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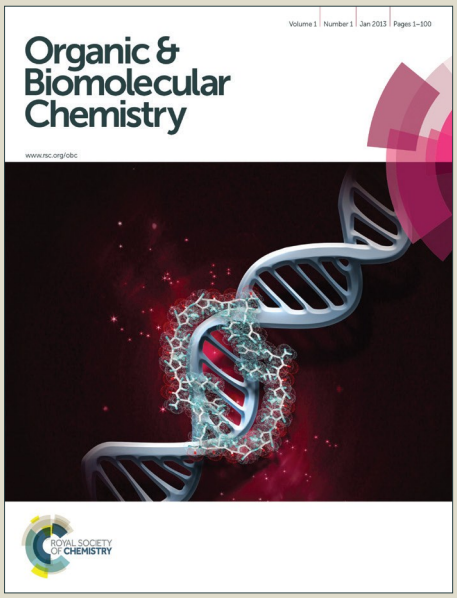

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A Selective Calix[6]arene-based Fluorescent Chemosensor for Phosphatidylcholine Type Lipids

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Abstract

The development of chemosensors that can selectively detect phosphatidylcholines (PCs) in biological samples is of medical relevance considering the importance of these phospholipids in cell growth and survival. Their selective sensing over phosphatidylethanolamines (PEs) is however a challenging task. We report here on the chemosensing capacities of calix[6]tris-pyrenylurea **1**, which is able to selectively interact with phosphatidylcholine-type lipids in organic media. Host **1** also binds them in a biphasic chloroform/water solution, opening the way to the design of selective chemosensors for these lipids in biological media. The results obtained by NMR, fluorescence spectroscopy and modelling studies show that the selectivity is the result of the high degree of complementarity between the lipids' zwitterionic phosphatidylcholine headgroup and the receptor's H-bonding donor site and hydrophobic pocket. The mode of recognition is reminiscent of natural systems, such as human phosphatidylcholine transfer proteins (PC-TPs), validating the biomimetic approach adopted in our work.

Keywords: Calixarenes – Supramolecular chemistry – Host-guest systems – Phospholipids – Chemosensors

Introduction

Phospholipids are a major class of zwitterionic lipids, present predominantly in cellular membranes.[1] These amphiphilic lipids possess a hydrophobic domain and a hydrophilic head linked together, either by a glycerol unit (glycerophospholipids) or by sphingosine (sphingolipids) (Figure 1). Their polar head is composed of a phosphodiester group and typically a nitrogenous moiety such as choline, ethanolamine or serine. Phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs) (Figure 1) are generally the most abundant membrane phospholipids and the ratio of PCs to PEs in the plasma membrane is, for example, reported as crucial for maintaining cellular growth and survival.[2] The quantification of these lipids in biological samples is thus of medical importance and is classically achieved by gravimetric[3] or colorimetric techniques.[4] Recently, a NMR method for phospholipids

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quantification has been reported.[5] The selective sensing of PCs over PEs remains however a challenge.

Natural protein receptors recognize and bind PCs and PEs with a high selectivity, thanks to specific interactions with the zwitterionic head. Typically, the phosphate group of the phospholipid is coordinated to a metal center[6] or interacts with a positively charged Arg or Lys residue through ionic and H-bonding interactions,[7] while the ammonium group interacts with a polyaromatic binding pocket through cation-π interactions.[8] A possible biomimetic approach for the selective sensing of phospholipids could consist in the use of heteroditopic receptors that associate a polar recognition site to a polyaromatic cavity.[9]

We have developed calix[6]arene-based systems bearing thiourea or urea moieties, that behave as heteroditopic receptors toward charged or neutral species, such as zwitterions, which could be interesting in the context of phospholipid sensing.[10] Very recently, we have reported that a calix[6]tube possessing two divergent hydrophobic cavities connected via three thiourea linkages (Figure 2, right) is able to selectively bind PCs over PEs in nonpolar solvents.[11] The binding of the phospholipids proceeds through multiple H-bonding interactions between their anionic moiety and the tris-thiourea groups of the host, as well as through π-cationic and CH-π interactions between their cationic $(CH_3)N^+$ group and one of the two polyaromatic calixarene cavity. It is to be noted that the fatty acid chains of the bound lipids do not protrude from the second calixarene cavity but from one of the three macrocycles formed by the (thio)urea arms. Also of interest is our recently developed fluorescent calix[6]tris-urea receptor **1** (Figure 2, left) that can be used for the sensing of anions and organic ion-pairs, and for which a cavity-based selectivity, in terms of size and shape of the guests, was observed with ammonium salts.[12] Considering these previous results, we wished to see if receptor **1** could be exploited for the chemosensing of zwitterionic phospholipids and in particular if it could distinguish between PCs and PEs.

