Anion binding and transport properties of cyclic 2,6-bis(1,2,3-triazol-1-yl)pyridines†

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A series of cyclic 2,6-bis-(1,2,3-triazolyl)-pyridine anion receptors with thiourea functionalities were synthesized by click reaction of 2,6-diazidopyridine with protected propargylamine followed by condensation of a bisthiocyanate derivative with a series of diamines. Their chloride binding affinities as well as their transport properties in POPC bilayers were examined. These receptors were found to function as anion carriers, which can mediate both Cl\(^{-}/NO_3\)^{−} antiport and H^+/Cl^− symport, and the transport activity of these hosts were dominated by their lipophilicity.

The development of synthetic transmembrane transporters is a rapidly expanding area of supramolecular chemistry.1–4 The transport of inorganic anions, such as chloride, across cell lipid bilayers is vital to a number of important biological processes. Malfunctioning of certain anion transport mechanisms can lead to serious diseases,5 most notably cystic fibrosis.6–8

Recently, there has been an increased interest in CH hydrogen bond donors.9,10 In particular, 1,2,3-triazoles proved to be interesting hydrogen bond donor groups for anion complexation11 due to their large polarity (dipole moment ∼5D), with the positive end of the dipole situated at the CH group.12 The practical utility of the 1,2,3-triazoles in anion receptor chemistry is further enhanced by the fact that they are readily accessible via the copper catalyzed Huisgen condensation or so-called ‘Click reaction’.13,14

In our studies, we have previously reported the synthesis and anion binding properties of a series of 2,6-bis-(1,2,3-triazolyl)-pyridine receptors, like 1 and 2 (Chart 1)15 with compound 2 showing a promising chloride binding affinity. Prompted by these findings, we investigated the binding and transport properties of some cyclic derivatives of this compound 2 and compared these results to the properties of the acyclic system.

Results and discussion

The cyclic receptors were synthesized starting from 2,6-difluoropyridine. In the first step of the synthesis, 2,6-diazidopyridine 3 was synthesized by treatment of 2,6-difluoropyridine with NaN_3 in DMF. Molecule 4 was then obtained as previously reported by a click reaction between 2,6-diazidopyridine 3 and N-Boc-propargylamine.15 In a next step, the diisothiocyanate compound 5 was synthesized by a deprotection of the Boc-groups of compound 4, followed by treatment of the obtained bisamine intermediate with thiophosgene, yielding the desired product 5. Finally, the macrocyclic receptors 6a–f were obtained in fair to good yield (18–65%) by combination of 5 with a number of α,ω-diaminoalkyl molecules with different chain lengths under high dilution conditions (Scheme 1).
Solution binding studies

The chloride binding affinities of the obtained cyclic hosts 6a–f were determined by $^1$H NMR titrations with tetra-n-butylammonium chloride in DMSO-$d_6$/$0.5\%$ H$_2$O. The data was fitted to a 1:1 binding model as confirmed by Job plot analysis (Fig. S1 in the ESI†) and the anion binding constants were calculated with HypNMR.17 Moderate binding affinities were obtained for all the hosts (Table 1), the highest binding constants were obtained for 6b and 6c, with log($K_a$) of respectively 1.75 ± 0.02 and 1.76 ± 0.02. For comparison, the binding affinity log($K_a$) of the open-chain receptor 215 was now also measured in DMSO-$d_6$/$0.5\%$ H$_2$O and found to be 1.20, much lower than the 3.4 reported when the less competitive acetonitrile was the solvent. We could unfortunately not use acetonitrile for studies of compounds 6 due to the low solubility.

Crystallography†

The structures of the complexes of compound 6a with the chloride salts of tetraethylammonium, 1-ethylpyridinium and 3-ethyl-1-methyl imidazolium were obtained by single crystal X-ray diffraction methods.18 The crystals were obtained by slow evaporation from DMSO in the presence of excess chloride salt.

