Anion Transport

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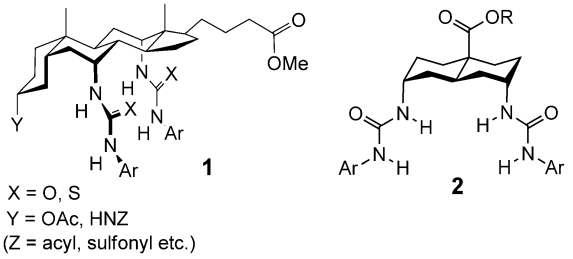
A Flexible Solution to Anion Transport: Powerful Anionophores Based on a Cyclohexane Scaffold**

*James A. Cooper, Steven T. G. Street, and Anthony P. Davis**

Abstract: *Transmembrane anion carriers (anionophores) have potential for biological activity, including the treatment of channelopathies such as cystic fibrosis. A new family of anionophores has been synthesized, in which three thiourea groups are mounted on a cyclohexane-based scaffold. Though conceptually related to earlier polycyclic systems, these molecules are simpler and far more accessible. Preorganization is somewhat reduced compared to earlier systems, and anion affinities are correspondingly lower. However, transport activities set new records. This surprising performance suggests a role for controlled flexibility in the design of transmembrane anion carriers.*

There is widespread interest in the design of anionophores, molecules capable of transporting anions across bilayer membranes.[1] Such agents would complement the well-known cationophore natural products (valinomycin, monensin, etc.), and may result in the discovery of new biological activities. In particular, there is hope that they could be used in "channel replacement therapy", for illnesses such as Best disease, Bartter's syndrome and (especially) cystic fibrosis.[1a] These genetic conditions result from defective anion channels,[2] and could perhaps be treated by providing alternative pathways for transmembrane anion transport.[3] If anion transporters are to serve practical purposes, it is clearly desirable to achieve high activities so that only small amounts are required. However, despite extensive work, there are still few systems which show proven effectiveness at low transporter loadings.[4,5] Among the most active are the cholapods **1**[4a,b] and the closely related diaxial diureidodecalins **2**.[4c] With optimal substitution, both series are capable of promoting chloride/nitrate exchange in synthetic vesicles at levels of transporter:lipid $\leq$ 1:250 000.

The success of **1** and **2** is obtained at a cost, as these rigid, polycyclic structures require lengthy syntheses. The cholapods **1** are prepared from the natural steroid cholic acid in 7 to 12 steps, depending on the variant. The diureidodecalins **2** require at least 7 steps from commercially available starting materials (depending on the choice of side-chain R). Herein we describe two new families of transporters, **3** and **4** (Figure 1), which are accessible in 3 to 4 steps from commercially available starting materials. Transporters **4**, in particular, are remarkably active and include the most powerful anionophore yet reported from our laboratory.



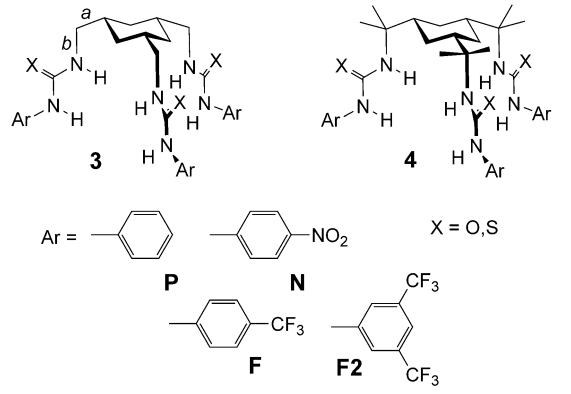


Figure 1. Cyclohexane-based tris-(thio)ureas discussed in this paper. The molecules are labeled according to scaffold (**3**/**4**), X (**O**/**S**) and aromatic substituent; for example **3OP** corresponds to the tris-*N*-phenylurea based on scaffold **3**. Side-chains are shown in the axial/NH-in conformation required for cooperative binding.

