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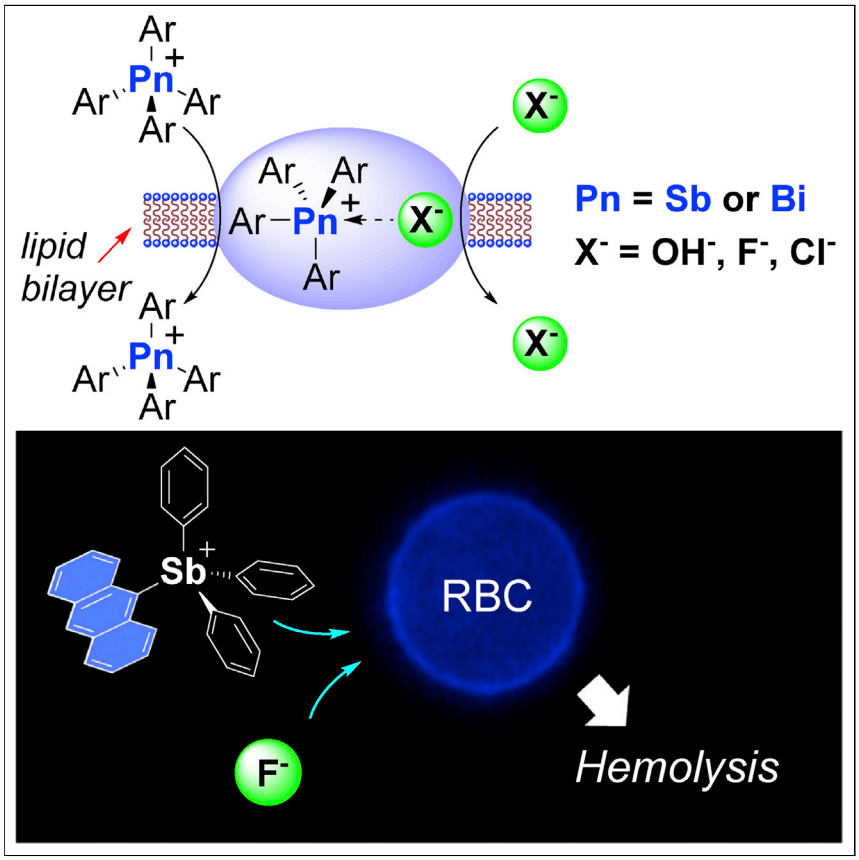
Heavy Pnictogenium Cations as Transmembrane Anion Transporters in Vesicles and Erythrocytes



This work introduces a new approach to transmembrane anion transport based on lipophilic and Lewis acidic tetraarylstibonium and tetraarylbismuthonium cations. The stibonium cations are particularly appealing given that they readily transport hydroxide, fluoride, and chloride anions in synthetic vesicles. When combined with human erythrocytes, the most active stibonium cation uncovered in this study quickly localizes in the cell membranes and induces hemolysis when fluoride is present. This effect is assigned to the transporter-facilitated influx of toxic fluoride anions.

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HIGHLIGHTS

Lewis acidic tetraarylstibonium and tetraarylbismuthonium cations as anion transporters

Transport of anions, including fluoride, across artificial membranes

Transporter-facilitated hemolysis of erythrocytes in the presence of fluoride anions

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Heavy Pnictogenium Cations as Transmembrane Anion Transporters in Vesicles and Erythrocytes

Gyeongjin Park,[1] Dakota J. Brock,[2] Jean-Philippe Pellois,[1,2,*] and François P. Gabbaï[1,3,*]

SUMMARY

Our work on the complexation of fluoride anions using group 15 Lewis acids has led us to investigate the use of these main-group compounds as anion transporters. In this paper, we report on the anion-transport properties of tetraarylstibonium and tetraarylbismuthonium cations of the general formula $[Ph_3PnAr]^+$, where Pn = Sb or Bi and Ar = phenyl, naphthyl, anthryl, or pyrenyl. Using egg yolk phosphatidylcholine (EYPC)-based large unilamellar vesicles, we show that these main-group cations transport hydroxide, fluoride, and chloride anions across phospholipid bilayers. A comparison of the properties of $[Ph_3SbAnt]^+$ and $[Ph_3BiAnt]^+$ (Ant = 9-anthryl) illustrates the favorable role played by the Lewis acidity of the central pnictogen element with respect to the anion transport. Finally, we show that $[Ph_3SbAnt]^+$ accelerates the fluoride-induced hemolysis of human red blood cells, an effect that we assign to the transporter-facilitated influx of toxic fluoride anions.

INTRODUCTION

The toxicity of fluoride at high doses derives, in part, from its enzyme-inhibiting properties.[1,2] Logically, the adverse properties of these anions also originate from its ability to penetrate cells, a step likely necessary before accessing vulnerable enzymes. Bacteria equipped with naturally occurring fluoride anion channels capable of exporting the toxic anion display a greater resilience when exposed to high doses of fluoride.[2–5] These findings have served as an inspiration for the development of synthetic derivatives that could be used to transport fluoride anions through cell membranes. While Matile and co-workers obtained initial evidence that synthetic ionophores based on naphthalene diimides are capable of transporting fluoride ions,[6,7] a team led by Gale has recently shown that strapped calix[4]pyrroles such as **A** behave as selective fluoride anion transporters, a property correlated to the favorable hydrogen bonds formed between the host and the anionic guest (Figure 1).[8] There is also growing interest in the transport of hydroxide anions, a process that could be used to defuse pH gradients across cellular membranes.[9,10] Such processes may be used to induce apoptosis, thus opening the door to applications in cancer therapy.[11–15]

While the use of organic receptors dominates the domain of anion transport, a series of recent reports indicate that Lewis acidic main-group compounds may constitute promising platforms, as in the case of the pnictogen-, chalcogen-, and halogen-bond-donor derivatives **B**,[16] **C**,[16] **D**,[17] and **E**,[18] which transport chloride ions through artificial lipid bilayers (Figure 1). These precedents, as well as the knowledge we have derived from our work on main-group Lewis acids as anion sensors,[19–22] led us to

The Bigger Picture

The transport of anions through human cell membranes usually occurs through channel proteins, which support numerous essential processes, including synaptic signal transmission, cell and organelle acidification, transepithelial salt transport, cell division, and apoptosis. With the view to mimic and possibly one day replace such channels for therapeutic purposes, the design of small molecules that facilitate the transport of anions through artificial phospholipid membranes has become a topic of active research. This article introduces an approach to transmembrane anion transport based on readily accessible group 15 cations. The results obtained in this study demonstrate that lipophilic tetraarylantimony and tetraarylbismuth cations are particularly well suited for the transmembrane transport of hard anions, such as the fluoride and hydroxide anions.

