Chemistry and Physics of Lipids 15 (1975) 95–104

CHEMICAL SYNTHESIS OF DIPHOSPHOINOSITIDE

V.P. SHEVCHENKO, M.L. TSIRENINA, Jul. G. MOLOTKOVSKY and L.D. BERGELSON

Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow, USSR

Received August 4, 1975, accepted September 3, 1975

A chemical synthesis of DL-1-O-(1′-palmitoyl-2′-oleoyl-*sn*-glycero-3′-phosphoryl)-*myo*-inositol-4-phosphate (diphosphoinositide) is described. Selective phosphorylation of DL-2,3; 5,6-di-O-cyclohexylidene-*myo*-inositol with diphenylphosphochloridate led to the corresponding 1-diphenylphosphate which was transformed into silver DL-4-O-acetyl-2,3; 5,6-di-O-cyclohexylidene-*myo*-inositol-1-(benzyl)phosphate. Condensation of the latter with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-iodohydrin gave a phosphotriester which after successive deacetylation, phosphorus oxychloride treatment and removal of the protective groups yielded diphosphoinositide. The intermediate DL-1-O-[1′-palmitoyl-2′-oleoyl-*sn*-glycero-3′-(benzyl)phosphoryl]-2,3; 5,6-di-O-cyclohexylidene-*myo*-inositol was used also for a new synthesis of phosphatidylinositol.

I. Introduction

The phosphoinositides – phosphatidylinositol, phosphatidylinositolphosphate and phosphatidylinositoldiphosphate – are important constituents of plant and animal tissues. They have been found to be metabolized at higher rates than any other phospholipid [1,2]. Recently the interest in these substances is growing rapidly since they have been shown to be involved in such processes as nerve conductivity [3–5], ion transport [6] and phagocytosis [7].

A high content of unsaturated acids is typical for many of the natural phosphoinositides. As individual molecular species of these phospholipids can not be obtained yet from natural sources, the elaboration of their chemical synthesis remains an actual task.

Several syntheses of racemic "single-acid" phosphatidylinositol and its monophosphate have been described [8–12]. Since all of them include a stage of hydrogenolysis for the removal of blocking phenyl groups, they are not suitable for the synthesis of molecular species with unsaturated fatty acid residues.

Recently the first total synthesis of an optically active unsaturated phosphatidylinositol has been accomplished in our laboratory [13].

In this paper we describe the synthesis of "mixed-acid" phosphatidylinositolphosphate, with an optically active unsaturated diglyceride moiety of natural con-

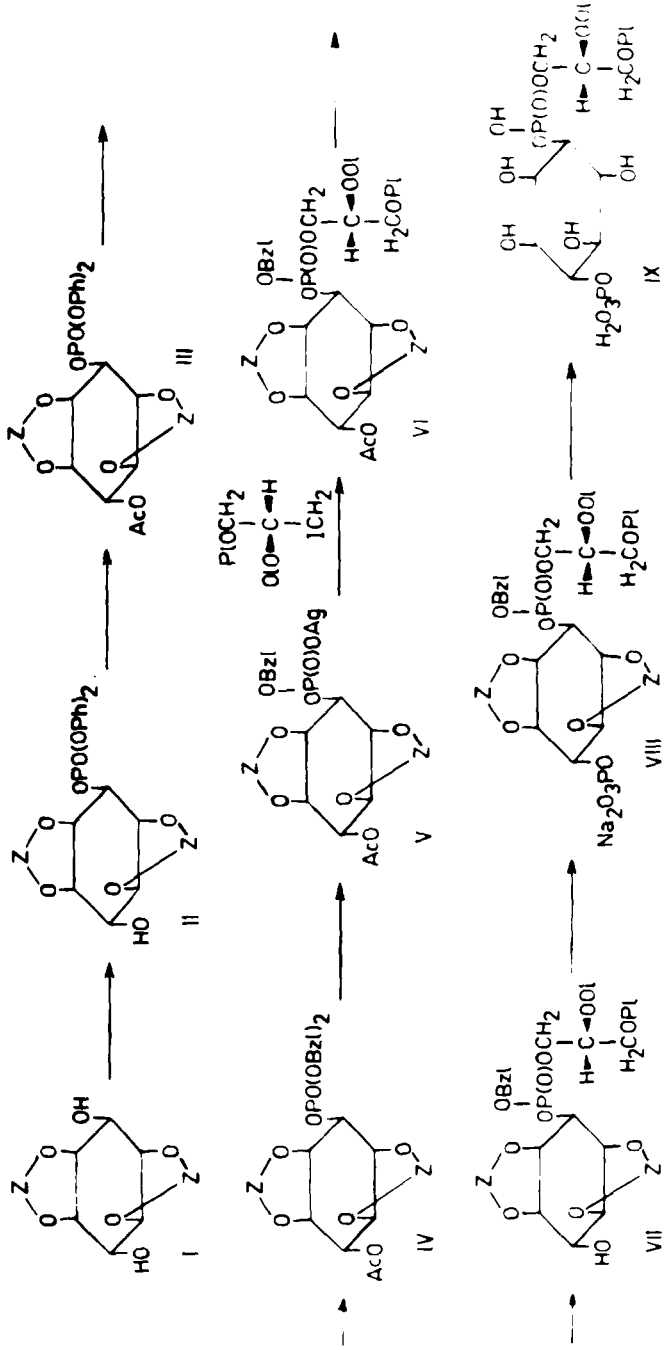
figuration. For protection of the phosphate linking the glycerol and *myo*-inositol residues we used the benzyl groups which can be removed by anionic debenzylation, without affecting double bonds.

