A tripodal tris-selenourea anion transporter matches the activity of its thio-analogue but shows distinct selectivity

Michael J. Spooner[1] and Philip A. Gale*[2]

[1]Chemistry, University of Southampton, Southampton, SO17 1BJ, UK
[2]School of Chemistry, The University of Sydney, NSW 2006, Australia

philip.gale@sydney.edu.au

Dedicated to Prof. Jerry Atwood on the occasion of his 75th birthday.

A tripodal tris-selenourea anion transporter matches the activity of its thio-analogue but shows distinct selectivity

We report the synthesis of a tripodal tris-selenourea transporter scaffold. The Cl^- and NO_3^- transport activity of the compound has been compared extensively with the analogous oxo- and thiourea compounds. We found that the selenourea demonstrates remarkably similar transport efficacy and mechanistic properties to the equivalent thiourea, but demonstrates flipped selectivity for Cl^- over NO_3^-.

Keywords: supramolecular chemistry, anion transport, hydrogen bonding, selenourea

Introduction

Supramolecular anion transport across lipid bilayers by synthetic carriers is a rapidly growing field, driven by the potential application of these compounds as therapeutics in the treatment of diseases such as cystic fibrosis or cancer. (*1*) New assays have been developed that allow insight into the activity of these molecules in live cells (*2*) and the anion transport mechanism. (*3*) As more families of transporters are being reported, (*4, 5*) so the rules governing the efficacy of this class of compounds are better understood and the number of tools available for transporter design increases.

Ureas have long been used as hydrogen bond donor motif in anion recognition, the two parallel N-H bonds being ideally arranged for the chelation of halides and Y-shaped oxoanions. (*6*) Their ease of synthesis and tunability of the N-H bond acidity through variation of the *N*-substituents make them an attractive moiety to work with in the development of anion receptors and organocatalysts. (*6-8*) Hence they have often been used as the anion binding motif in the design of compounds for supramolecular anion transport. (*4*)

There are however a number of moieties that are structurally-related to ureas that can be employed to boost the efficacy of anion receptors. This has been shown, for example, with the squaramide binding group, which improves anion recognition or catalytic activity over urea with its higher acidity and more convergent N-H hydrogen bond donors. (*9-12*) More recently, croconamide and deltamide structures have been suggested as potential donor units for anion receptors. (*13*)

Most commonly however, the urea group has been directly replaced by a thiourea binding moiety. This has been shown in several studies to increase transport efficacy in many scaffolds including steroidal cholapods, (*14*) tren-based tripodal structures (*15*) and simple small urea-based molecules. (*16-18*) Generally, it is accepted that the thiourea confers two advantages over urea; firstly the greater acidity of the thiourea group gives stronger interaction with the anions being studied, most commonly chloride, and secondly the larger, more charge-diffuse sulfur atom gives a lipophilic advantage, reducing the energy barrier for translocation of the receptor and its complexes through the lipid bilayer.

With this in mind, we wished to determine whether continuing the chalcogen series and incorporating selenourea in an anionophore could confer any further advantage over thiourea. Limited data on the electronic structure of selenourea suggests that it is electronically is very similar to thiourea, with the larger, more polarisable chalcogen affording slightly more delocalisation of electron density. (*19, 20*)

Until recently, there have been very few reports of selenoureas as receptors in anion recognition. Caltagirone and colleagues have recently reported a selenourea-based silica-supported chemosensor for CN^- and S^{2-} which relies on the formation of diselenide bonds between selenourea groups for its selective sensing mechanism. (*21*) The same group has also exploited diaryl-substituted selenoureas as molecular logic gates, through their ability to form either mono- or bi-coordinated adducts based on the shape of the anion complexed. (*22*) The recent report of a facile synthesis of an alkyl isoselenocyanate (*23*) allowed us to expand a previously reported series of alkyl-substituted tripodal chloride transporters (*15*) to include the selenourea analogue, and thus carry out an extensive evaluation of its transport abilities.

