827. *Phospholipids. Part VI.* Synthesis of Phosphatidic Acids and Cephalins.*

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Syntheses of (±)-1,2-distearoyl-phosphatidic acid, -phosphatidylaminoethanol, and -phosphatidylserine are described, 1,2-distearoylglycerol 3-(benzyl phosphite) being used as a common intermediate.

Syntheses in the phosphatide series should, ideally, take into account two important facts. The natural substances, in general, have unsaturation in the long-chain fatty acid residues, so that avoidance of hydrogenation steps would be advantageous. Secondly, these substances are all unsymmetrical dialkyl phosphates, so that a two-stage phosphorylation would be preferable in order to minimise the production of symmetrical by-products.

Many syntheses of phosphatides have been described in recent years.[1] The most highly developed are those due to Baer and his co-workers,[2] in which a 1,2-di-*O*-acylglycerol (ROH) is treated with phenyl phosphorodichloridate to yield the intermediate di-*O*-acylglycerol phenyl phosphorochloridate which, without isolation, is put into reaction with the second alcohol (R'OH; *e.g.*, choline chloride); the phenyl residue is then removed

$$R{\cdot}OH \longrightarrow RO{\cdot}PO(Cl){\cdot}OPh \longrightarrow RO{\cdot}PO(OR'){\cdot}OPh \longrightarrow RO{\cdot}PO(OR'){\cdot}OH$$

by platinum-catalysed hydrogenation. Only saturated phosphatides can be obtained by this means. For the synthesis of unsaturated members, phosphoryl chloride [3] is used as phosphorylating agent, the third chlorine residue being subsequently removed by mild hydrolysis. In both procedures symmetrical products [RO·PO(OH)·OR] are to be expected and are found. The success of the method depends on the fact that stepwise replacement of halogens by alkoxy-residues becomes progressively more difficult, although the differences are evidently small. Variants on the phosphoryl chloride route, in which the intermediate alkyl phosphorodichloridate can be isolated in a pure state, have been described.[4] The reaction between diacyloxypropyl iodides and the silver salts of dialkyl phosphates represents an independent route to phosphatides [5] and in principle can satisfy the two requirements mentioned above; yields of product, too, appear to be good.

A number of phosphorylation methods have been described recently, particularly in the nucleotide field,[6,7] which, in so far as they have been devised specifically for the synthesis of unsymmetrical dialkyl phosphates and pyrophosphates, should be applicable to phosphatide synthesis. The present experiments were designed to investigate the use of one of these, *viz.*, that using phosphite intermediates.[8] The syntheses described are models

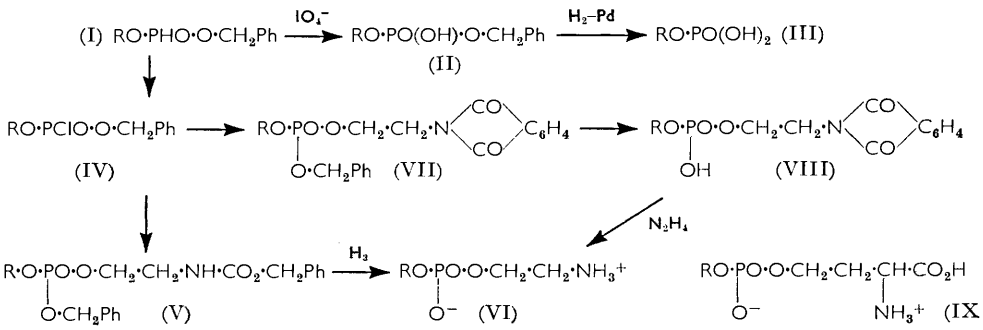
* Part V, *J.*, 1959, 3547.

[1] Malkin and Bevan, *Progr. Chem. Fats and Other Lipids*, 1957, **4**, 98.
[2] Baer, *Ann. Rev. Biochem.*, 1955, **24**, 135.
[3] Baer and Buchnea, *J. Amer. Chem. Soc.*, 1959, **81**, 1758.
[4] Hirt and Berchtold, *Helv. Chim. Acta*, 1957, **40**, 1928; Shapiro, Flowers, and Spector-Shafer, *J. Amer. Chem. Soc.*, 1959, **81**, 3743.
[5] Hessel, Morton, Todd, and Verkade, *Rec. Trav. chim.*, 1954, **73**, 150; Baylis, Bevan, and Malkin, *J.*, 1958, 2962.

in so far as the saturated (±)-1,2-di-*O*-stearoylglycerol [9] alone is used as starting material.

1,2-Di-*O*-stearoylglycerol when treated with *O*-benzylphosphorous *OO*-diphenylphosphoric anhydride yielded 1,2-di-*O*-stearoylglycerol 3-(benzyl phosphite) (I; R = distearoylglycerol residue throughout). The substance was never obtained analytically pure since phosphorylation generally proceeded to the extent of about 80% and it was not possible, because of the solubility properties of the materials, completely to remove the traces of unchanged distearoylglycerol. The crude phosphite could, however, be used satisfactorily in a number of syntheses. When oxidised by periodate [10] it gave di-*O*-stearoylglycerol benzyl phosphate [11] (II) which, on hydrogenation, yielded 1,2-di-*O*-stearoylphosphatidic acid (III).[12] The structure of the phosphite was thereby established.