II. Results

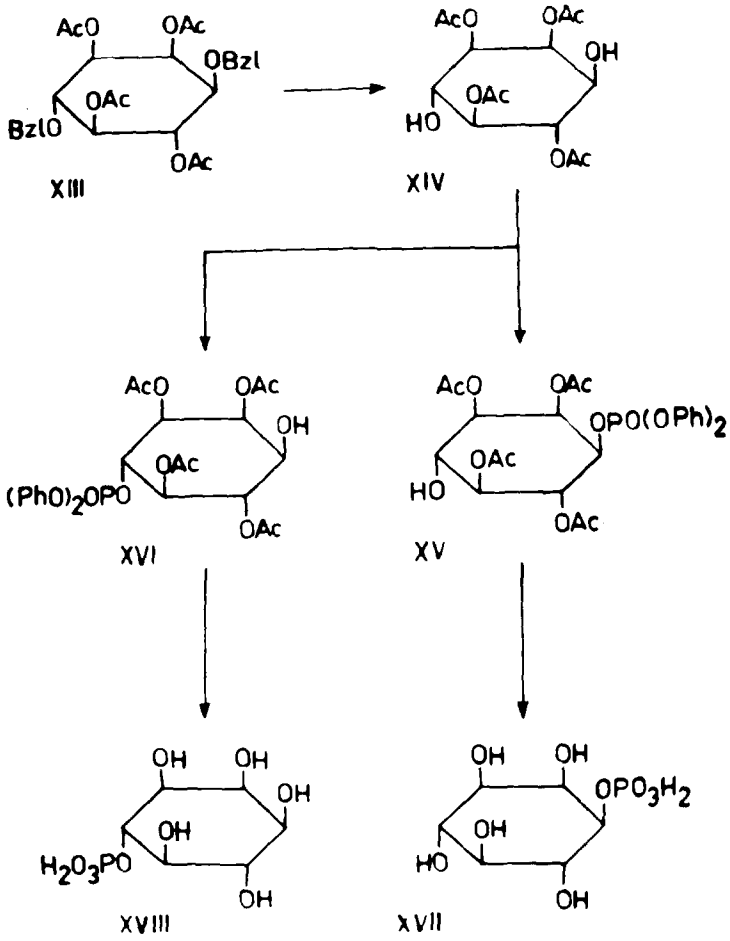
The pathway used for the synthesis of phosphatidylinositolphosphate is shown on scheme 1. When DL-1,2; 4,5-di-O-cyclohexylidene-*myo*-inositol (I) [14] was phosphorylated with diphenylphosphochoridata in dioxane, the 1-hydroxy group reacted predominantly. The phosphotriester (II) was purified by re-crystallization and acetylated by acetic anhydride in pyridine. The structure and homogeneity of the resulting DL-1-O-diphenylphosphoryl-2,3; 5,6-di-O-cyclohexylidene-4-O-acetyl-*myo*-inositol (III) were proved by its transformation into DL-*myo*-inositol-1-(dihydrogen phosphate) (XVII) by platinum catalyzed hydrogenolysis and subsequent basic and then acid hydrolysis. The phosphate obtained appeared to be identical by paper chromatography with authentic 1D-*myo*-inositol-1-(dihydrogen phosphate) [13] and differed distinctly from the isomeric DL-*myo*-inositol-4-(dihydrogen phosphate) (XVIII). This latter substance was synthesized from DL-1,4-di-O-benzyl-2,3-5,6-tetra-O-acetyl-*myo*-inositol (XIII) [15] by hydrogenolytic debenzylation over palladium black and subsequent phosphorylation with diphenylphosphochloridate (scheme 2). The main reaction product was DL-1-O-diphenylphosphoryl-2,3,5,6-tetra-O-acetyl-*myo*-inositol (XV) which after removal of the protective groups yielded DL-*myo*-inositol-1-(dihydrogen phosphate) (XVII). A minor product of the phosphorylation reaction was DL-4-O-diphenylphosphoryl-2,3,5,6,-tetra-O-acetyl-*myo*-inositol (XVI) which was transformed into substance (XVIII) by successive catalytic hydrogenolysis, alkaline deacylation and deionization (scheme 2).

