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Synthesis and conformational analysis of a novel carbohydrate-fused bis-crown ether: *crown-CyPLOS*

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ABSTRACT

A novel sugar-based macrocycle consisting of a phosphate-linked 12-membered disaccharide ring (cyclic phosphate-linked oligosaccharide, *CyPLOS*), fused to two 18-crown-6 ether residues, is here described. The synthesis of the target compound has been accomplished in 23% overall yield for 11 reaction steps, exploiting phosphoramidite chemistry for the dimerization and a classical phosphotriester methodology for the cyclization reaction. NMR-based conformational analysis studies have been carried out on the fully deprotected macrocycle, showing a characteristic arched-structure with C_2-symmetry.

1. Introduction

In order to achieve efficient recognition of a specific guest, most host agents in supramolecular chemistry exhibit pre-organized conformations, ensured by a well-defined three-dimensional presentation of the interacting moieties.[1] In the design of effective artificial hosts, the presence of chiral, amphiphilic and cyclic structural motifs is highly desirable, and cyclodextrins, calixarenes, cyclic peptides are typical examples in this frame. Among the most efficient and selective receptors for cations are crown ethers, which, in contrast, exhibit an achiral, rather simple backbone. Since the pioneering work of Pedersen,[2] crown ethers have attracted a great deal of attention over the last three decades, and a large number of diverse derivatives have been designed and synthesized for a variety of supramolecular applications. Bis- or poly-crown ether compounds have proven to be much more effective extraction agents - and more active transmembrane ion channels or mobile carriers - than their monomeric counterparts.[3] Relevant examples are, among others, crown ether-modified cyclodextrins,[4] calixarenes[5] and resorcinarenes,[6] as well as the synthetic ionophores developed by Gokel and coworkers.[7]

In a previous paper,[8] we have showed that cyclic phosphodiester-linked disaccharide **1** (Fig. 1), as the first member of a family of compounds we named CyPLOS (*Cyclic Phosphate-Linked OligoSaccharides*), preferentially adopts a concave conformation, potentially able to bind metal ions.

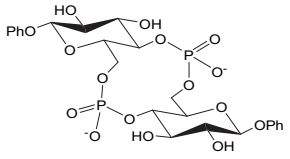

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Figure 1. Chemical structure of macrocycle **1**.

It was originally envisaged that **1** would provide a useful synthetic platform for the construction of new artificial receptors for transmembrane ion transport: the hydroxy groups at C-2 and C-3 of each saccharide residue can in fact be exploited as synthetic handles for the attachment of suitable lipophilic appendages assuring the insertion into the hydrophobic core of lipid systems. Following this design, by covalently linking alkyl or polyether linear tentacles onto the disaccharide macrocycle, a series of amphiphilic, jellyfish-shaped CyPLOS derivatives endowed with good ion transport activity have been prepared (**2a**–**c**, Fig. 2). It was also demonstrated that the presence of the disaccharide macrocycle was essential for the ionophore activity, probably furnishing a portal entry for ions at the water-membrane interface.[9]

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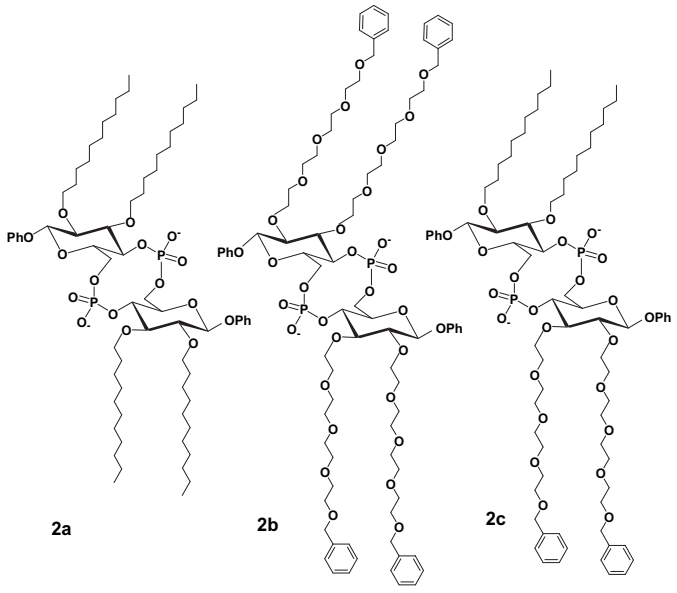


Figure 2. Chemical structure of macrocycles **2a**–**c**.

These promising results prompted us to explore other variants of functionalized CyPLOS, exhibiting diverse structural motifs useful for ion recognition. We here describe novel polycyclic derivative **3** (Fig. 3), carrying a cyclic phosphate-linked disaccharide skeleton fused to two 18-crown-6 units, which potentially offer additional complexing sites, thus contributing to the ionophore properties of the macrocycle.[10] Attachment of these moieties to the vicinal OH groups of a glucoside scaffold was considered to introduce only marginal rigidification into the crown ether backbone, thus leaving the six oxygen atoms in the cavity of each 18-crown-6 unit with optimal orientation to host cations.

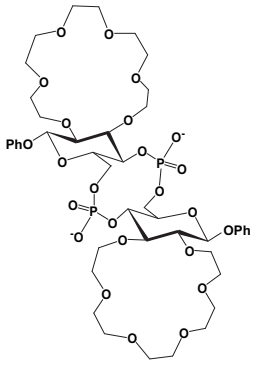


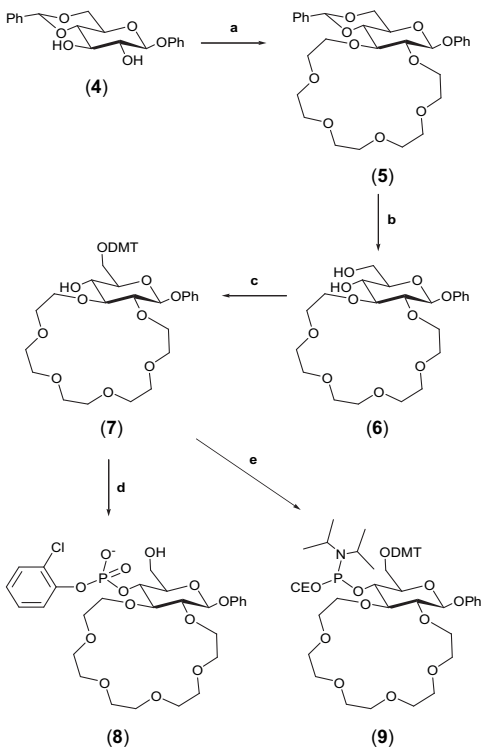
Figure 3. Chemical structure of macrocycle **3**.

Under the assumption that the binding abilities of the crown ether residues in derivative **3** are preserved, if two monopositive ions are bound, this compound results in an electrically neutral, internal salt, which could be spatially organized into a peculiar molecular architecture.

