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SYNTHESIS OF PHOSPHOLIPID ANALOGUES. VARIATION OF THE P–N DISTANCE

WALTER DIEMBECK and HANSJÖRG EIBL*

Max-Planck-Institut für biophysikalische Chemie, D-3400 Göttingen-Nikolausberg (F.R.G.)

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Phosphatidyl choline analogues with increased phosphate–trimethylammonium distance and phosphatidyl ethanolamine analogues with increased phosphate–ammonium distance were synthesised. The distance was varied by the incorporation of additional methylene groups, from 2 (natural) to 11 CH_2-units. The synthesis of the analogues was possible by phosphorylation of 1,2-dipalmitoyl-*sn*-glycerol with bromoalkylphosphoric acid dichlorides which were obtained from the respective bromoalkanols and phosphorus oxychloride. The resulting bromoalkylesters of 1,2-dipalmitoyl-*sn*-glycerol were subjected to direct amination with trimethylamine, dimethylamine, methylamine and ammonia to yield the respective phosphatidyl choline and ethanolamine analogues. Chromatographically pure products were obtained in yields of 50 to 70% of the 1,2-diacyl glycerol.

Introduction

The spontaneous arrangement of phospholipids into macromolecular structures such as bilayer vesicles or micelles is a direct consequence of their amphiphilic nature, that is, a polar and apolar region present in one and the same molecule. The polar moiety is of critical importance in determining the physical properties of the bilayer membrane and its significance can be investigated by maintaining the apolar part of the molecule constant [1]. For instance, a drop in the transition temperature of approx. 20°C has been observed when the negative charge per phospholipid molecule was reduced by one unit [2–7]. Similar changes in the transition temperature behaviour might be expected for diacyl-phosphocholines if the phosphate–trimethylammonium distance is increased, thus increasing the dipol moment in the head-group and possibly the intramolecular repulsive forces.

Therefore the aim of this work was the synthesis of a series of specific phospholipids which differ from their natural analogues by a greater charge separation. One such representative molecule has dipalmitoyl chains as the apolar part; the natural analogue being 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine which is

*To whom correspondence should be addressed.

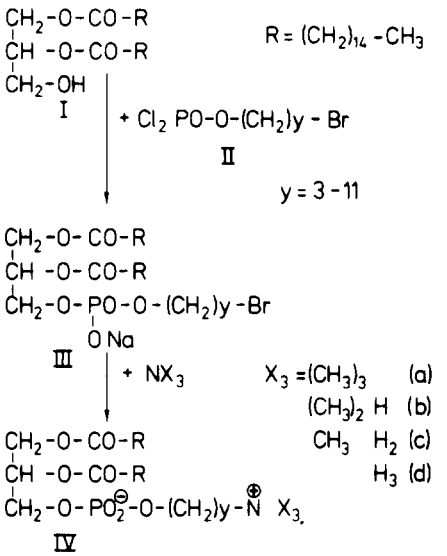

Fig. 1. Direct amination of phosphatidic acid bromoalkylesters to yield phosphatidyl cholines and ethanolamines with increased phosphate–trimethylammonium and phosphate–ammonium distance.

known to be the main lipid in lung surfactant [8–10]. This phospholipid has a phase transition temperature of 41°C [2] and the phosphate is separated from trimethylammonium by two methylene units (see Fig. 1; *IV*, $y = 2$).

This report describes the synthesis of phospholipid analogues which allow stepwise modifications of y from 3 to 11 and of X from *a* to *d* (see Fig. 1.) The transition temperature of the naturally-occurring phospholipid, 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine, occurs in the middle of the experimentally-accessible temperature range in water (0–90°C). It can therefore be used as a reference point from which to study the influence of the discussed structural modifications on the phase transition behaviour (W. Diembeck and H. Eibl, in preparation).

Materials and methods

Glycerol and hydrogen bromide (47% solution in water) were purchased from Merck (Darmstadt, Germany). Fatty acid chlorides, the alkandiols, 3-bromopropanol-(1) and the aminobases were products of Fluka (Switzerland). All solvents and reagents were of p.a. grade.

1,2-Dipalmitoyl-*sn*-glycerol (*I*) was prepared as previously described [11,12]. 1,2-Dipalmitoyl-*sn*-glycerol-3-phosphocholine as a reference was purchased from Medmark (München-Grünwald, Germany).