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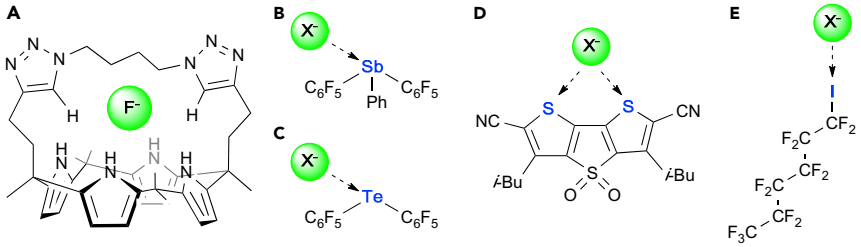

Figure 1. Selected Examples of Relevant Transmembrane Anion Transporters

speculate that Lewis acidic pentavalent pnictogen derivatives may be potent fluoride or hydroxide ion transporters. While contemplating this idea, it occurred to us that pnictogenium cations could be appealing targets because of the known cell-penetrating properties of their phosphorus congeners. Indeed, lipophilic phosphonium cations are known to quickly penetrate cell membranes, ultimately locating in the mitochondrion as a result of a negative electrostatic gradient (Figure 2).[23–25] Merging the concepts of cell penetration with those of Lewis acidity at the group 15 center, we have now decided to test whether pnictogenium cations would serve as a new type of fluoride and hydroxide anion transporters (Figure 2).

RESULTS AND DISCUSSION

Pnictogenium Cations

While the use of tetraarylphosphonium cations was initially tempting, we contended that the lack of Lewis acidity at the group 15 center might be problematic. Since simple stibonium or bismuthonium cations are known to react with fluoride anions to afford covalent λ^5-fluoro-stiboranes[19,22,26–28] or -bismuthanes,[29] respectively, we decided to focus on the use of these heavy pnictogen cations as potential transporters. The precedent that bismuth enjoys in medicine[30] and the ease of preparation of tetraarylbismuthonium cations served as an additional incentive for studying bismuth-based systems.[31,32] Bearing in mind that lipophilicity might play a significant role in the transport properties of these cations, we targeted cations $[\mathbf{1}]^+$–$[\mathbf{8}]^+$ (Figure 3) which could be readily prepared using existing protocols.[22,31] While $[\mathbf{6}]BF_4$ and $[\mathbf{7}]BF_4$ are new, we note that all other pnictogenium cations have been previously described.[19,22,33,34] All new salts have been fully characterized, and the solid-state structure of $[\mathbf{7}]BF_4$ has been crystallographically determined (see Figures S1–S10).

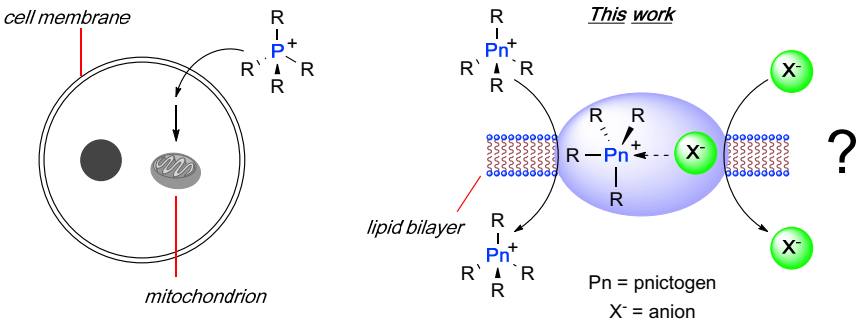

Figure 2. Cell-Penetrating and Anion-Binding Properties of Lipohilic Pnictogenium Cations

Left: Illustration of the ability of phosphonium cations to cross cell walls and target mitochondria. Right: Graphical summary of the hypothesis tested in this study.

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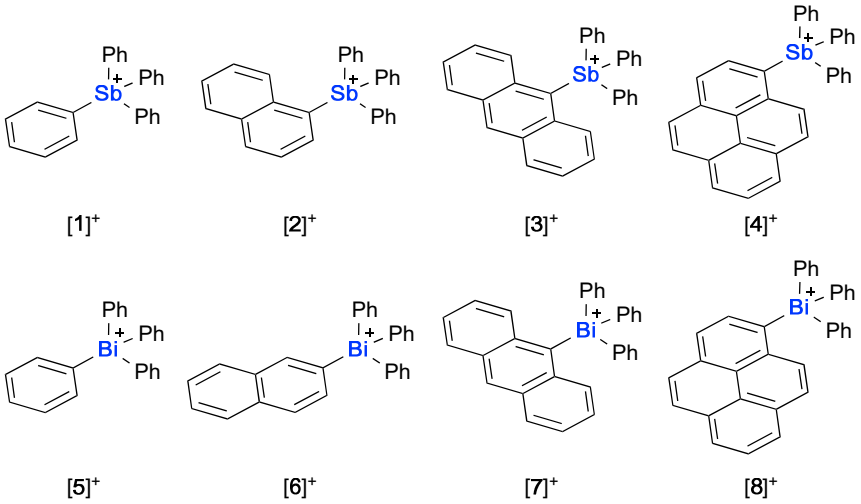
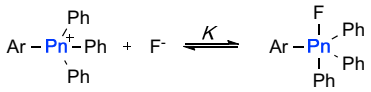


Figure 3. Structures of the Stibonium and Bismuthonium Cations Investigated as Anion Transporters

