Chemistry and Physics of Lipids, 33 (1983) 33–40
Elsevier Scientific Publishers Ireland Ltd.

A NEW SYNTHESIS OF BIS(DIACYLGLYCERO)PHOSPHATE

QUOC QUAN DANG* and WILHELM STOFFEL

Institut für Physiologische Chemie der Universität zu Köln, D-5000 Köln 41 (Federal Republic of Germany)

Received February 27th, 1982 revision received July 29th, 1982
accepted December 16th, 1982

The chemical synthesis of bis(diacylglycero)phosphate previously named bisphosphatidic acid, starting with a diacylglycerol and phosphatidic acid, is described. The phosphodiester bond formation is catalyzed by triisopropylbenzenesulfonylchloride. This simple approach allows the preparation of saturated as well as unsaturated bis(diacylglycero)phosphate species in one step without the use of any protecting group. The methods used until now yield only mono-acid species, or mixed-acid unsaturated species after many steps involving the introduction and the removal of protecting groups. The synthetic products have been characterized by component analysis and NMR-techniques.

Keywords: chemical synthesis; bis(diacylglycero)phosphate; triisopropylbenzenesulfonyl chloride; chemical and NMR spectroscopical characterization of products.

Introduction

Bis(diacylglycero)phosphate forms a group of acidic phospholipids, structurally related to cardiolipin. Besides their occurrence in developing plant tissues [1], their lysoderivatives have been identified as lysosomal storage products in certain lipidoses [2–11]. Nothing is known about their origin and biosynthetic pathway.

The synthesis of this phospholipid was reviewed by Hanahan [12], Van Deenen and De Haas [13] and recently by Kates [14]. Initially, saturated (1,2-diacyl-*sn*-glycero)phosphates with identical fatty acid residues were synthesized as a by-product [15]. They were obtained as main product with a reported almost quantitative yield [16], by phosphorylation of *sn*-1,2-diacylglycerols with phenylphosphoryldichloride to phenyl-bis(diacylglycero)phosphate, and subsequent hydrogenolysis for the removal of the phenyl protecting group. Baer and Buchnea synthesized mono-acid unsaturated bis(1,2-diacyl-*sn*-glycero)phosphates using *sn*-1,2-diacylglycerols and phosphorus trichloride [17] and de Haas et al. synthesized

**Present address:* INSERM, U 101. Biochimie des Lipides, 31059 Toulouse Cedex, France.

0009–3084/83/$03.00
Published and Printed in Ireland

mixed-acid unsaturated bis(1,2-diacyl-*sn*-glycero)phosphates [13,18] from phosphatidic acid mono-benzylester mono-silver salt and 1,2-diacylglycerol-iodohydrine. This last method required a multiple step synthesis via the protected phosphatidic acid salt, the phosphotriester, and finally the anionic removal of the benzyl protecting group.

We describe here an improved synthesis of bis(diacylglycero)phosphate. The phosphodiester bond was formed between a phosphatidic acid and a diacylglycerol by the activation of the phosphate group by triisopropylbenzenesulfonyl chloride (TPS), a reagent first used in the synthesis of polynucleotides by Lohrmann and Khorana [19] and applied to the synthesis of phosphoglycerides by Aneja et al. [20,21].

This pathway can be applied to the synthesis of unsaturated as well as saturated, mono-and mixed-acid bis(diacylglycero)phosphates with any fatty acid substitution, and any stereo chemical configuration. Moreover it is a simple one-step reaction starting from easily available materials, without need of introduction and removal of protecting groups.

In the previous syntheses and in ours, the species obtained had the tetracyl *sn*-glycero-3-phosphoryl-3′-glycerol configuration (although other configurations could not be excluded a priori). The naturally occurring bis(diacylglycero)phosphates or their diacylated derivatives could have this configuration or the unusual 1-*sn*-1′-*sn* configuration [4] or even the 3-*sn*-1′-*sn* configuration [14].

Materials and methods

Materials

The following diacylglycerols and PAs (analytical grade) have been purchased from Fluka Buchs, S.G., Switzerland: *rac*-1,2-dipalmitoylglycerol, 1,2-dipalmitoyl-*sn*-glycerol and the disodium salts of their respective PAs: *rac*-1,2-dipalmitoyl-3-glycerophosphate and 1,2-dipalmitoyl-*sn*-glycero-3-phosphate.

The free acid form of phosphatidic acid was generated from its disodium salt by the addition of equivalent amounts of aqueous 1 N HCl and extraction with ether/methanol, 10:1 (by vol.), removal of the solvents and drying in vacuo over P_2O_5. Its dipyridinium salt, more stable than the free acid, was obtained in crystalline form by addition of pyridine in a solution of phosphatidic acid in chloroform/methanol, 2:1 [14].

