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THE SYNTHESIS OF 3-PHOSPHATIDYL-1′-GLYCEROL

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The synthesis of 1-oleoyl-2-palmitoyl glycerol-3-phosphoryl-1′-glycerol is described. By means of a reaction between a 1.2-diacyl-glycerol iodohydrin and the silver salt of 2.3-isopropylidene glycerol-1-(benzyl)-phosphate and removal of the protecting groups phosphatidyl glycerol was obtained in the same stereochemical configuration as the naturally occurring substance. Confirmation of the structure of the synthesized compound was achieved with the aid of different enzymes and analytical procedures.

Introduction

After the discovery and structural identification of phosphatidyl glycerol in higher plants and algae by Maruo and Benson[1]), this phospholipid was found to occur also in bacteria[2–4]) and to a lesser extent in animal tissue[5–7]). Baer and Buchnea[8]) supposed that phosphatidyl glycerol, in analogy to other natural phospholipids, has the L-L-configuration (Fischer nomenclature). Benson and Miyano[9]), however, suggested that the natural substance has the L-D-configuration (3-phosphatidyl-1′-glycerol according to Hirschmann[10])). This latter stereochemical configuration was established by Haverkate and van Deenen[11–13]) by enzymic degradations of phosphatidyl glycerol isolated from bacteria and spinach leaves, while investigations on the biosynthesis of phosphatidyl glycerol in animal tissue and bacteria by Kennedy and coworkers[7,14]) pointed to the same configuration.

Chemical synthesis of phosphatidyl glycerol was first carried out by Baer and Buchnea[8]) by means of a reaction of a 1.2-diglyceride, 1.2-isopropylidene glycerol and phosphorus oxychloride. Removal of the protecting isopropylidene group gave 1.2-dioleoyl glycerol-3-phosphoryl-3′-glycerol (L-α-phosphatidyl-L-glycerol) in a yield of 90%. Kiyasu *et al.*[7]) repeated this synthesis with racemic isopropylidene glycerol with an overall yield of 11%.

A semi-synthetic preparation has been published by Coulon-Morelec and Giraud[15]). The mono potassium salt of phosphatidic acid isolated from germs of corn, was condensed with glycerol by means of dicyclohexylcarbodiimide. The free glycerol moiety of the obtained phosphatidyl glycerol was of course racemic. Another semi-synthetic method is the degradation of lecithin with phospholipase D in the presence of an excess of glycerol, as

was discovered by Benson[16]). This is a transphosphatidylation reaction which occurs in the presence of different alcohols. Phosphatidic acid, after incubation with phospholipase D and glycerol did not form any phosphatidyl glycerol. The free glycerol part of the so formed phosphatidyl glycerol turned out to be racemic*, which could be confirmed by us with the method used for the natural and synthetic compound.

