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Rational Design, Synthesis, and Application of a New Receptor for the Molecular Recognition of Tricarboxylate Salts in Aqueous Media

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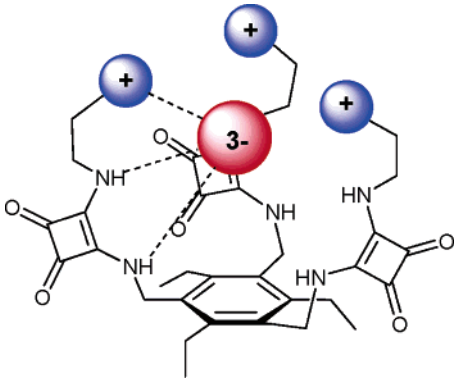

A rational design of a tripodal receptor for the molecular recognition of tricarboxylate salts in aqueous media, based on squaramide, has been performed using high-level DFT calculations (RI-BP86/SVP level of theory) in solution using the COSMO treatment, including some preliminary ab initio calculations at the higher RI-MP2/TZVP level of theory, comparing the ability of squaramide to bind carboxylate salts with two widely used guanidinium salts. The tripodal receptor has been synthesized using a new methodology that has been recently reported by some of us, and its capability of recognizing several mono-, di-, and tricarboxylate salts has been studied experimentally by means of microcalorimetry experiments in a very high competitive media, H_2O:EtOH 1:3. These experiments give enthalpic and entropic data, which are unfortunately scarce in the literature of molecular recognition of anions. Finally, a fluorimetric ensemble of the receptor with fluorescein has been found to be useful for the fluorimetric determination of zinc citrate in a commercial toothpaste using competition assays.

Introduction

Molecules which interact with one another in organized assemblies to perform useful functions are termed supramolecules and are found in their most refined form in biological systems. Advances in supramolecular chemistry[1] have given rise to the preparation of a great deal of molecular receptors which bear adequate functional groups to establish noncovalent interactions with molecular substrates. The use of computational methodologies for the design of such receptor molecules[2] has an important and increasing impact in different fields, such as analytical and medicinal chemistry, and in the development of catalytic substances mimicking enzymes, etc.

The relatively new field of anion coordination chemistry in comparison to cation coordination has become an established

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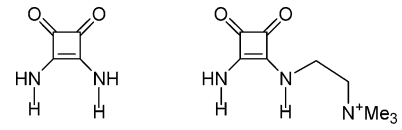
area in supramolecular chemistry, which is obviously reflected in the number of reviews published on this topic.[3] The design of anion receptors is especially challenging because, for instance, anionic species have a wide range of geometries. Therefore, a high degree of design is required to build receptors complementary to the anionic guest, and the receptors must work within the pH range of the anion. However, the number of excellent receptors present in the bibliography demonstrates that most of the challenges have been overcome. Solvent effects also play an important role in controlling the binding strength and selectivity of the anion guest. They are generally dominated by electrostatic interactions. Anions have free energies of solvation that are higher than those of cations;[4] therefore, strong interactions are required in water for a host to efficiently compete with the water molecules which are tightly bonded in the solvation sphere of the anion. The chemistry of life mainly takes place in water; therefore, the design and synthesis of receptors that are competitive in aqueous media are of special importance. Anion hosts active in aqueous solution are working models for natural anion binding systems (spermidine, carboxypeptidase A, phosphate binding protein, etc.), and they have a potential pharmaceutical value.[5]

One important part of the anion coordination chemistry is the selective complexation of carboxylate anions by natural and synthetic hosts,[6] which have interest in bioorganic chemistry because these species are involved in several molecular recognition phenomena of biological interest. For instance, the carboxylate recognition plays an important role in determining the biological activity of the vancomycin family of antibiotics.[7] Synthetic receptors for carboxylate anion recognition are usually based on charged guanidinium or amidinium groups,[8] neutral hydrogen bonding donor groups as amides,[9] ureas, thioureas,[10] and pyrroles,[11] and natural amino acids (biomimetic receptors).[12] Our group has used squaramido-based receptors for the molecular recognition of carboxylic acid and carboxylates.[13] Several theoretical studies have shown the superiority of squaramide over urea or thiourea as hydrogen bonding donor,[14] being one important reason for this, the increase of the aromatic character of the squaramide ring upon complexation of the anion.[15] Recently, some of us have used a combination of squaramide and one or two tetraalkylammonium groups to build receptors for carboxylates, sulfate, and hydrogen phosphate that are efficient in protic media.[16]

In our pursuit of designing receptors capable of competing in aqueous media, in this paper, we report the rational design and synthesis of a new receptor based on the squaramide−ammonium unit, which has been proven to be useful in the recognition of monocarboxylate anions in aqueous media.[13b] This receptor incorporates three squaramide−ammonium units to be used for the molecular recognition of tricarboxylates and very especially in the determination of citrate, a very common anion present in commercial soft drinks and certain medicines, such as sildenafil citrate (Viagra). In addition, an abnormal citrate level in urine has been used for the diagnosis of several diseases, such as prostate cancer and glycogen storage disease (GSD1a);[17] therefore, the development of citrate receptors and sensors is of current interest and significance. The rational design has been performed with the help of computational methods, first comparing the capability of the squaramide−ammonium unit with other charged binding units, such as guanidinium groups (energetic study), and analyzing the geometrical features of the host−guest interaction (geometrical study). Second, we have performed a computational study of the designed receptor with several tricarboxylates to confirm the suitability of the host. The synthesis of the receptor has been performed using a recent improvement that allows the synthesis of the spacer in four straightforward steps. The binding study has included a thermodynamic analysis of the complexes by calorimetric titrations and using an ensemble with fluorescein (fluorescent probe). Recently, two thematically related works on the selective binding of citrate and other tricarboxylates in water have been published by Schmuck et al.[18] and Fabbrizzi et al.,[19] and several studies and synthesis of citrate sensors using europium luminescence have been reported.[20]



squaramide squaramide-ammonium

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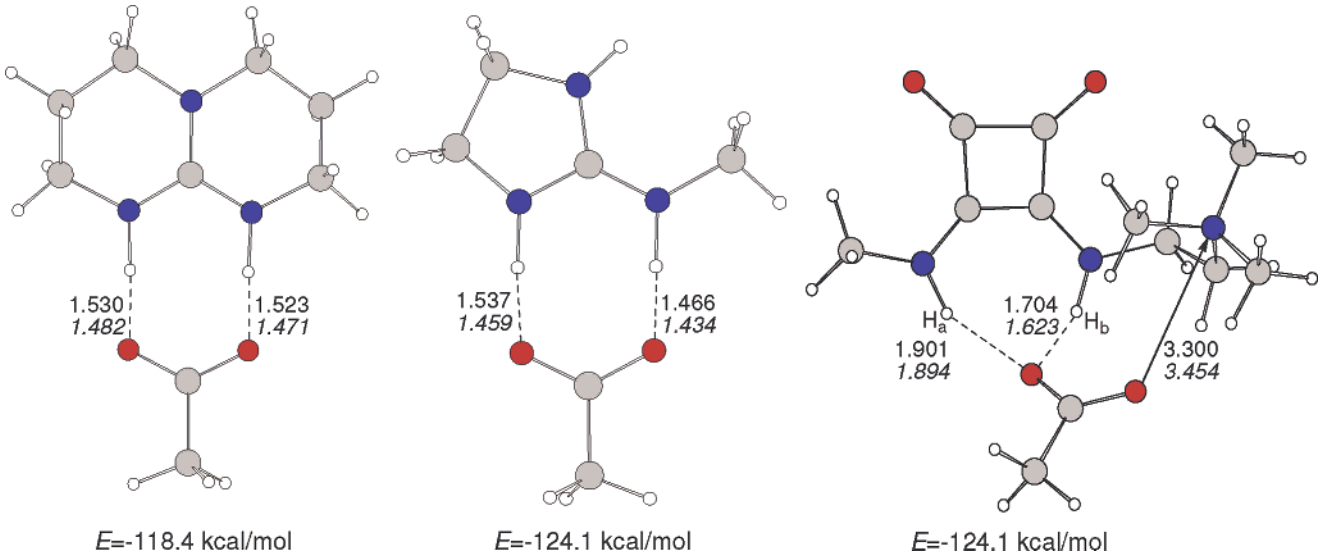

