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Hexakis (3,6-anhydro)-tetrakis [$2^{I,II,IV,V}$-*O*-(2-ethoxyethyl)] derivatives of (3,6-anhydro)-α-cyclodextrin exhibits novel cation affinities and tensioactive properties on membranes

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Received February 12, 2007, accepted April 19, 2007

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Pharmazie 62: 892–899 (2007) *doi: 10.1691/ph.2007.12.7048*

The synthesis of hexakis (3,6-anhydro)-tetrakis[$2^{I,II,IV,V}$-*O*-(2-ethoxyethyl)] cyclomaltohexaose (AEOE) was designed to obtain cation complexing properties. ^{1}H NMR study showed ionic radius dependence of AEOE cation affinity, markedly observed for Cs^+ and Rb^+. Besides, AEOE was found haemolytic (HC_{50}=9mM) and superficial tension measurements revealed positive tensio active properties. A ^{31}P and ^{2}HNMR study of phospholipid dispersions (dimyristoyl phosphatidyl cholin, DMPC) in the presence of AEOE was performed; it was found that, beside the typical lineshape of phospholipid bilayers, two new NMR lines were detected in the presence of AEOE: a) an isotropic line consistent with a detergent effect b) another isotropic resonance of 1 Hz linewidth over phase transition temperature (298 K), indicating a true solubilization. Coupling constant measurements confirmed that the main conformation at the polar head group level was close to that observed in chloroform/methanol solution. It was finally concluded that AEOE could form true solutions of DMPC, similarly to those induced by diethyl ether interactions with membranes, while giving soluble complexes.

1. Introduction

Cesium and iodine isotopes are radionuclides commonly found (Laylavoix et al. 1986) when a nuclear accident occurs, as observed after the explosion of the nuclear plant of Chernobyl (Ivanov et al. 2003). Due to the relatively long lifetime of the most common isotope ^{137}Cs (radioactive period of 30 years), radiobiological pollution by this radionuclide leads to a major environmental health problem. Cesium intestinal absorption is rapid in humans and its repartition almost complete in intracellular compartment (99%). Moreover, its metabolism is similar as that of potassium, leading to an important Cs^+ accumulation in muscles (Galle 1997). Cesium biodecontamination is thus an important challenge, with a special interest in the Cs^+ versus K^+ selectivity. Also, besides the high affinity and the selectivity requested for the toxic cations removal, tensio active properties would be of great interest in terms of surface decontamination. The first per(3,6-anhydro) cyclodextrins were designed and studied since the early 1990s (Gadelle 1991). These derivatives of low toxicity (Debouzy et al. 1998) exhibited cation complexation properties (Fauvelle et al. 1998) and were more or less amphiphilic. After having obtained lead and strontium complexation using the starting molecule, per(3,6-anhydro)-α-cyclodextrins (A36) (Baudin et al. 1998), supplementary permethylation of A36 on the six positions 2 resulted in a drastic enhancement of cation complexing properties, especially for lead and strontium, but also for other ions such as barium (Fauvelle 1999), while a reduction in the aqueous solubility was also observed. Later on, the substitution of per(3,6-anhydro)-α-cyclodextrins by octyl groups on four of the six positions 2 yielded the hexakis(3,6-anhydro)tetrakis($2^{A,B,D,E}$-*O*-octyl) cyclomaltohexaose (OCT). This molecule also exhibited high affinity for lead, mercury and uranyle (Debouzy et al. 2001a), while being completely insoluble in water (Debouzy et al. 2001b).

The persubstitution of ACD by ethyl groups on positions 2 gave hexakis(3,6-anhydro, 2-*O*-ethyl) cyclomatohexaose (E36). The good affinities obtained with Hg^{2+}, Pb^{2+} Sr^{2+} and Co^{2+} appeared well designed for the treatment of accidentally contaminated wounds, especially by Co^{2+} (Miltenberger et al. 1981), or Sr^{2+} (Hayek et al. 1970). This latter point was related with the tensioactive-soap-like properties of E36, enhancing complexation efficiency on/in membrane lipids, a major components of living tissues.

These results provided good arguments for further chemical modifications toward the final goal of decontamination: thus use substitutions on positions 2 of ACD to magnify amphiphilic properties (short-chained acyl groups) and to stabilize cation complexes by using negatively charged substituants – (Pelizzari et al. 1997; Fauvelle et al. 1996).

This led to synthesize a molecule bearing three clustering oxygen rings in its structure: the 3,6 ether and the interresidue glycosidic bonds (see Figure 1A). The third nega-

tively charged ring consisted of a side chain containing itself an oxygen on the positions 2. This structure was hexakis(3,6-anhydro)tetrakis($2^{A,B,D,E}$-*O*-) α-cyclodextrin (AEOE). This alkyl persubstitution was also expected to magnify cation selectivity (Pailler et al. 2005; Baudin et al. 2000; Debouzy et al. 2003) by sterical hindrance. The study of complexing and physico chemical properties of AEOE by ^{1}H NMR were the first topic of this paper. Haemolytic properties and AEOE interactions with membranes were then investigated using both natural red blood cells, and synthetic membranes in combination with ^{31}P (Roux 1987; Debouzy et al. 2002) and ^{2}H NMR (Douliez et al. 1996) methods.

