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A zinc–salophen/bile-acid conjugate receptor solubilized by CTABr micelles binds phosphate in water†

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Receptor 1, composed of two deoxycholic acid moieties appended to a Zn–salophen complex, was prepared, characterized and tested for anion binding by ^{1}H NMR and UV-vis spectroscopic techniques. While in polar DMSO, 1 is able to bind phosphate (K = ~700 M^{-1}), the addition of water severely diminishes the association. In a 1 : 9 water–DMSO mixture, the binding constant K is only *ca.* 20 M^{-1}. Notably, in an aqueous solution of CTABr micelles (CTABr 10 mM, cmc = ~1 mM), the zinc–salophen conjugate 1, due to its two non-polar bile-acid moieties, becomes solubilized and, most importantly, it almost completely recovers its binding ability towards phosphate, displaying a remarkable affinity (K = ~450 M^{-1}) in water.

Introduction

Anions are ubiquitous in the natural world and their recognition and binding constitute a central theme in Supramolecular Chemistry.[1] In particular, phosphates – major components in bio-mineralised materials, such as exoskeletons and bones,[2] and essential parts of DNA and RNA[3] – have prominent importance in biological processes, but also they represent significant environmental hazards for their widespread use as fertilizers. Hence, the development of species able to bind phosphate and phosphorylated molecules efficiently and selectively has attracted increasing attention in the last few decades.[4]

As evidenced by many authors, anion binding presents several additional challenges compared to cation binding, related to the greater size (compared to isoelectronic cations), the smaller charge density, and their varying shapes and geometries and pH sensitivities. Moreover, in protic solvents and water, solvation/hydration energies for anions are usually high and this represents a severe obstacle to binding, which explains the paucity of anion receptors able to be effective in water or aqueous media.[1a–c]

In general, among the plethora of different intermolecular interactions available, metal coordination is considered one of the most efficient a receptor could rely on. In organic solvents, metal sites are established as excellent binding centres for anions.[1,5] However, translating the same systems in water has turned out to be difficult. Firstly, simple neutral metal complexes are usually insoluble in water and this requires the introduction of synthetic modifications, which naturally should avoid any possible interference with the binding site. Furthermore, once the solubility issues are solved, the affinities observed could be lower than expected.[6] A completely different approach aims at constructing a "protective" environment around the receptor binding site that could serve as a shield for the binding event from the competing bulk water. Among a few successful attempts in this direction,[7] a demonstration of the above concept for the binding of anions was achieved by using aqueous CTABr micelles as carriers for a simple UO_2–salophen receptor.[7a] In that case, a strong binding to fluoride in water, unattainable without the micellar environment, was indeed observed.

Despite the effective strong Lewis acid character and anion binding aptitude of UO_2–salophen complexes,[8] uranium is mildly radioactive and this severely limits any possible exploitation of their properties in real-world applications. Hence, the need to study other metal centers able to bind anions, for instance, Zn(II) cation, is evident. It is well known that Lewis basic groups are excellent axial ligands for Zn–salen and –salophen complexes, and this explains why they have recently attracted attention in a number of structural,[9] catalytic[10] and host–guest chemistry studies.[11]

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†Electronic supplementary information (ESI) available: Additional information on the absorption and binding properties of **1** in the presence of phosphate and acetate anions in DMSO, a 9 : 1 DMSO–water mixture and water (10 mM CTABr); ^{31}P-NMR; details of the full NMR assignment and numbering of the compounds **1** and **4**. See DOI: 10.1039/c3ob40724a

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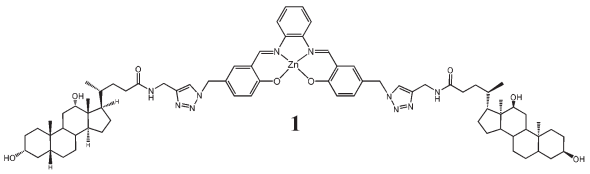


