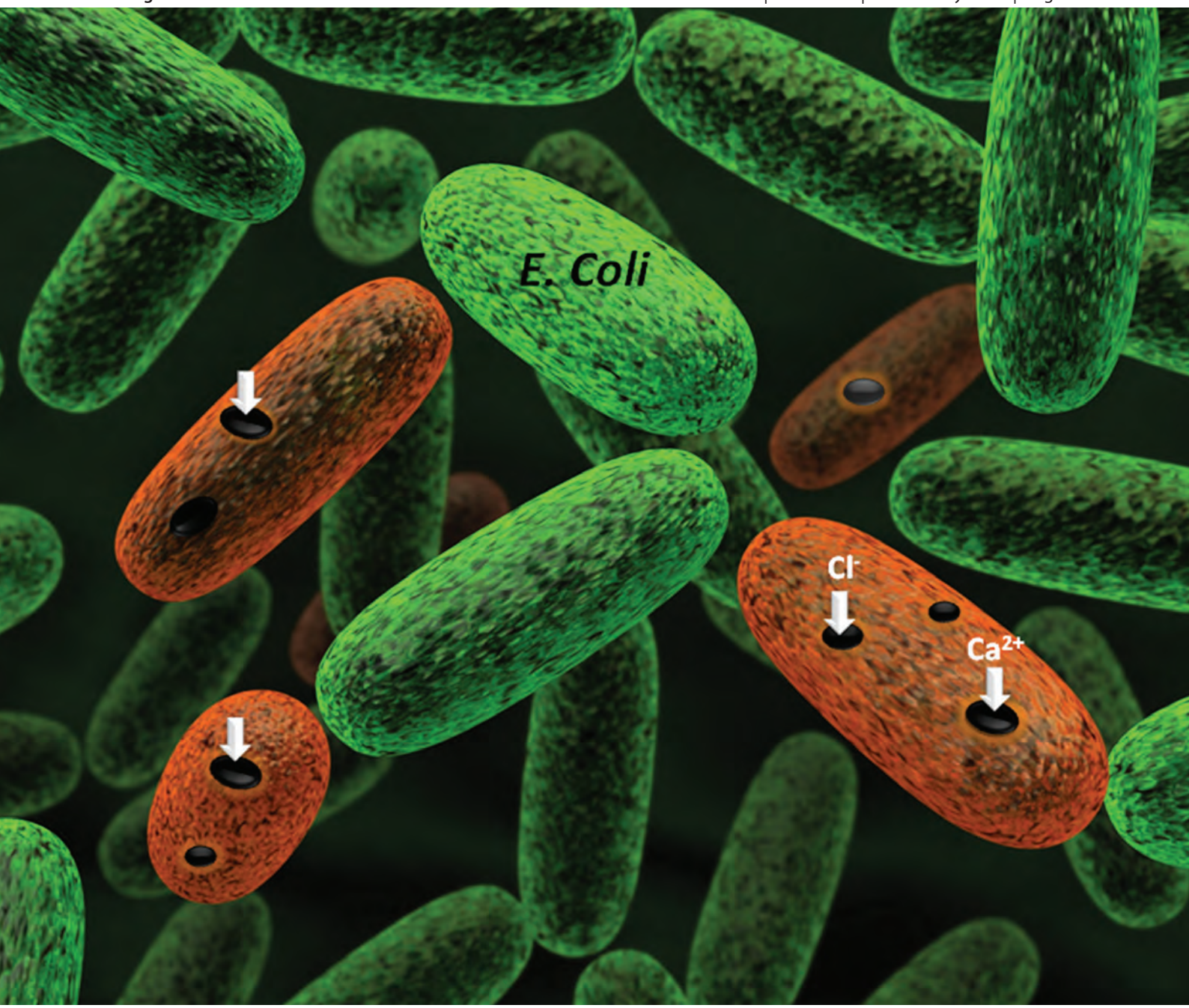
Organic & Biomolecular Chemistry

www.rsc.org/obc

Volume 11 | Number 6 | 14 February 2013 | Pages 861–1072



ISSN 1477-0520

RSC Publishing

PAPER

Andreea R. Schmitzer *et al.*

Benzimidazolium-based synthetic chloride and calcium transporters in bacterial membranes

1477-0520(2013)11:6;1-D

Organic & Biomolecular Chemistry

RSC Publishing

PAPER

View Article Online
View Journal | View Issue

Benzimidazolium-based synthetic chloride and calcium transporters in bacterial membranes†

Cite this: *Org. Biomol. Chem.*, 2013, **11**, 923

Received 9th October 2012,
Accepted 30th October 2012

DOI: 10.1039/c2ob26966j

www.rsc.org/obc

Claude-Rosny Elie,[a] Audrey Hébert,[a] Mathieu Charbonneau,[a] Adam Haiun[b] and Andreea R. Schmitzer*[a]

Herein, we present the first example of a benzimidazolium-based artificial transmembrane chloride transporter and a synthetic calcium ionophore that can regulate intracellular calcium concentrations in bacteria.

