Azacalixarene: synthesis, conformational analysis, and recognition behavior toward anions

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The synthesis of an azacalix[4]arene (**4**) having four primary amino groups on the upper rim has been achieved. The synthesis was accomplished by a one-step cyclization starting from two easily synthesized building blocks. ^{1}H NMR spectra indicate that this azacalixarene prefers a cone conformation, but that rapid interconversion among different conformations occurs at room temperature. It is shown that **4** is a useful receptor for sensing various anionic guests, such as inositol triphosphate (IP_3), in aqueous solution. In a competition binding assay, fluorescent probes bound within **4** were replaced by anionic guests in buffered aqueous solution, causing a modulation in the fluorescence intensity. NMR spectra of **4** in the presence of fructose 1,6-diphosphate suggest that this guest is bound inside the cavity which adopts a cone conformation.

Introduction

Calixarenes (**1**) and related macrocycles have received a lot of attention due to their molecular recognition properties.[1–4] In recent years, a few azacalixarenes have been synthesized[5–7] as part of a class of compounds called expanded calixarenes (Fig. 1 (**2**, **3**)). The azacalixarenes have nitrogens in the macrocyclic ring which could provide additional binding sites for guests. However, in contrast to calixarenes, the azacalixarenes have not proved as useful for developing receptors. For example, Hampton and co-workers reported that azacalixarene **2** showed no significant binding to alkali metal ions due to strong intramolecular hydrogen bonds between the hydroxy groups and the amines in the core of the macrocycle.[6] Azacalixarene **3**, created by Robson and co-workers, binds only metals such as Zn^{2+} and Co^{2+} ions as evidenced by co-crystal structures.[7]

We believe that azacalixarenes could have great utility as receptors for a large variety of guests. Specifically, they have a cationic and hydrophobic cavity which provides binding sites for anionic guests, and the amines could be modified to introduce additional functional groups. Furthermore, the CH_2–NH–CH_2 linkages introduce flexibility to the receptor, a feature that may be desirable for binding guests in an induced-fit fashion. Our interest in water soluble receptors for anionic guests[8] led us to develop the new azacalixarene receptor **4** (Fig. 1).

In this paper, we describe the synthesis, conformational analysis, and the recognition properties of **4** toward anionic guests, such as inositol triphosphate (IP_3). This is the first report of such studies using an azacalixarene. As a sensing strategy, we have applied a probe competition method.[9] Fluorescent active probes (**9** and **10**) were allowed to bind to **4** in aqueous solution in the absence of guests. Upon addition of other anionic guests, the probes were displaced from the binding cavity, resulting in absorption and fluorescence changes. We demonstrate the sensing of inositol triphosphate in the 10 μM range in aqueous solution.

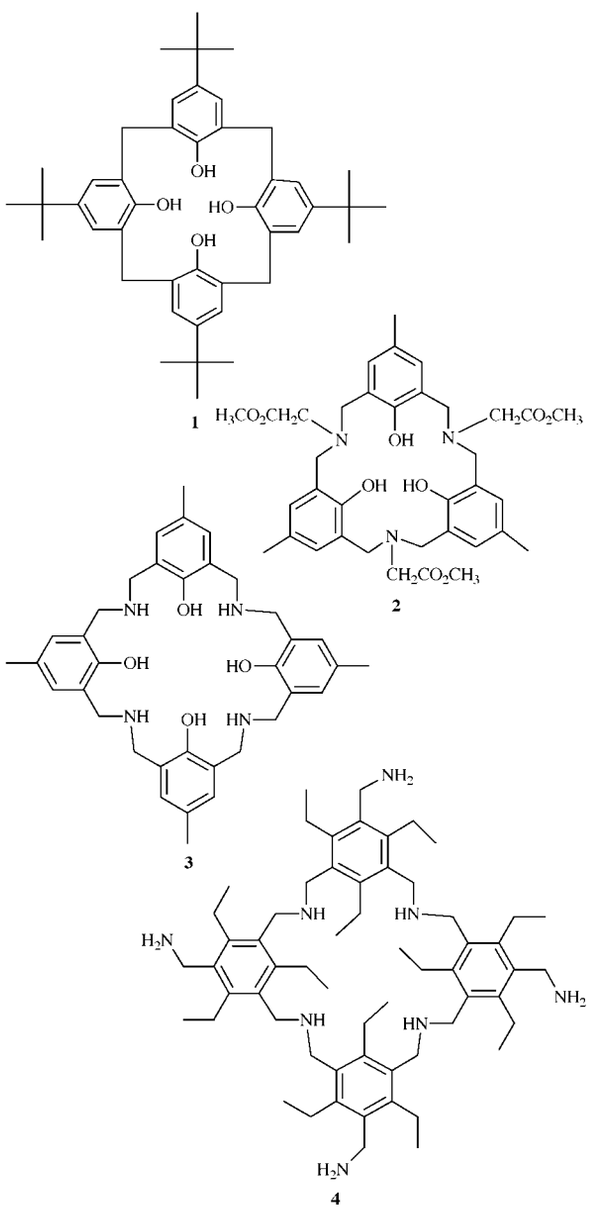


Fig. 1 Molecular structures of calix[4]arene and azacalixarenes.