Next, we measured the fluoride anion affinity of these cations in DMSO/H_2O 98:2 (v/v) using KF as a fluoride source and UV-visible spectroscopy as a monitoring method (Scheme 1; Figures S11–S18). All stibonium cations ($[1]^+$–$[4]^+$) display fluoride binding constants in excess of 10^7 M^{-1}, indicating essentially quantitative conversion into the corresponding λ^5-fluoro-stiborane under these experimental conditions. More progressive fluoride binding was observed with the bismuthonium cations ($[5]^+$–$[8]^+$). In this case, fitting the resulting titration data to a 1:1 binding isotherm afforded the binding constants K_a in Table 1. Because the binding constants of $[5]^+$ and $[6]^+$ are lower than those of $[7]^+$ and $[8]^+$, these results suggest a correlation between the hydrophobic character of the compounds and their fluoride ion affinity. Another important conclusion from these titration experiments is the higher Lewis acidity displayed by the antimony cations. While this conclusion may appear surprising at first, we note that it is readily reproduced by fluoride anion affinity calculations since the computed fluoride anion affinity of $[Ph_4Bi]^+$ is 3.9 kcal/mol lower than that of $[Ph_4Sb]^+$. We assign this difference to the more diffuse orbitals of bismuth, a factor that would weaken the covalent component of the Bi–F bond. We also note that relativistic effects in the sixth period lead to an anomalous increase of the electronegativity (χ = 2.01 for Bi versus 1.82 for Sb),[35] thereby lowering the Coulombic stabilization of the Bi–F bond. While the formation of fluorostiboranes by the reaction of fluoride anions with stibonium cations is well understood, knowledge about the corresponding bismuth species is more limited. To confirm that the bismuthonium cations interact with fluoride, we conducted ^{19}F NMR and ^1H NMR studies (Figures S20–S25). A resonance corresponding to the bismuth-bound fluorine atom was observed upon addition of $[6]^+$, $[7]^+$, or $[8]^+$ to solutions of tetra-*n*-butylammonium fluoride (TBAF) in d_6-DMSO. These ^{19}F NMR resonances appeared between –81 and –77 ppm, a range that can be compared to the value reported for pure Ph_4BiF in $CDCl_3$ (–84.6 ppm)[29] or DMSO-d_6 (–83.3 ppm, see Figure S19).



Scheme 1. Binding Equilibrium Occurring between a Pnictogenium Cation and a Fluoride Anion
K represents the equilibrium constant.

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Table 1. Partition Coefficients, Fluoride Binding Constants, and Fluoride-Transport-Activity Data Obtained for $[1]^+$-$[8]^+$

$[Pn]^+$	$\log K_{ow}$ [a]	K_a (M^{-1}) [b]	EC_{50} (mol %) [c]	n [d]
$[1]^+$	4.19	$>10^7$	6.91 (±0.70)	1.32 (±0.18)
$[2]^+$	5.09	$>10^7$	2.44 (±0.01)	0.98 (±0.05)
$[3]^+$	5.85	$>10^7$	0.41 (±0.05)	0.88 (±0.13)
$[4]^+$	6.26	$>10^7$	0.57 (±0.07)	1.03 (±0.13)
$[5]^+$ [e]	4.06	$6.7\ (\pm 0.2) \times 10^3$	–	–
$[6]^+$	4.94	$1.7\ (\pm 0.1) \times 10^4$	6.47 (±0.38)	0.99 (±0.06)
$[7]^+$	5.55	$1.1\ (\pm 0.3) \times 10^5$	1.24 (±0.03)	1.28 (±0.04)
$[8]^+$	6.04	$5.2\ (\pm 1.0) \times 10^4$	1.38 (±0.05)	1.50 (±0.08)

[a]Calculated octanol-water partition coefficient defined as $\log K_{ow}$ at 298 K.
[b]Fluoride anion binding constant in DMSO/H_2O 98:2 (v/v).
[c]Effective concentration needed to achieve 50% transport at t = 270 s (with valinomycin).
[d]Hill coefficient derived from the fluoride efflux data (with valinomycin).
[e]The low transport activity of this cation did not allow for Hill analysis at acceptable transporter concentrations.

Fluoride Anion Transport

To test the transport activities of $[1]^+$–$[8]^+$, we designed an assay in which a DMSO solution (7 μL) of the pnictogenium cation (10 mM) was added to a solution containing fluoride-loaded large unilamellar vesicles (LUVs) prepared with egg yolk phosphatidylcholine (EYPC) suspended in a buffered aqueous solution (5 mL, [EYPC] = 0.7 mM) (Figure 4; experiment 1).[8,17] Using a fluoride-selective electrode, we first verified that fluoride efflux was negligible in the absence of any transporter (Figure S26). With this baseline established, we tested the effect of DMSO alone and found that it induced a modicum of transport, as illustrated in Figure 5. Marked fluoride transport was detected in the presence of stibonium cations ($[1]^+$, $[2]^+$, $[3]^+$, or $[4]^+$), and the most hydrophobic derivatives $[3]^+$ and $[4]^+$ showed a higher activity. In the case of the bismuthonium cations, the most hydrophilic cation $[5]^+$, as well as $[6]^+$, showed no significant transport activity, suggesting that the higher Lewis acidity of the antimony cations is a favorable factor (Figure 5A). The importance of

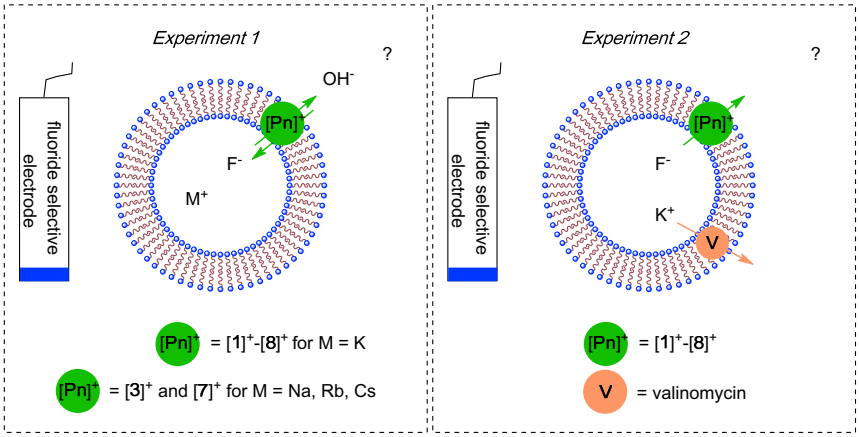

Figure 4. Schematic Representation of Selected Anion-Transport Experiments Showing an Idealized EYPC Unilamellar Vesicle