Crystals of the tetraethylammonium chloride complex of compound 6a (Fig. 1) were obtained by this method. The macrocyclic receptor was found to be present in two conformations within the crystal (approx. 73 : 27 occupancy) with the alkyl linker chain adopting a marginally different position in each case. In both conformations the chloride anion is bound in the centre of the macrocycle via six hydrogen bonds [N5⋯Cl1 3.260(1) Å; N6⋯Cl1 3.271(2) Å; N7⋯Cl1 3.382(1) Å; N8⋯Cl1 3.307(1) Å; C6⋯Cl1 3.531(1) Å; C19⋯Cl1 3.535(1) Å].

Crystals of the 1-ethylpyridinium chloride complex of compound 6a (Fig. 2) were also obtained as a hemihydrate. This structure contains two crystallographically independent molecules of 6a in a 2 + 2 arrangement via hydrogen bonding to two bridging chloride anions. One chloride anion bridges via two hydrogen bonds from each macrocycle (N5⋯Cl11 3.263(4) Å; N6⋯Cl11 3.263(4) Å; N107⋯Cl11 3.565(4) Å; N108⋯Cl11 3.167(4) Å). The other, along with the water molecule is disordered over two positions (approx. 62 : 38 occupancy) so that in the major component the chloride anion and water molecule each bridge via one hydrogen bond from each macrocycle (N7⋯Cl1 3.453(5) Å; N105⋯Cl1A 3.289(5) Å; N8⋯O1A 3.230 (9) Å; N106⋯O1A 2.939(7) Å) and in the minor component the...
chloride anion bridges via two hydrogen bonds from each macrocycle (N7⋯Cl1B 3.331(7) Å; N8⋯Cl1B 3.056(8) Å; N105⋯Cl1B 3.122(6) Å; N106⋯Cl1B 3.566(7) Å) with no involvement of the water molecule.

Interestingly the crystals of the 3-ethyl-1-methylimidazolium chloride complex of compound 6a (Fig. 3) give a structure which combines features from both the structures previously described. It is a Z′ = 2 structure with two crystallographically independent molecules of 6a which each adopt two conformations (approx. 88 : 12 occupancy). In one of the macrocycles one of the thioureas exhibits minor positional disorder, whereas in the other the disorder is more extensive involving both thioureas and the alkyl linker. In each conformation of both macrocycles a chloride anion is bound in the centre of the macrocycle via six hydrogen bonds (N5⋯Cl1 3.384(3) Å; N6⋯Cl1 3.317(3) Å; N7⋯Cl1 3.339(4) Å; N8⋯Cl1 3.320(4) Å; N7A⋯Cl1 3.31(2) Å; N8A⋯Cl1 3.50(3) Å; C6⋯Cl1 3.708(4) Å; C19⋯Cl1 3.638(4) Å and N105⋯Cl11 3.366(3) Å; N106⋯Cl11 3.327(5) Å; N107⋯Cl11 3.318(3) Å; N206⋯Cl11 3.45(4) Å; N207⋯Cl11 3.40(2) Å; N108⋯Cl11 3.348(3) Å; C106⋯Cl11 3.653(3) Å; C119⋯Cl11 3.660(4) Å).

**Transport studies**

The chloride transport activity of the receptors was initially assessed using an ion selective electrode (ISE) assay.19 We prepared a sample of unilamellar POPC vesicles loaded with 489 mM NaCl buffered to pH 7.2 with 5 mM sodium phosphate salts. The vesicles were suspended in 489 mM NaNO3 buffered to pH 7.2 with 5 mM sodium phosphate salts. A sample of the receptor (2 mol% w.r.t. lipid) was added in DMSO and the resulting chloride efflux was monitored using a chloride selective electrode. After 300 s the vesicles were lysed by the addition of detergent and the final electrode reading used to calibrate 100% chloride efflux. The results are shown below in Fig. 4. All of the thiourea containing receptors were shown to mediate chloride efflux from vesicles under these conditions. Compound 6b (C7 macrocycle) seemed to be the most efficient transporter, mediating the efflux of 81% of the encapsulated chloride after 270 s. Compound 2, which is an acyclic amide analogue of 6b showed no activity, highlighting the importance of the thiourea functionality.