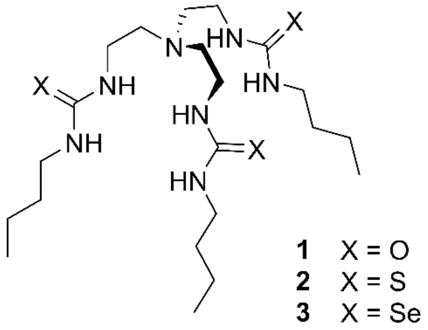

Figure 1. General structure of the tripodal compounds studied in this work.

Results and Discussion

Compounds **1** & **2** were prepared by variations on the literature methods. (*15*) The tripodal selenourea **3** was prepared from *n*-butylisoselenocyante **4** prepared via an adapted literature procedure. (*23*) The crude isoselenocyanate was dissolved in dichloromethane and treated with 0.33 equivalents of tris-(2-aminoethyl)amine. Compound **3** was obtained after stirring at room temperature for 4 hours and purification by flash chromatography. Full synthetic details and characterisation data are available in the ESI.

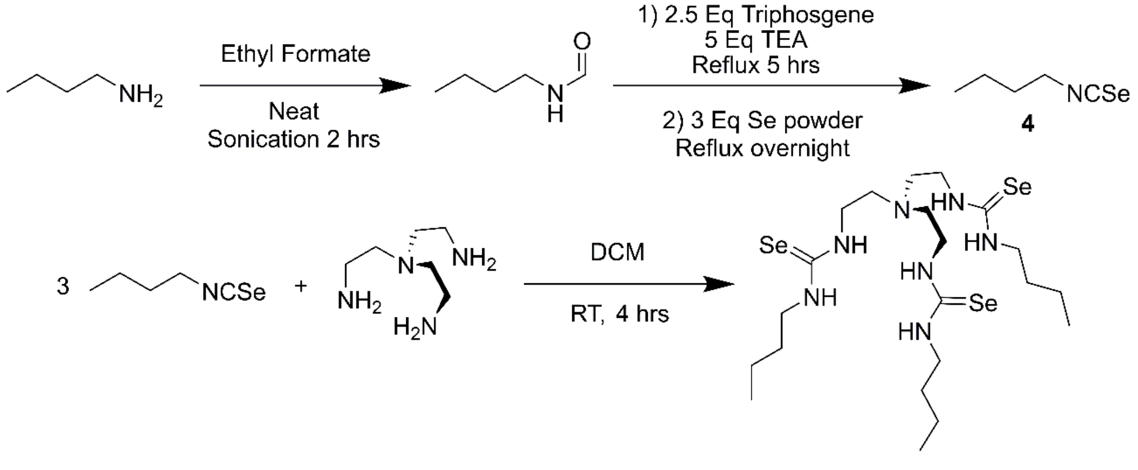

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Figure 2. Synthesis of the tripodal selenourea **3**, based on the reported synthesis of butylisoselenocyanate **4**.