The vesicles were loaded with a 300 mM alkali metalfluoride solution (MF) buffered at pH 7.2 (10 mM HEPES), and the external medium consisted of a 300 mM potassium gluconate solution also buffered at pH 7.2 (10 mM HEPES). A fluoride selective electrode was used for measuring fluoride efflux. EYPC concentration: 0.7 mM.

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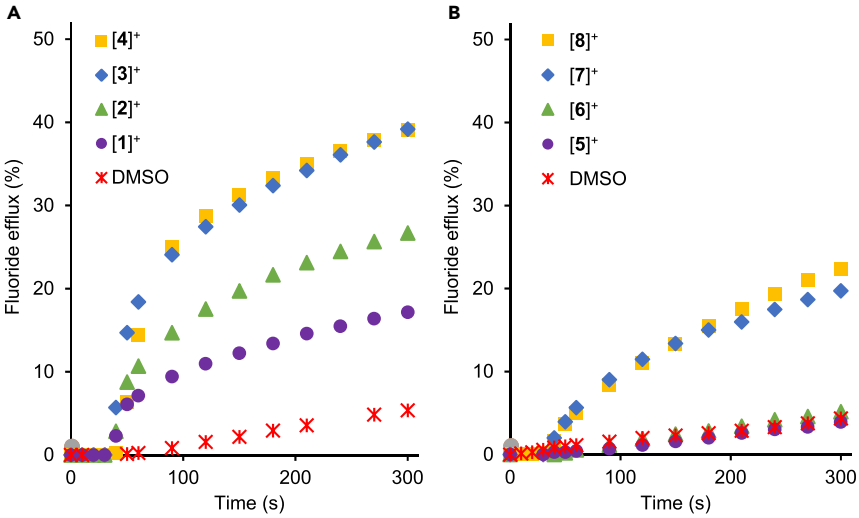

Figure 5. Transporter-Facilitated Fluoride Efflux in the Absence of Valinomycin

Fluoride efflux from EYPC vesicles triggered by the addition of a DMSO solution (7 μL) containing (A) only DMSO and $[1]^+$–$[4]^+$ and (B) only DMSO and $[5]^+$–$[8]^+$. Transporter concentration: 2 mol % with respect to the lipid concentration. The other experimental conditions are those described for experiment 1 in Figure 2.

hydrophobicity was also noted in the case of bismuth-based cations since the anthracene- and pyrene-based systems $[7]^+$ and $[8]^+$ induced significant fluoride efflux (Figure 5B). We also investigated the possible role played by the alkali cation and found that using NaF, RbF, or CsF instead of KF led to the same fluoride efflux. This result leads us to conclude that the fluoride transport activity of these pnictogenium cation does not depend on the nature of the alkali cation, allowing us to rule out metal-fluoride symport (Figure S27).

The transport activity observed in the absence of a cation transporter could be interpreted on the basis of two possibly complementary mechanisms. The first one would be a simple F^-/OH^- antiport mediated by the pnictogenium cation, which would lead to a drastic basification of the vesicle interior. Another relevant mechanism that does not involve accumulation of hydroxide ions within the vesicle would rely on the non-specific efflux of K^+ and/or F^- as a result of pnictogenium-induced membrane destabilization. Independently of which mechanism is at play, we reasoned that cation transport may be limiting anion efflux. For this reason, we became eager to test the effect of valinomycin, a well-known cation transporter (Figure 4; experiment 2).[36,37] Under these conditions, we observed drastically improved fluoride anion transport, especially in the case of the most hydrophobic pnictogenium cations $[3]^+$, $[4]^+$, $[7]^+$, and $[8]^+$ (Figures 6A, 6B, and S28). The resulting data were modeled by the Hill equation (Figures S29–S35), leading to the parameters compiled in Table 1.[38–40] The first conclusion that can be derived from the fitted parameters is that all cations act as mobile carriers for the fluoride anion. Indeed, the Hill coefficients ($n < 2$) calculated for $[2]^+$–$[4]^+$ were close to unity, suggesting that each cation transports one fluoride anion.[41] This conclusion is consistent with the formation of λ^5-fluoro-stiboranes or -bismuthanes as the critical species involved in fluoride shuttling.[29] The larger Hill coefficient observed for some of the cations could indicate the involvement of cooperative effects within the membrane where two molecules of the cationic transporter could chelate or exchange the fluoride anion.[20,42] In addition, comparison of the data obtained for the antimony and