We performed a Hill analysis20 for chloride transport under these conditions for all of the active transporters. This enabled us to determine the EC50 for each receptor – the concentration of receptor required to mediate 50% chloride efflux after...
The receptors tested in this study had a Hill coefficient of the stoichiometry of the transport process. All of these receptors were most likely to function as mobile carriers rather than channels. In order to gain further proof for this mechanism, we prepared a sample of vesicles composed of POPC–cholesterol (7:3). The vesicles contained NaCl and were suspended in NaNO₃ (both solutions buffered to pH 7.2 with 5 mM sodium phosphate salts). The addition of cholesterol to a bilayer is reported to reduce its fluidity—therefore the action of a mobile carrier, which is diffusion controlled, should be reduced.²¹ Correspondingly, the addition of 2 mol% of the receptors was found to mediate a reduced level of chloride efflux compared to experiments performed using vesicles composed of pure POPC lipid, thus indicating a mobile carrier mechanism is in effect.

During the Hill analysis, it was often observed that addition of high loadings of receptor did not result in increased chloride efflux, and in some cases, a precipitate was also observed. This indicates a loss of activity as a result of solubility issues.

The most efficient transporter in each experiment is compound 6b (C7 macrocycle). It has previously been observed that there may be an optimum lipophilicity for anion transport, as the transport process requires a balance between aqueous solubility (in order for the transporter to reach the lipid bilayer), and lipophilicity (as the receptor must efficiently partition with the bilayer to mediate anion transport).²² Without conducting a much larger quantitative structure activity relationship study, which is outside the scope of the current project, it is not possible to systematically determine which molecular parameters should be optimised to maximise the rate of transport.²³ However based on previous findings²² it is reasonable to assume that the optimum lipophilicity observed for 6b results from compounds having shorter chain lengths not being lipophilic enough to efficiently partition into the bilayer, and above this chain length the compounds become increasingly incompatible with delivery through the aqueous phase, resulting in precipitation—i.e. this is essentially a dependence on a (log P)² term. The trend in transport activity is summarized in Fig. 5.

**Symport vs. antiport?**

In order to investigate if the observed chloride transport was by a Cl⁻/NO₃⁻ antiport mechanism (as has been commonly observed for thiourea based transporters) or by a co-transport mechanism, we prepared a sample of vesicles containing 450 mM NaCl buffered to pH 7.2 with 20 mM sodium phosphate salts. The vesicles were suspended in 162 mM Na₂SO₄ buffered to pH 7.2 with 20 mM sodium phosphate salts. If a transporter functions solely by an anion antiport mechanism, it is expected that no transport will be observed if the extra-vesicular anion is sulfate, as the high dehydration penalty for

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**Table 2** Results of the Hill analysis. EC₅₀ values (concentration of receptor required to mediate 50% chloride efflux after 270 s) for Cl⁻/NO₃⁻ transport. Hill coefficients for each receptor

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC₅₀ Cl⁻/NO₃⁻</th>
<th>n</th>
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<tbody>
<tr>
<td>6a (C6)</td>
<td>2.7</td>
<td>1.2</td>
</tr>
<tr>
<td>6b (C7)</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>6c (C8)</td>
<td>3.0</td>
<td>1.1</td>
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<tr>
<td>6d (C9)</td>
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</tr>
<tr>
<td>6e (C10)</td>
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<tr>
<td>6f (C12)</td>
<td>48.0</td>
<td>0.7</td>
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**Fig. 4** Chloride efflux promoted by thioureas 6a–f (2% molar carrier to lipid) from unilamellar POPC vesicles loaded with 489 mM NaCl buffered to pH 7.2 with 5 mM sodium phosphate salts. The vesicles were dispersed in 489 mM NaNO₃ buffered to pH 7.2 with 5 mM sodium phosphate salts. At the end of the experiment, detergent was added to lysis the vesicles and calibrate the ISE to 100% chloride efflux. Each point represents the average of three trials.

