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# Cyclic phosphate-linked oligosaccharides (CyPLOS): Novel carbohydrate-based synthetic ion transporters*

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*Abstract*: Artificial ion transporters are synthetic molecules mimicking at a functional level the activity of naturally occurring ion channels or carriers. In the frame of cyclodextrin mimicry, we recently described the synthesis and conformational properties of new carbohydrate-based macrocycles having the glucoside units connected through 4,6-linked phosphodiester linkages, named CyPLOS (cyclic phosphate-linked oligosaccharides). The cyclic dimer was then adopted as a versatile synthetic platform to obtain a variety of analogs, carrying long linear alkyl or polyether chains. Diverse, jellyfish-shaped amphiphilic CyPLOS were thus obtained, with the compound carrying four tetraethylene glycol (TEG) tentacles acting as good ion transporter through lipid bilayers. A fine tuning of the properties and complexation abilities of these amphiphilic analogs was realized by introducing special reporter groups at the extremities of the TEG tentacles. Through the design of an azido-TEG functionalized key intermediate, a fluorescently labeled CyPLOS derivative was synthesized, showing a markedly increased ionophore activity, with the fluorescent tag also allowing the investigation of its mechanism of action and localization within the phospholipid bilayers. Incorporation of a spin label at the CyPLOS tentacles—to provide further insight into the study of their interactions with phospholipid membranes by electron spin resonance (ESR) spectroscopy—was also profitably achieved through a postsynthetic functionalization approach.

*Keywords*: amphiphilic oligosaccharides; glycomimetics; ion transporters; macrocycles; phosphate bonds; phosphate-linked macrocycles; self-assembly; synthetic ionophores.

## INTRODUCTION

Ion transport through phospholipid bilayers is a vital function. To achieve this, nature has evolved a large variety of different systems, based either on carriers or ion channels. In a very simplified picture, a carrier may be visualized as a sort of molecular "ferryboat", able to catch an ion at one interface with water, thus allowing it to float within phospholipids, and finally releasing it at the other interface. On the other hand, an ion channel is typically a system spanning the entire double layer, permanently residing in it so to result in a sort of tunnel or pore through which ions can easily pass. Naturally occurring ionophores are typically proteins, but can be also low-molecular-weight metabolites, which—in the case of an ion channel mechanism—exert their activity when structured in complex three-dimensional

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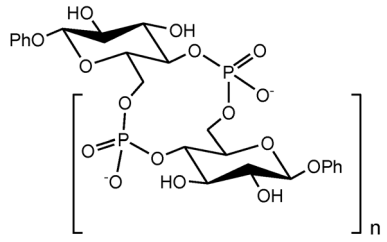
supramolecular architectures. Following Matile's definition, artificial ion transporters are synthetic molecules featuring significant structural motifs that do not occur in nature, able to mediate ion transport in lipid membranes [1]. In the last two decades, a large number of diverse artificial ionophores have been synthesized; many of these share a common design, based on a central macrocycle derivatized with long linear chains [2].

In this frame, cyclodextrins have been largely investigated as valuable starting material to produce efficient cation or anion transporters. The first example of cyclodextrin-based ion transporters was reported by Tabushi in 1982, who functionalized the lower rim of a β-cyclodextrin with four long, flexible tentacles [3]. This design was more recently reinvestigated by Gin and co-workers, who realized a very efficient anion channel by inserting seven oligobutylene glycol chains on the β-cyclodextrin core. [4] Selective modification of preformed cyclodextrins offers great synthetic opportunities but also severe chemical challenges, particularly if point mutations or backbone alterations are required [5,6]. On the other hand, the total synthesis approach, involving the stepwise synthesis of the linear oligomer followed by circularization, is a more general strategy for the preparation of modified macrocycles, giving access to a wider repertoire of novel structures. Remarkably, a wide range of diverse host molecules, with differently shaped internal cavities, have been obtained by replacement of the natural glycosidic bonds with alternative linkages, including *S*-glycosidic [7], acetylene [8], amide [9], amine [10], 1,2,3-triazole [11], or thiourea [12] groups.

