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Sulfate-Selective Recognition by Using Neutral Dipeptide Anion Receptors in Aqueous Solution

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Abstract: The synthesis of six small peptide anion receptors based on thiourea and squaramide recognition moieties is described. These new receptors bind to tetrahedral sulfate anions with remarkable affinity and selectivity in aqueous solution as shown by NMR spectroscopy. Molecular modelling suggests that selectivity is mediated by a hydrogen bond network incorporating the amide backbone protons in a manner similar to that found in the sulfate-binding protein.

Introduction

The design of new chemosensors for anionic species is an ever expanding field due to the ubiquitous nature of anions in a wide range of environmental, chemical, and biological processes.[1,2] In particular, receptors capable of selectively recognizing specific anionic guests in competitive solvents have potential uses in numerous biomedical and environmental applications. Sulfate anions are of particular relevance in this regard as they can interfere with proposed radioactive waste-treatment processes[3,4] and are often amongst a myriad of potentially toxic solutes found as ground water contaminants in mining-associated water pollution.[5] Indeed, a recent study evaluating the influence of mining on mine surrounded waterways observed SO_4^{2-} levels ranging from 11 mg L^{-1} for unmined streams to 1187 mg L^{-1} for streams in mined watersheds.[6]

In nature, the selective binding and transport of sulfate is achieved by the sulfate-binding protein (SBP), which binds to sulfate through seven hydrogen bonds, five of which are provided by main-chain amide groups.[7] In this manner, the SBP is capable of highly selective binding of sulfate with an association constant of approximately 10^6 M^{-1} in water (pH 5–8.1).[4] Although a number of approaches have been taken towards the selective binding of SO_4^{2-} with synthetic receptors based on macrocyclic[8,9] and interlocked structures,[3,10] tripodal scaffolds,[11–13] and metal ions,[14–16] the majority of these systems are only capable of binding sulfate in organic solvents, whereas for practical applications the binding of SO_4^{2-} in aqueous solutions is often desired.

In the field of synthetic anion receptors, amides, ureas, thioureas and, more recently, squaramides have been extensively employed to provide hydrogen bond donor sites,[17–21] and the use of either ureas or thioureas in combination with amides has been shown to provide significant enhancements in anion-binding affinity.[22,23] Indeed, we recently described a number of tripodal anion receptors based on a cyclic peptide scaffold functionalized with either urea or thiourea binding sites, which were highly selective for SO_4^{2-} and bound this ion with high affinities in aqueous solvent mixtures.[24–26] This selectivity and affinity is proposed to arise from a synergistic effect brought about by hydrogen bond donation from both the (thio)urea protons and the cyclic peptide backbone amides. Similarly, Kubik and co-workers have recently reported that a combination of cyclic peptide backbone hydrogen bond donors and charged side chain ammonium groups provides high affinity and selectivity for sulfate in aqueous buffer.[27] Although such cyclic peptide scaffolds have shown a high degree of selectivity towards their target anions, their synthesis is laborious and involves numerous purification steps. We envisaged that linear peptide scaffolds functionalized with suitable anion-recognition motifs would be readily synthesized and may also function as charge neutral sulfate-selective receptors in competitive solvents through a combination of peptide backbone and side chain binding sites, in a manner analogous to that of the SBP. Our recent observation that linear peptide-based bis[Zn^{II} dipicolylamino] receptors[28] showed similar anion selectivity to related cyclic peptide analogues[29–31] despite their lack of preorganization, suggested that the excellent sulfate selectivity previously observed for cyclic peptide scaffolds could be maintained for simplified linear analogues. To investigate this hypothesis we set about synthesizing a small family of linear peptide-based receptors in which we 1) varied the peptide length and number of binding sites available, 2) varied the nature of the recognition moiety, replacing thiourea groups with squaramides to provide enhanced hydrogen bond-donor strength, and 3) varied the stereochemistry of the amino acid side chains (Receptors **1**–**6**; Figure 1). To enable rapid access to

