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Synthesis of new bifluorophoric probes adapted to studies of donor-donor electronic energy transfer in lipid systems

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Abstract

A series of bifluorophoric fluorescent probes with identical pairs of chromophores have been synthesized. The fluorophores are 9-anthryl, 3-perylenyl and rhodamine 101, and they are separated by a long rigid (bisteroid) or flexible (dotriacontane) diol spacer. The probes are designed for studies of intra- and intermolecular electronic energy transfer in lipid systems such as model and biological membranes. They are shown to incorporate in phosphatidylcholine vesicles with an hitherto unknown orientation.

Keywords: Fluorescent bichromophoric lipid probes; Synthesis; Electronic energy transfer

1. Introduction

Electronic energy transfer, or fluorescence resonance energy transfer (FRET) is used widely in membrane studies. The main features of this process have been explored by Förster and Lakowicz [1,2] (for a recent review see [3]). Nevertheless, a general theory for applying FRET in complex systems such as membranes and proteins is yet not available. The complexity in accounting for inter- and intramolecular orientational distributions and motions of the interacting chromophores is a main reason for this.

Previously, we have studied energy transfer between identical molecules, or donor-donor (D-D) transfer, at lipid-water interphases by means of fluorescence depolarization experiments [4]. For a continuation of these studies we have developed D-D probes enabling intramolecular energy transfer over an essentially fixed distance. We have

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Abbreviations: $C_{12}EO_5$, pentaethyleneglycol mono-*n*-dodecyl ether; DCC, dicyclohexylcarbodiimide; DMSO, dimethylsulfoxide; DOPC, dioleoylphosphatidylcholine; DOTAC, dodecyltrimethylammonium chloride; EPC, egg yolk phosphatidylcholine; FRET, fluorescence resonance energy transfer; PMA, phosphomolybdic acid; TMS, trimethylsilyl; TPS, triisopropylbenzenesulfonyl chloride.

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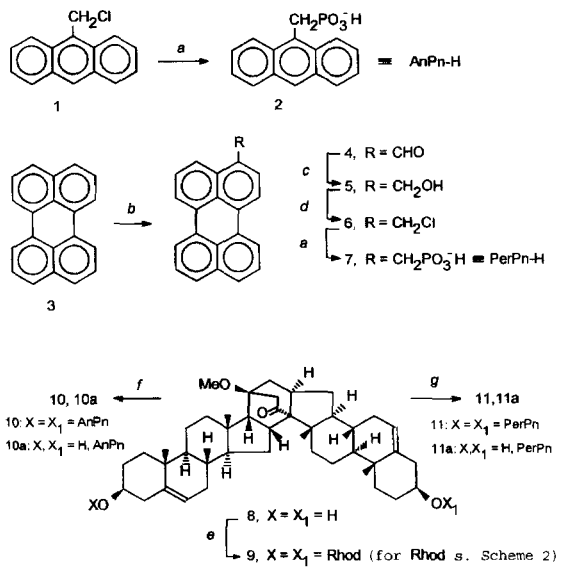


a, $(TMSO)_3P$, then H_3O^+; b, N-methylformanilide + $POCl_3$; c, $NaBH_4$; d, HCl gas; e, Rhodamine 101 + DCC; f, 2 + DCC; g, 7 + DCC.

Scheme 1

synthesized such probes of D-D type with two identical fluorophores (rhodamine 101, 9-anthryl or 3-perylenyl) separated either with the rigid decacyclic system (probes **9**–**11**) or with a long saturated hydrocarbon chain (probe **19**). In the first case, the 'bisteroid' **8** served as a spacer (Scheme 1); in the second — fluorophores were separated by the residue of 1,32-dotriacontanediol **18** (Scheme 2)[1].

These probes could be applied for various purposes. For example, the development of theories for analysing experiments involving energy migration within bichromophoric molecules demands well-defined model systems for testing such models. This is a topic of particular interest to us. Moreover, a bifluorophoric (bolaform, see below) probe spanning across a lipid bilayer reports on changes in local dynamics and order. However, it should also respond to changes in the distance between the fluorescent moieties. Thus, informations about the lipid bilayer thickness, as well as the packing properties of a bilayer could be extracted. 1,32-Dotriacontanediol esterified with the polar rhodamine 101 residues at both ends, serves as an example of such probe.

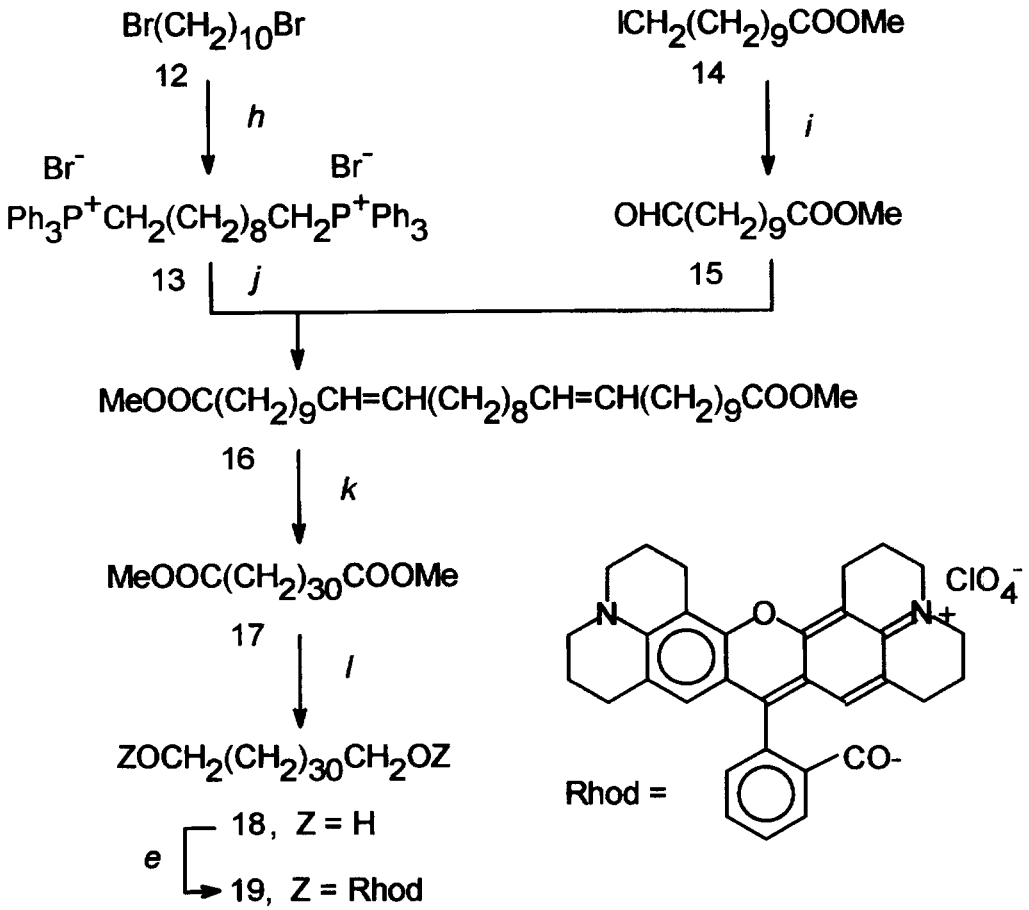
Some features of the probes behavior in model membranes, unilamellar vesicles prepared from egg phosphatidylcholine (EPC) or from dioleoylphosphatidylcholine (DOPC), and in detergent micelles, are quoted here.