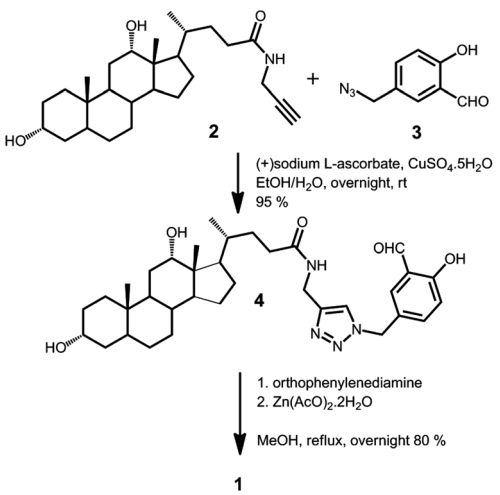
Scheme 1 Molecular formula of the Zn–salophen/bile-acid conjugate **1**.

Here we report on the novel zinc–salophen/bile-acid receptor **1** (Scheme 1) constituted by a central Zn–salophen complex, conjugated with two deoxycholate amido moieties at both sides linked by Huisgen copper-catalyzed click reaction. Receptor **1** is solubilized easily in aqueous CTABr micelles. Under such conditions, the **1**·CTABr system maintains its ability to bind phosphate by displaying a remarkable affinity. Receptor **1** represents the first example of a metal–salophen complex specifically designed to be incorporated into a micellar system. In this respect, bile acid moieties were chosen as ideal structural units due to their proven affinity for lipophilic membranes.[12] Huisgen copper-catalyzed click chemistry[13] was employed as a convenient method to connect the two structural elements, *viz.*, the metal–salophen unit and the bile acid moieties together into the new receptor **1**.

Results and discussion

Synthesis

5-Azidomethyl-2-hydroxybenzaldehyde (**3**) was prepared in two steps by transformation of salicylaldehyde to a corresponding chloromethyl precursor, which was subsequently converted into azide.[14] Propargyl amide **2** was prepared *via* amidation of deoxycholic acid by propargyl amine in the presence of DCC and DMAP. Compounds **2** and **3** were connected by Huisgen copper-catalyzed click chemistry to form the 1,2,3-triazole ring and give compound **4**. Two units of **4** were merged together by formation of bis-Schiff base with *ortho*-phenylenediamine in order to afford the final ligand. Subsequent addition of Zn(II) salt (one-pot) led to the receptor **1** in 80% yield (Scheme 2).



Scheme 2 Synthetic procedure for **1**.

Anion binding properties in DMSO

While in weakly coordinated solvents, tetra-coordinated Schiff base Zn(II)-complexes are known to auto-saturate their metal coordination sphere by formation of dimeric species and/or aggregates,[9] in coordinating solvents such as DMSO, they usually exist in the monomeric form and adopt a square pyramidal configuration at their metal centre, where the solvent is axially coordinated. The replacement of the solvent axial ligand by stronger donating species (amines, anions, *etc.*) is known to produce significant changes in the optical properties, which can be easily monitored by UV-vis spectroscopy. We initially investigated the anion binding properties of receptor **1** in DMSO, where it is soluble at 25 °C, by ^{1}H-NMR titration experiments in which the time averaged signals of the receptor were monitored as a function of increasing concentration of the target anion, added as TBA salt. This preliminary test allows also a direct comparison of the binding ability of **1** with other anion receptors reported in the recent literature.[1a–c] DMSO is becoming a very popular solvent to test binding since it might be considered as the upper limit in terms of competitiveness among common aprotic solvents. As an example of a typical experiment, the titration between a 2 mM solution of the receptor **1** and (TBA)H_2PO_4 is shown in Fig. 1a. Clearly, all