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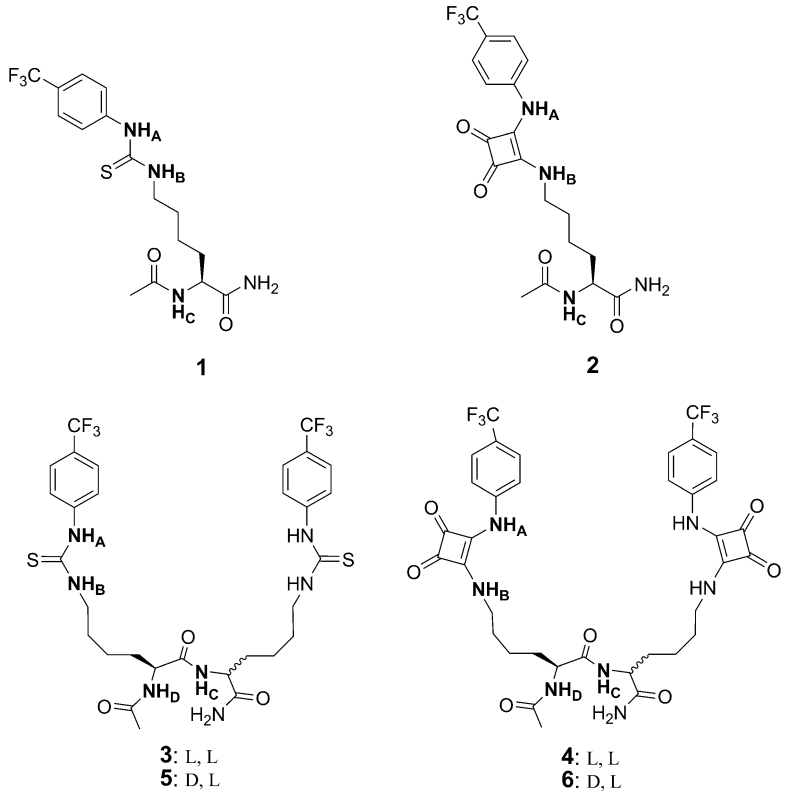
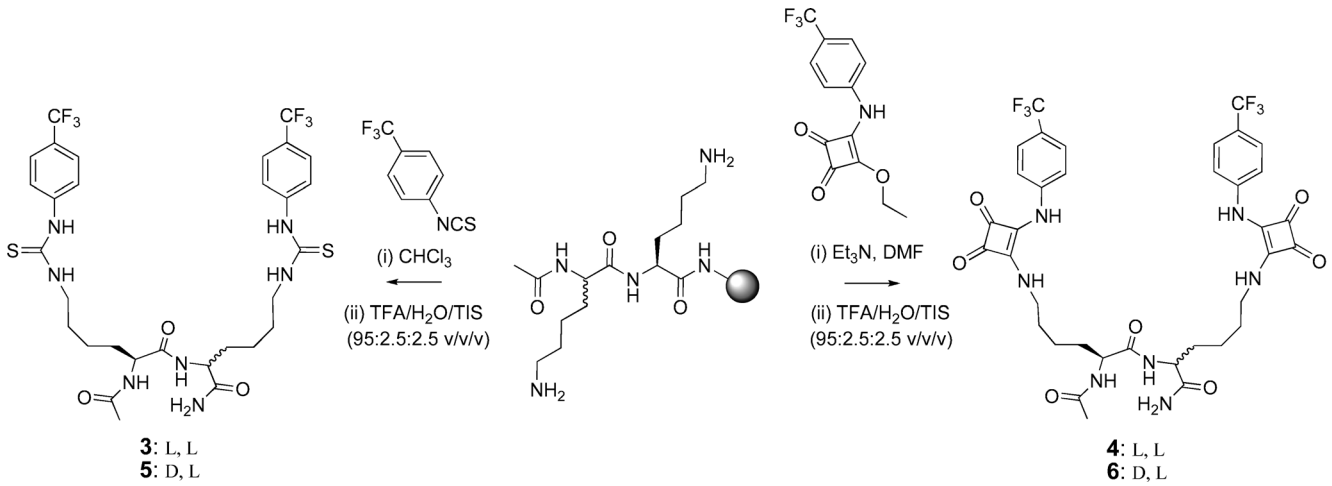


Figure 1. Structures of receptors **1**–**6**.

these and other linear peptide derivatives we designed a synthetic approach to enable the entire synthesis to be performed on the solid phase. The anion-binding properties of these receptors were then investigated.

Results and Discussion

The synthesis of **1**–**6** was carried out using a 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis (Fmoc-SPPS) strategy on a Rink amide resin (Scheme 1) with orthogonal allyloxycarbonyl (Alloc) protection of the side chain amino groups. Loading was achieved by treatment with L-Lys in the presence of tetramethyluronium hexafluorophosphate (HBTU) and *N*,*N*-diisopropylethylamine (DIPEA). Iterative deprotection (20% piperidine/DMF) and coupling (amino acid, HBTU/DIPEA) steps were followed by acetyl capping of the N-terminal amino acid by treatment with 20% acetic anhydride/pyridine. After assembly of the desired linear peptide scaffold, the Alloc groups were removed by treatment with $[Pd(PPh_3)_4]$ in the presence of acetic acid and morpholine.[32] Subsequent functionalization of the side chain amino groups was achieved by reaction with either 4-(trifluoromethyl)-phenyl isothiocyanate (**1**, **3**, and **5**) or 3-ethoxy-4-(4-(trifluoromethyl)phenylamino)cyclobut-3-ene-1,2-dione (**2**, **4**, and **6**) to install the thiourea and squaramide moieties, respectively. The entire assembly was finally cleaved from the solid support by treatment with a solution of TFA/triisopropylsilane (TIS)/H_2O (95/2.5/2.5) to afford the desired anion receptor peptides (**1**–**6**) isolated in yields of 28–36 and 58–76% for the thiourea- and squaramide-based receptors, respectively. Whereas it was possible to purify squaramide receptors **2**, **4**, and **6** by simple trituration with MeOH, the solubility of **1**, **3**, and **5** did not allow for this and HPLC purification was necessary for these ana-



Scheme 1. General synthetic route to receptors **1**–**6**.

logues resulting in lower yields of the isolated compounds **1**, **3**, and **5**.

To assess the anion-binding properties of this family of receptors, a number of ^{1}H NMR spectroscopic titration experiments were conducted, using the tetrabutylammonium salts of the anions to ensure their complete solubility. With the exception of the complex between compound **1** and acetate (data for this complex could not be fit to a suitable binding model), in all cases the observed changes to NH_A and NH_B were fitted to a 1:1 binding model by using Hyperquad[33] to give apparent stability constants, which are summarized in Table 1 (see the Supporting Information for fitted titration data). We first evaluated the binding ability of modified amino acids **1** and **2** to assess the effect that changing the binding motif (thiourea vs. squaramide) would have on binding affinity. Fabbrizzi et al. have recently reported that anion-binding affinities of squaramides are significantly higher than those for the analogous ureas[34] and as expected, squaramide derivative **2** was found to bind anions significantly more strongly than its thiourea counterpart (e.g., **1** + BzO^- $K_a = 76\ M^{-1}$, whereas **2** + BzO^- $K_a = 1300\ M^{-1}$). ^{1}H NMR spectroscopic titrations of both **1** and **2** revealed that they were capable of binding SO_4^{2-} with high affinity, whereas AcO^-, BzO^-, and Cl^- were bound less strongly (Table 1). Notably, for both **1** and **2**, the addition of sulfate ions resulted in significant downfield shifts ($\Delta\delta = 1.77$ and 1.67 ppm, respectively) of the amide protons (NH_C), indicating the formation of hydrogen bonds from these protons to sulfate. In contrast, the addition of other anions to **1** and **2** resulted in only minor shifts of these protons ($\Delta\delta < 0.2$ ppm), suggesting that the incorporation of additional amide hydrogen bond-donors might result in increased selectivity for sulfate over other anions. We therefore investigated dipeptide receptors **3**–**6**, which contain additional hydrogen bond donor sites.