2. Experimental

Fluorescent substances were handled under diffused light of incandescent lamps. Absorption spectra were recorded with a LKB Ultrospec II spectrophotometer. Mass spectra (EI ionisation) were registered with a Varian MAT 44 apparatus, FAB ionisation mass spectra with a Kratos MS-50TC spectrometer (Xe atoms, 6–8 keV; matrix: glycerol; positive ions registration), ^{252}Cf-plasma desorption time-of-flight mass spectra with a MSVK instrument ('Elektron' company, Sumy, Ukraine). ^1H-NMR spectra (δ values in ppm) were registered on a Bruker WM 500 spectrometer at 500 MHz in C^2HCl_3; ^{31}P-NMR spectra (^1H-decoupled) were obtained on the same instrument at 202.4 MHz with 85% H_3PO_4 as a standard. Melting points (corrected) were taken on a Kofler hot stage.

Solvents were purified and dried on standard procedures; all evaporations were performed in vacuo at temperatures less than 40°C. DOPC was from Avanti Polar Lipids (USA), dodecyltrimethylammonium chloride (DOTAC) from Eastman Kodak (USA), 9-chloromethylanthracene, *N,N'*-dicyclohexylcarbodiimide (DCC), perylene, 4-pyrrolidinopyridine, trimethylchlorosilane, trimethylorthoformate and triphenylphosphine were from Fluka; rhodamine 101 (perchlorate) from Lambda Physik (Germany), 1,10-dibromodecane, lithium aluminium hydride, *N*-methylformanilide, silica gel 60 and 100 for column chromatography and precoated plates for TLC (silica gel 60, and $RP\text{-}8_{254}$) from Merck; pentaethyleneglycol mono-*n*-dodecyl ether ($C_{12}EO_5$) from Nikko Chemicals (Japan); Sephadex LH-20 from Pharmacia; 3β-acetoxy-5,16-pregnadien-20-one from Sigma. Methyl 11-iodoundecanoate [5], 3-perylenealdehyde [6], potassium *tert*-pentoxide [7], dry trimethylamine *N*-oxide [8], and tris(trimethylsilyl)phosphite [9] were synthesized as described previously.

[1] A trivial name 'bisteroid' is used for this substance instead of the regular chemical name which is too complex and long.



e, Rhodamine 101 + DCC; *h*, Ph_3P; *i*, Me_3N;

j, *t*-$C_5H_{11}OK$; *k*, H_2/Pt; *l*, $LiAlH_4$.

Scheme 2

All solvent ratios are given by volume. Main systems for TLC: toluene/ethyl acetate, 9:1 v/v (A) and chloroform/methanol/conc. NH_4OH, 65:40:12 v/v (B) or 100:22:4 (C). Visualization: phosphomolybdic acid (*1*; universal), molybdenum blue [10] (*2*; for lipid phosphonates) and UV light (*3*; for fluorescent substances). HPLC was performed isocratically on an Altex 334 apparatus equipped with a model 153 UV detector (254 nm) or a Beckman 157 fluorescence detector.

2.1. Bisteroid **8**

Bisteroid **8** was prepared by the method described previously [11]. Firstly, starting from 3β-acetoxy-5,16-pregnadien-20-one (700 mg) bisteroid diacetate was obtained with 35% yield, m.p. > 350°C; ^{1}H-NMR: 0.78 (3H, s, 18-CH_3), 0.95 (3H, s, 18′-CH_3), 1.02 (3H, s, 19-CH_3), 1.04 (3H, s, 19′-CH_3), 2.03 (6H, s, CH_3CO), 3.23 (3H, s, CH_3O), 4.60 (2H, unresolved m, H-3, H-3′),

5.37 (2H, m, H-6, H-6'). This substance after alkaline hydrolysis [11] gave bisteroid **8** with 95% yield, m.p. >350°C, MS (FAB): *m/z* 643 (M^+). The substance was more than 95% homogeneous according to HPLC data of dibenzoate prepared by the action of benzoyl chloride in pyridine (5 μm Zorbax SIL 4.6 × 250 mm column, hexane/chloroform, 60:40 v/v, 1 ml/min, UV detector).