FIGURE 1. RI-MP2/TZVP optimized structures of the complexes between acetate and the three binding units compared in this study and the respective binding energies. The distances obtained at the MARIJ-BP86/SVP level of theory are in italics. Distances are in Å.

Theoretical Methods

The geometries of the receptors, guests, and complexes included in this study were fully optimized at the BP86 density functional level of theory using the SVP basis set (double-ζ with polarization). Because of the size of the receptors and consequently the time-consuming nature of the DFT calculations on these systems, we have used a computationally faster treatment than the traditional DFT method. This method is the multipole accelerated resolution of the identity DFT (MARIJ-DFT),[21,22] which uses an auxiliary fitting basis to avoid treating the complete set of two-electron repulsion integrals providing a fast evaluation of the Coulomb potential for electron densities. The calculation of the model compounds has been also carried out at a higher level of theory. We have used the RI-MP2 method using triple-ζ with polarization basis set (TZVP). The MARIJ-BP86 and RI-MP2 calculations without imposing any symmetry constrain were done using the program TURBOMOLE version 5.7.[23] The MARIJ-BP86 and RI-MP2 methods applied to the study of noncovalent interactions are considerably faster than the BP86 and MP2, respectively, and the interaction energies and equilibrium distances are almost identical for both methods.[24] We have also performed calculations using a continuum solvent model (COSMO)[25] to account for solvation effects. COSMO is a common method for incorporating solvation in the calculation, as energy derivatives are easily obtained and geometry optimization within the continuum is possible. COSMO calculations were performed at the MARIJ-BP86/SVP level and carried out to simulate the solvent mixture H_2O:EtOH 1:3 (v/v) using the default COSMO parameters of the TURBOMOLE/COSMO implementation. The efficiency and application of TURBOMOLE/COSMO for describing solvation effects has been studied by Schäfer et al.[26]

Results and Discussion

Preliminary Calculations. The rational design of a receptor for the molecular recognition of carboxylate salts was started by performing high-level ab initio calculations on some model systems. As stated in the Introduction, some of us have used squaramido-based neutral receptors for the molecular recognition of anions, and we have demonstrated that squaramide is a better hydrogen bonding donor than urea and thiourea. In addition, a series of charged squaramide−ammonium compounds have been used by our group to bind sulfate and hydrogen phosphate in ethanol−water mixtures. In Figure 1, we compare the 1:1 complex between the squaramide−ammonium compound and acetate with the 1:1 complexes of two guanidinium salts widely used in the molecular recognition of carboxylates. It can be observed that, while the guanidinium salts bind the acetate anion through a bidentate hydrogen bonding pattern, the squaramide−ammonium compound binds the acetate anion in a more complicated arrangement. The convergent directionality of the N−H groups in the squaramide molecule favors the simultaneous interaction of both groups with one oxygen atom of the anion, and the second oxygen atom forms three hydrogen bonds with three hydrogen atoms in α position with respect to the nitrogen atom of the ammonium group. From the energetic point of view, the interaction of the monocyclic guanidinium and the squaramide−ammonium compounds with an acetate anion is equivalent. For the bicyclic guanidinium, the computed interaction energy is less favorable by approximately 6 kcal/mol, in qualitative agreement with calorimetric experiments present in the literature, where the association constant obtained for the complex of TBA acetate with the monocyclic guanidinium is higher than that obtained for the bicyclic guanidinium.[27] It

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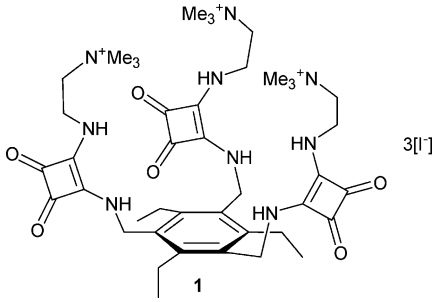
CHART 1. Designed Tripodal Host for Tricarboxylate Guests

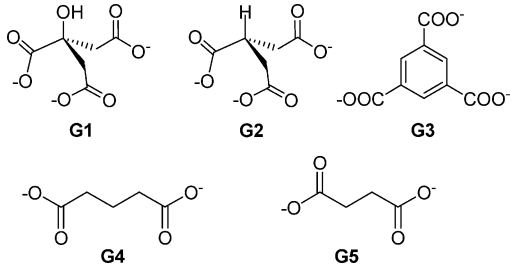


should be mentioned that the interaction energy difference between monocyclic and bicyclic guanidinium complexes with acetate measured experimentally (in DMSO) is lower than that predicted theoretically, probably due to solvent and counterion effects, which are not taken into account in the computation. The capability of the squaramide−ammonium compounds to interact with monocarboxylates has been demonstrated from this initial study since it is similar to the widely used guanidinium salts. An advantage of squaramide derivatives is their facile synthesis from the commercial diethyl squarate in mild conditions (room temperature), where chromatography purification is not needed in the workup procedure since the squaramide derivatives precipitate from the reaction mixture.

Design and Theoretical Analysis of the Receptor. Once demonstrated the potential utility of the squaramide−ammonium binding unit for the molecular recognition of monocarboxylates, we started the design of a tripodal receptor for tricarboxylates based on this binding unit. We chose a spacer that is a classical scaffold and has played an important role in host−guest recognition due to its excellent preorganization; it is 1,3,5-tris-(aminomethyl)-2,4,6-triethylbenzene.[28] Another important factor that has influenced its election is that some of us have recently improved its synthesis from inexpensive benzene (instead of relatively expensive 1,3,5-triethylbenzene) in four straightforward steps.[29] The designed receptor (**1**) is depicted in Chart 1, and it is based on the aforementioned spacer and three squaramide−ammonium binding units, which can be easily attached to the spacer by simple chemistry.

Previous to the synthesis, we have studied computationally the ability of this receptor to interact with di- and tricarboxylate guests using theoretical calculations at the MARIJ-BP86/SVP level of theory. In Chart 2, we represent the di- and tricarboxylate salts studied in this work, which are citrate (**G1**), tricarballate (**G2**), trimesoate (**G3**), glutarate (**G4**), and succinate (**G5**). We have optimized the complexes of the receptor with the different guests, and we have analyzed the host−guest hydrogen bonding interactions for each carboxylate group. The optimized complexes and the relative energies of **G1** and **G2** guests with the receptor are represented in Figure 2. The energies in solution have been computed by performing single point calculations of the gas-phase-optimized structures in a continuum solvation model (COSMO). It can be observed that the behavior of both complexes, regarding the binding mode of the guest, is the same in solution. As depicted in Figure 2, both guests prefer an interaction pattern with the host where the OH/H group/atom is pointing into the cavity (*endo*). In both complexes,

CHART 2. Anionic Guests Used in the Theoretical Study: Citrate (G1), Tricarballate (G2), Benzene-1,3,5-tricarboxylate (trimesoate) (G3), Glutarate (G4), and Succinate (G5)



each carboxylate group adopts a binding pattern with the squaramide−ammonium group similar to that observed in the model system studied in the preliminary calculations, indicating that the flexibility of the receptor is adequate for interacting with the guests.