The benzyl phosphite, when treated with *N*-chlorosuccinimide and 2-benzyloxycarbonylaminoethanol, gave a good yield of the phosphotriester (V). This type of reaction has hitherto been conducted by adding the *N*-chlorosuccinimide to the phosphite, allowing time for the formation of the corresponding phosphorochloridate, and then adding the alcohol.[8] In a reaction where adventitious moisture and the instability of benzyl phosphorochloridates [13] may combine to reduce yields, it was advantageous to add the *N*-chlorosuccinimide to a mixture of the phosphite and the alcohol in presence of the theoretical amount of tertiary base. When the fully protected ester (V) was hydrogenated, both the benzyl and the benzyloxycarbonyl group were removed and the 1,2-di-*O*-stearoylphosphatidylaminoethanol (VI) was isolated. Overall yields of 30% based on distearoylglycerol were obtained.



As an alternative route the benzyl phosphite was treated with phthalimidoethanol and *N*-chlorosuccinimide to give the triester (VII).* This, with sodium iodide in hot acetone, underwent debenzylation, and the product (VIII), without isolation, was then treated with hydrazine to remove the phthaloyl group. Di-*O*-stearoylphosphatidylaminoethanol (VI) was again isolated. This synthesis of the phospholipid accords with the requirements mentioned above in that a two-stage phosphorylation was used and no hydrogenation step was involved.

A synthesis of (±)-1,2-di-*O*-stearoylphosphatidyl-(±)-serine is also described. The benzyl phosphite (I) and (±)-*N*-benzyloxycarbonylserine benzyl ester were treated with *N*-chlorosuccinimide, and the intermediate triester immediately hydrogenated. The serine

* A Referee has drawn our attention to the preparation and debenzylation of this substance by Dr. P. J. C. Counsell, mentioned in a lecture by Dr. T. Malkin (*Fette, Seife, Anstrichmittel*, 1958, **60**, 930).

[6] Todd, *Proc. Nat. Acad. Sci. U.S.A.*, 1959, **45**, 1389.
[7] Smith, Moffatt, and Khorana, *J. Amer. Chem. Soc.*, 1958, **80**, 6204.
[8] Kenner, Todd, and Weymouth, *J.*, 1952, 3675.
[9] Howe and Malkin, *J.*, 1951, 2663.
[10] Brown and Hammond, preceding paper.
[11] Gielkens, Hoefnagel, Stegerhoek, and Verkade, *Rec. Trav. chim.*, 1958, **77**, 656.
[12] Uhlenboek and Verkade, *Rec. Trav. chim.*, 1953, **72**, 395; Baer, *J., Biol. Chem.*, 1951, **189**, 235.
[13] Atherton, Howard, and Todd, *J.*, 1948, 1106.

phosphatide (IX) was isolated as a powder by an extraction process similar to the method of Baer and Maurukas.[14]

Several attempts were made to prepare distearoyl-lecithin (phosphatidylcholine) by the phosphite route. It was clear that condensation of the di-*O*-stearoylglycerol benzyl phosphorochloridate with choline chloride occurred, since chromatograms indicated the presence of a substance responding both to phosphate and to choline reagents. Analytical values, although close to those required, were unacceptable. There was strong evidence that the initially formed chloride of di-*O*-stearoylglycerol benzyl choline phosphate was undergoing partial debenzylation, since benzyl chloride was always detected during isolation. This process should lead directly to distearoyl-lecithin itself, but it was not possible to isolate it in a pure state. Further experiments may allow clarification of this point and lead to a simple lecithin synthesis. It is hoped, too, to apply the above route to the synthesis of unsaturated phospholipids, and to more complex lipids.

Experimental

Evaporations were carried out *in vacuo* at, or near, room temperature.

1,2-*Di*-O-*stearoylglycerol* 3-(*Benzyl Hydrogen Phosphite*).—Monobenzyl phosphite (4·75 g., 1·6 mol.), diphenyl phosphorochloridate (5·60 ml., 1·5 mol.), and dry pyridine (2·2 ml., 1·5 mol.) were stirred together for 3 hr. with benzene (100 ml.) under anhydrous conditions. The solution was filtered from pyridine hydrochloride, into a dry solution of 1,2-di-*O*-stearoylglycerol (10 g., 1 mol.; m. p. 67—68°). Stirring was continued for 1 hr. and after filtration the solution was washed with water, 5% potassium hydrogen sulphate solution, and sodium hydrogen carbonate solution (100 ml. of each), and then dried (Na_2SO_4).

Paper chromatography showed that no benzyl phosphite, diphenyl phosphate, or monobenzyl phosphate were present, but phosphorus analyses gave evidence for incomplete phosphorylation (80%). The solution was used as such for further reactions, but a crude product could be isolated by precipitation with acetone.