2. Investigations and results

2.1. AEOE structure in solution and cation affinities

Mass spectroscopy (M=1156, M + Na=1177, ES+) and ^{1}H-/^{13}C NMR confirmed the tetrasubstitution of AEOE molecule. Hence, three ^{1}H distinct spins systems were identified, respectively corresponding to the following building blocks (see Fig. 1A), as follows:
(a) system: 5.29 ppm(d), H 1; 3.85(t)H 2; 4.43(t)H 3; 4.27(m), H 4; 4.05(m), H 5; 4.3(m)(H 6-6′) (b) system: 5.34ppm(d), H 1; 3.83(t)H 2; 4.44(t)H 3; 4.278(m), H 4; 4.05(m), H 5; 4.3(m)(H 6-6′) (c) system: 5.25ppm(d), H 1; 4.02(t)H 2; 4.39(t)H 3; 4.27(m), H 4; 4.05(m), H 5; 4.3(m)(H 6-6′).
As described above, per anhydro cyclodextrins have in common cation affinities, differing from one derivative to the other by their strength and selectivity.
In a first step, a coarse screening was used by recording ^{1}H NMR spectra on equimolar AEOE/cation mixtures (1 mM) and on sample containing excess of cation (see Experimental). In the absence of any spectral modification (mainly chemical shift, peak intensity and linewidth), the affinity was considered negligible and the study was not

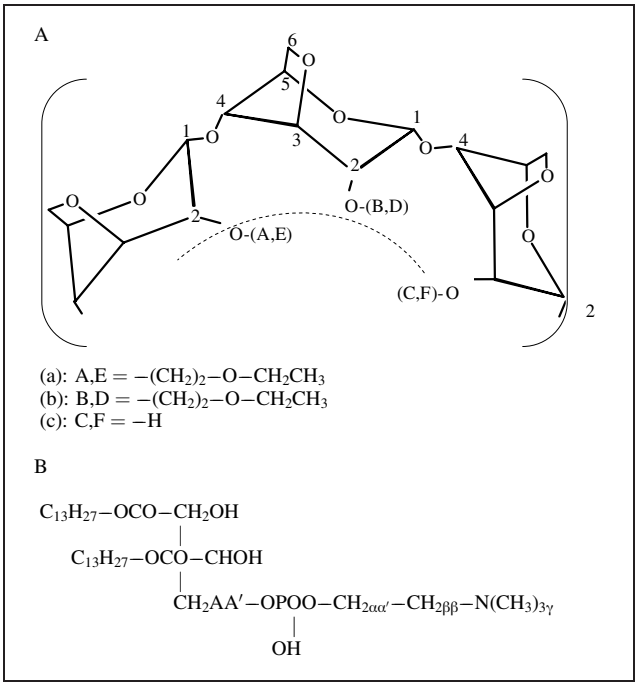

Fig. 1: A) Half representation and proton nomenclature of Hexakis (3,6-anhydro)-tetrakis[$2^{I,II,IV,V}$-*O*-(2-ethoxyethyl)]cyclomaltohexaose) (AEOE), showing the oxygen rings: A: interglycosidique O; B: ring of the 2′ positions; C: chain ether ring. B) Proton nomenclature used for DMPC molecule

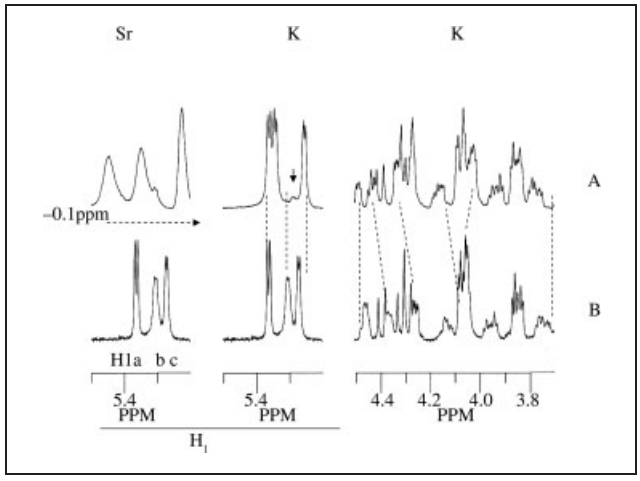

Fig. 2: ^{1}H NMR spectra of pure AEOE (bottom traces) and in the presence of equimolar Sr^{2+} (left trace; the spectrum was highfield shifted by 0.1 ppm to show all anomeric resonances; note the line broadening) and K^+ (two topleft traces; fast kinetics is ascertained by exclusive chemical shift variations). The arrow indicates the presence of 1% hexasubstituted derivative

followed further. Fast exchange kinetics were identified on ^{1}H NMR spectra by chemical shift variations of cyclodextrin peaks upon cation addition (Cs^+, Rb^+, K^+, Na^+, Ca^{2+}, Ba^{2+}, Co^{2+}, see Fig. 2A, right traces for K^+), and the classical method described by Job (Job 1925; Djedaini 1991) was used to draw the stoechiometry of the complex. Apparent affinity constants were calculated using Hildebrand-Benesy method (weak complex, Hildebrand and Benesi 1949) or mathematic program SIMPLEX (EXPREX, or MURIEL-X algorithms generously given by Bruno Perly, CEA Saclay, France, for strong complex). For Sr^{2+} only a coarse estimation could be proposed since a line broadening (up to 10 Hz, see Fig. 2A, left traces) was observed for high cation/AEOE ratio, indicating a borderline kinetics between fast and intermediate (Kaplan and Fraenkel 1980). As Ba^{2+} complexation kinetics was slow (both contributions of free cyclodextrin and the cyclodextrin-cation complex were simultaneously observed in the ^{1}H NMR spectra, without shift or line broadening), the apparent affinity constant was obtained by direct peak integration (Debouzy et al. 2001a).

