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Simple squaramide receptors for highly efficient anion binding in aqueous media and transmembrane transport

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A family of acyclic squaramide receptors (**L₁**-**L₅**) have been synthesised with the aim to bind anions in competitive solvent mixture and to evaluate how the presence of different heteroatoms on the aromatic substutent of the squaramide scaffold could affect the affinity towards anions . Receptor **L₂**, bearing an indole moiety (i.e. an additional and highly efficient H-bond donor group) was found to be the most efficient receptor of the series and was able to bind anions with high stability constants even in a mixture of DMSO-d_6/25 % water. **L₂** was also the only member of the series able to mediate transmembane anion transport

Anions play a crucial role in many biological and environmental processes. Thus, anion recognition, sensing and monitoring are high-impact goals of scientific interest in basic and applied fields.[1-5] During the past decades a wide range of synthetic neutral receptors able to interact with anion species has been reported. Amides, sulfonamides, ureas, thioureas and, more recently, selenoureas[6, 7] receptors exert their host-guest properties towards anions by forming hydrogen bonds through their NH moieties.[8]

In particular, squaramides are an important family of synthetic neutral anion receptors that are the subject of on-going studies.[9] Indeed, compared with the analogous ureas and thioureas, squaramides exhibit a greater acidity as well as structural convergence of their NH bonds, making them more suitable for anion binding.[10]

During the last decade several examples of squaramide receptors have emerged in the literature for applications in medicinal chemistry, catalysis, liquid crystalline self-assembly, and anion recognition.[11-13] Few examples of squaramide receptors with very high stability constants have been reported in the literature. Gale and co-workers have described mono, bis- and tris-squaramides bearing *p*-(trifluoromethyl)phenyl substituents able to bind chloride in DMSO-d_6/0.5% water with stability constants ranging from 433 M^{-1} to 8021 M^{-1}.[14] Li *et al*. reported a *N*-*tert*-Butyl sulfinyl squaramide with a 3,5-bis(trifluoromethyl)phenyl substituent with good affinity for chloride in DMSO-d_6/0.5% water (K_a = 820 M^{-1}).[15] One of the goals of supramolecular anion recognition is to develop neutral systems able to bind anions in competitive solvent media. In the case of neutral squaramide receptors most of the binding studies have been conducted in organic solvents such as MeCN, $CHCl_3$, and DMSO.[10, 16] Ballester, Costa and co-workers have reported one of the first examples of acyclic squaramides and a binding constant of 311 M^{-1} with acetate in DMSO-d_6/10% water was determined.[17] To the best of our knowledge, the highest stability constants in the presence of water has been reported by Jolliffe's group for a macrocyclic squaramide system able to bind sulphate in DMSO-d_6/33% water mixture with stability constants higher than 10^4 M^{-1}.[18]

Aiming at designing new systems capable of binding anions in a competitive solvent mixture, we report here a family of simple (acyclic) symmetric squaramides (**L₁**-**L₅**, Scheme 1) bearing aromatic substituents for anion recognition and transport to evaluate the possibility of tuning the binding/recognition event depending on the heteroatom in the aromatic substituent. The anion binding properties of the five receptors towards different anion guests (F^-, CN^-, AcO^-, BzO^-, $H_2PO_4^-$, and Cl^-) were studied by means of ^{1}H-NMR spectroscopy in DMSO-d_6/water and UV-Vis and fluorescence spectroscopies in DMSO and MeCN, as well as in the solid state. Moreover, in order to test the capability of **L₁**-**L₅** to mediate chloride transport across model lipid membranes, we investigated their transmembrane anion transport activity. It is worth noticing that we have recently described **L₁** and **L₃** as imaging agents towards non-tumoral and tumoral cells as free molecules and when encapsulated inside cubosomes.[19]

The reaction between squarate esters (methyl or ethyl) and the appropriate amine in the presence of $Zn(OTf)_2$ as a Lewis acid catalyst in a DMF/Toluene mixture (1/19: v/v) for **L₁**, **L₂**, **L₄** and **L₅** or MeOH (**L₃**) afforded the five receptors in 50-70% isolated

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Electronic Supplementary Information (ESI) available: [synthesis, NMR titrations, UV- Vis and spectrofluorimetric titrations, crystallographic data, details of transport studies, details on numerical model]. See DOI: 10.1039/x0xx00000x

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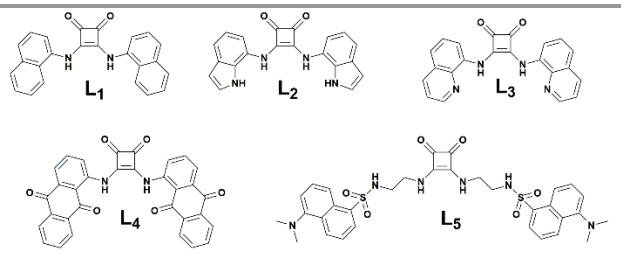
yields (see Electronic Supplementary Information, ESI, for synthetic details).[20]



Scheme 1 Receptors studied in this paper.