Initially, qualitative measurements with the dipeptide derivatives **3** and **4** were undertaken by using ^{1}H NMR spectroscopic screening experiments in which 10 equiv of a range of anions (AcO^-, BzO^-, $H_2PO_4^-$, SO_4^{2-}, Br^-, HSO_4^-, NO_3^-, TsO^-, and Cl^-as their tetrabutylammonium salts) were added to the receptors in solution (0.5% H_2O in [D_6]DMSO). These preliminary results indicated significant changes of the spectra of **3** and **4** in the presence of AcO^-, BzO^-, $H_2PO_4^-$, SO_4^{2-}, and Cl^-, culminating in large downfield shifts of both the thiourea/squaramide NH protons and, in some cases, the amide NH protons. Moreover, for the squaramide derivative **4**, there was also a large degree of peak broadening observed indicating the occurrence of slow exchange processes on the NMR timescale. Conversely only minor changes were observed in the presence of Br^-, HSO_4^-, NO_3^-, and TsO^- suggesting little interaction of these anions with either **3** or **4**. To investigate these effects and to probe the binding mode and affinities more closely, we conducted additional quantitative binding studies with **3** and **4** in the presence of AcO^-, BzO^-, SO_4^{2-}, and Cl^-. Unfortunately, titration of **3** and **4** with $H_2PO_4^-$ led to peak broadening, preventing an association constant from being determined. This behavior has also been observed for our (thio)urea functionalized tripodal cyclic peptides[24,25] and for diindolylureas reported by Gale et al. and may be indicative of a deprotonation process.[35]

Table 1. Apparent association constants (K_a, M^{-1}) and values (ppm) for **1**–**6** with various anions (added as their tetrabutylammonium salts) as determined by ^{1}H NMR spectroscopic titrations monitoring the thiourea or squaramide resonances NH_A and NH_B in 0.5% H_2O in [D_6]DMSO.[a]

	SO_4^{2-}			AcO^-			BzO^-			Cl^-		
	K_a	$\Delta\delta$ NH_A	$\Delta\delta$ NH_C	K_a	$\Delta\delta$ NH_A	$\Delta\delta$ NH_C	K_a	$\Delta\delta$ NH_A	$\Delta\delta$ NH_C	K_a	$\Delta\delta$ NH_A	$\Delta\delta$ NH_C
1	$>10^4$	2.51	1.77	ND[b]	2.95	0.19	76	2.84	0.18	38	0.56	0.03
2	$>10^4$	2.94	1.67	1724	3.00	0.12	1300	3.03	0.12	130	1.37	0.01
3	ND[b]	2.08	1.40	330	2.80	0.22	314	2.64	0.16	53	0.47	0.04
4	ND[b]	2.81	1.58	483	3.00	0.17	514	3.07	0.15	284	1.29	0.03
5	ND[b]	2.32	1.34	235	2.82	0.17	369	2.64	0.12	63.8	0.49	0.02
6	ND[b]	2.96	1.49	421	2.98	0.12	479	3.07	0.10	186	1.29	0.01

[a] Determined at 300 K. Where possible, data was fitted to a 1:1 binding model as confirmed by titrations. Job plot K_a values are an average obtained from monitoring NH_A and NH_B. Errors $<15\%$. [b] Titration data suggests strong binding however it could not be fitted to a suitable binding model.

The addition of AcO^-, BzO^-, SO_4^{2-}, and Cl^- to both **3** and **4** resulted in downfield shifts of the thiourea/squaramide NH proton signals (NH_A and NH_B) as well as varying degrees of downfield shifts for the backbone amides (NH_C and NH_D). Representative spectra for titration of **3** with $AcO^-$are shown in Figure 2(a), illustrating the significant downfield shifts of the thiourea proton signals. Similar effects were observed for squaramide derivative **4** in the presence of each of the anions measured, however, as seen for the amino acid receptors **1** and **2**, higher affinity for all anions was observed for **4** compared with the thiourea derivative **3** (e.g., **3** + Cl^- $K_a = 53\ M^{-1}$, whereas **4** + Cl^- $K_a = 284\ M^{-1}$). As observed for **1** and **2** above, on addition of AcO^-, BzO^-, and Cl^- the signals attributable to the peptide amide NH protons (NH_C and NH_D) were not significantly shifted, suggesting that minimal hydrogen bonding is occurring at these sites. The binding titrations for SO_4^{2-} with **3** and **4**, however, exhibited distinct behavior from the other anions measured and resulted in significant changes to NH_A, NH_B, NH_C, and NH_D as well as the aromatic phenyl protons. The ^{1}H NMR titration data for thiourea **3** with increasing equivalents of SO_4^{2-} is shown in Figure 2(b). Unfortunately, as has been previously observed for (thio)urea-functionalized tripodal cyclic peptides,[24,25] an accurate binding constant could not be calculated from the titration data due to a two-step binding profile. Such behavior has also been observed with acyclic indole and carbazole-based receptors and indicates the occurrence of more complex binding equilibria depending on the concentration of SO_4^{2-} present.[36] Addition of 0.2 to 1.4 equivalents of SO_4^{2-} resulted in significant signal broadening, however, further additions brought about well-resolved thiourea and amide signals that were significantly shifted downfield from their original positions ($\Delta\delta$ $NH_A = 1.83$ and