Herein, we report on the study by NMR and fluorescence spectroscopy of the interaction of chemosensor **1** with different phospholipids.

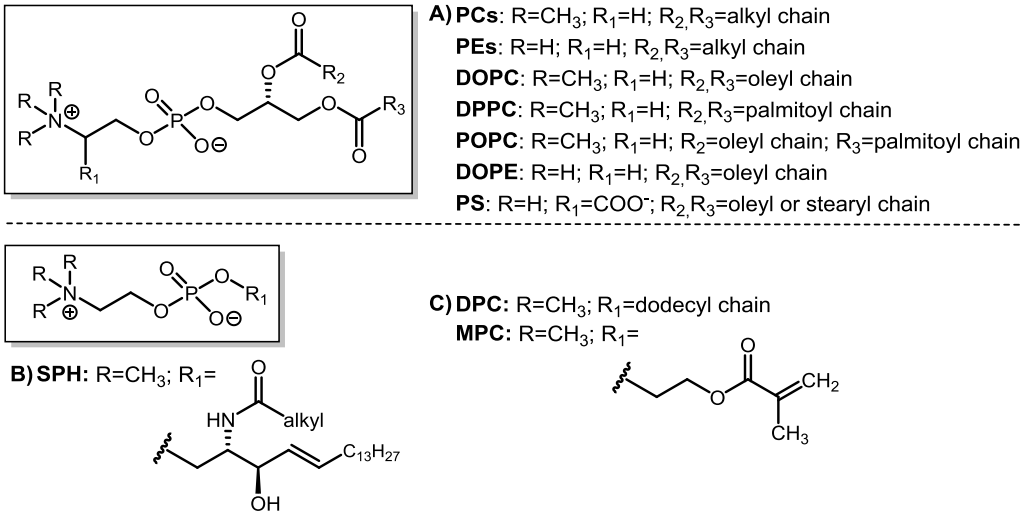

Figure 1. Structure of A) various glycerophospholipids, B) a sphingolipid and C) non-natural phosphocholines.

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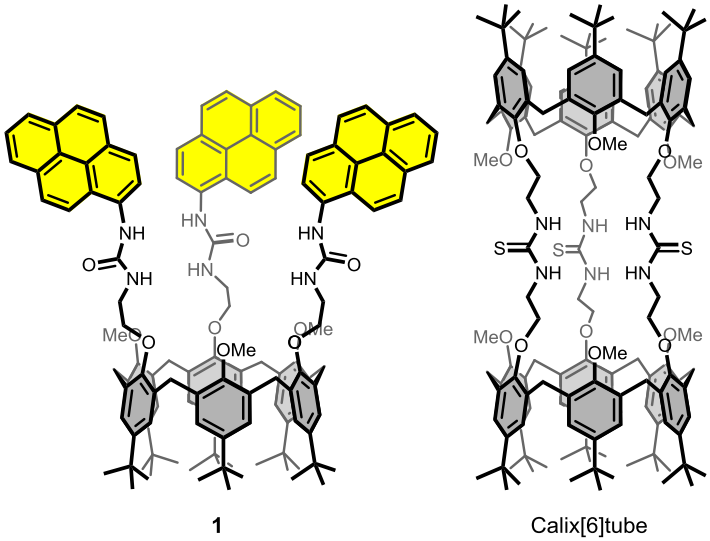

Figure 2. Structure of calix[6]tris-pyrenylurea **1** (left) and calix[6]tube (right).