The optimized structure of the complex of the trianion of the trimesic acid (**G3**) and the receptor is present in Figure 3 from two points of view. The optimized complex has approximately a C_3 symmetry axis. An additional driving force for the formation of this complex may be the establishment of a stacking interaction between the aromatic rings of the spacer and the guest. The computed distance in the complex is 4.7 Å, which is larger than the typical aryl−aryl stacking distance observed in crystal structures, 3.6−3.7 Å.[30] Therefore, this additional interaction is expected to have a negligible influence in the binding properties of this guest.

Dicarboxylate guests (dianionic forms of glutaric and succinic acids) **G4** and **G5** have been studied for comparison purposes. Their optimized complexes with **1** are present in Figure 4. In contrast to the rest of the complexes, where each carboxylate group interacts with one binding unit of each arm of the receptor, in these two complexes, one carboxylate interacts with two binding units and the other with one binding unit in order to establish the maximum number of hydrogen bonds.

The optimized geometries of dicarboxylate guests shown in Figure 4 allow one to predict a better binding constant for **G4** than for **G5** since the latter presents one carboxylate group at 4.20 Å from the tetraalkylammonium group while all three $N^+R_4\cdots O^-$ distances in the **1•G4** complex are shorter than 4.0 Å.

Synthesis of the Receptor. As stated in the Introduction, we have used a practical synthetic procedure, starting from inexpensive benzene, that yields 1,3,5-tris(aminomethyl)-2,4,6-triethylbenzene (**3**) in four steps. This procedure has recently been published by some of us,[29] and it is shown in the first part of Scheme 1. The other key intermediate is **2**, which is easily prepared in high yield (98%) from diethylsquarate and *N*,*N*-dimethylethane-1,2-diamine in ether at room temperature. The purification of this product is very easy since it precipitates in ether and chromatography is not required. The preparation of **4** is also carried out at room temperature in ethanol and, similar to the previous step, chromatography is not needed since **4** precipitates (complete precipitation requires the addition of pentane; see Experimental Section). The yield of this step is 83%. It is worth mentioning that the total synthesis of **4** from benzene and diethylsquarate is done in mild conditions of temperature, high yields, and inexpensive starting products,

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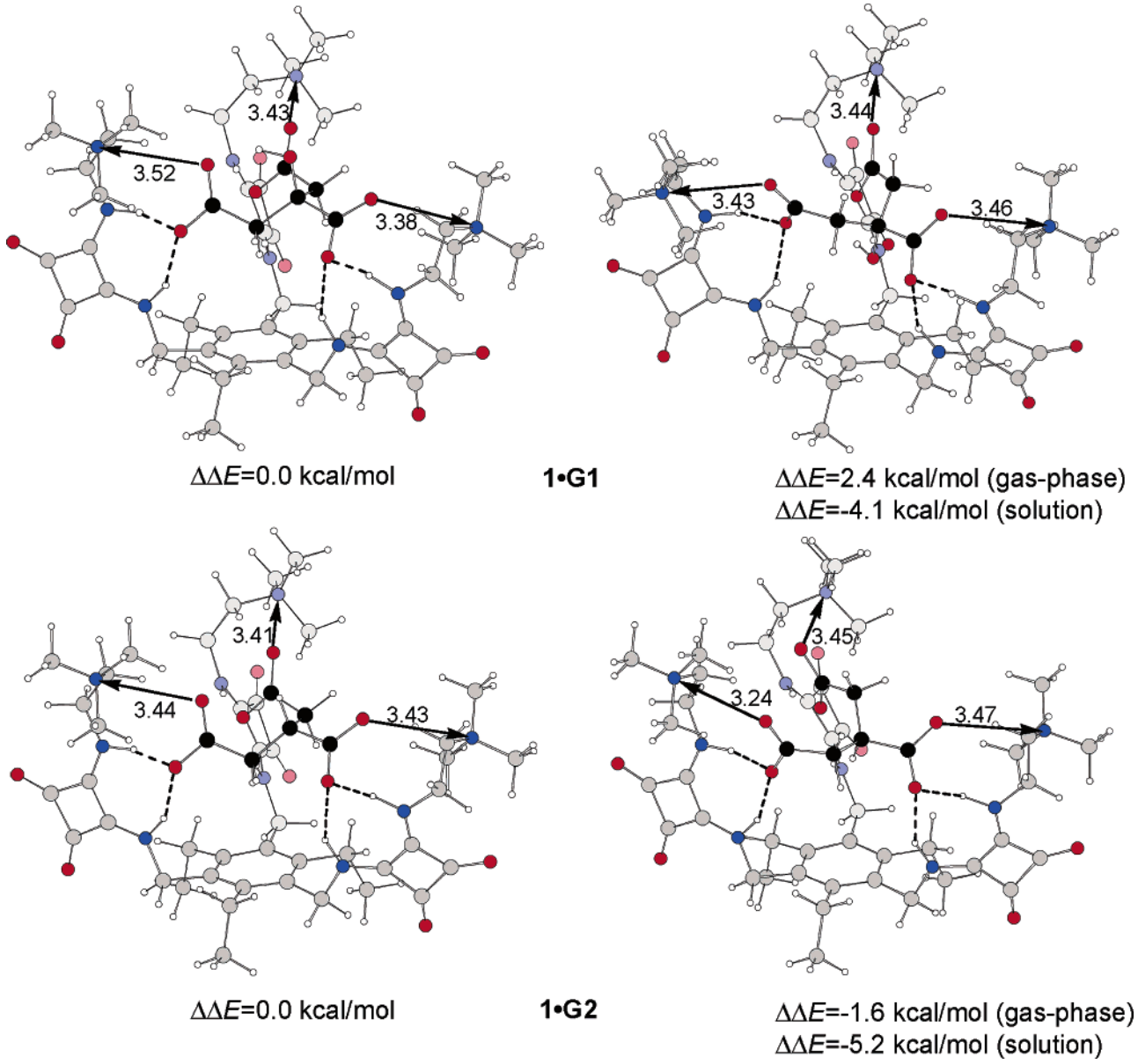

FIGURE 2. MARIJ-BP86/SVP optimized structures and relative energies of the complexes between **G1**/**G2** and the receptor. The carbon atoms of the guest are represented in black. The N−H···O hydrogen bonds of two of the three carboxylates are represented by dashed lines (the hydrogen bonds of the third carboxylate unit have been omitted for clarity). Distances are in Å.

satisfying three principles of "green chemistry", that is, atom economy, energy efficiency, and reduction of derivatives.[31] Only the final step requires refluxing conditions in a DMF/acetone mixture (1:2 v/v) to achieve the exhaustive methylation of **4** with methyl iodide, yielding receptor **1** (94%).