Introduction

Ion transport across cell membranes is the basis for generation of membrane potentials and provides the osmotic gradients for transmembrane and paracellular fluid transport. Chloride transport in the apical epithelial cell membrane is mainly mediated by the cAMP dependent Cystic Fibrosis Transmembrane Conductance Regulator [CFTR] chloride channel, which can move chloride into and out of the cell by calcium activated chloride channels. Calcium activated chloride channels complement the function of the CFTR in transmembrane chloride transport[1,2] where they seem to regulate each other's activity. For example, it was previously demonstrated that in biological systems, changes in ion concentration induced by opening a certain ion channel invariably affect the behaviour of other ion channels.[3,4]

To the best of our knowledge, the first and only example of an artificial chloride channel that can regulate intracellular calcium concentrations and the contraction of smooth muscle cells *via* modulating cell membrane potentials in living cells and tissues was reported by Yang *et al.*[5] The ability of synthetic chloride channels to perturb functions of other natural ion channels opens new perspectives for the applications of other synthetic ion channels in biological systems.

Research on synthetic ion transporters has led to the development of complex families of molecules[6–8] that rely on weak interactions[9] such as hydrogen bonds, cation–π and anion– π-interactions.[10] We previously exploited the anion–π--type interactions in the development of imidazolium-based synthetic chloride transporters.[11] In this case, anion–π interactions are due to the electron-withdrawing effect of imidazolium on the aromatic rings.[12,13] The effective anionic transport properties of these compounds were attributed to the formation of dimeric channel architectures penetrating into the liposome lipid membranes.[11,14] Nevertheless, the presence of multiple binding sites for ions along these dimers offers the possibility of multi-ion hopping as seen in biological ion channels responsible for fast and selective transport processes.[15] Our interest in improving the effectiveness of these imidazolium-type transporters for different parameters (*e.g.* EC_{50} and specificity constant) led us to develop new compounds by replacing the imidazolium unit with a benzimidazolium moiety (Fig. 1). These low molecular weight compounds still possess good amphiphilic character for spanning the lipid membrane and the presence of the benzimidazolium cation maximizes the supramolecular π-stacking properties. We describe here how this benzimidazolium cation has an impact on the efficiency of the chloride transport activity. We demonstrate that these benzimidazolium transporters can also ensure the transport of cations across the membranes of living *E. coli*, showing their "ditopic" properties: the ability to operate as an anion and cation symport mechanism. These new properties may be of interest to simultaneously transport $Ca^{2+}/2Cl^-$ in order to efficiently compensate the CFTR dysfunction.

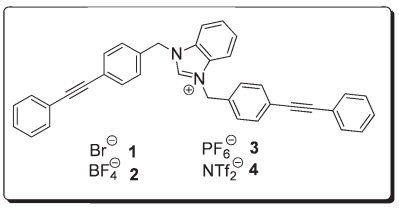
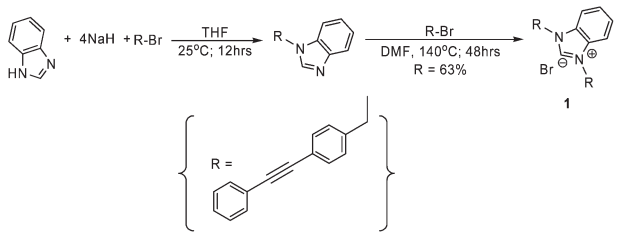


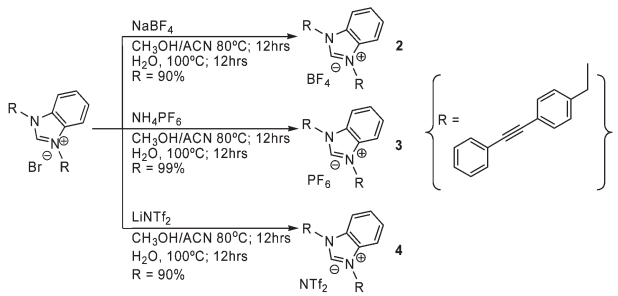
Fig. 1 Imidazolium salts studied for chloride transmembrane transport.