Results and Discussion

The ability of calix[6]tris-pyrenylurea **1**[12] to bind different phospholipids was investigated in $CDCl_3$ via ^1H NMR titration experiments. As shown previously, the ^1H NMR spectrum of **1** in $CDCl_3$ displays a broad NMR signature due to intramolecular self-association of the urea moieties (Figure 3a). The quantitative formation of a new species that displays well-defined NMR signals was however observed upon the addition of a few equivalents of either 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (see Figure 3b for the ^1H NMR spectrum of DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), *N*-acyl-4-sphingenyl-1-O-phosphocholine (SPH), dodecylphosphocholine (DPC) or 2-methacryloxyethyl phosphocholine (MPC) (see Figure 1 for the structures of the lipids).[13] As a representative example, the ^1H spectrum of host **1** in presence of *ca.* 3 equiv. of DOPC is shown in Figure 3c.

The following common features were observed for all the newly formed host-guest species:

i) The calixarene adopts a flattened C_{3v} symmetrical cone conformation ($\Delta\delta_{ArH} > 0.62$ ppm and $\Delta\delta_{tBu} > 0.61$ ppm) with the OMe groups expelled from the cavity ($\delta_{OMe} > 3.87$ ppm);

ii) The presence of strong H-bonding interaction between the urea groups NH_{pyr} and the bound lipid, as evidenced by the significant down-field shift of the NH_{pyr} signal;

iii) Intra-cavity complexation of the cationic head of the lipid, as clearly evidenced through HSQC and 1D EXSY experiments.[13] For instance, a signal at 1.54 ppm corresponding to the bound Me_3N^+ group is observed upon inversion of the Me_3N^+ signal of free DOPC (selective pulse excitation at 3.27 ppm) (Figure 4).

These NMR data suggest that host **1** behaves as a heteroditopic receptor capable of binding phosphatidylcholine type lipids through the establishment of specific interactions with their zwitterionic head. In all cases, two sets of signals were apparent over the course of the titration, indicating slow host-guest exchanges on the ^1H NMR chemical shift timescale. In the case of **1⊃DOPC**, all the ^1H signals belonging to the free and bound DOPC were assigned by 2D-NMR experiments (COSY, HSQC and ROESY),[13] and the complex-induced shifts (CISs) could be calculated (values given in the inset of Figure 3). The signals corresponding to the

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$\alpha(CH_3)_3N^+$ and $\beta(CH_3)_3N^+CH_2$ protons display negative CISs, which is coherent with the inclusion of the cationic part of DOPC in the heart of the calixarene cavity. In contrast, the γ, δ and ε protons exhibit positive CIS, in accordance with H-bonding interactions between the H-bond acceptor groups of DOPC (phosphate and esters) and the urea groups of **1**. The negative CIS of the ζ, η and θ protons suggest that they are positioned at the level of the pyrene moieties. All the other protons of DOPC experience negligible CIS, showing that the long oleyl chains protrude from the calixarene-based receptor.

Very interestingly, the ^{1}H spectrum of **1** remained unaffected upon the addition of either 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) or 1,2-diacyl-*sn*-glycero-3-phosphoryl-L-serine (PS).[13] This inertness toward PE type lipids implies that the presence of a quaternary ammonium group at the level of the polar head is mandatory for the recognition process, which would be the result of i) the establishment of CH–π and π–cationic interactions between the Me_3N^+ group and the aromatic walls of the calixarene and ii) the stronger self-association of PE type lipids in nonpolar solvents.[14]