2. Results and discussions

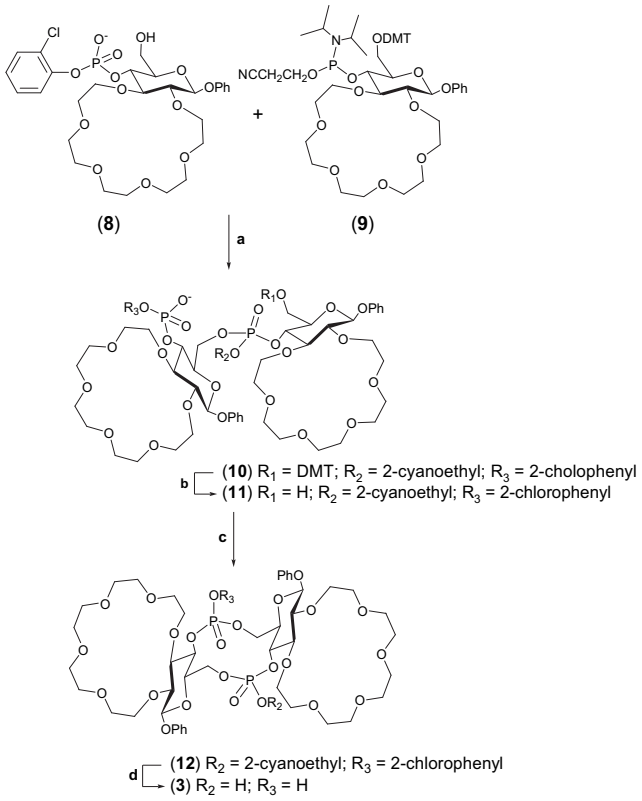
2.1. Synthesis

We reasoned that treatment of 4,6-benzylidene protected glucoside **4** [8] with a small excess of penta(ethylene glycol) ditosylate in the presence of a strong base as sodium or potassium hydride, i.e. under classical Williamson reaction conditions, would preferentially lead to the formation of the desired 18-crown-6 containing derivative. In fact, assuming the alkylation on the 2-OH group as the first event, the close proximity of the 3-OH associated with the template effect of the metal ion (Na^+ or K^+) should strongly favour the cyclization over undesired dimerization events.[11] Indeed, by reacting **4** with NaH in anhydrous THF for 30 min, then adding pentaethylene glycol ditosylate and, after additional 30 min, diluting the reaction system, the sole target compound **5** was obtained in very satisfactory yields (92% after chromatography). This building block was then elaborated to give the desired 4-phosphorylated and 4-phosphoramidite derivatives **8** and **9**, respectively, following the reactions described in Scheme 1.



Scheme 1. a) Pentaethylene glycol ditosylate, NaH, THF, reflux, 12 h, 92% yield; b) 10% TFA in CH_2Cl_2, 0 °C, 4 h, 99% yield; c) DMTCl, pyridine, rt, 12 h, 98% yield; d) 2-chlorophenyldichlorophosphate, 1,2,4-triazole, TEA, pyridine, rt, 3 h, followed by chromatographic purification on a column eluted with 0.1% TFA in a CH_2Cl_2/MeOH mixture, 92% yield; e) 2-*O*-cyanoethyl-*N*,*N*-diisopropylaminochlorophosphoramidite, DIPEA, CH_2Cl_2, r. t., 2 h, 96% yield.

This synthetic strategy essentially follows the route adopted for the preparation of CyPLOS derivatives **1** and **2a**–**c**.[8,9] Treatment of **5** with 10% TFA in CH_2Cl_2 at 0 °C for 4 h allowed the complete removal of the benzylidene protecting group, leading to **6** isolated in almost quantitative yields. Successive reaction of diol **6** with DMTCl in pyridine gave compound **7**, recovered in 98% yield after column chromatography. Phosphorylation of **7**, by treatment with 2-chlorophenyl-dichlorophosphate in the presence of 1,2,4-triazole and TEA in pyridine, followed by acidic work-up of the reaction mixture, gave, in a one-pot reaction, detritylated compound **8** in 92% yields for two steps. 4-Phosphoramidite derivative **9** was obtained in 96% yield, essentially following protocols well established in the elaboration of building blocks for oligonucleotide synthesis.[12] Linear dimer **10** was obtained through a phosphoramidite procedure, as described in Scheme 2, by coupling **8** and **9** in the presence of 0.25 M DCI in CH_3CN, followed by oxidation with 5.5 M *tert*-butylhydroperoxide (*t*-BuOOH) in *n*-decane. Upon DMT removal, linear dimer **11** was isolated as a stable compound, after column chromatography, in 50% overall yield for three steps.



Scheme 2. a) 0.25 M DCI in CH_3CN, rt, 2 h., then 5.5 M *t*-BuOOH in decane, rt, 30 min, 50% yield; b) 1% TCA in CH_2Cl_2 (v/v), rt, 30 min, 99% yield; c) MSNT, DMAP, pyridine, rt, 12 h, 83% yield; d) piperidine, 60 °C, 12 h, then aq ammonia, 60 °C, 12 h, 70% yield.

Cyclization was then achieved by exploiting a phosphotriester methodology,[13] well optimized both in solution and in the solid phase for the synthesis of cyclic oligonucleotides.[14] Using 1-mesitylensulfonyl-3-nitro-1,2,4-triazole (MSNT) as the condensing agent in pyridine in association with DMAP, under high dilution conditions (10^{-3} M), fully protected cyclic compound **12** was obtained in 83% yield. Final deprotection of the macrocycle was achieved in two steps, involving, first, a basic treatment with anhydrous piperidine (60 °C, 12 h) for the removal of the 2-cyanoethyl protecting group through β-elimination, followed by a basic hydrolytic treatment with aq ammonia at 60 °C to cleave the 2-chlorophenyl group. Final purification was carried out by gel filtration chromatography on a G25 Sephadex column eluted with H_2O/EtOH 4:1 (v/v), furnishing the pure target compound in 70% yield for the two deprotection steps. Following the described procedures, **3** was prepared in 11 steps and 23% overall yield from 4,6-protected phenyl-β-D-glucopyranoside **4**.

All the synthesized compounds were purified by column chromatography, in all cases allowing to isolate homogeneous compounds, and characterized by ^1H, ^{13}C (and ^{31}P, where present) NMR spectroscopy and mass analysis.

2.2. NMR analysis and structural studies

The final cyclic dimer compound proved to be a very hydrophilic tool, highly soluble in water and, to a lesser extent, in methanol. In contrast to our original expectations, **3** could not be investigated in transmembrane ion transport assays, since, differently from related analogue **2b**, it did not permeate lipid systems at all. Due to this marked hydrophilicity, the binding properties of host **3** towards cations could not be investigated through classical alkali metal picrate extraction experiments from water to chloroform. Therefore, a detailed NMR spectroscopic analysis was undertaken, in order to elucidate the conformational features of the synthesized compound, also in comparison with parent macrocycle **1**, and to determine its binding properties through ^1H NMR-based titrations.

^1H NMR spectra of **3** were recorded in water in the concentration range 0.1–2.0 mM. Upon varying the concentration, no significant change was observed in the shape or distribution of the ^1H NMR resonances, thus allowing to exclude aggregation phenomena under these conditions. As a completely deprotected macrocycle, it shows a high degree of internal symmetry, as expected for a flexible C_2-symmetric dimer. The ^1H NMR spectra show the two glucoside monomers as magnetically equivalent, with a unique signal present for each type of nucleus (Fig. 4). In fact, the 1D proton spectrum of **3** (700 MHz, D_2O, T=298 K) shows the presence of only one anomeric signal at δ_H 5.21 ppm. Additional four non overlapped signals are clearly discernible in the region between 3.7 and 4.3 ppm, successively assigned to H6$_a$ (δ_H 4.18), H4 (δ_H 4.03), H5 (δ_H 3.82), H3 (δ_H 3.70) of the sugar rings.