Table 1: Complexation stoichiometry, apparent association constant (Ka in M^{-1}) method of Ka determination and kinetics and ionic radii (Å) of the different ions tested

Ion	StoechC/i	Kapp(M-1)	Methode Ka	Kinetics	Ri(Å)
Cs	2/1	840	exprex2	F	1,6
K	1/1	50	Benesi	F	1,33
Na	0	0	0	0	0,97
Pb2+	1/1	?	?	insoluble	1,2
Sr2+	1/1	11	Benesi (H1c)	F/I	1,12
Ca2+	1/1	5	Benesi	F	0,99
Ba2+	1/1	25 000	Integration	S	1,34
Co2+	1/1	3	Benesi	F	0,72
Cu2+	0	0	0	0	0,72
Fe3+	0	0	0	0	0,74
UO2−	0	0	0	0	3
Mg2+	0	0	0	0	0,66
Hg2+	0	0	0	0	1,1
Rb2+	1	250	Benesi	F	1,47

S: slow kinetics; I, Intermediate, Fb, fast kinetics, Hildebrand-Benesi-method for constant determination,
Fs: fast kinetics, simplex method for constant determination; others ions tested were selected by prior Thin Layer Chromatography tests

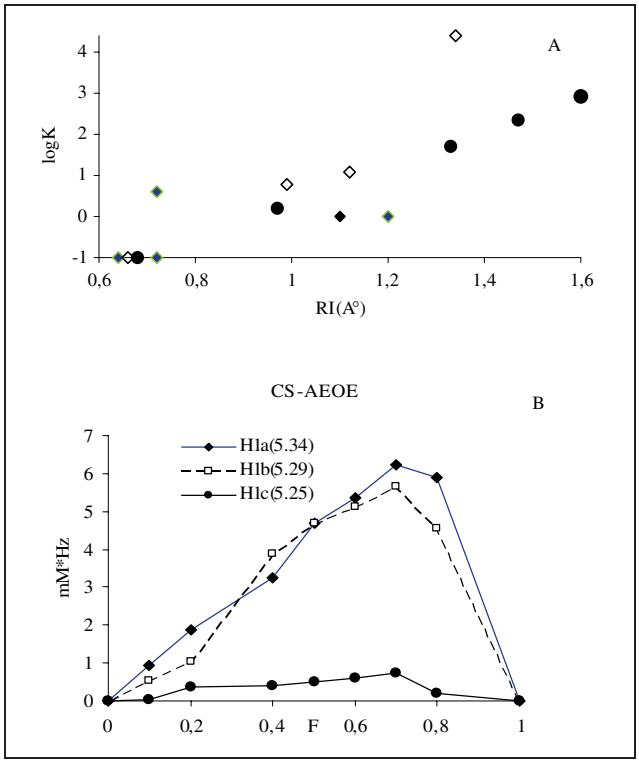

Fig. 3: A) Apparent affinity constant (logK) plotted versus ionic radius (Å); alcaline elements are plotted with full circles.; open diamonds represent Mg, Ca, Sr and Ba divalent ions, and full diamonds Cu, Hg, Pb divalent ions. B) Job plot construction built from chemical shift variations of AEOE anomeric protons H-1 a, b, c. in the presence of Cs^+

These results (Fig. 3 and Table 1) show that AEOE cation affinities strongly differ from those of all other anhydrocyclodextrins (Fauvelle et al. 1999; Debouzy et al. 2001a, 2001b). Hence, no affinity was found for heavy metals, or transition elements (except Pb^{2+}, that give unsoluble precipitates with AEOE), while some complexations with alkalis or alcaline earth were identified. Furthermore, the complexation constants of alkalis appeared clearly related with ionic radius, from 0 (Li) up to $\log K = 2.9$ (Cs) (see Fig. 3). In that latter case a 2 : 1 stoechiometry was identified for Cs complexation ($2Cs^+$/1AEOE).

From these features, AEOE appears as a promising starting structure for Cesium complexation, even if the the affinity constants are relatively weak. Under the scope of biological application, several tests had to be performed: this led us to investigate the haemolytic activity of AEOE, since numerous cyclodextrins exhibit haemolytic properties (Fauvelle et al. 1997)

2.2. *Haemolytic activity*

The haemolysis curve is shown in Fig. 4A. By comparison with natural α-cyclodextrin, AEOE haemolytic activity was found significantly higher, with a HC_{50} close to 9 mM (compared to more than 12 mM for natural alpha cyclodextrin). As such toxic properties would constitute a severe limitation in potential biological use, it was of great importance to investigate the molecular mechanism involved. Since the interactions of natural cyclodextrins (Markman et al. 1983) and also some anhydrocyclodextrins (Fauvelle et al. 1997) with membranes were related with their hemolytic properties, the physico chemical properties and interactions with membranes were then investigated.