[a]*Department of Chemistry, Université de Montréal, Montréal, Québec, Canada, H3C 3J7. E-mail: ar.schmitzer@umontreal.ca*
[b]*John Abbott College, Sainte-Anne-de-Bellevue, Québec, Canada*

†Electronic supplementary information (ESI) available: General information, general procedures, crystallographic data. CCDC XXXXXX. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c2ob26966j



Scheme 1 Synthesis of compound **1**.



Scheme 2 Synthesis of compounds **2–4**.

Results and discussion

Benzimidazolium ionophores were synthesised by the reaction of benzimidazole and sodium hydride with (4-phenylethynyl) benzyl bromide[11,14] followed by the reaction with an equivalent of (4-phenylethynyl)benzyl to afford benzimidazolium **1** in 63% yields (Scheme 1). The other compounds were obtained through anion metathesis of bromide affording **2**, **3** and **4** in 90%, 99% and 90% yields, respectively (Scheme 2).

The benzimidazolium salts **1–4** were first studied for their ability to promote the Cl^- transport across egg yolk phosphatidylcholine (EYPC) bilayers by directly monitoring the flow of ions in the presence of lucigenin.[11,14,16] Chloride efflux out of EYPC vesicles containing lucigenin[17] was measured and the resultant fluorescence traces show the change in emission when an aliquot of transporter is added to the lucigenin-containing vesicles. A cartoon representation of the liposome assay sequence is shown in the inset of Fig. 2. It appears, as depicted in this figure, that the injection of compound **4** induces an earlier increase of fluorescence. Benzimidazolium salts **2** and **3** present a similar activity, whereas the bromide (compound **1**) leads to a slower fluorescence increase, suggesting a weaker efflux of Cl^-. Furthermore, the Cl^- transport activity of salts **1–4** follows the same trend as we had previously observed for the imidazolium salts, according to the Hofmeister series. The preferential hydration of Br^- is due to its capacity to establish interactions with water molecules,[18] compared to BF_4^-, PF_6^- or NTf_2^-. The resulting hydrated salt is less prone to self-aggregate and to penetrate into the hydrophobic bilayer. Molecules with a highly chaotropic counter anion (*e.g.* NTf_2^-), which are less hydrated, are more likely to distribute into the liposome membrane and thus be more effective in the chloride transport process.

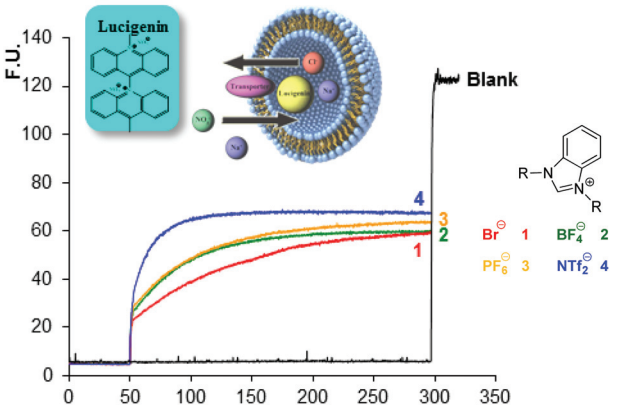


Fig. 2 Relative activity of compounds **1–4** in the lucigenin-based Cl^- transport assay. Intravesicular conditions: 100 mM NaCl, 10 mM phosphate buffer, 2 mM lucigenin; extravesicular conditions: 100 mM $NaNO_3$, 10 mM phosphate buffer (pH 6.4). 40 μM solutions of **1**, **2**, **3** and **4** were injected at t = 50 s; aqueous 10% Triton X-100 was injected at t = 300 s. Each curve represents the average of three trials. R = 4-phenylethylbenzyl.

We also examined the extent of Cl^- efflux out of fluid-phase EYPC vesicles as a function of time and ionophore content. By using similar procedures to those we previously described,[14] the mole percentages of **4** were varied from 1% to 12.5% corresponding respectively to 3 μM to 43 μM of transporter. The effectiveness of benzimidazolium **4** was characterized in the chloride transport process across the bilayer by its EC_{50} values, using dose–response analysis (see ESI†) based on data shown in Fig. 3a. The EC_{50} value for compound **4** in a Cl^-/NO_3^- system was 2.99% (relative to the EYPC concentration), a value slightly lower than the EC_{50} obtained for our previous imidazolium transporter (5.74%) or the phenylthioureas (3.05%) described by Gale *et al.*, and considered to be very effective synthetic transporters.[14,19]

Analysis of internal Cl^- efflux for different mole percentages of **4** as a function of time yielded the kinetic profile depicted in Fig. 3b. The observed pseudo-first-order rate constants (k_{obsd}) were found to have a second-order dependency on the concentration of **4** (Fig. 3b). Based on the mathematical approach we previously developed,[14] we can show here that

$$k_{obsd} = \frac{k_2\,[\text{monomer}]^n}{K_{diss}} + k_0 \qquad (1)$$

where K_{diss} is the equilibrium constant for dissociation of an assembly of n ionophore molecules into monomers, k_2 is an intrinsic rate constant and k_0 is the rate constant for ion transport in the absence of an ionophore. The linear correlation that was found between k_{obsd} and $[4]^2$ supports the hypothesis of transport-active dimers. Since thermodynamically stable systems might sometimes be undervalued,[18,20] the value of the Hill coefficient n, corresponding to the stoichiometry of the transport active aggregate, was also estimated by curve fitting (GraphPad Prism 6.0, GraphPad Software, Inc.) to be 2.092.

