# Transmembrane Transport of Phosphate by a Strapped Calix[4]pyrrole

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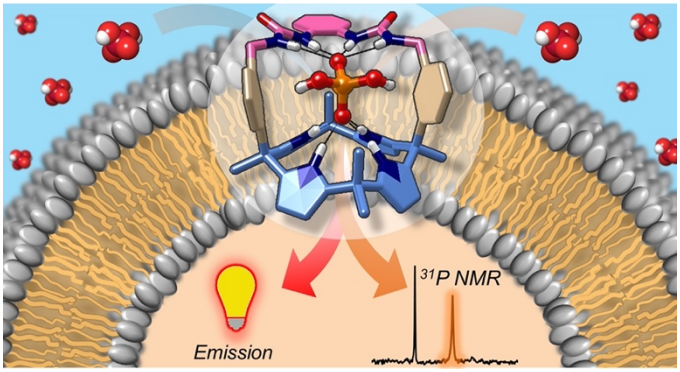
**ABSTRACT:**

Synthetic anion receptors are increasingly explored for the transport of anions across lipid membranes because of their potential therapeutic applications. A considerable amount of research focuses on the transport of chloride whereas the transmembrane transport of inorganic phosphate has not been reported to date, despite the biological relevance of this anion. Here we present a calix[4]pyrrole with a bisurea strap that functions as a receptor and transporter for $H_2PO_4^-$, relying on the formation of 8 hydrogen bonds and efficient encapsulation of the anion. Using a phosphate-sensitive lanthanide probe and $^{31}P$ NMR spectroscopy, we demonstrate that this receptor can transport phosphate into vesicles by $H_2PO_4^-/Cl^-$ antiport, $H_2PO_4^-$ uniport, and $Cs^+/H_2PO_4^-$ symport mechanisms. This first example of inorganic phosphate transport by a neutral receptor opens perspectives for the future development of transporters for various biological phosphates.




Inorganic phosphate is one of the most abundant intracellular anions and has essential roles in a wide range of biological processes, such as energy storage and bone mineralization.[1] Maintaining the correct levels of inorganic phosphate is crucial for the overall health and survival of organisms. At the cellular level, membrane transport proteins precisely control the transport of ions and molecules.[2] Phosphate homeostasis is maintained by the coordinated action of type II (SLC34 family) and type III (SLC20 family) sodium-dependent phosphate cotransporters and the phosphate export protein XPR1.[3-5] Malfunctioning phosphate transporters can cause a variety of diseases,[6] including osteoporosis and nephrolithiasis,[7] Fahr's syndrome,[8] and Fanconi syndrome.[9]

Synthetic molecules (ionophores) embedded in lipidic membranes have the potential to treat disorders derived from malfunctioning anion transport proteins by carrying specific anions across the lipid barrier. Despite numerous studies on synthetic transmembrane transporters,[10,11] including those for chloride,[12] bicarbonate[13] and sulfate,[14] to the best of our knowledge, there are no reports of inorganic phosphate transport across lipid bilayers mediated by synthetic carriers.[15] This can be attributed to the intrinsic properties of phosphate, such as its high hydration energy ($\Delta G_{hyd}$ = -473 kJ.mol$^{-1}$ for $H_2PO_4^-$ versus -340 kJ.mol$^{-1}$ for $Cl^-$),[16] speciation at neutral pH ($H_2PO_4^-$ and $HPO_4^{2-}$, $pK_a$ 7.2), and its tetrahedral geometry, which make its binding[17] and transport across an apolar lipid bilayer particularly challenging.

One approach for extracting and transporting such highly hydrated anions is using compounds which can form multiple H-bonds with the anion.[14,18-20] Strapped calix[4]pyrroles developed by the Sessler,[18] Gale,[19] and Ballester[20] laboratories have emerged as efficient anion carriers, with their selectivity and transport activity depending on the functionalisation of the calix[4]pyrrole scaffold and the nature of the "strap".[21-25] While there are reports on the encapsulation of phosphates with these synthetic receptors,[26,27] no transport of $H_2PO_4^-$ has been reported to date.