Unsaturated 1,2-diacylglycerols were prepared by phospholipase C (*Bacillus cereus*) hydrolysis of soy polyene phosphatidylcholine (kindly provided by Nattermann & Cie., D-5000 Köln) according to the standard assay procedure [22].

TPS, analytical grade, was purchased from E. Merck A.G. (D-6100 Darmstadt). All solid reactants for the phosphorylation were rigorously dried in vacuo over P_2O_5. All organic solvents were rigorously dried. Anhydrous pyridine (analytical grade) was obtained by distillation over CaH_2 [19].

Analytical methods

Phosphorus determination was carried out according to Rouser et al. [22].

The ratio fatty acyl/P was determined by quantitative gas liquid chromatography using methyl hendecanoate as an internal standard, as follows: saponification of the phospholipid with 0.5 N KOH in H_2O/MeOH (1:1, by vol), acidification with aqueous 6 N HCl, extraction of the fatty acid by petroleum ether/ether (1:1), esterification with BF_3–methanol for 30 min at 80°C, chromatography on silanized chromosorb P coated with 10% polyethylene adipate, 200 × 0.27 cm at 205°C.

Thin-layer chromatography was carried out on precoated silicagel G plates, 0.25 mm (E. Merck, A.G., D-6100 Darmstadt) using $CHCl_3/CH_3OH/H_2O$ (65:25:4, by vol.) as solvent system; lipids were visualized in an iodine chamber, then after evaporation of iodine, phosphorus-containing bands were stained with Zinzadze reagent [23].

Preparative TLC was carried out on silica gel G precoated Merck plates 0.25-mm thick, or silica gel H 0.5-mm thick; the bands were eluted using $CHCl_3/CH_3OH$ (2:1, by vol.).

Column chromatography was carried out on neutral silica gel 70-200 mesh (E. Merck, A.G., D-6100 Darmstadt) 50 times the weight of phospholipid. The compounds were eluted stepwise with 10 ml/g silica gel of each of the following solvent mixtures: $CHCl_3$; $CHCl_3/CH_3OH$, 9:1; $CHCl_3/CH_3OH$, 4:1 (by vol.).

NMR spectrometry: ^{1}H-NMR spectrometry was performed with a Varian 90 MHz NMR spectrometer: samples were dissolved in $CDCl_3$ at room temperature with tetramethylsilane (TMS) as an internal standard.

^{31}P-NMR spectra were recorded with a Bruker Fourier-transform spectrometer, model WH 90, in $CDCl_3$; D_2O/phosphoric acid (8.2) served as an external standard at 36.47 MHz.

General procedure for the synthesis of symmetrical and asymmetrical bis(diacylglycerophosphate)

Essentially, the procedure of Aneja et al. [21] was followed.

(1) Racemic bis(1,2-dipalmitoylglycero)-3-phosphate: (Fig. 1, IIIA) Dry racemic 1,2-dipalmitoyl glycero-3-phosphate (137 mg, 0.212 mmol) and racemic dipalmitoylglycerol (250 mg, 0.425 mmol) were dissolved in 0.5–1 ml anhydrous pyridine. A solution of TPS (160 mg, 0.640 mmol) in anhydrous pyridine (2 ml) was added and the reaction mixture incubated at room temperature for 6–8 h. The progress of the reaction was followed by analytical thin-layer chromatography on silica gel H (solvent system: $CHCl_3/CH_3OH/H_2O$, 65:25:4). Phosphatidic acid (R_f = 0.09) disappeared completely and the main phosphorus-containing compound appeared at $R_f \approx 0.72$ with trace contaminations at $R_f = 0.60$ and close to the solvent front (R_f = 0.97). Excess TPS was hydrolyzed with water (1.5 ml). The solvents were evaporated under reduced pressure and the residue extracted twice with ether

(5 ml). Yield of the ether-soluble crude product was 362 mg, giving the same chromatogram except that the origin is now phosphorus free. The products were separated and purified by preparative thin-layer chromatography on silica gel H plates (0.5 mm thickness) in the $CHCl_3/CH_3OH/H_2O$ solvent system. Bands were eluted with $CHCl_3/CH_3OH$ (2:1) [21]. Yield: 35 mg bis(dipalmitoylglycero)phosphate pure in TLC from 100 mg crude ether soluble reaction product, or 51.4% based on the starting phosphatidic acid.

A similar result was obtained by chromatographic separation on a silicic acid column (70–230 mesh (Merck), 5 g) with step-wise elution with chloroform (50 ml), chloroform/methanol, 9:1 (50 ml) and chloroform/methanol, 4:1 (50 ml).