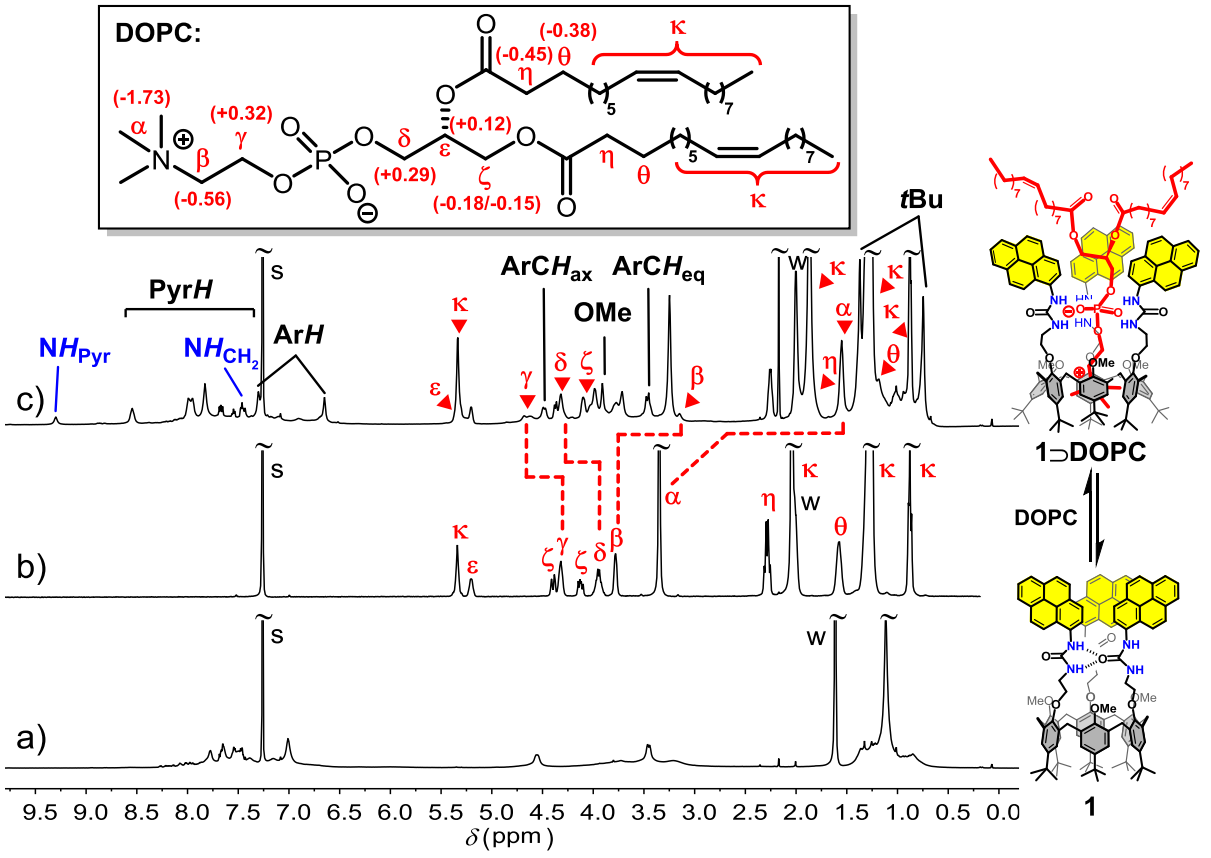

Figure 3. ^{1}H NMR spectra (600MHz, 298 K, $CDCl_3$) of: a) calix[6]tris-pyrenylurea **1**; b) DOPC; c) host **1** in presence of *ca.* 3 equiv. of DOPC. Inset: structure of DOPC with the CIS measured at 298 K. CIS defined as Δδ = δ (complexed DOPC) - δ (free DOPC). w: water; s: solvent.

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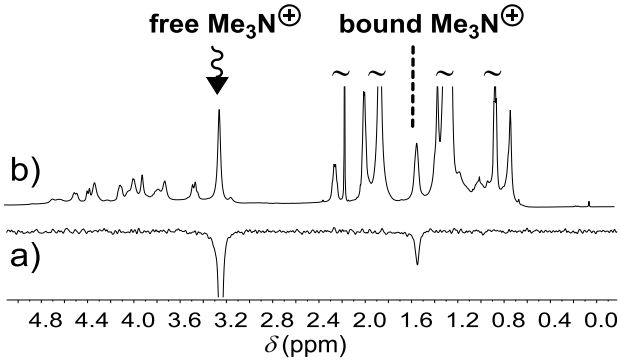

Figure 4. NMR spectra (600MHz, 298 K, $CDCl_3$) of host **1** in in the presence of *ca.* 3 equiv. of DOPC: a) 1D EXSY spectrum (mixing time = 25 ms) after selective excitation of the $^{+}NMe_3$ signal at 3.27 ppm; b) ^{1}H NMR spectrum.