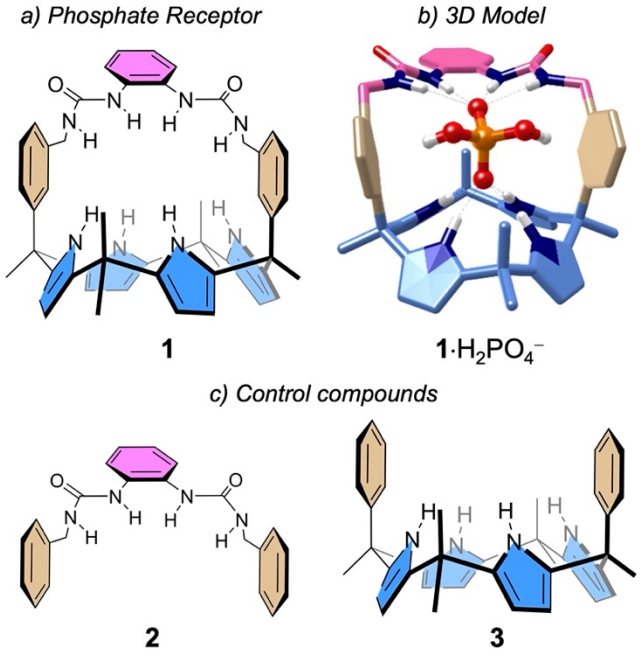


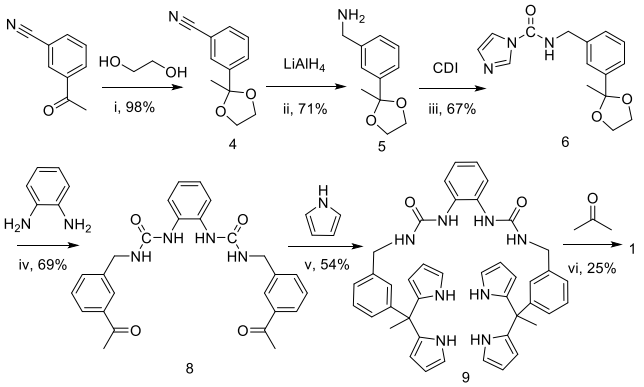

**Figure 1.** a) Novel phosphate receptor **1**; b) Energy minimised 3D model[28] of **1**·$H_2PO_4^-$; c) Control compounds used in the study.

Herein we report the first example of inorganic phosphate transport, using novel anion receptor **1**. The receptor design is based on two anion binding motifs: a calix[4]pyrrole and an *ortho*-phenylenebisurea strap, each providing 4 H-bond donors (Figure 1a,b). The benzylic linkers act as relatively rigid spacers, fixing the two binding motifs at a distance which should enable the cooperative binding of phosphate, but also ensuring the efficient shielding

of its charge from the hydrophobic membrane during transport. The transport of phosphate into liposomes by receptor **1** and the corresponding control compounds **2** and **3** (Figure 1c) was studied by two assays, the first exploiting the emission properties of an encapsulated phosphate-sensitive $Eu^{3+}$ probe,[29] and the second using $^{31}P$ NMR spectroscopy.

Receptor **1** was prepared from commercially available 3-acetylbenzonitrile, of which the ketone was protected, and the nitrile subsequently reduced to afford benzylamine **5** (Scheme 1).[30] After reaction with 1,1′-carbonyldiimidazole (CDI), monosubstituted carbamoyl imidazole **6** was reacted with *o*-phenylenediamine to give intermediate [**7**] and subsequently deprotected *in situ* to generate bis-urea strap **8** in good yield. Strap **8** reacted in neat pyrrole to form precursor **9**, which was subjected to a condensation reaction with acetone to afford strapped calix[4]pyrrole receptor **1** in moderate yield. Reported compounds **2**[31] and **3**[32] (Figure 1c) were synthesised as control compounds, representing either the strap (**2**) or the calix[4]pyrrole base (**3**) components of receptor **1** (Scheme S1).

**Scheme 1. Synthesis of transmembrane transporter 1.**




i) *p*TSA, toluene, 160°C, 3h; ii) THF (dry), 0-25°C; iii) $H_2O$, 0-25 °C, 2h; iv) $CHCl_3$, 66 °C, 72 h; then TFA, 25°C, 3h; v) TFA, 66°C, 2 h; vi) $BF_3 \cdot Et_2O$, 14 h.

