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Synthesis and intermembrane transfer of pyrene-labelled liponucleotides: ceramide phosphothymidines

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Abstract

Phospholipid conjugates of 3′-azido-3′-deoxythymidine (AZT) show activity against human immunodeficiency virus (HIV) in vitro. Here we report on the synthesis and characterization of two pyrene containing conjugates: 2-*N*-(4-(pyren-1-yl)butanoyl)ceramide 5′-phosphothymidine (Pbs–Cer–P–T) (XII) and 2-*N*-(10-(pyren-1-yl)decanoyl)ceramide 5′-phosphothymidine (Pds–Cer–P–T) (XIII). These fluorescent labelled conjugates served as model compounds to study incorporation of sphingoliponucleotides into membranes. The complex compounds were prepared by condensation of 3′-acetylthymidine and labelled ceramides using the phosphite triester coupling procedure. UV absorption, fluorimetry as well as ^{1}H-, ^{31}P-, ^{13}C-NMR analyses were used for structure confirmation of the synthesized substances. When incorporated into small unilamellar 1-palmitoyl-2-oleoyl-glycerophosphatidylcholine (POPC) vesicles and incubated with unlabelled acceptor POPC vesicles, the compounds (XII) and (XIII) exhibited spontaneous transfer. Kinetic data suggest that transfer from donor to acceptor vesicles occurred via the intervening aqueous phase. The non-specific lipid transfer protein from bovine liver stimulated the transfer of Pds–Cer–P–T between phospholipid vesicles in a concentration dependent manner.

Keywords: Nucleoside-phospholipid conjugates; Sphingophospholipids; Phosphite triester approach; Fluorescence spectroscopy; Spontaneous transfer; Protein mediated transfer

1. Introduction

Fluorescently labelled phospholipids carrying a fluorophore group in one of the hydrocarbon chains have become important and frequently indispensable tools in studies of the structure and dynamics of membranes. Since such probes retain head groups and resemble the molecular shape of native membrane lipids, they largely mimic the behaviour of their natural prototypes in biological membranes. These probes are applied in investiga-

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tions dealing with biophysical aspects of membranes, including lateral organization and mobility (Galla and Hartmann, 1980; Bergelson et al., 1985; Molotkovsky et al., 1991; Bredlow et al., 1992; Barenholz et al., 1996), and also in studies of intermembrane and transmembrane transfer of phospholipids (for reviews see Pownall and Smith, 1973; Pagano and Sleight, 1985; Nichols, 1993) and phospholipid metabolism (Pagano and Longmuir, 1983; Kok and Hoekstra, 1993; Gatt et al., 1996).

The lateral diffusion and intermembrane transfer of phospholipids have been the subject of numerous studies. Many of the physical and chemical determinants that govern these processes have been elucidated. In general, these studies have shown that the lateral diffusion of membrane lipids is strongly dependent on the fluidity and composition of the host membrane, but relatively independent of the chemical nature of the diffusing species. In contrast, intermembrane diffusion rates are strongly dependent on the composition and length (i.e. hydrophobicity) of the phospholipid acyl chains. A number of mechanisms have been proposed for transport of lipids among various membranes or plasma lipoproteins. These include (a) transport by specific carriers that are usually proteins, (b) fusion occurring at the point of contact between the donor and acceptor surfaces, and (c) passive transport via the surrounding aqueous phase. Phospholipid molecules spontaneously migrate between different artificial membranes and/or membranous particles (Roseman and Thompson, 1980; McLean and Philips, 1981; Massey et al., 1982a,b; Nichols and Pagano, 1982; McLean and Philips, 1984; Petrie and Jonas, 1984). The experimental criteria for this mechanism given by Charlton et al. (1976) are that the process is first order and independent of donor to acceptor concentration and of the identity of the acceptor. However, it has been demonstrated that phospholipid transfer between bilayer vesicles at higher vesicle concentrations is characterized not only by a first-order desorption rate (spontaneous migration) but also by a second-order process (collision) dependent on vesicle concentration (Jones and Thompson, 1989, 1990). The transfer kinetics of lipids labelled with markers different from pyrene had to be described by a sum of two exponential functions. The fast component was assigned to lipid transfer via soluble monomers and the slow component to transbilayer motion (Arvinte and Hildenbrand, 1984; Bai and Pagano, 1997). Pyrene lipid probes, while more bulky as compared to antrylvinyl and 7-nitro-2-1,3-benzoxadiazol-4-yl (NBD) fluorophores (Bredlow et al., 1992), were of considerable use for studies of kinetics and mechanism of the transfer because the spectroscopic properties of pyrene are concentration-dependent (Foerster, 1969; Pownall and Smith, 1973; Galla and Hartmann, 1980) and allow facile analysis of lipid exchange without separation of donor and acceptor particles (Galla et al., 1979; Roseman and Thompson, 1980; Correa-Freire et al., 1982; Massey et al., 1982a,b; Pownall et al., 1982; Frank et al., 1983; Pownall et al., 1983; Via et al., 1985; Homan and Pownall, 1988; Jones and Thompson, 1989, 1990; van Wijk et al., 1992). This makes pyrene-labelled lipid probes 'lipid-specific' in the sense that the structure and conformation of their polar head groups are identical or very close to those of corresponding natural lipids (Correa-Freire et al., 1982; Frank et al., 1983).

Formerly, we have synthesized *rac-erythro*-2-*N*-stearoyl-sphinganine 1-phospho thymidine (Oskolkova et al., 1996) via phosphite triester and H-phosphonate approaches and applied these methodologies for synthesis of ceramide phospho-3′-azidothymidine and ceramide phospho-2′,3′-didehydro-2′,3′-dideoxythymidine (Oskolkova et al., 1997). In this report we present the chemical synthesis and kinetic data for the rates of the intermembrane transfer of fluorescent nucleoside phospholipid conjugates- racemic ceramidephosphoryl thymidines containing 4-(1-pyrenyl)butanoic or 10-(1-pyrenyl)decanoic acids attached to the amine group of sphinganine. The synthesis directed towards both target compounds comprises two main stages: the acylation of a free amino group of racemic *erythro*-3-benzoyl sphinganine to give ceramide fluorescently labelled with pyrene fatty acids, and subsequent phosphitylation and condensation with the free hydroxy group at the 5′-end of protected thymidine using a phosphite triester approach followed by a depro-

tection procedure to give target *rac-erythro*-2-*N*-(4-(pyren-1-yl)butanoyl)sphinganine-1-*O*-phosphoryl-5′-thymidine (XII) and *rac-erythro*-2-*N*-(10-(pyren-1-yl)decanoyl)sphinganine-1-*O*-phosphoryl-5′-thymidine (XIII), respectively. Hydrophobicity of the latter compound closely resembles that of 2-*N*-palmitoyl-ceramide phosphothymidine. Liponucleotides (XII) and (XIII) were incorporated into POPC vesicles prepared by the ethanol-injection method (Batzri and Korn, 1973). Inclusion of the pyrene fluorophore enabled us to monitor continuously time-dependent changes in the concentration of labelled lipids in vesicle bilayers. The partially purified non-specific lipid transfer protein (ns-PLTP) from bovine liver stimulated the transfer of Pds–Cer–P–T between vesicles in a concentration dependent manner. In contrast, ns-PLTP had no effect on the transfer rate of the short-chain Pbs–Cer–P–T.