The (1,2-dipalmitoylglycero)phosphate recrystallized from benzene/acetone, melted at 59–62°C, had a TLC behavior and gave an IR spectrum identical with an authentic sample synthesized according to the method of Baer [16]. Analysis: P% found 2.70, calculated $C_{70}H_{135}O_{12}P$ (1200) 2.50%; acyl/P found 3.7.

Moreover, the product showed resonance lines in its ^{1}H-NMR spectrum, with chemical shifts and relative intensities in agreement with the expected structure: δ ppm/TMS 0.9 (triplet, 12H, CH_3); 1.2 (unresolved, 96H, $(CH_2)_{12}$); 1.5 (shoulder, 8H, CH_2 β to C=O); 2.3 (multiplet, 8H, CH_2–CO); 3.7–4.6 (broad signals, 8H, CH_2OCO, CH_2OP); 5.2 (broad signal, 2H, CH_2O–CO) [25]. The ^{31}P-NMR spectrum showed that the ^{31}P-peak of phosphatidic acid monoester, is shifted upfield by 63 Hz (1.7 ppm) and bis(dipalmitoylglycero)phosphate, a phosphodiester structure by 213 Hz (5.8 ppm) as compared to phosphoric acid.

(2) *Bis(1,2-dipalmitoyl-*sn*-glycero)-3-phosphate:* (Fig. 1, IIIA′) For the synthesis of bis(1,2-dipalmitoyl-*sn*-glycero)-3-phosphate the same procedure as described above was followed except that the pyridinium salt of the phosphatidic acid was used instead of the phosphatidic acid free acid form.

From the 1,2-dipalmitoyl-*sn*-glycero-3-phosphate dipyridinium salt (L-phosphatidic acid) (chromatographically pure, $[\alpha]_D^{23} = 3.5°$, C = 1 in C_6H_6/CH_3OH (10:1, by vol.), M_D 28.2°) and 1,2-dipalmitoyl-*sn*-glycerol (D-diglyceride), m.p. 68–69°C, $[\alpha]_D^{23} + 1.0°$ (C = 10 in $CHCl_3/CH_3OH$, 9:1, by vol.), bis(1,2-dipalmitoyl-*sn*-glycero-3)phosphate was separated by preparative TLC in 63% yield based on the starting phosphatidic acid salt.The product recrystallized in benzene/acetone, melted at 62–63°C, showed the same TLC behavior, optical rotation $[\alpha]_D^{23} = 6.7°$, c = 3 g/100 ml in benzene, $M_D = 81°$, IR and NMR spectra as an authentic sample prepared according to the method of Baer [16].

It is noticeable that phosphorus determination of the spots of analytical TLC of the ether extract indicated a 80–90% yield in bis(diacylglycero)phosphate.

(3) *Asymmetric bis(1,2-diacylglycero)-3-phosphate:* (Fig. 1, IIIB) The following asymmetric bis(1,2-diacyl-*sn*-glycero)-3-phosphate was synthesized following the general procedure described above. Polyene-phosphatidylcholine from soy was hydrolysed using *Bacillus cereus* phospholipase C [22]; *sn*-1,2-diacylglycerol with the following fatty acid composition was isolated: 16:0 25%, 18:0 4%, 18:1 10%, 18:2 56%, 18:3 5%. This diacylglycerol (2 mol) was condensed with *rac*-1,2-

dipalmitoylglycero-3-phosphate (1 mol) using TPS (3 mol) as catalyst to form the desired product (IIIB) in 30–40% yield.

The purified bis(diacylglycero)phosphate contained 2.38% P; calculated on the basis of a molecular weight of 1238 2.5%; acyl/P, 3.8. The fatty acid composition was: 16:0 57%, 18:0 2%, 18:1 5%, 18:2 33%, 18:3 3%, is in reasonable agreement with the structure of the desired product.

Results and discussion

Bis(diacylglycero)phosphates with different configuration of the substituted glycerol moieties were synthesized by the condensation of diacylglycerols and phosphatidic acids with TPS as outlined in Fig. 1.

The condensation reaction requires anhydrous conditions. Purification of bis-(diacylglycero)phosphate to homogeneity can be performed by either preparative thin-layer chromatography or silica gel column chromatography with chloroform/methanol mixtures as eluting solvents.

The reaction products were identified as bis(diacylglycero)phosphates by chemical degradation and quantitative analysis of phosphorus and the number of fatty acyl chains per atom of phosphorus, and in the case of asymmetric bis(diacyl-glycero)phosphate, of the fatty acid composition.