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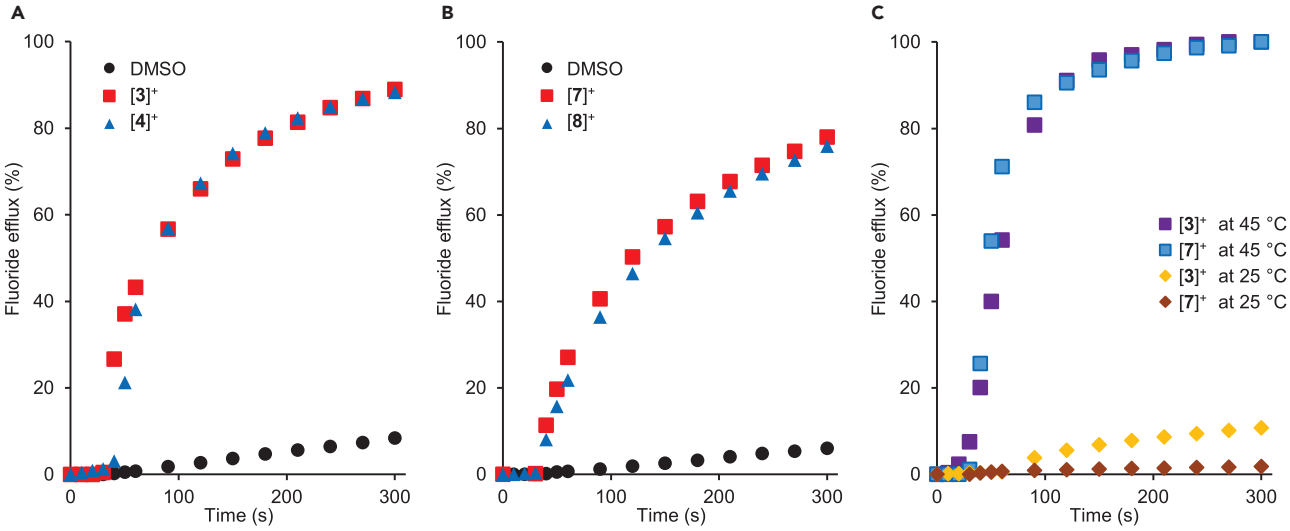

Figure 6. Transporter-Facilitated Fluoride Efflux in the Presence of Valinomycin

(A and B) Fluoride efflux from EYPC vesicles (0.7 mM) in the presence of valinomycin (0.1 mol % with respect to the lipid concentration). Efflux was triggered by the addition of a DMSO solution (7 μL) containing (A) only DMSO, $[3]^+$, and $[4]^+$ and (B) only DMSO, $[7]^+$, and $[8]^+$. Transporter concentration: 2 mol % with respect to the lipid concentration. The other experimental conditions are those described for experiment 2 in Figure 2. (C) Temperature-dependent transport experiments carried out with $[3]^+$ and $[7]^+$ and DPPC-based vesicles.

bismuth compounds points to the defining influence played by the elevated Lewis acidity and fluoride anion affinity of the stibonium cations which display significantly lower EC_{50} values. These data indicate that the tight complexation of fluoride anions by these antimony-based systems does not impede anion catch-and-release on either side of the membrane (Figures 6A and S28). The EC_{50} value of 0.41(±0.05) mol % (with respect to lipid concentration) measured for $[3]^+$ can be compared to the value of 0.9 mol % obtained by the Gale group for the Calix[4]pyrrole **A** using 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) vesicles rather than EYPC vesicles. While the difference in the nature of the phospholipids does not allow for a more detailed comparison, the low EC_{50} value obtained for $[3]^+$ validates our approach and shows that readily accessible main-group cations can be potent fluoride transporters. It could be argued that the transport observed in these experiments is the result of HF efflux coupled with transporter-facilitated hydroxide efflux. However, given that the fluoride concentration is 6 orders of magnitude higher that the hydroxide concentration, we see this scenario as highly unlikely.

To solidify our understanding of the role played by the lipophilicity of the pnictogenium cations, we computed the *n*-octanol/water partition coefficient K_{ow}, defined as $K_{ow} = [R_4Bi^+]_{octanol}/[R_4Bi^+]_{water}$ (see Figure S46).[43–45] Within the antimony and bismuth series, these partition coefficients increased gradually as the size of the polycyclic aromatic substituent increased (Table 1). Interestingly, the data in Table 1 reveal that the most lipophilic pnictogenium cations $[4]^+$ and $[8]^+$ are not the best transporters. We interpret these results as indicating that the high lipophilicity of $[4]^+$ and $[8]^+$ may force them to locate in the hydrophobic part of the membrane and thereby decrease access to hydrated fluoride anions. It is also possible that the more lipophilic pyrene ring may lower transmembrane mobility, also affecting fluoride anion transport. Such effects, which have been discussed previously for hydrogen-bond-donor anion transporters, indicate the subtlety that one should exert when optimizing the properties of these derivatives.[46] It is also possible that the more lipophilic cations can aggregate in the membrane or even partly precipitate from solution, thus lowering anion transport.[47–49]

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To verify our interpretation that these cations behave as mobile fluoride ion carriers, we tested the transport properties of $[3]^+$ and $[7]^+$ by using DPPC-based vesicles (DPPC = 1,2-dipalmitoyl-*sn*-glycero-3-phosphtidylcholine) (Figure 6C). This phospholipid is known to form temperature sensitive bilayers that undergo a phase transition between a gel and a liquid at 41°C.[48,49] It has been previously suggested that channel-type transporters are less affected by this phase transition.[50] However, in the case of $[3]^+$ and $[7]^+$, we observed a drastic decrease in transport when the temperature of the assay was lowered from 45°C to 25°C, indicating that transport is significantly affected by the fluidity of the bilayer. This observation asserts our interpretation that $[3]^+$ and $[7]^+$ behave as a mobile fluoride ion carrier rather than as a channel-type transporter.[51] Additionally, to rule out a pnictogenium cation-induced destabilization of the membrane as a cause of fluoride efflux, we carried out an assay based on carboxyfluorescein, a hydrophilic fluorescent dye that does not cross phospholipid membranes and that experiences self-quenching when confined to a vesicle. This dye is used as a marker of membrane integrity since its release into the medium, as a result of membrane destabilization, is accompanied by a marked fluorescence increase. Gratifyingly, no such effects were observed when $[3]^+$ or $[7]^+$ was used as a transporter at a concentration close to its EC_{50} value (Figure S38). The stability of the membrane in the presence of the pnictogenium cation was also assessed by dynamic light scattering (DLS) studies under the conditions employed in the electrode assay. These studies showed that the scattering intensity and the average size of the vesicles remained unchanged even 30 min after the addition of cation $[3]^+$ or $[7]^+$ (Figure S39), providing further evidence for the stability of the membrane.