Results and discussion

A. Design criteria

Receptor **4** provides a larger cavity than a normal calix[4]arene, allowing the binding of anionic carbohydrates such as inositol triphosphate. Three ethyl groups were introduced into each benzene ring to impart a preference for the amine groups to be

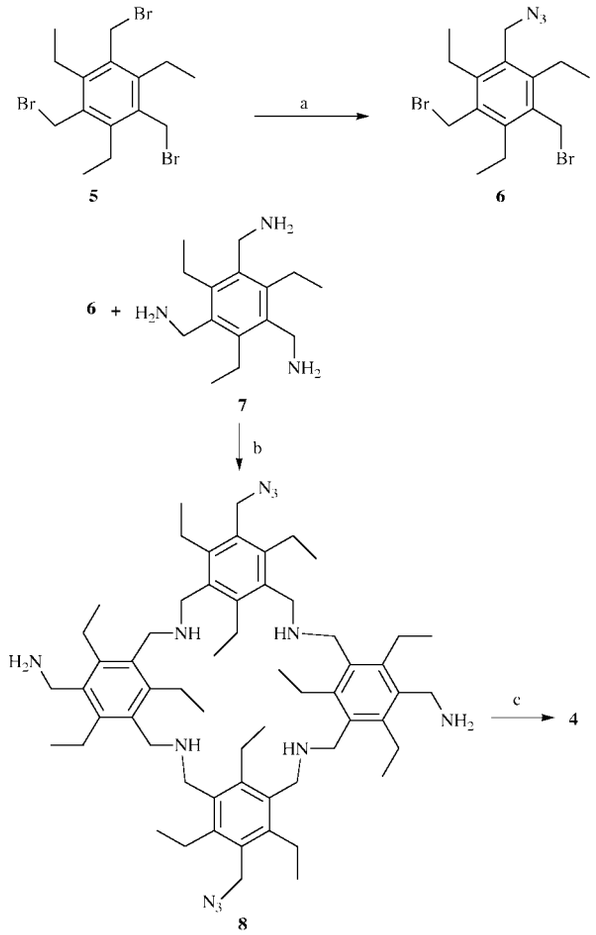


Fig. 2 Synthetic route to azacalixarene **4** a) NaN_3, DMF, 25 °C; b) TEA, CH_2Cl_2, 25 °C; c) triphenylphosphine, THF and H_2O, 25 °C.

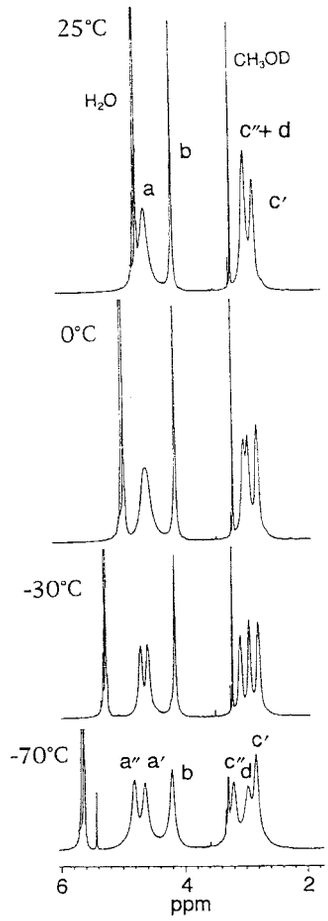


Fig. 3 ^1H NMR spectra (500 MHz) of receptor **4** in CD_3OD.

oriented toward the interior of the cavity.[10,11] The steric gearing imparted by the ethyl groups was predicted to lead to a cup conformation of the receptor. We predicted that this preorganization would give the receptor a high affinity for the target molecules. The four secondary amino groups on the lower rim were included to assist binding of anionic guests in the bottom of a hydrophobic cavity.

Normal calixarenes carrying amine groups on the top rim (*para*-position) exist as zwitterions because the phenols of calixarenes are stronger acids than monomeric phenols.[12] These intramolecular ion pairs may inhibit the binding of anionic guests. In contrast, our receptor does not have hydroxy groups on the rim, thus avoiding zwitterion formation.