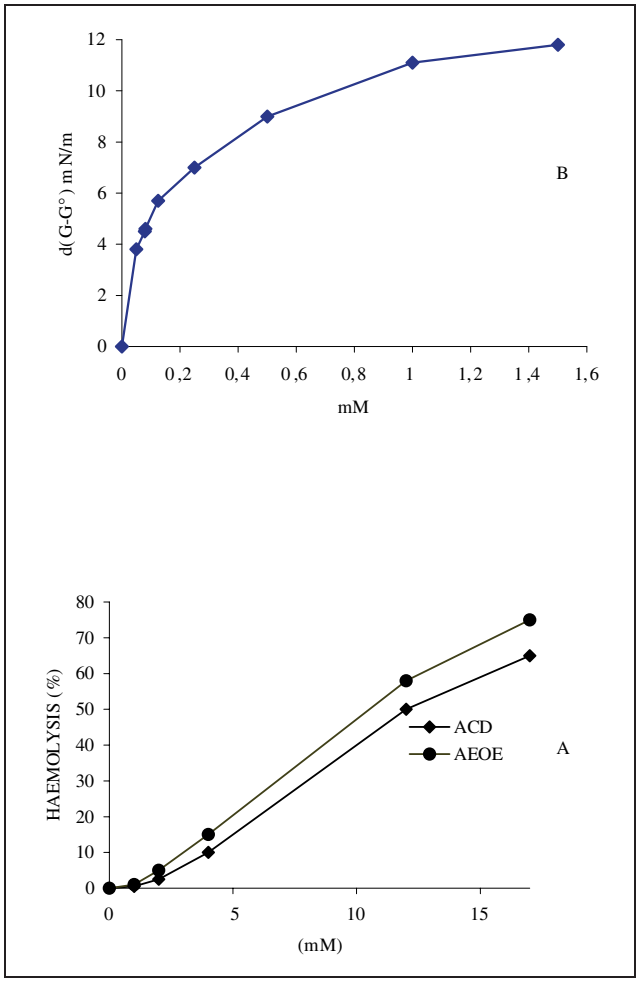

Fig. 4: A) Percentage of haemolysis following the concentration of AEOE (●) by comparison with natural alpha cyclodextrin (◆) at 25 °C. B) Superficial tension (dG-G°, mN/m) as a function of AEOE concentration (mM)

2.3. *Physico chemical properties*

Partition coefficient (LogP): The basic structure, per(3,6 anhydro) α cyclodextrin (A36) is a very hydrophilic molecule ($LogP = -8$). This property was exalted in alkaline medium due to the formation of alcoholate function. Previous work (Debouzy et al. 2001a) found that total or partial substitutions at the positions 2 resulted in continuous increases of LogP with the chain length. By comparison, the LogP value found for AEOE (−3.85) was relatively close to that of hexakis(3,6-anhydro) tetrakis($2^{A,B,D,E}$-*O*-propyl) cyclomalto hexaose (P36, $LogP = -3.1$). However, whereas AEOE is relatively soluble in water (up to 18 mM, as P36), the 3 Hz linewidths measured on AEOE ^1H NMR peaks for concentrations higher than 2.5 mM (compared to less than 0.3 Hz at 1 mM), indicated the presence of agregates rather than true solutions. This was reinforced by the macroscopic bubbling observed when AEOE solutions were stirred.

The surface properties of AEOE were then investigated (see Fig. 4B). By comparison with pure water ($\Delta G° = 78.5$ mN/m at 25 °C), a non linear surface pressure is observed even for small AEOE concentrations, then reaching a steady-state plateau of 12 mN/m for 1.5 mM. This result, consistent with the concentration dependence of NMR line widths indicates that AEOE exhibits positive tensioactive properties suggesting strong interactions with membranes.

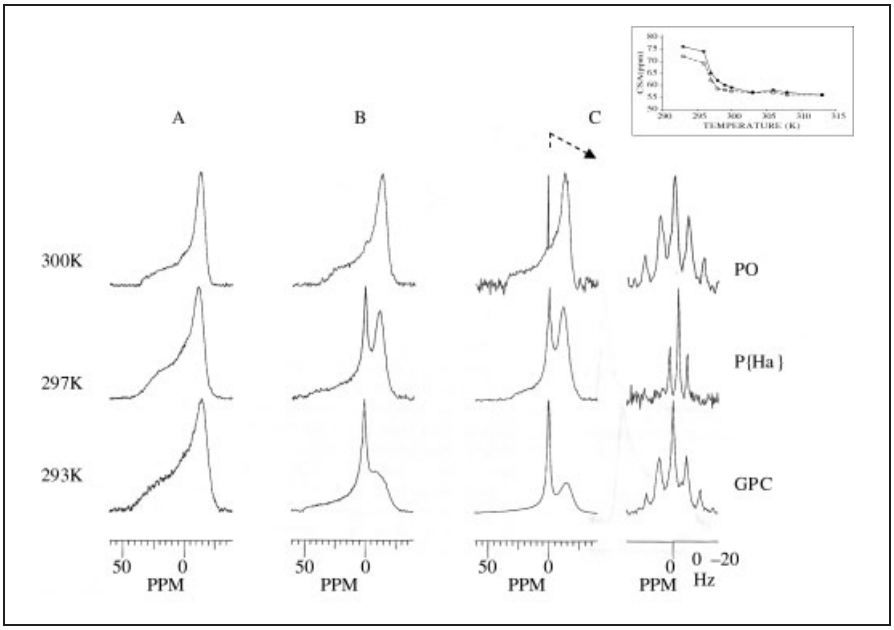

Fig. 5:
^{31}P NMR of DMPC: A) typical spectra of DMPC bilayers below (293 K), close to (298 K) and over (300 K) transition temperature B) Same conditions as A), in the presence of AEOE (R = AEOE/DMPC = 1/16M/M); C) Same as B) with R=1/8; fourth column: spectra of the supernatant after centrifugation (2000 rpm, 10 min), at 300 K top: Undecoupled ^{31}P NMR spectrum; middle: partially decoupled of glycerol protons bottom: GPC, 2mM, D_2O. Inset shows temperature dependence of the chemical shift anisotropy for pure DMPC (full line), and AEOE/DMPC systems (dashed line)

2.4. Membrane structure and dynamics study by ^{2}H and ^{31}P NMR

^{31}P and ^{2}H NMR spectroscopies of phospholipid dispersions (MLV) were used to observe the structural and dynamics consequences of the presence of AEOE at the polar head (^{31}P) and chain (^{2}H) levels of the membrane.