Anion-binding studies were conducted by means of ^{1}H-NMR titrations using DMSO-d_6/0.5% water as a solvent mixture. Stability constants from the obtained ^{1}H-NMR titration curves (see ESI, Figs S6-S27) were calculated by fitting the data to a 1:1 binding model using WinEQNMR[21] The results are shown in Table 1.

Table 1 Association constants (K_a/M^{-1}) for the formation of the 1:1 adducts of $\mathbf{L_1}$–$\mathbf{L_5}$ with anions added as their tetrabutylammonium salts in DMSO-d_6/0.5% water at 298 K determined following the signals of the squaramide NH protons. All errors estimated to be ≤14% (see ESI S6-S27)

	F^-	CN^-	AcO^-	BzO^-	$H_2PO_4^-$	Cl^-
L_1	deprot	deprot	400	600	1400	160
L_2	deprot	deprot	>10^4	n.d[a]	>10^4	1199
L_3	deprot	deprot	no interact	no interact	no interact	no interact
L_4	deprot	deprot	no interact	no interact	no interact	no interact
L_5	deprot	deprot	3700	1700	5500	n.d[b]

[a]: We were not able to fit the titration curves to a 1:1 or a 2:1 model; [b] the signal of the protons of the squaramide NH fragments undergoes a slight deshielding but remains hidden under the aromatic signals throughout the titration. As no other signals experience a significant shift, no association constant could be ascertained

Under the experimental conditions used, addition of 0.2 equiv. of both fluoride and cyanide resulted in the disappearance of the ^{1}H-NMR signal attributed to the protons of the squaramide NH fragments, accompanied by a dramatic color change of the solution. These observations suggest that the five receptors undergo a deprotonation process with these two anions.

Interestingly, as shown in Table 1, in the case of $\mathbf{L_3}$ and $\mathbf{L_4}$ no relevant changes are observed in the ^{1}H resonances of the NH protons in the presence of the other tested anion guests (AcO^-, BzO^-, $H_2PO_4^-$, and Cl^-) suggesting the lack of hydrogen bond interaction.

Additional ^{1}H-NMR experiments at different temperatures performed in the absence of the guests, pointed out to an upfield shift of the signals attributed to the squaramide NH protons as the temperature increases (see ESI, Figs. S28-S29). This is probably due to the presence of an intramolecular H-bond between the squaramide NHs and the heteroatoms in the quinoline and anthraquinone moieties in $\mathbf{L_3}$ and $\mathbf{L_4}$, respectively. The presence of the intramolecular H-bond in the case of $\mathbf{L_3}$ was also confirmed by the solid-state results (see ESI, Fig. S39 and discussion therein). On the other hand, $\mathbf{L_1}$, $\mathbf{L_2}$ and $\mathbf{L_5}$ coordinate oxo-anions (i.e. AcO^-, BzO^-, and $H_2PO_4^-$) with stability constants following the order $\mathbf{L_2}$>>$\mathbf{L_5}$>$\mathbf{L_1}$. (see ESI, Figs. S6-S25) As reported in Table 1, the stability constants of the 1:1 chloride adducts could only be determined in the case of $\mathbf{L_1}$ and $\mathbf{L_2}$, being remarkably high in the case of the latter.

The strong affinity of $\mathbf{L_2}$ for all the anion guests tested may be explained by considering the cooperative effect between the indole and the squaramide NHs in stabilising the anion adducts. Indeed, as shown in the stack-plot of the ^{1}H-NMR titration conducted with Cl^- (Figure 1A) the signal attributed to the squaramide NH protons undergoes a dramatic downfield shift (Δppm = 0.97) upon addition of the guest. The downfield shift of the signal attributed to the indole NH protons (Δppm = 0.15) is less pronounced. A significant downfield shift was also observed for the doublet at 7.2 (Δppm = 0.46), probably due to the effect of the formation of the strong H-bond between the squaramide NH and the anion guest.

A similar behaviour was also noticed in the presence of the other anions considered (see ESI Figures S10-12 for AcO^-, BzO^-, and $H_2PO_4^-$, respectively). Comparing the affinity of **L2** with the parent diphenyl squaramide[22], in the case of chloride (K_a = 260 M^{-1} in DMSO-d_6/0.5% water), it clearly appears the benefit of the presence of the additional H-bond donor of the indole moiety.