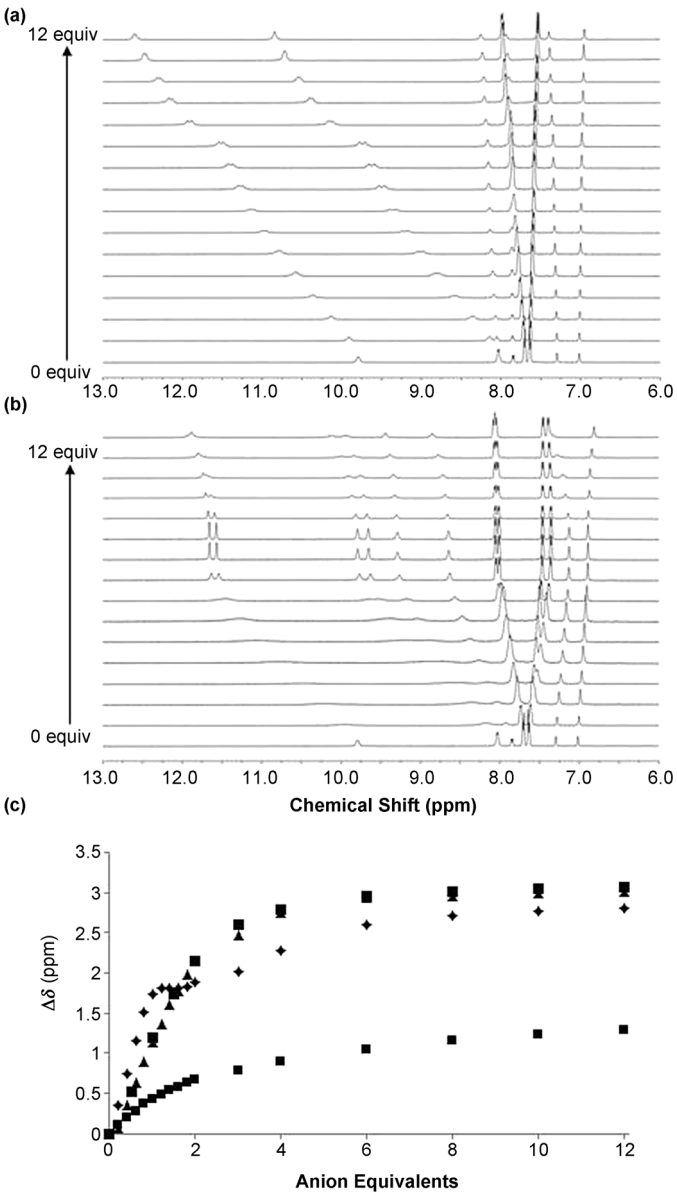


Figure 2. Stack plots of ^{1}H NMR spectra of **3** (2.5×10^{-3} M) upon addition of (a) TBAOAc and (b) $(TBA)_2SO_4$ (0–12 equiv) in 0.5% H_2O in $[D_6]$DMSO at 25 °C; (c) Comparison isotherms of **3** in the presence of increasing concentrations of SO_4^{2-} (◆), Cl^- (■), BzO^- (▲), and AcO^- (×).

$NH_C = 1.26$ ppm). Moreover, addition of SO_4^{2-} results in the poorly resolved aromatic and thiourea signals separating into two clearly distinct sets of peaks, and strongly suggests the formation of a host–guest complex in solution. Subsequent additions induced no further changes to the spectra until, after 4 equivalents of SO_4^{2-} had been added, the thiourea and amide signals began to shift downfield and broaden once again. This two-stage process suggests that initially **3** forms a strong 1:1 complex at low concentrations of SO_4^{2-} (i.e., less than two equivalents), whereas at higher concentrations of anion, more complex binding equilibria exist. Comparable behavior was observed for **4** in the presence of SO_4^{2-}, however, the resolution of the squaramide signals occurred after just 1 equiv had been added ($\Delta\delta$ $NH_A = 1.81$ and $NH_B = 1.71$ ppm) and these signals had completely disappeared before the addition of 2 equivalents of SO_4^{2-} suggesting a stronger interaction between squaramide **4** and SO_4^{2-} compared with that of the thiourea analogue **3**. As was the case with **3**, large $\Delta\delta$ values were measured for the amide protons of **4** over the course of the titration ($\Delta\delta$ $NH_C = 1.58$ and $NH_D = 1.33$ ppm), providing further evidence to suggest that the peptide backbone of these linear peptide scaffolds has a major role to play in the selective recognition of SO_4^{2-} in solution. Titrations were next carried out using **5** and **6**, to probe the effect of alternating stereochemistry on the binding behavior of the linear peptide scaffold. This structural change had only a minor influence on binding behavior as summarized in Table 1. Similar binding constants were determined for **5** and **6**, compared to those of **3** and **4**, respectively. Moreover, the $\Delta\delta$ values observed for the D,L derivatives **5** and **6** are very similar to those measured for their their L,L counterparts providing further evidence that the chirality of these particular receptors has little effect on their binding affinity for anionic species. Due to the large apparent stability constants obtained for these receptors with SO_4^{2-} ions in 0.5% v/v $H_2O/[D_6]$DMSO, we chose to investigate these systems in more competitive media by conducting further binding studies with AcO^-, BzO^-, and SO_4^{2-}. These studies were conducted in 20% v/v $H_2O/[D_6]$DMSO because the receptors were not soluble at higher H_2O concentrations. The binding data obtained reveals that moving to this more polar solvent mixture had a significant influence on the binding behavior of **1**–**6** (Table 2). Large decreases in the apparent stability constants were observed for binding of **1**–**6** to both $AcO^-$and BzO^-, whereas high affinity was retained for SO_4^{2-} for receptors **2**–**6**.

