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Synthesis and spectral properties of fluorescent linear alkylphosphocholines labeled with *all*-(*E*)-1,6-diphenyl-1,3,5-hexatriene

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ABSTRACT

A synthetic method has been designed to produce alkylphosphatidylcholine lipid molecules with the fluorescent group *all*-(*E*)-1,6-diphenyl-1,3,5-hexatriene (DPH) incorporated to the end of a polymethylene chain of variable length. The resulting compounds may be viewed as single-chain fluorescent lipid probes terminated with a phosphocholine (PHC) polar head-group. The method was applied to the synthesis of two lipids with different alkyl chain length, PHC-C4-DPH (**1**) and PHC-C6-DPH (**2**), as well as the corresponding alcohols OH-C4-DPH (**8**) and OH-C6-DPH (**9**). The absorption and fluorescence properties of these compounds in fluid solution were very similar to those of the well-known lipid probe propionic acid-DPH (PA-DPH). In addition, it was observed that the alkylphosphocholines were easily incorporated into unilamellar lipid vesicles, in which the DPH group would be positioned most likely near the centre of the bilayer. In these conditions, **1** shows a fluorescence quantum yield of 0.4 ± 0.1 and a relatively simple decay time, with τ_{av} close to 5.0 ns. Fluorescence polarization experiments also indicate that the reorientational motions of the DPH group of phosphocholine **1** are sensitive to the fluidity changes that accompany the main thermal phase transition of the bilayer. As a result, both compounds may be of utility as novel fluorescent lipid probes, characterized by a biomimetic zwitterionic polar head-group.

1. Introduction

The study of lipid function in natural or artificial systems could be greatly simplified by means of the current large array of experimental methods based on fluorescence spectroscopy. However, since lipids usually lack intrinsic fluorescence, the application of these methods requires first attaching a suitable emitting group to the lipid molecule, either at its hydrophilic head-group or at the hydrophobic alkyl chain [1]. As a result of that, the delicate amphipatic balance of the original molecule is altered, frequently perturbing one or several properties of the parent compound. We were interested in the application of fluorescence microscopy techniques to understand the antiparasite mechanism of single-chain alkyl-lipids, as the phosphocholine (PHC) ester of the fatty alcohol *n*-hexadecanol (miltefosine, Fig. 1), a drug used to treat human leishmaniasis infections and other diseases [2]. The PHC head-group of the molecule is important for its bioactivity and, therefore, fluorescence labeling of the alkyl-lipid can only be carried out at the polymethylene chain. Simple conjugated polyenes, like tetraene [3,4] or pentaene [5,6] groups, may be inserted in the saturated alkyl chain as emitting labels, hopefully minimally perturbing the original physico-chemical properties of the drug [1]. Unfortunately, the UV excitation (ca. 350 nm) required by these weakly emitting groups is inconvenient for living-cell fluorescence imaging methods, although in some instances two-photon excitation techniques may overcome this limitation [7]. Conjugated *phenyl*-polyenes show red-shifted absorption transitions and, in fact, a fluorescent analogue of miltefosine with potent antiparasite activity was produced by attaching a phenyltetraene chromophore to the parent drug [8]. Both, the excitation wavelength (ca. 380 nm) and the photochemical stability of this compound were more favorable than those of the simpler linear conjugated polyenes mentioned above. Further improvement in the photochemical and photophysical properties of the analogues may be achieved using *all*-(*E*)-1,6-diphenyl-1,3,5-hexatriene (DPH) as emitting tag.

The lipophilic DPH fluorophore became popular in lipid membrane research because of its large absorption coefficient and fluorescence yield, extensive dynamic characterization in fluid solution and lipid bilayers and, importantly, high photochemical stability [9–11]. A series of lipid probes have been produced by attaching simple polar head-groups to the DPH fluorophore, such as the trimethylammonium cation [12–15] or the negatively charged propionic acid group [16–18]. In this way, the transverse location of the fluorescent tag of the probe in a lipid membrane can be better