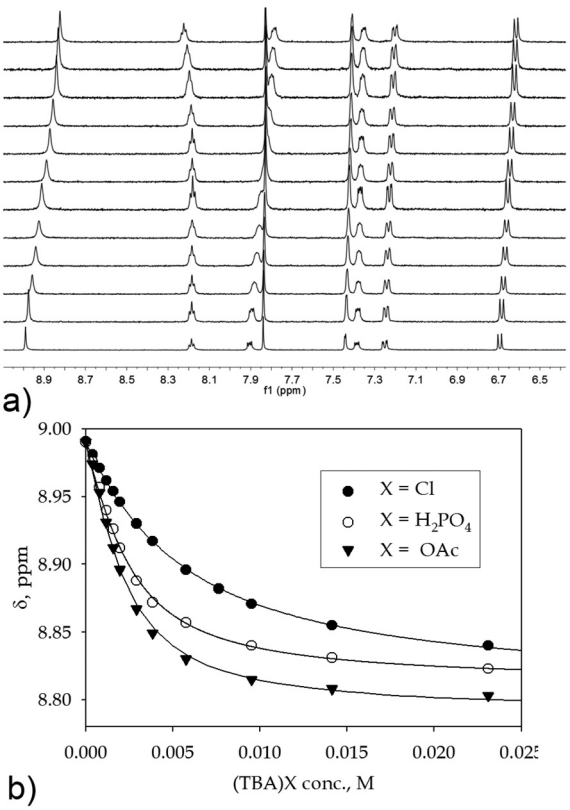


Fig. 1 (a) Chemical shift variations observed for signals of 2 mM **1** upon addition of increasing amounts of (TBA)H_2PO_4 (from bottom to top) in DMSO-d_6 at 300 K; (b) a plot of the chemical shift variations observed for the imine CH═N signal of **1** *vs.* (TBA)X concentration (X = Cl^-, AcO^-, $H_2PO_4^-$) in DMSO-d_6 at 300 K (lines represent best fit curves for each data set).

the receptor protons display a shift upon addition of increasing aliquots of the anion (from bottom to top). With the exception of the signal around 8.15 ppm,[15] the observed chemical shifts are shielded (upfield) and they were interpreted as due to the increase of electron density on the salophen framework upon formation of the negatively charged [**1**-phosphate]$^-$ complex. The anion is expected to interact with the Zn(II) centre *via* a coordinative bond thus forming a penta-coordinated square pyramidal complex.[11]

A plot of the observed δ of iminic protons *versus* the phosphate (○) concentration is shown in Fig. 1b, along with the δ variations observed upon addition of Cl^- (●) and acetate (▼) anions. Each data set can be easily fitted by making use of a non-linear least squares method and applying a 1 : 1 binding isotherm equation. Measurements were run in duplicate and the σ-weighted average of the association constants[16] for each anion is reported in Table 1. Acetate and phosphate anions are bound to **1** to a similar extent, while chloride displays a lower affinity. In the case of phosphate, the association constant was also confirmed by independent UV-vis titration experiments (see the ESI†). In the case of $(TBA)H_2PO_4$, ^{31}P NMR was also attempted but without success (see the ESI†).

Not surprisingly, addition of water to the DMSO solution strongly reduced the binding. For example, in a 1 : 9 water–DMSO mixture, the association constant K for phosphate drops to *ca.* 20 M^{-1} (see Fig. S1 in the ESI†). This finding, again, illustrates the intrinsic difficulty that anion receptors encounter in the presence of a strongly competing solvent. In pure water, had **1** been soluble, a negligible association could be expected.

Anion binding properties in CTABr–water

Although the Zn–salophen receptor **1** is insoluble in water, its two amphiphilic deoxycholic acid moieties enhance its lipophilic nature and strongly affect the solubility in organic pseudo-phases (*viz.*, micelle). Consequently, **1** can be solubilized into aqueous CTABr micelles. The solubilization process is availed by adding a concentrated DMSO solution of **1** into the CTABr–water solution to a final 99 : 1 water–DMSO mixture, which becomes coloured of an intense yellow. The solubilization of **1** in the presence of CTABr is effective only above the critical micellar concentration (cmc) of CTABr (*ca.* 1 mM), whereas sodium dodecyl sulphate (SDS) and sodium deoxycholate systems, below and above their cmc, do not yield appreciable results.