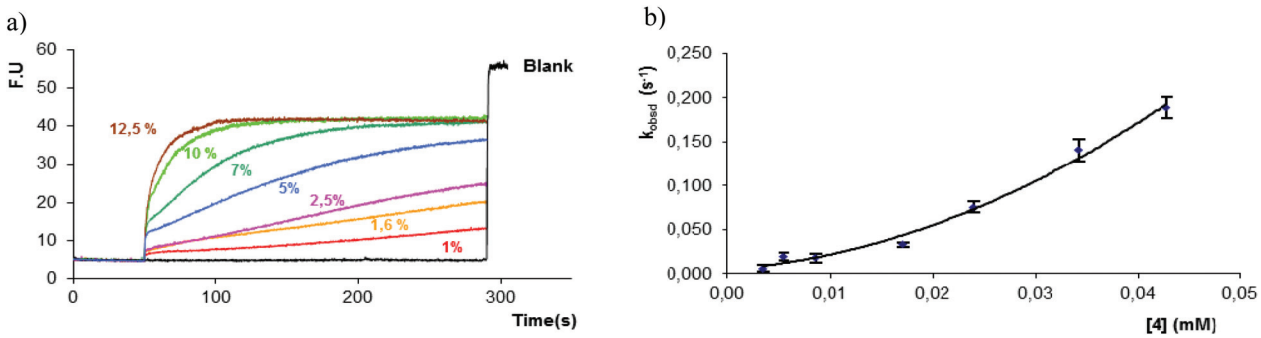


Fig. 3 (a) Efflux of Cl^- out of EYPC vesicles containing 1.0, 1.6, 2.5, 5, 7, 10 and 12.5 mol % of **4** (increasing rates, respectively) as a function of time at 37 °C. Each curve represents the average of three trials. (b) Plot of k_{obsd} *versus* concentration of **4**; the solid line represents a nonlinear least-squares fit of the data according to eqn (1), where $n = 2.0$. The data at each concentration of **4** are the average of 3 series of measurements.

This is, again, consistent with a dimerization process responsible for chloride transport.

According to our kinetic model, the value of k_2/K_{diss} (the slope), which measures the effectiveness of transporter **4**, increases 100 times compared to the previously reported imidazolium salt.[14] This ratio resembles a specificity constant, describing the transporter's ability to facilitate the diffusion of a given anion and the aggregation of **4**. Although it was not possible for us to separate the two terms in k_2/K_{diss}, their high ratio strongly suggests either a very high rate constant (k_2) or a very low dissociation process (K_{diss}). This last scenario is in agreement with the lower EC_{50}, supporting the idea that the EC_{50} and K_{diss} have proportional values.[18]

The organization of benzimidazolium transporters in the solid state was confirmed by X-ray diffraction. Crystals of **1** were grown by slow evaporation from acetonitrile and chloroform. The structure elucidated by single crystal X-ray diffraction is shown in Fig. 4 (see ESI† for details).

Crystal organization of the benzimidazolium cations is in close agreement with the theoretical organization predicted by molecular modeling.[11,14] The structure observed for **1** reveals the formation in one plane of a channeled structure, where the repeating unit is the benzimidazolium dimer. The supramolecular complex is self-assembled by C–H···π interactions. The dimeric structure forms a membrane-spanning channel which can promote the diffusion of the ions through the lipid bilayer. Remarkably, the dimer's length in the crystal structure is 42.998 Å, which corresponds to the thickness of an EYPC liposome bilayer (around 40 Å).[21] Additionally, the channel is flanked on its sides by two bent molecules having aromatic edge-to-face orientation at 5.987 Å distance. Benzimidazolium cations and bromide anions are held together by ionic interactions and H-bonds.

We have not been able to obtain crystals of compound **4**, but we believe bigger counter-anions (*e.g.* NTf_2^-) should yield larger channel diameters, as the bromide anions are positioned between the benzimidazolium rings. The activity of the transporter is probably not only due to the desolvation of the counter-anion, but also to the variable diameter of the channel, governed by the anion's size.