Herein we report on our original contribution to this field, centered on the study of novel cyclic oligosaccharide analogs, 4,6-linked through stable phosphodiester bonds (**1–3**, Fig. 1), which we named CyPLOS as the acronym of Cyclic Phosphate-Linked OligoSaccharides [13,14]. Successive synthetic elaboration of these macrocycles led to amphiphilic CyPLOS derivatives, endowed with ionophoric activity [15–18]. Relevant features of these compounds are here concisely summarized.




**1** (n=1); **2** (n=2); **3** (n=3)

**Fig. 1** Chemical structures of CyPLOS **1–3**.

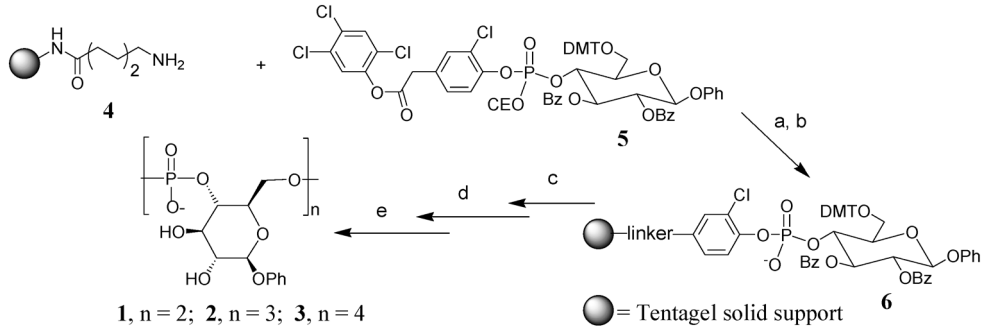
## RESULTS AND DISCUSSION

### CyPLOS: Synthesis and NMR conformational analysis studies

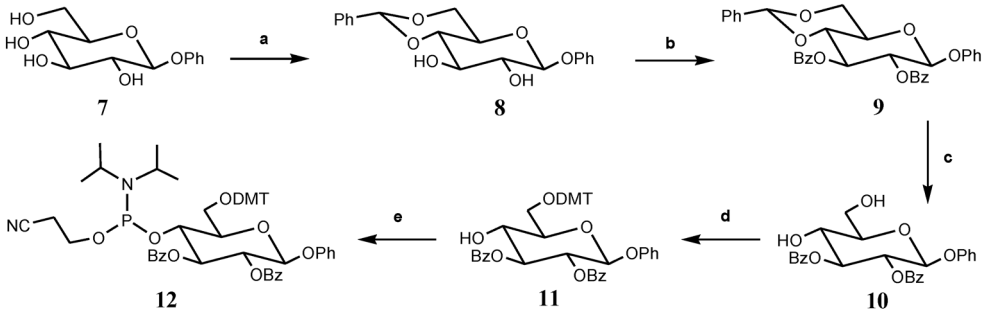
Aiming at oligosaccharide analogs containing chemically and enzymatically stable bonds, new macrocycles named CyPLOS—with two, three, and four phenyl-β-D-glucopyranoside residues, 4,6-linked through phosphodiester bonds—were designed (**1–3**, Fig. 1) [13]. Phosphate bridges, linking the saccharide monomers within the cyclic skeleton, may provide desirable features in glycomimetics: (i) chemical stability in a wide pH range; (ii) additional binding sites for cations; and (iii) easy synthetic access through effective, high-fidelity reactions, well optimized in oligonucleotide synthesis.

For the synthesis of **1–3**, a simple and efficient solid-phase protocol was developed (Scheme 1), based on derivatized phosphoramidite monomer **12**, obtained in five, high-yielding steps from commercially available phenyl-β-D-glucopyranoside **7** (Scheme 2).