Table 1 Association constants (K, M^{-1}) for complexes between Zn–salophen complex **1** and selected anions in DMSO-d_6 at 300 K

Anion	K ($\pm\sigma$)	$\Delta\delta$ (CH=N), ppm
Chloride	215 ± 5	−0.186
Phosphate	720 ± 30	−0.177
	(680 ± 30, $\Delta\varepsilon_{403} = -1740$)[a]	—
Acetate	830 ± 50	−0.205
Phosphate[a,b]	20 ± 3 ($\Delta\varepsilon_{403} = -5450$)	—

[a] By UV-vis spectroscopy; [b] in DMSO–water 9 : 1.

The UV-vis spectrum of compound **1** in water (1% DMSO, CTABr 10 mM) displays unstructured absorption with two main bands around 295 and 400 nm, hypsochromic shifted with respect to those observed in DMSO (299 and 403 nm, respectively). The close adherence to the Lambert–Beer law at different wavelengths (ESI†) indicates the absence of significant aggregation phenomena within the range of concentrations explored.

A plot of the UV-vis absorption changes of the **1**·CTABr system upon addition of $(TBA)H_2PO_4$ in water (10 mM CTABr, 1% DMSO) is shown in Fig. 2a. The most evident effect is related to the decrease of intensity of the characteristic band centered at 400 nm by increasing the anion concentration. Three isosbestic points (284, 321 and 372 nm) can be easily spotted, hinting at the presence of two species, *viz.*, **1** and [**1**-phosphate]$^-$ complex, responsible for the absorption. Profiles of the absorbance (at 355 and 400 nm) *versus* phosphate concentration are shown in Fig. 2b. Data analysis confirms the presence of a 1 : 1 equilibrium and affords an association constant K of 450 ± 30 M^{-1} ($\Delta\varepsilon$ = −8050 and 3240 at 400 and 355 nm, respectively).[17] This figure is, remarkably, of the same order of magnitude of the binding in DMSO (*ca.* 60%) and demonstrates that the micellar environment has an influence not only on the solubility of **1** in water, but also on its affinity

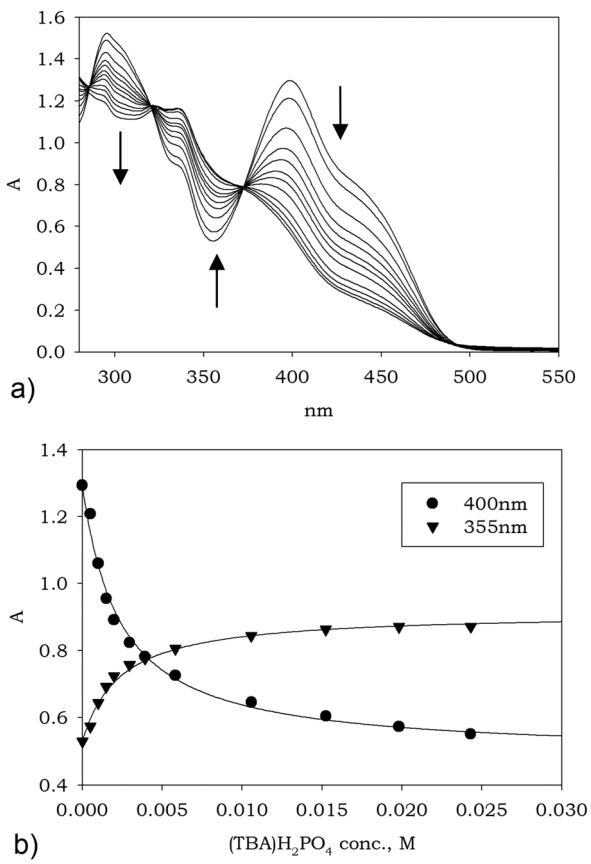


Fig. 2 (a) Absorption spectra taken over the course of the titration of a 1% DMSO–water solution of **1** (0.1 mM) with $(TBA)H_2PO_4$ in the presence of CTABr (10 mM) at 25 °C; (b) a plot of the absorbance (355 and 400 nm) *vs.* phosphate concentration and best fit curves, represented as full lines.

