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ORIGINAL ARTICLE

Mass spectroscopic investigation of bis-1,3-urea calix[4]arenes and their ability to complex *N*-protected α-amino acids

Sean P. Bew · Adam W. J. Barter · Sunil V. Sharma

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Abstract We report the ability of urea's appended onto the upper-rim of conformationally locked '*cone*' calix[4]arenes to show a preference for binding specific *N*-protected α-amino acids. Superior complexation (as judged by mass spectroscopy) between *N*-protected α-amino results and bis-1,3-*N*-benzylureas calix[4]arenes was observed when methylene bridges were present between the calix[4]arene 'host' and the urea motif. Interestingly we also demonstrate that subjecting mixtures of structurally diverse *N*-Fmoc-α-amino acids to a single bis-1,3-*N*-benzylurea derived calix[4]arene allows, in some cases, the calix[4]arene 'host' to selectively 'pick out' and complex a specific *N*-Fmoc amino acid from the mixture.

Keywords bis-1,3-Urea calix[4]arenes · Amino acids · Hydrogen bonding · Mass spectroscopy · Anion binding · Carboxylic acids

Introduction to anion receptors

In 1968 Simmons and Park [1, 2] reported the first anion receptor, i.e. **1** for the chloride anion. It was configured on a polyammonium cryptand that employed electrostatic and hydrogen bonding interactions to 'capture' the chloride anion within its cavity (Scheme 1).

Developing the idea further Lehn et al. reported in 1982 a polyammonium species that was capable of binding carboxylic acid anions. Synthesizing and employing species based on **3** (Scheme 2) Lehn et al. was able to show that these macrocycles encapsulated and bound (with varying strengths) dicarboxylic acids with different lengths of 'spacer' such as adipate, glutarate, oxalate, succinate, malonate and pimelate as well as *N*-acetyl-(L)-aspartate, *N*-acetyl-(L)-glutamate and *N*-acetyl-(L)-glutamyl anions [3].

Further work by Lehn et al. resulted in the synthesis of anion receptors based on guanidinium species **5** and quaternized nitrogen atoms that proved to be highly effective receptors for carboxylate or phosphate anions (Scheme 2) [4].

Beer et al. has pioneered the synthesis and applications of metallocene based calix[4]arenes as anion receptors [5]. Thus calix[4]arenes adorned with two, i.e. **7** or one, i.e. **8** cobaltocenium amide derivative (Fig. 1) are able to bind in the case of **7** dicarboxylate anions e.g. adipate and for **8** an acetate anion highly effectively via electrostatic and hydrogen bonding interactions.

Atwood et al. synthesized a series of electron-poor calix[4]arene cavities that have the capacity to bind anionic guests [6, 7]. Thus attaching cationic metal centers (**9**, Fig. 1) to the outer face of a calix[4]arene allowed size- and shape-selective complexation of anions such as tetrafluoroborate, triflate or hexafluorophosphate. It was noted that the tetrafluoroborate anion was held 'deep' within the cavity of **9**.

In addition to electrochemical methods for determining if a binding event has taken place (cf. [8]), fluorescence emission spectroscopy has also been utilized using ruthenium complexes bound to the lower rim of a calix[4]quinone, i.e. **10** (Fig. 2). Anion binding receptors based on **10** display selectivity for acetate over chloride and dihydrogen phosphate, with a 500% increase in emission for acetate

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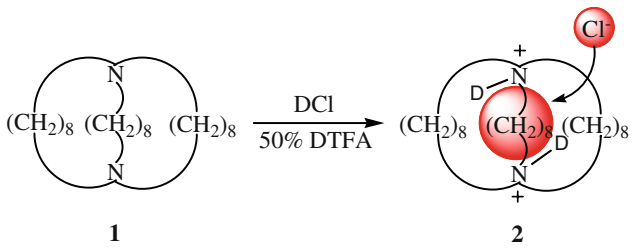
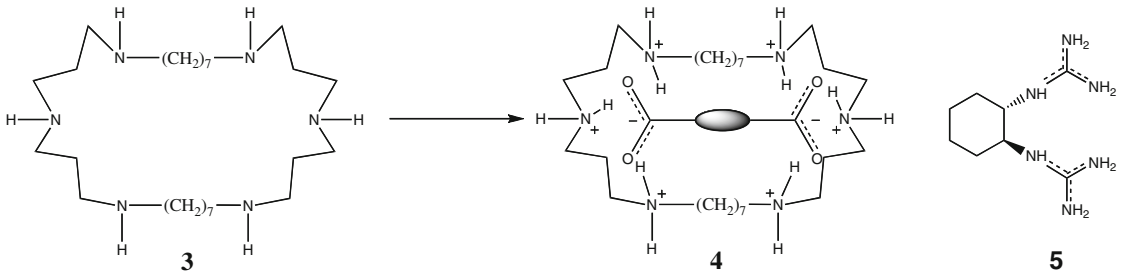
S. P. Bew (✉) · A. W. J. Barter · S. V. Sharma
School of Chemistry, University of East Anglia,
Norwich NR4 7TJ, UK
e-mail: s.bew@uea.ac.uk
URL: https://intranet.uea.ac.uk:443/cap/people/faculty/spb



Scheme 1 Encapsulation of DCl within **2**

and a 60% increase in emission for chloride binding. The increased emission was attributed to the anionic complex overcoming intramolecular quenching of the luminescence.

In 1993 Reinhoudt and co-workers reported the synthesis and application of an upper-rim derived tetrasulfonamide calix[4]arene, i.e. **11** (Fig. 2) that displayed selective binding for a tetrahedral sulfate anion, i.e. 10^2 over and above a dihydrogen phosphate, chloride, nitrate or perchlorate anion [9]. Interestingly the authors described



Scheme 2 Encapsulation of dicarboxylate anion in **4**

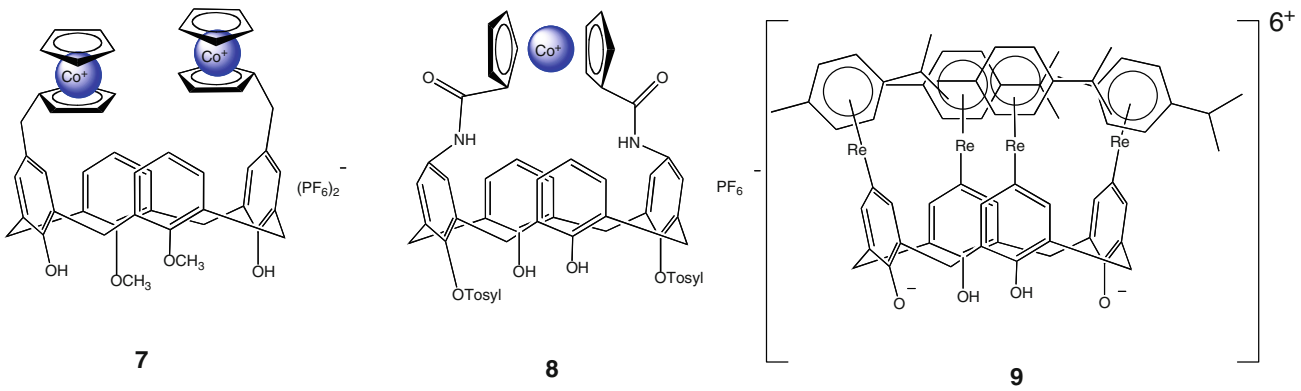


Fig. 1 Metallocene derived calix[4]arenes for anion recognition

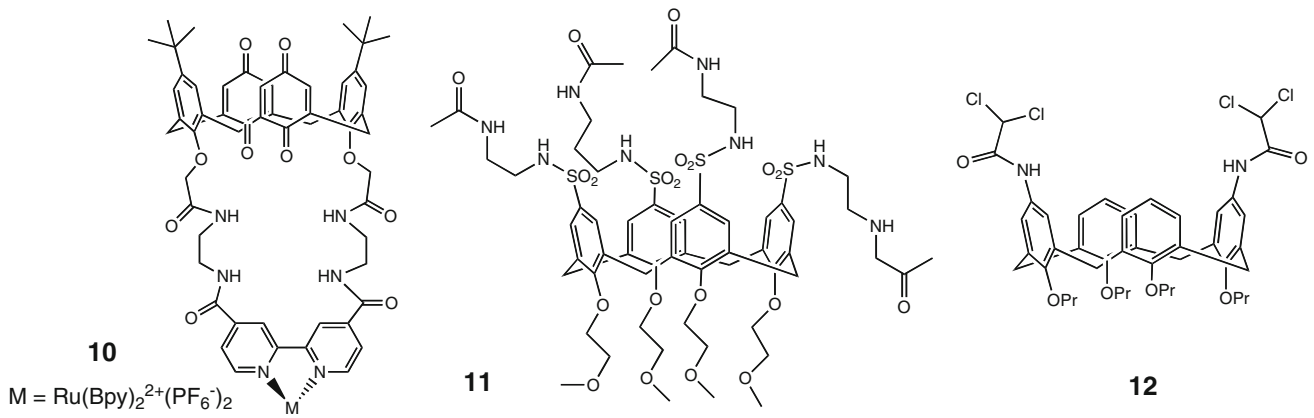


Fig. 2 Anion binding calix[4]arenes

how using FAB mass spectroscopy analysis on the solid complexes they were able to identify the signals corresponding to the tetra-sulfonamide calix[4]arene complex with the sulfate anion bound.