**Scheme 1** General procedure for the solid phase synthesis of CyPLOS **1–3**. a: coupling; b: CE removal; c: chain elongation and final DMT removal; d: MSNT-promoted cyclization; e: detachment from the support and deprotection. Bz = benzoyl; CE = 2-cyanoethyl; DMT = 4,4'-dimethoxytrityl; MSNT = 1-mesitylensulfonyl-3-nitro-1,2,4-triazole; Ph = phenyl.




**Scheme 2** Synthesis of phosphoramidite building block **12**. a: benzaldehyde dimethylacetal, *p*-toluensulfonic acid, DMF, 12 h, 50 °C (97 %); b: BzCl, pyridine, 12 h, r.t. (quant.); c: $I_2$, $Et_3SiH$ cat., $CH_3OH$, 12 h, r.t. (91 %); d: DMTCl, pyridine, 12 h, r.t. (95 %); e: 2-cyanoethyl-*N*,*N*-diisopropylaminochlorophosphoramidite, DIEA, $CH_2Cl_2$, 1 h, r.t. (96 %).

This was first incorporated into amino-functionalized Tentagel support **4** in the form of its activated derivative **5**, thus producing—after 2-cyanoethyl removal from the phosphate—supported 2-chlorophenylphosphodiester-linked monomer **6**.

Chain elongation was carried out starting from **6** on an automated DNA synthesizer by standard phosphoramidite chemistry, using **12** as the addition monomer. A classical phosphotriester protocol was then exploited for the cyclization, using 1-mesitylene-sulfonyl-3-nitrotriazole (MSNT) as the condensing agent. The target cyclic molecules were selectively released in solution by a mild basic treatment, under which unreacted linear oligomers were not detached. After a simple gel filtration chromatography, cyclic dimer **1**, trimer **2**, and tetramer **3** were isolated as pure compounds in overall yields ranging from 40 % for the dimer, to 5 % for the tetramer. Solution synthesis of **1** and **2** provided independently prepared samples, identical to those assembled on a solid support, as ascertained by high-performance liquid chromatography (HPLC), NMR, and electrospray ionization-mass spectroscopy (ESI-MS) analysis. A slightly modified protocol allowed the fully deprotected cyclic compounds still anchored on the Tentagel solid support, of interest for direct analytical assays in aq. solutions [14], to be obtained.

A detailed structural study of cyclic oligomers **1–3** has been carried out by NMR analysis [13]. Using $^1H$-$^1H$ and $^1H$-$^{31}P$ vicinal scalar couplings and nuclear Overhauser effect (NOE) data, as well as mechanic and dynamic calculations, cyclic dimer **1** was shown to have a two-fold symmetry, adopting

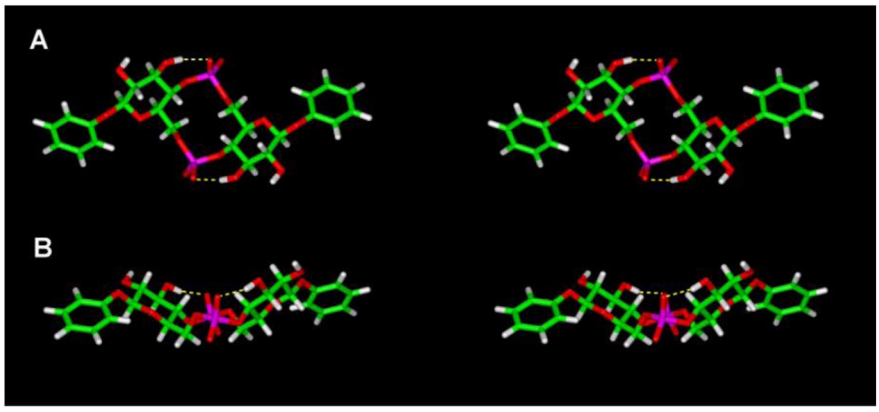



**Fig. 2** Top (A) and side (B) representations of the best NMR structure of **1**, depicted with carbons in green; oxygens in red; hydrogens in white; phosphorous in magenta. H-bonds are represented with yellow dashed lines.

a cradle-like preferential conformation in $D_2O$ and in dimethyl sulfoxide (DMSO), stabilized by strong H-bonding interactions between the phosphate groups and the adjacent 3-OH groups (Fig. 2).