The binding affinities toward the different PC type lipids in $CDCl_3$ were estimated via integration of the Me_3N^+ signal of the free lipid and of the PyrH signals of the host-guest complex. In all cases, the association constants (log K_{NMR}) were too high to be determined accurately by NMR (Table 1).[15] Upon addition of *ca.* 4% of CD_3OD to a solution of host **1** in the presence of 3 equiv. of DOPC in $CDCl_3$, *ca.* 5% of the host-guest complex **1⊃DOPC** was still detected.[13] In DMSO-d_6, a more competitive solvent, DOPC and DPC were however not recognized.[13]

The binding of POPC by receptor **1** was also investigated in $CDCl_3$ by ^{31}P NMR spectroscopy. The ^{31}P NMR spectrum of POPC exhibits a signal at -0.88 ppm (Figure 5a). Upon progressive addition of host **1**, the signal corresponding to the bound POPC was clearly detected at -2.18 ppm (Figure 5b-d). The significant CIS (-1.30 ppm) confirms the formation of H-bonding interactions between the phosphate group and the urea moieties. As expected, a high affinity was estimated through integration of the two signals (log $K_{NMR} > 4$) and no change in the ^{31}P NMR spectrum was observed upon addition of **1** to a DOPE solution.[13]

Table 1. Affinity of host **1** towards different lipids at 298 K.

Complex	Solvent	log K_{NMR}[a]	log K_{FLUO}[a]
1⊃DOPC	$CDCl_3$	>4	4.8 ± 0.1
1⊃DPPC	$CDCl_3$	>4	4.9 ± 0.2
1⊃POPC	$CDCl_3$	>4	4.6 ± 0.1
1⊃SPH	$CDCl_3$	>4	4.5 ± 0.6
1⊃DPC	$CDCl_3$	>4	4.5 ± 0.1
1⊃MPC	$CDCl_3$	>4	4.1 ± 0.3
1⊃DOPE	$CDCl_3$	nd[b]	nd[b]
1⊃PS	$CDCl_3$	nd[b]	nd[b]
1⊃DOPC	DMSO-d_6	nd[b]	-
1⊃DPC	DMSO-d_6	nd[b]	-

[a] K = [Complex]/([Host]×[Guest]). K_{NMR} and K_{FLUO} refer to binding constants determined by ^{1}H NMR spectroscopy and fluorescence spectroscopy respectively.
[b] No binding detected.

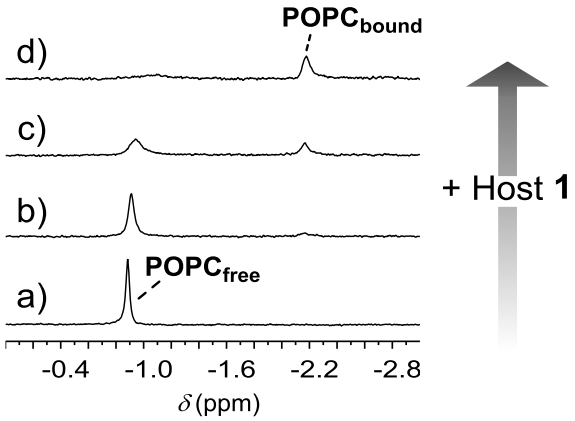

Figure 5. ^{31}P NMR spectra (400MHz, 298 K, $CDCl_3$) of: a) POPC (6.22 mM); b) after addition of *ca.* 0.1 equiv. of host **1**; c) after addition of *ca.* 0.3 equiv. of host **1**; d) after addition of *ca.* 0.6 equiv. of host **1**; triphenylphosphine used as internal reference.

To gain insight into the recognition process between host **1** and PC type lipids, the geometry of complex **1⊃DOPC** was optimized by molecular mechanics using the CHARM force field and the Smart Minimizer algorithm (RMS gradient 0.1).[16] The resulting energy minimized structure is in good agreement with the NMR results (Figure 6). The calixarene displays a flattened conformation with the OMe groups directed toward the outside of the cavity and interactions between host **1** and the zwitterionic head of the lipid are observed. Indeed, the Me_3N^+ group is buried inside the poly-aromatic cavity enabling it to establish π-cationic and CH-π interactions while the convergent urea groups stabilize the edifice through multiple H-bonding interactions. The two oleyl chains of DOPC are located between the three pyrenyl units where they are stabilized through CH−π interactions. From a biomimetic point of view, **1⊃DOPC** shows strong similarities with the complexes formed between PCs and their natural receptors such as the human phosphatidylcholine transfer protein (PC-TP).[17]