B. Synthesis

1,3,5-Tris(bromomethyl)-2,4,6-triethylbenzene (**5**) was synthesized according to a reported procedure.[10] The reaction of **5** with 0.8 equivalents of sodium azide in DMF for 24 h at 25 °C gave a mixture of mono-, bis-, and tri-azido substituted compounds (Fig. 2). The mixture was separated using silica gel chromatography to give a 20% yield of mono-azido compound **6**. The tri-azido analog was reduced with an excess of triphenylphosphine to the corresponding tris-amine compound **7** at 25 °C over 12 h. The reaction of **7** with half of an equivalent of **6** in CH_2Cl_2 for 2 days gave compound **8**. The purification was performed by silica gel chromatography to give a white powder (**8**) in a yield of 40%. Reduction of **8** to **4** is quantitative in a mixed solvent of THF and water. Compound **4** was dissolved in CH_2Cl_2 and extracted into a 1 M HCl aqueous solution. The acidic solution was lyophilized to give pure compound **4** as the octahydrochloride salt.

C. Conformational analysis

The conformation of **4** was determined by ^1H NMR spectra at various temperatures (Fig. 3). There is a significant amount of analytical data related to the conformation of calixarenes obtained using NMR techniques.[1–3] Our NMR spectra were compared to those reported for calixarenes. The NMR spectra observed for **4**, discussed below, support structures that are the cone conformation, but are interconverting rapidly among isomers on the NMR time scale at 25 °C (Fig. 4).

To determine the conformation of **4**, ^1H NMR measurements were carried out at 50 and 25 °C in DMSO-d_6 (not shown) and 25, 0, −30, and −70 °C in CD_3OD (Fig. 3). Proton letter assignments are shown in Fig. 4. The resonance arising from the methylene protons **a** (CH_2–NH–CH_2 at 4.8 ppm in methanol or 4.5 ppm in DMSO) was found to be a sharp singlet at temperatures near 50 °C and a broad singlet at around 0 °C. This resonance, however, separated into a pair of broad singlets at temperatures below −30 °C. We found that one of the **c** protons (**c**″) and **d** coincidently overlapped at 25 °C, and separated from each other below −30 °C. Upon cooling the **c**″ proton was shifted downfield.

Two possible scenarios explain the peak resolution of the **a** protons found at temperatures below −30 °C. First, two different conformations of **4** could exist coincidentally in a one-to-one ratio, where each separated peak for resonances **a** corresponds to one of the conformations. The second possibility is that a stable cone or 1,3-alternate conformation exists at low temperature and gives rise to separate chemical shifts for resonances **a** due to the diastereotopic nature of these centers (Fig. 4). Neither the 1,2-alternate nor the partial cone conformations are supported by the spectra shown in Fig. 3 because these conformations would give significantly more complex spectra, consisting of many more resonances. Also, the

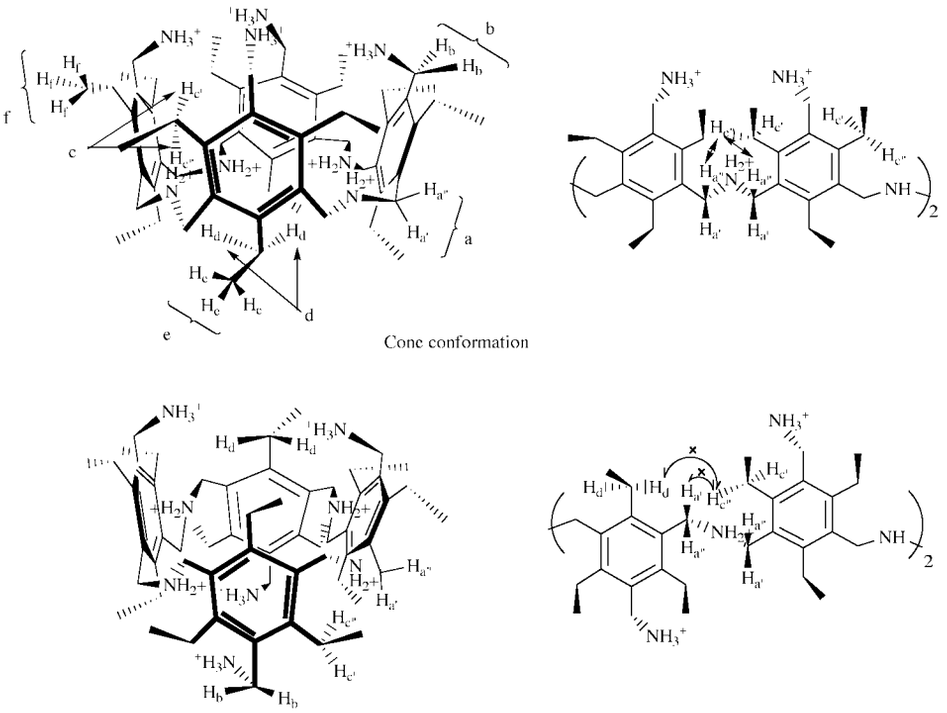


Fig. 4 Cone and 1,3-alternate conformation of receptor **4**.