The new designs extend our theme of exploiting 1,5-diaxial arrangements of H-bond donors to bind and transport anions. In the case of **1** and **2**, this positioning creates preorganized binding sites which are complementary to inorganic anions such as chloride, while also preventing intramolecular hydrogen bonding (see Figure 2a). We reasoned that if two binding groups with this arrangement were effective, then three should be better, and that in this case some flexibility might be allowable. In particular, the trisubstituted cyclohexanes **3** seemed interesting. Ab initio calculations suggested that the tris-(axial/NH-in) conformation shown can form six hydrogen bonds to Cl^- with lengths in the range 2.6–2.8 Å.[6] Although slightly longer than ideal,[7]

[*] J. A. Cooper, S. T. G. Street, Prof. A. P. Davis
School of Chemistry, University of Bristol
Cantock's Close, Bristol, BS8 1TS (UK)
E-mail: Anthony.Davis@bristol.ac.uk

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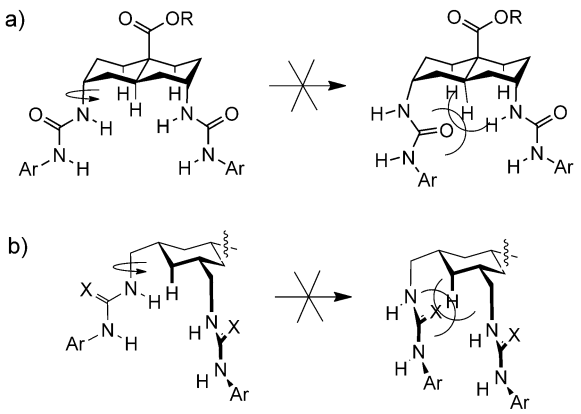


Figure 2. a) Conformational restrictions in diaxial diureidodecalins **2**. Rotation about the axial C–N bonds is strongly disfavored by steric interactions with axial CH. Intramolecular H-bonding is thus disallowed. b) Similar restrictions apply in **3** and **4**. Attempts to form intramolecular H-bonds are prevented by axial CH.

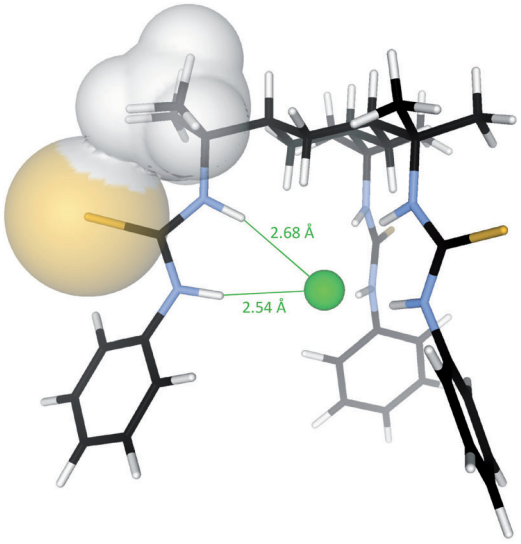
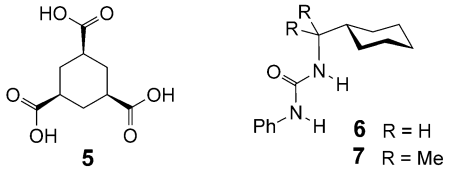


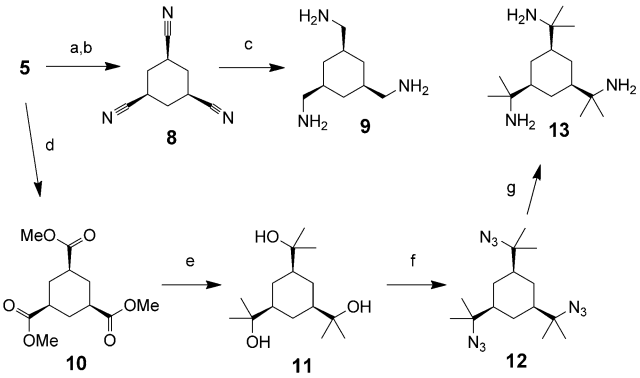
Figure 3. Calculated[6] C_3 symmetrical structure for chloride bound to **4SP**. NH···Cl^- distances are shown in green. A transparent surface highlights the Me···S interactions which compress the binding site, thus shortening and strengthening the H-bonds.