Trimer **2** showed a solvent-dependent conformational behavior, with the three constituting monomers magnetically equivalent in $D_2O$, while in DMSO different families of conformers, in slow equilibria on the NMR time scale, were present. For tetramer **3**, in all the investigated solvents many conformers in slow equilibria were observed.

The potential of these macrocycles as artificial cation receptors was investigated by NMR analysis. Preliminary results—obtained by $^1$H NMR titrations with several cations in $D_2O$—showed **1** having a marked preference for $Ca^{2+}$, bound with a 1:1 stoichiometry and an affinity constant in the range 80–100 $M^{-1}$ (unpublished data).

### Amphiphilic CyPLOS: Synthesis, self-aggregation properties, and ionophoric activity

With the purpose of exploring novel amphiphilic oligosaccharides as potential ion transporters, we then reasoned that the synthesized CyPLOS structures could be suitable synthetic platforms to obtain different jellyfish-shaped analogs. Particularly, the secondary 2- and 3-OH groups of the saccharide backbone could be exploited as handles for further, selective derivatization with a variety of different "tentacles". Amphiphilic macrocycles with a remarkable propensity toward self-aggregation were thus obtained by attaching to the CyPLOS backbone *n*-undecyl and tetraethylene glycol (TEG) residues as long chains with different lipophilicity (**13–15**, Fig. 3) [15]. In addition to the desired amphiphilicity, these functionalizations confer also a higher conformational freedom to the cyclic skeleton of **13–15** compared to parent dimer **1**. In fact, once permanently masked, the 3-OH groups of the pyranose residues are not available for H-bonding with the adjacent phosphate groups. Disruption of these H-bonds removes the structural motif further rigidifying the central cavity of the macrocycles, thus leading to more flexible structures, able to cover a wider conformational space in response to environmental stimuli.

The synthetic route for the preparation of these compounds, carried out in solution, is based on two differently 4-phosphorylated monomers: phosphoramidite **16** and 2-chlorophenylphosphate derivative **17**, so that phosphoramidite chemistry was exploited to obtain linear dimer **18**, and phosphotriester chemistry to provide cyclic compound **19** (Scheme 3). After final deprotection and column chromatography purification, target macrocycles **13–15** were characterized by NMR and ESI-MS data. Though ionic, the cyclic dimers proved to be highly lipophilic, with **13** soluble in $CHCl_3$ or $CH_2Cl_2$ and **14** and