Loeb and Cameron have reported the synthesis of a 1,3-bis amide derived calix[4]arene **12** (equipped with electron-withdrawing groups that enhanced the acidity of the amide proton, Fig. 2) and its ability to be an excellent 1:1 binder of carboxylate anions such as acetate and benzoate (K_{ass} 5160) [10].

A number of calix[4]arene derived peptide based anion sensing systems have been developed. Cyclic peptide derived calix[4]arene **13** (Fig. 3) displayed a strong binding potential for 4-nitrophenyl phosphate (K_{ass} 3,900 M^{-1}) [11]. Whilst the flexible peptide derived calix[4]arene **14** displayed weak binding for anions, subsequent synthesis of the constrained cyclic peptide derived calix[4]arene **15** afforded much higher binding affinities for anions such as acetate or benzoate. Thus the K_{ass} values for the binding of benzoate using **14** and **15** were 680 M^{-1} and 44,000 M^{-1}, respectively. With the selectivity displayed by **15** attributed to additional π–π stacking interactions with the aromatic capping moiety. Of particular interest to the research reported here (*vida infra*) Ungaro et al. was the first to report the use of a qualitative, competitive pre-screening technique for anion binding that employed mass spectroscopy [12–14].

The synthesis of upper-rim or lower-rim appended urea or thiourea derived calix[4]arenes and their application in anion sensing has been extensive. Early work focused on appending substituents to the lower rim, with calix[4]arene **16** showing selectivity for chloride over bromide and iodide (Fig. 4) [15]. The use of FAB mass spectroscopy and ^{1}H-NMR was critical to determining the effectiveness of the binding event and the stoichiometry of the host–guest complex, with bis-1,3-*N*-substituted urea calix[4]-arenes exhibiting higher binding and enhanced selectivity over tetra-*N*-substituted urea calix[4]arenes. The cyclic amide derived calix[4]arene **17** (Fig. 4) proved to be an effective receptor for binding carboxylates, with **17** showing increased selectivity for acetate over phosphate, sulfate or chloride anions [16]. The synthesis of hybrid calix[4]arene **18** equipped with two thiourea or urea motifs was reported by Yang et al. they demonstrated that these systems had significant potential and application in binding dicarboxylate anions such as adipate (K_{ass} 4,640 M^{-1}) and

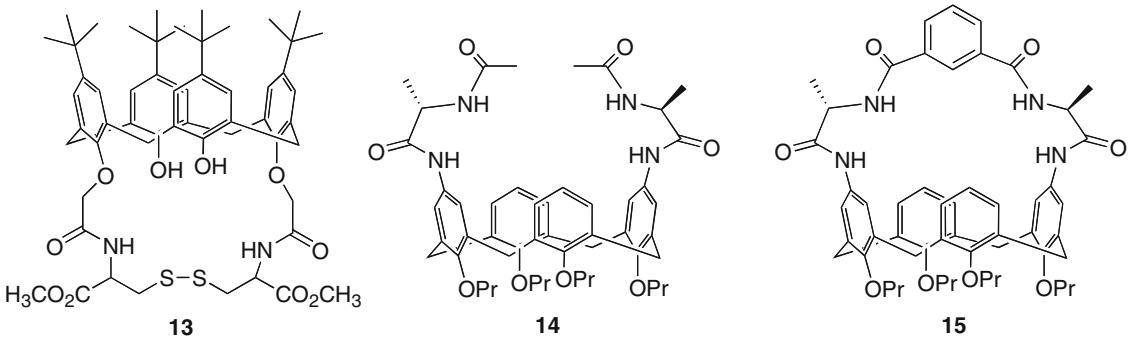
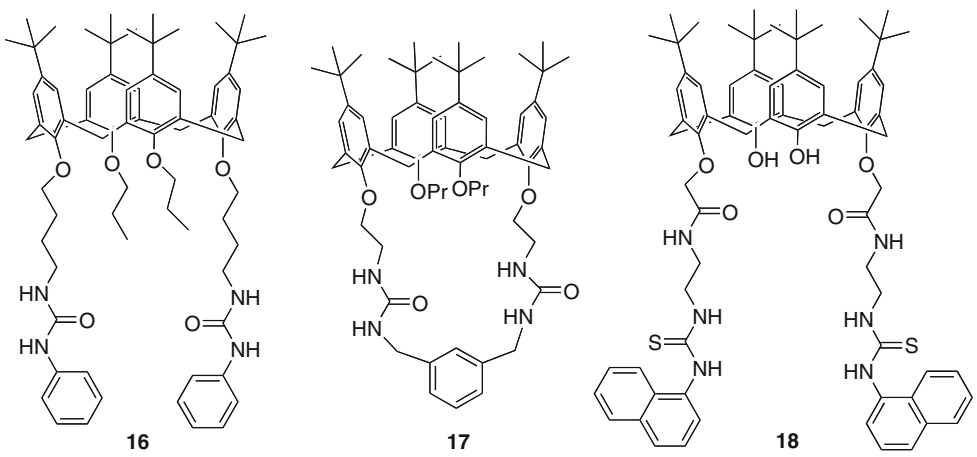


Fig. 3 Peptide based calix[4]arenes for anion recognition

Fig. 4 Halide and carboxylate binding calix[4]arenes



succinate which were bound significantly stronger than chloride or dihydrogen phosphate anions [17].

Binding of anions within upper-rim derived calix[4]-arenes especially those equipped with four urea species is complicated by the fact that these 'calixureas' are prone to dimerisation and capsule formation. Monourea calix[4]arene **19** (Fig. 5) has been synthesized and shown to be an efficient carboxylate binding species for benzoate, butyrate and *para*-nitrobenzoate, a fact that contrasts sharply with its inability to bind spherical anions such as chloride, bromide or iodide [18]. Similar to **19** bis-1,3-*N*-phenylurea calix[4]arene **20** did not bind spherical anions such as chloride, bromide or iodide it did however bind a variety of mono and dicarboxylate species, i.e. butyrate (K_{ass} 133 M^{-1}), *para*-phthalate (K_{ass} 200 M^{-1}), *meta*-phthalate (K_{ass} 230 M^{-1}), benzoate (K_{ass} 290 M^{-1}), *ortho*-phthalate (K_{ass} 300 M^{-1}) and acetate (K_{ass} 2200 M^{-1}). Extending the cavity on **21** via the inclusion of two aryl groups (Fig. 5) and rigidifying it via cyclisation of the two 1,3-diurea motifs was recently undertaken by Nakamura et al. The resulting system had the potential to selectively bind the *iso* phthalate dicarboxylic acid (K_{ass} 1100 M^{-1}) over benzoic acid (K_{ass} 43 M^{-1}) [19].

A consequence of the highly sensitive nature of fluorescence spectroscopy it is widely employed for studying host–guest binding. Employing titration studies it is often possible to gain a valuable insight into the location and nature of the anion-binding event. By way of example a recent report by Shuang et al. demonstrated that 'deep' calix[4]arene **23** (Fig. 6) was able to selectively bind a carboxylic acid species such as acetate or benzoate in preference to fluoride or dihydrogen phosphate via hydrogen bonding to the upper-rim carbamate species [20]. The anion binding and sensing properties of a series of calix[4]-arene derivatives were investigated using fluorescence titration (emission quenching) where a *N*-phenylcarbamate species was switched to a *N*-phenylamide species (**24**, Fig. 6). The bis-1,3-*N*-phenylamide (appended onto the upper-rim) derived calix[4]arene still displayed superior binding selectivities for acetate over fluoride but overall of **23** and **24** bis-1,3-*N*-phenylcarbamate calix[4]-arene **23** was still the most efficient carboxylic acid binder.