The crude products of the aminations were purified by column chromatography using Mallinckrodt Silic AR (100–200 mesh). Chloroform, methanol and aqueous ammonia (25%) were used for elution, starting with $CHCl_3/CH_3OH$/25% ammonia (200 : 15 : 1 by vol.) and increasing the polarity of the solvent stepwise up to 65 : 30 : 3 to elute the phosphatidylcholine or phosphatidyl ethanolamine analogues.

The end-products were characterised by microanalysis and by determination of the molar ratio of phosphorus : acyl : vicinal diol as described earlier [13]. Phosphate was determined according to Eibl and Lands [14] with the reagent kit supplied by Serva (Heidelberg, Germany). Vicinal diol was determined by periodate analysis by measuring the formed iodate directly as introduced by Eibl and Lands [15].

Experimental

Bromoalkanols

4-Bromobutanol-(1) and 5-bromobutanol-(1)

1,4-Butanediol or 1,5-pentanediol (0.25 mol), hydrogen bromide, 80 g (47% solution in water; 0.48 mol), 500 ml benzene and 10 ml petroleum benzene (b.p. 100–140°C) were heated under reflux for 7 h with continuous stirring.

ω-Bromoalkanols-(1)

The diols (0.25 mol), for instance hexandiol-(1,6) or undecandiol-(1,11), hydrogen bromide, 80 g (47% solution in water; 0.48 mol) and 1.5 l petroleum benzene (boiling range 100–140°C) were heated under reflux for the time intervals given in Table I.

The bromination process was followed by thin layer chromatography (TLC). After completion, the reaction products were found to be dissolved in the upper

TABLE I

ω-BROMOALKANOLS OBTAINED BY REACTION WITH HYDROGENE BROMIDE AS DESCRIBED IN THE TEXT

ω-Bromoalkanols	Reaction times (h)	Boiling points (°C at 53.4–80.0 Pa)	yields (%)
4-Bromo-butanol-(1)	6.5	58– 60	80
5-Bromo-pentanol-(1)	6	72– 74	83
6-Bromo-hexanol-(1)	1.5	85– 87	90
7-Bromo-heptanol-(1)	1.5	87– 89	95
8-Bromo-octanol-(1)	1	110–112	93
9-Bromo-nonanol-(1)	1	112–114	91
10-Bromo-decanol-(1)	0.5	124–126	95

phase and therefore the lower phase was discarded. The solvent was removed by evaporation and the residue distilled under reduced pressure to give colourless liquids up to 8-bromooctanol-(1) or white crystals for the longer chain derivates in yields ranging from 80% to 95% (based on the diol) as shown in Table I.

ω-Bromoalkylphosphoric acid dichloride (II)

Phosphorus oxychloride, 45 g (0.03 mol; freshly distilled, b.p. 105–107°C) was dissolved in trichloroethylene, 100 ml, and ω-bromoalkanol (0.02 mol) was added with stirring at 20°C. After 12 h of reaction, phosphorylation was completed as shown by TLC. After adding toluene, 50 ml, the solvents and excess phosphorus oxychloride were evaporated at 40°C under reduced pressure. The residual oil was used for the phosphorylation of 1,2-dipalmitoyl-*sn*-glycerol without further purification.

ω-Bromoalkylesters of 1,2-dipalmitoyl-sn-*glycerol-3-phosphoric acid* (III; y = 3–11)

Bromoalkylphosphoric acid dichloride (0.02 mol) in 60 ml trichloroethylene was cooled in an ice bath. After the addition of triethylamine, 8 g (0.08 mol), the mixture was thermostated at 25°C. With continuous stirring, 1,2-dipalmitoyl-*sn*-glycerol, 8 g (0.014 mol), dissolved in 60 ml trichloroethylene was added dropwise to the dichloride. TLC showed that the reaction was completed after 1 h. Then toluene, 120 ml, was added to the reaction mixture and the precipitated triethylammonium chloride 2 g (0.015 mol) was removed by filtration. The filtrate was evaporated and the residue dissolved in 100 ml tetrahydrofurane. Hydrolysis of the phosphoric acid monochlorides and of the excess ω-bromoalkyl-phosphoric acid dichlorides was initiated by the addition of 100 ml of sodium acetate (0.5 M, pH 5.0) with stirring. The hydrolysis was conveniently followed by TLC and found to be completed after 6 to 8 h. Ethylenediaminetetraacetate (5 ml, 0.5 M, pH 9.5) was added to the mixture and the pH of the solution was adjusted to 9.0 by the addition of sodium hydroxide. The products were extracted by the addition of 100 ml diisopropylether. To facilitate phase separation methanol, 20–40 ml, was also added.