these should provide substantial binding energy. Rotations would be possible about bonds *a* and *b* (see Figure 1), and the binding conformation would not be favored.[6] However, it could presumably be adopted in the presence of substrate. Intramolecular H-bonding would be prevented as for other 1,5-diaxial systems (Figure 2b). The tris-urea **3OP** was known, having been synthesized from commercial triacid **5** in just four steps.[8] We also realized that the hexamethyl analogues **4** could be still more promising. A short synthesis from **5** seemed realistic (see later). The lipophilicity of the added methyl groups would favor applications in membranes, and binding studies in organic solvents. Moreover, introduction of the methyl groups would promote binding in two ways. Firstly, they would alter the balance between side-chain conformations, favoring the axial/NH-in arrangement. This was demonstrated by calculations on the single-armed analogues **6** and **7**. Although not the ground state, the axial/



NH-in conformation for **7** was 3.6 kJ mol^{-1} closer to baseline than for **6**.[6] Secondly the methyl groups would strengthen the H-bonding by compressing the binding site, pushing the O/S downwards and thus moving the Ar-NH groups inwards. Calculations suggested that this effect could shorten some NH···Cl^- distances to 2.54 Å, within the optimum range predicted from crystallography[7] (see Figure 3).

The syntheses of **3** and **4** required practical routes to tris-(aminoalkyl)cyclohexanes **9** and **13**. The literature[8] preparation of **9** involved conversion of **5** to the corresponding tris-amide followed by reduction by $LiAlH_4$, proceeding in 10%



Scheme 1. Synthesis of triamines **9** and **13**: a) $SOCl_2$, DMF, 90°C, then NH_3, DCM, 97%; b) $SOCl_2$, DMF, 45°C, 60%; c) H_2 (1 atm), Raney Ni, 10% NH_4OH/MeOH, 75%; d) $SOCl_2$, DMF, 90°C, then MeOH, Pyridine, 70°C, 99%; e) MeMgBr, THF, 81%; f) $TMSN_3$, $BF_3{\cdot}Et_2O$, $CHCl_3$, 49%; g) H_2 (1 atm), Pd/C, EtOH, 93%.

overall yield. For the present work we developed a new route via hydrogenation of trinitrile **8**, as shown in Scheme 1.[6] Starting material **8** is commercially available, but can also be prepared from **5** by amidation and dehydration. The novel triamine **13** was synthesized in three steps from triester **10** (Scheme 1). Treatment of **10** with methylmagnesium bromide gave triol **11**, which was subjected to azide displacement using $Me_3SiN_3/BF_3{\cdot}Et_2O$ as described by Koziara and Zwierzak.[9] The product triazide **12**[10] was then converted to triamine **13** by catalytic hydrogenation. The overall yield from **10** to **13** was 37%. Triester **10** is commercially available, but may also be prepared economically from **5** by simple esterification ($SOCl_2$ then MeOH).

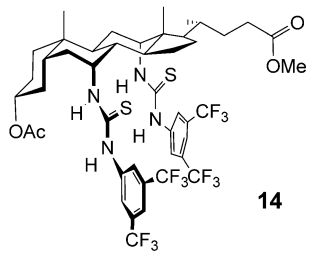
Triamines **9** and **13** were treated with aryl isocyanates and isothiocyanates to give the tris-(thio)ureas listed in Table 1.

Table 1: Chloride binding affinities (K_a, M^{-1}) and transport data for the compounds discussed in this paper.

Compound	K_a [M^{-1}][a]	Cl^-/NO_3^- exchange (transporter:lipid = 1:2500)[b]	
		I[c] [s^{-1}]	$t_{1/2}$[d] [s]
3OP	27	[e]	[e]
3OF2	57	[f]	[f]
3SF	68	0.00091	440
3SN	79	0.0014	340
3SF2	99	0.0046	120
4OP	180	[e]	[e]
4OF2	390	0.036	16
4SP	340	0.0017	190
4SF	400	0.029	22
4SN	480	0.01	66
4SF2	670	0.065	9
14	12000	0.093	6

[a] To $Bu_4N^+Cl^-$ in $[D_6]DMSO/H_2O$, 200:1, measured by ^1H NMR titration. $T = 298$ K. [b] In 200 nm vesicles formed from POPC/cholesterol (7:3) measured by following decay of lucigenin fluorescence F. [c] Initial rate of change of relative fluorescence, F/F_0. [d] Decay half-life of F/F_0. [e] Not measured due to low solubility. [f] Fluorescence decay indistinguishable from background.