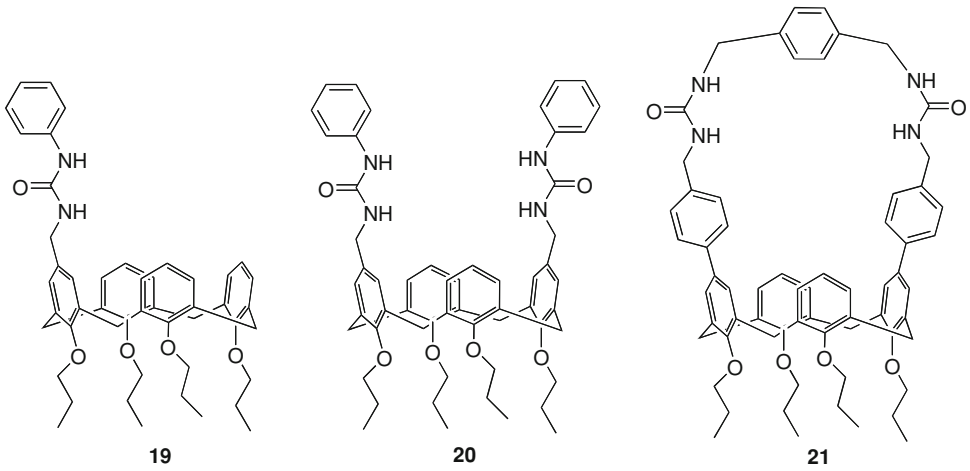


Fig. 5 Carboxylate binding calix[4]arenes

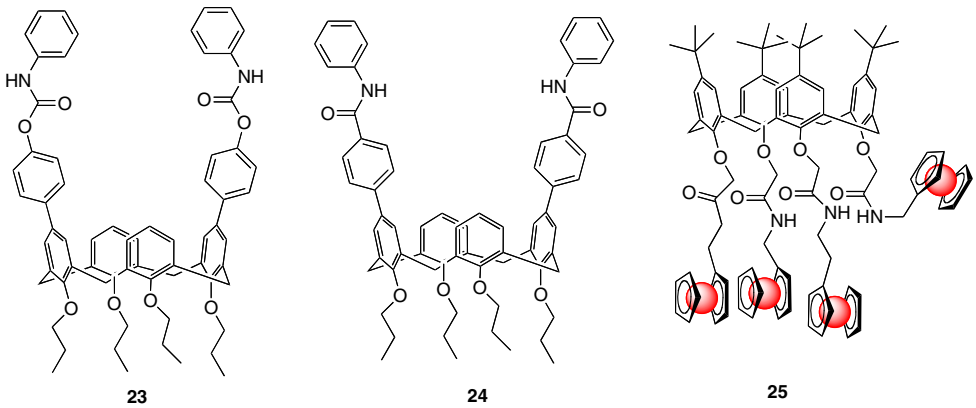


Fig. 6 Carboxylate, chloride and sulfate binding calix[4]arenes

Beer et al. have demonstrated that lower-rim appended ferrocene calix[4]arene **25** (Fig. 6) is capable of 'sensing' chloride, sulfate and dihydrogen phosphate with the latter anion producing the largest shift of the ferrocene/ferrocenium redox couple (160 mV) when titrated into a solution of **25**.

Results and discussion

Even though the *potential* of amino acid molecular recognition via the calixarene scaffold has long been recognized, the synthesis of functionalized calix[4]arenes [21–23] or resorcinarenes [24] and their subsequent investigation/application as amino acid molecular recognizing agents is not particularly common. Indeed a search of the literature[1] [25] afforded relatively few examples of amino acid recognition via binding to *upper-rim functional groups* appended onto calix[4]arenes [26]. Thus by way of example Nishimura et al. demonstrated that a conformationally constrained (on the upper-rim) calix[4]arene species that had been appended on the lower-rim with four pendant chiral esters derived from (*S*)-1-phenylethanol was able to selectively extract (from a biphasic system) certain α-amino acid esters and *N*-protected-α-amino esters from an aqueous solution into dichloromethane [27]. Nishimura demonstrated that glycine, L-alanine and *β*-alanine were not extracted, but that on the other hand L-phenylalanine and L-tryptophan were effectively and efficiently extracted with a preference for L-phenylalanine over L-tryptophan. This order of extraction efficiency can be explained by invoking a hydrophobicity effect, that follows the order: Phe > Trp > Ala > Gly. Furthermore the constrained calix[4]arene developed by Nishimura was able to mediate a *chiral molecular recognition* event if presented with a racemic mixture of Z-protected α-amino acids. The conformationally constrained calix[4]arene interacting with and 'selecting' L-amino acids over D-amino acids [11.0 to 73.1% excess (L − D/D × 100)]. The use, in any form, of mass spectroscopy to determine the ability of the α-amino acids to bind the calix[4]arenes was not discussed.

In 1999 Rebek et al. reported [28] on the ability of upper-rim derived tetra-urea calix[4]arenes to dimerise and within the resulting interior cavity accommodate a guest. Thus the synthesis of a series of homochiral α-amino acid derived calix[4]arene ureas appended with L-norleucine, L-leucine, L-isoleucine, L-valine, L-O-^{t}Bu-serine, L-phenylalanine, L-phenylglycine and L-alanine groups allowed Rebek et al. to show that the resulting calix[4]arene heterodimers generated via a 'seam of hydrogen bonds' generated the dimer in a non-racemic fashion with rotation solely in one direction. The result being that an asymmetric microenvironment within the calix[4]arene was generated. Rebek was able to show that the chiral heterodimers have a defined urea hydrogen bonding direction that upon addition of an enantiomeric guest produces two diasteromeric capsules.

Shuker et al. synthesized an upper-rim appended tetra-(*S*)-alanine calix[4]arene and was able to demonstrate that these species were able to perform a molecular recognition event between two calix[4]arenes species thus generating a dimer via hydrogen bonding. The ability to generate dimers in methanol, a solvent normally expected to *disrupt* hydrogen bonding, is remarkable. Interestingly the authors utilized mass spectroscopy to verify dimer formation and were able to demonstrate that they had been formed with an association constant of K_{ass} 29,000 M^{-1} in 24:1 CD_3OD/D_2O [29]. The authors report this as the first example of a calix[4]arene dimer that is thermodynamically stable in a polar solvent.

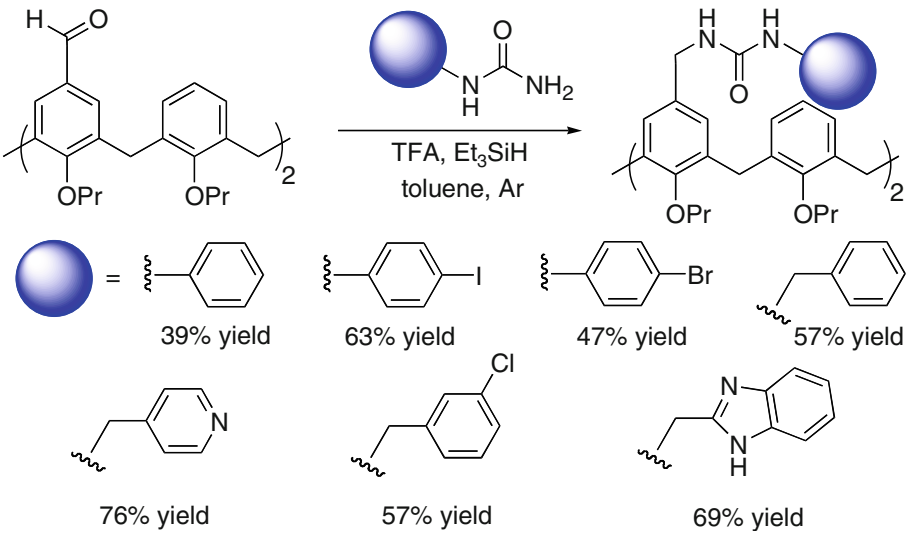
In 2003, Parisi et al. reported the synthesis of an upper-rim ureidocalix[5]arene that behaved as a remarkably efficient abiotic receptor of *ω*-amino acids and biogenic amines. Part of the *ω*-amino acids and biogenic amines were bound within the *π*-basic cavity and part bound via secondary hydrogen bonding to urea motifs [30]. The authors determined the binding affinities of the amine/calix[5]arene complexes using ^{1}H-NMR which indicated a 1 : 1 stoichiometry between the host and guest. The authors make no mention of the use of mass spectroscopy to investigate the host–guest relationship.