2.2. 3-Hydroxymethylperylene 5

A solution of 3-formylperylene **4** (1 g, 3.56 mmol) in 150 ml dioxane was added with stirring to 53 mg sodium borohydride dissolved in 4.5 ml isopropanol and left overnight. The excess of borohydride was quenched with 3% HCl, reaction products were extracted with chloroform and after drying (Na_2SO_4), separated by column chromatography on silica gel 60 under TLC control (system A: *1,3*). Elution with chloroform gave 0.87 g (86%) of **5** as a deep-yellow powder, m.p. 208–210°C (from chloroform/methanol), TLC: single component on silica gel plate, R_f 0.23 in system A; HPLC: >97% purity of **5** (column: Whatman Partisil ODS PXS 10/25, 4 × 250 mm; acetonitrile/water 85:15 v/v, 1 ml/min; UV and fluorescence detection) or its acetate (column: Macherey-Nagel Nucleosil 50-7, 4 × 250 mm; hexane/ethylacetate/isopropanol, 92:7:1 v/v, 1.3 ml/min; fluorescence detection). MS (EI): *m/z* 282 (M^+), 265 (M^+ − OH), 252 (M^+ − CH_2OH + H); UV in ethanol, nm (log ε): 254 (4.56), 390 (4.08), 412 (4.42), 438 (4.52); fluorescence in ethanol, λ_{max} 446, 475, 506 nm.

2.3. 3-Perylenylmethylphosphonic acid 7 (triethylammonium salt)

Slow stream of dry hydrogen chloride was bubbled in a solution of **5** (0.40 g) in 150 ml dry chloroform monitoring the reaction by TLC on silica gel (system A: *1,3*). After 3 h, the carbinol **5** (R_f 0.23) was completely converted to unstable 3-chloromethylperylene **6** (R_f 0.65, with tailing; if the plate with spots applied on the start are put aside for several min before developing, a spot of **5** will appear in the lane of **6**). The mixture was evaporated and the residue was evaporated in vacuo with two 10-ml portions of dry toluene to give crude **6** (0.43 g, an orange powder), MS (EI): *m/z* 300 (M^+), 265 (M^+ − Cl). It was used for the next step without purification.

A mixture of the above chloride **6** (0.20 g, 0.66 mmol) and tris(trimethylsilyl)phosphite (2.25 ml, 6,7 mmol) was heated at 120–140°C for 6 h under argon. After cooling, the excess of phosphite was removed in vacuo and the residue was stirred overnight with tetrahydrofuran/water, 5:1 (5 ml) and evaporated. The residue was dissolved in chloroform/methanol/water, 65:40:8 (120 ml), washed with water (3 × 25 ml), evaporated, dissolved in the same mixture (110 ml) and evaporated with thiethylamine (0.5 ml). The residue was chromatographed on a column with silica gel 60 in a gradient system chloroform/methanol/water/triethylamine (65:25:4:0.1 → 65:35:4:0.5) to yield 105 mg (37%) of **7** as thiethylammonium salt, a brownish powder with no definite m.p.; TLC (silica gel): single component, R_f 0.28 in system B, and 0.52 in chloroform/methanol/water/acetic acid, 65:35:4:4 (visualization: *1–3*); MS (EI): *m/z* 267 (M^+ − PO_3), 266 (M^+ − PO_3H); UV in ethanol, nm (logε): 254 (4.33), 398 (3.92), 418 (4.23), 444 (4.32); fluorescence in ethanol λ_{max} 454, 483, 513 (shoulder) nm (excitation 420 nm). As the acid **7** did not give mass spectrum with the molecular ion peak, a specimen of dimethyl ester of **7** was obtained by the action of diazomethane; MS (EI): *m/z* 374 (M^+), 265 (perylenyl-CH_2^+); ^{31}P-NMR in $^2H_5$-pyridine: δ 19.85 ppm.

2.4. 9-Anthrylmethylphosphonic acid 2 (pyridinium salt)

9-Chloromethylanthracene (0.58 g, 2.56 mmol) and tris(trimethylsilyl)phosphite (8.5 ml) gave in the analogous synthesis (see above; evaporation with pyridine) 0.56 g (59%) of **2** as an yellowish powder homogeneous on TLC with R_f values close to that of **7**. MS (FAB): *m/z* 273 (M^+ + 1); MS (EI): *m/z* 272 (M^+), 191 (M^+ − PO_3H_2); ^{31}P-NMR in $^2H_5$-pyridine: δ 21.85 ppm; UV in ethanol, nm (log ε): 256 (5.06), 334 (3.37), 350 (3.75), 370 (4.00), 390 (3.99); fluorescence in ethanol λ_{max} 395, 418, 443 nm (excitation 370 nm).

2.5. Bis(3-perylenylmethylphosphonate)bisteroid ***11*** *and mono(3-perylenylmethylphosphonate)bisteroid* ***11a*** *(ammonium salts)*

A mixture of phosphonate **7** (51 mg, 114 μmol) and bisteroid (40 mg, 62 μmol) were dried by evaporation in vacuo with dry pyridine (2 × 5 ml), dissolved in 5 ml dry pyridine under argon and treated with 140 mg of TPS (stirring for 30 min at 70°C), the mixture was cooled to room temperature and water (0.5 ml) was added. The mixture was stirred overnight, evaporated and dried by evaporation with toluene (2 × 10 ml). The residue was filtered through a Sephadex LH-20 column in chloroform/methanol/0.5 N NH_4OH, 65:25:2, to remove triisopropylsulfonate, and separated on a silica gel 100 column in a gradient system chloroform/methanol/7 N NH_4OH (100:5:0.5 → 100:22:4) with the TLC control (system C: *1–3*). This yielded 5.5 mg (7%) of ammonium salt of bis-derivative **11** as an amorphous solid, TLC: single component, R_f 0.23 in the above system; mass spectra of **11** (ammonium salt or free acid) showed no molecular peak. Bis-methyl ester of **11** (obtained by the action of diazomethane on free acid) gave MS (^{252}Cf) with *m/z* 1327 (M^+), 359 [perylenyl-$CH_2P(=O)(OH)OMe^+$]. UV of ammonium salt in ethanol, nm (log ε): 256 (4.62), 398 (4.20), 421 (4.51), 446 (4.58). Also 16 mg (27%) of mono-derivative **11a** (ammonium salt) was obtained; TLC: single component, R_f 0.45 in the same system, UV in ethanol, nm (log ε): 256 (4.33), 398 (3.91), 484 (4.21), 445 (4.28); MS (^{252}Cf): *m/z* 971 (M^+), 347 [perylenyl-$CH_2P(=O)(OH)_2^+$], 265 (perylenyl-CH_2^+); ^{31}P-NMR in $C^2HCl_3/C^2H_3O^2H$, 2:1: δ 22.20 ppm. Fluorescence spectra of 11 and 11a in ethanol show the same maxima at 455, 484 and 515 (shoulder) nm (excitation 420 nm) (see also Fig. 1).