The ether phase containing the sodium salts of the bromoalkylesters of phosphatidic acids was evaporated to dryness. The residue was dissolved in 20 ml of chloroform and precipitated by the addition of 200 ml of acetone. The precipitate was stored at 0°C and used directly for the aminations.

Amination of bromoalkylesters of 1,2-dipalmitoyl-sn-*glycerol-3-phosphoric acid* (IV; y = 3–11; X = a–d)

Amination with methylated amines (IV; y = 3–11; X = a–c)

Bromoalkylester (0.01 mol) was dissolved in 130 ml of chloroform/2-propanol/

TABLE II

ELEMENTAL ANALYSIS OF PHOSPHATIDYL CHOLINES AND PHOSPHATIDYL ETHANOLAMINES WITH INCREASED PHOSPHATE-TRIMETHYLAMMONIUM DISTANCE

Y indicates the number of methylene groups which separate phosphate and trimethylammonium. The letters *a* to *d* indicate the degree of N-methylation as shown in Fig. 1. The molecular weights of the phosphatidyl cholines are calculated for the monohydrates (3*a* to 11*a*).

$-(CH_2)_Y$	Formula (mol. wt)		%C	%H	%N	%P
3*a*	$C_{41}H_{84}NO_3P$	calc.:	64.28	11.05	1.83	4.04
	(766.10)	found:	63.26	10.49	1.52	3.86
b	$C_{40}H_{80}NO_8P$	calc.:	65.45	10.99	1.91	4.22
	(734.06)	found:	64.89	10.81	1.87	3.91
c	$C_{39}H_{78}NO_8P$	calc.:	65.06	10.92	1.95	4.30
	(720.04)	found:	64.66	10.79	1.85	4.01
d	$C_{38}H_{76}NO_8P$	calc.:	64.65	10.85	1.98	4.39
	(706.01)	found:	64.41	10.71	1.90	4.35
5*a*	$C_{43}H_{88}NO_9P$	calc.:	65.03	11.17	1.76	3.90
	(794.15)	found:	64.36	11.04	1.84	3.91
6*a*	$C_{44}H_{90}NO_9P$	calc.:	65.39	11.23	1.73	3.83
	(808.18)	found:	66.66	11.45	1.80	4.08
b	$C_{43}H_{86}NO_8P$	calc.:	66.54	11.17	1.81	3.99
	(776.14)	found:	66.24	11.01	1.75	3.71
c	$C_{42}H_{84}NO_8P$	calc.:	66.19	11.11	1.84	4.06
	(762.12)	found:	66.05	11.04	1.82	3.95
d	$C_{41}H_{82}NO_8P$	calc.:	65.83	11.05	1.87	4.14
	(748.09)	found:	65.66	10.94	1.81	4.07
7*a*	$C_{45}H_{92}NO_9P$	calc.:	65.74	11.28	1.70	3.77
	(822.20)	found:	64.90	11.16	2.02	4.59
8*a*	$C_{46}H_{94}NO_9P$	calc.:	66.07	11.33	1.68	3.70
	(836.23)	found:	65.15	10.91	2.30	4.60
9*a*	$C_{47}H_{96}NO_9P$	calc.:	66.39	11.38	1.65	3.65
	(850.26)	found:	66.28	11.43	1.85	3.85
11*a*	$C_{49}H_{100}NO_9P$	calc.:	67.01	11.48	1.59	3.53
	(878.31)	found:	67.19	11.15	1.58	3.64

dimethylformamide (3 : 5 : 5 by vol.) at 50°C and 70 ml of amine (40% solution of trimethylamine, dimethylamine or methylamine in water) was added. The reaction was completed after 4–6 h as shown by TLC. The volatile solvents were removed by evaporation at 40°C and 1.74 kPa. The residual water/dimethylformamide mixture was extracted by chloroform/methanol. The combined chloro-

form extracts were evaporated and the residue purified by silica gel chromatography (100 g of silica gel were used for the chromatography of 6–10 g of product).