2. Experimental procedures

2.1. Materials and general methods

Pyrene butanoic acid pentafluorophenyl ester was a gift from K. Balakin (Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Moscow). D,L-*erythro*-3-*O*-benzoyl-sphinganine sulfate was synthesized according to Shapiro (1969). D,L-*erythro*-3-*O*-benzoyl-sphinganine was obtained from its sulfate by shaking with a saturated sodium bicarbonate solution. The non-specific lipid transfer protein was isolated essentially as described by Bloj and Zilversmit (1983) and kindly provided by Dr. A. Fell (Technical University Graz).

Dichloromethane was distilled from phosphorus pentoxide and used freshly. Water-free pyridine was prepared by refluxing with calcium hydride for 12 h and distilled. Methanol was refluxed with magnesium methoxide and then distilled. Triethylamine (TEA) was purified by refluxing with ninhydrine and subsequent distillation. 3′-Acetylthymidine (Sigma) was dried by evaporation with absolute acetonitrile and then dried in vacuo. Aluminum chloride, sodium azide, Tris–HCl, EDTA (all from Merck), pyrene (Sigma), oxalyl chloride (Aldrich), sebacic acid monomethyl ester (Aldrich) were used as such. The solution of 1H-tetrazol (3.5%) in acetonitrile was obtained from Aldrich. TLC was carried out on silica gel 60 F_{254} aluminum sheets (0.2 mm, Merck) using the following developing solvents: petroleum ether/diethyl ether, 4:1, v/v (system 1); chloroform/methanol/acetone, 10:0.5:0.5, v/v/v (system 2); petroleum ether/ethyl acetate/TEA, 10:4:0.5, v/v/v (system 3); chloroform/methanol, 10:1, v/v (system 4); chloroform/methanol, 10:3, v/v (system 5); chloroform/methanol/water, 65:25:4, v/v/v (system 6). Short column chromatography was performed on Kieselgel 60 (230–400 mesh, Merck). Compounds with an aromatic moiety were visualized under UV-light (254 and/or 360 nm). Phosphorus containing material was detected using phosphomolybdic acid (Dittmer and Lester, 1964). All organic substances were detected by charring at 120°C after spraying with 50% sulfuric acid. Melting points were determined on a Büchi 510 apparatus (Switzerland) in open capillary tubes.

2.2. Chemical syntheses

2.2.1. 10-(1-Pyrenyl)decanoic acid

Sebacic acid monomethyl ester chloride was obtained by mixing of sebacic acid monomethyl ester with oxalyl chloride. The mixture was kept overnight, triple evaporated with water-free THF and then used without isolation. Pyrenedecanoic acid was prepared with modifications according to Galla et al. (1979) by a Friedel–Crafts reaction of pyrene and sebacic acid monomethyl ester chloride in presence of aluminum chloride in dichloromethane. After work up, pyrenoylnonanoic acid methyl ester was purified by column chromatography eluting with petroleum ether/ diethyl ether (6:1 by vol.). The yield was 48.9%, R_f 0.21 (system 1). Pyrenedecanoic acid was obtained from pyrenoylnonanoic acid methyl ester by a Kishner–Wolf reaction with hydrazine hydrate and potassium hydroxide in ethylene glycol. Purification was achieved on silica gel using a gradient of methanol (0–1%) in chloroform. A single spot was observed on TLC (R_f 0.31, system 2). Pyrenedecanoic acid was obtained with 89.0%

yield; m.p. 104–108.5°C (99°C (Galla and Hartmann, 1981), 110°C (Galla et al., 1979)).

2.2.2. 10-(1-Pyrenyl)decanoic acid p-nitrophenyl ester

The activated ester was obtained from pyrenedecanoic acid using a standard procedure by reacting the respective acid with *p*-nitrophenol in presence of dicyclohexylcarbobiimide in dichloromethane. Column chromatography on silica gel gave the pure product with 94.0% yield. R_f 0.55 (system 1), 0.82 (system 2), m.p. 99–103°C. ^{1}H-NMR ($CDCl_3$): 1.10–1.65 (m, 10H, 5CH_2), 1.65–2.05 (m, 4H, 3-CH_2, 9-CH_2), 2.58 (t, 2H, $J_{2,3}$ 7.42 Hz, 2-CH_2), 3.35 (t, 2H, $J_{9,10}$ 7.69 Hz, 10-CH_2), 7.83–8.31 (m, 13H, Ar).

2.2.3. 10-(1-Pyrenyl)decanoic acid pentafluorophenyl ester

Pentafluorophenyl ester of pyrenedecanoic acid was obtained as described above with 91.9% yield; R_f 0.94 (system 1), m.p. 80–81°C. ^{1}H-NMR ($CDCl_3$): 1.15–1.65 (m, 10H, 5CH_2), 1.65–2.00 (m, 4H, 3-CH_2, 9-CH_2), 2.64 (t, 2H, $J_{2,3}$ 7.37 Hz, 2-CH_2), 3.35 (t, 2H, $J_{9,10}$ 7.79 Hz, 10-CH_2), 7.82–8.35 (m, 9H, Ar).

2.2.4. 3-O-Benzoyl-2-N-(4-(1-pyrenyl)butanoyl)-D,L-erythro-sphinganine (II)

A mixture of racemic 3-benzoyl-sphinganine (I) (496.8 mg, 1.227 mmol) and pyrenebutanoic acid pentafluorophenyl ester (669.5 mg, 1.473 mmol) in 17 ml pyridine was kept at room temperature for 3.5 h, then concentrated in vacuo, the residue was subsequently co-evaporated with toluene (3 × 5 ml) to remove the rest of pyridine. The resulting oil was applied to a silica gel column and eluted with chloroform to give the crystalline product (II). Yield 375.6 mg (45.0%). R_f 0.61 (system 2), m.p. 91–94°C. ^{1}H-NMR ($CDCl_3$): 0.88 (t, 3H, CH_3-sphinganine (Sph)), 1.1–1.45 (m, 26H, CH_2–Sph), 1.55–2.00 (m, 4H, 4-CH_2–Sph, 3-CH_2–Pbs), 2.26 (dt, 2H, $J_{a,b} = J_{2\text{-Pbs},3\text{-Pbs}}$ 7.92 Hz, 2-CH_2–Pbs), 2.36 (dt, 2H, $J_{a,b} = J_{3\text{-Pbs},4\text{-Pbs}}$ 6.19 Hz, 3-CH_2–Pbs), 3.10 (bt, 1H, –OH), 3.41 (t, 2H, $J_{3\text{-Pbs},4\text{-Pbs}}$ 7.33 Hz, 4-CH_2–Pbs), 3.66 (m, 2H, 1-CH_2–Sph), 4.21 (m, 1H, 2-CH–Sph), 5.07 (dt, 1H, *J* 3.70 Hz, *J* 8.17 Hz, 3-CH–Sph), 6.30 (d, 1H, NH), 7.40–8.35 (m, 14H, Ar).