**Fig. 5** A representation of the relationship between the size of the macrocycle of receptors 6a–f and their Cl⁻/NO₃⁻ antiport activity (1/EC₅₀ as determined by a Hill plot analysis).
this anion prevents its transport in the vast majority of cases. However, on addition of a sample of the receptors (2 mol% w.r.t. lipid), significant chloride efflux was observed. We repeated these experiments but added a spike of NaHCO_3 to the extravesicular solution after 2 minutes – this was found not to increase the observed chloride efflux, indicating that these receptors do not mediate Cl^-/HCO_3^- antiport. The results for compound 6b are shown below in Fig. 6, and similar results were observed for all thiourea compounds tested.

The chloride efflux in the absence of a readily transported external anion initially indicated that some sort of co-transport process was occurring. We decided to further investigate this effect using the receptors that were found to mediate significant (>15%) chloride efflux after 390 s (6b, 6c, 6a and 6d). All salt solutions referenced in this section contain 20 mM phosphate buffer at pH 7.2 unless otherwise stated.

We considered the possibility of a M^+/Cl^- co-transport mechanism. We prepared a sample of vesicles encapsulating 450 mM CsCl and suspended them in 162 mM Na_2SO_4. We then monitored chloride efflux mediated by the receptors (2 mol%) using a chloride ISE, and compared the data to that collected using vesicles containing NaCl. The results are summarized below in Fig. 7. We found that there was no significant difference in the behaviour of these compounds when the encapsulated cation was changed from Na^+ to Cs^+, indicating the nature of the encapsulated cation does not affect the chloride transport properties of the receptors and hence that a M^+/Cl^- co-transport mechanism is not possible.

We then considered the possibility that these receptors could mediate a Cl^-/SO_4^{2-} antiport mechanism. It is usually assumed that SO_4^{2-} cannot be transported by synthetic ionophores as it is strongly hydrated. However, we have recently seen examples of extremely potent anion transporters that we believe to mediate this transport mechanism. We prepared a sample of vesicles with 100 mM NaCl inside and outside the vesicles, containing the halide sensitive fluorescent probe lucigenin (2 mM). In this experiment, there is no chloride gradient to drive chloride transport. We monitored the fluorescence intensity of the lucigenin in addition of (a) a spike of either NaNO_3, NaCl or Na_2SO_4 at 10 s followed by (b) the addition of the receptor in DMSO (2 mol%) at 40 s (Fig. 8). An increase to the fluorescence intensity of the lucigenin after the addition of

**Fig. 6** Chloride efflux promoted by compound 6b (2% molar carrier to lipid) from unilamellar POPC vesicles loaded with 450 mM CsCl buffered to pH 7.2 with 20 mM sodium phosphate salts. The vesicles were dispersed in 162 mM Na_2SO_4 buffered to pH 7.2 with 20 mM sodium phosphate salts. At t = 120 s, a solution of NaHCO_3 was added to give a 40 mM external concentration (blue markers) or the experiment was allowed to continue without the addition of NaHCO_3 (red markers). At the end of the experiment, detergent was added to lyse the vesicles and calibrate the ISE to 100% chloride efflux. Each point represents the average of three trials.

**Fig. 7** Chloride efflux promoted by 6a–6d (2% molar carrier to lipid) after 270 s from unilamellar POPC vesicles loaded with 450 mM CsCl buffered to pH 7.2 with 20 mM sodium phosphate salts. The vesicles were dispersed in 162 mM Na_2SO_4 buffered to pH 7.2 with 20 mM sodium phosphate salts. At the end of the experiment, detergent was added to lyse the vesicles and calibrate the ISE to 100% chloride efflux. Each point represents the average of three trials.