The phosphotriester (III) was subjected to hydrogenolysis and subsequent treatment with phenyldiazomethane forming the corresponding dibenzyl derivative (IV) which was converted into the silver salt (V) by treatment with sodium iodide in acetone and then with silver nitrate in acetone-methanol. Condensation of the silver salt (V) with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-iodohydrin [16] led to the fully protected phosphatidylinositol derivative (VI). As this substance appeared not to separate easily from contaminants it was without purification deacetylated by hydrazine hydrate to the compound (VII) with a free 4-hydroxy group. The latter was phosphorylated with excess phosphorus oxychloride in pyridine. After aqueous sodium bicarbonate treatment of the phosphorylation product, the sodium salt (VIII) was obtained. After its debenzylation with sodium iodide, splitting off the ketal groups by filtering through Dowex-50 cationite (H^+-form) and subsequent potassium oxalate impregnated silica gel column chromatography, individual 1-palmitoyl-2-oleoyl phosphatidylinositolphosphate (IX) was obtained in pure form as triammonium salt.

The synthetic phosphatide (IX) was undistinguishable from rat brain phosphatidylinositolphosphate [17], on silica gel TLC in different solvent systems. For the



Scheme 1. Synthesis of DL-1-O-(1′-palmitoyl-2′-oleoyl-*sn*-glycero-3′-phosphoryl)-*myo*-inositol-4-phosphate (IX) and DL-1-O-(1′-palmitoyl-2′-oleoyl-*sn*-glycero-3′-phosphoryl)-*myo*-inositol (X).



Scheme 2. Synthesis of DL-*myo*-inositol-1-(dihydrogen phosphate) (XVII) and DL-*myo*-inositol-4-(dihydrogen phosphate) (XVIII).

synthetic specimen, the phosphorus to fatty acid molar ratio was estimated as 1.00 : 0.91, and the oleic to palmitic acid ratio was found to be 1 : 1. The structure of the synthetic diphosphoinositide (IX) was proved by its transformation into the nonakis-trimethylsilyl derivative (XI) on basic deacetylation and subsequent exhaustive trimethylsilylation of the formed glycerophosphorylinositolphosphate. The trimethylsilyl derivative (XI) and a substance, obtained in the same way from brain diphosphoinositide were undistinguishable by GLC and had identical mass spectra coinciding completely with the mass spectrum of XI described earlier [17].

The intermediate phosphotriester (VII) was used also for a new synthesis of phosphatidylinositol. For this purpose VII was subjected to anionic debenzylation and subsequent acid hydrolysis to yield phosphatidylinositol (X) which appeared to be identical by TLC with a specimen of 1-palmitoyl-2-oleoyl-phosphatidylinositol synthesized earlier [13]. The phosphatide (X) gave after basic deacylation and trimethylsilylation an octakis-trimethylsilyl derivative, which by retention time and mass-spectrum coincided with the trimethylsilyl derivative (XII), obtained from authentic 1D-O-(*sn*-glycero-3′-phosphoryl)-*myo*-inositol.