Amination with ammonia (IV; *3d and 6d*)

Bromoalkylester (0.01 mol) was dissolved in 600 ml of chloroform/2-propanol/dimethylformamide (1 : 1 : 4 by vol.) at 40°C and 300 ml of ammonia (25% solution in water) were added. The reaction was completed after 6–10 h as shown by TLC. The volatile solvents were removed by evaporation at 40°C and 1.74 kPa. The produce was extracted from water/dimethylformamide by the addition of chloroform/methanol. The combined chloroform extracts were evaporated and the residue purified by silica gel chromatography as described in Materials and methods (100 g silica gel were used for the purification of 6–10 g of product).

Analytical data for the synthesised compounds and final yields are summarised in Table II.

Results and discussion

The synthesis of phospholipid analogues with increased phosphate-trimethylammonium and phosphate-ammonium distance is described. The synthesis is based on 1,2-dipalmitoyl-*sn*-glycerol which was converted to the respective bromoalkylesters of phosphatidic acid. The bromoalkylesters were used as general intermediates for the synthesis of phosphatidyl ethanolamines or cholines as described by Eibl and Nicksch [16].

Three routes were initially examined for the synthesis of bromoalkylesters of phosphatidic acids. Firstly the monosilver salt of the corresponding phosphatidic acid was refluxed with 1,4-dibromoalkanes to give unsatisfactory yields (<5%) of bromobutylester. Secondly the activation of 4-bromobutanol-(1) by triisopropyl benzene sulfonylchloride [17] followed by the condensation with phosphatidic acid was found to be somewhat better (yields approx. 30%). Thirdly, however, the reaction of 4-bromobutylphosphoric acid dichloride with 1,2-dipalmitoyl-*sn*-glycerol was superior and resulted in the formation of the intermediate 4-bromobutylester of phosphatidic acid in excellent yields (70–80%).

Bromoethylphosphoric acid dichloride has been used by different groups for the synthesis of phosphatidyl cholines [18]. It has been introduced by Hirt and Berchthold as a phosphorylating agent in lipid chemistry [19]. Bromopentylphosphoric acid dichloride was described by Eibl and Westphal [20] and bromopropylphosphoric acid dichloride by Kerscher et al. [21]. The final decision to use bromoalkylesters of phosphoric acid as phosphorylating agent was strongly influenced by good results with bromobutylphosphoric acid dichloride, earlier experiments with bromopentylphosphoric acid dichloride [20] and the extensive experience in our laboratory with bromoethylphosphoric acid dichloride [12,16]. However, distillation of the bromoalkylphosphoric acid dichlorides prior to phosphorylation was

omitted. The bromoalkylphosphoric acid dichlorides of longer chain bromoalkanols have higher boiling points and tend to decomposition. This is most pronounced for the respective 4-bromobutylesters and 5-bromopentylesters where degradation and formation of tetrahydrofurane and tetrahydropyrane was observed during distillation. Therefore excess phosphorus oxychloride was used for the preparation of bromoalkylphosphoric acid dichlorides and complete conversion of the bromoalkanols was confirmed by TLC. The unreacted phosphorus oxychloride was then removed from the bromoalkylphosphoric acid dichlorides by evaporation with toluene at reduced pressure. Care was taken that the temperature did not exceed 50°C.

Different procedures for the preparation of bromoalkanols have already been described [22–24]. The solvent extraction procedure of Degering and Boatright [24] seemed the most promising. Hexandiol-(1,6) was heated for three days with hydrobromic acid (47% solution in water) and petroleum benzene in a specially-devised extraction apparatus. According to our experience the extraction apparatus is not necessary and the reaction times can be considerably shortened. As shown in Table I the respective bromoalkanols are obtained in almost quantitative yields by simply refluxing the diols for several hours in correctly-selected solvent systems. Dibromoalkanes as by-products are not detected and the bromoalkanols are purified by distillation or recrystallisation from hexane. Melting points, boiling points and yields of the different bromoalkanols are summarized in Table I.

Solvent mixtures of tetrahydrofurane and toluene were used for the reaction of 1,2-dipalmitoyl-*sn*-glycerol with the different bromoalkyl-phosphoric acid dichlorides in the presence of triethylamine. This mixture of solvents led to an almost complete precipitation of the formed triethylammonium hydrochloride, whereas the phosphorylated product is completely dissolved at 20°C. This is shown by the weight of trimethylammonium hydrochloride, which perfectly corresponds to the stoichiometry of the reaction. In other solvents, such as chloroform and trichloroethylene, which were used in earlier studies, triethylammonium hydrochloride is only partly precipitated.

The filtrate is then directly hydrolysed in the tetrahydrofurane solution. Acetone is added and the precipitated bromoalkylesters are isolated in an almost pure state, 90–95% as judged from TLC.