for phosphate anion whose binding can be achieved also in water (having in mind that in a 1 : 9 water–DMSO mixture the affinity is *ca.* 22 times lower). The selectivity of the binding was also tested over chloride and acetate. Interestingly, analogous titration experiments with (TBA)Cl do not show any significant variation of the absorption of **1**, thus indicating absence of binding. Spectral variations in the visible region are observed upon addition of (TBA)AcO under the same experimental conditions. However, the data do not lend themselves to a simple analytical interpretation. Indeed, the initial linear dependence of Abs *vs.* AcO^- concentration is followed, at concentration above *ca.* 0.01 M, by a second linear tract having a smaller slope, but with no indication of reaching any plateau (see the ESI†). Such a trend is not consistent with a simple 1 : 1 binding event, despite the presence of isosbestic points. Leaving quantitative information aside, the comparison of the absorption response of **1** to the addition of acetate and phosphate strongly suggests a higher affinity for the latter anion (see Fig. 6S in the ESI†). However, preliminary data collected by DLS show that the addition of acetate (especially at concentration higher than 0.01 M) might have an effect on the size of the CTABr micelles. This introduces an additional variable to the supramolecular system which needs to be fully analyzed.

Conclusions

Anion binding in water by abiotic receptors remains a challenge, especially if the comparison with natural anion binders, such as anion binding proteins,[18] is elicited. Despite being not well investigated, one practicable strategy to obtain high affinities in water might rely on making the binding event to occur in a more favourable environment. Micellar interior and/or surface could provide an alternative environment to a given receptor, thus improving the affinity for the target analyte. Here, the Zn–salophen/bile-acid conjugate, **1**, insoluble in pure water and scarcely able to bind phosphate in 9 : 1 DMSO–water, becomes solubilized into aqueous CTABr micelles (10 mM). More importantly, in this novel environment, **1** is capable of binding phosphate with an affinity similar to that in pure DMSO and, remarkably, more than 20 times higher than that measured in 9 : 1 DMSO–water. Also, the selectivity over chloride anion improves significantly.

Considering the average aggregation number of micelles made of CTABr at 10 mM around 300 K (of the order of 10^2) and the relative concentration of **1** and the surfactants monomer (1 : 100), we expect that, approximately, each micelle contains no more than a single Zn–salophen/bile-acid conjugate **1**.

This work demonstrates the validity of a less conventional approach in the study of binding in solution, and highlights the importance of the **1**·CTABr system as an efficient phosphate receptor in water, also in the light of the possibility of its use in anion transport through lipophilic membranes.[19] Work is in progress to explicate the effect of different metal ions and formal charge (trivalent metals such as Al(III),[20] or Fe(III),[21] would generate a "+1" species) on the metal salophen moiety and to investigate the influence of different bile-acid derivatives either symmetrically or non-symmetrically decorating the central binding unit, aiming at an increased complex's solubility.

Experimental section

General methods

All the materials were purchased and used as received. 5-Azidomethyl-2-hydroxybenzaldehyde (**3**) was prepared by following a published procedure.[14] The ^1H and ^{13}C NMR spectra were recorded using Bruker Avance DPX250 and DRX500 FT NMR spectrometers. Mass spectra were recorded by ESI-TOF Bruker instrument model Micromass LCT. DLS studies were performed on the N5 Submicron particle size analyzer.

