, there was a dramatic difference in the reactivities of **2** and the tetraglucocalixarenes, and this difference was not associated with the charge state of the ions since the [M–H]$^-$ ions from **1** and **3** exchanged an almost maximum number of labile hydrogens within a reasonable reaction time. This difference in reactivity can be explained in terms of different reaction mechanisms or by differences in the accessibility of labile hydrogens for reaction (*e.g.*, conformation or structure of the ion). Indeed, we have shown that **2** appears in a *closed flattened*

cone structure where the volume between the glucose substituents is so small that efficient H/D exchange is improbable. Without concluding on the initiation mechanism of the H/D exchange, we note that the presence of glucose units on each of the four aromatic nuclei of the tetraglucosylthioureidocalix[4]arenes **1** and **3** ensures a seam of hydrogen bonds between adjacent sugars, which help to maintain an open C_4 symmetric *cone* conformation and allow easier migration of the deuterium from substituent to substituent than in the disubstituted compound.

Secondly, the number of possible isomeric forms increases with the number of glucosylthioureido groups, and simultaneously increases the possibility for a conformation with a reasonable reaction rate in H/D exchange. Interestingly, a distinct trimodal distribution is observed only for ions $[\mathbf{3} + \text{Trp}–2\text{H}]^{2-}$ and $[\mathbf{3} + \text{Tyr}–2\text{H}]^{2-}$, most likely resulting from at least two distinct positions of Trp and Tyr in the rigid ligand **3**. Both Tyr and Trp are able to form additional H-bonds through their side chains, which increases their capability to interact with **3** and, partly hindering the glucosyl units, decreases the deuterium incorporation rates.

The exchange rates of the ions were compared by their deuterium incorporation curves, since a large number of exchanges makes calculation of the site-specific rate constants impractical.[31] In the case of doubly charged ions of **1**, there were only minor differences in the exchange rates and the deuterium incorporation percentages (Fig. 8a). Maximum deuterium incorporations of doubly deprotonated **1** and the complexes of **1** settled near 80%.

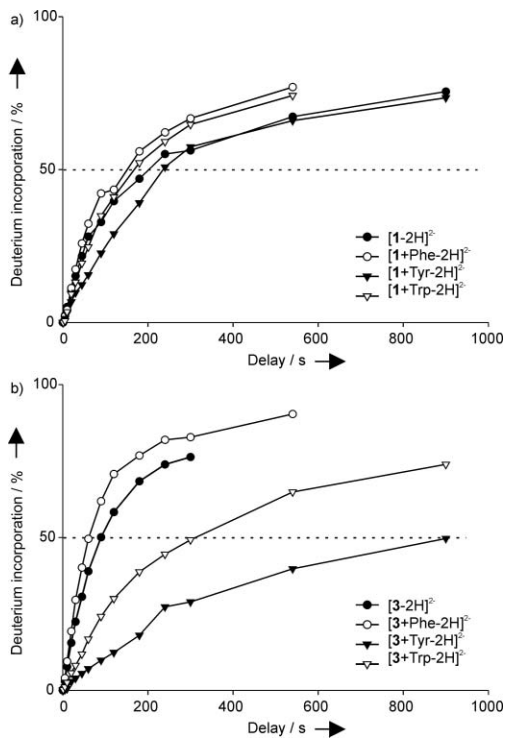


Fig. 8 Deuterium incorporation curves of a) **1** and b) **3**.

In contrast to **1**, there were noticeable differences in the deuterium incorporation curves of doubly deprotonated **3** and its complexes (Fig. 8b). Incorporation rates were lowest for the Tyr complex and highest for the Phe complex, but within the reaction delay of 900 s the maximum deuterium incorporation in no case exceeded 90%. Besides the deuterium distribution, the structure of the amino acid has a clear impact on the exchange rate, and the complexes formed with amino acids capable of forming additional H-bond through their side chains exhibited the slowest reaction. The difference in the H/D exchange behaviour of the two tetraglucocalixarenes is worth noticing. Both in deprotonated and complexed forms, the more flexible calixarene structure **1** exhibits clearly less selective H/D exchange reactions than the more rigid structure **3**. It seems that in complexes of **3** the rate of the reaction is inversely proportional to the number of interactions within the complex and the slowest rates are observed for the same complexes which showed trimodal H/D exchange distribution.