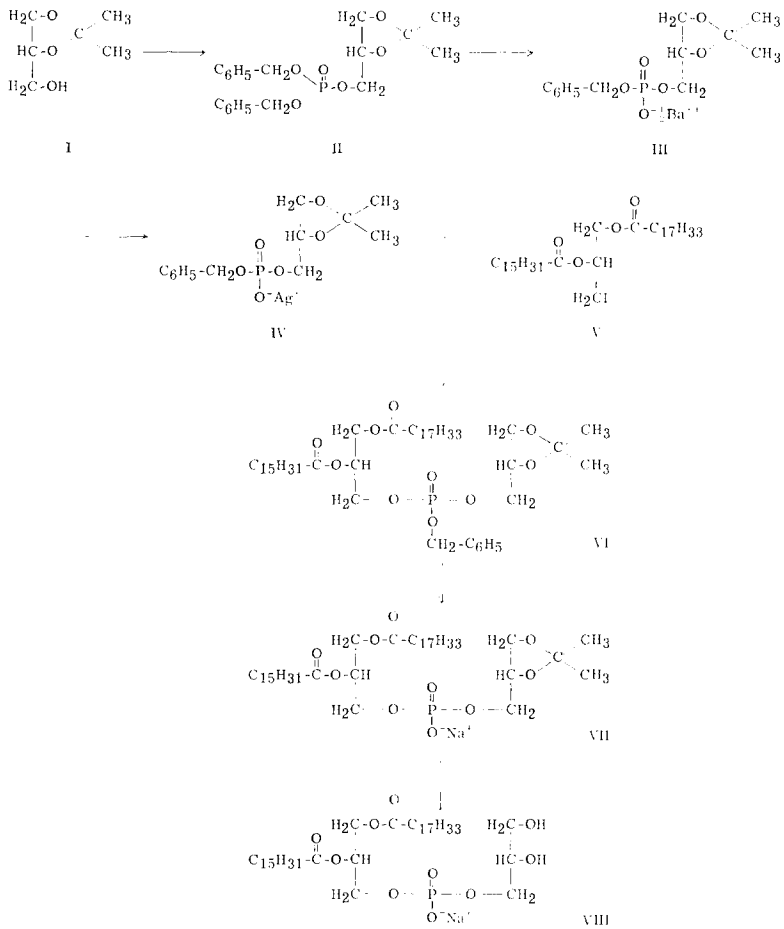
Since none of these procedures has led to the preparation of phosphatidyl glycerol with a stereochemical configuration identical with that of the natural product a *de novo* synthesis of such a compound was carried out.

In analogy with the synthesis of numerous phospholipids in our laboratory in which a silver salt was reacted with an iodo compound with, in general, very good results, this method seemed also suitable for our purpose. In principle two pathways are possible. Because of the dubious results of reactions of silver salts with isopropylidene iodohydrin and to the same extent in reactions in which a free hydroxyl group was adjacent to the iodo atom, preference was given to the pathway outlined in scheme 1. In order to obtain the free glycerol moiety of phosphatidyl glycerol in the right configuration, use was made of 2.3-isopropylidene glycerol (scheme 1, I) (L-aceton glycerol), which was phosphorylated according to Theodoropoulos and Souchleris[17]) with tetrabenzylpyro phosphate in the presence of imidazole. Debenzylation of the obtained phosphotriester (II) and conversion to a silver salt gave one of the desired starting products (IV). Reaction of the foregoing silver salt (IV) with a diacyl-glycerol iodohydrin (V) gave the fully protected phosphatidyl glycerol (VI). Debenzylation followed by the splitting off of the isopropylidene group with boric acid in trimethylborate, according to Mattson and Volpenhein[18]), furnished the endproduct after chromatographic purification over silica in an overall yield of 35–40%. At the same time a fully racemic phosphatidyl glycerol was synthesized following the reaction scheme of the optically active compound.

The purity of the synthesized product was checked by paper chromatography with the solvent system of Marinetti[19]), and on thin-layer chromatograms with different solvent systems. Only one spot could be detected with different staining methods, having the same R_F value as the natural compound.

The value found for the optical rotation ($+1.02°$ in chloroform) agrees very well with that found by Allen *et al.*[20]) for a natural specimen isolated from spinach leaves (as NH_4^+ salt, $+1.0°$ in chloroform). Apparently the contribution to the optical rotation of the free glycerol part of phosphatidyl glycerol is not so evident, for Baer and Buchnea[8]) found for the L-L-deriva-

* Personal communication of A. A. Benson.