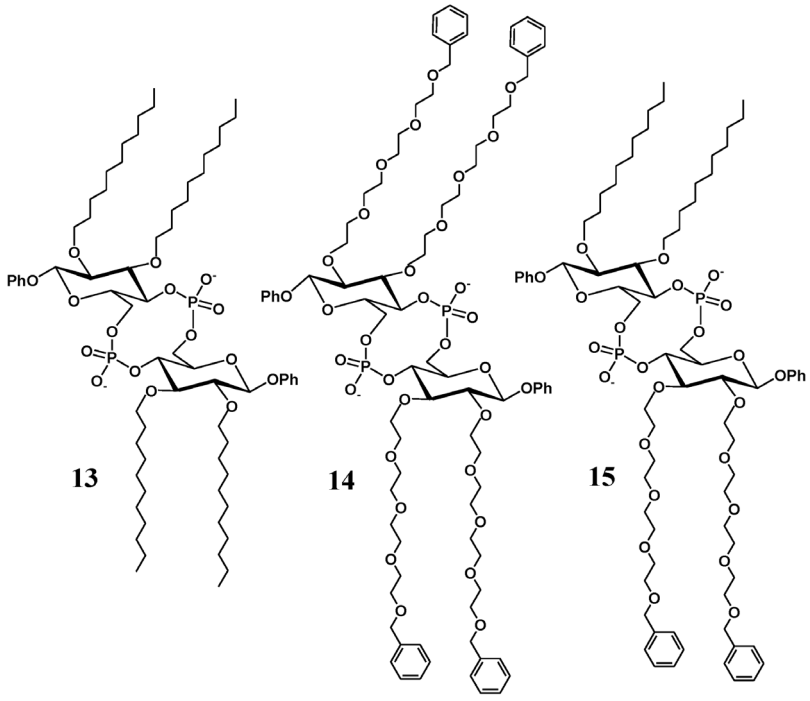
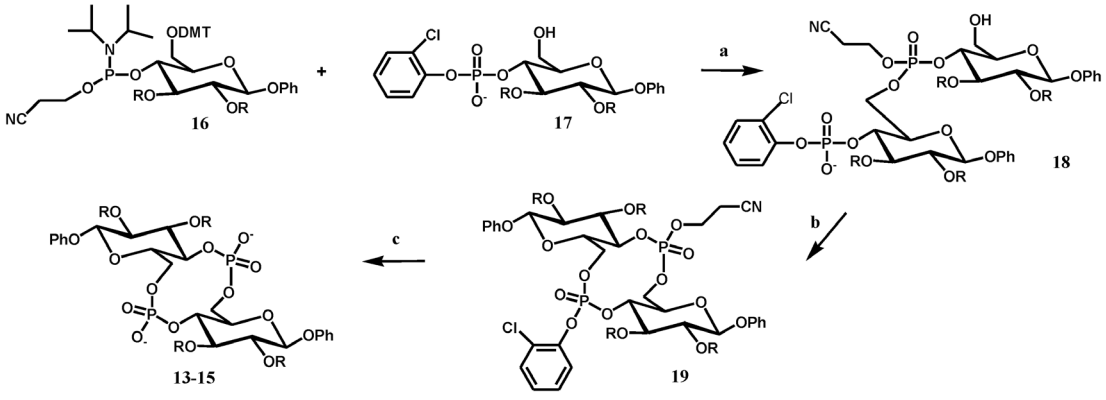



**Fig. 3** Chemical structures of amphiphilic CyPLOS **13–15**.




**Scheme 3** General procedure for the synthesis of CyPLOS **13–15**. a: dimerization; b: MSNT-promoted cyclization; c: phosphate deprotection. R = *n*-undecyl or Bn-TEG.

**15** soluble in all the most common organic solvents. Analyzed in $CDCl_3$, the final cyclic compounds **13–15** showed concentration-dependent NMR spectra, suggesting the presence of strong inter-molecular interactions [15].

Compound **13** gave spectra with two distinct sets of signals, as if in the presence of two different species, which finally coalesced into a single system upon dilution. On the other hand, NMR spectra of **14** in $CDCl_3$ showed dramatic line broadening, diagnostic of slow equilibria on the NMR time scale. Similar behavior was found in **15**, with very broad, badly resolved signals in the NMR spectra obtained already at the level of the linear precursor. Taken together, $^{1}H$ and $^{31}P$ concentration-dependent and

VT-NMR studies showed the following order in terms of increasing preference for self-aggregation in $CDCl_3$: **13** < **14** < **15**. We thus concluded that the TEG-containing compounds are able to generate large aggregates, responsible for the severe line broadening and chemical shift anisotropy in the NMR spectra, and critical aggregation concentrations in the mM to μM range, while the presence of the sole alkyl tails in **13** is not sufficient to generate large self-aggregated species.

A detailed characterization of the aggregative behavior of these compounds in aqueous media has been then carried out by means of a combined experimental strategy. Dynamic light scattering (DLS) measurements were performed to reveal the formation and size distribution of the CyPLOS aggregates, mainly forming large vesicles. Electron paramagnetic resonance (EPR) measurements, by using 5-doxyl stearic acid (5-DSA) as the spin-probe, indicated a lamellar microstructure of the aggregates, and allowed analysis of the local fluidity. Finally, small-angle neutron scattering (SANS) measurements allowed an estimate of the layer thickness of the vesicles. These results showed that the three CyPLOS analogs exhibited self-aggregation properties critically depending on the nature of the inserted tails [16].