Table 2. Apparent association constants (K_a, M^{-1}) and values (ppm) for **2**–**6** with various anions (added as their tetrabutylammonium salts) as determined by ^{1}H NMR spectroscopic titrations monitoring the thiourea or squaramide resonances NH_A and NH_B in 20% H_2O in $[D_6]$DMSO.[a]

	SO_4^{2-}			AcO^-			BzO^-		
	K_a	$\Delta\delta$ NH_A	$\Delta\delta$ NH_C	K_a	$\Delta\delta$ NH_A	$\Delta\delta$ NH_C	K_a	$\Delta\delta$ NH_A	$\Delta\delta$ NH_C
2	1116	ND[b,c]	0.68	12.3	ND[b,c]	0.03	26.9	ND[b,c]	0.02
3	1282	1.27	0.60	23.3	0.27	0.02	21.3	0.26	0.03
4	$>10^4$	1.44	0.66	44.9	ND[b,c]	0.07	17.1	ND[b,c]	0.04
5	775	1.31	0.75	17.3	0.36	0.06	17.4	0.35	0.05
6	$>10^4$	1.49	0.40	72.6	ND[b,c]	0.08	29.7	ND[b,c]	0.05

[a] Determined at 300 K. Where possible, data was fitted to a 1:1 binding model as confirmed by titrations. Job plot K_a values are an average obtained from monitoring NH_A and NH_B. Errors <15%. Anions added as their tetrabutylammonium salts. [b] Peak broadening prevented a value from being determined. [c] Titration displayed slow exchange on the NMR timescale and thus K_a values were obtained from monitoring NH_C.

In 20% v/v $H_2O/[D_6]$DMSO, the addition of SO_4^{2-} initially resulted in peak broadening of all of the signals. However, after one equivalent of the anion had been added, fast exchange processes on the NMR timescale were restored and well-resolved thiourea/squaramide signals were observed that were also shifted significantly downfield from their original posi-

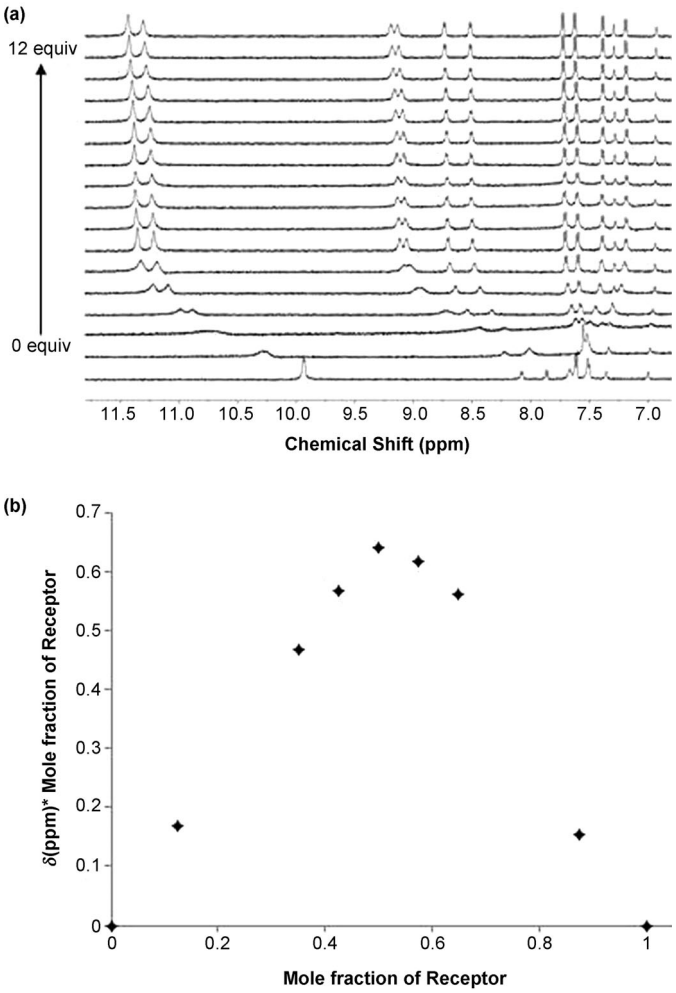


Figure 3. (a) Stack plot of ^{1}H NMR spectra of **4** (2.5×10^{-3} M) upon addition of $(TBA)_2SO_4$ (0–12 equiv) in 20% H_2O in $[D_6]$DMSO at 25 °C; (b) The corresponding Job plot analysis of **4** in the presence of $(TBA)_2SO_4$.

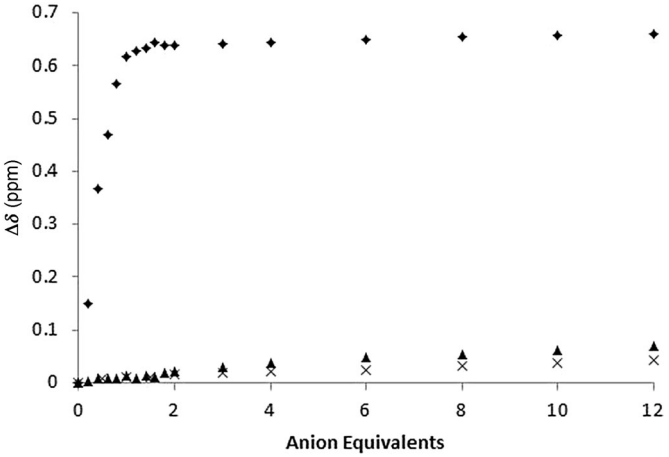


Figure 4. Comparison isotherm of **4** in the presence of increasing concentrations of SO_4^{2-} (◆), BzO^- (▲), and AcO^- (×) in 20% H_2O in $[D_6]$DMSO.