2.6. Bis(9-anthrylmethylphosphonate)bisteroid ***10*** *and mono(9-anthrylmethylphosphonate)bisteroid* ***10a*** *(ammonium salts)*

In the analogous reaction, pyridinium anthrylmethylphosphonate (27 mg, 77 μmol) and bisteroid (40 mg, 62 μmol) gave 12.8 mg (17%) of **10** and 23 mg (40%) of **10a** as amorphous solids. Bis-derivative **10**, TLC: single component, R_f 0.26 (system C: *1, 3*); MS (FAB): *m/z* 1151 ($M^+ - 2NH_3$); UV in ethanol, nm (log ε): 254 (5.17), 350 (4.02), 368 (4.26), 388 (4.23). Mono-derivative **10a**, TLC: single component, R_f 0.46 (system C: *1–3*); MS (^{252}Cf) of free acid: *m/z* 897 (M^+), 273 [anthryl-$CH_2P(=O)(OH)_2^+$], 191 (antyhryl-CH_2^+); ^{31}P-NMR in $C^2HCl_3/C^2H_3O^2H$ (2:1): 19.70 ppm; UV in ethanol, nm (log ε): 352 (3.72), 370 (3.95), 390 (3.91). Probes **10** and **10a** have the same fluorescence maxima in ethanol: 396, 418 and 444 nm (see Fig. 1).

2.7. Bis(rhodaminyl 101)bisteroid ***9***

A solution of diol the **8** (15 mg, 23 μmol), rhodamine 101 (42 mg, 71 μmol) and 4-pyrrolidinopyridine (20 mg, 135 μmol) in 3 ml dry chloroform was treated with 50 μl of the 50% DCC solution in trichloroethylene (25 mg, 2.05 mmol) for a day at 20°C under argon. Then the mixture was diluted with 20 ml chloroform, washed with 3% HCl, twice with water and with sat. NaCl (5 ml each) and dried (Na_2SO_4). Column chromatography of the residue on silica gel 100 in chloroform/methanol/7 N NH_4OH gradient system (92:8:0.7 → 75:25:2) gave 30 mg (78%) of **9** as a deep red gum; TLC: single component, R_f 0.30 on silica gel in chloroform/methanol/7 N NH_4OH, 80:20:1.2; and 0.35 on RP-8 in acetonitrile/acetic acid/water, 90:10:1; visualization: *1,3*. MS (^{252}Cf): *m/z* 1689 ($M^+ - ClO_4^-$), 1098 ($M^+ - ClO_4$ – rhodaminyl), 490 (rhodaminyl$^+$ – H); ^{1}H-NMR: peaks of steroidal part: 0.77 (3H, s, 18-CH_3), 0.93 (3H, s, 18′-CH_3), 1.00 (3H, s, 19-CH_3), 1.02 (3H, s, 19′-CH_3), 3.22 (3H, s, CH_3O), 4.52 (2H, unresolved m, H-3, H-3′), 5.19 and 5.23 (1H and 1H, unresolved triplets, H-6, H-6′); aromatic peaks make three groups of multiplets at 6.55, 7.74 and 8.24 ppm comprising for 12H. UV: in ethanol, nm (log ε): 578 (5.15); fluorescence: λ_{max} 600 nm, (excitation 570 nm) (see Fig. 1).