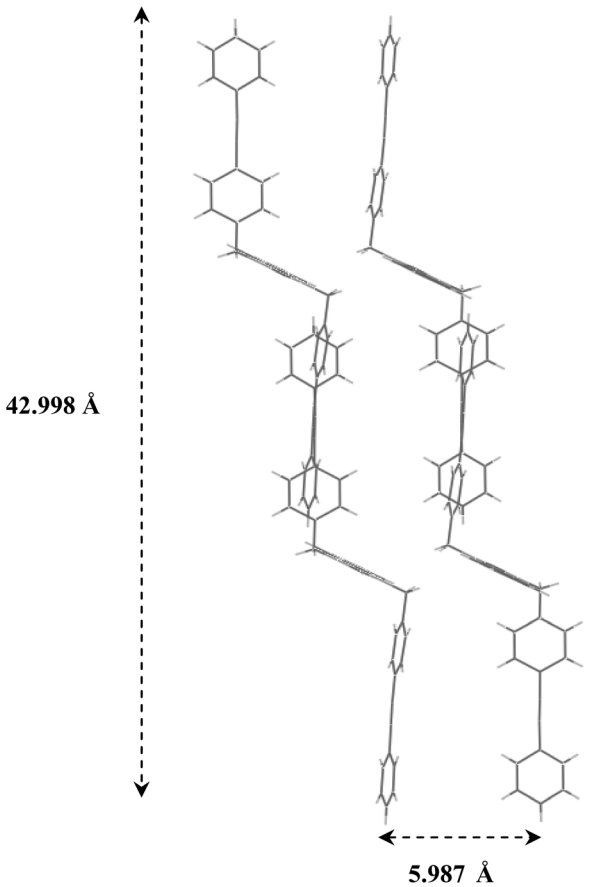


Fig. 4 Packing motif of **1** illustrating the dimerization process in the solid state. Br^- anions not shown for clarity.

In order to support the transmembrane channel hypothesis *versus* a mobile ionic transport mechanism, we measured the ionophoric activity of compound **4** in gel and fluid phase membranes.[22] For this purpose, we measured the efflux of Cl^- out of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) liposomes using the lucigenin-based assay, at temperatures above and below DPPC's transition phase (41 °C). As shown in Fig. 5, in the presence of 7 mol% **4** (relative to the DPPC

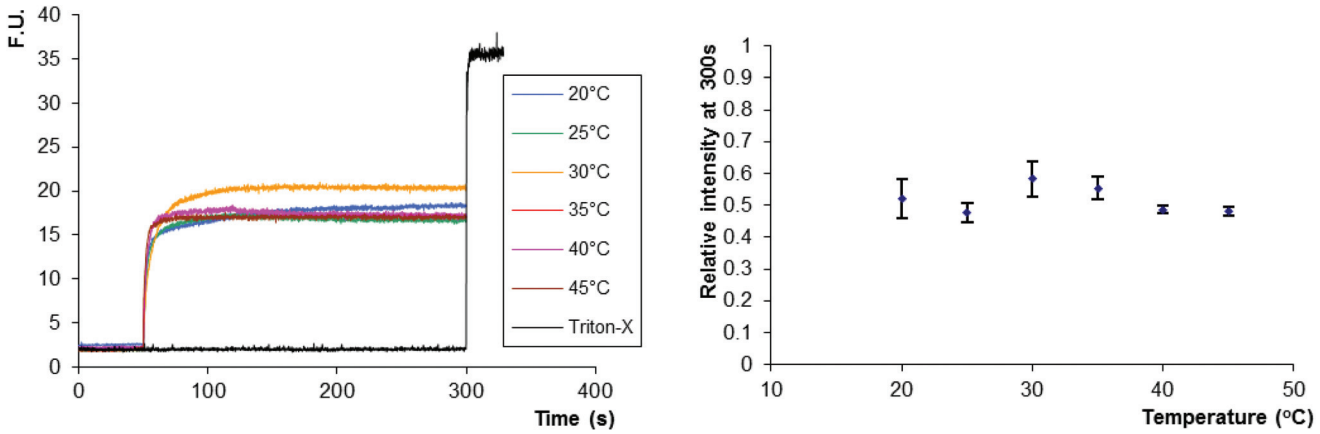


Fig. 5 Chloride efflux out of DPPC liposomes at 20 °C, 25 °C, 30 °C, 35 °C, 40 °C and 45 °C. The data at each temperature are obtained by using 7 mol% of **4** (relative to DPPC concentrations). The data at each temperature are the average of three series of measurements.

concentration), the results support a membrane-spanning mechanism, since the anionophoric activity of **4** remains independent of the fluidity of the DPPC membrane.