tions. Interestingly, the two-stage binding process observed for **3**–**6** with SO_4^{2-} in 0.5% v/v $H_2O/[D_6]$DMSO was completely suppressed in this more polar solvent (Figure 3(a)). This solvent-dependent behavior has previously been observed for cyclic peptides with thiourea arms and also with indole-based sulfate receptors.[25,36] Significant shifts were again observed for the backbone amide protons, suggesting that they are involved in hydrogen bonding to the SO_4^{2-} ions even in this highly competitive solvent. As was observed in the titrations carried out in 0.5% v/v $H_2O/[D_6]$DMSO, the addition of SO_4^{2-} results in increased resolution of the aromatic and thiourea/squaramide signals giving rise to four clearly resolved peaks. This observation suggests that anion/receptor complex formation significantly reduces the flexibility of the peptide, thereby placing these functional groups in chemically inequivalent environments. This effect was not observed upon addition of either AcO^-or BzO^-, indicative of the higher degree of flexiblity of these complexes, which can be attributed to the comparatively weak binding event (Figure 4). Notably, affinity for sulfate by the squaramide receptors **2**, **4**, and **6** was significantly higher than that observed for the analogous thioureas, **1**, **3**, and **5**, respectively, in this more competitive solvent mixture. The strong binding interaction with SO_4^{2-} was particularly evident in the cases of the dipeptide squaramide derivatives **4** and **6**, which displayed characteristic evidence to support the formation of a 1:1 receptor/anion complex in solution. These observations were supported by Job's plot analysis (Figure 3(b)) and apparent stability constants of $>10^4$ M^{-1} were calculated when fitted to a 1:1 binding model. Interestingly, under the same conditions, the stability constants obtained for AcO^- with **4** and **6** were seen to be at least an order of magnitude lower than the values obtained previously in 0.5% v/v $H_2O/[D_6]$DMSO demonstrating the high degree of selectivity that these receptors exhibit towards SO_4^{2-} even in highly competitive media.

To gain insight into possible modes of binding of SO_4^{2-} with this class of receptor, molecular modeling of **4** was performed by using Spartan 10 for Windows (Wavefunction, Inc.). The structure of **4** was energy minimized by using molecular mechanics before a SO_4^{2-} molecule was placed into the center of the receptor and the resulting complex was optimized by density functional theory (DFT) calculations at the B3LYP/6-31G* level of theory. Although such modeling of molecular docking cannot provide any quantitative results for interaction energies, it affords reasonable qualitative evidence for possible host/guest orientation in molecular complexes. As shown in Figure 5, modelling indicates that **4** wraps around the sulfate ion, binding through seven hydrogen bonds in a manner similar to the binding of SO_4^{2-} to the SBP. Two H-bonds are provided by each of the squaramide moieties, two further interactions from the backbone amide protons and a final H-bond from the carboxamide terminus. Moreover, the H-bond lengths observed between SO_4^{2-} and the squaramide NH groups were calculated as being between 1.740 and 1.928 Å in length; values that highlight the strong H-bond interaction with SO_4^{2-} and are shorter than distances recently observed in crystal structures of squaramides with $Cl^-$and DMSO.[34,37,38] Although not a conclusive result, the calculated structure corroborates the results observed in the NMR measurements, accounting for the observed shifts of all of the NH protons as well as the in-

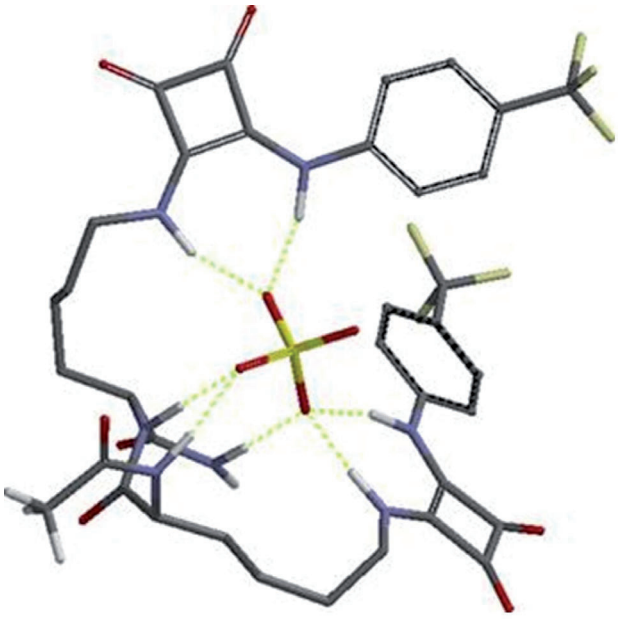


Figure 5. DFT calculated structure of the SO_4^{2-} complex of **4** showing the anion inserted into the binding cavity stabilized by 7 N–HO hydrogen bond interactions.

creased degree of chemical inequivalence seen between the squaramide NHs and the aromatic protons upon SO_4^{2-} addition.