The ability of compounds **1**-**3** to complex anions was evaluated by $^1H$ NMR titrations with $Cl^-$ and $H_2PO_4^-$ tetrabutylammonium salts in $d_6$-DMSO/$H_2O$ (0.5%). The binding affinities were derived from fitting a 1:1 binding curve to the changes observed in the chemical shifts of **1**-**3** over the course of the titration (Figures S31-S38). An association constant $K_a$ of 90±15 $M^{-1}$ was determined for the interaction between receptor **1** and $Cl^-$, which is higher than the values obtained for reference compounds **2** and **3** (Table 1). When titrating with $H_2PO_4^-$, significant broadening and shifts of the $^1H$ NMR signals of **1** and the appearance of new signals were observed (Figure S32), indicative of a loss of symmetry and intermediate exchange on the chemical shift NMR time scale. UV-Vis titrations were performed, from which a $K_a$ of 2.6(±0.4)·$10^4$ $M^{-1}$ was estimated (Figure S39). The $K_a$ of the bisurea **2** for $H_2PO_4^-$ was 20-fold lower than that of **1** and no interaction was observed with calix[4]pyrrole **3**.

These experimental findings agreed with the molecular modelling performed, which suggests 8 possible H-bonds for **1** with $H_2PO_4^-$ (1.76-2.13 Å, Figures 1b and S41a). In contrast, the distance between the bisurea and calix[4]pyrrole binding motifs of **1** is too large for efficient cooperative binding of $Cl^-$, as indicated by only 4 H-bonds to $Cl^-$ of 2.48-2.58 Å in the lowest energy conformation of **1**·$Cl^-$, similar to 4 H-bonds of 2.32-2.42 Å in each of the reference complexes **2**·$Cl^-$ and **3**·$Cl^-$ (Figure S41).

**Table 1. Association constants for receptor 1 and control compounds 2 and 3 as determined from titrations with tetrabutylammonium salts of $Cl^-$ and $H_2PO_4^-$ in DMSO with 0.5% $H_2O$ at 25 °C.**

| Anion | $K_a$ ($M^{-1}$) | | |
|---|---|---|---|
| | **1** | **2** | **3** |
| $Cl^-$ | 90±15 [a] | 20±3 [a] | <10 [a] |
| $H_2PO_4^-$ | (2.6±0.4)·$10^4$ [b] | (1.2±0.2)·$10^3$ [a] | - [c] |

[a] Determined by $^1H$ NMR spectroscopy; [b] Determined by UV-Vis spectroscopy; experimental errors are estimated to be 15%. [c] No interaction was observed.

Compounds **1**-**3** were tested as $Cl^-$ transporters with the commonly used HPTS assay (see SI for details) and **1** and **3**[32] were found to be active at 4 mol%. In contrast, **2** did not show any transport activity, despite its higher affinity for $Cl^-$ compared to **3**. This confirms that the strength of anion binding in an organic solvent and transport are not necessarily correlated.[12]

To study the phosphate transport properties of **1**-**3** by emission spectroscopy, the phosphate responsive lanthanide probe [Eu.*p*$BOH_2$]$^+$ (Figure 2) was encapsulated in large unilamellar vesicles (LUVs, 0.4 mM POPC, ~ 200 nm diameter) at pH 7. This water soluble probe, recently developed by Butler and co-workers, gives a selective emission enhancement in the presence of phosphate, with no response for a range of biological anions including chloride, sulfate, and bicarbonate.[29] The LUVs were prepared in a 100 mM N-methyl-D-glucamine hydrochloride ($NMDGH^+Cl^-$) solution buffered at pH 7 with 10 mM HEPES. This solution was used to provide $Cl^-$ anions that could participate in a $H_2PO_4^-/Cl^-$ antiport process, while avoiding the potential transport of cations ($NMDGH^+$ is a hydrophilic cation that is difficult to transport[33]).

Addition of 25 mM phosphate to LUVs without transporter did not give rise to an emission enhancement (Figure 2a, black curve), confirming the efficient encapsulation of the probe. In the presence of compound **1** (4 mol%, post-inserted from DMSO), a clear increase in the emission intensity was observed over time (Figure 2a, blue curve), signature of a $H_2PO_4^-/Cl^-$ antiport transport mechanism. The observed dependence of the transport rate on the concentration of **1** (0.5-4 mol%, Figure S44) further validates this assay, confirming that **1** transports phosphate. With control compounds **2** and **3** no phosphate transport activity was observed (Figure 2a), despite the activity of **3** as a $Cl^-$ transporter, supporting that phosphate is more difficult to transport than chloride.