Binding Properties of the Receptor. Isothermal titration calorimetry (ITC)[32] was utilized to study the association between **G1**−**G5** guests and receptor **1**. This technique provides a measure of association strength, stoichiometry of binding, as well as thermodynamic parameters from a single experiment. All host−guest complexes were studied in an aqueous mixture of H_2O:EtOH 1:3 (v/v). To regulate the pH of the aqueous mixtures, the 1 M Tris-HCl buffer system has been used (Tris is tris(hydroxymethyl)aminomethane), which provides an apparent pH of approximately 9, required to ensure the existence of the anionic form of the assayed guests. In addition, the association between **G1** and **1** was also studied in a H_2O:EtOH 1:1 mixture. As model examples of calorimetric titration, we show in Figure 5 the result obtained for the titrations of **G1** and **G3**. On the left, the heat evolving with each injection over the time is displayed. A binding isotherm is generated by integrating the peaks, which are corrected by the heat of dilution generated in a separate experiment. The nonlinear least-squares fit of the subtracted curve using a one-site binding model is shown (on the right). In one case (**G1**), the binding isotherm corresponds to a strong, exothermic 1:1 complex. The stoichiometry is evident from the inflection point of the curve at one equivalent. Very similar curves were obtained for the rest of complexes, apart from **G3**, where a different behavior has been found, and it is shown on the bottom of Figure 5. This opposite behavior will be discussed below.

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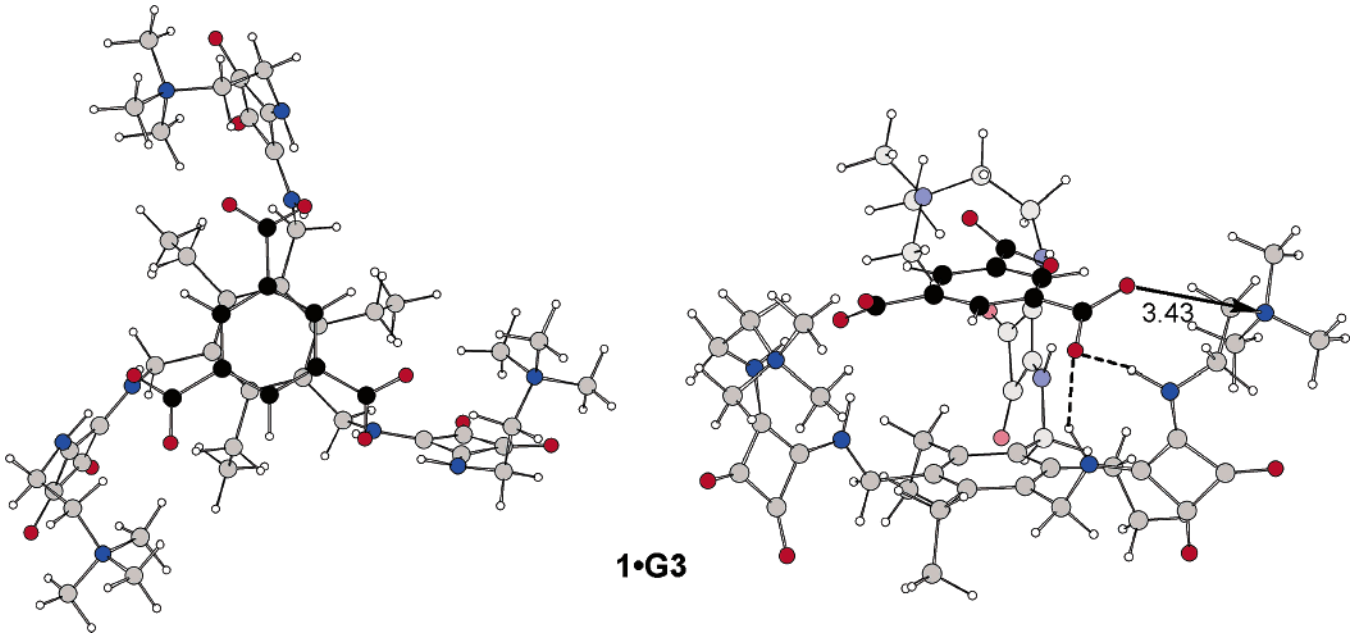

FIGURE 3. Zenithal and perspective views of the optimized complex between **G3** and the receptor. The carbon atoms of the guest are drawn in black. Distance is in Å.

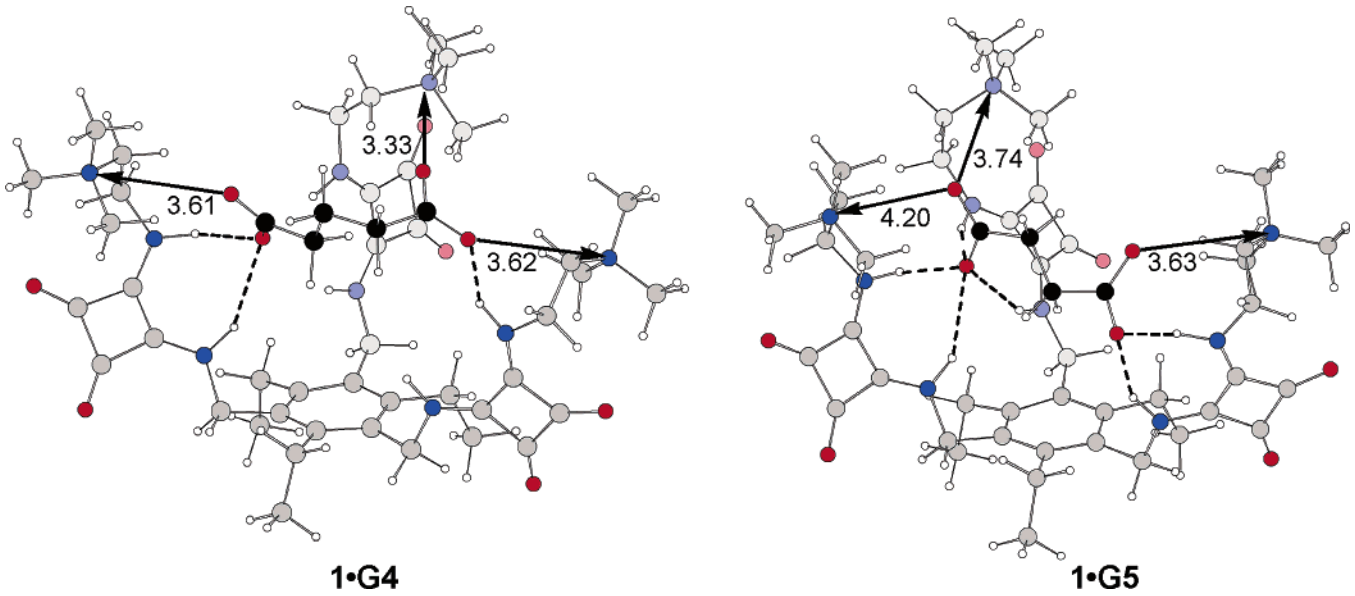

FIGURE 4. MARIJ-BP86/SVP optimized structures of the complexes between **G4**/**G5** and the receptor. The carbon atoms of the guest have are in black. The N−H···O hydrogen bonds are represented by dashed lines. Distances are in Å.