III. Experimental

A. Materials and methods

Melting points were determined on a Kofler block and are uncorrected; optical rotations were measured with a Perkin-Elmer 141 polarimeter at 18–22°C. TLC was carried out on KSK silica gel containing 5% gypsum as a binder, the spots were detected by charring with conc. H_2SO_4; TLC of the phosphomono- and diester was conducted on silica gel without binder. The solvent systems used were (v/v): (A) chloroform-ethyl acetate (10 : 3); (B) benzene-dioxane (5 : 1); (C) benzene-ethyl acetate (5 : 1); (D) benzene-ethyl acetate (1 : 1); (E) chloroform–methanol–conc. NH_4OH (65 : 25 : 4). Column chromatography was performed on KSK silica gel (100–150 mesh) or on activity III alumina (Reanal, Hungary).

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-iodohydrin [16] $[\alpha]_D$ + 6.8° (c 0.3; dioxane) and DL-2,3,5,6-tetra-O-acetyl-1,4-di-O-benzyl-*myo*-inositol [15] were prepared as described previously.

1. Deacylation of phospholipids

A solution of phospholipid (5 mg) in 0.1 ml chloroform was treated with 1 ml of 0.1 M KOH in 98% methanol for 30 min at 40°C and then neutralized with 0.1 ml of ethyl formate (40°C; 5 min). After cooling the hydrolysate was shaken with 5 ml of a chloroform–methanol–water (20 : 5 : 6) mixture and separated. The chloroform phase was analysed by GLC for fatty acid methyl esters. The phosphorus content of the upper phase was determined as described in [18].

2. Trimethylsilylation

Aliquots of solutions containing about 1 mg of glycerophosphorylinositol or its phosphate were dried at 40°C/15 mm. The residue was treated with 0.5 ml of pyridine-hexamethyldisilazane-trimethylchlorosilane (5 : 5 : 1) mixture at 70°C for 30 min and then analysed by GLC.

3. Gas-liquid chromatography

Analysis of trimethylsilylated phosphoglycerol derivatives was performed on a

Pye-104 series 24 apparatus equipped with a flame ionization detector and a column (1000 × 4 mm) packed with 1.5% JXR on 80–100 mesh silanized chromosorb W, and operated with initial temperature of 150°C and programming rate of 8°C to an upper temperature of 300°C.

GLC of fatty acid methyl esters was carried out on the same apparatus with a column (2000 × 4 mm) packed with 10% EGS on 80–100 mesh silanized chromosorb W, at 180°C.

4. Mass spectra

The mass spectra of trimethylsilylated derivatives were obtained on a LKB-9000 apparatus using direct insertion of samples into the ion source; the electron voltage was 70 eV.

B. Synthesis of phosphatidylinositolphosphate

1. DL-1-O-Diphenylphosphoryl-4-O-acetyl-2,3; 5,6-di-O-cyclohexylidene-myo-*inositol (III)*

To a stirred solution of 26.0 g of diketal (I) [14] in 150 ml dry dioxane and 12.1 ml pyridine, maintained at 0°C, 21.6 g of diphenylphosphorochloridate was added over a 3 hr period. The reaction mixture was left to stand overnight at room temperature, concentrated in vacuo, the residue was dissolved in ethyl acetate, washed successively with 1N HCl, water, saturated $NaHCO_3$ solution, water, dried over Na_2SO_4 and concentrated in vacuo. After five crystallizations of the residue from ethyl acetate 21.4 g (49%) of chromatographically pure DL-1-O-diphenylphosphoryl-2,3; 5,6-di-O-cyclohexylidene-*myo*-inositol (II) were obtained (TLC in systems A and B). This substance was treated at 0°C with 50 ml pyridine and 12.0 ml acetic anhydride and left at room temperature for 12 hr. Methanol was added with cooling, the mixture was concentrated in vacuo, the residue was dissolved in ethyl acetate, washed successively with 1N HCl, water, saturated $NaHCO_3$ solution and water, then dried over Na_2SO_4, filtered and concentrated in vacuo. Crystallization of the residue from ether gave 22.7 g (98.8%) of chromatographically pure phosphate (III), m.p. 167–169°C (TLC in system C).