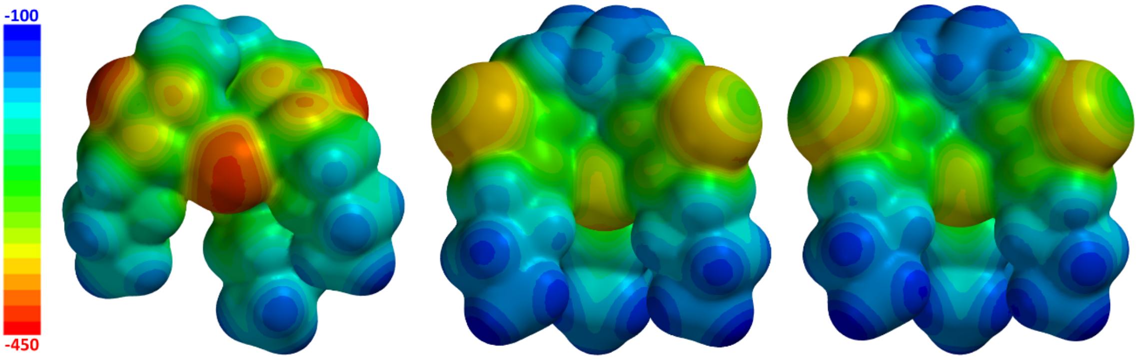
The anion binding properties of **3** were investigated by ^{1}H NMR titration in DMSO-*d*$_6$ (0.5 % H_2O). Titration of **3** with TBACl led to downfield shift of both selenourea NH protons, confirming Cl^- binding to **3**. However, the titration data was complicated by the broadening of the NH peak at 7.9 ppm (see Figure S3.2 in the ESI), preventing reliable determination of Cl^- binding constant in DMSO. To aid discussion of anion selectivity in transport (below), the nitrate and chloride binding constants for **2** & **3** were also determined in acetonitrile by UV-Vis titration. 20 μM solutions of the receptors were titrated with TBA Cl (0 - ~200 μM) and TBA NO_3 (0 – ~3 mM) and the absorbance spectrum between 220 and 320 nm was recorded for each titre. Binding constants for Cl^- were very high, 9.3×10^5 M^{-1} for **2** and 4.9×10^5 M^{-1} for **3**, as would be expected compared to values obtained in the more competitive DMSO.

The interaction with NO_3^- was weak, nevertheless a 1:1 binding constant of 1550 M^{-1} (± 3 %) for **3** could be obtained. In the case of **2**, background NO_3^- absorbance appeared to overlap the thiourea peak. The interferences was quantified in by taking the spectra of $TBANO_3$ acetonitrile solution and subtracting from the spectra of **2**.NO_3 before calculating binding constants. Fitting to a 1:1 binding model yielded a binding constant of 7821 ± 4 % M^{-1}. Full spectra and fittings are detailed in the ESI.

The transport ability and mechanism of the compounds was assayed using a variety of techniques. Firstly, the ability of the compounds to perform Cl^-/NO_3^- exchange from synthetic vesicles was determined using the previously reported chloride ion selective electrode (ISE) method. Briefly, synthetic POPC vesicles loaded with 500 mM NaCl buffered to pH 7.2 (5 mM phosphate salts) were suspending in a solution of buffered $NaNO_3$ (500 mM). The % efflux over 5 minutes of the total Cl^- from the vesicles was recorded using a Cl^- ISE at a variety of compound concentrations, and the values at 270 s fitted to the Hill Equation to obtain an $EC_{50\ (270\ s)}$ for each compound ($EC_{50\ (270\ s)} \equiv$ concentration required to obtain 50 % efflux after 270 s).

Compound **1** was too inactive in these experiments for Hill analysis, as has been previously reported (*15*), reaching a maximum of 1 % Cl^- efflux after 270 s at 5 mol %

loading (with respect to lipid, Figure S4.1). The $EC_{50\,(270\,s)}$ for thiourea **2** (0.138 mol %) and selenourea **3** (0.139 mol %), are within the margin in experimental error of each other. Hence in terms of the Cl^-/NO_3^- exchange ability, the selenourea appears to have the same activity as the thiourea, offering the same advantage over the oxourea, but no additional increase in activity over the thiourea. It should be noted however that related tripodal compounds have demonstrated Cl^- over NO_3^- selectivity previously, (*26*) thus it is unknown whether this value is limited by Cl^- or NO_3^- transport. The selectivity of these compounds has been explored in depth below.



1 2 3

Figure 3. Calculated electrostatic potential maps from DFT minimisations (M06/6-31G* level of theory in vacuum) for compounds **1** – **3** using Spartan '14 for Windows. (*27*) Values of electrostatic potential are given in kJ mol^{-1}.