2.2.5. 3-O-Benzoyl-2-N-((1-pyrenyl)decanoyl)-D,L-erythro-sphinganine (III)

A mixture of 111.0 mg (0.274 mmol) 3-benzoyl sphinganine (I) and 178.0 mg (0.361 mmol) pyrenedecanoic acid *p*-nitrophenyl ester in 3 ml pyridine and 50.0 μl (0.361 mmol) TEA was kept at room temperature for 5 h. The solvent was removed under reduced pressure, the residue was applied to a silica gel column and eluted with chloroform/petroleum ether (8:2, v/v), then with chloroform and with 1% methanol in chloroform to give 114.8 mg (55.1%) of ceramide (III). R_f 0.74 (system 2), m.p. 105–105.5°C (petroleum ether/diethyl ether, 2:1). ^{1}H-NMR ($CDCl_3$): 0.85 (t, 3H, CH_3–Sph), 0.97–2.10 (m, 40H, CH_2–Sph, Pds), 2.20 (t, 2H, $J_{2,3}$ 7.50 Hz, 2-CH_2–Pds), 3.28–3.61 (m, 4H, 1-CH_2–Sph, 10-CH_2–Pds), 4.16 (m, 1H, 2-CH–Sph), 5.08 (dt, 1H, *J* 3.94 Hz, *J* 7.95 Hz, 3-CH–Sph), 6.47 (d, 1H, $J_{2,NH}$ 8.52 Hz, NH), 7.40–7.63 (m, 5H, Bz), 7.80–8.30 (m, 9H, Ar).

An alternative procedure was carried out starting from 3-benzoyl sphinganine (121.8 mg, 0.300 mmol) (I) and 178.0 mg (0.361 mmol) pyrenedecanoic acid pentafluorophenyl ester. The purification was performed as described above giving 49.0% ceramide (III).

2.2.6. 3-O-Benzoyl-2-N-(4-(1-pyrenyl)butanoyl)-D,L-erythro-sphinganine-1-O-(N,N-diisopropylamino)-2-cyanoethylphosphine (IV)

To a solution of dried (by coevaporation with a dichloromethane-acetonitrile mixture (1:1 v/v, 3 × 3 ml)) 2-*N*-pyrenebutanoyl-ceramide (II) (100.0 mg, 0.148 mmol) and diisopropylammonium tetrazolide (17.0 mg, 0.099 mmol) in 1 ml dichloromethane, bis(*N*,*N*-diisopropylamino)-2-cyanoethylphosphine (0.1 ml, 0.315 mmol) was added. After completion of the reaction, methanol (0.1 ml) was added. After 15 min, the mixture was concentrated in vacuo, diluted with a 10% TEA solution in ethyl acetate (50 ml), washed with 10% sodium bicarbonate (2 × 10 ml) and water (2 × 10 ml). The organic layer was dried over sodium sulfate and the product was purified by flash chromatography on silica gel (elution with

petroleum ether/ethyl acetate/TEA, 10:4:0.5, v/v/v) to give 116.0 mg (89.2%) of oily phosphoramidite (X). R_f 0.28 and 0.33 (diastereoisomers on phosphorus) (system 3). ^{31}P-NMR ($CDCl_3$): 148.18 and 148.54 ppm.

2.2.7. 3-O-Benzoyl-2-N-(10-(1-pyrenyl)decanoyl)-D,L-erythro-sphinganine-1-O-(N,N-diisopropylamino)-2-cyanoethylphosphine (V)

The synthesis and isolation of amidophosphite (V) was performed under identical conditions as above, starting from the corresponding 3-benzoyl ceramide (III) (100.0 mg, 0.132 mmol). Yield 127.1 mg (75.9%) (oil). R_f 0.31 and 0.37 (diastereoisomers on phosphorus) (system 3). ^{31}P-NMR ($CDCl_3$): δ 148.05 and 148.79 ppm.

2.2.8. 3-O-Benzoyl-2-N-(4-(1-pyrenyl)butanoyl)-D,L-erythro-sphinganine-1-O-phospho-5′-(3′-O-acetyl)thymidine, triethylammonium salt (X)

Amidophosphite (IV) (200.0 mg, 0.228 mmol) and 3′-acetylthymidine (70.6 mg, 0.248 mmol) were dried by coevaporation with water-free dichloromethane (4 × 10 ml), dissolved in 4 ml dichloromethane, and a solution of 3.5% 1H-tetrazole in acetonitrile (0.64 ml, 22.4 mg, 0.320 mmol) was added. After 30 min at room temperature, the TLC analysis (system 3) showed the disappearance of the starting phosphoramidite (IV) and the formation of a new diastereomeric product of lower mobility. A 5.5 M solution of tert-butyl hydroperoxide (0.247 ml, 1.359 mmol) was added to the reaction mixture. After 40 min when the oxidation was complete, the solvents were evaporated and the reaction mixture was dissolved in 1ml of toluene and 1 ml triethylamine, kept at room temperature for 4 h, evaporated in vacuo, and co-evaporated with toluene (2 × 2 ml). The product was purified by column chromatography on silica gel by eluting isocratically with a chloroform/methanol/TEA mixture (100:5:1, v/v/v), affording homogenous (X) as oil (206.0 mg, 61.9%). R_f 0.12 (system 4), 0.37 (system 5), 0.63 (system 6). ^{1}H-NMR ($CDCl_3$–CD_3OD, 3:1): 0.72 (t, 3H, CH_3–Sph), 0.90–1.50 (m, 35H, CH_2–Sph, $3CH_3$–TEA), 1.59 (m, 2H, 4-CH_2–Sph), 1.70 (s, 3H, CH_3–thymidine (Thd)), 1.87 (s, 3H, CH_3–CO), 2.08 (m, 4H, 2-CH_2–Pbs, 2′-CH_2–Thd), 2.21 (m, 2H, 3-CH_2–Pbs), 2.73 (q, 6H, $3CH_2$–TEA), 3.26 (t, 2H, 4-CH_2–Pbs), 3.75–3.93 (m, 3H, 1-CH_2–Sph, 4′-H–Thd), the region 3.93–4.18 ppm was overlapped with a bright hydroxyl signal from methanol, 4.30 (m, 1H, 2-CH–Sph), 5.09, 5.18 (2 m, 2H, 3-CH–Sph, 3′-CH–Thd), 6.11 (2t, 1H, $J_{1',2'}$ 7.5 Hz, 1′-CH–Thd), 7.39–7.49 (m, 3H, Ar), 7.63–8.20 (m, 12H, Ar, 6-CH–Thd).