The ionophoric activity of amphiphilic CyPLOS **13–15** was monitored by the HPTS assay [17]. HPTS (8-hydroxy-1,3,6-pyrenetrisulfonic acid, trisodium salt) is a pH-sensitive dye exploited in a standard base–pulse assay that reports $H^+/OH^-$ transport through liposomes directly and cation/anion transport indirectly [18]. In essence, an increase in HPTS fluorescence emission indicates basification of the liposome inner water pools, which may be correlated to the ability of the ionophore to promote $H^+/OH^-$ transport and the associated cation/anion symport or antiport. This study showed **14** as the most effective compound in the investigated series, able to completely discharge the pH gradient across a liposomial membrane in less than 20 min at 2 % ionophore concentration. This activity was strictly correlated to the presence of TEG chains, with **14** being much more effective than **15** and tetra-alkylated derivative **13** almost completely inactive (Fig. 4). Analysis of the ionophoric properties of **14** in comparison with its linear and fully protected cyclic congeners proved that both the four TEG tentacles and the fairly rigid anionic macrocycle are structural motifs essential for activity.

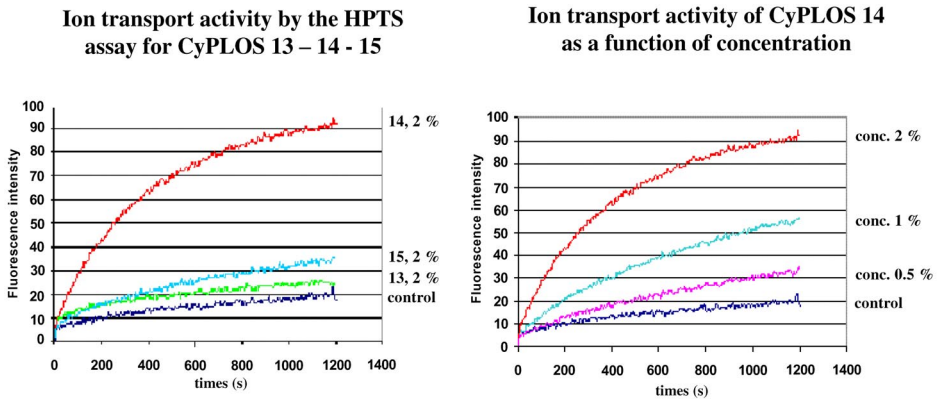



**Fig. 4** Normalized fluorescence change in HPTS emission (FI, $\lambda_{ex}$ 460 nm, $\lambda_{em}$ 510 nm) as a function of time after addition of 50 μL of 0.5 M NaOH, in the presence of 2 % conc. of ionophores **13–15** (left) and registered at different conc. of **14** (right), to 95:5 EYPC/EYPG liposomes loaded with HPTS (0.1 mM HPTS, 0.17 mM total lipid conc., 25 mM HEPES, 100 mM NaCl, pH 7.0, total volume 3 mL). The ionophore conc. is given in % with respect to the total conc. of lipid. (HPTS: 8-hydroxy-1,3,6-pyrenetrisulfonic acid, trisodium salt; HEPES: 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; EYPC: egg yolk phosphatidyl choline; EYPG: egg yolk phosphatidyl glycerol). From ref. [17].

When investigated for selectivity in ion transport, **14** did not show a sensibly different behavior on varying the cation; on the contrary, when tested with different anions, high transport activity was found with halogens, nitrate, and perchlorate; on the contrary, acetate, glutamate, and sulfate were not transported (Fig. 5). The lack of selectivity toward lipophilic anions was attributed to the formation of a poorly structured active species with little specificity toward the transported ions [17].