All could be dissolved in $[D_6]DMSO$ for binding studies (see below), but solubilities in less polar solvents varied considerably. In general solubility was favored by X = S (rather than O), methylated scaffold **4** (rather than **3**) and trifluoromethylated aryl groups, especially **F2**. Compound **4SF2** could be dissolved in chloroform to the level of ca. 1 mM. Cholapod **14** was synthesized as a comparison using previously reported procedures.[11]



14

The tris-(thio)ureas were assessed as receptors for chloride in $[D_6]DMSO/H_2O$, 200:1 through ^1H NMR titrations with $Bu_4N^+Cl^-$ as substrate.[6] In all experiments the most significant movement was observed for the two NH protons, which could be followed throughout. The titration data could be fitted to a 1:1 model using the HypNMR2008 computer program,[12] giving the results summarized in Table 1. As expected, the use of more electron-withdrawing aryl groups increases the chloride affinities of both series **3** and **4**, while the thioureas are nearly twice as powerful as the ureas. We were pleased to find that the affinities for **4** were higher than those for **3** by factors of 6–7, consistent with the conformational arguments discussed earlier (Figure 3). On the other hand the affinities were somewhat disappointing when compared to cholapod **14**. Tris-thiourea **4SF2**, which is the most powerful of the new compounds and the most comparable to **14**, was found to be 18-times weaker than the cholapod. Where solubility permitted, binding constants to $Et_4N^+Cl^-$ in chloroform were measured using a variant of Cram's extraction method,[13] as used previously for cholapods[14] and other powerful receptors.[4c] The values were considerably higher than those in wet DMSO (by ca. 5 orders of magnitude) but the trends were the same. For example **3SF2**, **4SF2** and **14** gave apparent[15] $K_a = 2.4 \times 10^6$, 3×10^7 and $4.3 \times 10^9\,M^{-1}$, respectively. Once again, it seemed that the methyl groups in **4** improved preorganization, but not enough to compete with the rigid steroidal scaffold.

As we planned to test for transport using chloride/nitrate exchange, the binding properties towards nitrate were also studied. Calculations on **3SP**·NO_3^- and **4SP**·NO_3^- revealed C_3 symmetrical structures with NH···O distances of 2.07–2.34 Å.[6] ^1H NMR titrations of **4SN** and **4SF2** with $Bu_4N^+NO_3^-$ in $[D_6]DMSO/H_2O$, 200:1 produced evidence of binding (downfield shifts of NH signals), but with affinities too small for meaningful analysis. This mirrors previous work which suggests that nitrate is especially difficult to bind in this medium.[5g] The affinities of **3SF2**, **4SF2** and **14** to $Et_4N^+NO_3^-$ in chloroform were measured by extraction, giving values of 5.2×10^5, 2.5×10^6 and $1.9 \times 10^8\,M^{-1}$, respectively. Thus all three receptors bound nitrate somewhat more weakly than chloride, in line with previous observations.[4b,14b]

Anion transport by the new compounds was investigated in large unilamellar vesicles (200 nm mean diameter) using the previously reported "lucigenin method".[4,6] Briefly, vesicles containing aqueous $NaNO_3$ (225 mM) and the halide-sensitive fluorescent dye lucigenin were prepared from a 7:3 ratio of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and cholesterol (7:3), with a small amount of added transporter. Suspensions of these vesicles in aqueous $NaNO_3$ (225 mM) were placed in a fluorescence spectrometer. An external pulse of sodium chloride (25 mM) was added and the influx of Cl^- was followed through the decay in lucigenin fluorescence. The transport rates were quantified in two ways. Firstly the initial rate of change of relative fluorescence I was determined by empirical curve fitting, and secondly the approximate half-life $t_{1/2}$ was estimated through fitting to a single exponential decay function.

An early series of experiments employed the (thio)ureas at loadings of transporter:lipid = 1:2500. Typical decay curves are shown in Figure 4, and the values of I and $t_{1/2}$ are listed in Table 1. Only one tris-urea, **4OF2**, gave positive results; **3OP** and **4OP** were not tested because of their very low solubilities, and **3OF2** gave no transport (probably again due to poor solubility). However all the tris-thioureas showed useful, measurable activity. For both series **3S** and **4S**, we observed the expected correlation between anion affinity and transport rate, with one exception in that **4SN** was less active than expected. We were pleased to find that the advantage of methylated scaffold **4** was retained. For example **4SF2** was ca. 14-times more active than **3SF2**. Most importantly the absolute levels of activity were surprisingly high, considering the modest chloride affinities. The most powerful, **4SF2**, showed $t_{1/2} < 10$ s, quite similar to cholapod **14**.