**Fig. 8** Unilamellar POPC vesicles were loaded with 100 mM NaCl and 2 mM lucigenin buffered to pH 7.2 with 20 mM sodium phosphate salts and dispersed in a 100 mM NaCl solution (buffered to pH 7.2). At t = 10 s, a solution of the appropriate anion was added (final concentration of 40 mM NaNO_3, 40 mM Na_2SO_4 or 40 mM NaCl). At t = 40 s, a DMSO solution of the compound 6b was added. At the end of the experiment (340 s), detergent was added to lyse the vesicles. The blank measurement refers to the addition of Na_2SO_4 followed by the addition of DMSO. Each point represents the average of three trials.
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Fig. 9 Intravesicular pH change promoted by 6a–6d (2% molar carrier to lipid) from unilamellar POPC vesicles loaded with 1 mM HPTS and 489 mM NaCl buffered to pH 7.2 with 5 mM sodium phosphate salts. The vesicles were dispersed in 162 mM Na2SO4 buffered to pH 7.2 with 5 mM sodium phosphate salts. At t = 10 s, a DMSO solution of the putative transporters was added to start the experiment. At the end of the experiment (t = 310 s), detergent was added to lyse the vesicles. Each point represents the average of three trials.

Conclusions

Macroyclic anion receptors 6a–f of different ring sizes, containing thiourea and 2,6-bistriazolopyridinium groups could be prepared by condensation of bisisothiocyanate and bisamine precursors. The binding constants with tetra-n-butylammonium chloride in the relatively competitive solvent DMSO-d$_6$/0.5% H$_2$O are modest but higher than for the open chain analogue. In conclusion, these receptors were found to function as anion carriers which can mediate both a Cl$^-$/NO$_3^-$ antiport and (to a lesser extent) H$^+$/Cl$^-$ symport. The most efficient carrier for Cl$^-$/NO$_3^-$ antiport was 6b, the structure of which provided the optimum balance between lipophilicity and hydrophilicity/solubility.

Experimental section

General experimental methods

NMR spectra were acquired on commercial Bruker instruments (300 and 400 MHz), and chemical shifts (δ) are reported in parts per million (ppm) referenced to tetramethylsilane (δH) or the internal (NMR) solvent signals (δC). Exact mass measurements were acquired in the EI (at a resolution of 10 000) or ESI (at a resolution of 15 000) mode. IR spectra were recorded on an FT-IR spectrometer with a universal sampling module. Melting points were determined by using a Reichert Thermovar apparatus and were not corrected. For column chromatography, 70–230 mesh silica 60 was used as the stationary phase. Chemicals received from commercial sources were used without further purification. Tetra-n-butylammonium salts were dried overnight under vacuum at 40°C prior to use.

2,6-Diazidopyridine (3)

A mixture of 2,6-difluoropyridine (0.3 ml, 3.3 mmol), NaN$_3$ (0.641 g, 9.86 mmol) and tetra-n-butylammonium bromide (0.424 g, 1.32 mmol) in DMF (5 ml) was stirred at 60°C for 48 hours. The crude product was purified by column chromatography (SiO$_2$, EtOAc) to afford 3 (0.145 g, 27%). $^1$H NMR (300 MHz, CDCl$_3$, ppm) δ: 7.55 (t, 1H, J = 7.8 Hz), 6.55 (d, 2H, J = 7.8 Hz); $^{13}$C NMR (75 MHz, CHCl$_3$, ppm) δ: 153.4, 140.9, 109.8; T$_{m}$: 73–75°C; FT-IR (cm$^{-1}$) 2109, 1562.

Di-tert-butyl[(1,1’-[pyridine-2,6-diyi]bis(1H-1,2,3-triazole-4,1-diyl)]bis(methylene)) dicarbamate (4)