2.4.1. The polar head group level

As shown in Fig. 5 (first column) the spectrum of pure DMPC dispersion (MLV) is typical of an axially symetric powder pattern, with a chemical shift anisotropy of 58 ppm, classical of DMPC bilayers in their liquid crystallin phase, below (293 K) and around (296–298 K) phase transition (Dennis and Plückhun 1984; Gorenstein 1984). The chemical shift difference between the lowfield and the highfield edges of the ^{31}P NMR spectrum is called Chemical Shift Anisotropy (CSA, ppm) and is directly related to the fluidity-reorientation at the polar head level where the phosphorus nuclei are located. On such spectra a mobile phosphorus group give a single narrow resonance (several Hz) as detected in true solution or for small structures (micelles), while solide state phosphorus gives extremely broad contributions (more than 100 ppm). Note that membrane fluidity increases (and CSA decreases) with temperature, with a special jump at the transition temperature between gel phase and liquid crystal structure (around 297 K for DMPC, see Fig. 5B). Thus the plot of CSA as a function of temperature provides a good overview of membrane dynamics at the polar head level where phosphorus nuclei are located, while the lineshape allows to identify the overall membrane organisation (bilayer, hexagonal, isotropic phases). Such plots are presented on the insert in Fig. 5 for pure DMPC dispersions and for AEOE containing MLV. As expected a CSA decrease (around 18–20 ppm) was observed on pure DMPC systems with the transition-related jump around 297 K.

Such was not the case for the spectra recorded under the same conditions on AEOE containing systems (Fig. 5) at various temperatures.

For the systems with AEOE/DMPC molar ratio of R = 1/16, an unresolved (500 Hz linewidth) contribution was detected at the "isotropic" position, as frequently observed when detergent effects occur. However, this contribution vanished with increasing the temperature over phase transition, where the corresponding spectrum only showed the main structure lineshape, indicating that a dynamic process occurred, leading to the apparent reintegration of the mobile structure in the main bilayer. Besides, the relative contributions corresponding to perpendicular versus parallel orientations of the spectrum (the lowfield shoulder and the high field resonance, respectively) increased below phase transition, indicating at least a partial orientation of the sample occurs. The presence of structural rearrangements was also supported by the increase in CSA at low temperature, with a normal transition temperature (297 K) and CSA values close to those of DMPC at higher temperatures (insert Fig. 5). Hence, the typical lineshape of DMPC dispersion was recovered when increasing temperature over transition temperature (297 K for DMPC). Finally, both lineshape and isotropic lines dependence with temperature were found completely reversible, indicating that dynamic exchange probably occurred, consistent with phase separation at low temperature, reversed by the dynamic rearrangements occurring around transition temperature.

The spectra recorded under transition temperature in the presence of high amounts of AEOE (R = 1/8) (Fig. 5 third column), showed a similar broad contribution (except that

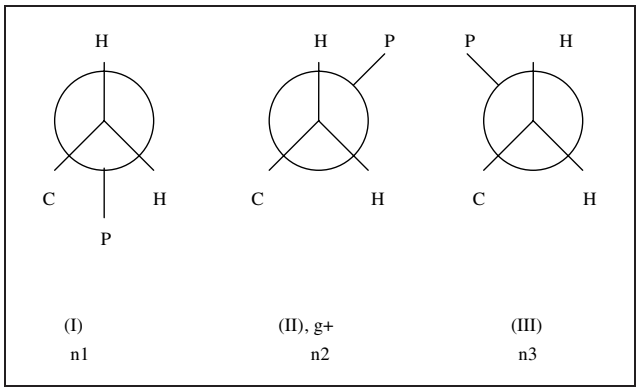

Fig. 6: Fischer's representation of possible rotamers in phosphodiester bond P–O–CH_2; $n_{1,2,3}$ are the relative contributions of each species, g: gauche, t, trans

Table 2: Overall splitting and linewidths of ^{31}P NMR undecoupled spectra, residual coupling and relative g contribution (n_2, %) of partially decoupled spectra for aqueous solutions od AEOE/DMPC (R = 1/8, supernatant), PC, GPC, and for DMPC solutions in c chloroform or chloroform/methanol (9/1, V/V) at 300

^{31}P NMR	Undecoupled spectra Splitting/linewidth (Hz)	J $POCH_2\alpha\alpha'$ P{H_A} (Hz)	n2 $POCH_2\alpha\alpha'$	J $POCH_{2AA'}$ P{Hα} (Hz)	n2 $POCH_{2AA'}$
AEOE/DMPC	25.6 (1.5)	3.9	1*	4.4	1*
GPC	24.3 (1.8)	4	1*	4.5	1
PC	20 (2.5)	–	–	4.5	–
DMPC $(CDCl_3/MeOD)9/1$	27 (5)	4.8	0.95	5.9	0.85
DMPC (MeOD)	27.3 (1.8)	5.2	0.93	5.1	0.94

P{H_A}: $H_{AA'}$ irradiated, ^{31}P observed; P{Hα}: Hαα′ irradiated, ^{31}P observed
*: mean dihedral domain corresponding to 0–120° region

the relative contribution was more important) and oriented line shape. However, above 297 K, this trace disappeared and was replaced by an extremely resolved line (1.5 Hz linewidth), revealing that rapidly motioning systems had been formed. The centrifugation of the sample (2000 rpm, 10 min) allowed the separation of two components: i) a bilayer structure close to that of pure DMPC, with similar lineshape and CSA, while slightly more fluid; ii) a mobile structure giving exclusively a 1.5 Hz width line; the undecoupled ^{31}P NMR spectrum recorded a on this fraction consisted of a resolved quintuplet, related with the phosphorus coupling with the 4 protons present in DMPC headgroup (glycerol-Ha/a′ protons and cholin Hα/α′ protons, see Fig. 1B for proton nomenclature). This was ascertained by recording partially $H_{AA'}$ or $H_{\alpha\alpha'}$ decoupled $^{31}P\{^1H\}$ NMR spectra showing typical triplets. (see Fig. 5, 4th column). The coupling constants were then estimated from residual splitting and compared with those corresponding at the polar head group alone in D_2O (glycerophosphorylcholine, GPC) and DMPC in methanol (see table II), or methanol/chloroform (1/9, V/V).