In Table 1, we summarize the results derived from the ITC titration curves of **G1**−**G5** anions and receptor **1**. It can be observed that in all cases, apart from **G3**, the binding process is clearly exothermic and in all cases is entropically favored. For all complexes, the value of the stoichiometry parameter "*n*" ranges between 0.9 and 1.1, in good agreement with a 1:1 binding mode. The high association constants obtained for the tricarboxylate guests are a clear indication of the binding ability of the receptor in a competitive medium. For **G1**, we have also determined the association constant in a mixture of H_2O:EtOH 1:1. The increase in the water ratio, from 25 to 50%, gives rise to a decrease in the magnitude of the association constant by 1 order of magnitude, from 1.1×10^5 to 3.9×10^4 M^{-1} (Table 1, entry 2). Moreover, the association constant is modestly dependent on the counterion of the guest. For instance, when the sodium salt of **G1** is used, the association constant experiences a moderate decrease (Table 1, entry 3). The experimental association constants determined for dicarboxylate guests **G4** and **G5** are smaller, especially **G5**, which is 2 orders of magnitude lower than that of **G4** (entries 6 and 7). In one case, we have further characterized the complex by mass spectrometry. In Figure 6, the electrospray ionization (ESI+) mass spectrum of a 1:1 mixture of receptor **1** with Na_3citrate in H_2O:EtOH 1:3 (v/v) is shown. It presents signals at m/z = 982.5295 amu, corresponding to a $[\mathbf{1{\bullet}G1}+H]^+$ species, and at m/z = 1004.5309 amu, which corresponds to a $[\mathbf{1{\bullet}G1}+Na]^+$ species, confirming the formation of the proposed 1:1 complex.

SCHEME 1. Synthetic Route to 1 from Benzene and Diethylsquarate

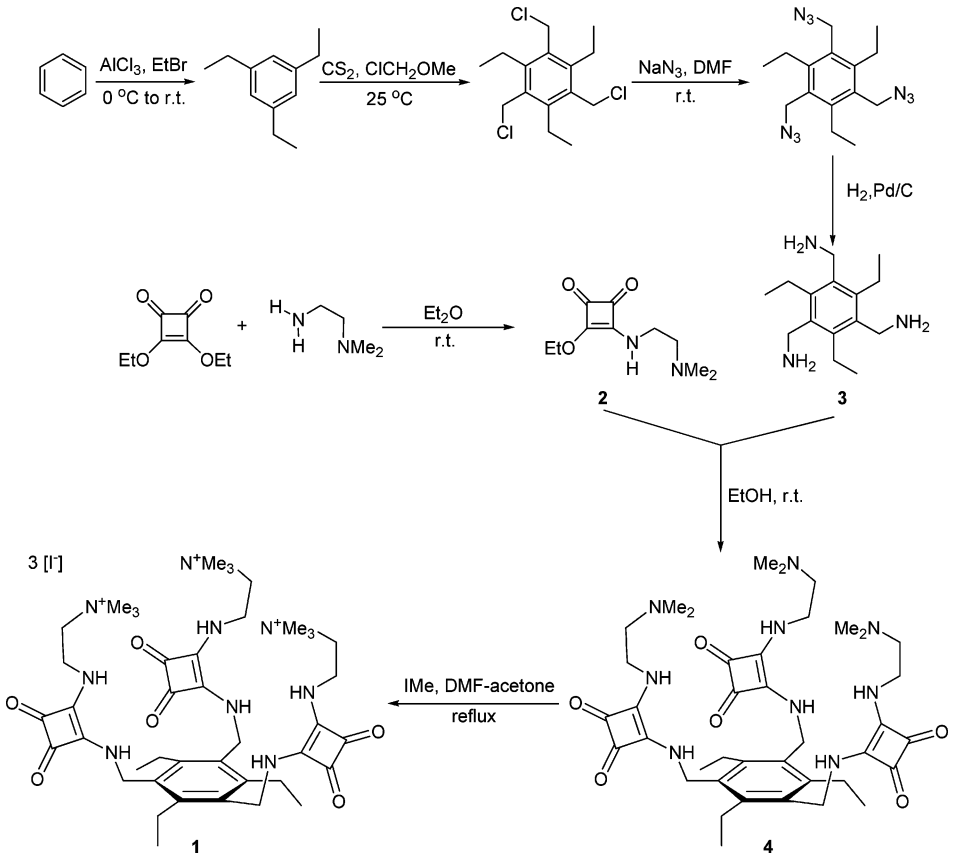

TABLE 1. Binding Constants (K_{as}, M^{-1}), Association Enthalpy (ΔH, kcal/mol), and Association Entropy ($T\Delta S$, kcal/mol) for Guests G1−G5 and Receptor 1 in H_2O:EtOH 1:3 v/v at 294 K

entry	complex	K_{as}	ΔG	ΔH	$T\Delta S$	n
1	**1•G1**	$(1.1 \pm 0.1) \times 10^5$	−6.7	−2.9	3.9	0.9
2	**1•G1**[a]	$(3.9 \pm 0.4) \times 10^4$	−6.1	−2.8	3.3	0.9
3	**1•G1**[b]	$(8.2 \pm 0.6) \times 10^4$	−6.6	−2.3	4.3	1.0
4	**1•G2**	$(1.5 \pm 0.2) \times 10^5$	−6.9	−1.9	5.0	0.9
5	**1•G3**	$(4.5 \pm 0.5) \times 10^4$	−6.2	2.3	8.5	0.9
6	**1•G4**	$(2.2 \pm 0.2) \times 10^4$	−5.8	−0.4	5.4	0.9
7	**1•G5**[c]	$\sim 2.8 \times 10^2$	~ -3.3	–	–	–
8	**1**•isocitrate[b]	$(1.5 \pm 0.2) \times 10^4$	−5.6	−0.6	5.0	1.1
9	**1**•AcO^-[c]	$<1.0 \times 10^2$	–	–	–	–

[a] H_2O/EtOH (1:1 v/v). [b] Sodium salt. [c] Excessively small for being measured by ITC.

Complexes of higher stoichiometry were not found in the ESI+ mass spectrum.

In Table 1, we have also included the results of two experiments that have not been studied theoretically. First, the association constant of receptor **1** with isocitrate (entry 8), which gives a value of K_{as} 6-fold lower than that of citrate in the same conditions (entry 3). The only difference between both guests is the location of the hydroxyl group. To give an explanation to this fact, we have computed the solvation energy of both guests using COSMO. As a result, the computed solvation energy of isocitrate is 1.1 kcal/mol more negative than **G1**, which explains the difference in the association constant since it comes principally from the ΔH term (see Table 1). The association constant of **G4** (dicarboxylate anion) is similar to that determined for isocitrate. This result can be considered as a drawback for the selectivity of the receptor toward tricarboxylate guests. However, a direct comparison between both experiments cannot be done since the counterion is different. Second, the experiment using acetate as guest (entry 9) indicates that the complexation of **1** with monocarboxylate anions is not favored.