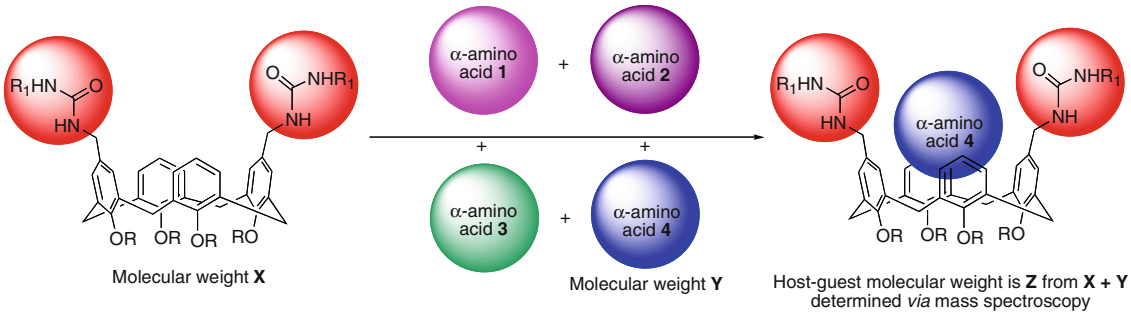
Using a convergent synthesis protocol Ito et al. synthesized a series of homocalix[4]arene species, each one incorporating a different α-amino acid, i.e. L-valine, L-phenylalanine, L-tyrosine, L-typtophan and L-alanine. Using these 'chiral cavity' calix[4]arenes a quaternary ammonium species was able to bind inside the *π*-basic cavity and undergo an interaction.

In an ongoing project focused on generating a new protocol towards the synthesis of upper-rim derived urea calix[4]arenes [26] we wanted to extend our interests and investigate the possibility that mass spectroscopy (LCMS) be used to detect, in a qualitative sense, host–guest complexes between *N*-protected α-amino acids and bis-1,3-*N*-substituted urea calix[4]arenes that have been fixed in the *cone* conformation. Thus in a recent publication we reported an uncomplicated protocol for synthesizing hybrid calix[4]arene urea and carbamate motifs via an experimentally straightforward ionic hydrogenation procedure (Scheme 3). The method is fully amenable to generating structurally diverse bis-1,3-*N*-substituted urea calix[4]arenes, some of which were able to undergo further elaboration via transition-metal-mediated transformations.

[1] We searched SciFinder Scholar and Scopus for the key words 'calixarene' and 'amino acid recognition' returned 56 and 55 'hits' respectively.

Scheme 3 Ionic hydrogenation for synthesis of bis-1,3-urea calixarenes





Scheme 4 Selective molecular recognition of amino acids using urea appended calix[4]arenes

For the purposes of the research programme outlined here, our hypothesis was straightforward and could be divided into two parts: (1) is it possible that *N*-protected α-amino acids have the ability to 'bind' to bespoke upper-rim appended bis-1,3-*N*-substituted urea calix[4]arene scaffolds such that subsequent ESI mass spectroscopy allows the 'supramolecular complex' to be detected and (2) if this proves possible, would it be possible if a *mixture* of *N*-protected α-amino acids were presented to a *single* bis-1,3-*N*-substituted urea calix[4]arene hybrid for that calix[4]arene to specifically 'pick out' *one* *N*-protected α-amino acid and generate a supramolecular host–guest complex that can be identified from its unique host–guest mass to be comprised of the *N*-protected α-amino acid and bis-1,3-*N*-substituted urea calix[4]arene. The idea of selective complexation (using calix[4]arene with a molecular weight **X**) of an α-amino acid with molecular mass **Y** with concomitant formation of a supramolecular species with molecular weight **Z** is shown schematically in Scheme 4.

The application of mass spectroscopy techniques for investigating anion-binding events within macrocyclic systems, and for the purposes of this research we have focused on calixarenes, has received scant attention in comparison to NMR and fluorescence techniques. Thus although mass spectroscopy has proved itself invaluable to chemists across all aspects of chemistry, its application as a sensitive tool for investigating calixarene based molecular recognition events have been relatively few.

With our hypothesis in mind (*vida supra*) we initiated our investigation using two simple bis-1,3-*N*-phenylurea calix[4]arenes, i.e. **26** and **27** (Fig. 7). Both afforded strong 'control' mass peaks when subjected to ESI (negative mode) mass spectroscopy, with **26** generating a M_{wt} of 860.4 and **27** a M_{wt} of 1140.3 (See ESM, Fig. 3).

Subsequent addition of a molar equivalent of *N*-Boc glycine (**28**, M_{wt} 175) to the sample of **26** and re-running the ESI afforded a new mass peak at 1035, i.e. correct mass for **26** and *N*-Boc-glycine, although the intensity of this peak was relatively low,[2] furthermore it is interesting to note that mass spectral evidence for non-complexed **26** was

[2] From a qualitative point of view we have attempted to 'standardized' the mass spectroscopic data of the 'host–guest' calix[4]arenes complexes by taking the observed mass intensity of the bis-1,3-*N*-urea calix[4]arene amino acid complex and dividing it by the observed mass intensity of the bis-1,3-*N*-urea calix[4]arene amino acid, this is a crude but relatively effect way of allowing us to compare all the data sets generated.

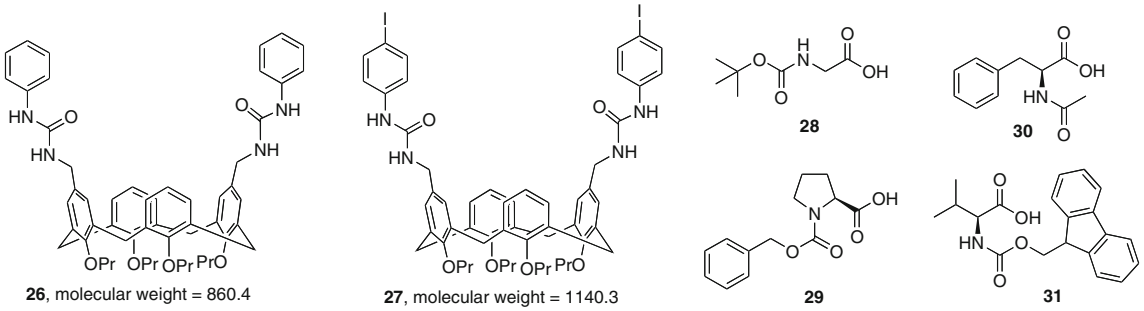


Fig. 7 Ureas and amino acids used in molecular recognition study

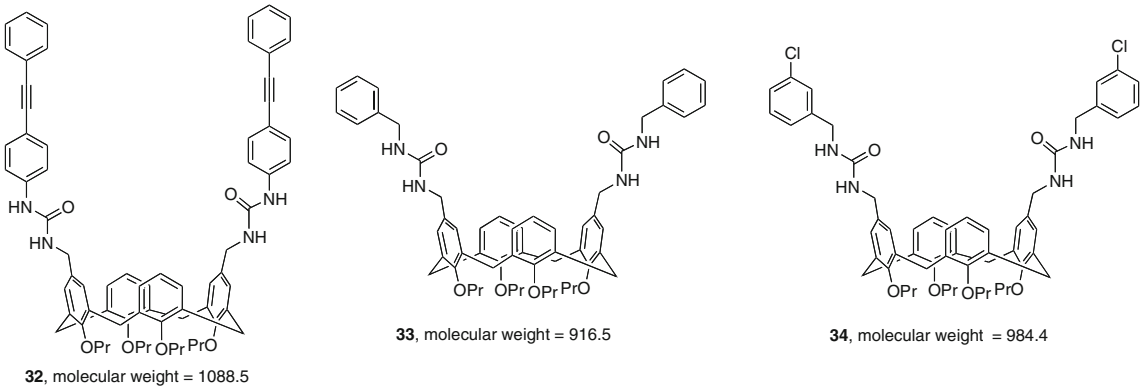


Fig. 8 Ureas used in molecular recognition study

also present in the spectrum. Performing the same procedure[3] with **26** but switching to *N*-Cbz-proline (**29**, M_{wt} 249) a more intense mass peak at 1110.1 was observed (see ESM, Fig. 4), which again correlates to the formation of a host–guest adduct between **26** and **29**. Encouraged by these results we pursued the application of aromatic α-amino acid **30**, i.e. *N*-acetyl-(*S*)-phenylalanine, anticipating that the aromatic group may aid formation of the calix[4]arene host–guest complex via possible inclusion of the phenyl ring of the α-amino acid within the calix[4]arene cavity. Gratifyingly when an equimolar quantity of **30** (M_{wt} 207) was added to **26** a strong mass peak correlating to the formation of the anticipated host–guest complex was observed, i.e. 1067 (see ESM, Fig. 5), finally repeating this process with *N*-Fmoc-(*S*)-valine **31** afforded a strong mass peak that correlated well with the expected mass for the formation of a host–guest complex, i.e. 1199 (see ESM, Fig. 6). It seemed from these preliminary results that 'strong' complex formation between **26** and a few select *N*-protected α-amino acids was observed, this appeared to be particularly so when aromatic groups were present on the α-amino acid component.