2.2.9. 3-O-Benzoyl-2-N-(10-(1-pyrenyl)decanoyl)-D,L-erythro-sphinganine-1-O-phospho-5′-(3′-O-acetyl)thymidine, triethylammonium salt (XI)

The synthesis of protected ceramide phosphothymidine (XI) was carried out identically as described above, starting from phosphoramidite (V) (114.0 mg, 0.119 mmol) and 3′-acetylthymidine (36.8 mg, 0.129 mmol). The product was purified by column chromatography on silica gel, elution with chloroform/methanol/TEA (100:1:1, v/v/v). Yield 78.1 mg (54.5%), oil. R_f 0.23 (system 4), 0.52 (system 5), 0.66 (system 6). ^{1}H-NMR ($CDCl_3$): 0.88 (2t, 3H, CH_3–Sph), 1.00–1.50 (m, 45H, CH_2–Sph, CH_2–Pds, $3CH_3$–TEA), 1.52–1.92 (m, 6H, 4-CH_2–Sph, 3-CH_2–Pds, 9-CH_2–Pds), 1.97 (s, 3H, CH_3–Thd), 2.04 (s, 3H, CH_3–CO), 2.12–2.35 (m, 4H, 2-CH_2–Pds, 2′-CH_2–Thd), 2.99 (q, 6H, $3CH_2$–TEA), 3.32 (2t, 2H, J 1.83 Hz, J 7.71 Hz, 10-CH_2–Pds), 3.84 (t, 2H, $J_{1,2}$ 5.66 Hz, 1-CH_2–Sph), 3.93–4.20 (m, 3H, 5′-CH_2–Thd, 4′-CH–Thd), 4.41 (m, 1H, 2-CH–Sph), 5.30 (m, 2H, 3′-CH–Thd, 3-CH–Sph), 6.35 (2t, 1H, $J_{1',2'}$ 8.13 Hz, 1′-CH–Thd), 7.30–8.42 (m, 17H, Ar, NH–Sph, NH–Thd, 6-CH–Thd).

2.2.10. 2-N-(4-(1-pyrenyl)butanoyl)-D,L-erythro-sphinganine-1-O-phospho-5′-thymidine (XII)

To 3-benzoyl-pyrenebutanoyl phosphoceramide 3′-acethylthymidine (X) (261.8 mg, 0.233 mmol) in diethyl ether/methanol (1:1 by vol., 6 ml), a 0.2N solution of sodium methoxide (6 ml) was added. The mixture was kept at room temperature for 1 h, then evaporated in vacuo. The residue was chromatographed on silica gel,

elution with chloroform–methanol from 10:1 to 10:4 (by vol.). Yield of product (XII) was 184.0 mg (90.1%). R_f 0.51 (system 6). ^{1}H-NMR ($CDCl_3$–CD_3OD, 3:2): 0.85 (t, 3H, CH_3–Sph), 1.08–1.40 (m, 26H, CH_2–Sph), 1.52 (m, 2H, 4-CH_2–Sph), 1.88 (2s, 3H, CH_3–Thd), 2.18 (m, 2H, 3-CH_2–Pbs), 2.38 (t, 2H, J 6.64 Hz, 2-CH_2–Pbs), 3.36 (t, 2H, J 7.51 Hz, 4-CH_2–Pbs), 3.64 (m, 2H, 2′-CH_2–Thd), 3.87–4.02 (m, 5H, 1-CH_2–Sph, 5′-CH_2–Thd, 4′-CH–Thd), 4.25 (m, 1H, 2-CH–Sph), 4.44 (m, 1H, 3′-CH–Thd), the signal of 3-CH–Sph overlapped with a bright signal of water, 6.24 (t, 1H, 1′-CH–Thd), 7.64 (d, 1H, J 8.01 Hz, 6-CH–Thd), 7.82–8.35 (m, 9H, Ar). ^{13}C-NMR ($CDCl_3$–CH_3OH–H_2O, 65:25:4): 12.02 (CH_3–Thd), 14.04 (CH_3–Sph), 22.71 (17-CH_2–Sph), 25.74 (5-CH_2–Sph), 27.88, 29.40, 29.70, 31.95, 32.96 (CH_2–Sph), 33.76 (16-CH_2–Sph), 36.16 (3-CH_2–Pbs), 39.44 (2-CH_2–Pbs), 54.22 (2-CH–Sph), 57.52 (4-CH_2–Pbs), 64.98 (1-CH_2–Sph, 5′-CH_2–Thd, 2′-CH_2–Thd), 70.00, 70.10 (ds, 3-CH–Sph, J_{C-P} 7.47 Hz), 70.48, 70.70 (2s, 3′-CH–Thd), 85.05 (1′-CH–Thd), 85.42, 85.52 (d, 4′-CH–Thd, J_{C-P} 7.25 Hz), 123.32, 124.80, 124.86, 124.95, 125.90, 126.73, 127.28, 127.41, 127.49 (CH–Ar), 136.71 (6-CH–Thd). ^{31}P-NMR ($CDCl_3$–CH_3OH–H_2O, 3:2:1): 1.17 and 1.26 ppm.

2.2.11. 2-N-(10-(1-pyrenyl)decanoyl)-D,L-erythro-sphinganine-1-O-phospho-5′-thymidine (XIII)