Activity toward Other Anions

As noted above, the observation that fluoride efflux occurred in the absence of valinomycin suggests the possible involvement of a OH^-/F^- antiport mechanism. Such a scenario is certainly supported in the case of the stibonium cations, which displayed a high affinity for hydroxide anions.[19,52] To add credence to this interpretation, we tested the hydroxide transport properties of $[1]^+$ by using the pH-sensitive dye 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS), which was loaded inside EYPC vesicles (Figure 7A). This cation was selected because it shows good transport properties with respect to fluoride and does not absorb at 410 or 460 nm, the two wavelengths used to excite HPTS in ratiometric pH fluorescence measurements. Transport was initiated by the addition of TBAOH (5 mM) to the vesicle solution (TBA = tetra-*n*-butyl ammonium), which resulted in a base pulse of +1.36 pH unit of the buffered solution (10 mM HEPES, starting pH 7.0).[10] Hydroxide transport inside the vesicle, facilitated by the membrane permeable TBA^+ cation, was manifested by a rapid change in the HPTS fluorescence profile and the normalized fractional fluorescence intensity I_f (Figure 7B). An initial slope analysis of the I_f versus time data collected between 5 and 20 s showed that higher concentrations of $[1]^+$ correlated with an increased hydroxide influx (Figures S40–S45). This hydroxide transport experiment supports the proposal that OH^-/F^- antiport is at least partly responsible for the fluoride efflux observed in the electrode-monitored assay in the absence of a cation transporter. Finally, we also tested the ability of $[3]^+$ and $[7]^+$ to transport chloride anions by using conditions analogous to those in experiment 2 (Figure 4) but with KCl instead of KF (Figures S36 and S37). Monitoring the anion transport with a chloride selective electrode showed that these two cations were also active in the transport of chloride anions, as indicated by EC_{50} values of 0.61 ($\pm$0.04) mol % (with respect to lipid concentration) obtained for $[3]^+$ and 3.77 ($\pm$0.23) mol % (with respect to lipid concentration) obtained for $[7]^+$. We note that these EC_{50} values were higher than those obtained for fluoride anions, indicating that these transporters remain selective for fluoride anions by a factor of 1.5 in the case of $[3]^+$ and 3.0 in the case of $[7]^+$.[8]

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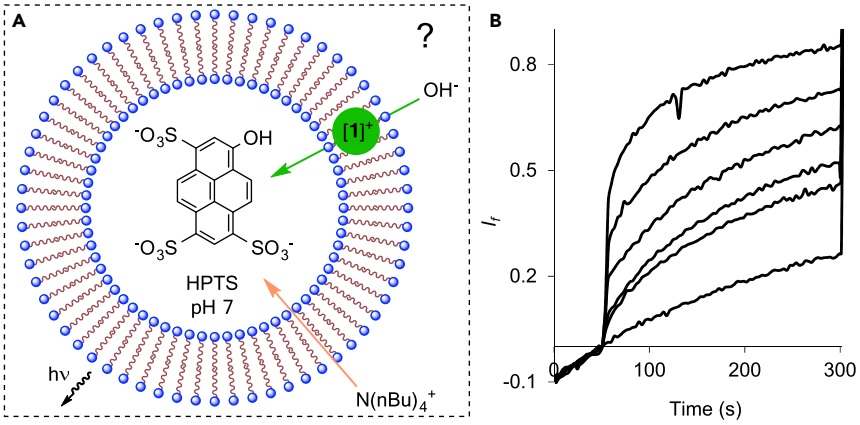

Figure 7. Hydroxide Transport Experiments with $[1]^+$ as a Transporter

(A) Schematic representation of the hydroxide anion transport assay involving EYPC vesicles (EYPC concentration: 0.1 mM). The vesicles were loaded with an HPTS (1 mM)/potassium gluconate (100 mM) solution buffered at pH 7.0 (10 mM HEPES), and the external medium contained only potassium gluconate and the buffer at the same concentrations and pH value. Transport was initiated by the addition of TBAOH (5 mM). Hydroxide influx was monitored by measuring the fluorescence intensity of the HPTS molecule (λ_{em} = 510 nm) at two excitation wavelengths simultaneously (λ_{ex} = 410 and 460 nm).

(B) The graph shows the normalized fluorescence intensity as a function of time. Transporter $[1]^+$ was added as a DMSO solution 50 s after the base. $[1]^+$ concentrations: 0 (pure DMSO), 0.5, 1, 2, 4, and 6 mol % with respect to the lipid concentration.

Application to Fluoride Anion Transport in Biological Systems

With the anion-transport ability of tetraarylstibonium and tetraarylbismuthonium established in model systems, we became eager to also assess the properties of these new transporters in biological systems. In search of a model system, we were drawn by a series of reports showing that fluoride induces hemolysis in murine erythrocytes as a result of oxidative stress.[53–55] We hypothesized that these toxic effects, which could be accelerated in the presence of a fluoride anion transporter, could serve as a marker of activity for the cationic transporters described in this study. To test this possibility, we suspended freshly obtained human erythrocytes in PBS buffer (6.8% by volume) and first tested their behavior in the presence of 100 μM concentrations of $[3]^+$ and $[7]^+$. The experiment was monitored by microscopy, which revealed a different behavior for these two cations. Indeed, whereas the erythrocytes remained visually unchanged upon addition of the antimony cation $[3]^+$, the bismuth cation $[7]^+$ induced the rapid appearance of cell ghosts, indicating lysis of the erythrocytes (Figure S47). This process was so fast that no intact cells remained after 3 min. By contrast, the appearance of ghosts was not observed with $[3]^+$, even after 25 min (Figure 8A). The toxicity of $[7]^+$, which probably arises from the known oxidative properties of tetraarylbismuthonium cations,[56,57] shows that such bismuth-based cations are not well suited for application in biological systems. We therefore decided to focus our efforts on $[3]^+$, which appeared to be more innocuous. As argued above, $[3]^+$ should be lipophilic, an attribute that should drive its localization in the cell membrane. We tested this possibility by using fluorescence cell imaging and anticipated that the anthracene fluorophore of $[3]^+$ would provide a detectable signal. Because of the weakly emitting nature of $[3]^+$, we decided to carry out these experiments by using a relatively high concentration of 100 μM. Images collected soon after the addition showed a clear enhancement of fluorescence of the cell membranes, suggesting that the transporter indeed localizes in the cell. Images taken 25 min