Utilising **27** we set about investigating the effect that halogens, namely iodine, would have on the ability of the bis-1,3-*N*-*para*-iodophenylurea calix[4]arene to undergo host–guest complexation. We repeated the process outlined for **26** with *para*-iodophenylurea calix[4]arene **27**. Interestingly from the mass spectroscopy results it seemed all four *N*-protected α-amino acids **28**–**31** (See Fig. 7), were only very poorly accommodated or complexed by the *para*-iodo substituted calix[4]arene. Seemingly the presence of the two sterically encumbered large iodine atoms inhibited host–guest formation (Chart 1).

Synthesising [26] 'deep' calix[4]arene **32** (Fig. 8) we investigated formation of the corresponding host–guest complexes with **28**–**31** (See ESM Figs. 11–14), similar to **27** only weak interactions were detected with the uncomplexed host (**32**) also present. The application of hosts with 'deep' and extended sides did not seem to offer any increased potential for generating urea appended calix[4]arenes with superior molecular recognition properties.

Changing tact slightly we considered the possibility that introducing a methylene spacer between the urea motif and the aryl group may afford an *N*-benzylurea calix[4]arene species that has greater capacity due to increased flexibility for binding an *N*-protected α-amino acid. Synthesizing **33** we investigated its potential to bind, individually, the α-amino acids **28**–**31** using mass spectroscopy as our investigative tool. Interestingly incorporating a bis-1,3-*N*-benzyl urea system onto the calix[4]arene seemingly

[3] Undertaking the mass spectra experiments was straightforward and quick, see ESM for details.

restored (compared to **27** and **32**) and increased (judged against **26**) the capacity of the calix[4]arene to bind the *N*-protected α-amino acids **28–31** (See ESM, Figs. 15–18). Again similar to **26**, **33** had a preference for aromatic containing α-amino acids, i.e. **30** and *N*-Fmoc derived α-amino acids, i.e. **31** (See Chart 1).

Investigating what effect incorporating a *meta*-chlorobenzyl group would have on the ability of the resulting calix[4]arene to bind **28–31** we synthesized **34**. From Chart 1 it is evident that both **33** and **34** were significantly better at binding **28–31** than the *N*-phenylureas **26** and **27**. Furthermore and similar to **33** the chlorine containing **34** displayed broadly comparable and impressive selectivity for cyclic peptide **29**, aromatic α-amino acid **30** and *N*-Fmoc derived α-amino acid **31**, this was countered by a decreased for **33** and **34** to bind *N*-Boc glycine **28**.

Delighted that *N*-benzyl derivatives **33** and **34** did indeed seem to be able to bind to specific *N*-protected α-amino acids we synthesized three more calix[4]arene variants, i.e. **35–37** (Fig. 9). Using these substrates we repeated the mass spectroscopy process outlined above using the same α-amino acids **28–31**; an interesting and striking result was obtained (Chart 1). Of the four α-amino acids employed *N*-acetyl-(*S*)-phenylalanine and Fmoc-(*S*)-valine were of particular interest. Focusing on **35** and these two amino acids particularly strong mass peaks for complexation were observed with commensurate very small mass peaks for the host, i.e. **35** (See ESM, Figs. 23–26). This intriguing result suggests that with suitable *N*-substituted urea motifs appended onto conformationally fixed calix[4]arene scaffolds their ability to form host–guest complexes that are stable and detectable via mass spectroscopy is viable.

Anticipating that benzimidazole **36** may form extended hydrogen bonding arrays with **28–31** we were disappointed that only weak binding was observed in all of the four amino acids investigated (Chart 1). Changing tact slightly we synthesizing **37** a sterically encumbered secondary benzylamine derived bis-1,3-urea calix[4]arene. Investigating its potential to bind **28–31** we were disappointed that only weak binding of **28–31** (See ESM, Figs 27–30) was observed (Chart 1), it would seem that increasing the steric bulk around the carbon adjacent to the two proximal ureas on **37** serves only to inhibit binding of the *N*-protected α-amino acids (See ESM, Figs 31–34).

Intrigued by the fact that *N*-Fmoc-(*S*)-valine **31** displayed what appeared to be enhanced binding towards calix[4]arenes **33–35** (Chart 1) we opted to investigate the possibility that it was the Fmoc group that was critical to the calix[4]arene binding event. With this in mind we tested (individually) a further three *N*-Fmoc protected α-amino acids, i.e. glycine, (*S*)-proline and (*S*)-phenylalanine (**38–40** respectively) for their ability to bind to bis-1,3-*N*-substituted urea calix[4]arenes **26**, **32–37**. Employing our standard mass spectroscopy analysis protocol (see Experimental section) *N*-phenyl derivatives **26** and **32** behaved in a manner similar to previously tested *N*-protected α-amino acids **28–31**, the results suggested that there was not much evidence of a strong host–guest relationship (see Chart 2). On the contrary, screening **38–40** for their ability to undergo host–guest relationships with **33–35** afforded interesting and generally positive results (See ESM, Figs. 35–43), with the aromatic *N*-Fmoc-(*S*)-phenylalanine **40** displaying a particularly strong propensity to bind to *para*-pyridine derived urea **35**. Interestingly as seen in the previous study, substituting *N*-Cbz-(*S*)-proline (**29**) for *N*-Fmoc-(*S*)-proline (**39**) demonstrated a significant improvement in the ability of the *N*-Fmoc derivative to bind to the same two calix[4]arenes **34** and **35** (Chart 2). *N*-Fmoc-glycine **38** also appeared to have a greater affinity for **34** and **35** than the corresponding *N*-Boc glycine derivative **28** (compare Charts 1, 2), overall these results tend to confirm that incorporating an *N*-Fmoc group did have a positive influence on amino acid binding and complex formation.

Similar to **28–31** *N*-benzimidazole **36** displayed no real binding affinity for **38–40** (See ESM, Figs. 44–46), on the contrary and for reasons not yet fully understood *N*-Fmoc α-amino acids **38–40** do have a reasonable affinity for **37**, with the cyclic α-amino acid **39** standing out (Chart 2) amongst the three tested cf. *N*-Fmoc-(*S*)-valine **31** (Chart 1).

The interesting results obtained with the *N*-Fmoc protected derivatives **38–40** prompted us to investigate the effect of changing the α-amino acid component and

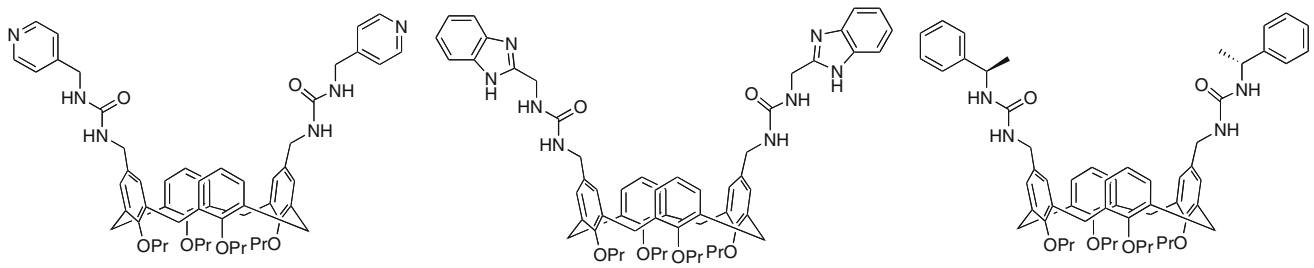


Fig. 9 Ureas used in molecular recognition study

Chart 1 Mass spectroscopic interaction of bis-1,3-urea calix[4]arenes **26**, **27**, **32–37** with amino acids **28–31** screened on an individual basis