The ability of the squaramide−ammonium binding block to interact with a carboxylate moiety in aqueous media has been confirmed experimentally, giving reliability to the theoretical study. It is worth mentioning that the complexation of **G3** is entropy-driven. The ΔH term is positive, indicating that the complexation process is endothermic. In polar solution, supramolecular aggregation can be endothermic due to the reorganization of the solvent upon complexation.[33] The positive value obtained for the enthalpy is ascribed to the energy needed to desolvate the charged groups, which overrides the negative enthalpic contribution due to the formation of the complex. Surprisingly, this effect is only observed in the **1•G3** complex. A likely explanation is that the **1•G3** complex is different from **1•G1** and **1•G2** in terms of the cavity that is formed between the aromatic ring of the spacer of the host and the aromatic ring of the guest (see Figure 3). This cavity, which is probably desolvated, is not generated in the **1•G1** and **1•G2** complexes and provokes an enthalpic−entropic compensating effect, that is, an enthalpically unfavorable process due to the desolvation of solvent molecules and an entropically favorable process due to the release of highly ordered solvent molecules to the bulk solvent. This explanation has been also used in guest encapsulation processes where positive values of ΔH and ΔS are observed

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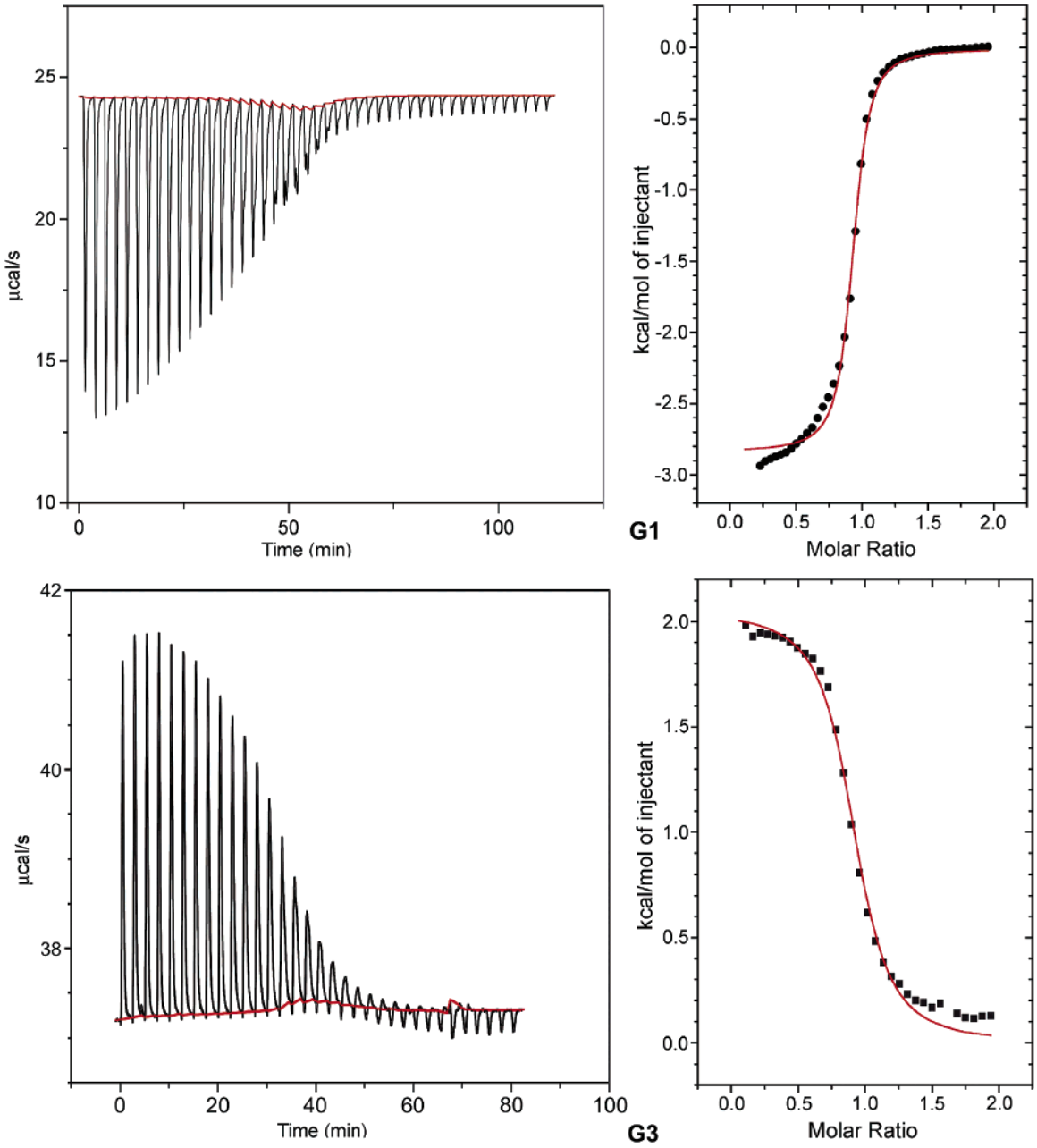

FIGURE 5. Top: on the left, the heat evolved with each injection of **G1** is shown. On the right, the ITC binding curve from addition of **G1** to **1** in H_2O:EtOH 1:3 at 294 K is represented. Bottom: on the left, the heat evolved with each injection of **G3** is shown. On the right, the ITC binding curve from addition of **G3** to **1** in H_2O:EtOH 1:3 at 294 K is represented.

in aqueous medium,[34] in cleft-type receptors for dicarboxylates,[35] and in the molecular recognition of tetraanionic peptides.[36] In fact, the ITC experiment (Table 1, entry 5) shows a positive variation of ΔH and a very important contribution of $T\Delta S$ that compensates the endothermicity of the process. As a result, a value of ΔG comparable to the that of the rest of tricarboxylate guests is observed. Moreover, another plausible explanation arises from the fact that **G3** is more rigid than **G1** and **G2** and the larger entropic contribution in **G3** complexation may possibly come up from the entropic conformation cost induced upon complexation of the more flexible anions **G1** and **G2**. Last, for dicarboxylate guests **G4** and **G5**, the prediction of the theoretical calculations was that **G5** is worse than **G4** because one electrostatic interaction is not effective in the **1•G5** complex, as shown in Figure 4, in complete agreement with the experimental results.

For the sake of comparison, some recent works on the same topic must be mentioned: first, a tricationic guanidiniocarbonyl pyrrole receptor that binds citrate and other tricarboxylate anions with very high association constants of $K_{as} > 10^5\ M^{-1}$ in water determined by UV spectroscopy;[18] second, a Cu-containing trifurcate receptor that recognizes citrate in water. The negatively