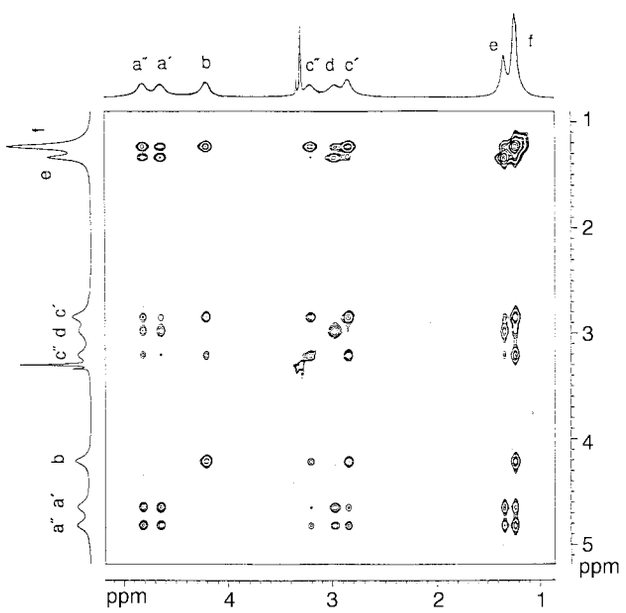


Fig. 5 NOESY spectrum of receptor **4** at −70 °C in CD_3OD.

orientations of the $CH_2NH_3^+$ groups must all be the same (not mixtures of all-in, all-out, in and out) due to the simplicity of the spectra at this temperature. Further, in other highly charged systems we find alternation of the six groups around the benzene as presented in our depiction of **4**.[9]

In order to distinguish if two conformations exist, or if a single conformation exists that is either a cone or 1,3-alternate, a NOESY spectrum of receptor **4** was recorded at −70 °C (Fig. 5). Before examining the evidence for or against two conformations, we explain the reasons behind assigning chemical shifts. The resonance for **d** correlates with **a** and **e** since they are close in space. One of the **c** protons (labeled as **c**′) correlated stronger with **b** than the other **c** proton (labeled as **c**″). Thus, the proton located adjacent to **b** should be **c**′. One of the **a** protons (labeled as **a**′) correlated stronger with **d** than the other **a** (labeled as **a**″). This supports **a**′ being adjacent to **d**.

Using these correlations and chemical shifts, it is clear that two conformations do not exist. Two **a** peaks (**a**′ and **a**″) correlate with each other, and two **c** peaks (**c**′ and **c**″) also correlate with each other. These correlations suggest that receptor **4** has one conformation at −70 °C rather than existing as a mixture of two different conformations.

Judging from the NOESY spectrum, we conclude that the conformation of receptor **4** at −70 °C is in the cone conformation. There are three pieces of the data that support this conclusion. First, proton **c**″ (one of the diastereotopic **c** protons, see lettering on Fig. 4) correlates stronger with **a**″ but only weakly or not at all with proton **a**′. In a cone conformation, protons **a**″ are always located above protons **a**′ along a horizontal axis through receptor **4**, whereas in a 1,3-alternate conformation protons **a**″ and **a**′ alternate up and down relative to a horizontal axis through the receptor. Therefore protons **c**″ correlate with **a**″ in a cone conformation. In contrast, in a 1,3-alternate conformation, protons **c**″ should correlate with **a**′ which does not occur here. None of these correlations involves protons which undergo chemical exchange, and thus the correlations reflect proximity effects.

Further, there are no NOE cross peaks between **c**″ and **d** protons. The space filling model of receptor **4** indicates that in a cone conformation, **c**″ and **d** are located far away from each other as evidenced by the absence of cross peaks in the NOE. In contrast, in a 1,3-alternate conformation, **c**″ is close in space to **d** on adjacent benzene rings (Fig. 4). These cross peaks would be observed in a 1,3-alternate conformation.

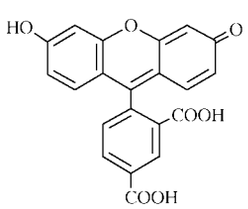
As final evidence, the **c**″ protons are largely shifted downfield (+0.25 ppm) from 25 to −70 °C. Since protons **c**″ are located between adjacent benzene rings in a cone conformation, their chemical shift should be affected by a strong diamagnetic anisotropy effect, leading to a downfield shift. If the conformation were 1,3-alternate, the **c**″ protons would not be affected because the adjacent benzenes alternate up and down.

The azacalixarene **4** has a longer spacer, CH_2–NH–CH_2, between benzene rings than the one methylene found in calixarenes. Due to these longer spacers, azacalixarene **4** should be more flexible than calixarenes. Furthermore, in calixarenes, intramolecular hydrogen bonding among the OH groups on the

benzene rings contributes to the preference of the cone conformation in organic solvents.[14] In water, as is our case, there is no intramolecular hydrogen bonding to stabilize the cone conformation. The increased flexibility and lack of hydrogen bonding could bring the different conformational isomers closer in energy. Nevertheless, **4** is frozen in the cone conformation at low temperature. We attribute this to the ethyl groups attached to the benzene rings, which sterically obligate the secondary amino groups at the lower rim to be oriented in the same direction.