The signals in the aromatic region at δ_H 7.29, 7.05 and 7.03 ppm account for the phenyl protons. A severe overlapping of signals was observed at δ_H 3.6 ppm, due to the methylene groups of the crown ether moieties. In addition, a unique signal is apparent for the two phosphorus nuclei in the ^{31}P NMR spectra. Proton (700 MHz, T=298 K) and carbon (175 MHz, T=298 K) assignments were obtained through an in-depth analysis of two-dimensional PE-COSY, TOCSY, HSQC, and HMBC NMR experiments. Furthermore, 2D ROESY experiment allowed confirmation of the assignments and gave us

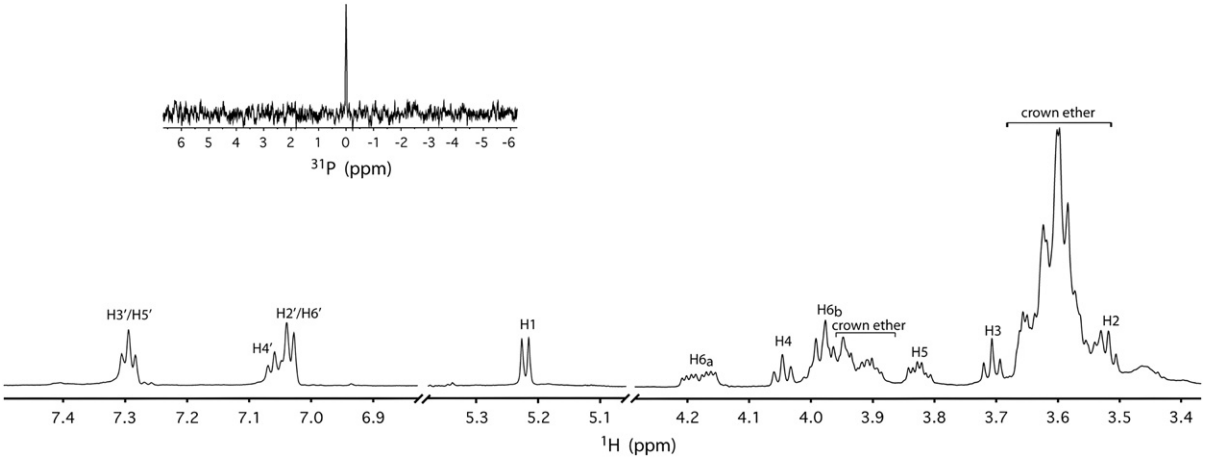


Figure 4. Selected regions of the ^1H NMR (700 MHz, D_2O) and–in the inset–of the ^{31}P NMR (161.98 MHz, D_2O) spectrum of **3**.

structural information as well. Essentially all ROEs are between 1,3-diaxial protons within the sugar, thus suggesting that the β-D-glucopyranoside moieties adopt a classical $^4C_1$ chair conformation and are not distorted. This observation is also confirmed by the analysis of the proton coupling constants (9 Hz) measured for the sugar ring protons (H1, H2, H3, H4, and H5). Relevant structural information relative to the central 12-membered ring could be derived from the analysis of the multiplicity and coupling constants of the H4 and $H6_a$ (for their couplings with P) and H5 (for its couplings with $H6_a$ and $H6_b$) resonances. $H6_a$ resonates as a double-double-doublet, characterized by coupling constants of 22, 12 and 5 Hz. The first coupling constant (22 Hz) is due to the coupling with the phosphorus, the coupling constant of 12 Hz is indeed generated by geminal coupling with $H6_b$, and the smallest coupling constant is relative to the coupling with H5. The signal attributed to H5 appears as a double triplet and exhibits a coupling constant of 5 Hz (due to the coupling with $H6_a$), and a coupling constant of 9 Hz (generating the triplet), due to the coupling with H4 and $H6_b$. This suggests that both the dihedral angles H5-C5-C6-$H6_b$ and H5-C5-C4-H4 are close to either 0° or 180°. Analogously, the dihedral angle $H6_a$-C6-O6-P is very close to 180° ($^3J_{HP}$=22 Hz), while no information could be retrieved for the dihedral angle $H6_b$-C6-O6-P. Differently from what observed for **1**, H4 is characterized by a triplet multiplicity, due to the scalar couplings (9 Hz) with H3 and H5. This means that no coupling is present with phosphorus and that the dihedral angle H4-C4-*O*-P is close to 90° (or 270°). Three-dimensional structures of **3**, satisfying all the ROEs and dihedral angle constraints ($^3J_{HH}$ and $^3J_{HP}$), were built by restrained simulated annealing calculations. An initial structure of **3** was generated and minimized to eliminate any possible conformational bias. Restrained simulations were carried out in vacuo for 230 ps. The dynamics started at 1000 K using the consistent valence force field, as implemented in the Discover software (Accelrys, San Diego, USA). Thereafter, the temperature was decreased stepwise to 250 K. The final step was to energy-minimize and refine the structures obtained by using the steepest descent and the quasi-Newton-Raphson (VA09A) algorithms. Out of 500 structures generated, 50 structures with the lowest energies were selected. An excellent superimposition of the 50 structures with root mean square deviation (RMSD) values of 0.18±0.08 for all heavy atoms could be obtained. The lack of violations and the low RMSD value suggest that the minimized conformation of **3**, reported in Figure 5, is consistent with the experimentally determined restraints. As expected, the three-dimensional structure obtained for **3** is very different from the one previously derived for **1** (see Fig. 5). In fact, macrocycle **3** adopts an arched structure, in which the two phosphate functionalities point out towards the crown ether groups forming, with the crown ethers themselves, a pocket capable to comfortably host cations.

In order to test the complexing abilities of compound **3**, a series of ^{1}H NMR-based titrations were performed. However, when alkali metal cations–i.e., sodium, potassium, cesium or rubidium, given in the form of chlorides or perchlorates–were progressively added to the host compound solution up to a large excess, only marginal changes in the ^{1}H NMR signals of the substrate were observed in all cases, both at the level of the chemical shifts and *J* values. Very small downfield or upfield shifts should only account for the effects due to the increased ionic strength, ruling out the possibility of effective binding, as would be explained in the case of prevailing competing solvation effects. Alternatively, these results can also be interpreted assuming that sodium, incorporated during the NaH-mediated crown ether formation in compound **5**, is never lost in the successive synthetic elaborations leading to the final macrocycle, nor successively displaced by other cations. The close proximity of two negatively charged phosphate groups could indeed dramatically increase the affinity of the 18-crown-6 ether derivatives for sodium cations, which may be assumed to be tightly bound in the crown ether pockets in **3** independently from the successive addition of external cations.[15] In order to establish whether Na^+ can actually be hosted in the crown ethers and simultaneously interact with the phosphate groups present in **3**, a novel set of molecular dynamic and mechanic calculations was performed in the presence of sodium cations. These calculations were carried out by using the same constraints and procedures used for those performed in the absence of cations. In order to keep the Na^+ ions close to the crown ethers, we have introduced additional constraints into the calculations. In particular, we have used a range of distances between the oxygens of the crown ethers and the sodium ions of 2.4–5.0 Å. The width of this range, if necessary, guarantees the Na^+ ions to freely move towards the negatively charged phosphate groups, and, at the same time, to remain in proximity of the crown ethers. The calculations show structures which are almost superimposable to the ones obtained in the absence of ions. Interestingly, the ions are well accommodated within the two crown ether residues and, as a matter of fact, they are very close (2.7 Å) to the phosphates (see Fig. 6).