Conclusion

We have developed a solid-phase synthetic strategy that is readily applicable to the preparation of libraries of peptide-based receptors and allows facile functionalization with different recognition motifs. We have synthesized and evaluated the anion-binding affinity and selectivity of a small family of such peptide-based anion receptors, all of which show remarkable binding to SO_4^{2-}in 0.5% v/v H_2O/[D_6]DMSO with significantly higher affinity for this anion than for AcO^-, BzO^-, $H_2PO_4^-$, SO_4^{2-}, Br^-, HSO_4^-, NO_3^-, TsO^-, and Cl^-. The inclusion of a squaramide anion-recognition motif was found to give rise to a stronger anion affinity than the thiourea analogues, with 1:1 receptor/anion complexes formed after the addition of just one equivalent of SO_4^{2-}. Studies to probe the effect of side chain stereochemistry revealed little preference for one diastereoisomer over the other, suggesting that with a flexible lysine side chain, the dipeptide stereochemistry is not a major contributor to selectivity or affinity. Receptors **1** and **2**, which comprise a single amino acid, exhibited significant binding affinity for SO_4^{2-}. However, receptors **3**–**6** with additional hydrogen bond donor sites exhibited enhanced binding affinity and sulfate selectivity compared with the amino acid analogues, especially when measured in 20% v/v H_2O/[D_6]DMSO. This suggests that at least two amino acid residues are required for optimal sulfate binding. The selectivity for sulfate appears to arise from a synergistic interaction between both the amide backbone and the thiourea/squaramide NH protons that is not observed with AcO^-, BzO^-, or Cl^-. Such results, taken in combination with the recent observations of Kubik et al., which indicate that oxygen atoms of the sulfate anion can form hydrogen bonds to the NH groups along a cyclopeptide ring,[27] suggest that exploiting such interactions is a viable new approach to the future design of sulfate-selective receptors. Taken together, the NMR spectroscopic analyses and molecular modeling studies suggest a 1:1 receptor/SO_4^{2-} complex for these flexible dipeptide receptors, which is brought together by a network of hydrogen bonds in a manner similar to that of the SBP. Future work will focus on analogues with increased water solubility and the introduction of additional binding sites for the fourth O atom of the sulfate residue to provide the maximum number of 12 hydrogen bonds ideal for coordination of sulfate.

Experimental Section

Synthesis

General peptide synthesis: As the syntheses for all the compounds were similar, general outlines of the procedures used and example characterization of receptors **3** and **4** are given below. Full details of all synthetic procedures and anion-binding studies are provided in the Supporting Information.

Iterative peptide assembly (Fmoc-SPPS): Rink amide resin (0.41 mmol g^{-1} as stated) was swollen in dry CH_2Cl_2 for 1 h. The resin was drained, then washed with DMF (×5), CH_2Cl_2 (×5), and DMF (×5). The resin was treated with 10% piperidine/DMF (2× 3 min) and subsequently washed with DMF (×5), CH_2Cl_2 (×5), and DMF (×5). A solution of appropriate Fmoc-protected amino acid (2 or 4 equiv relative to resin capacity for Lys or other amino acids, respectively), HBTU (1.1 equiv relative to peptide), and iPr_2NEt (2 equiv relative to peptide) in dry DMF (0.1 M) was added and the mixture was agitated at RT for 2 h. The resin was then washed with DMF (×5), CH_2Cl_2 (×5) and DMF (×5). *Deprotection*: The resin was treated with 10% piperidine/DMF (2×3 min) and washed with DMF (×5), CH_2Cl_2 (×5) and DMF (×5). *Amino acid coupling*: A preactivated solution of protected amino acid (2 or 4 equiv relative to resin capacity for Lys or other amino acids, respectively), HBTU (1.1 equiv relative to peptide), and iPr_2NEt (2 equiv relative to peptide) in dry DMF (0.1 M) was added to the resin and agitated at RT for 2 h. The resin was then washed with DMF (×5), CH_2Cl_2 (×5) and DMF (×5). *Acetylation*: Upon removal of the Fmoc protecting group, the resin was treated with 20% acetic anhydride/pyridine (3×4 min), followed by washing with DMF (×5), CH_2Cl_2 (×5) and DMF (×5).

Allyloxycarbonyl (Alloc) deprotection: All Alloc-deprotected peptides were prepared following a modification of the method described by Kates et al.[32] The resin was swollen at RT for 15 min in $CHCl_3$/morpholine/acetic acid (90:5:5). Tetrakis(triphenylphosphine)palladium (1.05 equiv relative to peptide) was added to the suspension, and the syringe was shielded from light and agitated for 2 h. The resin was drained then washed with $CHCl_3$ (×5) and a palladium-chelating cocktail (DMF/diethyldithiocarbamic acid-3-water/triethylamine 25 mL:225 mg:250 μL). Traces of the chelating cocktail were removed by a basic wash (0.5% triethylamine in DMF, ×5). The resin was then washed with MeOH (×5), DMF (×5), CH_2Cl_2 (×5), and DMF (×5). *Thiourea functionalisation*: The resin was swollen in dry DMF at RT for 30 min before the addition of 4-(trifluoromethyl)-phenyl isothiocyanate in $CHCl_3$ (12 equiv relative to loading). The

suspension was agitated at RT for 24 h, drained and washed sequentially with DMF (5×10 mL), CH_2Cl_2 (5×10 mL), DMF (5×10 mL), and CH_2Cl_2 (5×10 mL). *Squaramide functionalization*: The resin was swollen in dry DMF at RT for 30 min before the addition of 3-(4-trifluromethylphenylamino)-4-ethoxycyclobut-3-ene-1,2-dione[38] (3 equiv relative to loading) and triethylamine (6 equiv relative to loading). The suspension was agitated at RT for 24 h, drained, and washed sequentially with DMF (5×10 mL), CH_2Cl_2 (5×10 mL), DMF (5×10 mL), and CH_2Cl_2 (5×10 mL). *Cleavage*: The resin was treated with a solution of trifluoroacetic acid/H_2O/triisopropylsilane (95:2.5:2.5 v/v/v) for 1 h. The resin was drained and then washed with trifluoroacetic acid (×4). The cleavage solution and acid washes were combined and concentrated in vacuo.