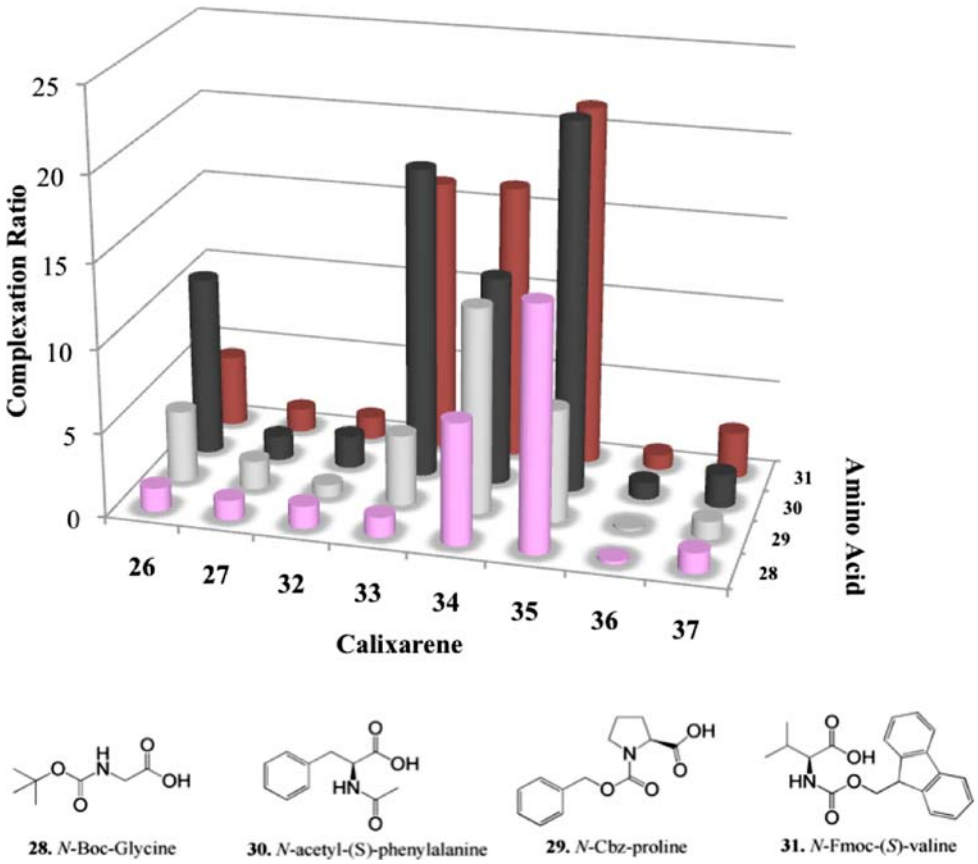
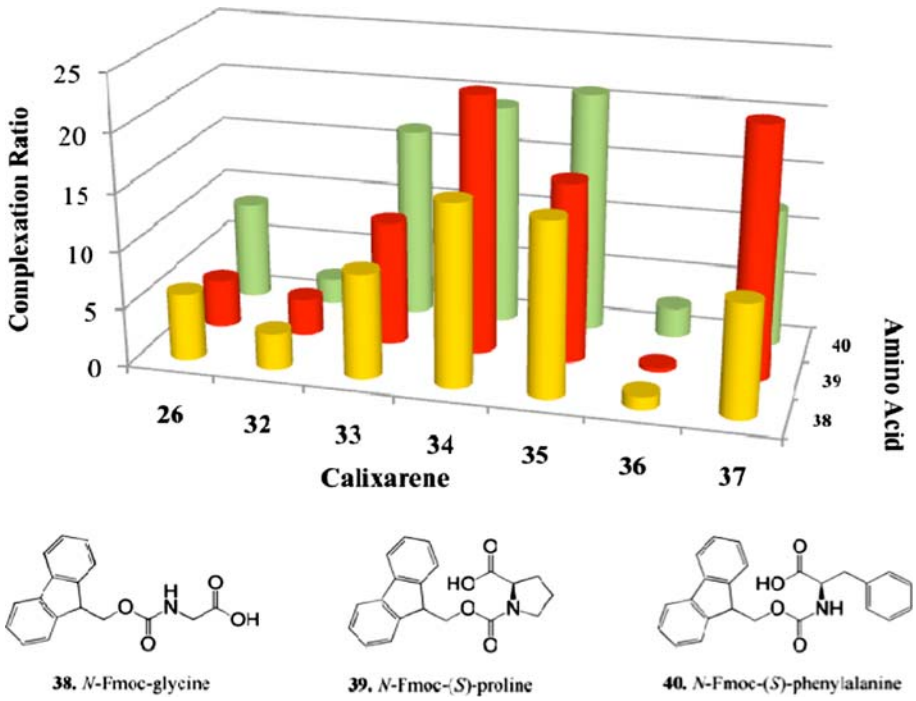


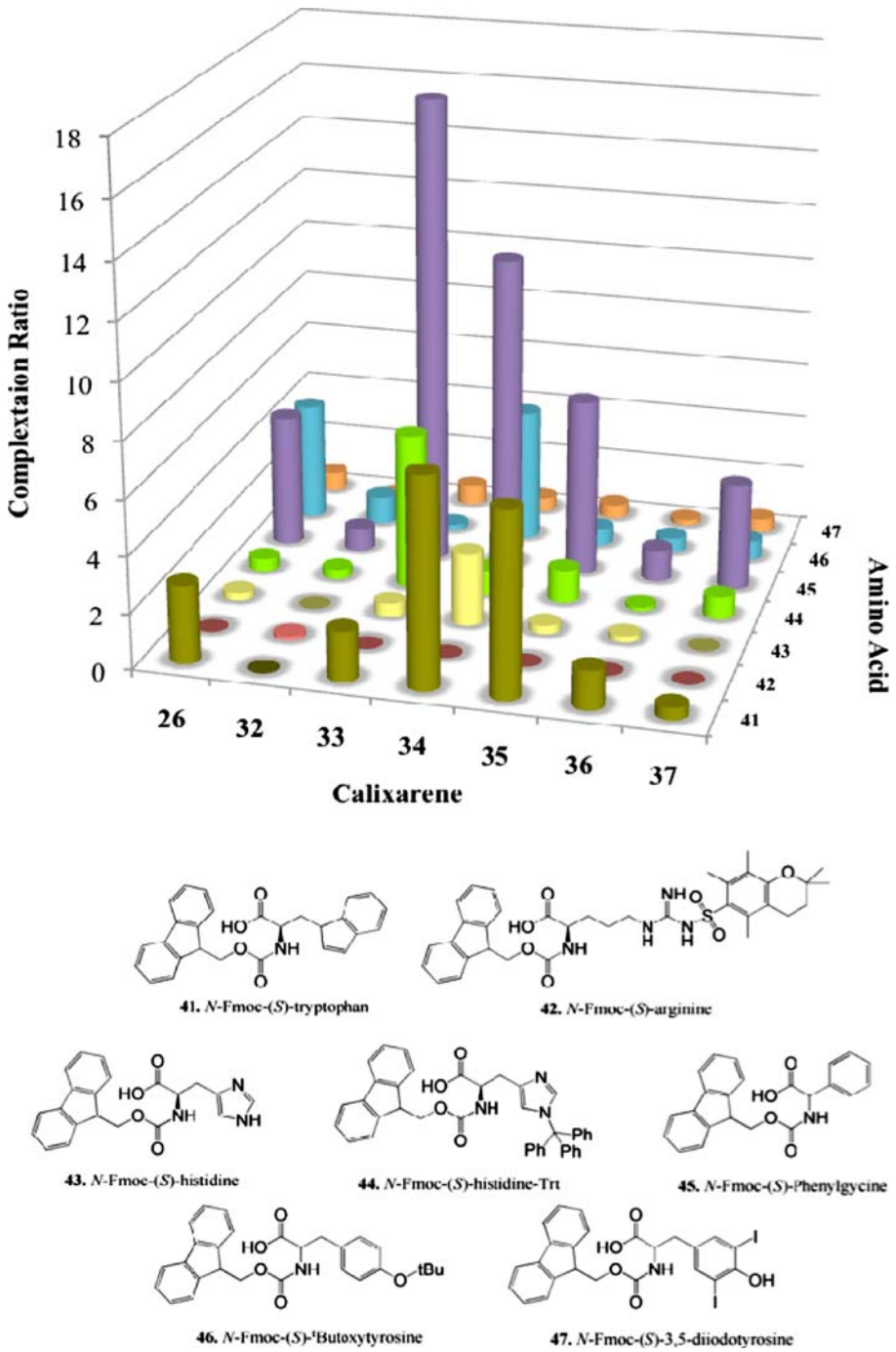
Chart 2 Mass spectroscopic interaction of bis-1,3-urea calix[4]arenes **26**, **32–37** with amino acids **38–40** screened on an individual basis



determining their ability to generate host–guest complexes that are detectable via ESI mass spectroscopy with **26**, **32–37**.