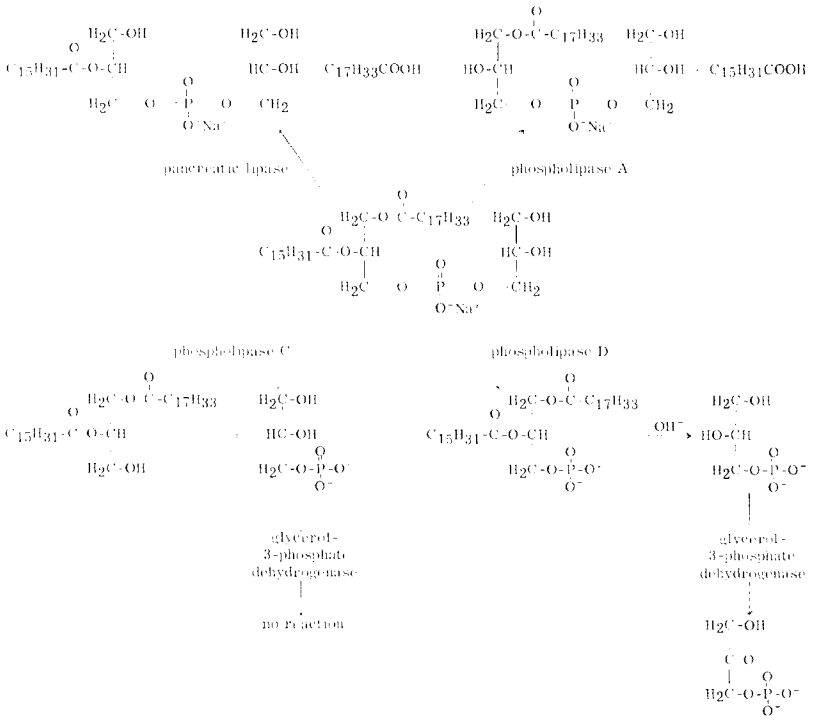
Scheme 1. Synthesis of 1-oleoyl-2-palmitoyl-glycerol-3-phosphoryl-1′-glycerol.

tive a rotation of +2.0° (as acid in chloroform). Coulon-Morelec and Giraud[15]), on the other hand, reported an unaccountably high value for their semi-synthetic product in which the free glycerol part was racemic (+7.72° as K-salt in ethanol). The infrared spectrum was identical with that of phosphatidyl glycerol isolated from spinach leaves[21]), except an absorption at 10.3 μ in the latter that has to be attributed to a trans-double bond. The synthetic compound contains only oleic- and palmitic acid in a ratio of 1:1. Alkaline hydrolysis according to Dawson *et al.*[22,23]) revealed one spot on paper chromatograms with propanol–ammonia–water (6:3:1, v/v/v) as solvent system, positive with the molybdate reagent and periodate Schiff reagent and with the same R_F value as glyceryl phosphoryl glycerol. Periodate

oxydation of the optically active compound following the method of Kiyasu *et al.*[7]) showed the presence of 99% of vicinal hydroxyl groups.

As was mentioned already the final elucidation of the stereochemical configuration of naturally occurring phosphatidyl glycerol was achieved by means of different enzymes[11-13]), which are in many cases of excellent use in the establishment of structures. On the other hand, the substrate may be valuable in the determination of substrate specificity of the enzymes, as it was in the case of synthetic phosphatidyl glycerol, which was available as optically active compound and racemic as well. Phospholipase A (EC 3.1.1.4) from *Crotalus adamanteus* hydrolyses the fatty acid attached at the 2-position of 3-phosphatidyl-1′-glycerol quantitatively (scheme 2). Only palmitic acid could be detected as free fatty acid, while in the lyso compound exclusively oleic acid was present. The fully racemic compound was hydrolysed for about 50% even after 24 hr.

Pancreatic lipase (EC 3.1.1.3) is known to degrade triglycerides as well as phospholipids[24]), in the latter case acting on the C-1 attached fatty acid. This enzyme does not react stereo-specifically, however. Even from the fully racemic component the C-1 attached fatty acid was rapidly released quantitatively.



Scheme 2. Enzymic hydrolysis of 1-oleoyl-2-palmitoyl-glycerol-3-phosphoryl-1′-glycerol.