These findings suggest that the studied molecule is indeed well pre-organized for hosting cations and, particularly, can be realistically complexed with Na^+. Definitive confirmation of the presence of bound cations in **3** was expected from X-ray crystallography studies, but all the attempts carried out so far to obtain good quality crystals have been unsuccessful.

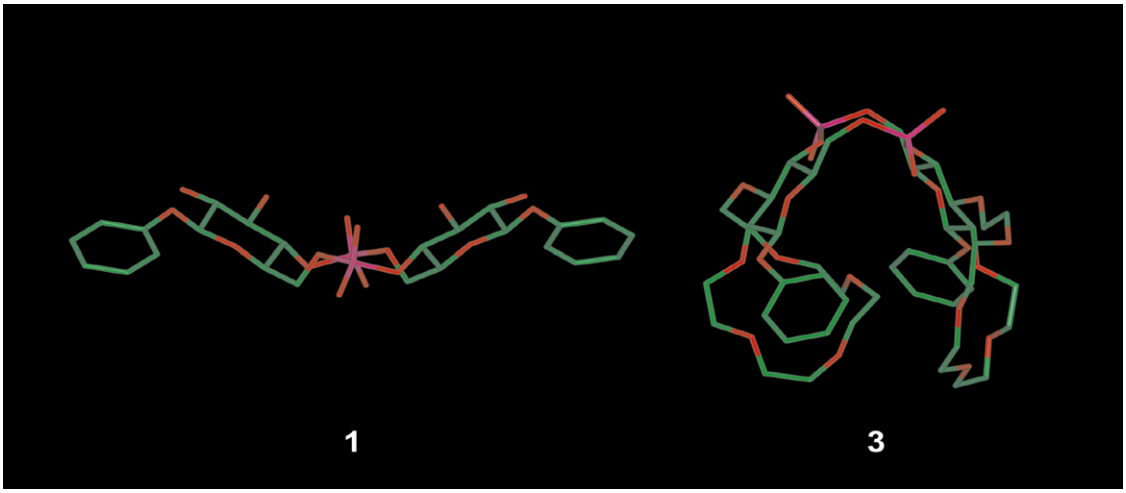


Figure 5. Minimized conformations for compounds **1** (Ref. 8) and **3**.

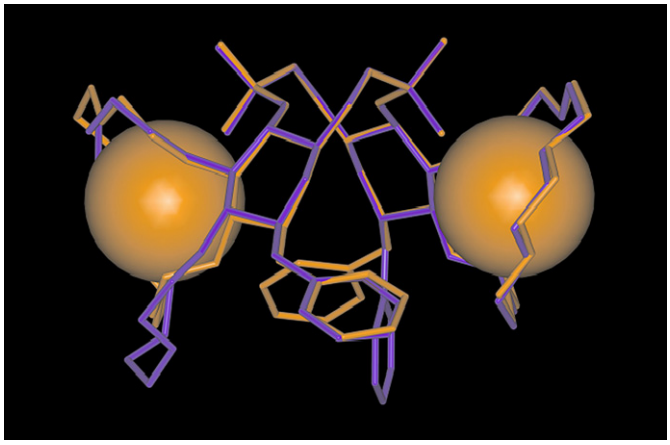


Figure 6. Superimposition of the structures obtained for **3** with (yellow) and without (magenta) sodium ions.

Circular dichroism analysis is consistent with the NMR data for **3**; CD spectra of substrate **3** dissolved in water in the absence and in the presence of 2 equiv. NaCl were found to be absolutely superimposable, showing no evident conformational difference due to a prolonged contact of **3** with added sodium cations (see Supplementary data).

Experiments are in progress to investigate the antimicrobial, antifungal and antitumoral profile of this novel macrocycle.

3. Conclusions

A novel C_2-symmetric basket-shaped macrocycle with a central phosphate-linked disaccharide skeleton, decorated with two 18-crown-6 ether moieties (*crown-CyPLOS*), has been prepared in 11 steps with 23% yields. Conformational preferences in solution of the bis-crown ether-fused compound have been investigated by a detailed NMR analysis, suggesting that the supramolecular capacities of the crown ethers into this macrocycle can be dramatically enhanced by the close interaction with negatively charged phosphate groups, presumably leading to a stable, internal salt, tightly binding monopositive ions. Such a circumstance is indeed detrimental for ionophore activity, where loose binding is required for effective ion exchange in different media. Even though the synthesized macrocycle did not prove to be a useful candidate for transmembrane ion transport experiments, these data are useful to guide future studies, addressed to the synthesis of related polycyclic analogues, finely tuning the saccharide decorations so to obtain more effective ionophores and biologically active compounds.

4. Experimental section

4.1. Materials and methods

TLC analyses were carried out on silica gel plates from Merck (60, F254). Reaction products on TLC plates were visualized by UV light and then by treatment with a 10% $Ce(SO_4)_2/H_2SO_4$ aqueous solution. For column chromatography, silica gel from Merck (Kieselgel 40, 0.063–0.200 mm) was used. HPLC analyses were performed on a Beckman System Gold instrument equipped with a UV detector module 166 and a Shimadzu Chromatopac C-R6A integrator. By HPLC analysis on a Nucleosil 100–5C18 Supelco analytical column (250×4.6 mm, 5 μm), eluted with a linear gradient from 0 to 100% in 30 min of CH_3CN in H_2O, flow=0.8 mL/min, detection at λ=264 nm, all the synthesized compounds were more than 98% pure. For the ESI MS analyses, a Waters Micromass ZQ instrument–equipped with an Electrospray source–was used in the positive and/or negative mode. MALDI TOF mass spectrometric analyses were performed on a PerSeptive Biosystems Voyager-De Pro MALDI mass spectrometer in the Linear mode using 2,5-dihydroxybenzoic acid as the matrix. NMR spectra for the characterization of compounds **5**–**12** were recorded on Bruker WM-400 or Varian Inova 500 spectrometers. All the chemical shifts are expressed in ppm with respect to the residual solvent signal; *J* values are in Hz. The following abbreviations were used to explain the multiplicities: s=singlet; d=doublet; t=triplet; q=quartet; m=multiplet; dd=double doublet; ddd=double double doublet. ^{31}P NMR spectra have been registered using 85% H_3PO_4 as the external reference. UV measurements were carried out on a Jasco V-530 UV spectrophotometer equipped with a Jasco ETC-505 T temperature controller unit. Circular dichroism (CD) experiments were registered on a JASCO J-715 spectrophotometer equipped with a thermostatically controlled cuvette holder (JASCO PTC-348), in a 0.1 cm path length cuvette. CD spectra were collected from 200 to 300 nm, at 20 nm/min, with a response time of 4 s and at 1 nm bandwidth.