Evidence of the similar nature of the thio- and seleno- analogues was obtained by calculating the electrostatic potential of the Cl^- complexes of the three compounds from DFT minimised structures (Figure 3). The charge distribution of **2** and **3** are remarkably similar, with the negative charge (red/orange) spread over the larger S/Se atoms and a larger spread of more neutral blue colour across the molecule. This is also demonstrated numerically by the differences between the electrostatic potential minima being minimal (-381.65 kcal mol^{-1} for **2** and -373.86 kJ mol^{-1} for **3**). As similar electrostatic potential surfaces would be presented to the bilayer, the energy barriers to crossing the tail region are likely to be very similar. In contrast, **1** demonstrates a high polarisation of the C=O bond (electrostatic potential minimum of -440.58 kJ mol^{-1}), with a high negative charge density on the oxygen atom, which would disfavour interaction with the tail region of the bilayer.

The similar lipophilicity of **2** & **3** is also clearly demonstrated in their calculated log P values (4.58 and 4.94 respectively, cf. 2.96 for **1** (*28*)). This is confirmed experimentally by considering the retention factor (k`) of the compounds on a C18 HPLC column. Under isocratic elution conditions, log k` is directly proportional to log P. (*29, 30*) Compounds **2** & **3** have the same log k` values, (log k` = -0.14, their retention time under the employed conditions was exactly the same, and the compounds co-eluted when run together) indicating a very similar lipophilicity. This is in stark contrast to **1**, which is not retained by the column (retention time equal to the column dead time, hence log k` is undefined) as it is significantly more polar. These values demonstrate a large difference in affinity for the bilayer, and thus Cl^-/NO_3^- transport activity, between oxourea **1** and thio- and seleno- analogues **2** & **3**.

The anion shuttling mechanism of the anionophores was probed using a valinomycin-/ monensin-coupled assay. (*3*) POPC vesicles are loaded with KCl and suspended in a solution of potassium gluconate (KGluc; gluconate is a large, polar anion that we assume cannot be transported). Experiments are run in the presence of valinomycin or monensin, which transport K^+ through the membrane by different mechanisms. The ability of the anion transporters to couple to these processes to facilitate KCl efflux (detected by Cl^- ISE) determines the ability of the anionophore to effect electrogenic Cl^- transport or electroneutral HCl cotransport.

Valinomycin (Vln) is a strict electrogenic K^+ uniporter, thus compounds must facilitate electrogenic Cl^- transport to couple to this process and effect KCl efflux without the build-up of a pH gradient or membrane potential. Both thiourea **2** and selenourea **3** couple strongly to Vln (Figure 3), indicating they are both good electrogenic Cl^- transporters.

Monensin (Mon) is a H^+/K^+ antiporter, thus anionophores being tested must facilitate HCl cotransport (or functionally equivalent Cl^-/OH^- exchange) in order to avoid building up a pH gradient to facilitate overall KCl efflux. Again, both thiourea **2** and selenourea **3** couple to Mon, indicating that they are also capable of HCl co-transport, although the efflux is markedly reduced in comparison to that achieved with Vln, indicating they may be limited by their H^+/OH^- transport ability.

Oxourea **1** was also tested using this experiment, but its limited activity necessitated increasing its concentration to 5 mol % with respect to lipid molecules. In this case, it appeared to be able to couple to Mon but not Vln (Figure S4.4) suggesting limited ability to facilitate electrogenic transport, although the efflux achieved after 5 minutes reached only 6 %.