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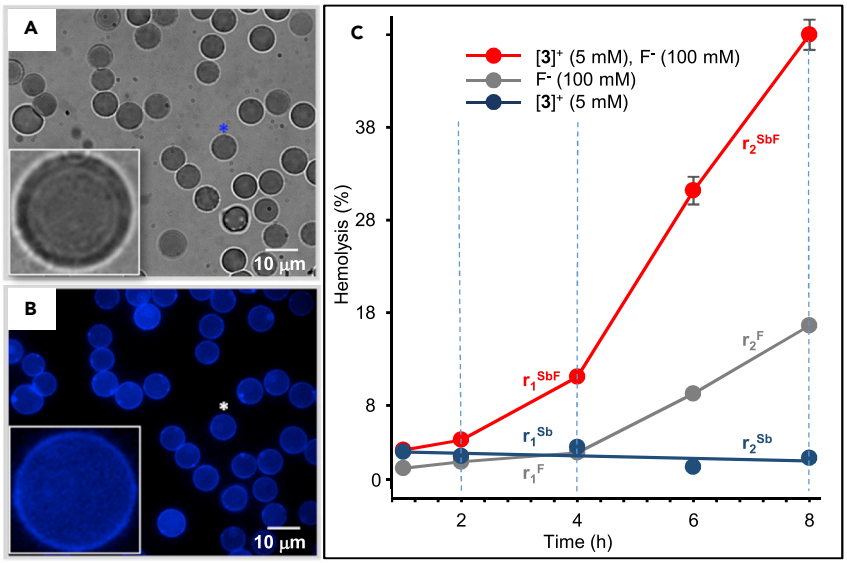

Figure 8. Effect of $[3]^+$ on Human Erythrocytes in the Absence and Presence of Fluoride Anions

(A and B) Bright-field and fluorescence images of a sample of human erythrocyte (0.2% by volume) 25 min after the addition of [3]OTf (100 μM). The insets show a magnified view of the cell marked by an asterisk. The dark contrast of the cells in the bright-field image shows that the cells were intact, and the fluorescence image shows localization of $[3]^+$ in the membrane. The DAPI filter cube (from Chroma Technology, $\lambda_{ex}/\lambda_{em}$ = 300–388 nm, 425–488 nm) was used to obtain the image in (B). Scale bars, 10 μm.

(C) A hemolysis assay carried out with human erythrocytes shows the cooperative effects occurring when $[3]^+$ and fluoride were combined. The cells were suspended in PBS buffer (HyClone). $[3]^+$ was administered as the OTf^- salt, and fluoride was administered as NaF. The rate of hemolysis is denoted as r_n^X, where n = 1 or 2 and X = Sb, F, or SbF, as noted on the graph (n = 1 corresponds to the rates observed in the 2–4 h window, and n = 2 corresponds to the rates observed in the 4–8 h window; X = Sb stands for $[3]^+$, X = F stands for F^-, and X = SbF stands for $[3]^+$ and F^- combined). Each experiment was carried out in triplicate, and the data points represent mean percentages. The triplicated data showed high reproducibility such that some of the error bars were too small to discern.

after the addition showed an even clearer contrast with a halo defining the cell membrane of most cells (Figure 8B). These features, particularly the observation of a halo, are characteristic of red blood cells (RBCs) stained by lipophilic fluorescent dyes.[58,59] We confirmed the absence of autofluorescence by imaging RBCs (0.2% by volume) in the absence of $[3]^+$ (Figure S48).

Next, we decided to test whether transporter $[3]^+$ would increase the sensitivity of the RBCs of erythrocyte toward fluoride as a result of facilitated anion transport. We investigated this possibility by carrying out a hemolysis assay, the results of which are presented in Figure 8C. We first tested the independent toxicity of the fluoride and $[3]^+$ over the course of 8 h. When a 100 mM concentration of fluoride was used, hemolysis did not appear to progress to any appreciable extent for the first 4 h of the experiment. After this point, however, hemolysis started to increase and reached 16.6% after 8 h. The long induction period may reflect the progressive influx and accumulation of fluoride inside the cell. The addition of transporter $[3]^+$ led to a drastically different hemolysis profile. When the transporter was used in a 5 μM concentration, the point taken at 2 and 4 h showed an increasing extent of hemolysis. This trend became even clearer at 6 and 8 h, when hemolysis reached 31.2% and 48%, respectively. Analysis of the rate of hemolysis showed that the effect observed was cooperative rather than additive. For the 2–4 h window, the

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hemolysis rate measured in the presence of $[3]^+$ and fluoride (r_1^{SbF} = 3.4%/h) was 9.7 times higher than the sum of the rates (r_1^{Sb} + r_1^{F} = 0.4%/h) obtained when $[3]^+$ (r_1^{Sb} = −0.1%/h) and fluoride were used alone (r_1^{F} = 0.49%/h). It follows that $[3]^+$ greatly shortened the toxicity induction period, which we propose resulted from facilitated fluoride transport into the cell. Analysis of the data in the 4–8 h window of the experiment provided corroborating results. Indeed, the rate of hemolysis in the presence of $[3]^+$ and fluoride (r_2^{SbF} = 9.2%/h) was 2.8 times higher than the sum of the rates (r_2^{Sb} + r_2^{F} = 3.3%/h) obtained when $[3]^+$ (r_2^{Sb} = −0.1%/h) and fluoride (r_2^{F} = 3.4%/h) were used alone. To a lesser degree, accelerated hemolysis was also observed when $[3]^+$ was used at a 1 μM concentration, as illustrated in Figure S49.

Conclusions

This paper introduces a new approach to transmembrane anion transport based on lipophilic and "fluoridophilic" pnictogenium cations. Evolved from the knowledge that phosphonium cations possess biological membrane translocating properties while their heavier analogs are Lewis acidic at the group 15 center, this approach and its preliminary implementation illustrate how the structure of the ligands and the nature of the Lewis acidic element govern the transport properties of these main-group compounds. The stibonium cations are particularly appealing given that they readily transport hydroxide, fluoride, and chloride anions in synthetic vesicles. Finally, we also investigated the behavior of $[3]^+$ and $[7]^+$ toward human erythrocytes. While the bismuthonium cation $[7]^+$ proved to be very toxic, the stibonium cation $[3]^+$ quickly localized in the cell membranes but did not induce notable hemolysis when used in micromolar concentrations. However, when $[3]^+$ and fluoride were co-administered, the cells underwent accelerated hemolysis. We propose that this phenomenon results from the transporter-facilitated influx of toxic fluoride anions.