$C_{32}H_{39}O_{10}P$	Found: C 62.7; H 6.3; P 4.2
	Calc.: C 62.5; H 6.4; P 5.0

2. DL-2,3,5,6-Tetra-O-acetyl-myo-inositol (XIV)

DL-2,3,5,6-Tetra-O-acetyl-1, 4-di-O-benzyl-*myo*-inositol (XIII) [15] (0.5 g) was hydrogenated in 5 ml acetic acid over 70 mg of Pd black for 6 hr, the catalyst was filtered off and the filtrate concentrated in vacuo. Crystallization of the residue from ether gave 0.33 g (98.8%) of XIV, m.p. 183–185°C (TLC in system B).

	Found: C 47.9; H 5.8
$C_{14}H_{20}O_{10}$	
	Calc.: C 48.3; H 5.8

*3. DL-4-O-Diphenylphosphoryl-2,3,5,6-tetra-O-acetyl-*myo*-inositol (XVI) and DL-1-O-diphenylphosphoryl-2,3,5,6-tetra-O-acetyl-*myo*-inositol (XV)*

To a stirred solution of 2.0 g of the tetraacetate (XIV) in 6 ml pyridine a solution of diphenylphosphorochloridate (1.9 g) in 50 ml dry chloroform was added over a 1 hr period at −20°C. The mixture was then maintained at 50°C for 8 hr, the course of the reaction being checked by TLC in system D. The reaction mixture was concentrated in vacuo at 50°C, dissolved in ethyl acetate, filtered, washed successively with water, 1N HCl solution, water, saturated $NaHCO_3$ solution and water, then dried over $MgSO_4$, filtered and concentrated in vacuo. The residue was chromatographed on a silica gel column using benzene–ethyl acetate mixtures as eluents (control by TLC in system D). 150 mg (4.5%) of DL-4-O-diphenylphosphoryl-2,3,5,6-tetra-O-acetyl-*myo*-inositol (XVI) were obtained first, m.p. 150–153°C (from ethyl acetate–ether).

$C_{26}H_{29}O_{13}P$ Found: C 53.3; H 5.3; P 5.4
Calc.: C 53.6; H 5.0; P 5.3

Subsequently 1.70 g (51%) of the isomer (XV) were eluated, m.p. 168–170°C (from ethyl acetate–ether).

*4. DL-*myo*-inositol-1-(dihydrogen phosphate) (XVII) and DL-*myo*-inositol-4-(dihydrogen phosphate) (XVIII)*

A solution of 5 mg of phosphotiester (III) in 0.3 ml of 80% acetic acid was maintained at 50°C for 2.5 hr and concentrated in vacuo. The residue was subjected successively to hydrogenolysis, alkaline deacylation and deionization as described in [19]. Samples of the phosphotriesters (XV) and (XVI) were worked up in the same way. All the specimens of DL-*myo*-inositol-dihydrogen phosphate were submitted to descending PC (Filtrak 3, DDR; solvent system iso-propanol–water–28% NH_4OH 7 : 2 : 1, 20°C, 20 hr, detection with Zinzadze reagent [20]) together with authentic 1D-*myo*-inositol-1-dihydrogen phosphate [13] and *sn*-glycerol-3-(dihydrogen phosphate) the latter being used as a reference substance to which relative mobilities (R_{GP}) of other compounds were listed. The samples of DL-*myo*-inositol-1-dihydrogen phosphate obtained from III and XV had identical mobility (R_{GP} 0.32) with authentic 1-phosphate but differed noticeably from the 4-isomer (XVIII) (R_{GP} 0.44).

*5. DL-1-O-Dibenzylphosphoryl-4-O-acetyl-2,3; 5,6-di-O-cyclohexylidene-*myo*-inositol (IV)*

7.0 g of diketal (III) were hydrogenated in 200 ml dioxane with 5% acetic acid over Pt black (from 1.0 g of PtO_2) for 16 hr (control by TLC in system E). The residue was dissolved in chloroform and treated with phenyldiazomethane solution until a pink colour persisted. Chromatography of the product on a column packed with 75 g of alumina using benzene-ether mixtures as eluents (control by TLC in

system C) yielded 5.27 g (72%) of phosphotriester (IV), m.p. 149–150°C (from ether).