Phospholipase C (EC 3.1.4.3) from *Bacillus cereus* gave with the optically active product a rapid complete degradation, giving a 1.2-diglyceride and glycerolphosphoric acid, from which the stereochemical configuration was determined with glycerol-3-phosphate dehydrogenase (EC 1.1.1.8). No trace of glycerol-3-phosphoric acid could be detected. The racemic compound was hydrolysed rapidly until about 50% and then the velocity diminished considerably, just as was found in other substrates[21]).

Phospholipase D (EC 3.1.4.4.) from savoy-cabbage degraded 3-phosphatidyl-1′-glycerol to phosphatidic acid and glycerol. The phosphatidic acid obtained in this way was hydrolysed with alkali according to Dawson *et al.*[22,23]), giving glycerolphosphoric acid which after glycerol-3-phosphate dehydrogenase treatment appeared to be practically 100% glycerol-3-phosphoric acid. With the racemic compound the same behaviour with phospholipase D was observed as with phospholipase C. After rapid hydrolysis of 50% of the amount of phosphatidyl glycerol, the velocity diminished seriously. All these results compared with those obtained by Haverkate *et al.*[11–13,25]) on phosphatidyl glycerol from bacteria and spinach leaves show that the synthetic compound is structurally identical and has the same stereochemical configuration as naturally occurring phosphatidyl glycerol.

Experimental part

General methods

Micro-analyses were carried out in the Analytical Department of the Laboratory of Organic Chemistry, University of Groningen. Melting points were determined on a Kofler hot plate and are uncorrected. Gas–liquid chromatographic analyses were carried out as described previously[26]). Optical rotations were measured in a Lichtelektrisches Präzisions polarimeter 0.005°, Carl Zeiss.

The purity of intermediates and end product were checked by thin-layer- and paper chromatography as described earlier[21]). Enzymic degradations were carried out following established procedures[11,24]).

Materials

2.3-isopropylidene glycerol (scheme 1, I) (L-aceton glycerol) was synthesized from L-arabinose (purchased from Light & Co.) via the phenyl hydrazon of L-mannose according to Sowden and Fischer[27]), via L-mannitol following the method of Vargha *et al.*[28]) via the diisopropylidene derivative of L-mannitol according to Baer and Fischer[29]), finally leading to 2.3-isopropylidene glycerol as described by LeCocq and Ballou[30]). Dibenzyl phos-

phoric acid was prepared according to Atherton *et al.*[31]). 1-oleoyl-2-palmitoyl-glycerol iodohydrin was obtained as described previously[32]).

2.3-isopropylidene glycerol-1-dibenzylphosphate (II)

32.5 g of dibenzyl phosphoric acid and 12.0 g of dicyclohexyl carbodiimide were dissolved in chloroform to give tetrabenzylpyro phosphate. After 1 hr a solution containing 8.8 g of 2.3-isopropylidene glycerol (I) and 7.95 g of imidazole in chloroform was added dropwise. The mixture was stirred for 48 hr at room temperature. After filtration of dicyclohexylurea the solvent was evaporated, the residue dissolved in ether and washed in succession with an icy-cold 10% hydrochloric acid and four times with water. The ethereal solution was dried over sodium sulphate and evaporated *in vacuo*, giving II as a colourless viscous oil in a yield of 55–65%. A small amount of the substance was chromatographed over silica with benzene-ether as eluents for elemental analysis and optical rotation. $[\alpha]_D^{20} = -3.65°$ (*c* 10 in chloroform).