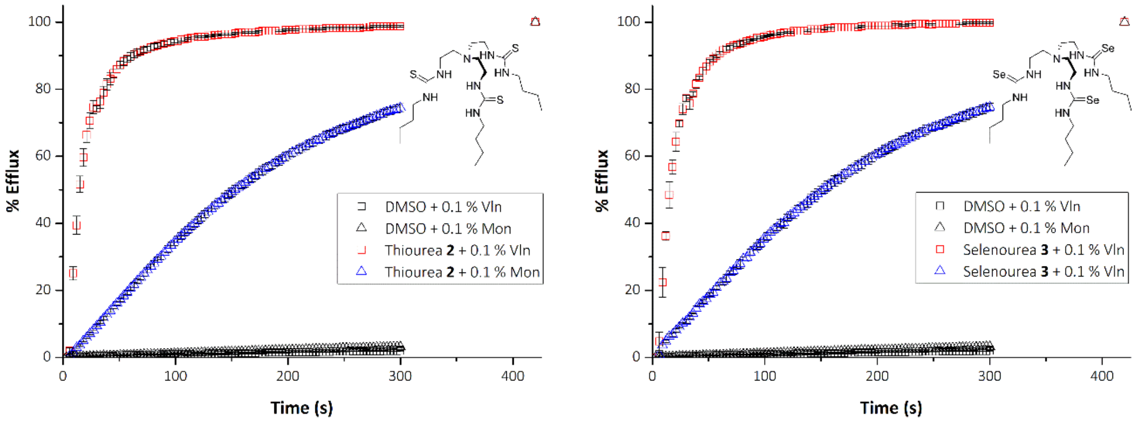


Figure 4. Cl^- efflux from POPC vesicles loaded with 300 mM KCl $_{(aq)}$ suspended in 300mM KGluc $_{(aq)}$ for thiourea **2** (**A**) and selenourea **3** (**B**) at 1 mol % loading in the presence of 0.1 mol % valinomycin (red squares) or 0.1 mol % monensin (blue triangles). Black markers: 5 µl DMSO control.

As previously mentioned, due to the potential selectivity of these compounds, the Cl^-, NO_3^- and H^+/OH^- selectivity of the compounds was probed using a pH-driven HPTS assay. (*3, 26*) POPC vesicles were suspended in a solution of NMDG.HCl or $NMDG.HNO_3$ at pH 7.2 (NMDG = N-methyl-D-glucamine, protonated at physiological pH forming a large hydrophilic cation that cannot be transported) and the anionophores to be tested were added to the membrane as a solution in DMSO. The experiment was commenced by the addition of the free NMDG base to raise the external pH to pH 8, the anionophore is able to dissipate the pH gradient by performing HCl co-transport (or functionally equivalent Cl^-/OH^- exchange). The experiment is repeated in the presence of 0.1 mol % of the proton channel gramicidin (Gr), which provides a fast flux of protons through the membrane. Anionophores which are limited by their H^+/OH^- transport ability, should be enhanced under these conditions as they only need to carry out electrogenic Cl^- transport to balance the charge as fast H^+ flux to dissipate the pH gradient is provided by the channel.

Table 1. $EC_{50\ (270\ s)}$ data obtained for compounds **2** & **3** in the NMDG.HCl and NMDG.HNO_3 assays, together with calculated transport selectivities, and Cl^- over NO_3^- binding selectivity calculated by the ratio of Cl^- to NO_3^- 1:1 binding constants from UV-Vis titrations in acetonitrile (20 µM compound concentration).

	$EC_{50\ (270\ s)}$ / mol %				**Transport Selectivity (S)**			**Binding Selectivity**
Compound	**NMDG.HCl**	**NMDG.HCl + Gr**	**NMDG.HNO_3**	**NMDG.HNO_3 + Gr**	**Cl^-/H^+**	**NO_3^-/H^+**	**Cl^-/NO_3^-**	**Cl^-/NO_3^-**
2	0.032 ± 0.0095	0.0039 ± 0.00073	0.022 ± 0.0023	0.0010 ± 0.00017	8.3	21.4	0.26	119
3	0.031 ± 0.0037	0.0024 ± 0.00021	0.034 ± 0.0089	0.0054 ± 0.000021	13.0	6.1	2.4	316