D. Recognition behavior

a) Binding behavior toward indicators. Understanding the binding characteristics of azacalixarene **4** with anionic guests is our next point of interest. We have previously explored a chemosensing technique using a competition assay with fluorescent indicators for the detection of specific guests.[9] The idea is that an indicator complexed with a receptor may be replaced by added guest in solution. Replacement of the indicator leads to a modulation of the fluorescence or absorbance intensity. For this purpose we used 5-carboxyfluorescein (**9**) and 1-hydroxypyrene-3,6,8-trisulfonate (HPTS, **10**) as indicators.



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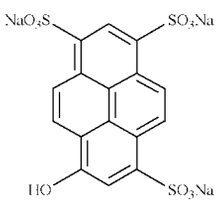


10

Both of these probes are pH indicators which are very sensitive to their surrounding microenvironment.[15,16] We tested whether these indicators bind to receptor **4**. Fig. 6 shows the absorbance change upon addition of receptor **4** to the individual solutions of **9** and **10** (HEPES buffer, 10 mM, pH 7.2). The intensity of λ_{max} (490 nm) of **9** decreased with increasing concentration of receptor **4**, whereas that for **10** increased (Fig. 6). In both cases, UV–Vis spectra revealed an isosbestic point, which supports a one-to-one complex between the probes and the receptor. The association constants for the complexes were determined using the Benesi–Hildebrand method,[17] giving constants of 9.3×10^3 and 50×10^3 M^{-1} for **4** with **9** and **10** respectively.

We also conducted a ^{1}H NMR study to confirm the binding stoichiometry in methanol. The chemical shift of proton **b** was plotted as a function of mole ratio [**4**]/[**10**]. The change in chemical shift decreased linearly with increasing [**10**]. At a one-to-one host–guest ratio, the line cleanly changed to a slope of zero, indicating that **10** binds to receptor **4** in a one-to-one complex with an association constant larger than can be determined using ^{1}H NMR.

b) Binding behavior toward phosphorylated carbohydrates. The fluorescence intensity of **10** decreased with increasing [**4**] when **10** was excited at 390 nm (Fig. 7(a)). This decrease in fluorescence intensity can be explained by a decrease in the protonation state of the hydroxy group on HPTS after binding to the receptor **4**.[16]

As anticipated, based upon the difference in the spectroscopy of **10** when it is bound to **4** *versus* when it is free in solution, addition of inositol triphosphate (IP_3) to a solution of receptor **4** and **10** resulted in an increase of fluorescence (Fig. 7(b)) due to the release of **10** into the solution. Fig. 8 shows the fluorescent change of **10** as a function of guest concentration of four different anionic guests. As anionic guests, we chose IP_3 (**11**), fructose 1,6-diphosphate (**12**), gluconic acid (**13**), and adamantane-1,3-dicarboxylic acid (**14**). From a space filling model, we predicted that these guests could be bound inside the cavity of the cone conformation. The fluorescence intensities

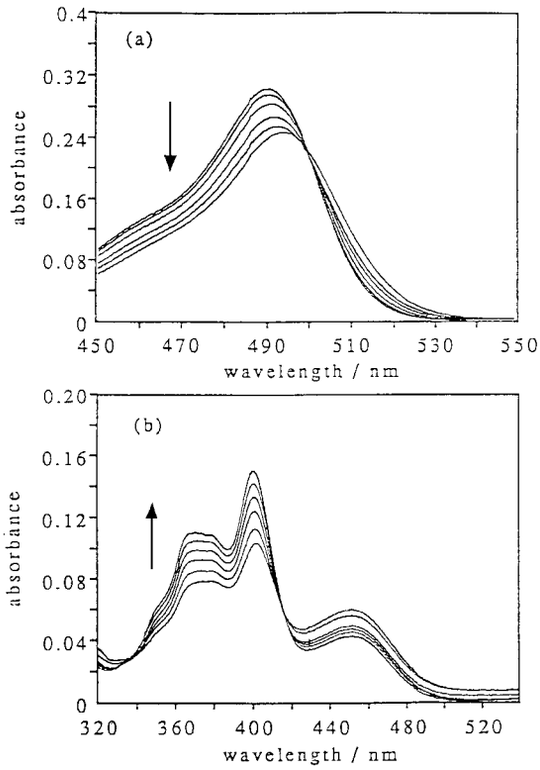


Fig. 6 (a) UV–visible absorption spectra of **9** upon addition of **4**. (b) **10** upon addition of **4**. All in 10 mM HEPES buffer (pH = 7.2), [**9**] = [**10**] = 7.5 μM. [**4**] = 0–50 μM.