To evaluate the performance of **1** as phosphate uniporter or symporter, $Cl^-$ was replaced by gluconate, whose high polarity impedes its transport, and $NMDGH^+$ with $K^+$. When both **1** and the $K^+$ transporter valinomycin (**V**)[34] were added, efficient phosphate transport was observed (Figure 2b, purple curve), which can be attributed to $H_2PO_4^-$ uniport by **1** combined with $K^+$ transport by **V**.

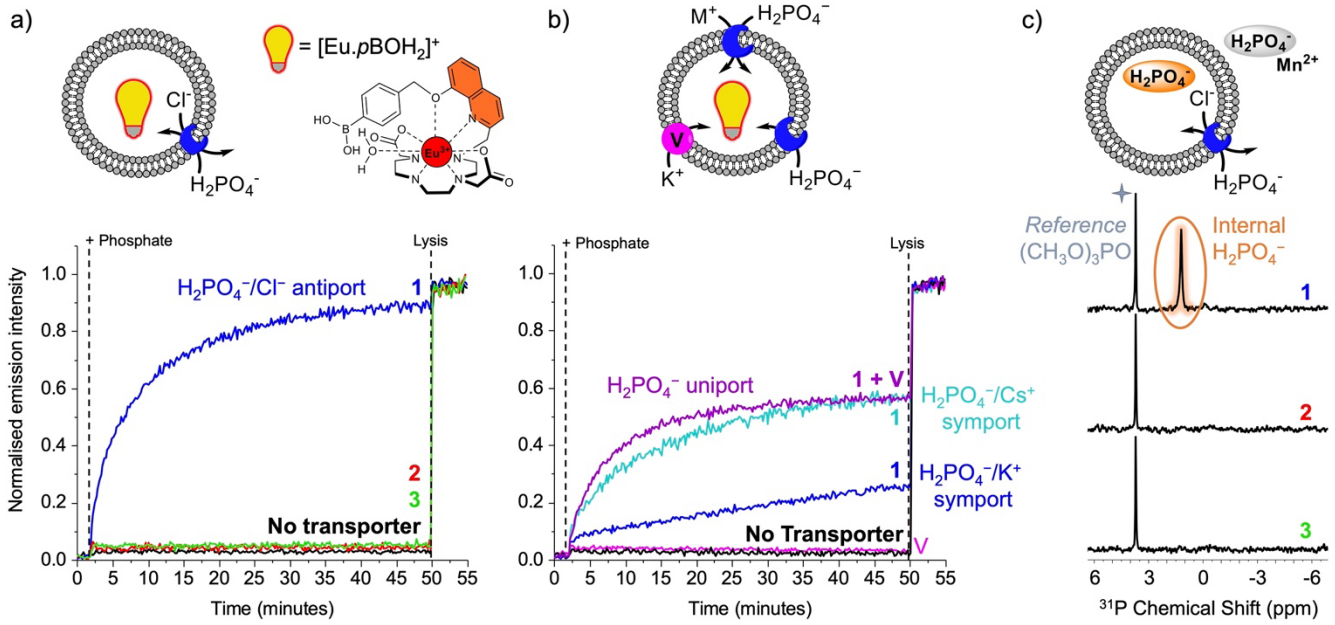



**Figure 2.** Normalised transport curves of compounds **1-3** (4 mol% transporter to lipids ratio) obtained with the [Eu.*p*BOH$_2$]$^+$ assay in a) NMDGH$^+$Cl$^-$ and b) KGluconate or CsGluconate at pH 7, monitoring the emission intensity of the phosphate sensitive europium(III) probe [Eu.*p*BOH$_2$]$^+$ at 615 nm upon excitation at 322 nm with a time-gate applied.[35] Transport was initiated by adding a 25 mM phosphate pulse after 2 min and the LUVs were lysed after 50 min using Triton X-100; c) $^{31}$P NMR spectra recorded 1h after the addition of NaH$_2$PO$_4$ to the exterior of LUVs with **1-3** preincorporated in the lipid bilayer (1 mol%) in NaCl at pH 5. Before recording the spectra, the paramagnetic agent MnSO$_4$ was added and trimethylphosphate was used as external reference (3.7 ppm).