The high affinity of $\mathbf{L_2}$ observed in DMSO-d_6/0.5% water prompted us to perform further anion binding studies with $\mathbf{L_2}$ in more competitive solvent mixtures (i.e. DMSO-d_6 with 10% and 25% of water, see ESI, Figs. S14-S21). The results reported in Table 2 point out the remarkable high affinity of the receptor for the oxo-anions as well as the significant affinity for chloride in these conditions, with a stability constant >10^3 for the oxo-anions and >10^2 for chloride at 10% water content of the solvent mixture. When the experiments were carried out in a DMSO-d_6/25% water mixture the formation of the 1:1 adduct with both oxoanions and chloride was still detected.

When comparing the results obtained with $\mathbf{L_2}$ with those Gale and co-workers reported for 1,3-diindolylureas,[6] we observed for $\mathbf{L_2}$ in a DMSO-d_6/25% water mixture constants of one order of magnitude higher, thus demonstrating the remarkable binding properties of this ligand as compared to the analogous urea derivative.

Crystals structures of the 1:1 adducts of $\mathbf{L_1}$ with TBABzO [($\mathbf{L_1}$BzO)TBA)] (**1**) and of $\mathbf{L_2}$ with TBACl [($\mathbf{L_2}$Cl)TBA)] (**2**) were obtained by slow evaporation of a solution containing the receptors and an excess of the tetrabutylammonium salts of the anions in DMSO (Figure S40 and 1B, for structures **1** and **2**, respectively). It is worth noting that in structure **2** the chloride anion fits perfectly into the psudo-cavity of $\mathbf{L_2}$ and this can be an explanation of the almost 8-fold increase observed in the association constant when compared to that of receptor $\mathbf{L_1}$.

The presence of fluorogenic fragments in the receptors spurred us to test them also as sensors for optical anion recognition (see ESI for discussion).

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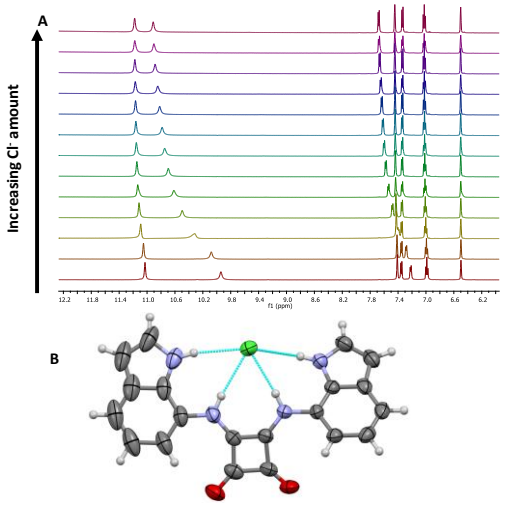

Figure 1 A) Stack plot of the ^{1}H-NMR spectra of $\mathbf{L_2}$ upon addition of increasing amount of TBACl in DMSO-d_6/0.5% water; **B**) [($\mathbf{L_2}$Cl)TBA)] (**2**) as seen in its crystal structures. Ellipsoids are drawn at the 50% probability level; hydrogen atoms are shown as spheres of arbitrary radii. Dashed blue lines show the hydrogen bonds. TBA counter cations are omitted for clarity.

Table 2 Comparison of association constants (K_a, M^{-1}) of the 1:1 adducts formed between $\mathbf{L_2}$ and $H_2PO_4^-$, AcO^-, BzO^-, and Cl^- (added as tetrabutylammonium salts) at 298 K in DMSO-d_6/water (various percentage), Errors estimated to be no more than 15% (see ESI, Figs. S14-S21).

Anionic species	K_a /M^{-1}		
	DMSO-d_6/0.5% water	DMSO-d_6/10% water	DMSO-d_6/25% water
$H_2PO_4^-$	>10^4	2000	1700
AcO^-	>10^4	1900	220
BzO^-	n.d.*	3800	120
Cl^-	1200	100	20