The following abbreviations were used throughout the text: AcOEt=ethyl acetate; *t*-BuOOH=*tert*-butylhydroperoxide; CE=2-cyanoethyl; DCI=4,5-dicyanoimidazole; DIPEA=*N*,*N*-diisopropylethylamine; DMAP=*N*,*N*-dimethyl-aminopyridine; DMT=4,4′-dimethoxytriphenylmethyl; EtOH=ethanol; *Glu*=glucose residue; MeOH=methanol; MSNT=1-mesitylensulfonyl-3-nitro-1,2,4-triazole; TCA=trichloroacetic acid; TEA=triethylamine; TFA=trifluoroacetic acid; THF=tetrahydrofuran.

4.2. Nuclear magnetic resonance

NMR spectra for compound **3** were determined in D_2O (Aldrich, Milwaukee, USA). The NMR samples were prepared at a concentration range of 0.1–2.0 mM. NMR spectra were recorded with a Varian ^{Unity}INOVA 700 MHz and 500 MHz spectrometers. Phase sensitive ROESY spectra[16] were recorded with mixing times in the range 250–500 ms (T=298 K). TOCSY spectrum[17] with mixing time of 100 ms was recorded. ROESY and TOCSY were recorded using TPPI[18] procedure for quadrature detection. {^{1}H-^{13}C}-HSQC[19] and {^{1}H-^{13}C}-HMBC[20] were optimized for $^1J_{C-H}$=135 Hz and $^{2,3}J_{C-H}$=10 Hz, respectively. In all the 2D experiments, time domain data consisted of 2048 complex points in t_2 and 400–512 fids in t_1 dimension. The relaxation delay was kept at 1.2 s for all the experiments. The spectra were calibrated relative to HDO (4.75 ppm) as the internal standard. The NMR data were processed on a SGI Octane workstation using FELIX 98 software (Accelrys, San Diego, USA) and on a iMAC using the software iNMR (www.inmr.net).

4.3. Structure calculations

Cross peak volume integrations were performed with the program FELIX 98, using the ROESY experiment collected at mixing time of 250 ms and with a relaxation delay of 3 s. The NOE volumes were then converted to distance restraints after they were calibrated using known fixed distances of $H6_a$ and $H6_b$ of the glucose subunit. Then an NOE restraint file was generated with three distance classifications as follows: strong NOEs (1.5 Å$\leq r_{ij} \leq$2.5 Å), medium NOEs (2.0 Å$\leq r_{ij} \leq$3.5 Å) and weak NOEs (3.0 Å$\leq r_{ij} \leq$5.0 Å). A total of 20 NOE-(10 for each symmetric moiety) derived distance restraints were used. Proton/proton ($^3J_{HH}$) and proton/phosphorus ($^3J_{HP}$) coupling constants were measured from 1D proton spectra and from PE-COSY spectrum. Dihedral angles were calculated using the *J*-values extracted by solving the Karplus equation[21] with coefficients A=6.98, B=−1.38, C=1.72 for $^3J_{HH}$,[22] and A=15.3, B=−6.2, C=1.5 for $^3J_{HP}$.[23] Only those angles yielding two real roots when solving the Karplus equation were used during structural calculation. Three-dimensional structures, which satisfy NOE and dihedral angle constraints were constructed by simulated annealing calculations. All the calculations used a distance dependant macroscopic dielectric constant of 80*r

and an infinite cut-off for nonbonded interactions to partially compensate for the lack of the solvent.[24] Initial structures of **3**—in the absence and in the presence of sodium ions—were built using a completely random array of atoms. Using the steepest descent followed by quasi-Newton-Raphson method (VA09A) the conformational energy was minimized. Restrained simulations were carried out in vacuo for 230 ps at 1000 K using the CVFF force field as implemented in Discover software (Accelrys, San Diego, USA). Then, the temperature was decreased stepwise until 250 K. The final step was again to energy-minimize so to refine the structures obtained, using successively the steepest descent and the quasi-Newton-Raphson (VA09A) algorithms. Both dynamic and mechanic calculations were carried out by using a 10 (kcal/mol)/Å^2 flatwell restraints. A total of 50 structures were generated. Illustrations of structures were generated using the INSIGHT II program, version '98 (Accelrys, San Diego, USA). All the calculations were performed on a SGI Octane workstation.

*4.3.1. Synthesis of derivative **5**.* Derivative **4**[8] (250 mg, 0.725 mmol) was dissolved into anhydrous THF (20 mL) and treated with NaH (104 mg, 4.35 mmol, 60% mineral oil dispersion); after 10 min, pentaethyleneglycol ditosylate (540 mg, 1.08 mmol) was added and the resulting mixture left under vigorous stirring at room temperature. After 20 min, an additional volume of anhydrous THF (12 mL) was added and the reaction left at reflux for 12 h. The reaction was quenched by addition of a few drops of MeOH, the resulting mixture was concentrated under reduced pressure, diluted with $CHCl_3$, transferred into a separatory funnel and washed twice with a satd aq NaCl solution. The organic layer was collected, taken on anhydrous sodium sulfate, filtered, concentrated under reduced pressure and then purified on a silica gel column. Elution of the column with $CHCl_3$ containing increasing amounts of MeOH (from 0% to 5%) allowed to recover pure **5** (364 mg, 0.666 mmol), in 92% yield.

*4.3.1.1. Compound **5**.* oil, R_f=0.6 ($CHCl_3$/CH_3OH, 98:2, v/v); ν_{max} (liquid film) 1490, 1452, 1354, 1234, 1059, 1028, 1012 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 7.49–7.03 (complex signals, 10H, aromatic protons), 5.55 (s, 1H, Ph-C*H*), 5.06 (d, *J*=7.5 Hz, 1H, H-1), 4.35 (dd, *J*=5.0 and 10.5 Hz, 1H, H-4), 4.09–4.00 [overlapped signals, 5H, 2x(CH_2CH_2O-*Glu*) and H-6$_a$], 3.79 (t, *J*=10.0 and 10.0 Hz, 1H, H-3), 3.73–3.58 [overlapped signals, 17H, 4x(OCH_2CH_2O) and H-6$_b$], 3.56–3.47 (overlapped signals, 2H, H-2 and H-5); δ_C (125 MHz, $CDCl_3$): 157.0, 137.1, 129.6, 129.4, 128.8, 128.1, 127.8, 125.9, 122.8 and 116.8 (aromatic carbons), 101.7 (C-1), 101.0 (Ph-*C*H), 82.3 (C-2), 81.5 and 80.8 [2x(CH_2CH_2O-*Glu*)], 72.7 and 72.6 [2x(CH_2CH_2O-*Glu*)], 70.8, 70.7, 70.6, 70.5 and 70.4 [3x(OCH_2CH_2O) and, overlapped, C-6], 68.5 (overlapped signals, C-4 and C-3), 66.0 (C-5); ESI-MS (positive ions): calcd for $C_{29}H_{38}O_{10}$, 546.25; *m*/*z*, found 568.59 (M+Na$^+$), 584.57 (M+K$^+$); HRMS (MALDI-TOF, positive ions): *m*/*z*: calcd for $C_{29}H_{38}O_{10}Na$: 569.2363; found 569.2390 (M+Na$^+$).