Conclusions

Mass spectrometric data for three glucocalixarenes showed that an increase in the number of substituents from two to four has two major impacts on their properties. First, the greater the number of substituents the greater is the number of possible isomeric forms. According to H/D exchange data, the tetraglucocalixarenes **1** and **3** exist in several non convertible isomeric forms with divergent H/D exchange rates. In calixarene **3**, where the bulky propyl groups prevent the interconversion from *cone* to *partial cone* or *1,3-alternate* conformation, it is reasonable to assume that the populations of ions with different H/D exchange rates do not originate from different conformations of the calixarene but from different orientations of the glycosyl substituents. Solution-phase studies showed that the solvent has a clear impact on the conformations of the glycosylcalixarenes. However, gas-phase studies indicated that, since differences in H/D exchange behaviour between the tetrasubstituted calixarenes are small, even the mobile **1** (which could also adopt the *partial cone* conformation) exists predominantly in *cone* conformation in gas phase as also supported by *semi*-empirical calculations. The residual mobility of **1** may nevertheless enable fine-tuning of its conformation, which gives rise to structurally similar complexes regardless of the amino acid as observed in the gas phase by the decreased selectivity of the H/D exchange reactions. On the other hand, the complex formation with **3** results (especially in the case of Tyr and Trp) in rigid isomeric structures, which show a trimodal H/D exchange distribution and a decreased rate of deuterium incorporation due to a limited accessibility of MeOD to the initiation sites of the H/D exchange reaction.

All three 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. ESI-MS enantiomeric-labelling studies used to assess the enantioselectivity for the recognition of D- *vs.* L-amino acids showed selectivity ranging from 0.61 to 2.58.

Experimental

Synthesis

Moisture-sensitive reactions were carried out under a nitrogen atmosphere. Dry solvents were prepared according to standard procedures and stored over molecular sieves. Melting points were determined in an electrothermal apparatus in capillaries sealed under nitrogen. ^{1}H and ^{13}C NMR spectra (300 and 75 MHz, respectively) were obtained on a Bruker AV300 spectrometer; partially deuterated solvents were used as internal standards.

Mass spectra for the characterization of compounds **1** and **6** were recorded in ESI mode on a single quadrupole Micromass ZMD instrument (capillary voltage 3 KV, cone voltage 30-160 V, extractor voltage 3 V, source block temperature 80 °C, desolvation temperature 150 °C, cone and desolvation gas (N_2) flow-rates 1.6 and 8 l min^{-1}, respectively). TLC was performed on silica gel Merck 60 F_{254}, and flash chromatography using 32–63 μm, 60 Å Merck silica gel. 5,17-Bis(β-D-glucopyranosylthioureido)-25,26,27,28-tetrapropoxycalix[4]arene **2**,[19] 5,11,17,23-tetrakis-(β-D-glucopyranosylthioureido)-25,26,27,28-tetrapropoxycalix-[4]arene **3**[19] and 5,11,17,23-tetramino-25,26,27,28-tetramethoxycalix[4]arene **4**[21] were prepared according to the literature.

5,11,17,23-Tetrakis[(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-thioureido]-25,26,27,28-tetramethoxycalix[4]arene. (6). A solution of 5,11,17,23-tetramino-25,26,27,28-tetramethoxycalix[4]arene **4** (75 mg, 0.139 mmol) and tetracetyl-β-D-glucosylisothiocyanate **5** (432 mg, 1.111 mmol) in 10 mL of dry CH_2Cl_2 was stirred at 50 °C in a sealed tube under nitrogen. After 24 h the solvent was removed under reduced pressure and the residue was purified by flash chromatography (SiO_2: from hexane–AcOEt 6 : 4 to hexane–AcOEt–MeOH 6 : 4 : 1). Yield: 52%; M.p.: 174–177 °C. ^{1}H NMR (300 MHz, DMSO-d_6, 363 K): δ 9.55 (bs, 4H, ArN*H*), 7.63 (bs, 4H, CHN*H*CS), 7.04 (bs, 8H, Ar), 5.88 (bs, 4H, H1), 5.32 (t, 4H, J = 9.6 Hz, H3), 5.01–4.91 (m, 8H, H4, H2), 4.18 (dd, 4H, J = 12.6, 5.1 Hz, H6a), 4.10–3.90 (m, 8H, H5, H6), 3.90–3.35 (bs, 20H, ArCH_2Ar, OCH_3), 2.09, 2.01, 1.96 (3 s, 48H, CH_3CO); ^{13}C NMR (75 MHz, $CDCl_3$): δ 181.9, 181.3 (CS), 170.5, 169.7, 169.4 (CO), 157.5, 156.4, 137.2, 136.4, 134.9, 133.0, 129.8, 128.5, 127.6, 126.1, 125.2 (Ar), 83.0 (C1), 73.4 (C5), 72.6 (C3), 70.5 (C2), 68.1 (C4), 61.6 (C6), 61.3, 61.2, 60.3, 59.7 (OCH_3), 35.5, 34.9, 30.1, 29.9 (ArCH_2Ar), 20.9, 20.7, 20.5 (CO*C*H_3); ESI-MS: m/z 2120.9 [100%, $(M + Na)^+$], m/z 1071.8 [80%, $(M + 2Na)^{2+}$].