n.d.*= not determined

Finally, we decided to explore the anion transport properties of receptors $\mathbf{L_1}$-$\mathbf{L_5}$. We used chloride-containing liposomes composed of 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (POPC). Facilitated chloride efflux from these vesicles can be monitored using a chloride-selective electrode.[23] Lysing of the vesicles by adding a detergent at the end of the experiments allows normalization of the results. The studied compounds are added as DMSO aliquots and the external isotonic solution can be varied to evaluate the impact of these changes in the chloride efflux (see ESI for details). Our results identified $\mathbf{L_2}$ as a very potent transmembrane transporter (see ESI, Fig. S42). Very limited albeit detectable chloride efflux was observed for $\mathbf{L_1}$ and $\mathbf{L_3}$ (see ESI, Fig. S41). The chloride efflux induced by $\mathbf{L_2}$ was found concentration- dependent. The EC_{50} was calculated to be 0.55 μM and 3.35 μM (0.11% and 0.67% molar ratio to POPC) in the exchange assays when nitrate or sulfate/bicarbonate are used in the external solution, respectively (see ESI, Table S5). These results are consistent with an anion exchange mechanism in which the nature of the external solution impacts the rate of chloride efflux. The lack of response in the carboxyfluorescein-based assay ruled out membrane disruption or unspecific transport processes induced by these compounds (see ESI, Fig. S48). The ability of $\mathbf{L_2}$ to rectify a pH gradient across the vesicles was also tested with entrapped HPTS (8-Hydroxypyrene-1,3,6-trisulfonic acid). Efficient vesicle basification after addition of an external NaOH pulse to induce a pH gradient was observed (see ESI, Figs. S45-S46). This activity was found to be similar in the concentration range explored, being very efficient at concentrations down to 0.1 mol% $\mathbf{L_2}$ to POPC. A much more limited response was observed for $\mathbf{L_1}$, for which 1 mol% was required to observe a significant response. Squaramide derivatives bearing aryl substituents decorated with fluorine atoms or fluorinated pendant groups such as -CF_3 have been shown to be extremely active as transmembrane anion transporters due to a combination of increased lipophilicity and anion binding strength.[14, 24]. Overall, with the present series of compounds we observed, that the reduced transport activity due to the absence of fluorinated substituents, could be overcome by decorating the substituent at the squaramide moiety with additional hydrogen bond donors that cooperate with the squaramide NHs in the anion binding.

In order to describe the transport process in detail and aiming at deriving a quantitative model to correlate transport performance to molecular descriptors, we wrote an algorithm to simulate chloride release *vs*. time. As far as we are aware, a comprehensive numerical model trying to take both the thermodynamics and kinetics of the whole process into account, has not attempted yet.[25] Here, we present our first trial by using $\mathbf{L_2}$ in the presence of nitrate as counter-diffusing ion as benchmark case. The main features and assumptions behind our model, together with the first tests, are reported (see the ESI for further information). Several parameters were estimated, and these can be certainly improved with appropriate experimental determinations. At this initial stage, we wanted to keep the model as simple as possible (i.e. using only few parameters and considering only the main estabilished equilibria) and report the results of our first trials. As expected, the release rate increases with increasing the carrier concentration. According to our model, most of the carrier is always located outside the vesicles. The free carrier in solution is always in equilibrium with the membrane bound state by virtue of the partition constant, and the membrane bound complex increases alongside with chloride ions being released from the vesicles (Figure S49). However, first trials were performed with the same value of the diffusion constant through the lipids for both the free carrier and the complex. Simulations showed that the model reached maximum release much faster than the experimental data. Actually, diffusion constant through the lipid bilayer can be expected to be lower for the negatively charged complex than for the neutral carrier. When we implemented a fitting Monte Carlo scheme and we used the two values as independent parameters, results were much more satisfactory as shown in Figure 2. The obtained

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curves show that our model is reliable if the two diffusion constants are not kept equal.

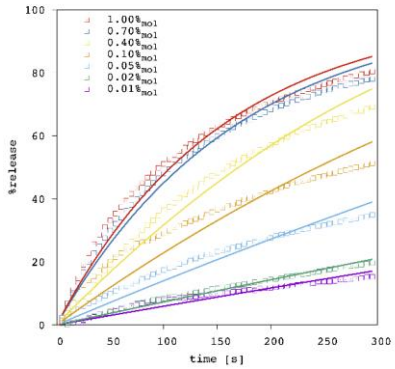

Figure 2 Curve fitting (lines) and experimental data points (empty squares) for different carrier concentrations. Fitting parameters were the diffusion constant of the free carrier and the complex and are reported in the ESI (Table S5).

In conclusion, in this work we have demonstrated that in the design of anion receptors the cooperativity between different H-bond donor groups could dramatically improve the anion recognition properties also in in aqueous media. H-bond acceptors (such as nitrogen or oxygen in the quinoline or anthraquinone moieties in the case of $\mathbf{L_3}$ and $\mathbf{L_4}$, respectively) hamper the possibility of binding anion guests. On the other hand, supplementary H-bond donor groups such as sulphonamides ($\mathbf{L_5}$) or indole ($\mathbf{L_2}$) substituents improve the anion binding affinities of the receptors. In particular, the indole substituents in $\mathbf{L_2}$ lead to the formation of the 1:1 anion adducts also in competitive aqueous media with the highest stability constants ever observed in solution for simple squaramide receptors. Moreover, we demonstrated that $\mathbf{L_2}$ acts as a very potent chloride transmembrane transporter and we proposed, for the first time, a simple numerical model to fit and analyze the experimental data of chloride transport

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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