With this in mind we screened structurally diverse *N*-Fmoc protected α-amino acids **41–47** (Chart 3). It is clear from Chart 3 that (*S*)-arginine derivative **42** (containing the

Chart 3 Mass spectroscopic interaction of bis-1,3-urea calix[4]arenes **26**, **32–37** with amino acids **41–47** screened on an individual basis



pentamethylchroman sulfonyl *N*-protecting group or PMC), (*S*)-histidine **43** and **44** did not generally form complexes that were detectable or stable enough to be identified via ESI mass spectroscopy. *N*-Fmoc-(*S*)-tryptophan **41** displayed improved complexation properties with **34–35** and similar to previous *N*-Fmoc α-amino acids tested, i.e. **28–31** and **38–40** was poor at binding to *N*-phenyl derived calix[4]arenes **26** and **32**. Switching to (*S*)-tyrosine derivatives **46** and **47** there was limited evidence, the only exception being between pyridine derivative **34** and **46**, of any binding to the calix[4]arenes. This indicates that the addition of the sterically bulky *tert*-butyl group to the phenolic hydroxyl in **46** essentially shuts down the formation of complexes with all but one of the hybrid calix[4]arenes. A similar situation was observed with *N*-Fmoc protected 3,5-diiodo-(*S*)-tyrosine derivative **47**, with essentially no complexation observed with **33–37** (orange bars). The situation changes somewhat however when *N*-Fmoc-(*S*)-phenylglycine (**45**) is employed where what appears to be quite strong and selective complexation (purple bars,

Chart 3) between **33–35** and *N*-Fmoc-(*S*)-phenylglycine is observed and specifically between phenylglycine derivative **45** and bis-1,3-*N*-benzylurea calix[4]arene **33** (See ESM, Figs. 47–53).

From results attained thus far it seemed, that at least from a qualitative point of view, specific bis-1,3-*N*-substituted urea's did have the ability to selectively bind *N*-protected α-amino acids. Introducing a further level of complexity we considered the possibility of presenting to single bis-1,3-*N*-substituted urea calix[4]arenes mixtures of *N*-protected α-amino acids, thus allowing us to probe the possibility that given competition for binding to the bis-1,3-substituted urea calixarenes the host molecule does indeed demonstrate a selective binding process.

In the first instance we screened the original *N*-protected α-amino acids, i.e. **28–31** against calix[4]arenes **26**, **32–37**. If Charts 1 and 4 are compared it is clear that employing a mixture of *N*-Fmoc-α-amino acids results in a considerable change in Fmoc amino acid selectivity, furthermore it is evident that a number of striking similarities are observed but also that anomalies become apparent. From Chart 4 the most obvious feature is the seemingly very high propensity for **26**, **33**, **34** and **37** to complex with *N*-Fmoc-(*S*)-valine **31** even if they have competition from amino acids **28–30**. Interestingly complexation of amino acids **28–30** with **26**, **27**, **32–37** seems to have undergone a global *reduction* if compared with the individual screenings outlined in Chart 1. The first and last calix[4]arenes, i.e. **26** and **37** are interesting. Comparing the individual screenings of **28–31** outlined in Chart 1 with **26** there is a considerable difference between the mixtures **28–31** (Chart 4) and the observation in Chart 1 for the individual amino acids, furthermore the *substantial increase* in **37** binding to **31** (Chart 4) (See ESM, Figs. 61–67) when presented with the mixture of α-amino acids **28–31** is surprising. A rational explanation that accounts for these observations is difficult to establish. Needless to say these preliminary results spurred us onto try alternative α-amino acid mixtures.

Earlier work indicated that individual *N*-Fmoc derived α-amino acids such as **28–31** displayed higher affinity for bis-1,3-*N*-substituted urea calix[4]arenes such as **33–35** and **37** than *N*-Boc or *N*-Cbz cf. Charts 1 and 2. With this and the preliminary, interesting results from the mixture of α-amino acids in mind we elected to investigate the possibility that individual calix[4]arenes **26**, **32–37** may when presented with a mixture comprising four *N*-Fmoc α-amino acids (**31**, **38–40**) selectively bind to one of them. Employing a procedure essentially identical to that outlined for α-amino acids **28–31** an interesting observation resulted, see Chart 5. Similar to individually tested amino acids (Chart 2) calix[4]arenes **34** and **35** had a propensity to complex *N*-Fmoc-(*S*)-phenylalanine **40** but at the expense of forming host–guest complexes with **38**, **39** and **31** (See ESM, Figs 68–69).

Comparing the individual α-amino acid study reported (Chart 3) with a mixture study utilizing **41–42** and **43–44** we screened these α-amino acid mixtures against bis-1,3-*N*-substituted urea calix[4]arenes **26**, **32–37**. The only significant difference between the 'single' versus 'mixture' study was the slight drop in the affinity of **41** for **34** and **35** and the increase in complexation between **41** and **37** (compare Chart 3 with Chart 6) (See ESM, Fig. 70). Focusing on the individual α-amino acids **43** and **44** in Chart 3 with the results from the mixtures outlined in Chart 7 for the same two *N*-Fmoc-(*S*)-histidines there is relatively little difference between using single amino acids versus a mixture (See ESM, Figs. 71–72).

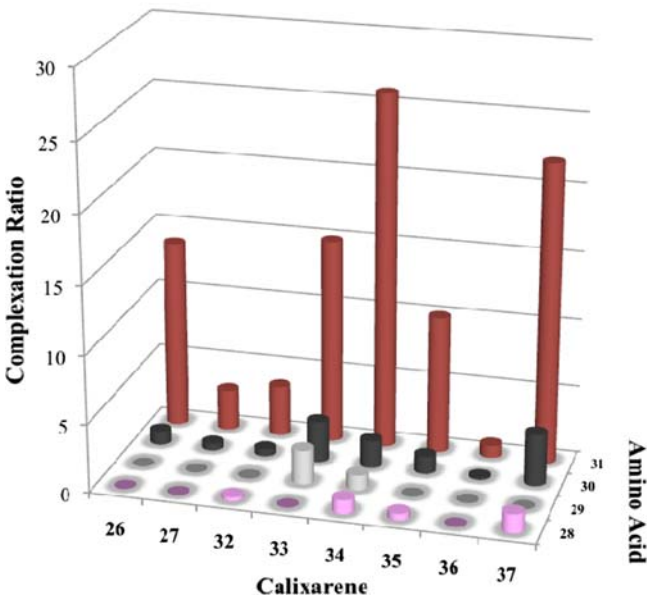


Chart 4 Investigating via mass spectroscopy the interaction of bis-1,3-urea calix[4]arenes **26**, **27**, **32–37** with mixtures of amino acids, i.e. **28–31**

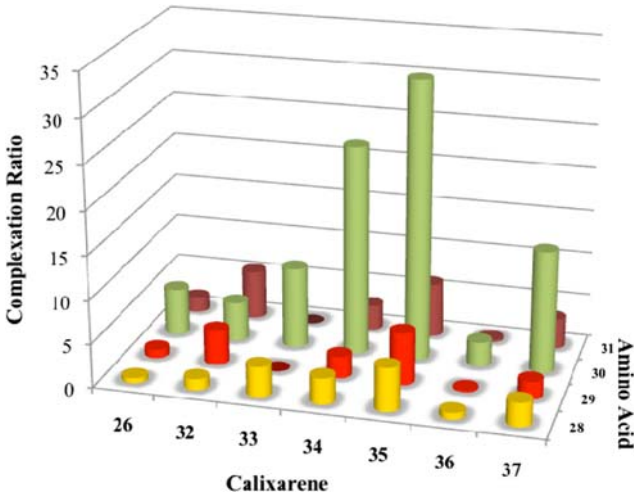


Chart 5 Investigating the mass spectroscopic interaction between bis-1,3-urea calix[4]arenes **26**, **32**–**37** and mixtures of amino acids, i.e. **31** and **38**–**40**

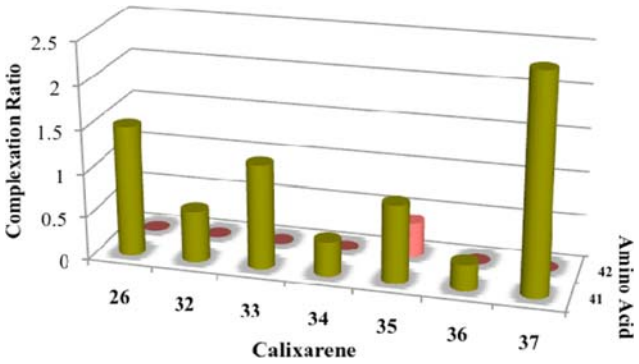


Chart 6 Investigating the mass spectroscopic interaction between bis-1,3-urea calix[4]arenes **26**, **32**–**37** and mixtures of amino acids, i.e. **41** and **42**