$C_{34}H_{43}O_{10}P$ Found: C 63.7; H 6.7; P 5.0
Calc.: C 63.6; H 6.7; P 4.8

*6. Silver salt of DL-2,3; 5,6-di-O-cyclohexylidene-*myo*-inositol-4-O-acetyl-1-(benzyl) phosphate (V)*

A solution of 500 mg of phosphotriester (IV) and 146 mg NaI in 6 ml dry acetone was heated in a sealed tube at 80°C for 2 hr and cooled to room temperature. The precipitate was filtered off, washed with dry acetone, crystallized from chloroform-methanol and dried over P_2O_5 yielding 440 mg (99.0%) of chromatographically pure (TLC in system E) sodium DL-4-O-acetyl-2,3; 5,6-di-O-cyclohexylidene-*myo*-inositol-1-(benzyl)phosphate, m.p. 322–324°C (decomp.).

$C_{27}H_{36}O_{10}NaP$ Found: C 55.7; H 6.6; P + Na 17.7
Calc.: C 56.4; H 6.3; P + Na 17.8

To a solution of the sodium salt (430 mg) in 5 ml methanol, a solution of 131 mg $AgNO_3$ in 0.5 ml water and 1 ml methanol was added, the mixture was diluted with 10 ml acetone and set aside for 30 min at room temperature. The precipitate was filtered off, washed with acetone and dried over P_2O_5 at 0.3 mm yielding 360 mg (73.0%) of silver salt (V), m.p. 249–251°C (decomp.). All manipulations with the silver salt (V) were performed in weak diffused light.

*7. DL-1-O-[1′-Palmitoyl-2′-oleoyl-*sn*-glycero-3′-(benzyl)phosphoryl]-2,3; 5,6-di-O-cyclohexylidene-*myo*-inositol (VII)*

A mixture of 406 mg 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-iodohydrin, 360 mg of the silver salt (V) and 10 ml of dry toluene was boiled for 2.5 hr with vigorous stirring in the dark, cooled, filtered and concentrated in vacuo. Chromatography of the residue on a column packed with 60 g of silica gel using benzene-ether mixtures as eluents (control by TLC in system C) gave 450 mg of DL-1-O-[1′-palmitoyl-2′-oleoyl-*sn*-glycero-3′-(benzyl)phosphoryl]-2,3; 5,6-di-O-cyclohexylidene-4-O-acetyl-*myo*-inositol (VI) slightly contaminated with an unidentified substance. The above mixture was dissolved in 20 ml of 95% ethanol and treated with 33 mg hydrazin hydrate overnight at room temperature. After evaporation of the solvent in vacuo the residue was subjected to column chromatography on 50 g of silica gel with benzene-ether mixtures as eluents (control by TLC in system A) to afford 218 mg of unchanged acetate (VI) and 163 mg of the phosphotriester (VII) as a light-yellow gum, $[\alpha]_D$ + 2.3° (c 2.0; $CHCl_3$).

$C_{62}H_{103}O_{13}P$ Found: C 68.2; H 9.8; P 3.1
Calc.: C 68.5; H 9.5; P 2.8

*8. DL-1-(1'-Palmitoyl-2'-oleyl-*sn*-glycero-3'-phosphoryl)-*myo*-inositol-4-phosphate (diphosphoinositide) (IX)*

To a stirred solution of 0.13 ml phosphorus oxychloride in 1 ml dry chloroform a solution of 160 mg of phosphotriester (VII) and 0.3 ml pyridine in ml dry chloroform was added at 0°C for 30 min and left to stand overnight at room temperature. The reaction mixture was evaporated at 0.2 mm, the residue stirred with 0.5 ml of saturated $NaHCO_3$ solution for 10 min at 0°C and again concentrated in vacuo. The residue was dissolved in 15 ml of chloroform, washed three times with water (phase separation by centrifugation) and evaporated in vacuo. The residue was dried at 50°C/0.1 mm over P_2O_5, dissolved in 10 ml of dry methyl ethyl keton, boiled for 2 hr with 50 mg of dry NaI and cooled to room temperature. The precipitate was filtered off, washed with dry acetone, dissolved in 25 ml of a chloroform–methanol–water (10 : 10 : 1) mixture and stirred for 5 hr with 20 ml of Dowex-50 (H^+). The resin was filtered off and washed twice with 20 ml of the same solvent. After evaporation of the filtrate in vacuo the residue was subjected to column chromatography on 7.0 g of silica gel impregnated with potassium oxalate [21] using mixtures of chloroform–methanol–water (9 : 7 : 2) and chloroform–methanol–4N NH_4OH (9 : 7 : 2) as eluents. Fractions were checked by TLC in the system chloroform–4N NH_4OH (9 : 7 : 2) (R_f, 0.54), diphosphoinositide from rat brain being used as a reference substance (R_f, 0.54). 36 mg(25%) of pure phospholipid (IX) were obtained as tri-ammonium salt, m.p. 187°C (sealed capillary; from $CHCl_3$-methanol), $[\alpha]_D$ + 1.8° (c 0.3; $CHCl_3$).