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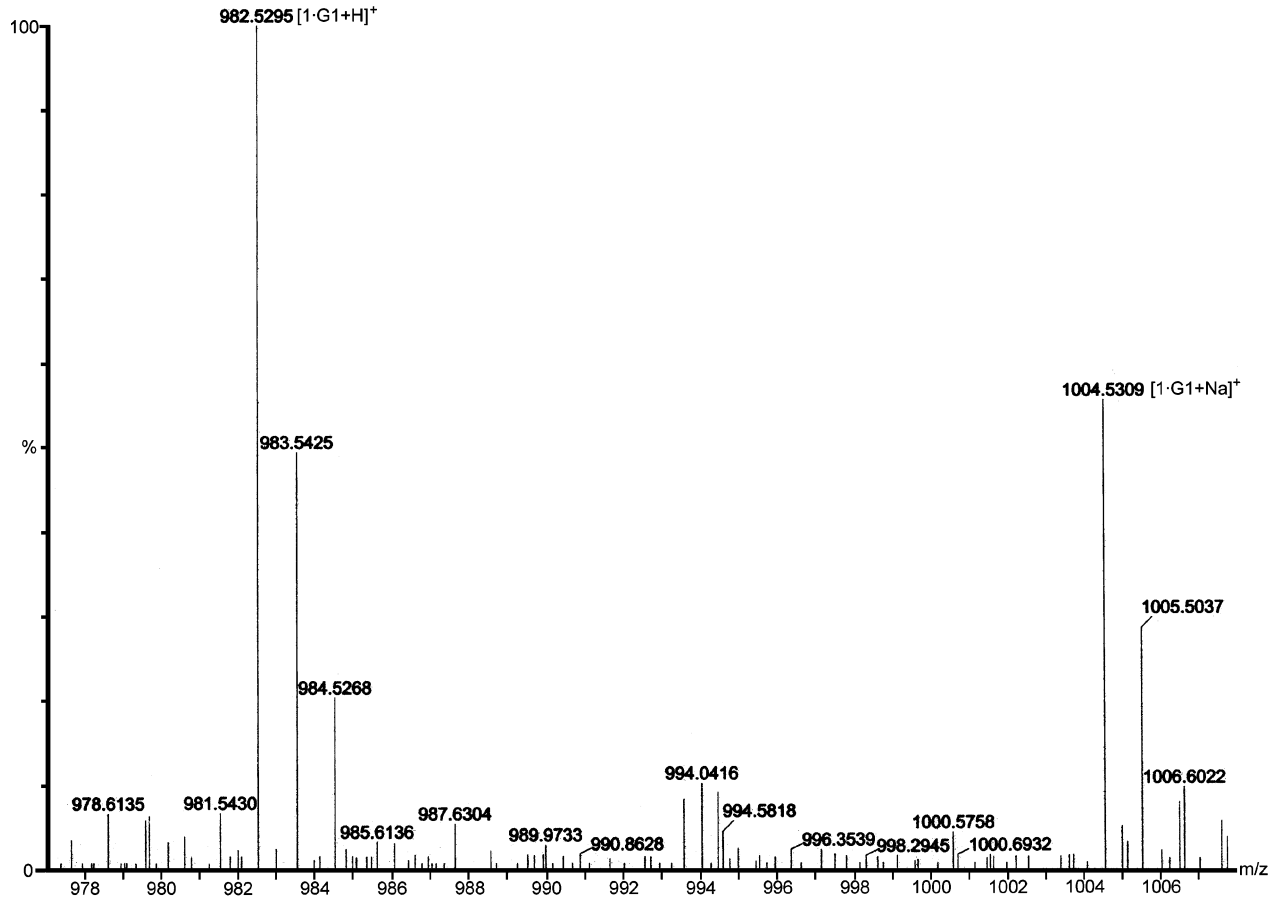

FIGURE 6. ESI+ HRMS spectrum of the complex of receptor **1** with **G1** is shown. The peaks corresponding to the [**1**•**G1**+H]$^+$ and [**1**•**G1**+Na]$^+$ species are labeled.

charged oxygen atoms of the guest interact with three coordinatively unsaturated Cu^{II} centers.[19] The association constants are also greater than 10^5 M^{-1} in water. Unfortunately, in both works, ITC experiments were not reported.

Fluorimetric Assays of 1 with Citrate. Competition assays can be used as a relatively simple method for determining association constants. The introduction of an indicator to the receptor establishes an equilibrium of binding between the indicator (I), the receptor (H), and the I−H complex, resulting in a particular optical response. The addition of the guest (G) to the I−H ensemble perturbs the equilibrium. This change in the equilibrium is dependent on the degree of association between G and H. This approach is widely present in the literature and allows synthetic receptors to act as sensors without introducing additional covalent architecture.[37] Taking advantage of this approach, we have used a sensing ensemble of **1** and fluorescein. Addition of receptor **1** to a solution of fluorescein disodium salt in 25% (v/v) water in ethanol results in a **1**−fluorescein ensemble that is not fluorescent as a consequence of an effective quenching of the fluorescein emission band in the complex, probably due to a quenching produced by the squaramide rings of the receptor via a photoinduced electron transfer (PET) process. First, we have determined the association constant of **1** with fluorescein by measuring the change in the fluorescence intensity of fluorescein in the presence of increasing amounts of **1** in the same conditions used for the calorimetric experiments (ITC), that is, 25% (v/v) water in ethanol with 1 M Tris-HCl buffer. The association constant determined by titration for the formation of the **1**−fluorescein ensemble was found to be $K_{as} = 6.3 \times 10^3$ M^{-1} using a 1:1 binding algorithm. This binding constant, which is about 2 orders of magnitude lower than the K_{as} obtained for citrate using the same conditions by ITC experiments, allows the use of competition assays for evaluation of citrate in aqueous solution. Second, we have obtained a calibration plot at $\lambda = 525$ nm (maximum of fluorescence emission band) for the **1**−fluorescein ensemble upon addition of citrate; see Figure 7 (left). The evolution of the intensity of the emission band upon incremental additions of citrate is shown the same figure, on the right. The progressive addition of citrate displaces the fluorescein from the receptor, restoring the original fluorescence and signaling the presence of citrate.

To check the potential applicability of the **1**−fluorescein ensemble for the fluorimetric quantitative determination of citrate, we have used it for the quantitative determination of zinc citrate in a commercial toothpaste (GINGI KIN B5).[38] The sample was prepared suspending 0.3 g of paste in 5 mL of

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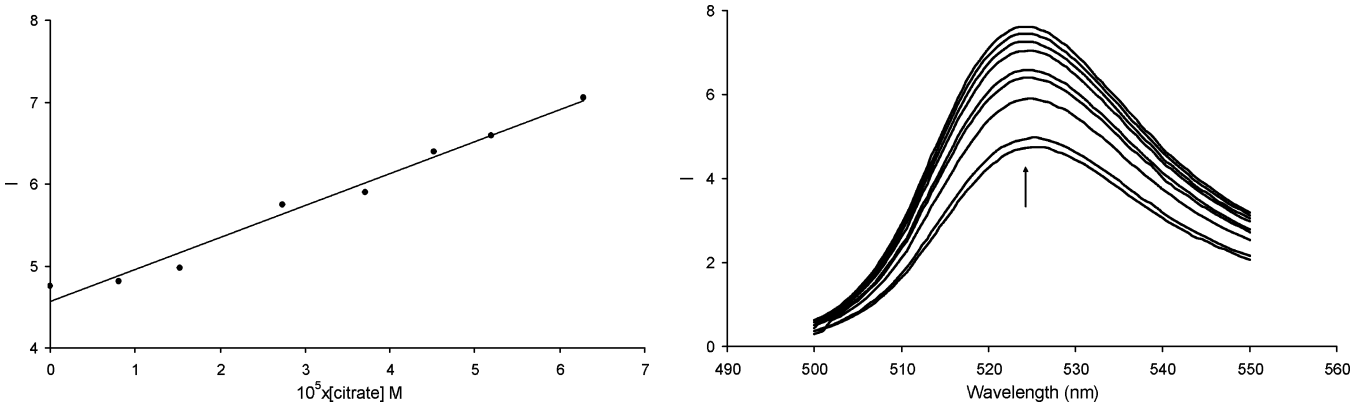

FIGURE 7. On the left: calibration plot at 525 nm for the ensemble of **1** and fluorescein upon addition of citrate (25% water in ethanol (v/v), 1 M Tris-HCl). On the right: fluorescence spectra obtained for the ensemble of **1** and fluorescein, showing the progressive increase in the emission intensity (*I*, arbitrary units) as citric acid is added.