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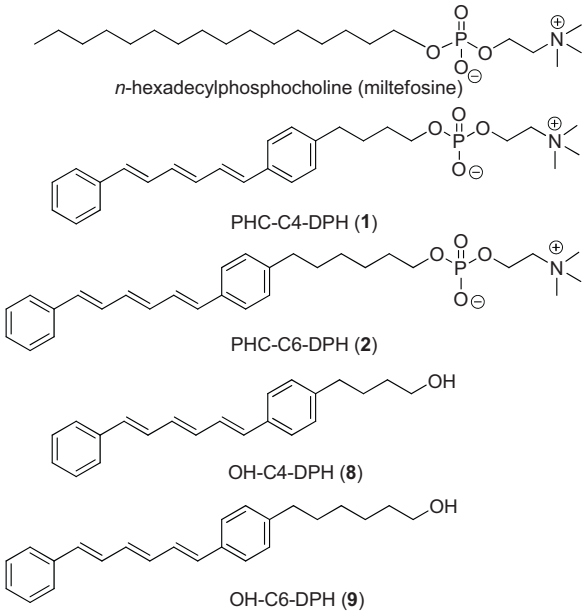


Fig. 1. Structure of the fluorescent alkylphosphocholines **1** and **2**, the corresponding alcohols **8** and **9** and of miltefosine.

defined [19]. In addition, the DPH fluorophore has been incorporated to one of the acyl chains of different glycerophospholipids with the same purpose [20].

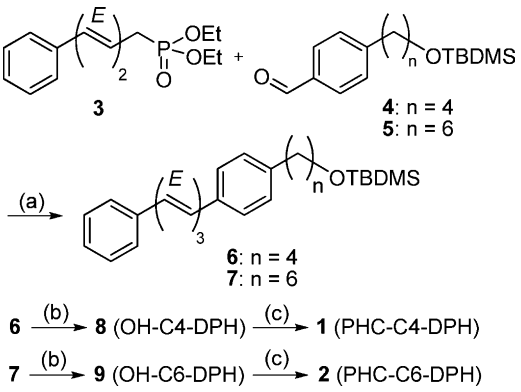
Herein we report the synthesis and spectral properties of fluorescent alkylphosphocholines with a structure that is intermediate between these two types of compounds. In these novel single-chain DPH-tagged lipids, the emitting group is bound to a saturated C_4 or C_6 alkyl chain tethered to a phosphocholine head-group (Fig. 1). The *all*-(*E*) stereochemistry of the three conjugated double bonds was selected to minimize the perturbation of the lipid arrangement in the membrane. The data presented here indicate that these compounds may be of general utility as probes of lipid function and structure in natural and artificial membranes.

2. Experimental

2.1. Methods

UV/vis absorption spectra were recorded on a Varian CARY 3E spectrophotometer and corrected for scattering when necessary (lipid vesicles suspensions). Steady-state fluorescence excitation and emission spectra and anisotropy were recorded in an ISS PC1 photon counting fluorimeter and corrected for instrumental factors. Relative fluorescence quantum yields of optically diluted samples were determined by the comparative method [21], by reference to that of quinine sulfate in 0.05 M H_2SO_4 ($\Phi_F = 0.51$) [22], with an uncertainty of 10–20%. Fluorescence lifetimes were recorded in a picosecond laser spectrometer described elsewhere [23], using the time-correlated single-photon counting technique. Data analysis was carried out by a non-linear least-squares formalism using a commercial package (Globals, Laboratory for Fluorescence Dynamics, Univ. California, Irvine).

Large unilamellar vesicles (LUV) of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC, 99.0% purity, from Avanti Polar Lipids, USA) in 10 mM pH 7 aqueous phosphate buffer were prepared by extrusion of a suspension of multilamellar vesicles through 0.1 μm filters, as described elsewhere [23]. The average LUV diameter was ca. 80 nm, as determined by standard light-scattering techniques. Total lipid concentration was determined by the phosphomolybdate method [24]. Two different procedures were used to incorporate the fluorescent alkylphosphocholines to the LUV bilayer. In the first case, a few microliters of a concentrated ethanol solution of the compound were added to the aqueous LUV suspension. In a second method, the phosphocholine was dissolved at the start with DMPC, before LUV preparation. Scattering and background emission contributions were subtracted from the absorption and fluorescence anisotropy spectra, respectively, using blank LUV suspensions free of the emitting compound.