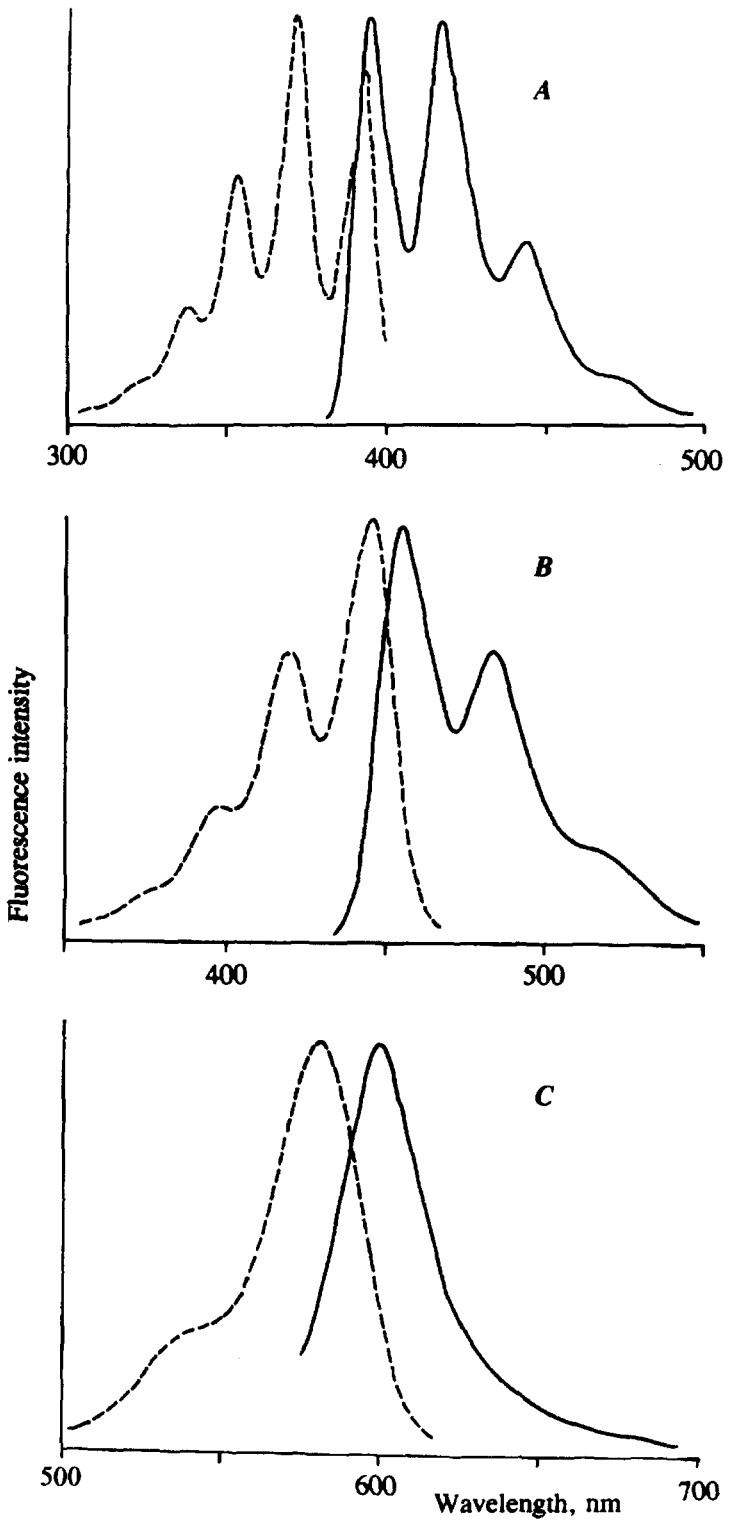


Fig. 1. Normalized fluorescence excitation (- - -) and emission (——) spectra of D-D probes in ethanol. (A) bis(9-anthrylmethylphosphonate)bisteroid **10**; (B) bis(3-perylenylmethylphosphonate)bisteroid **11**; (C) bis(rhodaminyl 101)bisteroid **9**. Excitation spectra: λ_{em} 440 (A), 482 (B) and 620 (C) nm. Emission spectra: λ_{exc} 336 (A), 420 (B) and 570 (C) nm. Concentration 8–12 μM; temperature 20°C.

2.8. 1,10-Bis(triphenylphosphonio)decane dibromide **13**

A solution of 1,10-dibromodecane **12** (11.0 g, 36.7 mmol) and triphenylphospnine (25 g, 95 mmol) in 40 ml dry acetonitrile was refluxed for 30 h at 100°C (bath) and evaporated. The residue was twice crystallized from chloroform/ethyl acetate to give 22.0 g (73%) of white powder, m.p. 248–250°C. Anal. calc. for $C_{46}H_{50}Br_2$: C 67.00, H 6.11, Br 19.38%; found: C 67.3, H 6.3, Br 19.6%.

2.9. Methyl 11-oxoundecanoate **15**

To a solution of dry trimethylamine *N*-oxide (2 g, 2.6 mmol) in 15 ml dry chloroform, a solution of methyl 11-iodoundecanoate **14** (3,5 g, 1,07 mmol) in 5 ml chloroform was added in four equal portions with 1-min intervals under argon atmosphere and stirring. The mixture was then stirred 1 h at 60°C up to nearly complete conversion of **14** (TLC control on silica gel: hexane/methylene chloride/ethyl acetate, 20:20:1, visualization: I and 2,4-dinitrophenylhydrazine). The mixture was diluted with 50 ml benzene containing 20 mg butylated *p*-hydroxytoluene (antioxidant), washed with water, 3% HCl, water and sat. NaCl (20 ml each), dried (Na_2SO_4) and evaporated. The residue after column chromatography over silica gel 60 in hexane/chloroform/ethyl acetate (90:9:1 → 60:36:4) gave the aldehyde **15** as a colorless oil; TLC: single component, R_f 0.60 in the above system; 2,4-dinitrophenylhydrazone, m.p. 56–58°C, MS (EI) *m/z* 394 (M^+), 364 (M^+ – NO). Anal. calc. for $C_{18}H_{26}N_4O_6$: N 14.21%, found N 14.4%.

2.10. Dimethyl 1,32-dotriacontanedioate **17**

To a suspension of phosphonium salt **13** (1 g, 1.21 mmol) in 10 ml dry dimethylformamide stirred in argon atmosphere, 2.4 ml of 0.97 M potassium *tert*-pentoxide in toluene was added by syringe in 2 min, the mixture color turned into orange. After 15-min stirring, a solution of aldehyde **15** (0.78 g, 3.64 mmol) in 3 ml dry dimethylformamide was added at once (the orange color faded quickly) and the mixture was set aside overnight. Then it was diluted with 100 ml ether, washed three times with water and with conc. NaCl (30 ml each), dried (Na_2SO_4) and evaporated. Chromatography of the residue over silica gel 60 in benzene/ether mixtures (monitoring by TLC, system A: *1*) gave 0.54 g (83%) of dimethyl dotriaconta-11,21-diene-1,32-dioate **16** (mixture of *E,Z*-isomers) as an oil; MS (FAB) *m/z* 535 (M^+); ^{1}H-NMR: 1.22–1.38 (36H, broad m, aliphatic protons), 1.62 (4H, m, $C\underline{H}_2CH_2CO$), 2.01 (8H, m, $C\underline{H}_2CH{=}CH$), 2.30 (4H, t, $CH_2C\underline{H}_2CO$, ^3J 7.3 Hz), 3.66 (6H, c, CH_3), 5.2–5.39 (4H, m, *E,Z*-CH=CH).

This diester (450 mg) was hydrogenated over pre-reduced platinum oxide (50 mg) in 15 ml freshly distilled dioxane until process ceased. The catalyst was filtered off and fitrate was evaporated. The residue was twice crystallized from chloroform/isopropanol to give 250 mg (56%) of diester **17**, m.p. 85.5–87°C; MS (FAB) *m/z* 539 (M^+). Anal. calc. for $C_{34}H_{66}O_4$: C 76.06, H 12.02%; found: C 75.8, H 12.2%.