To a solution of 2,6-diazidopyridine 3 (0.120 g, 0.74 mmol) in dry THF (5 ml) was added N-Boc-propargylamine (0.18 ml, 2 mmol), [(CH$_3$CN)$_2$Cu]PF$_6$ (0.056 g, 0.15 mmol) and 0.03 ml Et$_3$N and the reaction mixture was stirred at 50°C for 3 hours. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (SiO$_2$, EtOAc) to afford 4 (0.306 g, 87%). $^1$H NMR (300 MHz, DMSO-d$_6$, ppm) δ: 8.94 (s, 2H), 8.38 (t, 1H, J = 8.3 Hz, 8.21 (d, 2H, J = 7.9 Hz), 7.45 (br s, 2H), 4.32 (d, 4H, J = 5.6 Hz), 1.40 (s, 18H); $^{13}$C NMR (75 MHz, DMSO-d$_6$, ppm) δ: 155.6, 147.3, 146.7, 143.8, 120.2, 112.9, 78.05, 35.5, 28.2; HRMS: (ESI$^+$) calculated mass for C$_{21}$H$_{29}$N$_9$O$_4$Na: 494.2235 g mol$^{-1}$ [M + Na$^+$], found: 494.2240 g mol$^{-1}$; T$_{m}$: 165–166°C; FT-IR (cm$^{-1}$): 3407, 1687, 1519.
product was redissolved in 12 mL DCM. Subsequently, thiophosphogine (0.65 mL, 6.8 mmol) and 12 mL saturated KHCO₃ solution were added and the reaction mixture was stirred at room temperature for 16 hours. The product was extracted with DCM (3 x 50 mL), the combined organic layers were dried over Na₂SO₄ and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (SiO₂, CHCl₃ + 1% MeOH) to afford 5 (0.603 g, 65%).¹¹H NMR (300 MHz, DMSO-d₆, ppm): δ: 8.90 (s, 2H), 8.40 (t, 1H, J = 8 Hz), 8.23 (d, 2H, J = 8 Hz), 7.84 (br s, 2H), 7.58 (br s, 2H), 4.83 (s, 4H), 3.39 (m, 4H), 1.52 (m, 4H), 1.32 (m, 6H), 1.23 (m, 2H);¹³C NMR (75 MHz, DMSO-d₆, ppm): δ: 183, 147.4, 146.7, 143.8, 120.1, 113.3, 43.7, 43.6, 28.0, 25.7; MS (ESI+): m/z 822 [M + Na]; Tₘ: 267–268 °C (decomposition upon melting); FT-IR (cm⁻¹): 3250, 3065, 2924, 2851, 1607, 1556.

2,3,4,7,9,16,18,21,22,23,28-Unodecaazatetracyclo- [23.3.1.1.5,1.20,23]tritriaconta-1(28),3,5(30),20(29),21,24,26-heptaene-8,17-dithione (6a)

General procedure for the synthesis of cyclic pyridine receptors. Dry CHCl₃ (30 mL) was refluxed. At reflux, 1,6-diaminohexane (0.030 g, 0.26 mmol) (0.03 mL, 0.25 mmol) and 5 (0.091 g, 0.26 mmol), both dissolved in dry CHCl₃ (10 mL), were simultaneously and slowly added to the CHCl₃ solution over 4 hours. The reaction mixture was further refluxed over night and the obtained precipitate was collected by filtration. This precipitate was then further purified by column chromatography (SiO₂, CHCl₃ + 1% MeOH) to afford 6a (0.036 g, 18% yield) of receptor 6a was obtained.

2,3,4,7,9,17,19,22,23,24,29-Unodecaazatetracyclo- [23.3.1.1.5,1.20,23]tetraconta-1(32),3,5(34),24(33),25,28,30-heptaene-8,21-dithione (6e)

Synthesis according to general procedure 1: 0.100 g 5 (0.28 mmol), 0.041 g 1,8-diaminooctane (0.28 mmol). 0.092 g (62% yield) of receptor 6e was obtained.

2,3,4,7,9,22,24,27,28,29,34-Unodecaazatetracyclo- [28.3.1.1.5,1.20,23]hexatriaconta-1(34),3,5(36),26(35),27,30,32-heptaene-8,23-dithione (6f)

Synthesis according to general procedure 1: 0.100 g 5 (0.28 mmol), 0.048 g 1,10-diaminoundecane (0.28 mmol). 0.027 g (18% yield) of receptor 6f was obtained.

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Notes and references