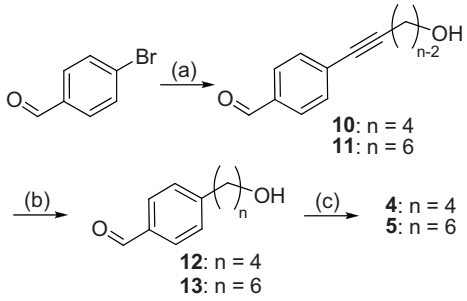


Scheme 1. Reagents and conditions: (a) MeONa, THF, 0 °C to r.t., 1 h, 98% (**6**), 80% (**7**); (b) tetrabutylammonium fluoride, THF, r.t., 50 min, 96% (**8**), 97% (**9**); (c) 2-chloro-1,3,2-dioxaphospholane-2-oxide, NMe_3, MeCN, r.t., 3 h, then 70 °C, 4 h, 38% (**1**), 17% (**2**).

2.2. Synthesis

Compounds PHC-C4-DPH (**1**) and PHC-C6-DPH (**2**) were designed as to preserve the linear dimension of the parent lipid *n*-hexadecylphosphocholine. They were obtained through a stereoselective Horner–Wadsworth–Emmons (HWE) coupling, a procedure used previously for the synthesis of a conjugated phenyltrienyne alcohol analogue of *n*-hexadecylphosphocholine [6]. In brief, diethyl [(2*E*,4*E*)-5-phenylpenta-2,4-dienyl]phosphonate (**3**) – prepared in four steps from (2*E*)-cinnamaldehyde, as described previously [18] – was made to react with the silylated aldehydes **4** and **5**, yielding with good yields the corresponding silylated *all*-(*E*) compounds **6** and **7** (Scheme 1). ^{1}H NMR spectra of **6** confirmed the (*E*) stereochemistry of the generated double bond, with less than 1% of the corresponding (*Z*) isomer. Irradiation of the proton signals of the central double bond of the DPH system allowed the simplification of the two AB systems assigned to the protons of the new double bond and of the Ph–CH=CH– double bond, with coupling constants close to 15 Hz and, hence, with (*E*) configuration. This agrees with previous results on the simulated ^{1}H NMR spectrum from another DPH compound [18]. The silyl group was separated with tetrabutylammonium fluoride in THF, yielding the corresponding alcohols **8** and **9** with good yields. Eventually, the introduction of the phosphocholine moiety by reaction with 2-chloro-1,3,2-dioxaphospholane-2-oxide and trimethylamine [8] provided the target compounds PHC-C4-DPH (**1**) and PHC-C6-DPH (**2**), respectively, both with all-(*E*) stereochemistry, as confirmed by ^{1}H NMR spectra. This synthetic method is easily scalable and allows the preparation of other analogues with different alkyl chain length and different terminal polar groups, as well as with different substituents in the terminal phenyl group.

Both phosphochlines **1** and **2**, as well as the corresponding alcohols **8** and **9**, are photostable as DMF, DMSO or MeOH solutions. In solid form and kept at −20 °C in the dark, they are stable for years. While analogues **1** and **2** are soluble only in polar solvents (MeOH, EtOH, DMSO, DMF), the corresponding alcohols **8** and **9** are rather soluble (>10^{-3} M) in chloroform, dichloromethane, diethyl ether, THF, ethyl acetate and acetonitrile.



Scheme 2. Reagents and conditions: (a) 3-butyn-1-ol ($n=4$) or 5-hexyn-1-ol ($n=6$), $Pd(PPh_3)_2Cl_2$, CuI, THF, Et_3N, r.t., 48 h, 91% (**10**), 85% (**11**); (b) Pd/C (10%), 40 psi, AcOEt, r.t., 3 h, 95% (**12**), 70% (**13**); (c) TBDMSCl, imidazole, THF, r.t., 3 h, 95% (**4**), 100% (**5**).