2.11. 1,32-Dotriacontanediol **18**

A solution of diester **17** (150 mg, 0.28 mmol) and 150 mg $LiAlH_4$ in 30 ml dry ether was refluxed for 4 h (control: TLC on silica gel, benzene/chloroform/isopropanol, 10:10:1, UV visualization after spraying with 0.02% rhodamine 6G solution in ethanol). Then $LiAlH_4$ (100 mg) was added and refluxing was continued for 5 h. After cooling, the excess of hydride was carefully decomposed with saturated aqueous ammonium chloride, the mixture was filtered and the sediment was washed thoroughly with warm chloroform/isopropanol, 5:1. After evaporation of combined filtrates, the residue was crystallized from chloroform/pentane and chloroform/methanol to yield 86 mg (64%) of **18**, as a white powder poorly soluble in common solvents, m.p. 115–117°C (Lit. data: m.p. 98°C [12]; 94°C [13]). Diacetate: m.p. 80–81°C (from dry ethanol); MS (FAB) *m/z* 567 (M^+); ^{1}H-NMR: 1.20–1.39 (56H, broad m, aliphatic protons), 1.64 (4H, m, $C\underline{H}_2CH_2O$), 2.07 (6H, s, CH_3CO), 4.10 (4H, t, ^3J 7.1 Hz, CH_2O).

2.12. 1,32-Bis(rhodaminyl 101-oxy)dotriacontane **19**

From diol **18** (20 mg, 41 μmol) and rhodamine 101 (95 mg, 160 μmol), by the method described for the derivative **9** synthesis, probe **19** was obtained as a deep red amorphous solid, yield 43 mg (69%); TLC: single component, R_f 0.26 on silica gel in chloroform/methanol/ 7 N NH_4OH, 80:20:1.2, and 0.40 on RP-8 in acetonitrile/acetic acid/water, 90:10:1, visualization: *1,3.* MS (^{252}Cf): *m/z* 1529 ($M^+ - ClO_4$), 1429 ($M^+ - H^+ - 2ClO_4^-$), 491 (rhodaminyl$^+$); ^{1}H-NMR: 1.65–1.90 (56H, broad m, aliphatic protons), 2.00 (4H, m, $C\underline{H}_2CH_2OOC$), 4.62 (4H, t, ^3J 7.2 Hz, CH_2OOC), aromatic protons produce three groups of multiplets centered at 7.88, 8.37 and 8.88 ppm (total 12H); UV in ethanol, nm (log ε): 578 (5.18); fluorescence: λ_{max} 600 nm (excitation 570 nm) (Fig. 1).

2.13. Membrane experiments

Unilamellar vesicles were prepared by the injection method [14]: ethanol solution of EPC (30 mg/ml, 750 μl) was injected quickly by a Hamilton syringe into 10 ml of severely agitated 150 mM NaCl/10 mM Tris–HCl buffer, pH 7.4, under argon stream at room temperature. Then liposome suspension was dialyzed overnight against 1 l of the same buffer at 4°C, centrifugated (3000 × *g*, 15 min) and 10-fold diluted with the above buffered saline up to the final lipid concentration 225 μg/ml. A total of 95% of the vesicles obtained had mean diameter within the 60–75 nm range as estimated by the light scattering on a Coulter N4MD submicron laser analyzer. Probes were added to the vesicle suspension as solutions in DMSO (0.4 mg/ml) to the mol. probe/lipid ratio of 1:200–1:400 with subsequent incubation at 30°C under constant fluorimetric monitoring.

DOPC vesicles for the fluorescence lifetime measurements were prepared by sonication as described earlier [4]. In these experiments, a probe was added to the starting mixture before vesicles (or micelles) preparation, at DOPC (or detergent) to probe molar ratio of 5000:1.

Fluorescence spectra (steady-state) were recorded on a Hitachi F-4000 spectrofluorimeter equipped with a thermostated cell, in quartz cuvette 10 × 10 mm, band width 2 nm for excitation and 5 nm for emission.

A PRA 3000 system (Photophysical Research Assoc., Ontario, Canada) was used for the time-correlated single-photon-counting measurements of the fluorescence decay. The excitation source was a thyratron-gated flash lamp (Model 510C, PRA) filled with deuterium gas and operated at about 30 kHz. The excitation and emission wavelengths were selected by interference filters (Omega/Saven AB, Sweden) and longpass filters (KV series, Schott, Germany). The excitation wavelengths for the 9-anthryl derivatives were 351 and 389.9 nm, and the emission was monitored at wavelengths > 391 and 450 nm, respectively. For the 3-perylenyl derivatives excitation was at 420 and 450 nm, and emission at the wavelengths > 470 and 550 nm, respectively. The rhodamine 101 derivatives were excited at 545 nm, and the emission was observed above 610 nm. In all experiments, the absorbance was kept low (< 0.07) to avoid reabsorption.

For reconvolutions, a non-linear least-square analysis was used [4]. The quality of fits was judged by the χ^2-values calculated, as well as by residual plots. The convolution was carried out on a Silicon Graphics work station (IRIS Indigo).

3. Results and discussion

3.1. Synthesis

For synthesis of the probes with a rigid spacer, the bisteroid **8** was prepared by condensation of 16-dehydropregnenolone acetate (3β-acetoxy-5,16-pregnadien-20-one) with trimethylorthoformate followed by acidic hydrolysis and subsequent alkaline deacetylation [11,15]. The configuration of bicyclic system joining two dehydropregnenolone blocks in bisteroid **8** is shown (Scheme 1) as it has been found by Latt et al. [11]. These authors have studied the electronic energy transfer between different fluorophores (*p*-methoxyphenyl-acetyl, 1-naphtoyl and 9-anthroyl)

which were linked to HO groups of the bisteroid. From steady-state fluorescence experiments they estimated a distance of about 19–21 Å between the donor and acceptor residues. For restricting the localization in lyotropic systems (micelles, vesicles, liquid crystals) of our bisteroid probes, we have linked the fluorophores to the bisteroid through an ester or a phosphonic group. The polarity of these groups makes a localization of the probes in the lipid/water interface more probable.