This compound was prepared as described for (XII) starting from *N*-pyrenedecanoyl phosphoceramide 3′-acethylthymidine (XI) (78.1 mg, 0.065 mmol). Yield of (XIII) 55.8 mg (89.9%). R_f 0.55 (system 6). ^{1}H-NMR ($CDCl_3$–CD_3OD, 3:1): 0.71 (t, 3H, CH_3–Sph), 0.95–1.58 (m, 40H, CH_2–Sph, CH_2–Pds), 1.76 (s, 3H, CH_3–Thd), 1.95–2.30 (m, 4H, 9-CH_2–Pds, 2-CH_2–Pds), the signals of 10-CH_2–Pbs (3.12–3.34) and of 3′-CH–Thd (3.95–4.22) overlapped with the signals of methanol, 3.45, 3.60, 3.85 (3m, 8H, 2′-CH_2–Thd, 1-CH_2–Sph, 5′-CH_2–Thd, 4′-CH–Thd, 2-CH–Sph), 4.29 (m, 1H, 3-CH–Sph), 6.11 (m, 1H, 1′-CH–Thd), 7.37 (s, 1H, 6-CH–Thd), 7.68–8.18 (m, 9H, Ar). ^{13}C-NMR ($CDCl_3$–CD_3OD, 3:1): 12.48 (CH_3–Thd), 14.41 (CH_3–Sph), 23.11 (17-CH_2–Sph), 26.20 (5-CH_2–Sph), 26.50, 28.16, 29.80, 29.97, 30.15, 30.29, 31.63, 32.36, 32.44, 34.05, 36.93, 39.94, 40.00 (CH_2-Sph, CH_2–Pds), 54.62 (2-CH–Sph), 61.81 (10-CH_2–Pds), 65.20 (1-CH_2–Sph, 5′-CH_2–Thd, 2′-CH_2–Thd), 70.19, 70.31 (d, 3-CH–Sph, J_{C-P} 9.28 Hz), 70.97, 71.23 (2s, 3′-CH–Thd), 85.30, 85.36 (2s, 1′-CH–Thd), 86.04, 86.17 (d, 4′-CH–Thd, J_{C-P} 9.28 Hz), 123.95, 125.06, 125.22, 126.22, 126.90, 127.54, 127.73, 127.99 (CH–Ar), 137.17 (6-CH–Thd). ^{31}P-NMR ($CDCl_3$–CH_3OH, 2:1): 1.52 and 1.60 ppm.

2.3. Spectroscopic analyses

2.3.1. NMR measurements

^{1}H-NMR spectra were measured in deuterated solvents at 199.97 MHz, using a Varian Gemini 200 impulse spectrometer. Chemical shifts (δ) are given in ppm relative to tetramethylsilane as a standard. ^{31}P- and ^{13}C-NMR spectra were recorded on a Bruker MSL 300 spectrometer. ^{31}P-NMR spectra were measured proton-noise decoupled at 121.49 MHz; chemical shifts (δ, ppm) are reported relative to 85% orthophosphoric acid as external standard. The ^{13}C-NMR spectra were recorded at 75.47 MHz using DEPT sequence; chemical shifts are given in ppm (δ) related to deuterated solvents.

2.3.2. Absorbance and fluorescence measurements

A molar extinction coefficient of 42,000 M^{-1} cm^{-1} (at 342 nm) in ethanol was used to determine the pyrene lipid concentration (measurements were performed on a Hitachi U-3210 spectrophotometer). Fluorimetry was registrated on a Shimadzu RF-5301PC spectrofluorophotometer equipped with a stirring device and thermostated cuvette holder. Fluorescence spectra were recorded at excitation wavelength of 342 nm with 3 nm band-passes for both excitation and emission. For transfer experiments, excitation and emission wavelengths were 342 and 378 nm, respectively with corresponding slit widths of 1.5 and 5.0 nm. The small excitation band-pass ensured minimal photodecomposition of the pyrene probes.

2.4. Donor vesicle preparation

Single-walled phosphatidylcholine vesicles were prepared by ethanol injection (Batzri and Korn, 1973). Final ethanol concentrations were adjusted to 1% by vol. A mixture of POPC/Pbs–Cer–P–T (85:15 mole%) or Pds–Cer–P–T (85:15 mole%) was evaporated to dryness under a gentle stream of argon. The dried lipids were redissolved in ethanol at a final concentration of 250 μM of total lipids. For measurements of excimer to monomer fluorescence intensity at different concentrations of pyrene phospholipids, aliquots of above-described solutions were diluted with a 250 μM solution of POPC to give two sets of stock solutions with different molar fractions of pyrene probes. Aqueous lipid dispersions were prepared by injecting a 42 μl ethanolic phospholipid solution through a Hamilton syringe into 4158 μl of an intensively stirred buffer solution. During injection, the temperature was kept at 37°C. The buffer solution used throughout this work was 10 mM Tris–HCl, 1 mM EDTA, and 0.02% sodium azide adjusted to pH 7.40. For one measurement of intermembrane transfer, 1 ml of vesicle solution (9 mole% of the pyrene-labelled conjugates) was used. Dynamic light scattering analysis (Orthaber and Glatter, 1998) of obtained liposomes (Pbs- and Pds-phospholipids, 9 mole% for both, 37°C) revealed a size distribution of vesicles of average diameter about 60 nm.

2.5. Acceptor vesicle preparation

Acceptor vesicles consisting of POPC were prepared by the ethanol injection method (final lipid concentration 25 or 100 μM) in analogy to the procedure outlined above. In transfer experiments, the concentration of the acceptor sample was either ten or 40 times greater than the donor vesicle concentration. This protocol minimized the contribution of reverse transfer to the signal.

2.6. Transfer assays

After a stable fluorescence signal originating from the donor vesicles was observed, a 10- or 40-fold excess of unlabelled acceptor vesicles (1.0 ml, 25 or 100 μM respectively) was added to a stirred solution of the donor vesicles (1.0 ml). The spontaneous transfer of labelled ceramide phosphothymidines from donor to acceptor vesicles was monitored continuously by measuring the pyrene monomer fluorescence increase at 378 nm (excitation at 342 nm) as a function of time up to 480 s. Protein-mediated transfer was measured upon addition of ns-PLTP after a stable signal of spontaneous transfer was achieved (see legends to the figures). For the transfer rate calculation, the linear range during first seconds after addition of acceptor vesicles or protein was covered.

3. Results and discussion

3.1. Chemical syntheses

The synthetic routes leading to the nucleotide–lipid conjugates are outlined in Fig. 1. The starting compound for the preparation of pyrene-labelled ceramide phosphorylthymidines (XII) and (XIII) was racemic 3-*O*-benzoyl-sphinganine (I) with both free amino- and hydroxyl-groups. The most crucial step in the synthesis of lipids carrying fluorescently labelled fatty acids is the choice of the selective method for acylation of the amino-group. For the synthesis of ceramides acylation could be carried out using an acid anhydride (Singh and Schmidt, 1989), acid-halides (Shapiro, 1969; Vunnam and Radin, 1979; Dokolina et al., 1983; Zimmerman et al., 1988), mixed carbodiimide (Hammarstroem, 1971), *N*-hydroxy succinimidates (Julina et al., 1986) or mixed anhydrides formed by ethylchloroformate and a fatty acid (Acquotti et al., 1986). Alternative methods, using coupling reagents such as dicyclohexylcarbodimide (Cohen et al., 1984; Kiso et al., 1986; Molotkovsky et al., 1991) or diethylphosphoryl cyanide in the presence of triethylamine (Anand et al., 1996), have also been applied. The procedures involving these acylation or activation agents have