Aldehydes **4** and **5** were prepared by a three-step sequence (Scheme 2) starting with the reaction between 4-bromobenzaldehyde and 3-butyn-1-ol or 5-hexyn-1-ol, respectively, under Sonogashira–Hagihara cross-coupling conditions [25], yielding the corresponding compounds **10** and **11**. After triple-bond hydrogenation in ethyl acetate, in order to reduce the hydrogen reactivity and, hence, to preserve the aldehyde group, *p*-hydroxyalkylbenzaldehydes **12** and **13** were obtained. Their reaction with *tert*-butyldimethylsilyl chloride (TBDMSCl) yielded **4** and **5**, respectively. See Supplementary Data for additional information on the synthesis of all the former compounds.

3. Results

The absorption and fluorescence spectra of PHC-C4-DPH (**1**) in dimethylformamide (DMF) solution are shown in Fig. 2. The absorption and emission spectra of the other DPH compounds obtained in this work (Fig. 1) are very similar to those in Fig. 2, and only the spectral maxima are reported in Table 1, together with the values of the fluorescence quantum yield (Φ_F) and lifetime (τ_F). The decay of the fluorescence intensity in DMF solution is complex and requires a multi-exponential best-fitting function. This is illustrated for the case of PHC-C4-DPH in Fig. 3, that shows overlaid the experimental data and a three-exponential fitting function. All these spectral characteristics remind those described for the free DPH chromophore, and do not differ substantially from those observed before in other DPH-labeled compounds. Thus, the shape of the absorption band centered at ca. 361 nm is nearly identical to

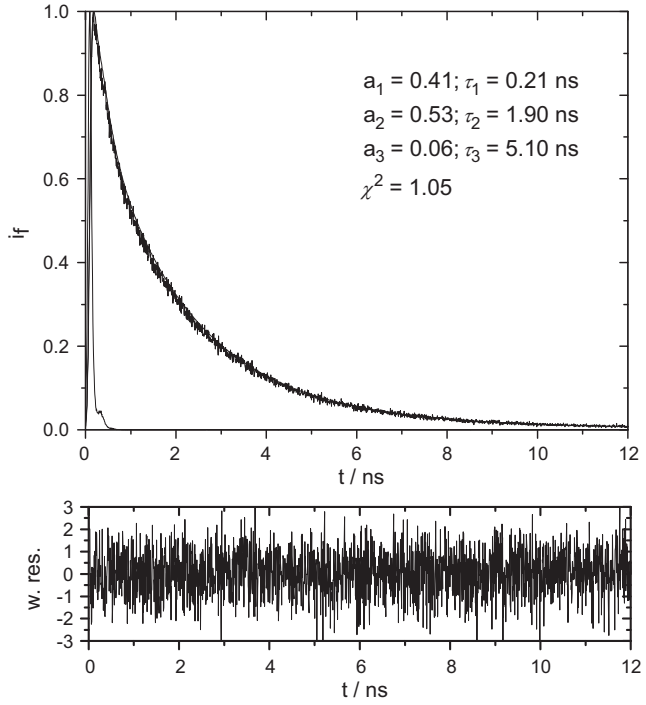


Fig. 3. Fluorescence lifetime of PHC-C4-DPH (**1**) in dimethylformamide solution ca. 10^{-6} M. Up: experimental decay and three-exponential best-fitting function (overlaid). Down: weighted residuals distribution; $\lambda_{exc}=372$ nm, $\lambda_F=535\pm8$ nm, $T=23\,^{\circ}C$.

that of the unsubstituted DPH chromophore, but is red-shifted relative to that of the spectrum recorded in non-polar solvents. This shift is due to the sensitivity of the DPH main absorption transition to solvent polarity and polarizability [11].

The emission yield and lifetime of alkylphosphocholines **1** and **2**, and those of the corresponding alcohols **8** and **9**, are very similar, indicating very small perturbation of the spectroscopic properties of the pending chromophore by the polar head-group in DMF solution. These fluorescence properties are also very similar to those of the parent DPH fluorophore in polar solvents, as e.g. ethanol [11].