Probe **9** having two rhodamine 101 residues has been obtained (see Scheme 1) by an acylation of bisteroid **8** with rhodamine 101 in the presence of DCC. Although the ester group is of moderate polarity (e.g., anthroyloxy fatty acids have their fluorophore together with ester group located in the hydrophobic region of bilayer [16]), the rhodamine moiety is a quaternary ammonium salt which is polar enough to reside in the lipid/water interface of a membrane, and this is the case even if it is linked to a long apolar chain [17].

For linking the hydrophobic anthryl or perylenyl residues to the bisteroid, the ester group is less suitable. In this case, one would expect a less well-defined localization in a lipid bilayer. Therefore, we have linked these apolar fluorophores to the bisteroid by methylphosphonic groups. The phosphonic residue was chosen because of its higher stability against hydrolysis as compared to phosphates. This is of importance when applying these probes to systems like liposomes, micelles or lyotropic liquid crystalline phases.

9-Anthrylmethylphosphonic acid **2** was obtained from 9-chloromethylanthracene **1** and tris(trimethysilyl)phosphite in the Arbuzov reaction [9]. Condensation of the acid **2** with the bisteroid **8** under action of TPS gave bis-9-anthrylmethyl-phosphonic probe **10** separated chromatographically as ammonium salt (Scheme 1). Also the mono-9-anthrylmethylphosphonic derivative **10a** was obtained. In applications, this compound could be of interest as a probe of comparison and for interpretations of experimental data obtained with **10**. It should be noted that two hydroxy groups of bisteroid **8** are not equivalent because of asymmetry of the central cycle of the molecule. This is revealed by the presence of two pairs of singlets in the ^{1}H-NMR spectrum corresponding to two pairs of non-equivalent 18- and 19-CH_3 groups (see Experimental section). Thus, the probe **10a** constitutes a mixture of the two isomers having nearly the same properties. So far, we have not succeeded in separating them by HPTLC or HPLC.

Probe **11** was obtained in a similar way. Formulation of perylene **3** with *N*-methylformanilide led to 3-formylperylene **4** [6] which was reduced by sodium borohydride to 3-hydroxymethylperylene **5**. It should be mentioned here that formylation of perylene proceeds with the high regioselectivity. Whereas Friedel-Crafts succinylation of perylene gave 3-(3-perylenoyl)propionic acid with ~ 10% admixture of the positional isomer [18], the carbinol **5** was isomerically pure as judged by HPLC data. This compound has been transformed by the action of dry hydrogen chloride in toluene into the unstable chloride **6** (it decomposes partly during TLC on a silica gel plate in neutral aprotic solvent system). In the Arbuzov reaction, compound **6** gave 3-perylenylmethylphosphonic acid **7** which we have linked to the spacer **8** to obtain the bis-3-perylenylmethylphosphonic probe **11**. The mono-3-perylenylmethylphosphonic derivative **11a** was also obtained (see Scheme 1).

In the D-D probe **19**, two rhodamine 101 chromophores are separated by a saturated hydrocarbon chain of 32 C-atoms. Starting the synthesis, we have kept in mind that this spacer has no firm rigidity but have supposed that it would expose predominantly the stretched conformation in lyotropic systems which should be thermodinamically preferable to the U-shaped one. The length of $(CH_2)_{32}$-chain is close to the thickness of the apolar region of a bilayer built from common phospholipids, e.g. EPC or 1-palmitoyl-2-oleoylphosphatidylcholine. Therefore, the probe **19** could span across a lipid bilayer so that the rhodamine residues reside in the lipid/water interface and are located on opposite sides of the bilayer. Recently, strong evidence for this has been found [19]. Actually, this probe mimicks the bolaform lipids which are built up by two polar head groups separated by one or two hydrocar-

bon chain(s). The bolaform lipids are well known both as natural [20] and synthetic [21–25] compounds (see also papers cited in these references). The orientation of these lipids in membranes depends strongly on their structure. The bolaamphiphiles which contain a single saturated hydrocarbon chain can take both a transmembrane and an U-shaped conformation [21,23], while lipids with a rigid inset in the apolar chain(s) are predominantly oriented across the bilayer [25]. The bolaform lipids can form lipid bilayers by themselves [20] or in mixtures with other phospholipids or cholesterol [21]. However, this does not imply that all bolaform lipids exclusively take the extended conformation, but merely suggests that this conformation could be expected.

The diol **18** was described previously [12,13]. Here we have elaborated our own variant of the synthesis. It starts (see Scheme 2) from 1,10-dibromodecane **12** which was converted into the bistriphenylphosphonium salt **13**. The last in the Wittig synthesis with methyl 11-oxoundecanoate **15** (obtained by oxidation of the iodoester **14**) gave the dienic diester **16** as a mixture of *E,Z*-isomers. It was hydrogenated over platinum catalyst to produce dimethyl 1,32-dortiacontanoate **17** which was reduced by lithium alumohydride into the diol **18**. This compound has been obtained by us with m.p. 115–117°C, which differs substantially from the values of 98°C and 94°C, reported by Moss et al. [12] and Furukawa et al. [13], respectively. We do not know the reasons for this discrepancy which could be caused by crystal polymorphism. The mass and ^{1}H-NMR spectra of the diacetate of our compound (the diol itself has poor solubility) are consistent well with the structure of 1,32-dotriacontanediol **18** (see Experimental section).

Esterification of diol **18** with rhodamine 101 in the presence of DCC led to the probe **19** (see Scheme 2). It is interesting that in its ^{1}H-NMR spectrum, resonance signals of the aliphatic protons are shifted downfield by ca. 0.5 ppm in comparison with the corresponding peaks in the spectrum of diol **18** diacetate. This is most likely caused by deshielding from the rhodamine 101 aromatic system.

3.2. Spectral and membrane properties

The absorption and fluorescence spectra of the bichromophoric probes **9–11** and **19** are similar to those of the corresponding monochromophoric 9-anthryl [26], 3-perylenyl [18] and rhodamine 101 [17] derivatives. The fluorescence excitation and emission spectra of the bisteroid probes **9–11** are displayed in Fig. 1. These spectra extend over a wide spectral range, from near UV to visible wavelengths. This allows studies of interactions of the probes with a variety of other probes and fluorogenic substances (e.g. proteins).