The reaction of the bromoalkylesters with trimethylamine was straightforward and the products were purified by column chromatography in solvent systems containing ammonia. Thus the choline bromides were converted into the trimethylammonium form without the previously-used treatment with silver acetate [12].

The synthesised compounds, phosphatidyl choline analogues with increased phosphate trimethylammonium distance, were used in model membrane studies. The influence of the stepwise increase in the charge distance on the phase transition is to be described elsewhere (Diembeck and Eibl, in preparation).

It has already been shown that phospholipase D catalyses the exchange of various

primary alcohols for the choline moiety of phosphatidyl cholines [25]. The reaction depends upon the chain length of the acceptor alcohol whereas the choline moiety of the donor molecule was kept constant. This series of phospholipids with alterations in the P–N distance now allows study of phospholipase activities as a function of the head-group composition. Preliminary experiments have shown that phospholipase A_2 can tolerate rather large changes in the polar region of phosphatidyl cholines.

Thus, the synthetic phosphatidyl choline analogues not only permit examination of membrane structure of phospholipid bilayers but also allow investigation of substrate-enzyme interaction with respect to chemical and steric modifications in the polar regions of phospholipids.

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References

1 H. Eibl, in W.H. Kunau and R.T. Holman (Eds.), Polyunsaturated Fatty Acids, Am. Oil Chem. Soc. Champaign, IL, U.S.A., (1977) p. 229.
2 H. Träuble and H. Eibl, Proc. Natl. Acad. Sci. U.S.A., 71 (1974) 214.
3 H. Träuble and H. Eibl, in F.O. Schmitt, D.M. Schneider and D.M. Crothers (Eds.), Functional Linkage in Biomolecular Systems, Raven Press, (1975) p. 59.
4 H. Träuble, M. Teubner, P. Woolley and H. Eibl, Biophys. Chem., 4 (1976) 319.
5 A.J. Verkleij, B. de Kruyff, P. Ververgaert, J.F. Tocanne and L.L.M. van Deenen, Biochim. Biophys. Acta, 339 (1974) 432.
6 A. Watts, K. Harlos, W. Maschke and D. Marsh, Biochim. Biophys. Acta, 510 (1978) 63.
7 H. Eibl and A. Blume, Biochim. Biophys. Acta, in press.
8 R.J. King and J.A. Clements, Am. J. Physiol., 223 (1972) 715.
9 T.E. Morgan, T.N. Finley and H. Fialkow, Biochim. Biophys. Acta, 106 (1965) 403.
10 H. Träuble, H. Eibl and H. Sawada, Naturwissenschaften, 61 (1974) 344.
11 E. Baer and H.O.L. Fischer, J. Biol. Chem., 128 (1939) 475.
12 H. Eibl, D. Arnold, H.M. Weltzien and O. Westphal, Liebigs Ann. Chem., 709 (1967) 226.
13 H. Eibl, and W.E.M. Lands, Biochemistry, 9 (1970) 423.
14 H. Eibl and W.E.M. Lands, Anal. Biochem., 30 (1969) 51.
15 H. Eibl and W.E.M. Lands, Anal. Biochem., 33 (1970) 58.
16 H. Eibl and A. Nicksch, Chem. Phys. Lipids, 22 (1978) 1.
17 R. Aneja, J.S. Chadha and A.P. Davies, Tetrahedron Lett., 48 (1969) 4183.
18 A.J. Slotboom and P.P.M. Bonsen, Chem. Phys. Lipids, 5 (1970) 301.
19 R. Hirt and R. Berchthold, Pharm. Acta Helv., 33 (1958) 349.
20 H. Eibl and O. Westphal, Liebigs Ann. Chem., 738 (1970) 170.
21 P. Nuhn, H.J. Rüger, P. Kertscher, K. Gawrisch and K. Arnold, Pharmazie, 33 (1978) 181.
22 R.S. Tipson, J. Org. Chem., 9 (1944) 235.
23 F.L.M. Pattison, J.B. Strothers and R.G. Woolford, J. Am. Chem. Soc., 78 (1956) 2255.
24 E.F. Degering and L.G. Boatright, J. Am. Chem. Soc., 72 (1950) 5137.
25 S. Kovatchev and H. Eibl, in S. Gatt, L. Freysz and P. Mandel (Eds.), Enzymes of Lipid Metabolism, Plenum Publishing Co., 1978, p. 221.