several disadvantages including the requirement for a large molar excess of fatty acid, low yield, long reaction time, and undesirable side reaction such as *O*-acylation. In our case, this methodology must be neither too complicated nor include the use of acyl chlorides or anhydrides because of the possible degradation of pyrene fatty acids during preparations of their derivatives. On the other hand, the capacity of *p*-nitro- and pentafluorophenyl esters of carboxylic acids to acylate under certain conditions the amino group of various types of compounds—for example, serine, threonine, and hydroxylamine (Lapidot et al., 1967); sphingosine-1-phosphocholine (Ahmad et al., 1985); sphingoid bases or their derivatives (Tkaczuk and Thornton, 1981; Dokolina et al., 1983; Bruzik, 1988; Ohashi et al., 1989; Shibuya et al., 1992) led us to utilize these reagents in the acylation of 3-*O*-benzoyl-sphinganine (I). Thus, we performed this procedure with activated esters of pyrene fatty acids in water-free pyridine. The efficacy of the acylation of the amino group with *p*-nitrophenyl ester or with pentafluorophenyl ester of pyrenedecanoic acid, or with pentafluorophenyl ester of pyrenebutanoic acid was comparable and did not exceed 50% yields. This points to equivalent efficiency of *p*-nitrophenyl ester and pentafluorophenyl ester during derivatization. The use of other solvents or addition of small amounts of triethylamine did not significantly improve the yields. However, acylation of the amino group of sphingolipids with *p*-nitrophenyl oleate (Tkaczuk and Thornton, 1981), *p*-nitrophenyl esters of myristic or lauric acids (Groenberg et al., 1991), *p*-nitrophenyl stearate (Kann et al., 1991), or *p*-nitrophenyl palmitate (Ohashi et al., 1989; Shibuya et al., 1992) have been shown to be more effective. The low yields

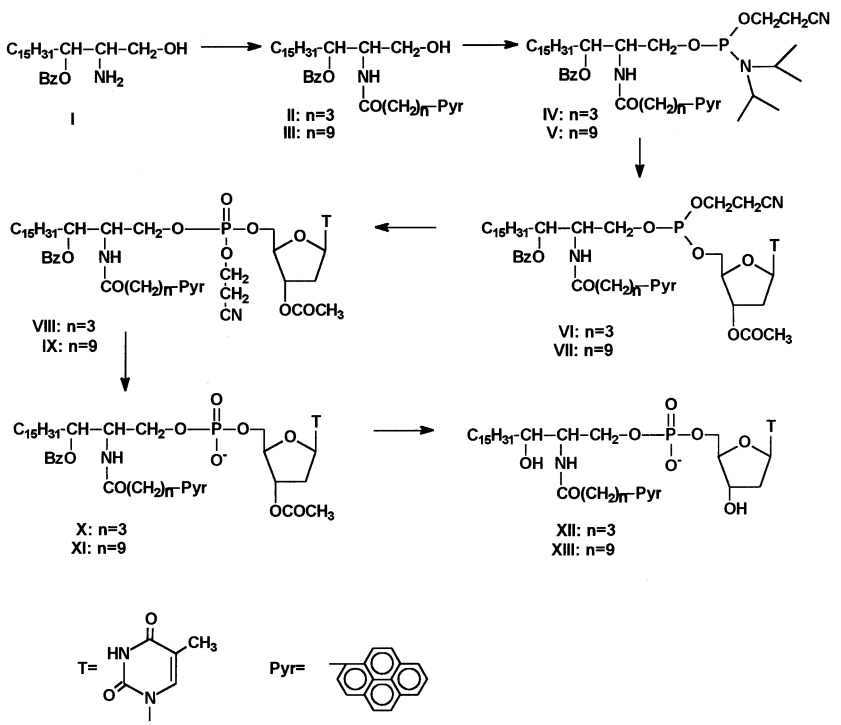

Fig. 1. Synthrtic routes of pyrene labelled ceramide phosphothymidines (XII) and (XIII).

of acylation observed with pyrene-labelled acid esters could be explained by the presence of the bulky pyrene group at the terminus of the fatty acids.

To create the phosphodiester structure, we chose the mild phosphitylation procedure, which had previously been used for the successful synthesis of ceramide phosphothymidine and its antiviral analogs (Oskolkova et al., 1996, 1997) as well as for glycerophospholipid synthesis (Morillo et al., 1996) and other substance classes (Beaucage and Iyer, 1992, 1993a,b). The corresponding pyrene-labelled ceramides (II, III) were converted with good yields (89.2 and 75.9%, respectively) using the bifunctional reagent, bis(*N*,*N*-diisopropylamino)-2-cyanoethyl phosphine in the presence of diisopropylammonium tetrazolide as catalyst. The ^{31}P-NMR spectra of purified phosphoramidites (IV) and (V) were indicative of the presence of two diastereoisomers each (148.18 and 148.54 ppm for (IV), and 148.05 and 148.79 ppm for (V)). 1H-tetrazole catalyzed the condensation of phosphoramidites (IV) and (V) with the free hydroxyl group of 3′-*O*-acetylthymidine. The resulting phosphite triesters (VI) and (VII) were oxidized in situ with tert-butyl hydroperoxide to give the phosphate triesters (VIII) and (IX), respectively. These compounds were partly deblocked without isolation by the action of triethylamine to form phosphodiesters (X) and (XI) with good yields. The structure of the two latter compounds was verified by ^{1}H-NMR spectroscopy data. The proton signals of both, lipid and nucleoside moieties, were fully present in the spectra. Finally, removal of benzoyl- and acetyl-protective groups by the action of sodium methylate in methanol furnished with 90.1 and 89.9% yields the target ceramide phosphothymidines (XII) and (XIII), respectively. The ^{31}P-NMR data of (XII) and (XIII) exhibit two signals (1.17 and 1.26 ppm for (XII); 1.52 and 1.60 ppm for (XIII)), which most likely belong to D- and L-diastereoisomers at the sphinganine residue. Analogous pairs of signals have been found in ^{31}P-NMR spectra of unlabelled ceramide phosphothymidines (Oskolkova et al., 1996, 1997). For phosphates (XII) and (XIII), ^{1}H- and ^{13}C-NMR spectra were recorded, which were in agreement with the structures of the synthesized ceramide phosphothymidines.

3.2. Spectral properties

The absorption (342 nm) and emission (378, 398 nm) wavelengths found in the UV- and fluorescence spectra in dilute solutions in ethanol of the synthesized substances (XII) and (XIII) were typical for pyrene-labelled phospholipids described earlier (Pownall et al., 1982; van Wijk et al., 1992) and revealed only monomer fluorescence.