The fluorescence lifetimes (τ) have been measured for the probes synthesized in this work. The τ-values found for the probes in solutions, micelles and lipid vesicles are summarized in Table 1. The detergents forming the micellar systems were pentaethyleneglycol mono-*n*-dodecyl ether ($C_{12}EO_5$) and dodecyltrimethylammonium chloride (DOTAC). Small unilamellar vesicles were formed from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC).

The fluorescence relaxation of the 9-anthryl derivatives and the rhodamine 101 derivatives is a simple exponential in all systems studied. We have found lifetimes of about 12 $\pm$ 2 ns for the 9-anthryl compounds, which corresponds to fluorescence quantum yield of ca. 80%. For the rhodamine probes, the corresponding values are nearly 100%. The fluorescence relaxation of the 3-perylenyl derivatives is less straightforward, but can be fitted to a sum of two exponential functions. The shorter lifetime, typically about 5 ns is expected, as judged from a comparison with perylene itself and its aliphatic derivatives [27]. The reason for the longer lifetime of ca. 10 ns is not yet established. From an analysis of the fluorescence relaxation monitored at different excitation and emission wavelengths we conclude that solvent relaxation is not a likely explanation.

We have obtained the Förster radii of the An-B and the An-B-An probes, and the R-C_{32}-R probe to be 26.8 $\pm$ 1 Å (in 1,2-propanediol) and 59 $\pm$ 1 Å (in DOPC vesicles), respectively. From time-resolved fluorescence depolarization experiments on An-B and An-B-An in 1,2-propanediol, we find that the rate of intramolecular energy trans-

Table 1
Fluorescence lifetimes (τ_1) obtained by time-correlated single-photon counting experiment on bisteroid derivatives with mono- and bis-9-anthryl (**10a** and **10**, denoted An-B and An-B-An, respectively) and mono- and bis-3-perylenyl (**11a** and **11**, denoted by Pe-B and Pe-B-Pe, respectively) groups, and also on bis-rhodaminyl derivative of dotriacontanediol (**19**, denoted by R-C_{32}-R)

Probe	System	τ_1	τ_2
An-B (**10a**)	1,2-Propanediol	11.3 ± 0.2	
An-B-An (**10**)	1,2-Propanediol	11.2 ± 0.2	
An-B (**10a**)	DMSO	10.5 ± 0.5	
An-B-An (**10**)	DMSO	10.8 ± 0.5	
An-B (**10a**)	$C_{12}EO_5$	13.2 ± 0.3	
An-B-An (**10**)	$C_{12}EO_5$	12.9 ± 0.3	
An-B (**10a**)	DOTAC	13.6 ± 0.3	
An-B-An (**10**)	DOTAC	13.2 ± 0.3	
Pe-B (**11a**)	1,2-Propanediol	4.7 ± 0.1 (0.95)	11.0 ± 1.3
Pe-B-Pe (**11**)	1,2-Propanediol	4.5 ± 0.1 (0.87)	13.2 ± 1.3
Pe-B (**11a**)	DMSO	4.4 ± 0.1 (0.95)	9.0 ± 0.9
Pe-B-Pe (**11**)	DMSO	3.8 ± 0.3 (0.75)	11.7 ± 1.2
Pe-B (**11a**)	$C_{12}EO_5$	5.8 ± 0.2 (0.87)	9.9 ± 1.0
Pe-B-Pe (**11**)	$C_{12}EO_5$	5.9 ± 0.2 (0.92)	12.8 ± 1.0
Pe-B (**11a**)	DOTAC	5.5 ± 0.1 (0.60)	8.0 ± 1.3
Pe-B-Pe (**11**)	DOTAC	5.5 ± 0.1 (0.68)	9.7 ± 1.3
R-C_{32}-R (**19**)	Ethanol	4.3 ± 0.1	
R-C_{32}-R (**19**)	1-Decanol	4.2 ± 0.1	
R-C_{32}-R (**19**)	DOPC vesicles	4.8 ± 0.1	

The figures within parentheses refer to contribution of τ_1 to the total fluorescence intensity. The quality of fits were judged the χ^2-parameter, where only values of $0.8 \leq \chi^2 \leq 1.2$ were accepted

fer is about $3 \cdot 10^8\ s^{-1}$. From this rate we have been able to calculate the intramolecular distance between 9-anthryl groups, which is found to be in excellent agreement with the values obtained from molecular models [28].

We are currently studying the intramolecular energy transfer in R-C_{32}-R solubilized in DOPC vesicles. Fluorescence depolarization studies show that the transfer rates are in the order of $10^9\ s^{-1}$.

The probes incorporate slowly into phospholipid bilayers after adding a probe (dissolved in DMSO) to unilamellar vesicles of EPC. This process can be monitored by the increasing fluorescence intensity with time. For the bichromophoric molecules **9**–**11** and **19**, the maximum plateau intensity is reached within 3 to 4 h at 30°C. The corresponding time for the mono-derivatives **10a** and **11a** is about 1.5 to 3 h.

The orientation of these probes in lipid bilayers is not known. It is of interest to know whether they orient parallel or perpendicular to the normal of the bilayer. Preliminary fluorescence quenching experiments with the iodide ion [29] show that 50% of the bis-anthryl and bis-rhodaminyl probes (**10** and **9**) in EPC vesicles are accessible for quenching. These data are compatible with an orientation of the probes parallel to the normal of the bilayer. But, since the distance between the phosphonic groups is about 20 Å (in **10**) and the lipid bilayer apolar zone has thickness of about 40 Å, this would suggest that the lipid acyl chains around the probe molecules of opposite monolayers are interdigitated. However, the quenching data can also be explained by an orientation of the probes perpendicular to the normal of the bilayer provided that the transmembrane migration (flip-flop) occurs within a few hours. For solving this and other questions concerning the interaction between these probes and lipid membranes, further experimental studies are needed. We are currently investigating these probes in various lipid bilayer systems by means of different polarized light spectroscopic methods.

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