EXPERIMENTAL PROCEDURES

General Procedure Adopted for the Synthesis of [5]BF_4-[8]BF_4

This procedure was adapted from the literature.[31] BF_3•OEt_2 (0.5 mmol) was transferred to a cooled (0°C) CH_2Cl_2 solution (10 mL) containing triphenylbismuth difluoride (0.3 mmol) and the arylboronic acid (0.4 mmol). The resulting solution was stirred for 2 h at room temperature. An aqueous solution of $NaBF_4$ (2.0 mmol) was then added. The resulting mixture was stirred vigorously for 30 min. The aqueous phase was separated and extracted with CH_2Cl_2. The combined organic extracts were dried over $MgSO_4$ and evaporated under reduced pressure to afford a solid residue, which was taken up in CH_2Cl_2 (2 mL) and precipitated with Et_2O (20 mL). After filtration, the solid product was washed with two portions of Et_2O (20 mL, for [5]BF_4 and [6]BF_4) or THF (10 mL, for [7]BF_4 or [8]BF_4).

Vesicle Preparation

The vesicles were prepared according to a previously established method.[60] A thin film of the lipid was prepared by evaporation of a solution of EYPC (30 mg) dissolved in a 1:1 mixture of MeOH/$CHCl_3$ (2 mL). This film was dried under vacuum overnight. A buffered KF solution (1 mL, 10 mM HEPES, 300 mM KF [pH 7.2]) was then added, resulting in a suspension that was then subjected to nine freeze-thaw cycles (liquid N_2, 40°C water bath) and extruded 27 times through a 200 nm polycarbonate membrane. To remove any extravesicular component, the vesicle suspension was passed through a size exclusion column (Sephadex G-50) using a buffer solution (10 mM HEPES, 300 mM KGlc [pH 7.2]) as an eluent. The DPPC vesicles were prepared in a similar fashion, except that the water bath used during the freeze-thaw cycles

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was elevated to 50°C. The same temperature was also used during the vesicle extrusion.

Fluoride Efflux in the Presence of Valinomycin

The following assay was adapted from a previous report.[8] A solution with a final phospholipid concentration of 0.7 mM was obtained by combining an aliquot of the KF-loaded EYPC vesicles with an aqueous solution (5 mL, pH = 7.2, [HEPES] = 10 mM) containing KGlc (300 mM). The fluoride-selective electrode was immersed in this solution. After the signal of the voltage had stabilized (~60 s), the measurement was initiated. At t = 0 s, valinomycin dissolved in DMSO (0.7 mM) was added to the assay such that the final valinomycin concentration was 0.1 mol %. At t = 30 s, the bismuthonium or stibonium salt ([**1**]-[**3**]OTf, [**4**]Br, and [**5**]BF_4-[**8**]BF_4) was added to the assay. At t = 300 s, 50 μL of a Triton X-100 solution (10:1:0.1 H_2O:DMSO:Triton X-100 (v/v/v)) was added to lyse the vesicles, triggering complete release of the fluoride cargo. The value corresponding to 100% fluoride efflux was recorded at t = 420 s, 2 min after the vesicles were lysed. The DPPC-based assay was carried out according to the same protocol and the same concentrations as those employed for the EYPC-based assay. The only difference is that the transport experiments were carried out at 25°C and 45°C.

Erythrocyte-Based Assays

Whole blood was purchased from the Gulf Coast Regional Blood Center (Houston, TX). Erythrocytes were isolated from whole blood by centrifugation for 10 m at 1,250 × *g*. The erythrocyte pellet was resuspended in PBS (HyClone) and this washing procedure was repeated three times to remove plasma and the buffy coat. To observe membrane localization of [**3**]$^+$ by fluorescence microscopy, RBCs were diluted to 2% volume in PBS and treated with 100 μM [**3**]$^+$ for 5 or 25 min at 37°C. The solution was then rapidly diluted to 0.2% by volume in PBS and added to a glass-bottom 96-well plate for imaging. Fluorescence microscopy was performed with an inverted microscope (Olympus IX-81) with a ×100 objective as well as a heated stage (37°C), and images were taken with a Rolera-XR back-illuminated electron-multiplying CCD camera (Qimaging). For fluorescence imaging, a DAPI ($\lambda_{ex}/\lambda_{em}$ = 300–388 nm, 425–488 nm) filter cube was used (Chroma Technology). The bright-field and fluorescence images of cells were obtained with SlideBook 4.2.0.7 software (Intelligent Imaging innovations). For hemolysis assays, RBCs were diluted to 6.8% volume in PBS and treated with 100 mM NaF and/or 1 or 5 μM [**3**]$^+$ as indicated, and ion transport was allowed to continue for 1–8 h at 37°C. At each time point, intact RBCs and ghosts were sedimented by centrifugation for 2 min at 1,250 × *g*. The supernatant containing the heme was then transferred to an optical bottom 96-well plate for measurement of free heme by measuring the absorbance (λ = 488 nm) using a GloMax-Multi+ detection system plate reader (Promega). Triplicate experiments were performed, measured, and subjected to normalization. To normalize hemolysis results, a positive control was conducted in tandem by treating RBCs with 1% Triton X-100.

DATA AND CODE AVAILABILITY

Crystallographic data for [**7**]BF_4 have been deposited in the Cambridge Crystallographic Data Centre and are available under accession number CCDC: 1863621.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.chempr.2019.06.013.

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AUTHOR CONTRIBUTIONS

G.P., D.J.B., J.-P.P., and F.P.G. conceived the study. G.P. carried out all experiments involving the synthesis of all compounds, their characterization, and the use of the transporters in synthetic vesicles. D.J.B. carried out all RBC-based assays.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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