The absorption and emission spectra of PHC-C4-DPH (**1**) incorporated into unilamellar lipid vesicles (LUV) of DMPC are shown in Fig. 4, and the corresponding spectral parameters are listed in

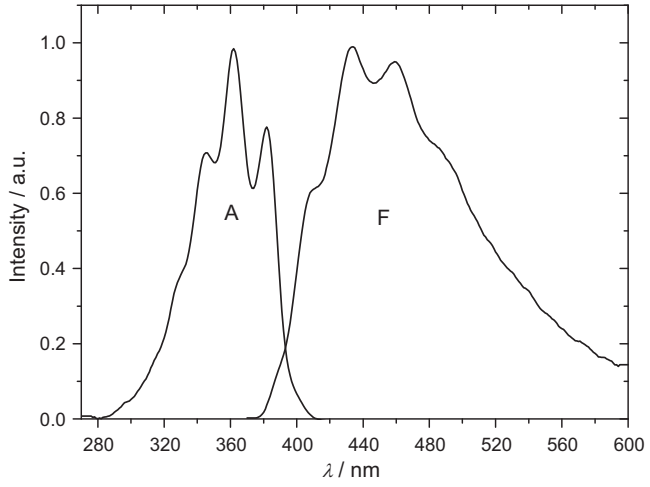


Fig. 2. Absorption (A) and corrected fluorescence (F) spectrum of PHC-C4-DPH (**1**) in dimethylformamide solution ca. 10^{-6} M; $\lambda_{ex}=360\pm2$ nm, $T=23\,^{\circ}C$.

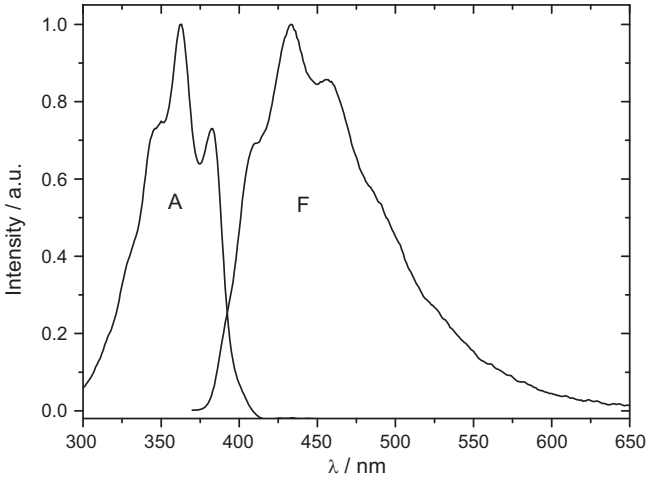


Fig. 4. Absorption (A) and corrected fluorescence (F) spectrum of PHC-C4-DPH (**1**) incorporated into DMPC large unilamellar lipid vesicles. pH 7.0 phosphate buffer, probe:lipid molar ratio 1:300, [DMPC] = 6×10^{-4} M, $\lambda_{exc}=380\pm1$ nm, $T=35\,^{\circ}C$.

Table 1
Absorption (λ_{abs}) and fluorescence (λ_F, Φ_F, τ_F) properties of lipid analogues containing the *all*-(*E*)-1,6-diphenyl-1,3,5-hexatriene (DPH) emitting group. T = 23 °C.

Compd.	Solvent[a]	λ_{abs}/nm[b]	λ_F/nm	Φ_F	a_1	τ_1/ns	a_2	τ_2/ns	a_3	τ_3/ns	τ_{av}/ns[c]
PHC-C4-DPH	DMF	344, 361, 382	434	0.16	0.40	0.22	0.54	1.9	0.06	5.1	2.5
	DMPC, 35 °C	347, 362, 381	433	0.36[d]	–	–	0.14	1.3	0.86	5.0	4.9
	DMPC, 15 °C	347, 363, 383	433	0.38[d]	–	–	0.15	2.0	0.85	5.5	5.3
OH-C4-DPH	DMF	344, 361, 382	433	0.21	0.40	0.24	0.56	1.9	0.04	5.0	2.2
PHC-C6-DPH	DMF	345, 361, 381	458	0.17	0.30	0.23	0.55	1.8	0.15	5.2	3.2
OH-C6-DPH	DMF	345, 361, 381	460	0.16	0.30	0.22	0.53	1.8	0.17	5.1	3.3

[a] DMF: dimethylformamide; DMPC: large unilamellar vesicles (LUV) of dimyristoylphosphatidylcholine.
[b] Spectrum maximum underlined.
[c] Average lifetime, $\tau_{av} = \sum a_i \tau_i^2 / \sum a_i \tau_1$.
[d] Uncertainty 30%.