Found	C 60.6	H 6.4	P 7.6
Calculated for $C_{20}H_{25}O_6P$ (M = 392.37)	C 61.18	H 6.42	P 7.88

Barium salt of 2.3-isopropylidene-glycerol-1-(benzyl) phosphate (III)

Debenzylation of the foregoing product (II) was effected by barium iodide in dry acetone at boiling temperature for 4 hr. The precipitated barium salt (III) was filtered off and washed several times with acetone. The product revealed one distinct spot on thin-layer chromatograms; yield 93%; m.p. over 300 °C; $[\alpha]_D^{20} = +1.27°$ (*c* 10 in water)

Found	C 42.3	H 5.1	P 8.5
Calculated for $C_{13}H_{18}Ba_{0.5}O_6P$ (M = 369.94)	C 42.24	H 4.90	P 8.37

Silver salt of 2.3-isopropylidene-glycerol-1-(benzyl) phosphate (IV)

In the dark an aqueous solution of an equivalent amount of silver sulphate was added to a solution of the barium salt (III) in water. After centrifugation of barium sulphate, the clear solution was evaporated *in vacuo*. The silver salt (IV) was obtained as a white powder in a yield of 100%; m.p. 194–198 °C; $[\alpha]_D^{20} = +1.40°$ (*c* 10 in water)

Found	C 38.0	H 4.4	P 7.7
Calculated for $C_{13}H_{18}AgO_6P$ (M = 409.14)	C 38.16	H 4.43	P 7.64

1-oleoyl-2-palmitoyl-glycerol-3-(benzyl)phosphoryl-1'-(2'.3'-isopropylidene) glycerol (VI)

4.1 g of 1-oleoyl-2-palmitoyl-glycerol iodohydrin (V) was reacted with 3.1 g (30% excess) of the silver salt (IV) in toluene at a bath temperature of 100°. After 1 hr the mixture was cooled, the silver iodide removed by centrifugation

and the solution evaporated *in vacuo*. The residue was dissolved in pentane and washed subsequently with a bicarbonate solution and water. After drying over sodium sulphate the solution was evaporated *in vacuo*. The yellowish oily residue was chromatographed over silica with 5% ether in benzene, giving VI as a colourless oil in a yield of 75% based on V; $[\alpha]_D^{20} = +0.75°$ (*c* 10 in chloroform).

Found	C 68.30	H 10.1	P 3.5
Calculated for $C_{50}H_{87}O_{10}P$ (M = 879.17)	C 68.30	H 9.97	P 3.52

Sodium salt of 1-oleoyl-2-palmitoyl-glycerol-3-phosphoryl-1′-(2′.3′-isopropylidene) glycerol (VII)

The triester (VI) was debenzylated with barium iodide in boiling acetone for 3 hr. The precipitate was centrifuged and crystallized from chloroform-acetone (three times). The barium salt was transformed in the sodium salt by washing an ethereal solution of the barium salt with a solution of sodium sulphate. After drying over sodium sulphate and evaporation of the solvent a waxy solid remained; yield 95%; $[\alpha]_D^{20} = +3.44°$ (*c* 5 in chloroform)

Found	C 63.4	H 9.8	P 3.5
Calculated for $C_{43}H_{80}NaO_{10}P$ (M = 811.04)	C 63.67	H 9.94	P 3.82

Sodium salt of 1-oleoyl-2-palmitoyl-glycerol-3-phosphoryl-1′-glycerol (VIII)

0.6 g of the isopropylidene derivative (VII) was dissolved in 10 ml trimethylborate, 1.5 g of boric acid was added and dissolved by refluxing on a water bath for 5 min. The solvent was evaporated and the residue heated at 100 °C for 10 min with a rotary evaporator. After cooling the product was dissolved in ether and washed with a saturated sodium chloride solution. After drying over sodium sulphate and evaporation of the solvent, the product was purified by chromatography over silica with chloroform-methanol (85:15, v/v) as eluent. The pure phosphatidyl glycerol obtained was crystallized as the sodium salt from chloroform-acetone, giving VIII as a white powder in a yield of 85%; m.p. 176–179 °C; $[\alpha]_D^{20} = +1.02$ (*c* 10 in chloroform)

Found	C 62.2	H 10.0	P 3.8
Calculated for $C_{40}H_{76}NaO_{10}P$ (M = 770.98)	C 62.31	H 9.94	P 4.02

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