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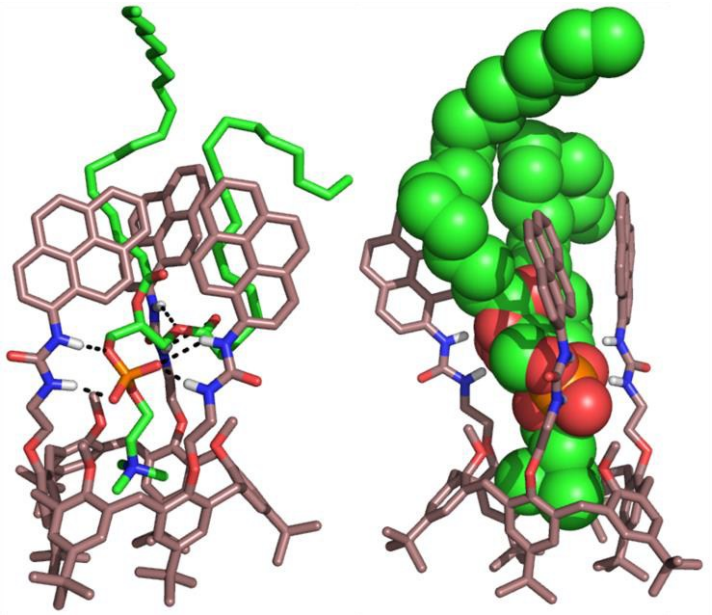

Figure 6. Energy minimized structure of **1⊃DOPC**; Hydrogen bonds are indicated by dashed lines. Selected distances (Å): N(host)−O(DOPC): 2.97, 3.00, 3.01, 3.37, 3.38, 3.51; N^+(host)-(π-centroids): 4.24, 4.29, 4.32. With the exception of the NH of the host, all the hydrogen atoms of **1** and of DOPC are omitted for clarity. Two perpendicular orientations of the structure of **1⊃DOPC** are presented.

The complexation of the different lipids by host **1** was also investigated by fluorescence spectroscopy. No significant changes were observed in the fluorescence spectra of host **1** during the titrations with the PE type lipids (DOPE and PS).[13] With the PC type lipids, an increase of the monomer emission (at 398 and 420 nm) and a decrease of the excimer emission (at 486 nm) were clearly observed over the course of the titrations (see Figure 7 for DOPC).[13] These spectral changes are the signature of the separation of the self-associated pyrenyl urea groups upon lipid complexation. Association constants (log K_{FLUO}) of the same order of magnitude were determined for all the PC type lipids (Table 1). Such a lack of selectivity shows that the recognition of these lipids is mostly due to specific interactions with their phosphorylcholine polar head. The normalized increase in fluorescence intensity of host **1** at 420 nm in the presence of *ca.* 35 equiv. of the different lipids is shown in Figure 8. For MPC, a much weaker variation is observed in comparison with all the other PC type lipids. The different behaviour of this lipid may likely originate from the absence of long alkyl chains that could play a role in separating the pyrene moieties. All these results show that host **1** constitutes a selective chemosensor for lipids bearing a phosphorylcholine head.

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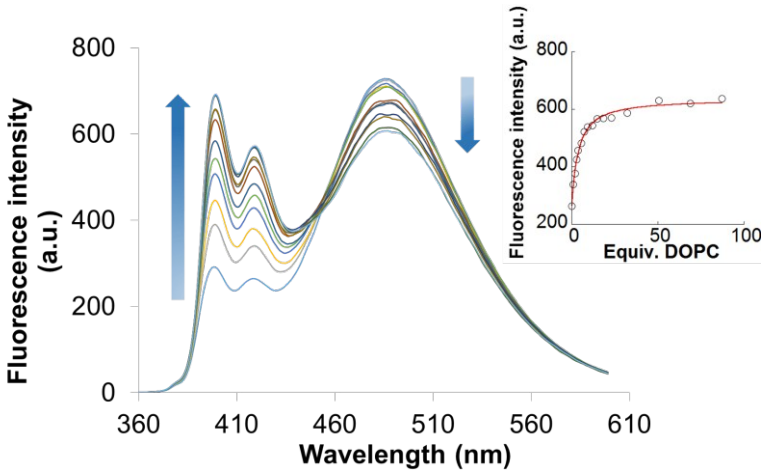

Figure 7. Emission spectra of **1** upon addition of DOPC (0 to 85 equiv.) in chloroform. $[\mathbf{1}]_0$ = 5.0 µM and λ_{ex} = 345 nm. Inset: variation of fluorescence intensity at 420 nm upon the addition of DOPC. Solid line: fitting to a 1:1 binding model.