To obtain more meaningful comparisons, we performed a second series of measurements at lower transporter load-

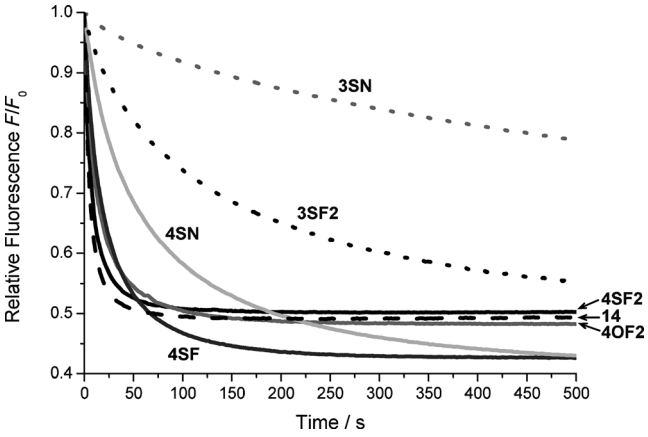


Figure 4. Chloride transport by **3** and **4** at receptor:lipid = 1:2500, detected by the lucigenin method (see text). These traces were used to obtain the *I* and $t_{1/2}$ values listed in Table 1.

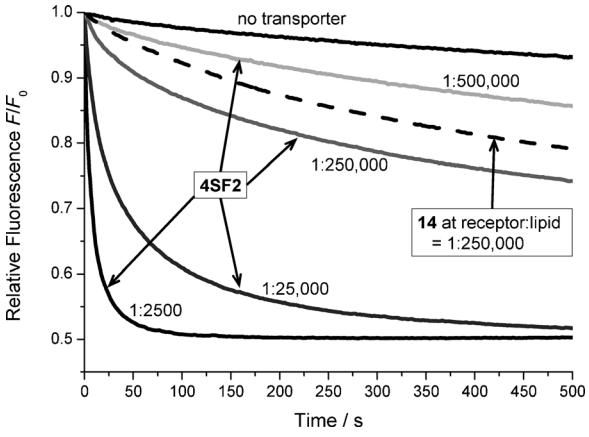


Figure 6. Chloride transport by **4SF2** at different receptor:lipid ratios (lucigenin method). A trace for **14** at receptor:lipid = 1:250000 is included for comparison.

ings, focusing on the most active systems from each series (**3SF2** and **4SF2**) as well as control cholapod **14**. Results from experiments at transporter:lipid = 1:25000 are shown in Figure 5 and Table 2. **4SF2** and **14** were also compared at transporter:lipid = 1:250000 (Table 2), and **4SF2** was tested at the very low loading of transporter:lipid = 1:500000. The traces for **4SF2** at all loadings, with that of **14** at 1:250000, are shown in Figure 6. As expected, the advantage of **4** over **3** was confirmed, the methylated **4SF2** proving to be ca. 10-times more active than **3SF2** at transporter:lipid = 1:25000 (Table 2, Figure 5). More significantly, **4SF2** was found to overtake **14** at these concentrations (possibly reflecting some self-association at higher loadings). At transporter:lipid = 1:25000 the cyclohexane-based system was roughly 3-times more powerful than the cholapod (Table 2, Figure 5). Indeed, **4SF2** was exceptional by historic standards. Transport was clearly detectable at transporter:lipid = 1:500000 (Figure 6), and comparison with archived traces implied higher activity than all previous systems reported from this laboratory (see Figure S48, Supporting Information).

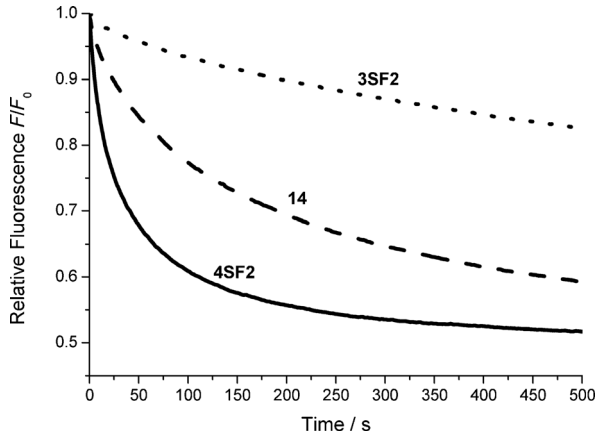


Figure 5. Chloride transport by **3SF2**, **4SF2** and cholapod **14** at receptor:lipid = 1:25000 (lucigenin method). These traces were used to obtain the *I* and $t_{1/2}$ values listed in Table 2.