As mentioned earlier, the benzimidazolium salts studied here possess the structural requirements to favour anion–π and cation–π interactions. With all the kinetic and structural information on the chloride transmembrane transport, we further studied the capacity of our benzimidazolium salts to promote $Ca^{2+}/2Cl^-$ symport processes, in the interest of compensating CFTR dysfunction. To explore the potential applications of compound **4** in biological and pharmacological sciences, we investigated whether compound **4** can modulate bacterial cell permeability for calcium cations.

For these studies, we used *Escherichia coli* (*E. coli*) strains, overexpressing a mutant citrine protein with post-translational modifications.[22] Citrine is a genetically encoded Ca^{2+} indicator located in the subcellular environment. The mutant we used possessed a Q69M mutation, presenting improved properties in particular with regard to its photostability, its pH sensitivity and its expression at 37 °C.[22] In this way, citrine was used as a non-ratiometric indicator of the increase in intracellular calcium concentrations because citrine's fluorescence increases in the presence of Ca^{2+}. Transport tests were carried out in a quartz cuvette by adding a 1.6 ml aliquot of the *E. coli* bacterial culture and $CaCl_2$ (1 mM). The fluorescence intensity of citrine was then recorded as a function of time. After 2 minutes, an aliquot of 150 µl of the ionophore **4**, or of MeOH, was injected (ESI† for details).

Fig. 6 clearly shows a sudden increase in cytosolic citrine fluorescence when compounds **2–4** are added, compared to the control (MeOH). This fluorescence increase represents a massive influx of calcium into the *E. coli* under the effect of the ionophores. This supports the hypothesis that benzimidazolium transporters are able to facilitate the diffusion of Ca^{2+} cations across *E. coli* membranes, similar to the symport process observed in liposomes. Calcium transport efficiency follows the same order in bacteria as in liposomes, with compound **4** being the most active Ca^{2+} transporter. This could be

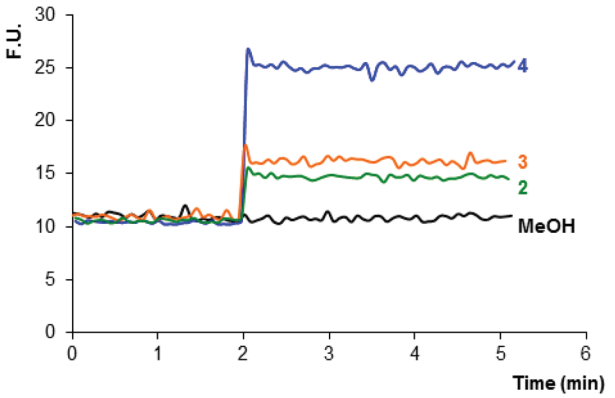


Fig. 6 Fluorescence changes in *E. coli* cells expressing cytosolic citrine after given stimulations with MeOH, **2**, **3** and **4** (0.45 mM) in the presence of extracellular $CaCl_2$ (0.38 mM) (λ_{ex} = 516 nm; λ_{em} = 529 nm).

directly related to the size of the channel opening. Moreover, their ability to transport ions through bacterial membranes provides an additional support to channel formation. As explained above, the transport efficiency of a channel is virtually independent of the nature of the membrane (gel/fluid state) in which it is inserted. The increase in fluorescence is not due to bacterial lysis, which would have had, as a consequence, a decrease of the optical density. The measure of the bacterial population, better known as optical density or OD_{600}, of an aliquot of *E. coli* in the presence of **4** indicates the same value as an aliquot of living bacteria (OD_{600} = 1.3) (see ESI† for details).

Conclusion

In summary, we herein described the ability of small benzimidazolium compounds to act as good ionophores and identified one lead compound **4** that functions in liposomes and living bacteria. This latter combines a higher k_2/K_{diss} constant and lower EC_{50} than its previous imidazolium analogue.[14] We have

also presented the first example of a benzimidazolium-based compound that can transport anions and cations across liposomes, as well as bacterial membranes, *via* a channel formation pathway. The ability of **4** to drill transient channels through the robust wall of bacterial cells should make it attractive as a paradigm for designing future antibiotic transporters across resistant bacterial membranes. Characterization of the membrane-spanning channel's size and its ability to transport other molecules need further insight and are currently being investigated in our laboratory.

Acknowledgements

We are grateful to the Natural Sciences and Engineering Research Council of Canada (NSERC), Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT), Centre in Green Chemistry and Catalysis (CCVC), Canada Foundation for Innovation (CFI) and Université de Montreal for financial support. We thank Prof. J.N. Pelletier for access to her bacterial incubator. We also thank colleagues for a careful reading and discussion of this manuscript.