*4.3.2. Synthesis of derivative **6**.* Compound **5** (352 mg, 0.645 mmol) was treated with a 10% TFA solution in CH_2Cl_2 (v/v, 10 mL total volume) under stirring at 0 °C for 4 h. The reaction was then quenched by addition of a satd aq $NaHCO_3$ solution, the resulting mixture diluted with $CHCl_3$, transferred into a separatory funnel and washed twice with a satd aq NaCl solution. The organic layer was collected, taken on anhydrous sodium sulfate, filtered, concentrated under reduced pressure and the residue purified on a silica gel column. Elution of the column with $CHCl_3$ containing increasing amounts of MeOH (from 0% to 10%) allowed to obtain pure **6** (295 mg, 0.645 mmol) in an almost quantitative yield.

*4.3.2.1. Compound **6**.* Oil, R_f=0.3 ($CHCl_3$/CH_3OH, 95:5, v/v); ν_{max} (liquid film) 3398 (br), 1591, 1493, 1452, 1348, 1230, 1066, 1028 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 7.29–6.99 (complex signals, 5H, aromatic protons), 4.99 (d, *J*=10.0 Hz, 1H, H-1), 4.29–4.25 [m, 1H, (CH_2CH_aO-*Glu*-C-2)], 4.06–3.83 [overlapped signals, 4H, H-6$_a$, (CH_2CH_2O-*Glu*-C-3) and (CH_2CH_bO-*Glu*-C-2)], 3.79–3.60 [overlapped signals, 18H, 4x(OCH_2CH_2O), H-4 and H-6$_b$], 3.48 [overlapped signals, 2H, H-2 and H-5], 3.40 (m, 1H, H-3); δ_C (125 MHz, $CDCl_3$): 157.0 129.5, 122.5 and 116.3 (aromatic carbons), 101.2 (C-1), 84.6 (C-3), 82.1 (C-2), 75.3 (C-5), 72.8 (CH_2CH_2O-*Glu*-C-2), 71.7, 71.5 and 71.4 [(CH_2CH_2O-*Glu*-C-3), 2x(CH_2CH_2O-*Glu*)], 70.6, 70.5, 70.3, 70.1 and 70.0 [3x(OCH_2CH_2O) and, overlapped, C-4], 62.6 (C-6); ESI-MS (positive ions): calcd for $C_{22}H_{34}O_{10}$, 458.21; *m*/*z*, found 480.59 (M+Na$^+$), 496.56 (M+K$^+$); HRMS (MALDI-TOF, positive ions): *m*/*z*: calcd for $C_{22}H_{34}O_{10}Na$: 481.2050; found 481.2060 (M+Na$^+$).

*4.3.3. Synthesis of derivative **7**.* Compound **6** (295 mg, 0.645 mmol), dissolved in anhydrous pyridine (3.0 mL), was treated with 4,4′-dimethoxytriphenylmethylchloride (DMTCl) (283 mg, 0.839 mmol) for 12 h at room temperature. The reaction mixture was then concentrated under reduced pressure and purified on a silica gel column. Elution with CH_2Cl_2, added with 1% pyridine (v/v), containing increasing amounts of MeOH (up to 5%) allowed to recover pure **7** (480 mg, 0.632 mmol) in 98% yield.

*4.3.3.1. Compound **7**.* Yellow oil, R_f=0.8 (CH_2Cl_2/CH_3OH, 95:5, v/v); ν_{max} (liquid film) 3390 (br), 1056, 1037, 1006 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 7.45–6.75 (complex signals, 18H, aromatic protons), 4.95 (d, *J*=8.0 Hz, 1H, H-1), 4.27–4.22 [m, 1H, (CH_2CH_aO-*Glu*-C-2)], 4.12–4.08 [m, 1H, (CH_2CH_aO-*Glu*-C-3)], 4.02–3.98 [m, 1H, (CH_aCH_2O-*Glu*-C-3)], 3.93–3.88 [m, 1H, (CH_aCH_2O-*Glu*-C-2)], 3.77 (s, 6H, 2×OCH_3 of DMT group), 3.73–3.62 [overlapped signals, 16H, 4x(OCH_2CH_2O)], 3.58 (apparent t, *J*=10.0 and 8.5 Hz, 1H, H-4), 3.53–3.48 [overlapped signals, 2H, H-2 and H-5], 3.45–3.42 (m, 1H, H-6$_a$), 3.37–3.31 (overlapped signals, 2H, H-3 and H-6$_b$); δ_C (125 MHz, $CDCl_3$): 158.3, 157.2, 144.8, 135.9, 130.0, 129.3, 128.1, 127.7, 126.6, 122.4, 116.8 and 113.0 (aromatic carbons), 101.3 (C-1), 86.1 (quaternary C of DMT group), 84.9 (C-3), 82.2 (C-2), 74.5 (C-5), 72.9 (CH_2CH_2O-*Glu*-C-2), 71.8, 71.3 and 71.2 [(CH_2CH_2O-*Glu*-C-3) and 2x(CH_2CH_2O-*Glu*)], 70.9, 70.8, 70.6, 70.5, 70.4 and 70.2 [2x(OCH_2CH_2O) and, overlapped, C-4], 63.6 (C-6), 55.1 [2x(OCH_3 of DMT group)]; ESI-MS (positive ions): calcd for $C_{43}H_{52}O_{12}$, 760.35; *m*/*z*, found 782.16 (M+Na$^+$); HRMS (MALDI-TOF, positive ions): *m*/*z*: calcd for $C_{43}H_{52}O_{12}Na$: 783.3356; found 783.3370 (M+Na$^+$).

*4.3.4. Synthesis of derivative **8**.* To compound **7** (152 mg, 0.201 mmol), dissolved in anhydrous pyridine (2.0 mL), 1,2,4-triazole (111 mg, 1.61 mmol), previously dried in vacuo, was added. To the solution taken at 0 °C, TEA (225 μL, 1.61 mmol) and 2-chlorophenyldichlorophoshate (130 μL, 0.804 mmol) were sequentially added. The reaction mixture was left under stirring at room temperature for 3 h, then the solution was concentrated under reduced pressure, the resulting mixture redissolved in $CHCl_3$, transferred into a separatory funnel and washed twice with a satd aq NaCl solution. The organic layer was collected, taken on anhydrous sodium sulfate, filtered, concentrated under reduced pressure and the residue purified on a silica gel column. Elution of the column with CH_2Cl_2, added with 0.1% of TFA (v/v), containing increasing amounts of MeOH (up to 10% in volume) allowed to isolate pure **8** (120 mg, 0.185 mmol) in 92% yield.

*4.3.4.1. Compound **8**.* Yellow oil, R_f=0.3 (CH_2Cl_2/CH_3OH, 95:5, v/v); ν_{max} (liquid film) 3322 (br), 1642, 1480, 1227, 1059, 1028, 1012 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 7.71–6.85 (complex signals, 9H, aromatic protons), 4.96 (broad signal, 1H, H-1), 4.64 (m, 1H, H-4), 4.10–3.36 [overlapped signals, 25H, H_2-6, H-2, H-5, 5x(OCH_2CH_2O) and H-3]; δ_C (125 MHz, $CDCl_3$): 162.3, 157.2, 149.3, 129.3, 127.6, 124.9, 123.2, 122.3 and 116.5 (aromatic carbons), 101.4 (C-1), 83.1 (C-3), 79.6 (C-2), 75.9 (C-5), 70.9, 70.7, 70.5, 69.8, 69.3, 68.9 and 68.8

[5x(OCH_2CH_2O) and C-4], 60.3 (C-6); δ_P (161.98 MHz, $CDCl_3$): −3.1; ESI-MS (negative ions): calcd for $C_{28}H_{38}ClO_{13}P$, 648.17; *m*/*z*, found 646.85 $(M-H)^-$. HRMS (MALDI-TOF, negative ions): *m*/*z*: calcd for $C_{28}H_{37}ClO_{13}P$: 647.1660; found 647.1690.