5,11,17,23-Tetrakis[(β-D-glucopyranosyl)thioureido]-25,26,27,28-tetramethoxycalix[4]arene (1). The protected glucosylthioureido calixarene **6** was suspended in dry CH_3OH, and the pH was adjusted to 8–9 by addition of a solution of CH_3ONa in CH_3OH. The reaction was stirred at room temperature for 45 min and quenched by the addition of Amberlite IR120 (H+) resin until neutral pH. The resin was filtered off, and the solvent was removed under reduced pressure to obtain deprotected glucocalixarene **1**. The product was purified by trituration in ethanol. Yield: 85%; M.p.= 70 °C (dec.); ν_{max}/cm^{-1} (KBr) 3415, 1218; ^{1}H NMR (300 MHz, D_2O): δ 7.29 and 7.24 (2 s, 4H each, Ar), 5.57 (d, 4H, J = 9 Hz, H1), 3.94 (d, 4H, J = 12.3 Hz, H6a), 3.88 (bs, 8H, ArCH_2Ar) 3.77 (dd, 4H, J = 12.3, 5.1 Hz, H6b), 3.68 (t, 4H, J = 7.8 Hz, H3), 3.67-3.60 (m, 4H, H5), 3.60 (s, 12H, OCH_3), 3.55-3.45 (m, 8H, H2, H4); ^{13}C NMR (75 MHz, D_2O): δ 182.8 (CS), 136.4, 128.3 (Ar), 84.6 (C1), 77.8 (C5), 76.8 (C3), 72.3 (C2), 69.8 (C4), 60.9 (OCH_3), 58.8 (C6), 35.9 (Ar*C*H_2Ar); ESI-MS: m/z 735.9 [100%, $(M + 2Na)^{2+}$], 1447.7 [70%, $(M + Na)^+$]. Exact mass: $[M + H]^+$, m/z 1425.425210 (ESI-FTICR MS), mass accuracy 0.77 ppm from the theoretical mass of $C_{60}H_{81}N_8O_{24}S_4$.

ESI–FTICR MS

The calixarenes were dissolved in methanol and their final concentration in samples was 2 μM. Host/guest ratios of 1 : 3 were used, and the final concentration of the guest molecules in the samples was 6 μM.

All measurements were performed in pure methanol using negative polarisation on a Bruker Daltonics (Billerica, MA, USA) APEX Qe Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer equipped with 4.7 T superconducting magnet, Infinity® ICR cell, AP2 electrospray ion source and pre-cell quadrupole interface. The required 1 × $10^{-9/-10}$ torr vacuum was obtained by using rotary vacuum and turbomolecular pumps supplied by Edwards (West Sussex, UK). The sample was introduced to the ion source through a capillary with a 70° off–axis sprayer at the end. The flow rate of 1.5 μl min^{-1} was obtained by using a Cole–Parmer (Cole–Parmer Instrument Company, IL, USA) syringe pump and Hamilton (NV, USA) syringes. Nitrogen gas was used for nebulisation (0.2 psi) and for drying (5.0 Ls^{-1}) and the ion source was heated to either 180 or 240 °C. Ion source voltages varied from 3.2 to 3.5 kV to end plate and from 3.7 to 4.0 kV to capillary. Capillary exit voltages varied from –250 to –375 V depending on the sample. Because of the FT-ICR mass discrimination, for wide-range MS scans different parameters had to be applied for reliable detection of the singly and doubly charged ions in different m/z ranges. In most cases a total of 16 scans were collected and the whole operation was performed using Apollo II control version 1.3 and XMASS 7.0.8 software, which was also used for data processing.