As classically admitted (Makryannis et al. 1990) these values are related with the local conformation, i.e. the dihedral angles formed by the bounds in the two different $POCH_2$ blocks ($POCH_2$(A,A′) and $POCH_2(\alpha,\alpha')$. From experimental considerations (Bushby et al. 1990; Hansbro et al. 1992) the observed coupling constants J P-CH_2 are resulting from the averaging of the values corresponding at 3 possible conformations I ($J_{gauche}+$), II ($J_{gauche}-$) and III (J_{trans}) (see Fig. 6). Thus the relative population of each rotamer can be deduced from the apparent coupling constant

$$J\ P\text{-}CH_2 = n_1/2 \cdot (Jg + Jt) + n_2 \cdot Jg + n_3 \cdot (Jg + Jt), \quad (1)$$

with $n_1 + n_2 + n_3 = 1$, the relative population of each rotamer and, by replacing the reference Jg (4.5 Hz) and Jt (23.5 Hz), <J P-CH_2> estimated, and $n_1 + n_3 = 1 - n_2$

$$\begin{aligned} <J\ P\text{-}CH_2> &= 14 \cdot n_1 + 4.5n_2 + 14n_3 \\ &= 14(1 - n_2) + 4.5n_2, \end{aligned} \quad (2)$$

the relative proportion of g population is given by:

$$n_2 = [14 - <J\ P\text{-}CH_2>]/9.5 \quad (3)$$

These values are presented in Table 1 for PC, GPC and the complex AEOE/DMPC in the water, and for pure DMPC in methanol or chloroform/methanol (9/1 V/V) solution.

As expected the preference of phosphate esters to *gauche* conformations was found for pure DMPC samples (glycerol $C_A-O-P-O$ ester perpendicular to the surface, and $O-P-O-C_\alpha$ bended towards the surface). Conversely, the small coupling constants observed for both GPC in solution and AEOE/DMPC association led to consider either an exclusive g conformation, or rather a fast reorienting position in the g−/g+ angular distribution within each pair H_A/H_A and $H_\alpha/H_{\alpha'}$ ($n_2 \geq 1$!) consistent with free motional averaging (as in solution). This hypothesis was also supported by the very similar linewithedths (2–2.5 Hz) and coupling constant J P-O-CH $\alpha_2 = 4.5$ Hz measured on undecoupled spectra of phosphocholine in aqueous solution.

Acyl chain contribution in AEOE-DMPC interactions was then observed by recording ^{2}H NMR experiments on chain perdeuterated DMPC (DMPC-d_{54}) dispersions in the same conditions.

2.4.2. The acyl chain level

Figure 7 (bottom) shows the spectrum of DMPC-d_{54} (dimyristoyl phosphatidyl choline with perdeuterated chains) dispersions. This spectrum is typical of phospholipid bilayers in the liquid crystal phase (temperature of 303 K, as shown on the partially dePaked spectrum) (Douliez et al. 1996; Fauvelle et al. 1997). Such a spectrum appears as a superimposition of symetrical doublets, each doublet corresponding to a methylenic CD_2 group of the acyl chain. For a given doublet, the splitting (quadrupolar splitting, $\Delta\nu Q$) is directly related to the local order following the relation:

$$\Delta\nu Q = [A^*(3^* \cos^2 \theta - 1)]/2,$$

where A is 170 kHz (for the CD_2 bound in DMPC) and θ the averaged value of the solid angle of reorientation. This splitting can be used in a first approximation as an order parameter. As the acyl chain fluidity decreases from the terminal methyl group (CD_3) to the methylenic groups close to the polar head of the lipids (the so called "plateau region", from C-2 to C-8 of the chain), the resulting spectrum consists of i) an inner doublet with a quadrupolar splitting of 3200 Hz attributed to the CD_3 methyl group, a is found, ii) doublets with increasing quadrupolar splittings assigned to successive CD_2 groups from C14 to C9; iii) the external edge doublet, attributed to the deuterium of the C2–C8 plateau region where a 28 kHz quadrupolar splitting is measured.

The main spectrum recorded under the same conditions (303 K) in the presence of AEOE (R = 1/8 M/M) was similar as this of natural DMPC-d_{54}, both in quadrupolar splittings and in main linewidths (middle trace Fig. 7). However, a supplementary contribution was found at the isotropic position ($\delta = 0$ Hz) with a linewidth close to 500 Hz. Such a line are sometimes detected in pure systems, especially when natural water is present (mobile nat-