4.3.5. Synthesis of derivative ***9***. Compound **7** (175 mg, 0.231 mmol) was dissolved in anhydrous CH_2Cl_2 (2.0 mL) and left in contact with activated 3 Å molecular sieves. DIPEA (320 μL, 1.85 mmol) and 2-*O*-cyanoethyl-*N*,*N*-diisopropylaminochlorophosphoramidite (205 μL, 0.93 mmol) were sequentially added dropwise under anhydrous nitrogen atmosphere. After 2 h at room temperature the reaction mixture was concentrated under reduced pressure and the resulting residue purified on a silica gel column. Elution of the column with AcOEt/*n*-hexane 7:3 (v/v), added with 1% TEA, containing increasing volumes of AcOEt (up to 100%) allowed to isolate pure **9** (213 mg, 0.221 mmol) in 96% yield.

4.3.5.1. Compound ***9***. Oil, mixture of diastereomers, R_f=0.8 (CH_2Cl_2/CH_3OH, 95:5, v/v); ν_{max} (liquid film) 1066, 1037, 1012 cm^{-1}; δ_H (400 MHz, $CDCl_3$): 7.45–6.70 (complex signals, 36H, aromatic protons), 4.99 [m, 2H, 2x(H-1)], 4.20–3.87 [overlapped signals, 8H, 2x(CH_2CH_2O-*Glu*-C-2) and 2x(CH_2CH_2O-*Glu*-C-3)], 3.75 [s, 12H, 4x(OCH_3 of DMT group)], 3.80–3.46 [overlapped signals, 42H, 2x(H-4), 8x(OCH_2CH_2O), 2x(OCH_2CH_2CN) and 2x(H_2-6)], 3.43–3.20 {overlapped signals, 10H, 2x(H-3), 2x(H-2), 2x(H-5) and 2×N[$CH(CH_3)_2]_2$}, 2.75–2.55 [m, 4H, 2x(OCH_2CH_2CN)], 1.32–0.84 {overlapped signals, 24H, 2×N[$CH(CH_3)_2]_2$}; δ_C (100 MHz, $CDCl_3$): 158.2, 157.2, 157.1, 145.0, 144.9, 136.2, 136.1, 136.0, 135.9, 130.1, 130.0, 129.3, 128.2, 128.1, 127.5, 126.5, 122.3, 116.7, 116.6 and 112.8 (aromatic carbons), 117.6 and 117.2 (CN), 101.0 and 100.9 (C-1), 85.8 and 85.7 (quaternary C of DMT group), 84.8 and 84.1 (C-3), 82.5 and 82.4 (C-2), 75.3 and 75.2 (C-5), 72.6, 72.5, 72.3 and 72.2 [2x(CH_2CH_2O-*Glu*-C-2) and 2x(CH_2CH_2O-*Glu*-C-3)], 70.8, 70.7, 70.6, 70.5, 70.5 and 70.4 [8x(OCH_2CH_2O)], 70.2 [2x(C-4)], 63.6 and 63.2 (C-6), 58.4 and 58.2 (OCH_2CH_2CN), 55.0 [4x(OCH_3 of DMT group)], 42.9 and 42.7 {N[$CH(CH_3)_2]_2$}, 24.3 and 24.2 {N[$CH(CH_3)_2]_2$}, 22.8 and 22.7 (CH_2CH_2CN); δ_P (161.98 MHz, $CDCl_3$): 151.1 and 150.4; ESI-MS (positive ions): calcd for $C_{52}H_{69}N_2O_{13}P$, 960.45; *m*/*z*, found 983.19 ($M+Na^+$). HRMS (MALDI-TOF, positive ions): *m*/*z*: calcd for $C_{52}H_{69}N_2O_{13}PNa$: 983.4435; found 983.4398 ($M+Na^+$).

4.3.6. Synthesis of linear dimer ***11***. Compound **8** (120 mg, 0.185 mmol) and compound **9** (213 mg, 0.222 mmol), coevaporated several times with anhydrous CH_3CN, were reacted with a 0.25 M DCI solution (6.0 mL) in CH_3CN, previously left in contact with activated 3 Å molecular sieves. The resulting mixture was left under stirring at room temperature for 2 h, then a 5.5 M *t*-BuOOH solution (425 μL) in *n*-decane was added. After 30 min the reaction mixture was concentrated under reduced pressure and the resulting residue redissolved in CH_2Cl_2, transferred into a separatory funnel and washed twice with a satd aq NaCl solution. The organic layer was collected, taken on anhydrous sodium sulfate, filtered, concentrated under reduced pressure and the residue purified on a silica gel column. Elution of the column with CH_2Cl_2, added with 1% pyridine (v/v), containing increasing amounts of MeOH (from 0% to 5%) allowed to obtain the pure DMT-protected linear dimer **10** (140 mg, 0.092 mmol) in 50% yield for the two steps. This was then treated with a 1% TCA solution (4 mL) in CH_2Cl_2 (v/v) for 30 min at room temperature. The reaction mixture was concentrated under reduced pressure and the resulting residue purified on a silica gel column eluted with CH_2Cl_2 containing increasing volumes of MeOH (from 2% up to 10%). Pure compound **11** (113 mg, 0.092 mmol) could be isolated in an almost quantitative yield.

4.3.6.1. Compound ***11***. Yellow oil, R_f=0.3 (CH_2Cl_2/CH_3OH, 95:5, v/v); ν_{max} (liquid film) 3402 (br), 1582, 1474, 1351, 1237, 1053 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 7.77–6.98 (overlapped signals, 14H, aromatic protons), 4.85 [broad signal, 2H, 2x(H-1)], 4.65–3.32 [overlapped signals, 48H, 2x(CH_2CH_2O-*Glu*-C-2) and 2x(CH_2CH_2O-*Glu*-C-3), 2x(H-4); 2x(H_2-6), 8x(OCH_2CH_2O) and (OCH_2CH_2CN)], 3.07–2.50 [overlapped signals, 8H, 2x(H-2), 2x(H-5), 2x(H-3) and (OCH_2CH_2CN)]; δ_C (125 MHz, $CDCl_3$): 156.9, 156.8, 149.8, 135.7, 129.5, 127.4, 123.6, 122.8 and 116.9 (aromatic carbons), 117.3 (CN), 101.8 and 101.6 (C-1), broad signal centered at 81.8 [2x(C-3) and 2x(C-2)], broad signal centered at 70.4 [10x(OCH_2CH_2O), 2x(C-5) and 2x(C-4)], 65.3 (C-6 adjacent to the phosphate), 62.0 (C-6 adjacent to OH), 60.8 (OCH_2CH_2CN), 25.6 (OCH_2CH_2CN); δ_P (161.98 MHz, $CDCl_3$): overlapped, broad signals, extended from +7.6 to −4.4; MALDI-MS (positive ions): calcd for $C_{53}H_{73}ClNO_{25}P_2$, 1220.36; *m*/*z*, found 1243.67 ($M+Na^+$); HRMS (MALDI-TOF, positive ions): *m*/*z*: calcd for $C_{53}H_{73}ClNO_{25}P_2Na$: 1243.3533; found 1243.3587 ($M+Na^+$).