Synthesis

***N*-(Prop-2-yn-1-yl)-3α,12α-dihydroxy-5β-cholan-24-amide (2).** Deoxycholic acid (1.92 g; 4.9 mmol) was dissolved in THF (40 mL), and then DMAP (59 mg; 0.5 mmol) and propargyl amine (0.84 mL; 12.2 mmol) were added under stirring. After cooling down the reaction mixture with an ice-water bath, DCC (1.31 g; 6.4 mmol) was added. The reaction mixture was stirred overnight at room temperature and then filtered off and urea precipitated. The mixture was diluted with dichloromethane (30 mL) and washed with 1 M HCl (15 mL), sat. $NaHCO_3$ (10 mL) and brine (20 mL). The organic layer was dried over Na_2SO_4 and the solvents were removed under reduced pressure. The crude product was purified by column chromatography on silica gel using acetone–dichloromethane 3 : 1 as an eluent. The analytical data for compound **2** were similar to those already published.[22]

***N*-{[1-(3-Formyl-4-hydroxybenzyl)-1*H*-1,2,3-triazol-4-yl]methyl}-3α,12α-dihydroxy-5β-cholan-24-amide (4).** (+)Sodium L-ascorbate (26 mg; 1×10^{-4} mol) and $CuSO_4 \cdot 5H_2O$ (42 mg; 0.2 mmol) were added in one portion to a stirred mixture of propargyl deoxycholanamide (**2**) (300 mg; 0.7 mmol) in water (2 mL) and ethanol (4 mL). Subsequently, a solution of **3** (124 mg; 0.7 mmol) in ethanol (1 mL) was added. The reaction mixture was stirred for 2 h at room temperature and then left under reflux until TLC showed the complete conversion. The solvent was evaporated. Product **4** was obtained in 95% yield, after column chromatography, using dichloromethane–methanol 10 : 1 as an eluent. ^1H NMR (500 MHz, $CDCl_3$): δ = 11.04 (s, 1H, C3′-OH), 9.87 (s, 1H, H-1′), 7.55 (s, 1H, H-27), 7.52 (d, $J_{7',5'}$ = 2.3 Hz, 1H, H-7′), 7.46 (dd, $J_{5',4'}$ = 8.5 Hz, $J_{5',7'}$ = 2.3 Hz, 1H, H-5′), 6.99 (d, $J_{4',5'}$ = 8.5 Hz, 1H, H-4′), 6.56 (t, $J_{NH,25}$ = 5.4 Hz, 1H, –NH–), 5.47 (s, 2H, H-8′), 4.45 (d, $J_{25,NH}$ = 5.4 Hz, 2H, H-25), 3.94 (br. s, 1H, H-12), 3.60 (m, 1H, H-3), 1.99–2.33 (2 × m, 2H, H-23), 0.95–1.95 (steroidal 26H), 0.91 (d, $J_{20,21}$ = 6.1 Hz, 3H, H-21), 0.90 (s, 3H, H-19), 0.64 (s, 3H, H-18); ^{13}C NMR ($CDCl_3$): δ = 196.0 (C-1′), 173.8 (C-24), 162.5 (C-3′), 145.3 (C-26), 136.2 (C-5′), 134.6 (C-7′), 127.5 (C-2′), 123.5 (C-27), 120.0 (C-6′), 118.8 (C-4′), 73.1 (C-12), 71.7 (C-3), 53.4 (C-8′), 48.3 (C-14), 46.9 (C-17), 46.5 (C-13), 42.1 (C-5), 36.4 (C-8), 36.0 (C-4), 35.2 (C-1, C-20), 34.7 (C-25), 34.1 (C-10), 33.6 (C-9), 33.1 (C-23), 31.5 (C-22), 30.5 (C-2), 28.7 (C-11), 27.5 (C-16), 27.1 (C-6), 26.1 (C-7), 23.6

(C-15), 23.1 (C-19), 17.4 (C-21), 12.7 (C-18) ppm; MS (ESI-TOF) *m*/*z* (%): 629.36 (100) $[M + Na]^+$, 1235.82 (8) $[2M + Na]^+$.