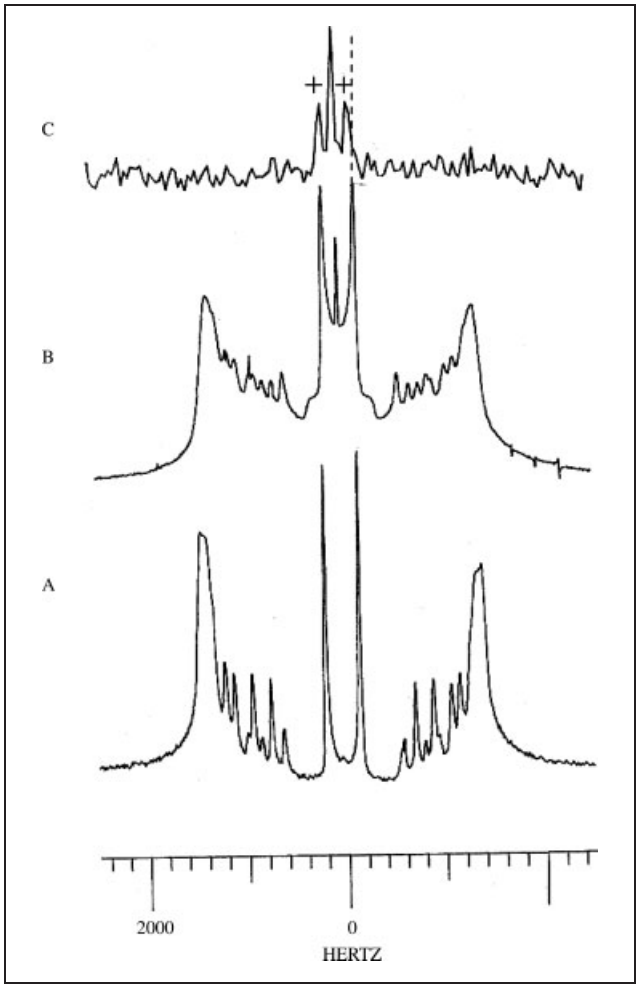

Fig. 7: ^{2}H NMR spectrum of A) pure DMPC-d_{54} dispersions at 300 K (the spectrum is partially de-paked to show the splittings), B) in the presence of AEOE (R = 1/8 M/M) spectrum; C) Same conditions as in B) recorded on the supernatant of the centrifugation

ural water deuterons are also located at the isotropic position). Supplementary spectra were then recorded after centrifugation of the sample, giving a spectrum of pure DMPC-d_{54} dispersions (corresponding to the pellet), while the supernatant forwarded both the isotropic line and a poorly resolved membrane contribution (see stars in Fig. 7 top); On such a trace, the observation of a doublet with $\Delta\nu Q = 2.6$ kHz revealed the presence of significant motional averaging. This value (compared to the 3.2 kHz of CD_3 doublets of pure DMPC-d_{54} dispersions) indicates that the terminal methyl groups of the membrane experienced a significant fluidification.

3. Discussion

AEOE was synthesized for selective complexation and/or decontamination of toxic cations (with special interest for Cesium). Thus, Cs Complexation was observed while relatively weak (or no) affinity was identified for alkali and alkaline earths, including physiological cations (Ca, Na, K...). AEOE appears then as a promising model to entrap big cations such as Cs. Considered the affinity dependence on ionic radius, one can reasonably expect dramatic affinity and selectivity enhancement by modulating the sterical hindrance, i.e. by changing the chain length and ether bond position in the side chain at the positions 2.

However, AEOE was found of significant hemolytic activity, that would limit possible biological use. Besides, the tensioactive properties identified led us to investigate AEOE membrane interactions. From coupling constants and linewidths considerations, a detergent effect with macroagregate formation as not involved, except for low amounts of AEOE, as revealed by the relatively broad ^{31}P NMR line. Both the resolved lines and the small averaged ^{31}P^{1} H coupling constants also precluded the existence of any stable conformation. Finally, the temperature and concentration dependent mechanism of interaction would be summarized as follows:

– For low concentrations of AEOE and below phase transition, the broad isotropic contribution to ^{31}P NMR spectra is quite similar as that classically observed when solvents (Waikite et al. 1992) or small molecules (Markman et al. 1993; Makryanis et al. 1990) integrate the membrane and could be described as local fluidizing or detergent effect;
– Over phase transition and for low AEOE amounts, the overall membrane fluidity allows an homogenous repartition of AEOE in the main structure and the isotropic contribution is no longer detectable;
– For high amounts over transition temperature, the excess of AEOE precludes a complete distribution in the membrane, while the fluidity (or the energy conferred by temperature rise) allows the solubilization of some phospholipids and a phase separation; the result is the superimposition of a very resolved line (the solubilized DMPC molecules) and of normal bilayer structure (with normal CSA values).

The role of membrane fluidity is supported by results obtained in SUV (not shown); in this case, the isotropic resolved line only progressively appears after several hours evolution time (final state reached after 24 h); the small SUV diameter (150 nm) results in membrane curvature and rigidity dramatically higher than those of dispersions, and finally a supplementary physical barrier to overcome for membrane interactions.

^{31}P NMR undecoupled spectra and ^{2}H NMR patterns obtained with DMPC dispersions suggested an important conformational averaging of phosphodiester bonds, associated with an overall fluidization at the chain level. Furthermore, NOESY experiments (from 70 to 300 ms, not shown) revealed no spatial connectivity, thus precluding the presence any stable conformation.

At this step, we have obtained a cation complexing structure with a relative selectivity for alkaline earths, especially cesium ions. Furthermore, the mechanism of the hemolytic activity of AEOE was identified as a true membrane solubilization. The perspectives are presently to enhance this selectivity by varying the chain length and/or ether bond position inside the side chain. This would also allow to control the hydrophobic/hydrophilic balance (i. e. logP) in order to obtain either soluble short chained derivatives, or more amphiphilic molecules more adapted to surface and biological use.