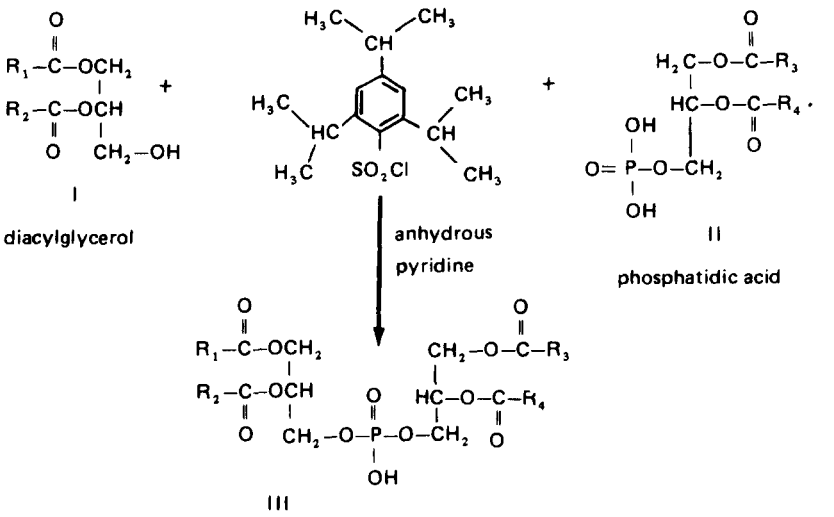
The ratio of fatty acids/phosphorus was close to four in all species synthesized so far.

Additional proof of the structure came from proton and ^{31}P spectroscopic analyses.

In order to obtain configurationally pure bis(diacylglycero)phosphate, we preferred to start from the pyridinium salt of the PA than from the free acid form. The salt form is more stable than the free acid [14]. The pyridinium dipalmitoyl-phosphate as well as the bis(1,2-dipalmitoyl-*sn*-glycero)-3-phosphate obtained showed optical activities in good agreement with previous data (pyridinium 1,2-dipalmitoylphosphatidate $[\alpha]_D^{25} = 3.8°$ in chloroform (c = 4.4), $M_D = +27.6°$ [26]; bis(1,2-dipalmitoyl-*sn*-glycero)-3-phosphate $[\alpha]_D^{23} = + 6.7°$ in benzene (c = 4), $M_D = + 80.2°$ [16].

The yield of the phosphorylation step measured by phosphorus determination of the spots of the thin-layer chromatogram of the ether extract was 80–90% based on the starting phosphatidic acid. Important lowering of the yields accompany the separation steps. This may be explained by the relative instability of acidic phospho-lipids at least in their free acid form, as reported earlier [18,26,27], specially when unsaturated [27].

It should be mentioned that repeated attempts to reproduce the phosphorylation reaction of Baer (1,2-dipalmitoylglycerol + phenylphosphoryldichloride), with careful exclusion of moisture, did not give better yields than 50% in phenyl-bis-(1,2-dipalmitoyl-*sn*-glycero)-3-phosphate; the mono-substituted product phenyl (1,2-dipalmitoyl-*sn*-glycero)-3-chlorophosphate was formed in 25% yield (Dang et al.,



A, A′ : $R_1 = R_2 = R_3 = R_4 = C_{15}H_{31}$

B : R_1, R_2 = mixtures $C_{15}H_{31}$, $C_{17}H_{35}$, $C_{17}H_{33}$, $C_{17}H_{31}$.
$R_3 = R_4 = C_{15}H_{31}$

I_A *rac*-1,2-dipalmitoylglycerol

II_A *rac*-1,2-dipalmitoylglycero-3-phosphoric acid

III_A bis(1,2-dipalmitoylglycero-3) phosphoric acid

$I_{A'}$ 1,2-dipalmitoyl-*sn*-glycerol

$II_{A'}$ 1,2-dipalmitoyl-*sn*-glycero-3-phosphoric acid

$III_{A'}$ bis(1,2-dipalmitoyl-*sn*-glycero-3) phosphoric acid

I_B 1,2-diacyl-*sn*-glycerol from soya phosphatidylcholine

II_B *rac*-1,2-dipalmitoyl-glycero-3-phosphoric acid

III_B mixed acid bis(1,2-diacyl-glycero-3) phosphoric acid

Fig. 1.

unpublished data). This is in agreement with the statement of Brown [28] that the two chlorine atoms of phenyl phosphoryl dichloride are not equally reactive to acyl substitution.