Zn–salophen complex (1). Compound **4** (200 mg; 0.3 mmol) was dissolved in methanol (3 mL) and the solution was refluxed at 50 °C for 30 min. Subsequently, *ortho*-phenylenediamine (18 mg; 0.2 mmol) in methanol (1 mL) and $Zn(AcO)_2{\cdot}2H_2O$ (80 mg; 0.4 mmol) in methanol (1 mL) were added. The reaction mixture turned orange after the addition of the amine compound and then yellow after the addition of the zinc salt. The reaction mixture was stirred and left under reflux (55 °C) overnight. The product was observed on a TLC plate as a yellow spot fluorescent at 365 nm and it precipitated out from the reaction mixture after cooling it down (0 °C, in the fridge). Product **1** was obtained as a yellow crystalline solid in 80% yield. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.99 (s, 2H, H-1′), 8.23 (t, $J_{NH,25}$ = 5.5 Hz, 2H, –NH–), 7.90 (dd, $J_{11a',11b'}$ = 3.4 Hz, $J_{10',11a'}$ = 6.1 Hz, 2H, H-11′), 7.85 (s, 2H, H-27), 7.44 (d, $J_{7',5'}$ = 2.3 Hz, 2H, H-7′), 7.38 (dd, $J_{11a',11b'}$ = 3.4 Hz, $J_{10',11a'}$ = 6.1 Hz, 2H, H-10′), 7.25 (dd, $J_{5',4'}$ = 8.8 Hz, $J_{5',7'}$ = 2.3 Hz, 2H, H-5′), 6.69 (d, $J_{4',5'}$ = 8.8 Hz, 2H, H-4′), 5.39 (s, 4H, H-8′), 4.45 (d, $J_{OH,12}$ = 4.2 Hz, 2H, C-12-OH), 4.24 (d, $J_{25,NH}$ = 5.5 Hz, 4H, H-25), 4.16 (d, $J_{OH,3}$ = 4.0 Hz, 2H, C-3-OH), 3.77 (br. s, 2H, H-12), 3.33 (m, 2H, H-3), 1.96–2.10 (2 × m, 4H, H-23), 0.97–1.79 (steroidal 48H), 0.88 (d, $J_{20,21}$ = 6.1 Hz, 6H, H-21), 0.83 (s, 6H, H-19), 0.55 (s, 6H, H-18); ^{13}C NMR (DMSO-d_6): δ = 172.6 (C-24), 172.0 (C-3′), 162.5 (C-1′), 145.3 (C-26), 139.3 (C-9′), 136.2 (C-7′), 134.6 (C-5′), 127.5 (C-10′), 123.5 (C-4′), 122.3 (C-27), 120.0 (C-6′), 118.8 (C-2′), 116.6 (C-11′), 71.0 (C-12), 69.9 (C-3), 52.6 (C-8′), 48.6 (C-14), 46.1 (C-17), 46.0 (C-13), 41.6 (C-5), 36.3 (C-8), 35.6 (C-4), 35.1 (C-20), 35.0 (C-1), 34.2 (C-25), 33.8 (C-10), 32.9 (C-9), 32.3 (C-23), 31.6 (C-22), 30.2 (C-2), 28.6 (C-11), 27.2 (C-16), 27.0 (C-6), 26.1 (C-7), 23.5 (C-15), 23.1 (C-19), 17.1 (C-21), 12.4 (C-18) ppm; MS (ESI-TOF) *m*/*z* (%): 405.10 (100), 875.48 (48), 1369.83 (10) $[M + Na]^+$.

NMR titration. Titrations were performed by addition of increasing small volumetric aliquots of a concentrated solution of the (TBA)X salts (X = Cl^-, $H_2PO_4^-$ and AcO^-) *via* a Hamilton syringe to 0.6 ml of a fresh solution of **1** (2 mM) placed in the NMR tube. All spectra were recorded on a Bruker Avance DRX500 spectrometer (500 MHz) at 300 K. Relaxation delay = 5 s; SW = 12 ppm; TD = 32 K, LB = 0.2 Hz; NS = 32. RG was adjusted at high TBA salt concentration.

UV titration studies. They were carried out on a LAMBDA 850 UV-vis spectrophotometer stabilized at 300 K. A DMSO stock solution of **1** was added to 2 mL of a 10 mM CTABr water solution in the quartz cuvette obtaining a final concentration equal to 0.1 mM. Increasing aliquots of a concentrated solution of the anion, in the form of tetrabutylammonium salt, were added and the spectra recorded.

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