4.3.7. Synthesis of derivative ***12***. Compound **11** (30 mg, 0.024 mmol) was dissolved in anhydrous pyridine (25 mL) and then reacted with DMAP (3.0 mg, 0.020 mmol) and MSNT (218 mg, 0.74 mmol). The reaction mixture was left at room temperature under stirring for 12 h, then concentrated under reduced pressure and the resulting residue redissolved in CH_2Cl_2, transferred into a separatory funnel and washed twice with a satd aq NaCl solution. The organic layer was collected, taken up in anhydrous sodium sulfate, filtered, concentrated under reduced pressure and the residue purified on a silica gel column. Elution of the column with CH_2Cl_2 containing increasing volumes of MeOH (from 0 to 15%) allowed to isolate pure **12** (24 mg, 0.020 mmol) in 83% yield.

4.3.7.1. Compound ***12***. Yellow oil, mixture of diastereomers, R_f=0.5 (CH_2Cl_2/CH_3OH, 95:5, v/v); ν_{max} (liquid film) 1563, 1518, 1471, 1420, 1379, 1310, 1221, 1177, 1094, 1012 cm^{-1}; δ_H (400 MHz, CD_3OD): 7.49–6.87 (complex signals, 28H, aromatic protons), 5.23–5.08 [overlapped signals, 4H, 4x(H-1)], 4.59–4.34 [overlapped signals, 12H, 4x(CH_2CH_2O-*Glu*-C-2) and 2x(OCH_2CH_2CN)], 4.18–3.40 [overlapped signals, 96H, 4x(CH_2CH_2O-Glu-C-3), 4x(H-3), 4x(H-4), 16x(OCH_2CH_2O), 4x(H_2-6), 4x(H-2) and 4x(H-5), 2.78 [m, 4H, 2x(OCH_2CH_2CN)]; δ_C (100 MHz, CD_3OD): 165.1, 158.7, 147.2, 141.1, 140.8, 138.7, 133.3, 132.2, 131.5, 131.1, 129.7, 128.4, 123.8, 123.2, 124.5, 118.4, 118.2 and 116.9 (aromatic carbons), 117.1 (CN), 101.9 and 101.4 (C-1), 83.0, 81.8, 80.0, 79.7 and 79.3 (C-3 and C-2), 76.9 and 76.7 (C-5), 74.0, 73.5, 71.9 [20x(OCH_2CH_2O) and C-4], 69.9, 68.4, 68.0 and 67.5 (C-6), 64.9 [2x(OCH_2CH_2CN)], 31.1 [2x(OCH_2CH_2CN)]; δ_P (161.98 MHz, $CDCl_3$): -1.25, -3.10, -3.59 and −5.70; MALDI-MS (positive ions): calcd for $C_{53}H_{72}ClNO_{24}P_2$, 1203.36; *m*/*z*, found 1226.21 ($M+Na^+$), 1242.37 ($M+K^+$); HRMS (MALDI-TOF, positive ions): *m*/*z*: calcd for $C_{53}H_{72}ClNO_{24}P_2Na$: 1226.3506; found 1226.3459 ($M+Na^+$).

4.3.8. Synthesis of ***3***. Derivative **12** (24 mg, 0.020 mmol) was dissolved in anhydrous piperidine (500 μL) and left under stirring for 12 h at 60 °C. After TLC monitoring confirmed the total disappearance of the starting compound, the reaction mixture was concentrated under reduced pressure, redissolved in aq ammonium hydroxide solution (500 μL) and left under stirring for 12 h at 60 °C. The reaction mixture was concentrated under reduced pressure, redissolved in H_2O and purified on a Sephadex G25 column, eluted with H_2O/EtOH, 4:1 (v/v). From UV spectrophotometric measurements, fractions absorbing at λ=264 nm were collected and taken to dryness, yielding pure **3** (15 mg, 0.014 mmol) with 70% yield for the two steps.

4.3.8.1. Compound ***3***. Colourless oil. R_f=0.3 (CH_2Cl_2/CH_3OH, 85:15, v/v); ν_{max} (liquid film) 1553, 1492, 1454, 1356, 1097, 1068, 1015 cm^{-1}; δ_H (700 MHz, D_2O): 7.32–7.02 (complex signals, 10H, aromatic protons), 5.21 [d, *J*=9.0 Hz, 2H, 2x(H-1)], 4.18 [ddd, 2H, 2x($H\text{-}6_a$)], 4.03 [t, *J*=9.0 and 9.0 Hz, 2H, 2x(H-4)], 3.98–3.85 [overlapped signals, 10H, 2x(CH_2CH_2O-*Glu*-C-2), 2x(CH_2CH_2O-*Glu*-C-3 and 2x($H\text{-}6_b$)), 3.82 [m, 2H, 2x(H-5)], 3.70 [t, *J*=9.0 and 9.0 Hz, 2H,

2x(H-3)], 3.67–3.55 [overlapped signals, 32H, 8x(OCH_2CH_2O)], 3.51 [m, 2H, 2x(H-2)]; δ_C (175 MHz, D_2O): 158.8, 132.7, 126.4 and 119.3 (aromatic carbons), 103.3 (C-1), 83.5 (C-3), 83.4 (C-2), 80.4 (C-4), 74.5, 74.4 and 72.8 [10x(OCH_2CH_2O)], 69.9 (C-5), 69.0 (C-6); δ_P (161.98 MHz, D_2O): 0.08; ESI-MS (negative ions): calcd for $C_{44}H_{66}O_{24}P_2$, 1040.34; *m/z*, found 1038.79 $(M-H)^-$; 517.82 $(M-2H)^{2-}$; HRMS (MALDI-TOF, negative ions): *m/z*: calcd for $C_{44}H_{65}O_{24}P_2$: 1039.3341; found 1039.3293.

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tet.2009.09.100.

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15. To further corroborate this hypothesis, which would well explain the irresponsiveness of **3** towards the addition of metal cations, two kinds of experiments were carried out. The first one involved the treatment of **3** with competing binders as EDTA, which was left in contact with the bis-crown derivative for 72 h; however no apparent change was observed in the ^1H NMR resonances in **3** as such, nor when successively left in contact with metal cations. Similar results were obtained treating **3** with strong cation exchange resins, as Chelex-100. In a second set of experiments, the apolar, fully protected macrocycle **12** was filtered through basic alumina, reported also by other authors (*cf.r.* ref. 6b and references cited therein) as a standard chromatographic procedure in crown ether chemistry for cations removal. Indeed **12**, originally only soluble in methanol, after filtration on alumina was converted into a chloroform soluble compound, with a higher R_f, still having the same MS features. However, if it could be reasonably concluded that this treatment was effective in removing bound cations from the crown ether pockets in **12**, no different behaviour was then observed when analyzing the resulting **3**. It is to be noted, however, that **3** is derived from **12** through two deprotection steps, with the latter one carried out in aq ammonia, requiring for the purification a prolonged contact with aq solutions. So, even if efficiently removed from **12**, Na^+ or K^+ ions could be successively extracted from aq solutions and/or from glassware, so to give a stable complex of **3**, which is then obviously unreactive towards additional metal ions.
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