Conclusions

The simple procedure for the synthesis of bis(diacylglycero)phosphate, described here, consists of the one-step condensation of a diacylglycerol and a phos-

phatidic acid moiety, using TPS as catalyst, originally successfully applied to the formation of phosphoric acid ester bonds in nucleic acid chemistry [19] and later in phospholipid syntheses [20,21]. The bis(diacylglycero)phosphate is separated in a satisfactory yield (30–65% of theory); the phosphorylation step itself proceeds in a much higher yield (80–90% of theory). The advantage and potential of this simple TPS catalyzed condensation lies in the flexibility of starting materials. No hydrogenolysis of protecting group is required and therefore not only saturated but also unsaturated D- and L-diglycerides or D- and L-phosphatidic acids with unsaturated fatty acyl substituents can be used as starting material. Therefore any desired bis(diacylglycero)phosphate can be obtained.

Treatment of the bis(diacylglycero)phosphate with phospholipase A_2 may yield the corresponding bis(monoacylglycero)phosphate present in lysosomes of liver in certain lipidoses [2–11].

Bis(diacylglycero)phosphate though not isolated from mammalian tissues so far form a class of acidic phospholipids, closely related to cardiolipin. This lipid class, easily accessible by chemical synthesis, may prove suitable for studies in which cardiolipin (bisphosphatidylglycerol) not easily in larger amounts, may be mimicked by bis(diacylglycero)phosphate.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft and INSERM, France. We are grateful to Dr. B. Tunggal for recording the NMR-spectra and Dr. G. Tschöpe for the GLC-analysis.

References

1 E.M. Stearns, Jr. and W.T. Morton, Lipids, 12 (1977) 451.
2 G. Rouser, G. Kritchevsky, A. Yamamoto, A.G. Knudson, Jr. and G. Simon, Lipids, 3 (1968) 287.
3 P.N. Seng, H. Debuch, B. Witter and H.R. Wiedemann, Hoppe-Seyler's Z. Physiol. Chem., 352 (1971) 280.
4 J. Brotherus, O. Renkonen, O. Herrmann and W. Fischer, Chem. Phys. Lipids, 13 (1974) 178.
5 A. Joutti and O. Renkonen, Chem. Phys. Lipids, 17 (1975) 254.
6 P. Somerharju, J. Brotherus, K. Kahma and O. Renkonen, Biochim. Biophys. Acta, 487 (1977) 154.
7 A. Joutti and O. Renkonen, J. Lipid Res., 20 (1979) 230.
8 A. Joutti, Biochim. Biophys. Acta, 575 (1979) 10.
9 B.J.H.M. Poorthuis and K.Y. Hostetler, J. Biol. Chem., 250 (1975) 3297.
10 B.J.H.M. Poorthuis and K.Y. Hostetler, J. Biol. Chem., 251 (1976) 4596.
11 B.J.H.M. Poorthuis and K.Y. Hostetler, J. Lipid Res., 19 (1978) 309.
12 D.J. Hanahan, Lipid Chemistry, Wiley and Sons, New York, 1960, p. 165.
13 L.L.M. Van Deenen and G.H. De Haas, Adv. Lipid Res. (1964) 167.
14 M. Kates, Methods Membr. Biol., 8 (1977) 251.
15 E. Baer and M. Kates, J. Am. Chem. Soc., 72 (1950) 942.

16 E. Baer, J. Biol. Chem., 198 (1952) 853.
17 E. Baer and D. Buchnea, Arch. Biochem. Biophys., 78 (1958) 294.
18 G.H. De Haas, P.P.M. Bonsen and L.L.M. Van Deenen, Biochim. Biophys. Acta, 115 (1966) 114.
19 R. Lohrmann and H.G. Khorana, J. Am. Chem. Soc., 88 (1966) 829.
20 R. Aneja and A.P. Davies, Chem. Phys. Lipids, 4 (1970) 60.
21 R. Aneja, J.S. Chadra and A.P. Davies, Biochim. Biophys. Acta, 218 (1970) 102.
22 R.F.A. Zwaal and B. Roelofsen, Methods Enzymol., 32 (1974) 154.
23 G. Rouser, A.N. Siakotos and S. Fleischer, Lipids, 1 (1966) 85.
24 J.C. Dittmer and R.L. Lester, J. Lipid Res., 5 (1964) 126.
25 D. Chapman and A. Morrison, J. Biol. Chem., 241 (1966) 5044.
26 E. Baer, J. Biol. Chem., 189 (1951) 235.
27 M. Faure and Coulon-Morelec, Ann. Institut Pasteur, 104 (1963) 246.
28 D.M. Brown, in: R.A. Raphael (Ed.), Advanced Organic Chemistry, Vol. 3, 1967, p. 75.