The competition experiments were performed bilaterally, by including only two guests in a sample, in order to avoid nonspecific complexation and to keep the resulting spectra as unambiguous as possible. Competition experiments were performed with a host/$guest_1$/$guest_2$ ratio of 1 : 3 : 3. In the case of **1** and **3**, which formed a variety of singly and doubly charged complexes, all observed complexes were included in the calculations of the relative abundance. Competitions between amino acid enantiomers were performed using commercially available multiply deuterated L-amino acids, and they included 5, 7 and 8 deuterium atoms for L-tryptophan, L-tyrosine and L-phenylalanine, respectively. Each competition experiment was carried out on five parallel samples and each sample was measured five times. Variance was calculated from the standard deviation of the five samples (s_1) and from the standard deviation of the most deviated series of five measurements (s_2) with the equation $s^2 = s_1^2 + s_2^2$.[32]

In the energy-resolved CID experiments, the precursor ions were isolated in the ICR cell by using correlated harmonic excitation fields. After isolation, a short pumping delay (3.0 s) was used. Isolated ions were excited by RF excitation and allowed to collide with argon gas, which was leaked to the ICR cell through a pulsed valve. Normalised intensities were used in calculation of $E_{0.5}^{com}$ values ($[Complex]_{E=X}/[Complex]_{E=0}$). The $E_{0.5}^{com}$ values represent the activation energy required for dissociation of the complex to its half-intensity.

In H/D exchange ion-molecule reactions with polyfunctional compounds, the proton affinity differences between the reagent and the analyte should be in close proximity.[28] Deuterated methanol (MeOD) was therefore selected as a reagent. H/D exchange reactions were performed to singly and doubly deprotonated glucocalixarenes and their complexes formed with aromatic amino acids. Deuterated methanol (MeOD) was introduced to the ICR cell from a separate volume through an adjustable needle valve. MeOD gas flow, from volume to cell, was set to keep the cell

pressure at elevated state of 1×10^{-7} Torr. Isolation was performed just as for the CID experiments except that single frequency excitation shots were used to obtain monoisotopic isolation of the precursor ion. Isolated ions were allowed to react with MeOD by ranging the pumping delay times from 0.001 to 900 s. Sixteen scans were collected when reaction delays were 60 s or less, eight scans when reaction delays were longer than 60 s and two scans when reaction delays were 900 s. The variation in the number of scans did not influence the information gathered from the resulting spectra. In activated H/D exchange reactions, the ions were activated by an on-resonance pulse near their threshold energies prior to H/D exchange.

Computational details

The complexation of **2** with different (L-) amino acids was investigated by using the BP86 functional. The structures of **2** and **3** were optimized starting from initial structures obtained by *semi*-empirical molecular mechanics (PM3 level). Additionally, an initial structure, which was stepwisely constructed from optimized calixarene skeleton, optimized lower rim and optimized glycosyl substituents was used. The amino acid complexes of **2** were optimized starting from different orientations of amino acids, including an orientation in which the aromatic part is directed towards the cavity. These did not, however, result in different complex structures. Neutral non-zwitterionic amino acids were used in calculations since amino acids in this form have been reported to mainly exist in gas phase.[26] Unfortunately, due to increased resource requirements (when the number of flexible glycosyl substituents increases from two to four) it was impractical to optimize amino acid complexes of **1** and **3** by using an adequate level of theory. The Karlsruhe split-valence basis set with polarization functions (SVP)[33] was applied with the Density functional (DFT) method. Geometry optimizations and energy calculations were performed with the TURBOMOLE 5.9 Program Package[34] using the efficient resolution of identity (RI) technique. The interaction energies for the complexes were obtained by using BP86/SVP level of theory. As has been noted,[34] the DFT and MP2 theories describe the interactions involved in the complexes in a slightly different way, and here MP2 theory would have better described the noncovalent interactions involved in the complexes.

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