References

1 J. Eggermont, *Proc. Am. Thorac. Soc.*, 2004, **1**, 22–27.
2 C. Hartzell, I. Putzier and J. Arreola, *Ann. Rev. Physiol.*, 2005, **67**, 719–758.
3 C. M. Armstrong and B. Hille, *Neuron*, 1998, **20**, 371–380.
4 B. Hille, *Sinauer Associates*, Sunderland, MA, 2001.
5 X. Li, B. Shen, X.-Q. Yao and D. Yang, *J. Am. Chem. Soc.*, 2009, **131**, 13676–13680.
6 (*a*) O. Lawal, K. S. J. Iqbal, A. Mohamadi, P. Razavi, H. T. Dodd, M. C. Allen, S. Siddiqui, F. Fucassi and P. J. Cragg, *Supramol. Chem.*, 2009, **21**, 55–60; (*b*) J. C. Iglesias-Snchez, W. Wang, R. Ferdani, P. Prados, J. de Mendoza and G. W. Gokel, *New J. Chem.*, 2008, **32**, 878–890; (*c*) O. A. Okunola, J. L. Seganish, K. J. Salimian, P. Y. Zavalij and J. T. Davis, *Tetrahedron*, 2007, **63**, 10743–10750; (*d*) Y. Tanaka, Y. Kobuke and M. Sokabe, *Angew. Chem.*, 1995, **107**, 717–719, (*Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 693–694).
7 (*a*) J. K. Chui and T. M. Fyles, *Beilstein J. Org. Chem.*, 2011, **7**, 1562–1569; (*b*) S. Matile, A. Vargas Jentzsch, A. Fin and J. Montenegro, *Chem. Soc. Rev.*, 2011, **40**, 2453–2474; (*c*) U. Devi, J. R. Brown, A. Almond and S. J. Webb, *Langmuir*, 2011, **27**, 1448–1456; (*d*) F. Otis, C. Racine-Berthiaume and N. Voyer, *J. Am. Chem. Soc.*, 2011, **133**, 6481–6483; (*e*) H. Cho, L. Widanapathirana and Y. Zhao, *J. Am. Chem. Soc.*, 2011, **133**, 141–147; (*f*) J. T. Davis, P. A. Gale, O. A. Okunola, P. Prados, J. C. Iglesias-Sanchez, T. Torroba and R. Quesada, *Nat. Chem.*, 2009, **1**, 138–144; (*g*) A. P. Davis, D. N. Sheppard and B. D. Smith, *Chem. Soc. Rev.*, 2007, **36**, 348–357.
8 (*a*) G. L. Reddy, T. Iwamoto, J. M. Tomich and M. Montal, *J. Biol. Chem.*, 1993, **268**, 14608; (*b*) M. Oblatt-Montal, G. L. Reddy, T. Iwamoto, J. M. Tomich and M. Montal, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 1495; (*c*) D. P. Wallace, J. M. Tomich, J. W. Eppler, T. Iwamoto, J. J. Grantham and L. P. Sullivan, *Biochim. Biophys. Acta (Biomembranes)*, 2000, **1464**, 69; (*d*) K. E. Mitchell, T. Iwamoto, J. Tomich and L. C. Freeman, *Biochim. Biophys. Acta (Biomembranes)*, 2000, **1466**, 47; (*e*) J. R. Broughman, K. E. Mitchell, R. L. Sedlacek, T. Iwamoto, J. M. Tomich and B. D. Schultz, *Am. J. Physiol.: Cell Physiol.*, 2001, **280**, C451.
9 (*a*) R. E. Dawson, A. Hennig, D. P. Weimann, D. Emery, S. Gabutti, J. Montenegro, V. Ravikumar, M. Mayor, J. Mareda, C. A. Schalley and S. Matile, *Nat. Chem.*, 2010, **2**, 533–538; (*b*) J. Míšek, A. Vargas Jentzsch, S. Sakurai, D. Emery, J. Mareda and S. Matile, *Angew. Chem.*, 2010, **122**, 7846–7849, (*Angew. Chem., Int. Ed.*, 2010, **49**, 7680–7683).
10 A. V. Jentzsch, D. Emery, J. Mareda, S. K. Nayak, P. Metrangolo, G. Resnati, N. Sakai and S. Matile, *Nat. Commun.*, 2012, DOI: 10.1038/ncomms1902.
11 C. R. Elie, N. Noujeim, C. Pardin and A. R. Schmitzer, *Chem. Commun.*, 2011, 1788–1790.
12 R. R. Dykeman, N. Yan, R. Scopelliti and P. J. Dyson, *Inorg. Chem.*, 2011, **50**, 717–719.
13 (*a*) A. Frontera, P. Gamez, M. Mascal, T. J. Mooibroek and J. Reedijk, *Angew. Chem.*, 2011, **123**, 9736–9756, (*Angew. Chem., Int. Ed.*, 2011, **50**, 9564–9583); (*b*) L. M. Salonen, M. Ellermann and F. Diederich, *Angew. Chem.*, 2011, **123**, 4908–4944, (*Angew. Chem., Int. Ed.*, 2011, **50**, 4808–4842); (*c*) H. T. Chifotides, B. L. Schottel and K. R. Dunbar, *Angew. Chem.*, 2010, **122**, 7360–7365, (*Angew. Chem., Int. Ed.*, 2010, **49**, 7202–7205); (*d*) O. B. Berryman, V. S. Bryantsev, D. P. Stay, D. W. Johnson and B. P. Hay, *J. Am. Chem. Soc.*, 2007, **129**, 48–58; (*e*) D. QuiÇonero, C. Garau, C. Rotger, A. Frontera, P. Ballester, A. Costa and P. M. Deya, *Angew. Chem.*, 2002, **114**, 3539–3542, (*Angew. Chem., Int. Ed.*, 2002, **41**, 3389–3392); (*f*) M. Mascal, A. Armstrong and M. D. Bartberger, *J. Am. Chem. Soc.*, 2002, **124**, 6274–6276; (*g*) I. Alkorta, I. Rozas and J. Elguero, *J. Am. Chem. Soc.*, 2002, **124**, 8593–8598.
14 C. R. Elie, M. Charbonneau and A. R. Schmitzer, *Med. Chem. Commun.*, 2012, DOI: 10.1039/c2md20107k.
15 (*a*) J. P. Galligan and D. A. Dougherty, *Proc. Natl. Acad. Sci. U. S. A*, 1999, **96**, 9459–9464; (*b*) S. Mecozzi, A. P. West and D. A. Dougherty, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **93**, 10566–10571; (*c*) J. C. Ma and D. A. Dougherty, *Chem. Rev.*, 1997, **97**, 1303–1324.
16 (*a*) P. Davis, D. N. Sheppard and B. D. Smith, *Chem. Soc. Rev.*, 2007, **36**, 348–357; (*b*) V. Sidorov, F. W. Kotch, J. L. Kuebler, Y. F. Lam and J. T. Davis, *J. Am. Chem. Soc.*, 2003, **125**, 2840–2841; (*c*) B. A. McNally, A. V. Koulov, B. D. Smith, J. B. Joosb, A. P. Davis, W. A. Harrell Jr., M. L. Bergmeyer, P. Y. Zavalij and J. T. Davis, *Chem. Commun.*, 2010, 3950–3952; (*d*) B. A. McNally, A. V. Koulov, B. D. Smith, J. B. Joos and A. P. Davis, *Chem. Commun.*, 2005, 1087–1089.