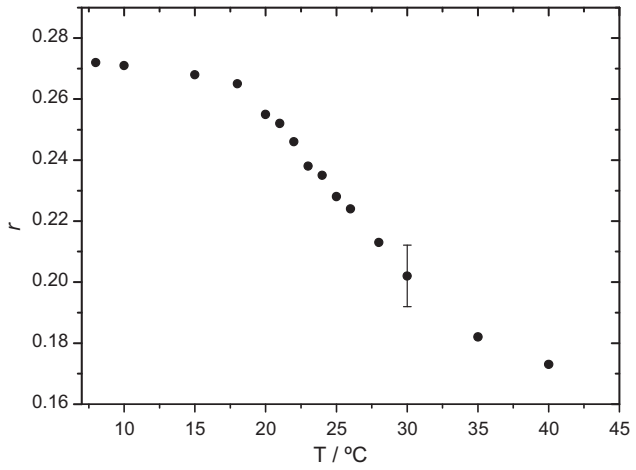


Fig. 5. Steady-state fluorescence anisotropy (r) as a function of temperature of PHC-C4-DPH (**1**) incorporated into DMPC unilamellar lipid vesicles. [DMPC] = 5×10^{-4} M, probe:lipid = 1:200, λ_{exc} = 360 ± 1 nm, λ_F = 450 ± 10 nm, pH 7.0 phosphate buffer.

Table 1. The contribution due to LUV scattering in the absorption spectrum has been subtracted. The spectra in the lipid bilayer are similar to that reported above in DMF solution, although the fluorescence yield and average lifetime increased by ca. 100%. These larger values are due to the suppression of the subnanosecond decay channel, accompanied by a large increase of the fractional contribution of the 5 ns lifetime component (Table 1). At temperatures below the DMPC gel/liquid-crystalline transition (T_m = 23 °C), the alkylphosphocholine fluorescence is strongly polarized (Fig. 5). By increasing the temperature of the LUV suspension, the value of the emission anisotropy of the phosphocholine decreases substantially, revealing the bilayer fluidification process.

4. Discussion

The emitting lipids studied here have in common with natural fatty acids and acylphospholipids the main physical and chemical properties. Thus, for example, the estimated length of the C4-DPH chain in a fully extended conformation is ca. 20 Å, which is identical to that of the palmitoyl residue, and similar to the average half-width of a fluid lipid bilayer. As a result of that, DPH-phosphocholine esters **1** and **2** may show enough compatibility with natural lipids to probe a large variety of lipid physical phenomena. For that purpose, some understanding of the fundamental photophysical properties of these compounds would be of utility. As noted above, the main absorption band of these compounds is due to the DPH group, and it is not affected by the type of polar head-group. The small red-shift (2–3 nm) of this band relative to that of *all*-(*E*)-diphenylhexatriene in the same solvent [13] is similar to that observed in other alkyl-substituted DPH compounds [26]. By analogy, this absorption band can be assigned to the symmetry-allowed transition to the S_2 electronic state, as was established [27] for the parent DPH (i.e. $1^1B_u \leftarrow 1^1A_g$). This is consistent with the red-shift of the alkylphosphocholine absorption in DMF solution relative to that of unsubstituted DPH in hydrocarbon solvents (e.g. λ_{max} = 355 nm in *n*-hexane [9d]), due to lowering of the energy of the 1^1B_u state in high-polarizability solvents. The absorption coefficient of PHC-C4-DPH (**1**) in ethanol solution (60100 ± 2000 M^{-1} cm^{-1}, λ_{abs} = 356 nm) is ca. 25% lower than that of the parent DPH, but very similar to that of the propionic acid-DPH probe (PA-DPH) [16]. The fluorescence emission of the alkylphosphocholines **1** and **2** shows a large Stokes shift and lack of mirror symmetry, indicating that the emission should take place largely from the lowest S_1 excited state (the symmetry-forbidden 2^1A_g state in DPH). The complex fluorescence decay of these compounds in DMF (Table 1) is likely due to interactions between the fluorophore excited-states promoted by solvent-induced decrease of the S_1–S_2 energy gap. These aspects of the fluorescence of the DPH group have been studied in great detail by Saltiel and coworkers [28]. It is interesting that the average fluorescence lifetime of PHC-C4-DPH (**1**) in DMF solution reproduces that of PA-DPH in the same solvent [16].