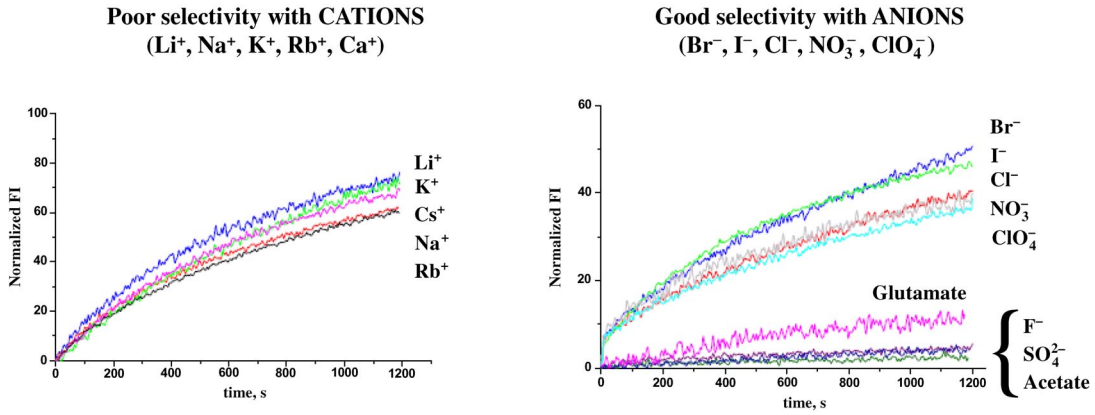



**Fig. 5** Cation selectivities for ionophore **14** (1 % conc.), using the HPTS assay (100 mM MCl, pH 7.0, base pulse by addition of 50 µL of 0.5 M MOH, with M = $Li^+$, $Na^+$, $K^+$, $Cs^+$, or $Rb^+$); (c) Anion selectivities for ionophore **14** (0.5 % conc.), by the HPTS assay (100 mM NaX, pH 7.0, base pulse by addition of 50 µL of 0.5 M NaOH with $X^-$ = $F^-$, $Cl^-$, $Br^-$, $I^-$, $NO_3^-$, $SO_4^{2-}$, $ClO_4^-$, acetate glutamate).

## Amphiphilic CyPLOS carrying specific reporter groups: An insight into the mechanism of their ionophoric activity

Insight into the mechanism of action of an artificial ion transporter through membranes can be achieved if labeled compounds, allowing information on the interactions between the label and the lipid bilayer, are available. Fluorescence and EPR spectroscopy are the techniques of choice to obtain these data. To this purpose, two new analogs of **14**, respectively, carrying a fluorescent tag and a spin label, have been designed and synthesized. In the first study, dansyl-CyPLOS **20** (Fig. 6) was investigated, showing a 3-fold increase in ionophore activity compared to **14** [19].

The presence of dansyl groups at the extremities of CyPLOS tentacles gave precious information on the insertion mode of the ionophore in membranes (Fig. 7).

In particular, in EYPC/EYPG liposomes, the ionophore appears to be active as a monomer with the polar macrocyclic head lying on the surface of the membrane and the TEG chains inserted in the bilayer. The TEG chains, apparently not deeply inserted in the phospholipid bilayer, are located in the mid-polar region, between the polar surface and the hydrocarbon core.