Millipore water, and the mixture was sonicated for 5 min in a glass screw-top vessel and followed by centrifugation to eliminate the solids in suspension. The concentration of citrate in the sample was determined from the calibration plot (see Figure 7, left). The predicted concentration of citrate was found to be 0.91 ± 0.01 mM, which is the average value plus the standard deviation of three measurements. The real concentration obtained from the composition of the commercial toothpaste was 1.01 mM, which is in agreement with the concentration obtained using indicator displacement assays of the **1**−fluorescein ensemble. It is worth mentioning that it is compatible with other compounds present in the composition of the toothpaste, such as sodium fluoride, 5-chloro-2-(2,4-dichlorophenoxy)-phenol (triclosan), provitamine B_5 (2,4-dihydroxy-*N*-(3-hydroxypropyl)-3,3-dimethylbutanamide, and xylitol (1,2,3,4,5-pentol), emphasizing the selectivity of **1**.

Conclusion

In summary, we have used DFT and ab initio calculations to design a tripodal receptor that is able to form stable complexes with tricarboxylate anions. We have synthesized it using "green" chemistry and evaluated its binding properties using ITC titrations with several guests, and we have determined its association constants. The calorimetric experiments also give information about the thermodynamic characteristics of the complexation, which is favorable both enthalpically and entropically, apart from **G3**, which presents an endothermic complexation process.

In addition, we have shown that a sensing ensemble of **1** and fluorescein is able to determine the citrate concentration of a commercial toothpaste. This shows that this class of receptors can be easily applied, using competition assays, for the determination of citrate. Finally, the study demonstrates that rational design can lead to selective receptors for tricarboxylate anions (hydrophilic guests) in highly competitive media.

Experimental Section

Titration Conditions: 40−45 injections (6 μL each) of a 8−10 mM solution of the anion (**G1**−**G5**), in H_2O:EtOH 1:3 (v/v) 1 M Tris-HCl buffer system (apparent pH ∼ 9), were introduced into a sample cell at 294 K containing 1.5 mL of a 1.0 mM solution of **1** in H_2O:EtOH 1:3 (v/v) 1 M Tris-HCl buffer system. The heats of dilution were subtracted prior to data analysis by Origin MicroCal software. In all cases, the *c* parameter, defined as $c = K_a[\mathbf{1}]_t n$, was kept between 10 and 1000. Errors were calculated at a confidence level of 95%. K_{as}, ΔH, and ΔS were obtained at 294 K by curve fitting using Origin 5.0 software as implemented by MicroCal.

Synthesis of 3-(3-(Dimethylamino)ethylamino)-4-ethoxy-3-cyclobutene-1,2-dione (2): This compound was prepared by dropwise addition of 3-(dimethylamino)ethylamine (0.388 g, 4.41 mmol) in diethyl ether (20 mL) to a solution of diethylsquarate (0.5 g, 2.94 mmol) in diethyl ether (3 mL). The precipitated was collected from the reaction mixture by filtration, washed with cold diethyl ether (2 × 10 mL), and dried, yielding squaramide **2** as a white solid (94% yield). Mp = 107−110 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.44 (br s), 7.27 (br s), 4.75 (d, J(H,H) = 6.6 Hz, 2H), 3.74 (br s, 0.7 H), 3.48 (br s, 1.3 H), 2.51 (br s), 2.25 (s, 6 H), 1.45 (t, J(H,H) = 7.1 Hz, 3H) ppm. ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 189.5, 183.3, 177.6, 172.8, 69.8, 59.2, 58.5, 45.3, 42.0, 41.6, 16.0. FTIR (KBr): ν = 3172, 2976, 2940, 2824, 2766, 1801, 1699, 1596, 1332, 1039 cm^{-1}. HRMS-ESI$^+$ found *m*/*z* 213.1239, calculated for $C_{10}H_{16}N_2O_3$ M^+ 213.1239.

Synthesis of 1,3,5-Tris[(3-aminomethyl)-4-(2-*N*,*N*-dimethylaminoethylamino)-3-cyclobutene-1,2-dione]-2,4,6-triethylbenzene (4): A solution of 1,3,5-tris(aminomethyl)-2,4,6-triethylbenzene **3** (0.22 g, 0.89 mmol) in ethanol (20 mL) was added dropwise to a solution (0.66 g, 3.1 mmol) of **2** in ethanol (20 mL). The reaction mixture was stirred at room temperature for 15 h. The precipitate was collected from the reaction mixture by filtration, washed with cold ethanol (2 × 10 mL), and dried, yielding the desired product **4** (0.55 g, 83%). Mp > 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.56 (s, 3H), 7.38 (s, 3H), 4.98 (d, J(H,H) = 4.5 Hz, 6H), 3.69 (q, J(H,H) = 5.6 Hz, 6H), 2.84 (q, J(H,H) = 7.3 Hz, 6H), 2.47 (t, J(H,H) = 5.6 Hz, 6H), 2.23 (s, 18H), 1.22 (t, J(H,H) = 7.3. Hz, 9H) ppm. ^{13}C NMR (75.4 MHz, DMSO-d_6): δ = 188.3, 173.3, 172.6, 149.3, 138.3, 64.9, 50.8, 45.9, 28.5, 22.3 ppm. FTIR (KBr): ν 3288, 3202, 2955, 1801, 1641, 1576, 1532 cm^{-1}. HRMS-ESI$^+$ found *m*/*z* 748.4515 (0.7 ppm), calculated for $C_{39}H_{58}N_9O_6$ M^+ 748.4510.

Synthesis of 1,3,5-Tris[(3-aminomethyl)-4-(2-*N*,*N*,*N*-trimethylammoniumethylamino)-3-cyclobutene-1,2-dione]-2,4,6-triethylbenzene triiodide salt (1): MeI (40 μL, 0.12 mmol) was added via syringe to a solution of **4** (0.10 g, 0.13 mmol) in acetone (20 mL) and DMF (4 mL). This solution was heated to reflux for 12 h under argon atmosphere. The precipitate was collected from the reaction mixture by filtration, washed with cold acetone (2 × 10 mL), and dried, yielding the desired product **1** (0.24 g, 94%). Mp = 260−262 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.65 (s, 3H), 7.39 (s, 3H), 4.98 (s, 6H), 4.07 (q, J(H,H) = 6.5 Hz, 6H), 3.64 (t, J(H,H) = 6.5 Hz, 6H), 3.23 (s, 27 H), 2.84 (q, ^3J(H,H) =

(38) GINGI KIN B5. Laboratorios Kin S.A., Barcelona, Spain (www.kin.es).

7 Hz, 6H), 1.22 (t, J(H,H) = 7 Hz, 9H) ppm. ^{13}C NMR (75.4 MHz, DMSO-d_6): δ = 188.7, 188, 173.1, 172.8, 149.5, 137.8, 70.5, 58.5, 43.2, 36.4, 28.4, 22 ppm. FTIR (KBr): ν 3463, 3318, 3216, 2965, 1802, 1654, 1541 cm^{-1}. HRMS-ESI$^+$ found m/z 1046.3263 (3.6 ppm), calculated for $C_{42}H_{66}N_9O_6I_3$ [M − I]$^+$ 1046.3226.

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Supporting Information Available: General experimental methods, Cartesian coordinates and energies of MARIJ-BP86/SVP fully optimized complexes **1•G1**−**1•G5**, ITC binding curves of the complexation processes not shown in Figure 5 (Figures S1−S6), and ^{1}H NMR spectra of compounds **1**, **2**, and **4** (Figures S7−S9). This material is available free of charge via the Internet at http://pubs.acs.org.

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