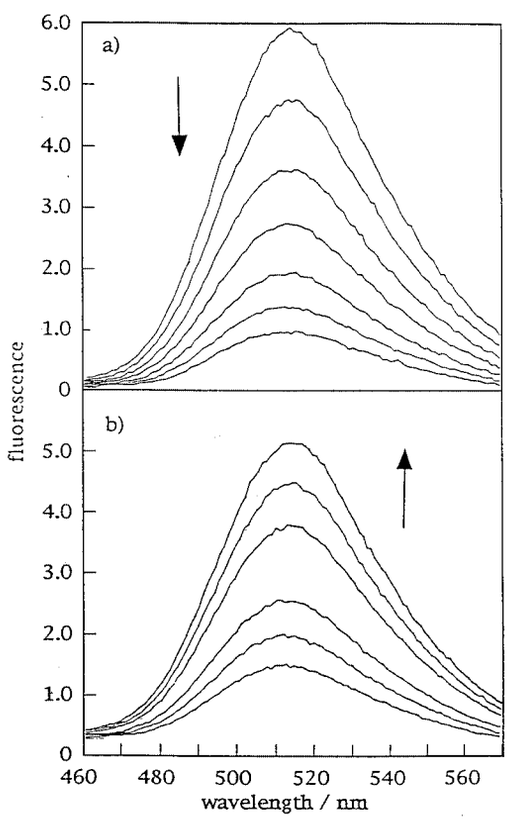


Fig. 7 (a) Fluorescence spectra of **10** upon addition of **4** (0, 1.3, 4, 9, 23, 31, 50 μM). (b) **10** upon addition of IP_3 (0, 3.6, 9.7, 15.7, 22, 40 μM) in the presence of **4**. All in 10 mM HEPES buffer (pH = 7.2). [**4**] = 30 μM, [**10**] = 7.5 μM, Excited at 390 nm.

Table 1 Binding constants (K_a) of anionic guests with receptor **4** in HEPES buffer (10 mM, pH 7.2)

Guest	$K_a/10^3$ M^{-1}
9	9.3
10	50
11	240
12	12
13	3.0
14	<1.0

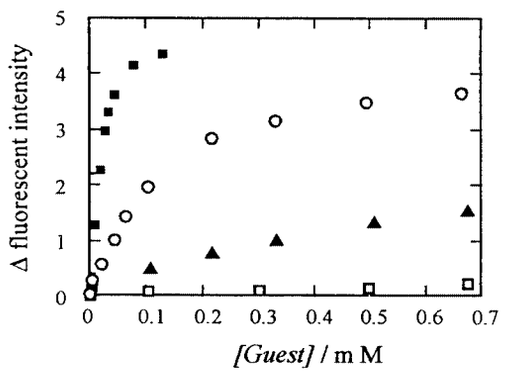
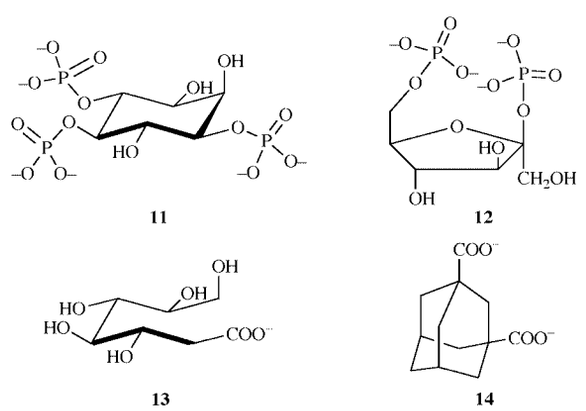


Fig. 8 Fluorescence spectral changes at 450 nm of **10** upon addition of anionic guest in HEPES buffer, 10 mM, pH 7.2. [**4**] = 30 μM, [**10**] = 7.5 μM, Excited at 390 nm: ■ = **11**, ○ = **12**, ▲ = **13**, and □ = **14**.



11 **12**

13 **14**

became saturated with increasing guest concentrations. According to reported procedures treating competition assays,[9,17] these curves were transferred to lines. From the slopes, binding constants were calculated. Table 1 shows the binding constants of receptor **4** for these anionic guests in HEPES buffer, 10 mM, pH 7.2. Although receptors having multiple amino groups tend to lose the specificity for anionic guests[9b,18] and often precipitate, receptor **4** can strongly associate with anionic guests in water.