4. Experimental

4.1. Materials

4.1.1. Synthesis of hexakis (3,6-anhydro) tetrakis (2^{A,B,D,E}-O-ethyl-O-ethyl) cyclomalto hexaose (AEOE)

AEOE was prepared from intermediate hexakis (3,6-anhydro) α-cyclodextrin according to Gadelle and Defaye (1987). Hexakis (3,6-anhydro)α-cyclodextrin (864 mg, 1 mmol) was dried at 105 °C (3 h – vacuum pomp). The product was stirred in a mixture of DMSO (10 ml) and DMSO-NaH (6 ml, 2N) under argon. After 3 h, 2-bromoethyl ethyl ether (1.17 ml,

11 mmol) was added. The solution was heated to 45 °C. After 48 h solvents were removed under reduced pressure. The solid residue dispersed in water was ultrafiltrated to remove mineral material (Millipore, YCO5 – 2L water). Hexakis (3,6-anhydro) tetrakis ($2^{A,B,D,E}$ – ethyl ethyl ether) α-cyclodextrin was the main product in the crude mixture. The solution freeze-dried was purified on a silica gel chromatography column (MeOH: $CHCl_3$, 1 : 4 – rf 0.25). The crystalline product (322 mg, 28%) was controlled by NMR (see section 2.1 for peak attribution) and MS (ES+) analysis.

4.1.2. Chemicals

Cation nitrate salts, dimyristoylphosphatidylcholine (DMPC, proton nomenclature is given on the Fig. 1B), glycerophosphorylcholine (GPC), phosphatidylcholine from egg yolk (PC) and deuterated solvents were purchased from Sigma (La Verpillère, France) and were used as received. Chain perdeuterated DMPC-d_{54} was from Interchim, Montluçon, France.

4.1.3. Multibilayers (MLV)

DMPC liposomes for ^{31}P experiments were prepared by successive freezing and thawing cycles (Roux 1987) until an homogenous milky sample was obtained (Debouzy et al. 1988). The suspensions were degassed under nitrogen gas then introduced into NMR tubes and sealed. The final lipid concentration was 50 mM, while AEOE/DMPC in mixed systems was ranged from 1/16 to 1/8, M/M. The same procedure was used for multilayers for ^2H NMR experiments, except that 25% DMPC with perdeuterated chains were used (DMPC-d_{54}) to build liposomes.

4.2. Methods

4.2.1. Haemolytic activity

All procedures were in accordance with the standards for animal care established by our institute and were approved by our animal use ethic committee (decree 87–848 19 October 1987).

Blood from male Sprague-Dawley rats was collected in heparinated and washed twice using isotonic NaCl solution; the hematocrit was then brought to 10%. 4 mL cuves were filled with the cyclodextrin solutions to test, and 100 μL of the diluted blood were added. The samples were stocked for 1 h at 37 °C, then centrifuged at 2400 rpm, 4 °C for 10 min. Absorption measurements were finally performed on a Shimazu MCS-2000 absorption spectrometer at 540 nm, as described elsewhere (Debouzy et al. 1998; Ribarov et al. 1980).

The haemolytic activities were expressed in terms of HC_{50}, the concentration giving 50% haemolysis as referenced to i) the total haemolysis induced by triton X-100 addition or on sonicated samples ii) the absence of any haemolysis (0% haemolysis) evaluated on samples where only isotonic NaCl (0.9% W/W) solution was added.

4.2.2. NMR experiments

All NMR experiments were recorded on a Brüker AM-400 spectrometer. ^1H NMR spectra were acquired at 293 K using a presaturation of the water resonance and a spectral width of 10 ppm. The chemical shifts were referenced by setting the water resonance at 4.75 ppm. ^1H NMR attribution was performed as classically described using 1D and 2D (COSY, TOCS, Sanders 1989) experiments at 293 K, 2 mM, D_2O.

In ^1H NMR complexation screening experiments, the concentration of AEOE was 1 mM, and that of the cation tested was 1 mM (stoechiometry 1/1), 5 and 20 mM (saturation).

Dipolar correlation experiments were recorded at 298 K on the centrifugation (2000 rpm, 10 min), supernatant.

^{31}P NMR experiments were performed at 162 MHz. Phosphorus spectra were recorded using a dipolar echo sequence (π/2-t-π-t) (Mavromoustakos et al. 1999) with a t value of 12 μs and a broadband two levels proton decoupling. Phosphoric acid (85%) was used as external reference. Undecoupled spectra and partial continuous wave proton low level decoupling (24 L) were also used to measure phosphorus-proton coupling constants.

^2H NMR experiments were performed at 61 MHz. Deuterium spectra were recorded by using a quadrupolar echo sequence (π/2-t-π/2-t) with a t value of 20 μs. The free induction decay was shifted by fractions of the dwelling time to ensure that its effective time for the Fourrier transform corresponds to the top of the echo (Dufourcq 1986).

4.2.3. LogP estimation

LogP was determinated by two methods; a first estimation was obtained by the classical octanol/water partition method (Schlechter 1990) using ^1H NMR of the H_1 integrated resonances in the water and in octanol. Briefly, starting from 1 mM solutions of the given derivative in water, and in octanol, ^1H NMR spectra were recorded as reference. Both solutions were then mixed and the mixture centrifugated to allow phase separation. Finally, ^1H NMR spectra of these phases were recorded in quite identical conditions, and H-1 resonance intensities (integral) were used to establish the respective concentrations of cyclodextrin in octanol and water, which gave the partition coefficient LogP. A direct structure-based calculation of LogP was used using SMILES coded structures issued from Chemdraw, and introduced in the procedure found in the website http://www.daylight.com/dayhtml/.

4.2.4. Surface tension measurements

Measurements were done on a Tensiometer CSC-Du Nouy (CSC N°70535) using the ring method of measurement. AEOE was dissolved in DMSO and measurements were made in 20 ml of water. Pure water from MilliQ (18.2 MΩ.cm) was used as reference (75.8 mN/m at 293 K).

Acknowledgements: Thanks to Bruno Perly for SIMPLEX COMPLEX program, to Prof. J. Defaye and Dr F. Estour for helpfull corrections, to Pr G. R. Hausbadern for stimulating discussions and to Bertrand Lefebvre for mass spectra.

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