Table 2: Anion transport data for thioureas **3SF2**, **4SF2**, and **14** at low transporter loadings.[a]

Compound	Transporter:lipid = 1:25000		Transporter:lipid = 1:250000	
	I [s^{-1}]	$t_{1/2}$ [s]	I [s^{-1}]	$t_{1/2}$ [s]
3SF2	0.00097	408		
4SF2	0.013	54	0.0023	214
14	0.0037	147	0.00095	309

[a] Details as for Table 1.

The surprisingly high activities observed for **3** and **4** suggested that they might be acting as self-assembled channels rather than mobile carriers. To test this possibility, **4SF2** was tested for chloride/nitrate exchange in vesicles composed of dipalmitoylphosphatidylcholine (DPPC), which undergoes a gel–liquid phase transition at 41 °C. At 45 °C rapid chloride transport was observed, but at 25 °C **4SF2** showed no activity (see Figure S49). Like the cholapods[4b] it seems that **4SF2** is sensitive to bilayer viscosity, suggesting a mobile carrier mechanism.[16,17]

Finally we were interested to know whether, like some other systems,[4c,5c,g,11] the new transporters might also be effective for the biologically important anions bicarbonate and sulfate. As these hydrophilic substrates are resistant to transport, they can be studied by pairing with chloride in antiport experiments and following the change in Cl^- concentration (bicarbonate/sulfate transport being the rate-limiting process). Transporter **4SF2** was tested in vesicles prepared using 1) aqueous Na_2SO_4 and 2) aqueous $NaHCO_3$. In the former case the rate of fluorescence decay was negligible after an initial small drop (see Figure S51). It therefore seems that **4SF2** does not transport sulfate at an appreciable rate. The initial drop may be due to unbalanced chloride influx, creating a potential across the membrane. With the vesicles formed using $NaHCO_3$ an initial drop in fluorescence was followed by slow long-term decay, suggesting that bicarbonate can be transported but only very poorly.

The remarkable effectiveness of these cyclohexane-based transporters may provide new insight into anionophore design. Studies thus far have suggested that binding affini-

ty[1h,4] and lipophilicity[18] are major factors determining activity, and trends within the **3**/**4** series are consistent with both effects. Lipophilic balance (an even distribution of lipophilic groups)[19] and molecular size[18a] also seem to be relevant. However, the difference between **4SF2** and **14** is difficult to rationalize on the basis of these factors. The two molecules possess similar sizes and *c*log*P* values[6] and are not obviously different in terms of lipophilic balance. Nonetheless, **4SF2** is 3-times *more* active than **14** as a transporter, but 18-times *less* powerful as a receptor. A possible explanation[20] may be the difference in flexibility. The cholapods **1** (and decalins **2**) possess preorganized NH groups which are well-suited for binding but perhaps less so for transport, where on–off rates could be critical. The conformational freedom of **3**/**4** may lower affinities but, to compensate, accelerates binding kinetics. The methyl groups in **4** promote transport by improving binding without substantially affecting flexibility.

In conclusion, we report a new series of cyclohexane-based anion carriers which are less preorganized but more accessible than earlier systems. While their flexibility reduces anion affinities, transport rates remain high. Especially notable is the hexamethyl system **4** in which acyclic conformational control improves binding and transport, and which sets new records for carrier activity. The results suggest that control of flexibility could be an important parameter for the design of future anionophores.

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[6] For details of calculations, synthetic procedures, binding experiments and transport studies, see the Supporting Information.

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[20] As suggested by a referee, the possibility that the earlier transporters bind too strongly should also be considered (see: A. V. Jentzsch, D. Emery, J. Mareda, P. Metrangolo, G. Resnati, S. Matile, *Angew. Chem.* **2011**, *123*, 11879; *Angew. Chem. Int. Ed.* **2011**, *50*, 11675; J. P. Behr, M. Kirch, J. M. Lehn, *J. Am. Chem. Soc.* **1985**, *107*, 241). However, within closely related series we have generally found that affinities correlate with transport activity, even for very powerful receptors. It thus seems that we have not yet reached the zone where improved binding inhibits transport.