We investigated the ^{1}H NMR spectra of receptor **4** upon addition of fructose 1,6-diphosphate (**12**) in methanol. Chemical shifts of protons **a**, **b**, **c**, and **d** were followed with increasing concentration of **12** and mole ratio plots created (Fig. 9). The resonances for the methylene protons (**a**) (same lettering system as given in Fig. 4) were largely shifted upfield. However, the resonance for **b** was not shifted significantly. Upfield shifts of the methylene protons on the bottom rim suggest that **12** sits on the bottom rim and binds to the cyclic secondary amines. The lack of a chemical shift in the **b** protons implies that **12** does not contact the primary amines at the upper rim. Macrocyclic polyamines have been reported to provide high affinities for anions such as phosphate ions.[19,20] Only a conformation of receptor **4** can provide the cyclic secondary amines for binding sites because flipping of benzene rings could cause steric

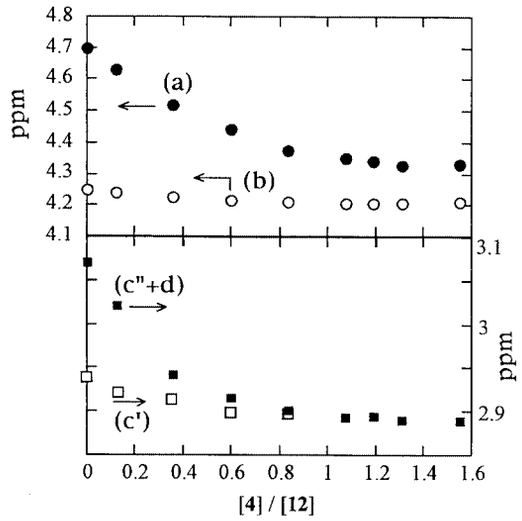


Fig. 9 NMR chemical shifts of receptor **4** upon addition of fructose 1,6-diphosphate (**12**) function of concentration ratio, [**4**]/[**10**]. [**4**] = 8.4 μM. See Fig. 5 for proton positions, (**a**), (**b**), (**c**), and (**d**).

hindrance between the guest and ethyl groups of **4** (see 1,3-alternate conformation as an example).

Upon addition of **12**, the overlapped resonance peak for the **c**″ and **d** protons was shifted upfield, and also the resonance for the **c**′ protons was slightly shifted. This suggests that the **c**″ and **d** protons interact with **12** more strongly than the **c**′ protons. In a cone conformation these three protons face the cavity and the **c**″ and **d** protons are located deeper in the cavity than the **c**′ protons (Fig. 4). This can be explained by an interaction of **12** with receptor at the bottom of the cavity of a cone conformation while the phosphates of **12** are bound to the amines of the lower rim.

Conclusion

A new azacalixarene has been designed and synthesized. Azacalixarene **4** is a flexible molecule at 25 °C. Receptor **4** bound anionic indicators in a one-to-one fashion and the indicators can be replaced by anionic guests. This replacement was detected as changes in both the UV–vis and fluorescence spectra of the anionic indicators. This demonstrates the usefulness of azacalixarenes as receptors for anionic guests. We further demonstrate that the probe competition method can be widely applied to create sensing systems for receptors that do not contain covalent chromophores. Modification of the *N*-substituents is currently being investigated to generate new receptors for the inclusion of organic and inorganic molecules.

Experimental

1) General considerations

All solvents and reagents were purchased from Aldrich Chemical Co., and were used as received. NMR spectra were recorded on 300 or 500 MHz Varian Unity instruments. Chemical shifts were referenced to solvent peaks. UV–vis spectra were recorded on a Beckman DU70 spectrometer and fluorescence spectra were recorded on an SPF-5000 C™ spectrofluorometer. Melting points were measured on a Thomas Hoover capillary melting-point apparatus and are uncorrected. Preparative flash chromatography was performed on Whatman 60 Å 230–400 mesh silica gel. Reactions were run under N_2 atmospheres.

2) Conformational analysis

Azacalixarene **4** as its chloride salt (10 mg) was dissolved in methanol-d_4 and DMSO-d_6. ^{1}H NMR (500 MHz) measurements were carried out at 50 and 25 °C for DMSO-d_6 solutions

and at 25, 0, −30, −70 °C for methanol-d_4 solutions. NOESY spectra were recorded at −70 °C in methanol-d_4. Resonance signals from solvents were used as a standard reference.

3) Binding studies

A solution of receptor **4** (30 μM) was titrated into a 1.5 ml solution of 5-carboxyfluorescein (**9**) or HPTS (**10**) (2 μM) in HEPES buffer (pH 7.2, 10 mM) at 25 °C. Absorbances at 490 nm for **9** and 400 nm for **10** were measured. Standard curves of absorbance as a function of concentration of the receptor were transferred to Benesi–Hildebrand plots.[17] From their slopes, we obtained the binding constants of probes for receptor **4**. As a competition method, concentrated aqueous solutions of anionic guests **11**, **12**, **13**, and **14** were respectively added to a solution of receptor **4** (10 μM) and HPTS (2 μM) in HEPES buffer (10 mM, pH 7.2). The solutions were excited at 390 nm and emission spectra at 513 nm were collected upon addition of anionic guests. Binding constants were obtained according to previously published procedures.[9,13]

An NMR titration was carried out for fructose 1,6-diphosphate. Receptor **4** (5 mg) was dissolved in methanol-d_4 (800 μl) in an NMR tube. Fructose 1,6-diphosphate (35 mg) was dissolved in 1 ml D_2O. Aliquots of the guest aqueous solution were added to the receptor solution in an NMR tube.