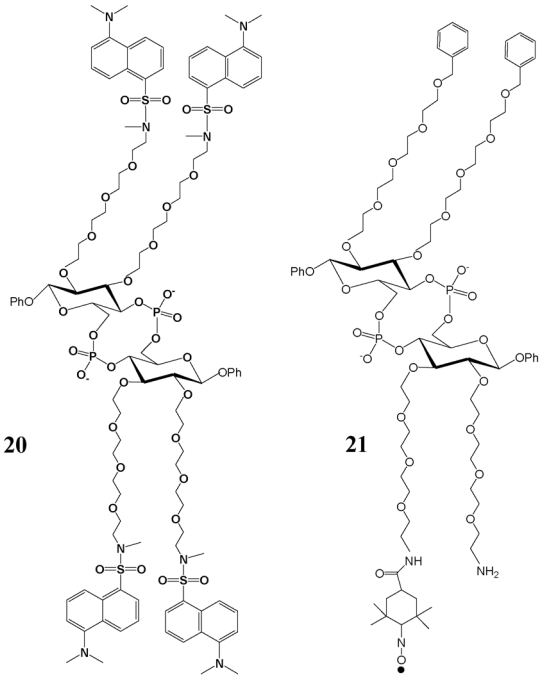



**Fig. 6** Chemical structures of labeled amphiphilic CyPLOS analogs **20** and **21**.

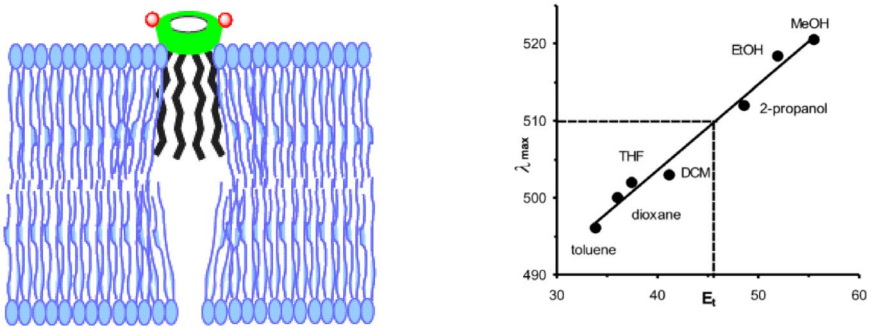
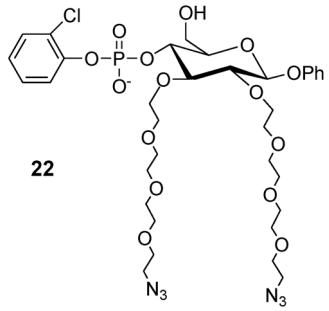



**Fig. 7** Representation of the insertion of CyPLOS derivatives within phospholipids (left) and plot of fluorescence emission maxima for **20** at 1.7 μm conc. (right) vs. the polarity of different solvents, expressed in terms of the Dimroth–Reichardt parameter $E_t$ [20]. The dotted lines correspond to the emission maxima of **20** at conc = 3.4 μm and excitation wavelength $\lambda_{max}$ = 340 nm, measured in an EYPC/EYPG suspension (95:5, [total lipid] = 0.17 mM, 25 mM HEPES, 100 mM NaCl, pH 7.0).

In order to obtain more detailed data on the mechanism of action of CyPLOS analogs and their interaction with membranes, novel spin-labeled CyPLOS **21** was prepared (Fig. 6) [21] and studied in its ability to interact with cell membranes by ESR spectroscopy [22]. The synthesis of **21** was profitably achieved exploiting a postsynthetic functionalization procedure based on azido-TEG functionalized monomer **22** (Fig. 8). This intermediate can be exploited as a "convertible" monomer for the end-functionalization of CyPLOS derivatives with a large variety of reporter groups.




**22**

**Fig. 8** Chemical structure of "convertible" monomer **22**, used for the synthesis of CyPLOS **21**.

## CONCLUSIONS

In these studies, novel cyclic glycomimetics CyPLOS, in which the sugar monomers are connected through phosphodiester bonds, have been described. These macrocycles have been prepared through an efficient and versatile synthetic strategy, giving access to analogs with a large degree of intrinsic chemical diversity. Indeed, the repertoire of the accessible CyPLOS derivatives can be significantly expanded by ad hoc modulating the nature, ring size, and stereochemistry of the selected building blocks. Amphiphilic CyPLOS **14** and **20** proved to be very efficient ionophores by the HPTS assay, with good selectivity toward anions. Fluorescence and ESR spectroscopy confirmed that CyPLOS interact with phospholipid bilayers, adsorbing on their surfaces. This interaction causes a destabilization of the local lipid packing, thus allowing small ions to pass through the membranes. The synthesis of a second generation of CyPLOS analogs is currently in progress, aiming at increasing their ionophoric selectivity profile and studying their biological properties in detail.

## ACKNOWLEDGMENTS

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