Ac-Lys(thiourea)-Lys(thiourea)-NH_2 (3): Receptor **3** was synthesized on Rink amide resin (0.610 g, 0.250 mmol, resin capacity 0.41 mmol g^{-1}), utilizing the general methods for Fmoc-SPPS, Alloc deprotection, and thiourea functionalization. Cleavage from the resin was then achieved through treatment with a solution of TFA, triisopropylsilane, and water (95:2.5:2.5 v/v/v) for 1 h. The crude peptide was then purified by preparative RP-HPLC (0 to 50% B over 40 min). The appropriate fractions were lyophilized, affording the linear dipeptide receptor **3** as a TFA salt, which was treated with basic anion exchange resin to give the desired dipeptide receptor **3** (t_R = 18.6 min) as a white solid. Yield: 58 mg, (32%); $[\alpha]_D$ = −11.7 (c = 0.05 in MeOH); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 9.81 (br s, 2H, 2×thiourea), 8.03–8.05 (m, 3H, amide and 2×thiourea), 7.84–7.86 (d, J = 8.2 Hz, 1H, amide), 7.70–7.72 (d, J = 8.5 Hz, 4H, ArH), 7.62–7.64 (d, J = 8.6 Hz, 4H, ArH), 7.30 (s, 1H, carboxamide), 7.02 (s, 1H, carboxamide), 4.15–4.25 (m, 2H, 2×α-CH), 1.85 (s, 3H, CH_3), 1.51–1.75 (m, 10H, CH_2), 1.23–1.39 ppm (m, 6H, CH_2); ^{13}C NMR (100 MHz, ([D$_6$]DMSO): δ = 180.2, 173.6, 171.6, 169.5, 143.4, 125.7, 125.6, 123.0, 121.7, 52.8, 52.2, 43.7, 31.7, 31.5, 28.0, 27.9, 22.9, 22.8, 22.5 ppm; IR (thin film): $\tilde{\nu}$ = 3280, 3269, 3250, 1660, 1502, 1325, 1207, 1164, 1123 cm^{-1}; HRMS (ESI): m/z calcd for $C_{30}H_{37}F_6N_7O_3S_2Na^+$: 744.2196; $[M+Na]^+$; found: 744.2196.

Ac-Lys(squaramide)-Lys(squaramide)-NH_2 (4): Receptor **4** was synthesized on Rink amide resin (0.254 g, 0.105 mmol, resin capacity 0.41 mmol g^{-1}), utilizing the general methods for Fmoc-SPPS, Alloc deprotection, and squaramide functionalization. Cleavage from the resin was then achieved through treatment with a solution of TFA, triisopropylsilane, and water (95:2.5:2.5 v/v/v) for 1 h. The crude peptide was then purified by trituration with MeOH affording the linear dipeptide receptor **4** as a white solid. Yield: 48 mg, (58%). $[\alpha]_D$ = −1.1 (c = 0.54 in DMF); ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 9.91 (s, 2H, NH), 8.02 (d, J = 7.3 Hz, 1H, NH), 7.83 (d, J = 8.0 Hz, 1H, NH), 7.72 (s, 2H, NH), 7.66 (d, J = 8.3 Hz, 4H, ArH), 7.59 (d, J = 7.6 Hz, 4H, ArH), 7.30 (s, 1H, NH), 7.02 (s, 1H, NH), 4.15–4.25 (m, 2H, 2×α-CH), 3.58 (d, J = 5.3 Hz, 4H, 2 × CH_2), 1.84 (s, 3H, CH_3), 1.2–1.76 ppm (m, 12H, 6×CH_2); ^{13}C NMR (125 MHz, [D$_6$]DMSO): δ = 184.7, 180.0, 173.5, 171.6, 169.7, 169.6, 162.9, 142.6, 126.7, 125.6, 123.4, 122.3 (q, J = 32.0 Hz, CF_3), 117.9, 52.7, 52.1, 43.7, 31.5, 31.4, 30.3, 22.5, 22.4, 22.3 ppm; IR (thin film): $\tilde{\nu}$ = 3173, 1657, 1557, 1455, 1336, 1116, 1073, 834 cm^{-1}; HRMS (ESI): m/z calcd for $C_{36}H_{37}F_6N_7O_7Na^+$: 816.2558 $[M+Na]^+$; found: 816.2556

NMR Binding studies

NMR spectroscopic titrations were performed by additions of aliquots of the putative anionic guest as the tetrabutylammonium (TBA) salt (0.15–0.2 M), in a solution of the receptor (2.5×10^{-3} M) in either 0.5% H_2O in [D$_6$]DMSO or 20% H_2O in [D$_6$]DMSO to a 2.5×10^{-3} M solution of the receptor in either 0.5% H_2O in [D$_6$]DMSO or 20% H_2O in [D$_6$]DMSO. Typically, up to 9–12 equivalents of the anion were added to the solution. Both salt and receptor were dried under high vacuum prior to use. ^{1}H NMR spectra were recorded on a Bruker Avance III 500 spectrometer at a frequency of 500.13 MHz and calibrated to the residual proton solvent peak in [D$_6$]DMSO (δ = 2.50 ppm). Stack plots were made using MestReNova Version 6.0. Where possible, and when the change in chemical shift was larger than $\Delta\delta$ = 0.02 ppm, non-linear curve fitting of the experimentally obtained titration isotherms (equivalents of anion vs. chemical shift of the squaramide NH protons or amide NH protons) by using the commercially available software program HypNMR (Hyperquad package) enabled the calculation of association constants (K_a (M^{-1})) using a 1:1 model.

Molecular modelling

Molecular modelling of **4** was performed using Spartan 10 for Windows (Wavefunction, Inc. Irvine, CA). The structure was energy minimized by using molecular mechanics before a SO_4^{2-} molecule was placed into the center of the receptor and optimized by density functional theory (DFT) calculations at the B3LYP/6–31G* level of theory.

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