Synthesis

[3,5-Bis(bromomethyl)-2,4,6-triethylphenyl]methyl azide (6). 1,3,5-Tris(bromomethyl)-2,4,6-triethylbenzene (**5**) (19.1 g, 44 mmol) and sodium azide (2.2 g, 35 mmol) were dissolved in DMF (100 ml). This mixture was stirred at room temperature for one day. The solvent was removed by a rotary evaporator. The residue was taken up in ether (200 ml) and washed with brine three times. The organic layer was dried with Na_2SO_4, filtered, and evaporated. The crude solid was purified by flash column chromatography with gradient elution of 1% to 5% methanol in DCM. The compound at $R_f = 0.8$ was collected as a white powder. Yield 18%; mp 103 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.47 (2H, s, CH_2N$_3$), 4.58 (4H, s, CH_2Br), 2.88 (6H, m, CH_2CH$_3$), 1.30 (9H, m, CH$_2$CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 15.8, 15.9, 23.2, 23.3, 48.5, 131.1, 133.0, 145.1, 145.5; HRMS(FAB) 401.009 ($C_{15}H_{21}N_3Br_2$, calcd. 401.010).

[23-(Aminomethyl)-3,11,19,27-tetraaza-15,31-bis(azidomethyl)-6,8,14,16,22,24,30,32,33,34,35,36-dodecaethylpentacyclo[27.3.1.1^{5,9}.1^{13,17}.1^{21,25}]hexatriaconta-1(33),5,7,9(34),13,15,17(35),21,23,25(36),29,31-dodecaen-7-yl]methylamine (8). 1,3,5-Triethyl-2,4,6-tris(aminomethyl)benzene (**7**, 1.5 g, 6.0 mmol) and triethylamine (5 ml) were dissolved in CH_2Cl_2 (125 ml). Compound **6** (1.5 g, 15 mmol) dissolved in CH_2Cl_2 (50 ml) was added dropwise for one hour. This ratio of reactants gave the best yields. The mixture was stirred for 2 days at 25 °C. During stirring, a white precipitate formed. The resulting solution was washed with brine three times, and the organic layer was dried with Na_2SO_4, filtered, and evaporated to give a white solid. The crude material was purified by flash column chromatography with 1% methanol in CH_2Cl_2 as eluent. The compound at $R_f = 0.8$ was collected. mp: decomposed over 200 °C; yield 40%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.39 (4H, s, CH_2N$_3$), 3.80 (20H, s, CH_2NHCH_2 and CH_2NH$_3$), 2.84 (24H, m, CH_2CH$_3$), 1.35 and 1.24 (36H, m, CH$_2$CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 48.7, 48.6 (CH_2–NH–CH_2), 48.1 (CH_2N$_3$), 39.5 (CH_2NH$_2$), 22.7 and 22.5 (CH_2CH$_3$), 17.1 and 16.5 (CH$_2$CH_3), 128.7, 133.7, 141.2 and 143.4 (Ar); HRMS(FAB): 981.763 ($C_{60}H_{96}N_{12}$, calcd. 981.764).

[3,11,19,27-Tetraaza-6,8,14,16,22,24,30,32,33,34,35,36-dodecaethyl-15,23,31-tris(aminomethyl)pentacyclo[27.3.1.1^{5,9}.1^{13,17}.1^{21,25}]hexatriaconta-1(32),5(34),6,8,13(35),14,16,21(36),22,24,29(33),30-dodecaen-7-yl]methylamine (4). Compound **8** (160 mg, 0.16 mmol) and triphenylphosphine (500 mg, 1.9 mmol) were dissolved in THF (100 ml) and H_2O (10 ml). The mixture was stirred at 25 °C for one day. The solvent was evaporated and the residue was taken up in CH_2Cl_2 (100 ml) to which was added 0.1 M HCl (100 ml). The aqueous layer was washed CH_2Cl_2 three times and lyophilized twice to give 210 mg of pure white compound. Yield: quantitative; mp: decomposed over 250 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 4.7019 (16H, bs, CH_2NHCH_2), 4.297 (4H, bs, CH_2NH$_3$), 3.0742 and 2.9319 (24H, bs, CH_2CH$_3$), 1.361 and 1.253 (36H, bs, CH$_2$CH_3); ^{13}C NMR (125 MHz, CD_3OD) δ 8.144 (CH$_2$NH$_3$), 26.167 and 27.42 (CH_2CH$_3$), 18.11 and 17.3 (CH$_2$CH_3), 127.451, 129.415 and 149.386 (Ar); HRMS(FAB): 934.062 ($C_{60}H_{101}N_8$, calcd. 934.1059).

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