In the absence of **V**, a small increase in emission intensity was also observed (Figure 2b, blue curve). To test if **1** could act as H$_2$PO$_4^-$/M$^+$ symporter, the experiment was repeated, replacing K$^+$ with Na$^+$ or Cs$^+$ (Figure S47). The higher response with Cs$^+$ (Figure 2b, turquoise curve), compared to K$^+$ and Na$^+$, can be attributed to H$_2$PO$_4^-$/Cs$^+$ symport. These results were not surprising given the reports on symport by calix[4]pyrroles, commonly with a selectivity for Cs$^+$.[36,21-23]

At pH 7, H$_2$PO$_4^-$ and HPO$_4^{2-}$ are the main phosphate species present. Given the double negative charge of the dibasic HPO$_4^{2-}$ and its higher hydration energy ($\Delta G_{hyd}$ = -1089 kJ mol$^{-1}$ for HPO$_4^{2-}$,[37] compared to -473 kJ mol$^{-1}$ for H$_2$PO$_4^-$ [16]) we hypothesized that the phosphate transport observed by emission spectroscopy was due to H$_2$PO$_4^-$ rather than HPO$_4^{2-}$ crossing the membrane. To verify this we set up a $^{31}$P NMR assay, reminiscent of protocols used to monitor ion transport by $^{35}$Cl, $^{33}$S, and $^{13}$C NMR spectroscopy,[38,14,13,39] in which intra- and extra-vesicular phosphate can be distinguished upon the addition of Mn$^{2+}$, a paramagnetic agent, to the exterior of the liposomes.

The $^{31}$P NMR experiments were first performed at pH 5, where H$_2$PO$_4^-$ is the predominant phosphate species (~97%).[40] Compound **1** was pre-incorporated (1 mol%) in the lipid bilayer of LUVs (50 mM POPC, ~200 nm) prepared in 400 mM NaCl buffered using 5 mM MES. When a phosphate pulse (100 mM) was added to the exterior of the LUVs, a large signal was observed in the $^{31}$P NMR spectrum at 1.05 ppm. Addition of MnSO$_4$ (2 mM) to the LUV suspension resulted in the full relaxation of the extravesicular phosphate signal. After 1 h of transport followed by 1 h of data acquisition, a clear $^{31}$P NMR signal was visible at 1.2 ppm (Figure 2c),[41] providing further evidence of the ability of **1** to transport H$_2$PO$_4^-$.

The transport by **1** was then monitored at pH 7 (~25% H$_2$PO$_4^-$)[40] and a significantly smaller signal appeared for the intravesicular phosphate, under the same conditions as above. The intensity of the $^{31}$P NMR signals increased over 12 hours, to a value which is about a fourth of the one observed after 12h at pH 5 (Figure S57). At pH 8 (~3% H$_2$PO$_4^-$)[40] no internal signal appeared, even after 12h. The amount of transported phosphate correlates with the concentration of H$_2$PO$_4^-$ in solution, confirming our hypothesis that phosphate is predominantly transported in the form of H$_2$PO$_4^-$. Control compounds **2** and **3** did not give rise to an internal $^{31}$P signal, even after 12h at pH 5, confirming the results obtained in the [Eu.*p*BOH$_2$]$^+$ assay.

In conclusion, we present the first example of inorganic phosphate transmembrane transport using the neutral bisurea-strapped calix[4]pyrrole **1**. The unique ability of receptor **1** to transport phosphate was demonstrated using two complementary assays in which the entry of phosphate into vesicles is monitored directly using emission spectroscopy ([Eu.*p*BOH$_2$]$^+$ assay) and $^{31}$P NMR spectroscopy. Receptor **1** was found to be active as a H$_2$PO$_4^-$/Cl$^-$ antiporter, a H$_2$PO$_4^-$ uniporter, and a Cs$^+$H$_2$PO$_4^-$ symporter. The higher transport activity observed in the $^{31}$P NMR assay at pH 5 compared to pH 7 and 8 indicates that phosphate is mainly transported in the form of H$_2$PO$_4^-$. This work represents a breakthrough for the development of new phosphate transporters, as well as a milestone towards transmembrane transport of other biological phosphates.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures, synthesis and characterization of compounds, UV-Vis and NMR anion binding studies, molecular modeling, and all anion transport experimental details for the assays used in this work (PDF).

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### Notes

The authors declare no competing financial interests.

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