Peptides

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.[3, 4] 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.[1, 2] In particular, receptors capable of selectively recognizing sulfate in aqueous solutions is often desired.

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).[6]

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-recognizing 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[ZnII dipicolylamino] receptors[28] showed similar anion selectivity to related cyclic peptide analogues[29–31] despite their lack of preorganized 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...
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(PPh₃)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)/H₂O (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-

Figure 1. Structures of receptors 1–6.

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 1H 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 solved thiourea and amide signals that were significantly shifted 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\(^-\), HS\(_O\)^-\(^-\), 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\(_2\)PO\(_4\) 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.\(^{[25]}\)

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 and 4 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\(_D\) = 53 m\(^-1\), whereas 4 + Cl\(^-\) K\(_D\) = 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\(_C\) NH\(_B\) NH\(_D\) as well as the aromatic phenyl protons. The 1H 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 acrylic 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\(_B\) = 1.83 and

<table>
<thead>
<tr>
<th>(K_D)</th>
<th>SO(_4)^2-(^-)</th>
<th>AcO(^-)</th>
<th>BzO(^-)</th>
<th>Cl(^-)</th>
</tr>
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<tbody>
<tr>
<td>(&gt;10^4)</td>
<td>2.51</td>
<td>1.77</td>
<td>ND</td>
<td>2.95</td>
</tr>
<tr>
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<td>1.67</td>
<td>1724</td>
<td>3.00</td>
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<td>1.40</td>
<td>330</td>
<td>2.80</td>
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<td>2.81</td>
<td>1.58</td>
<td>483</td>
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<td>2.96</td>
<td>1.49</td>
<td>421</td>
<td>2.98</td>
</tr>
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</table>

(a) Determined at 300 K. Where possible, data was fitted to a 1:1 binding model as confirmed by titrations. Job plot \(K_D\) 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.

Table 1. Apparent association constants (\(K_D\) m\(^-1\)) and values (ppm) for 1–6 with various anions (added as their tetrabutylammonium salts) as determined by 1H NMR spectroscopic titrations monitoring the thiourea or squaramide resonances NH\(_A\) and NH\(_B\) in 0.5% H\(_2\)O in (D\(_6\)DMSO)\(^{[24]}\).
complex at low concentrations of SO$_4^{2-}$ suggesting a stronger interaction between squaramide and SO$_4^{2-}$ compared with that of the thiourea analogue 3. As was the case with 3, large $\Delta\lambda$ values were measured for the amide protons of 4 over the course of the titration ($\Delta\lambda$ NH$_A$ = 1.58 and NH$_B$ = 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 altering 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 $\Delta\lambda$ values observed for the d,l derivatives 5 and 6 are very similar to those measured for 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$_2$O/[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$_2$O/[D$_6$]DMSO because the receptors were not soluble at higher H$_2$O 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$_2$O in [D$_6$]DMSO.$^{[14]}$

<table>
<thead>
<tr>
<th>Anion</th>
<th>SO$_4^{2-}$</th>
<th>AcO$^-$</th>
<th>BzO$^-$</th>
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<tr>
<td></td>
<td>$K_a$</td>
<td>$\Delta\lambda$ NH$_A$</td>
<td>$\Delta\lambda$ NH$_B$</td>
</tr>
<tr>
<td>2</td>
<td>1116 ND$^{[4]}$</td>
<td>0.68</td>
<td>12.3 ND$^{[4]}$</td>
</tr>
<tr>
<td>3</td>
<td>1728 1.27</td>
<td>0.60</td>
<td>23.3 0.27</td>
</tr>
<tr>
<td>4</td>
<td>$&gt;10^4$ 1.44</td>
<td>0.66</td>
<td>44.9 ND$^{[4]}$</td>
</tr>
<tr>
<td>5</td>
<td>775 1.31</td>
<td>0.75</td>
<td>17.3 0.36</td>
</tr>
<tr>
<td>6</td>
<td>$&gt;10^4$ 1.49</td>
<td>0.40</td>
<td>72.6 ND$^{[4]}$</td>
</tr>
</tbody>
</table>

[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$_B$.

NH$_A$ = 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 ($\Delta\lambda$ 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 and SO$_4^{2-}$ as compared with that of the thiourea analogue 3. As was the case with 3, large $\Delta\lambda$ values were measured for the amide protons of 4 over the course of the titration ($\Delta\lambda$ NH$_A$ = 1.58 and NH$_B$ = 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 altering 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 $\Delta\lambda$ values observed for the d,l derivatives 5 and 6 are very similar to those measured for 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$_2$O/[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$_2$O/[D$_6$]DMSO because the receptors were not soluble at higher H$_2$O 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.

In 20% v/v H$_2$O/[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-
tions. Interestingly, the two-stage binding process observed for 3–6 with SO$_4^{2−}$ in 0.5% v/v H$_2$O/[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$_4^{2−}$/C$_0$ ions even in this highly competitive solvent. As was observed in the titrations carried out in 0.5% v/v H$_2$O/[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 flexibility 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$_2$O/[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$_4^{2−}$/C$_0$ 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; 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

Figure 3. (a) Stack plot of $^1$H NMR spectra of 4 (2.5 × 10$^{-1}$ mol) upon addition of (TBA)$_2$SO$_4$ (0–12 equiv) in 20% H$_2$O in [D$_6$]DMSO at 25 °C; (b) The corresponding Job plot analysis of 4 in the presence of (TBA)$_2$SO$_4$.
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$_2$O/[D$_6$]DMSO with significantly higher affinity for this anion than for AcO$^-$, BzO$^-$, H$_2$PO$_4^-$, $SO_4^{2-}$, 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$_2$O/[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,[39] 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 SBF. 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$_2$Cl$_2$ for 1 h. The resin was then washed with DMF (×5), CH$_2$Cl$_2$ (×5), and DMF (×5). The resin was treated with 10 % piperidine/DMF (2 × 3 min) and subsequently washed with DMF (×5), CH$_2$Cl$_2$ (×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 4-Pr$_3$NET (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$_2$Cl$_2$ (×5) and DMF (×5). Deprotection: The resin was treated with 10 % piperidine/DMF (2 × 3 min) and washed with DMF (×5), CH$_2$Cl$_2$ (×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 4-Pr$_3$NET (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$_2$Cl$_2$ (×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$_2$Cl$_2$ (×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/acetonic acid (90:5:5). Tetrais(triethylphosphine)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/diethylidithiocarbamic 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$_2$Cl$_2$ (×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 x 10 mL), CHCl₃ (5 x 10 mL), DMF (5 x 10 mL), and CHCl₃ (5 x 10 mL). Squaramide functionalization: The resin was swollen in dry DMF at RT for 30 min before the addition of 3-(4-trifluoromethylphenylamino)-4-ethoxycyclobut-3-ene-1,2-dione (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 x 10 mL), CHCl₃ (5 x 10 mL), DMF (5 x 10 mL), and CHCl₃ (5 x 10 mL). Cleavage: The resin was treated with a solution of trifluoroacetic acid/H₂O/triisopropylsilane (95:2.5:2.5 v/v/v) for 1 h. The resin was drained and then washed with trifluoroacetic acid (x4). The cleavage solution and acid washes were combined and concentrated in vacuo.

Ac-Lys(thiourea)-Lys(thiourea)-NH₂ (3): Reception 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 squaramide functionalization. Cleavage from the resin was then achieved through treatment with a solution of TFA, trifluoroacetic acid, 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 as a white solid. Yield: 48 mg, (58%).

Keywords: NMR spectroscopy, peptides, receptors, squaramide, sulfate, thiourea

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