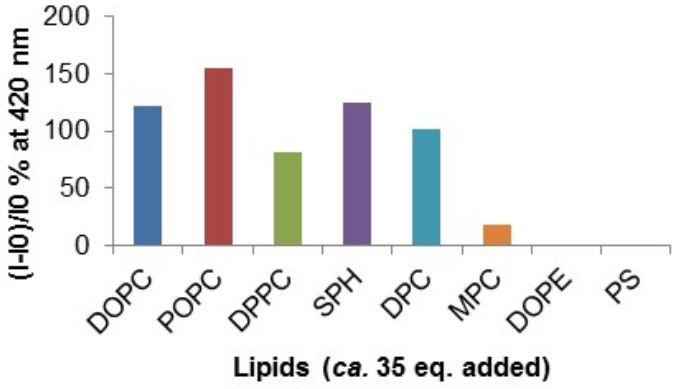

Figure 8. Fluorescence intensity changes ($(I - I_0)/I_0 \times 100$ %) of **1** (*ca.* 5 µM) in $CHCl_3$ upon the addition of various lipids (λ_{ex} = 345 nm). I_0 is the fluorescence emission intensity at 420 nm for free host **1**, and I is the fluorescence emission intensity after addition of *ca.* 35 equiv. of the lipid.

Preliminary experiments were also undertaken to evaluate the possibility of using host **1** for the chemosensing by fluorescence spectroscopy of PC type lipids in an aqueous environment. Liquid-liquid extraction experiments were undertaken where 20 µL of aqueous solutions of different known DOPC concentrations were added to 2 mL of a micromolar solution of **1** in chloroform.[13] The fluorescence spectra of the chloroform solution recorded after stirring showed the characteristic signature of **1⊃DOPC**, with the intensity depending on the initial concentration of DOPC in the aqueous solution. The formation of **1⊃DOPC**, following liquid-liquid extraction experiments, was confirmed by NMR highlighting that the receptor can still efficiently complex DOPC even in a biphasic chloroform/water solution. The affinity of **1** in chloroform for DOPC is not significantly affected by the presence of a large amount of water, and remains larger than 10^4 (log $K \sim 4.5$). These results are encouraging and suggest that the detection and quantification of PC type lipids in biological media using host **1** could be envisaged.

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Conclusion

Calix[6]tris-pyrenylurea **1** behaves as an efficient heteroditopic chemosensor able to strongly interact with lipids in organic media (log $K > 4$). A high selectivity for phosphatidylcholine type lipids over the closely related phosphatidylethanolamines is observed, highlighting the importance of specific interactions between the receptor and the zwitterionic head of the lipid. This mode of recognition is reminiscent of natural systems such as human phosphatidylcholine transfer proteins (PC-TPs), validating the biomimetic approach adopted in our work, that consists in designing receptors that associate a H-bonding donor site to a hydrophobic pocket in order to achieve a high degree of complementarity with the two ionic groups of zwitterions. Interestingly, liquid-liquid extraction experiments showed that host **1** can still bind phosphatidylcholine type lipids in a biphasic chloroform/water solution, opening the way to the design of selective chemosensors for lipids in biological media.

Experimental Section

General experimental methods. DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), SPH (*N*-acyl-4-sphingenyl-1-O-phosphocholine, MPC (2-methacryloxyethyl phosphocholine), DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) and PS (1,2-diacyl-*sn*-glycero-3-phosphoryl-L-serine) (all ≥ 99%) were purchased from Sigma-Aldrich (Missouri, USA). DPC (dodecylphosphocholine) (≥ 99%) was purchased from Avanti Polar Lipids (Alabama, USA). DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) (≥ 99%) from Bachem (Bubendorf, Switzeland). Chloroform (both deuterated and non-deuterated) was filtered, prior to use, over a short column of basic alumina to remove traces of HCl/DCl. ^{1}H spectra were recorded at 400 MHz (9.4 T) or 600 MHz (14.1 T). 2D NMR spectra (COSY, HSQC, and ROESY) were recorded to complete signal assignments. 1D EXSY experiments were recorded with an iburp2 pulse sequence. NMR parameters (acquisition time, recycling times, and signal accumulation) were chosen to ensure that quantitative data could be obtained from signal integration in the 1D ^{1}H spectra. Traces of residual solvent were used as an internal chemical shift reference. Chemical shifts were quoted on the δ scale. The NMR and fluorescence spectra were recorded at 298 K unless otherwise stated.