$C_{43}H_{91}N_3O_{16}P_2$

Found: C 53.4; H 8.5; P 6.6

Calc.: C 53.3; H 9.5; P 6.4

C. Synthesis of phosphatidylinositol

The phosphotriester (VII) (5 mg) was debenzylated by NaI treatment as described above, the reaction mixture was concentrated in vacuo, dissolved in 2 ml chloroform, filtered and evaporated in vacuo. The residue was treated with 2 ml of 80% acetic acid for 2.5 hr at 50°C and evaporated in vacuo. The DL-1-O-(1'-palmitoyl-2'-oleoyl-*sn*-glycero-3'-phosphoryl)-*myo*-inositol (X) obtained did not differ from yeast phosphatidylinositol on TLC in the solvent systems chloroform–methanol–water–28% NH_4OH 130 : 70 : 8 : 0.5 (R_f 0.37); chloroform–methanol–water–acetic acid 50 : 25 : 8 : 4 (R_f 0.48) and chloroform–methanol–water 65 : 35 : 8 (R_f 0.45).

References

[1] G. Hauser, J. Eichberg and F. Gonzalez-Sastre, Biochim. Biophys. Acta 248 (1971) 87
[2] S. Steiner and R.L. Lester, Biochim. Biophys. Acta 260 (1972) 82
[3] A.C. Birnberger, K.L. Birnberger, S.G. Eliasson and P.C. Simpson, J. Neurochem. 18 (1971) 1291

[4] J.G. Salway and I.E. Hughes, J. Neurochem. 19 (1972) 1233
[5] E.G. Lapetina and R.H. Michell, FEBS Letters 31 (1973) 1
[6] M. Kai and J.N. Hawthorne, Annals New York Acad. Sci. 169 (1969) 761
[7] Tou Jen-Sie and R.L. Stjenholm, Arch. Biochem. Biophys. 160 (1974) 487
[8] A.V. Lukyanov, A.I. Lutik, V.I. Shvets and N.A. Preobrazhenskii, Dokl. Akad. Nauk SSSR 165 (1965) 121
[9] S.M. Kozlova, N.B. Tarusova and N.A. Preobrazhenskii, Zh. Obshch. Khim. 39 (1969) 2463
[10] A.I. Lutik, M.S. Sadovnikova, V.I. Shvets and R.P. Evstigneeva, Zh. Obshch. Khim. 44 (1974) 2573
[11] B.A. Klyashchitskii, E.G. Zhelvakova, V.V. Pimenova, V.I. Shvets, R.P. Evstigneeva and N.A. Preobrazhenskii, Zh. Obshch. Khim. 41 (1971) 1386
[12] P.A. Gent, R. Gigg and C.D. Warren, Tetrahedron Letters (1970) 2575
[13] Jul. G. Molotkovsky and L.D. Bergelson, Chem. Phys. Lipids 11 (1973) 135
[14] S.I. Angyal, M.E. Tate and S.D. Gero, J. Chem. Soc. (1961) 4116
[15] A.I. Lutik, V.I. Krylova, S.M. Kozlova, B.A. Klyashchitskii, V.I. Shvets, R.P. Evstigneeva and E.S. Zhdanovich, Zh. Obshch. Khim. 41 (1971) 2747
[16] G.H. de Haas and L.L.M. van Deenen, Rec. Trav. Chim. 80 (1961) 951
[17] T.J. Cicero and W.R. Sherman, Biochem. Biophys. Res. Commun. 42 (1971) 428
[18] E. Gerlach and B. Deuticke, Biochem. Z. 337 (1963) 477
[19] D. Mercier, J.E.G. Barnett and S.D. Gero, Tetrahedron 25 (1969) 5681
[20] R.S. Bandurski and B. Axelrod, J. Biol. Chem. 193 (1951) 405
[21] P.H. Cooper and J.N. Hawthorne, J. Chromatogr. 87 (1973) 267