The solubility of compounds **1** and **2** in water and in aqueous phosphate buffers is very low. In the 10^{-5} to 10^{-6} M concentration range, these phosphocholines form non-emitting aggregates, which are detected by increased scattered light (data not shown). In these conditions, it is very likely that these compounds are present as micellar aggregates, since they are essentially zwitterionic surfactant molecules. In that case, the DPH chromophores would be oriented towards the micelle core, favoring electronic interactions that would quench the fluorescence. The critical micelle concentration (c.m.c.) of the parent compound *n*-hexadecylphosphocholine (Fig. 1) has been determined several times using a variety of techniques [29]. Although the reported values span a wide range (2–160 μM), most of them cluster around the lower end of this range. The c.m.c. of **1** and **2** should be even lower, considering the strong lipophility of the pending DPH group.

When added to a DMPC vesicle suspension, the DPH-phosphocholines bind quickly to the lipid vesicles, as detected by the increased fluorescence emission of the bound-form (data not shown), while the fraction remaining in the aqueous buffer is virtually non-fluorescent. Preliminary measurements of the absorption and fluorescence spectra of PHC-C4-DPH embedded in LUV (Fig. 4 and Table 1) show values similar to those of the PA-DPH probe in lipid vesicles. The quantum yield of PHC-C4-DPH embedded in the LUV lipid bilayer went up to ca. 0.4 (the PA-DPH value in similar conditions is 0.73 [16]). There is also a large change in the fluorescence lifetime of phosphocholine **1** from DMF solution to the lipid bilayers. In the LUV, the decay becomes simpler, and can be described by a biexponential function dominated by a large fraction (85%) of the 5 ns component (Table 1). As indicated above, phosphocholine **1** was incorporated into LUV in two different ways. In

the first one, compound **1** was added to the LUV buffer suspension, while in the second compound **1** was dissolved together with DMPC before preparing the vesicles. In the first case compound **1** would be associated only with the external lipid monolayer, due to the slow flip-flop rate, while in the second procedure the probe would be randomly distributed between the external and internal bilayer leaflets. Nevertheless, the spectral properties of PHC-C4-DPH recorded here were virtually the same in both cases. In the case of the hydroxy-terminated compounds **8** and **9** the rate of interchange between the two bilayer surfaces is expected to be faster.

The polarized fluorescence of PHC-C4-DPH (**1**) is very sensitive to changes in lipid order and fluidity that take place at the bilayer main thermal transition (Fig. 5), which indicates that the emitting molecules are incorporated to the membrane. The relatively low anisotropy value in the gel phase and the broadened temperature range of the recorded change have been also observed for other probes in small unilamellar vesicles [30]. These experiments indicate that the chromophore is very likely buried in the most fluid part of the lipid bilayer, as would also be expected from the phosphocholine chain length. To determine more precisely the transverse location of the DPH group in the bilayer additional experiments would be necessary [19].

5. Conclusions

A general method for the synthesis of fluorescent alkylphosphocholines is detailed here. It can be used to prepare single-chain lipids with different length and polar head-group, incorporating DPH or its derivatives as fluorescent tag of the alkyl chain. This procedure was used to obtain two single-chain emitting alkylphosphocholines incorporating the DPH chromophore, PHC-C4-DPH (**1**) and PHC-C6-DPH (**2**), as well as the corresponding alcohols. The spectroscopic and photochemical properties of **1** and **2**, both in solution and in unilamellar vesicles of DMPC, are similar to those of the lipid probe propionic acid-DPH and well adapted for a variety of lipid-related studies. Moreover, these compounds present the added advantage that contain a biocompatible phosphatidylcholine head-group, which would be in zwitterionic form at physiological pH. Overall, the data presented here show the potential utility of these alkylphosphocholines and warrant a more detailed assessment of PHC-C4-DPH and PHC-C6-DPH as fluorescent molecular probes of lipid structure and dynamics in bilayers and cell membranes.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jphotochem.2010.09.010.

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