The two compounds were used to study their membranotropic features as components of phospholipid vesicles. Both conjugates (XII) and (XIII) were readily incorporated into donor vesicles prepared by ethanol-injection. At low concentrations of pyrene ceramide phosphothymidines in single bilayer POPC vesicles, the fluorescence spectra were similar to those recorded in ethanol. At higher concentrations (above 0.5 mole%) of the labelled lipids, an excimer fluorescence band centered at 478 nm appeared. This indicates that ceramide phosphothymidines (XII) and (XIII) maintain the characteristic features of pyrene groups displaying a different emission spectrum depending on the degree of self-interactions. Particularly, the prevalence of the two emission peaks at 398 and 378 nm, observed in organic solvent solution, is typical of non-interacting pyrene molecules, while the preponderance of the wide peak at 478 nm, observed at higher concentrations, indicates interactions within the hydrophobic tails carrying the pyrene group. The intensity of the excimer fluorescence at 478 nm (E) relative to the monomer fluorescence at 398 nm (M) of each pyrenyl derivative increased with its microscopic concentration in POPC vesicles. The relationship between the E/M ratio and the molar% concentration of Pyr–Cer–P–T in vesicles was linear up to about 4 mole% for both short and long-chain pyrene derivatives (Fig. 2), which is in agreement with published data (Massey et al., 1982b; Somerharju et al., 1985; Ollman et al., 1987). However, pyrene-labelled phospholipids

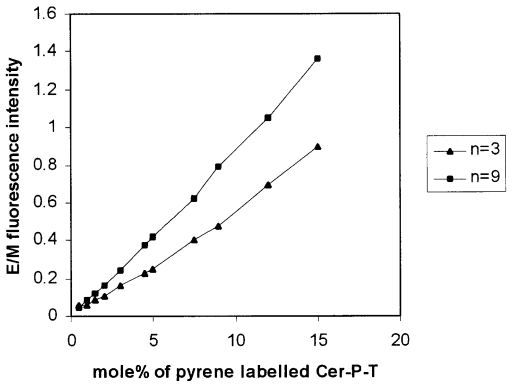

Fig. 2. Dependence of the excimer (478 nm) to monomer (398 nm) intensity ratio on the Pyr-Cer-P-T (XII, $n=3$) (▲) and (XIII, $n=9$) (■) concentration in POPC vesicles at 37°C. Each data point is an average value out of three determinations of the E/M fluorescence intensity (SD $\pm \leq 5\%$). For details see Section 2.4.

don't always show the linear E/M dependence upon their concentration, thus supporting evidence for regular distribution of labelled phospholipids in vesicles (Viani et al., 1988; Tang and Chong, 1992; Chong et al., 1994) due to the immiscibility of pyrene lipids in phospholipid bilayers (for example, pyrenedodecanoyl sphingomyelin in stearoyl sphingomyelin or in POPC (Barenholz et al., 1996)). On the other hand, pyrene-labelled phosphatidylcholine in dimyristoylphosphatidylcholine membranes is an example of a system with nearly ideal mixing (Hresko et al., 1986).

The Fig. 2 shows the higher tendency of the long-chain derivative (XIII) to form excimer structures. This is apparent from the higher E/M intensities compared to E/M ratio observed with the short chain compound (XII) at the same concentrations and temperature. Within a phospholipid bilayer, pyrene residues of long chain acyl rests are located close to the boundary between two membrane leaflets and are therefore more mobile and the probability of excimer formation is increased. In contrast, with the short-chain compound (XII) the pyrene label experiences lower mobility and is thus less likely to form excimers.

Our data are in agreement with published results (Barenholz et al., 1996) supporting the notion that the attachment of a paraffinic chain to pyrene reduces its ability to form excimers, which may be related to lower mobility and/or steric restriction, which decreases the ability to give excimers.

3.3. Spontaneous transfer studies

As a measure of the capacity of Pyr–Cer–P–T (XII) and (XIII) to exchange between membranes, we determined their transfer from donor unilamellar vesicles to a 10- and 40-fold excess of acceptor vesicles. Fig. 3 shows the spontaneous transfer of pyrenebutanoyl- and pyrenedecanoyl ceramide phosphothymidines (lines I and II, respectively) at 37°C. Compared to Pds–Cer–P–T (XIII), the spontaneous transfer of Pbs–Cer–P–T (XII) is approximately 26-fold faster, with estimated transfer rates of 14.89 pmol/min for (XII) and 0.57 pmol/min for (XIII) at the donor to acceptor ratio 1:10. Therefore, the short-chain lipid conjugate (XII) was less stably integrated in a POPC bilayer compared to its long-chain counterpart (XIII). The intermembrane transport (Fig. 3) could be described as a first order kinetic process. Furthermore, the transfer rate for both lipids was independent of acceptor concentration. These lines of evidence support the notion that the lipid transfer occurs via rate-limiting desorption of the pyrene probe from the donor surface into the surrounding aqueous phase, followed by a rapid diffusion-controlled association of pyrene lipid with the acceptor (Galla et al., 1979; Roseman and Thompson, 1980; McLean and Philips, 1981; Correa-Freire et al., 1982; Massey et al., 1982a,b; Nichols and Pagano, 1982; Pownall et al., 1982, Frank et al., 1983; Pownall et al., 1983; Arvinte and Hildenbrand, 1984; McLean and Philips, 1984; Via et al., 1985; Homan and Pownall, 1988). Thus, our data conform to the criteria for lipid transfer occurring via the aqueous phase. Another liponucleotide analogue, 3′-deoxythymidine diphosphate diglyceride, has been shown to be transported by the same mechanism (van Wijk et al., 1992). The rate of intermembrane transfer is determined by several factors: (1) insolubility of the bulky polar headgroup in the hydro-

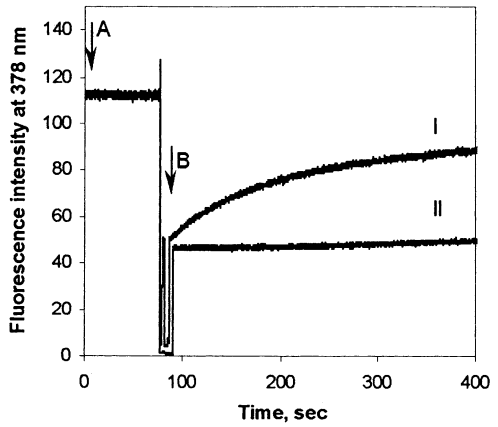

Fig. 3. Spontaneous intermembrane transfer of fluorescently labelled ceramide phosphothymidines (XII) and (XIII). Donor vesicle lipids (10 μl, total lipid concentration 250 μM, 9 mole% of the labeled lipids) were injected into buffer (990 μl) (arrow A). After 3 min of intensive stirring, a 10-fold excess of acceptor vesicles (1 ml, 25 nmol) was added (arrow B). Traces: I, Pbs-Cer-P-T (XII); II, Pds-Cer-P-T (XIII). The temperature was held constant at 37°C.

phobic membrane interior results in a slow flip–flop rate in lipid model systems; (2) bulkiness and charge of the polar headgroup facilitate phospholipid transmembrane transport; (3) the length of fatty acid chains is responsible for the solubility of lipids in an aqueous phase. The latter factor is a major determinant as it affects the first stage of spontaneous transport, namely dissociation of lipids from donor vesicles (McLean and Philips, 1981; Ferrell et al., 1985). Manipulations of the acyl chain lengths greatly affects the $t_{1/2}$ values for spontaneous transfer.