The HPTS assays were repeated at multiple concentrations of receptors, and Hill fittings were used to obtain $EC_{50\ (270\ s)}$ values as with the Cl^-/NO_3^- exchange assay (Table 1). Note that these values are significantly lower due to the increased sensitivity of this assay caused by the much lower concentration of ions that must be transported to achieve 100% response. Selectivity (S) values for X^- over H^+ over OH^- were obtained by dividing the EC_{50} for that anion in the absence of Gr to that in its presence. The S value for Cl^- over NO_3^- was obtained by dividing the EC_{50} from the NMDG·HNO_3 + Gr by that from NMDG·HCl + Gr.

These data are summarised in Table 1, with the Hill plots reproduced in the ESI. Compound **1** was again too inactive for Hill analysis, a single-point screen suggested slight Cl^-/NO_3^- selectivity, with little gramicidin enhancement in either case suggesting poor electrogenic transport ability, in agreement with the Vln/Mon experiments (Figure S4.5).

Firstly it should be noted that all the EC_{50} values measured in the absence of Gr are the same within error. Thus in these conditions the compounds are rate-limited by their H^+/OH^- transport ability, which is equal for **2** & **3**. This is in agreement with the Vln/Mon experiments which showed that the compounds' electroneutral HCl cotransport ability was lower than their electrogenic Cl^- uniport ability.

The EC_{50} values obtained for the electrogenic (with Gr) transport of Cl^- and NO_3^- show some interesting trends. **2** demonstrates a selectivity for NO_3^- over Cl^- ($S = 0.26$). In contrast, **3** demonstrates faster electrogenic transport for Cl^- over NO_3^- ($S = 2.4$). It has been previously reported that Cl^- over NO_3^- selectivity is well correlated with Cl^- over NO_3^- binding selectivity. (*26*) This can be calculated by dividing the 1:1 binding constants for Cl^- by that for NO_3^- from the UV-Vis titration data; these values are also reported in Table 1.

As discussed above, **2** binds NO_3 much more strongly than **3**, thus its selectivity for Cl^- over NO_3^- binding is much lower despite having a higher absolute affinity for Cl^-. The 100-fold binding selectivity is not enough to override the Hofmeister bias towards NO_3^- selectivity in transport, hence the compound's selectivity. In contrast, the binding selectivity of **3** is 3 times higher, significant enough to flip the selectivity. This is in agreement with previous studies, where compounds that exhibited 100-fold selectivity or less were selective for nitrate, whereas those with > 10^3 fold selectivity are able to mediate Cl^- selective transport. It should be noted that previous studies on a pentyl-substituted tripodal thiourea suggested that this compound showed the opposite selectivity (Cl^- over NO_3^- preference) compared to **2**, (*26*) suggesting a single carbon difference in the length of the chain is sufficient to increase the encapsulation of the anion enough to flip the selectivity of the thiourea analogues.

Finally, it is interesting to consider the EC_{50} for the electrogenic uniport process from the NMDG assays which would be the rate-limiting in the Cl^-/NO_3^- assays (i.e., NMDG.HCl + Gr for NO_3^- selective **2** and NMDG.HNO_3 + Gr for Cl^- selective **3**). These values are the same within error (Table 1), hence the same EC_{50} values for both compounds are observed for Cl^-/NO_3^- exchange.

Conclusions

We have extended the series of alkyl tripodal tris-ureas with the synthesis of a selenourea analogue **3**. In exchange assays, it matches the performance of the previously reported thiourea analogue **2**, maintaining a significant lipophilic advantage over oxourea **1**. On more specific analysis of the compounds' selectivity it was shown that the replacing the thiourea group with a selenourea, flipped the selectivity of the scaffold from a NO_3^- to a Cl^- preference. Thus, despite the additional synthetic difficulty, selenourea may offer an additional advantage in the design of more specific transporter scaffolds in applications where selectivity for Cl^- is key.

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