17 B. A. McNally, A. V. Koulov, T. N. Lambert, B. D. Smith, J.-B. Joos, A. L. Sisson, J. P. Clare, V. Sgarlata, L. W. Judd, G. Magro and A. P. Davis, *Chem.–Eur. J.*, 2008, **14**, 9599–9606.
18 S. Matile and N. Sakai, in *Analytical Methods in Supramolecular Chemistry*, ed. C. A. Schalley, Wiley, Weinheim, 2nd edn, 2012, pp. 711–742.
19 N. J. Andrews, C. J. E. Haynes, M. E. Light, S. J. Moore, C. C. Tong, J. T. Davis, W. A. Harrell Jr. and P. A. Gale, *Chem. Sci.*, 2011, **2**, 256–260.
20 S. Bhosale and S. Matile, *Chirality*, 2006, **18**, 849.
21 J. Sabin, G. Prieto1, J. M. Ruso1, R. Hidalgo-Alvarez and F. Sarmiento, *Eur. Phys. J. E*, 1998, **20**, 401–408.
22 (*a*) S. Krasne, G. Eisenman and G. Szabo, *Science*, 1971, **174**, 413; (*b*) G. Boheim, W. Hanke and H. Eibl, *Proc. Natl. Acad. Sci. U. S. A.*, 1980, **77**, 3403; (*c*) V. Koulov, T. N. Lambert, R. Shukla, M. Jain, J. M. Bood, B. D. Smith, H. Y. Li, D. N. Sheppard, J.-B. Joos, J. P. Clare and A. P. Davis, *Angew. Chem., Int. Ed.*, 2003, **42**, 4931; (*d*) S. Krasne, G. Eisenman and G. Szabo, *Science*, 1971, **174**, 414.