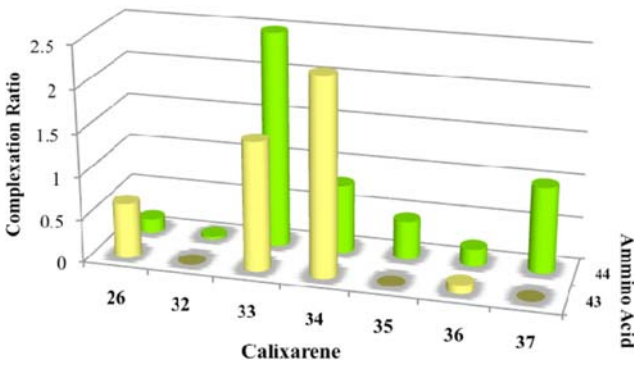


Chart 7 Investigating the mass spectroscopic interaction between bis-1,3-urea calix[4]arenes **26**, **32**–**37** and mixtures of amino acids, i.e. **43** and **44**

Switching our attention to a mixture comprising Fmoc derivatives **45**–**47** a number of differences are apparent, again a global *reduction* in binding affinity seems to have occurred, with the intensity of the majority of the peaks in Chart 8 greatly reduced to the many of those observed in Chart 3.

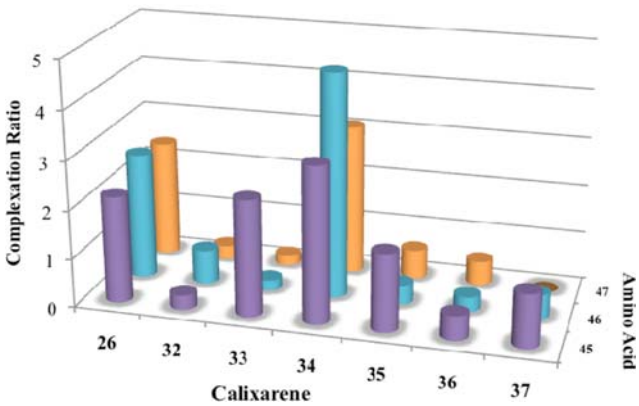


Chart 8 Investigating the mass spectroscopic interaction between bis-1,3-urea calix[4]arenes **26**, **32**–**37** and mixtures of amino acids, i.e. **45** and **47**

Interestingly, calix[4]arene **26** appears to have generated approximately equal quantities of host–guest complexes between all three α-amino-acids (including **47**) that comprise the mixture, comparing this to the single amino acids screen in which **47** did not appear to have any great propensity to form a host–guest complex with **26**. Similarly calix[4]arene **34** when presented with a mixture of **45**–**47** (See ESM, Figs. 73–74) seems to have an increased affinity for **47** (Chart 8).

In summary, preliminary evidence for the ability of *N*-protected α-amino acids to form host–guest relationships with bis-1,3-*N*-substituted urea calix[4]arenes fixed in the cone conformation has been accumulated. Although specific, single α-amino acids seem to associate quite well, the situation with hybrid calix[4]arenes and their ability to generate bespoke host–guest complexes with specific α-amino acids 'extracted' from within a mixture is considerably more complicated and requires further investigation.

Experimental section

General protocols

All reactions requiring anhydrous conditions were conducted in flame-dried glass apparatus under an atmosphere of nitrogen or argon. Water refers to distilled water. All commercially available chemicals, reagents and salts were used as supplied. Melting points were recorded using open capillary tubes on a Gallenkamp melting point apparatus and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer Paragon 1000 FT infrared spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded in Fourier

transform mode at the field strength specified either on a Varian Gemini 300, 400 or 500 spectrometer. Unless otherwise stated, deuterated chloroform was used as solvent. The ^{1}H-spectra were recorded in ppm and referenced to the residual $CHCl_3$ signal located at δ 7.24 ppm. ^{13}C-NMR spectra were recorded in ppm and referenced to the residual $CHCl_3$ signal found at δ 77.00. Multiplicities in the NMR spectra are described as: *s* singlet, *d* doublet, *t* triplet, *q* quartet, *m* multiplet, *br* broad; coupling constants are reported in Hz. Mass spectra were run on either a Shimadzu LCMS-2010A, Micromass Quattro II, Finnigan MAT95 or MAT 900 spectrometer. Ion mass/charge (*m/z*) ratios are reported as values in atomic mass units. Thin layer chromatography was performed on Merck aluminium plates coated with 0.2 mm silica gel-60 F_{254}. Flash column chromatography was performed on silica gel (Kieselgel 60). All the commercially available amino acids were used without any further purification. All the sample solutions were prepared using HPLC grade dichloromethane or methanol as required. The synthesis of **27**, **32**–**36** has been reported [26].

Typical synthesis of bis-1,3-*N*-substituted ureas is outlined below for **37** [26]

To a solution of bis-1,3-formyl-tetra-*O*-propoxycalix[4]arene (120 mg, 0.18 mmol) and *N*-[(*R*)-α-methylbenzyl]urea (95 mg, 0.58 mmol) in anhydrous toluene (5 mL), was added trifluoroacetic acid (116 μL, 1.5 mmol), followed by triethylsilane (180 μL, 1.14 mmol). The reaction mixture was stirred at room temperature for further 24 hours under nitrogen. The reaction was subsequently diluted with ethyl acetate (30 mL) and washed with aqueous saturated sodium bicarbonate (5 mL) and followed by brine. The organic phase was dried over magnesium sulphate, filtered and the solvent was removed under reduced pressure. Purification via flash chromatography (hexanes/diethyl ether, gradient 50-100%) afforded **37** as a white solid (130 mg, 76%). Mp (decomp.) 211–216 °C (dichloromethane); ^{1}H NMR ($CDCl_3$, 500 MHz, Fig. 1) δ 7.35–7.25 (m, 10H), 6.89 (dd, 4H, $J = 12.3$, 7.44 Hz), 6.74 (t, 2H, $J = 7.44$ Hz), 6.11 (d, 4H, $J = 8.66$ Hz), 5.25 (bs, 2H), 5.05 (bs, 2H), 4.83 (m, 2H), 4.39 (d, 4H, $J = 13.10$ Hz), 3.96 (m, 4H), 3.79 (q, 4H, $J = 15.1$ Hz), 3.64 (t, 4H, $J = 7.02$ Hz), 3.07 (d, 4H, $J = 13.10$ Hz), 2.04–1.79 (m, 8H), 1.44 (d, 6H, $J = 6.9$ Hz), 1.05 (t, 6H, $J = 7.44$ Hz), 0.91 (t, 6H, $J = 7.49$ Hz) ppm; ^{13}C NMR ($CDCl_3$ + 5% CD_3OD, 100 MHz, Fig. 2) δ 158.4, 157.3, 154.9, 144.8. 136.1, 134.0, 132.6, 128.7, 128.6, 127.1, 126.1, 125.7, 122.0, 77.1, 76.7, 49.5, 42.9, 31.1, 23.5, 23.2, 15.5, 10.8, 10.1 ppm; FT–IR (neat) 3315, 2922, 2866, 1710, 1655, 1461, 1377, 1212, 722 cm^{-1}; HRMS (ES) *m/z* 967.5326 $[M+Na]^+$ (calculated for $C_{60}H_{72}N_4O_6Na$ 967.5344).

ESI–MS experiments

The ESI–MS experiments were performed on a Shimadzu LCMS-2010A instrument (mass range 10–2000 amu) equipped with Q-array ion optical-quadrupole detector system, SIL 20A autosampler and LCMS 2010EV detector. The calix[4]arene stock solutions were prepared by dissolving appropriate amounts of sample in a mixture of dichloromethane-methanol (5% v/v) to give a final concentration of 1 mM. Individual amino acid solutions (1 mM) were prepared in methanol. Calix[4]arene-amino acid mixture samples were prepared by mixing 20 μL each of the required stock solution and diluting up to 1.2 mL with methanol. The samples were introduced as 10 μL injection using the autosampler and at a flow rate of 0.5 mL/min eluting with methanol. For guest competition experiments 20 μL solutions of calix[4]arene with 1 equivalent of each guest amino acid were prepared and analysed as detailed above. A ratio of the intensity of mass ion peaks of the host–guest complex to the molecular ion peak of host was used as a qualitative indicator of the relative binding ability.

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