^{1}H NMR titration experiments. Lipids were purchased All experiments were undertaken following a similar protocol: a known volume (*ca.* 600 µL) of solution of known concentration of host (*ca.* 2 mM) was placed in a NMR tube and the ^{1}H NMR spectrum was recorded. Aliquots (*ca.* 5 µL corresponding to 0.25 equiv. of host) of a stock solution of guest were successively added and a ^{1}H NMR spectrum was recorded after each addition. Aliquots were added until no change was observed for the host signals.

^{31}P NMR titration experiment. A known volume (*ca.* 600 µL) of solution of known concentration of POPC (*ca.* 6 mM) was placed in a NMR tube and the ^{31}P NMR spectrum was recorded. An external capillary tube containing a known concentration of triphenylphosphine was used to provide the reference peak for quantification. Aliquots (*ca.* 5 µL corresponding to 0.25 equiv. of guest) of a stock solution of host **1** were successively added and a ^{31}P NMR spectrum was recorded after each addition.

Fluorescence titration experiments. All experiments were undertaken following a similar protocol: a known volume (*ca.* 2 mL) of solution of known concentration of host (*ca.* 5.0×10^{-6}

M) was placed in a cell and the absorbance and emission spectra were recorded. Aliquots (*ca.* 5 µL corresponding to 0.5 equiv. of host) of a stock solution of guest were successively added and a spectrum was recorded after each addition. Aliquots were added until no change was observed in the spectrum. The values obtained for emission were corrected for dilution.

^{1}H NMR characterization of the host-guest complex 1⊃DOPC: ^{1}H NMR ($CDCl_3$, 298K, 600 MHz: δ (ppm) 0.70-0.81 (m, 27H, *t*Bu), 0.87 (m, 6H, DOPC$^{\kappa,in}$), 1.17 (m, 4H, DOPC$^{\theta,in}$), 1.21-1.45 (m, 44H, DOPC$^{\kappa,in}$), 1.35-1.44 (m, 27H, *t*Bu), 1.54 (m, 9H, DOPC$^{\alpha,in}$), 1.80 (m, 4H, DOPC$^{\eta,in}$), 2.00 (m, 4H, DOPC$^{\kappa,in}$), 3.15 (m, 2H, DOPC$^{\beta,in}$), 3.46 (m, 6H, $ArCH_2^{eq}$), 3.77 (br s, 6H, CH_2N), 3.90 (m, 9H, OMe), 3.96 (m, 1H, DOPC $^{\zeta,in}$), 4.06 (br s, 6H, CH_2O), 4.21 (m, 1H, DOPC$^{\zeta,in}$), 4.30 (m, 2H, DOPC$^{\delta,in}$), 4.48 (m, 6H, $ArCH_2^{ax}$), 4.66 (m, 2H, DOPC$^{\gamma,in}$), 5.34 (m, 1H, DOPC$^{\varepsilon,in}$), 5.34 (m, 4H, DOPC$^{\kappa,in}$), 6.64 (s, 6H, ArH^{Urea}), 7.30 (s, 6H, ArH^{OMe}), 7.41-8.14 (m, 21H, PyrH), 7.44 (s, 3H, *NH*CH_2). 8.57 (m, 6H, PyrH), 9.31 (s, 3H, *NH*Pyr). The majority of the signals of DOPC^{in} were assigned thanks to the COSY, HSQC and 2D ROESY spectra.

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Supporting information: ^{1}H NMR spectra, ^{31}P NMR spectra and fluorescence studies of the complexation properties of **1**.

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[15] It is noteworthy that the signal corresponding to the $(CH_3)_3N^+$ protons of the free lipid shift downfield as the lipid concentration increases. This suggests that the free lipid is in exchange between different environments. This is not simply the signature of a lipid monomer-dimer equilibrium as this signal shifts upfield with lipid concentration in the absence of any host.

[16] Structure of host **1**, was built on the basis of previous structures or related calix[6]tube molecules (references 10a and 10c). Starting conformation for the **1⊃DOPC** complex has been manually build using Discovery Studio 2016 (BIOVIA, *Dassault Systèmes*). Geometry was further optimized with the same suite of programs using the CHARM force field and the Smart Minimizer algorithm (RMS gradient 0.1).

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