Pownall et al. (1982) have reported data on spontaneous transfer of *N*-(9-(1-pyrenyl)-nonanoyl)sphingomyelin between single-bilayer vesicles composed of DMPC, beef brain sphingomyelin, or 1-palmitoyl-2-palmitoleoylphosphatidylcholine. It was found that the transfer of bovine brain sphingomyelin was about an order of magnitude slower below the phase transition of this lipid than above it. Where comparable, however, respective half-times were about an order of magnitude smaller than those described by others (Frank et al., 1983). The origin of this difference is due to the different lengths of pyrenyl fatty acids which were used for sphingomyelin labeling. The transfer half-times increase with increasing chain length of the amide-linked fatty acid in sphingomyelins. A similar acyl chain length dependence has been noted for a variety of phospholipids labelled with the NBD fluorophore (Nichols and Pagano, 1982) as well as for pyrene-labelled glycerophospholipids, cerebrosides and other amphiphiles (Massey et al., 1982a; Pownall et al., 1983; Via et al., 1985). Studies performed by Homan and Pownall (1988) have shown that an increase of the *sn*-1-acyl chain length by two methylene groups reduced the rate of spontaneous transfer approximately 8-fold.

3.4. Protein-mediated transfer

The effect of an enriched non-specific lipid transfer protein preparation on the rate of transfer of Pbs- (XII) and Pds–Cer–P–T (XIII) was tested using a 10-fold excess of acceptor. The ns-PLTP from bovine liver was shown to accelerate transfer of the ceramide phosphothymidine bearing the pyrenedecanoic acid moiety (XIII) (Fig. 4). In contrast, the protein did not affect the

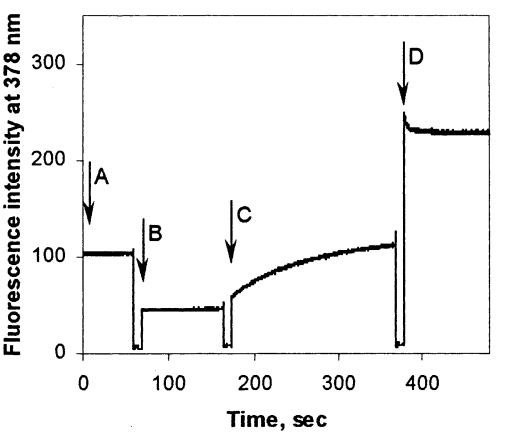

Fig. 4. Protein-mediated transfer of Pds-Cer-P-T (XIII) catalyzed by non-specific phospholipid transfer protein from bovine liver. The arrow A shows addition of the donor vesicles (1 ml, total lipid content 2.5 nmol, 9 mole% of the labelled lipid in POPC). At arrow B, acceptor vesicles (1 ml, 25 nmol) were added and the spontaneous transfer was monitored. At arrow C, lipid-transfer protein (20 μl, 1 mg protein/ml) was added. After addition of Triton X-100 (10 μl, 20%) (arrow D) the cesicles were completely disintegrated.

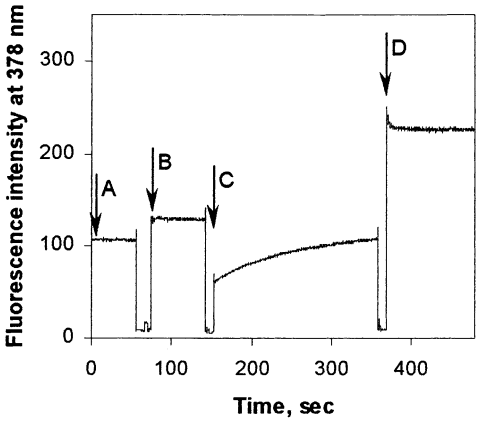

Fig. 5. Binding of Pds-Cer-P-T by ns-PLTP. After a stable signal from donor vesicles (1 ml, 2.5 nmol total lipid content, 9 mole% of label) (arrow A) was obtained, ns-PLTP was added (20 μl, 3 mg protein/ml) (arrow B), followed by 1 ml (25 nmol) acceptor vesicles (arrow C). At arrow D, Triton X-100 (10 μl, 20%) was added.

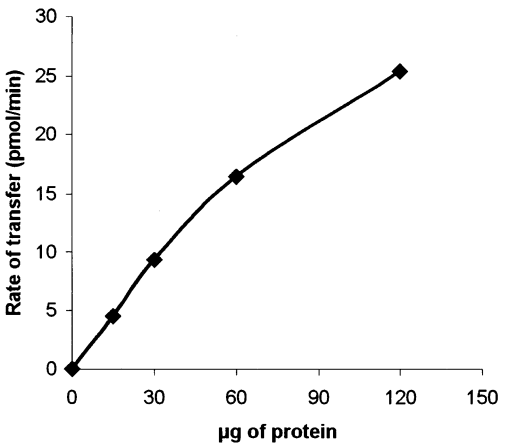

Fig. 6. Dependence of protein mediated transport of Pds-Cer-P-T (XIII) from donor to acceptor vesicles on the protein content. For details see Section 2.

transfer rate of the short-chain Pbs–Cer–P–T (data not shown), most likely due to the considerably higher rate of spontaneous transfer of the short-chain labelled lipid (XII). Upon addition of ns-PLTP to the donor vesicle dispersion containing the liponucleotide (XIII), the monomer fluorescence intensity increases instantaneously (Fig. 5). Upon addition of the acceptor vesicles, a further gradual increase occurs. This experiment demonstrates that upon mixing ns-PLTP and Pds–Cer–P–T the lipid is rapidly bound by the protein and then transferred to the acceptor vesicles. ns-PLTP from bovine liver stimulated the transfer of Pds–Cer–P–T between vesicles in a concentration dependent manner (Fig. 6). It was previously demonstrated that this protein was capable of stimulating the transfer of the CDP-DG analogue 3′-deoxythymidine diphosphate diglyceride between vesicles (van Wijk et al., 1992). Our data demonstrate that ns-PLTP also catalyzes the intermembrane transfer of ceramide phosphothymidine.

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