1,2-*Di*-O-*stearoylglycerol* 3-(*Benzyl Hydrogen Phosphate*).—To the above phosphite (from 2 g. of distearoylglycerol) in carbon tetrachloride (5 ml.), dioxan (20 ml.) and 2,6-lutidine (5 ml.), was added 3·6M-periodic acid (1 ml.; 1·1 mol.) in water (4·5 ml.). The solution was stirred for 2 hr. and shaken with 5% potassium hydrogen sulphate solution (100 ml.) and chloroform (100 ml.). The potassium hydrogen sulphate washing (water-washing caused troublesome emulsions) was repeated twice and the chloroform solution was dried (Na_2SO_4) and evaporated. The product was recrystallised from acetone and then several times from pentane to constant phosphorus analysis. It had m. p. 54° (lit.,[11] 52°) (yield 0·6 g.) [Found, in material dried at 35°/0·1 mm. over P_2O_5: C, 69·5; H, 10·5; P, 3·7%; equiv. (by titration against tetrabutylammonium hydroxide in benzene–methanol), 790. Calc. for $C_{46}H_{83}O_8P$: C, 69·5; H, 10·5; P, 3·9%; equiv., 795].

1,2-*Di*-O-*stearoylglycerol* 3-(*Dihydrogen Phosphate*).—The above benzyl ester (0·5 g.) was hydrogenated over palladium black (0·2 g.) in chloroform (5 ml.) and hexane (20 ml.). Uptake of hydrogen was rapid, and after 1 hr. the product was isolated by evaporation of the centrifuged solution. After two crystallisations from hexane (80 ml.) it (0·31 g.) had m. p. 70—71° (lit.,[12] 71°) (Found, in material dried at 40°/0·1 mm. over P_2O_5: C, 66·2; H, 10·4; P, 4·5. Calc. for $C_{39}H_{77}O_8P$: C, 66·5; H, 10·9; P, 4·4%).

1,2-*Di*-O-*stearoylglycerol* 3-(*Benzyloxycarbonylaminoethyl Benzyl Phosphate*).—The phosphite (from 2·0 g. of distearoylglycerol), benzyloxycarbonylaminoethanol (0·625 g., 1 mol.), 2,6-lutidine (0·72 ml., 2 mol.), and *N*-chlorosuccinimide (0·427 g., 1 mol.) were dissolved in benzene (75 ml.), and the solution was stirred. After 16 hr. the white precipitate was filtered off, and the solution taken to dryness at room temperature. The solid residue was extracted with boiling light petroleum (b. p. 40—60°) (4 × 10 ml.). The extract was evaporated and the *phosphate* extracted from the residue by hot, dry ethyl acetate (4 × 10 ml.) from which it crystallised on cooling. It formed a microcrystalline powder (0·77 g.), m. p. 62—63° (Found, in material dried for 2 hr. at room temperature/0·5 mm. over P_2O_5 and wax: C, 69·0; H, 9·7; N, 1·7; P, 3·3. $C_{56}H_{94}NO_{10}P$ requires C, 69·2; H, 9·7; N, 1·4; P, 3·2%).

[14] Baer and Maurukas, *J. Biol. Chem.*, 1955, **212**, 25.

1,2-*Di*-O-*stearoylphosphatidylaminoethanol*. *Route* (a).—The above phosphate (0·77 g.) in glacial acetic acid (30 ml.) and chloroform (5 ml.) was hydrogenated over pre-reduced platinum oxide (0·2 g.) and palladium black (0·2 g.). After 2 hr., consumption of hydrogen was complete and chloroform (10 ml.) was added to bring the product into solution. Catalysts were removed by centrifugation and the solution was evaporated *in vacuo*. The product was isolated as a white powder (0·55 g.) by dissolving the residue in chloroform (5 ml.) and precipitating it with acetone (40 ml.). It was washed with dry ether and dried (P_2O_5 and wax), and had m. p. 170° with previous softening (Found: C, 66·2; H, 10·9; N, 2·1; P, 3·9. Calc. for $C_{41}H_{82}NO_8P$: C, 65·8; H, 11·0; N, 1·9; P, 4·0%) and $\nu_{max.}$ 3410, 2925, 2855, 1735, 1640, 1472, 1380, 1179, and 719 $cm.^{-1}$.

1,2-*Di*-O-*stearoylglycerol* 3-(*Phthalimidoethyl Benzyl Phosphate*).—The crude distearoyl benzyl phosphite (1 g., above), *N*-2-hydroxyethylphthalimide (0·245 g.), 2,6-lutidine (0·288 ml.), and *N*-chlorosuccinimide (0·172 g.) were dissolved in dry benzene (75 ml.), and the solution was stirred at room temperature for 16 hr. The *phosphate* (0·2 g.) was isolated as for the benzyloxycarbonylaminoethyl ester, above, and had m. p. 63—64° (Found: C, 69·1; H, 10·4; P, 3·4. $C_{56}H_{90}NO_{10}P$ requires C, 69·4; H, 9·3; P, 3·2%), $\lambda_{max.}$ (in $CHCl_3$) 267, 294 mμ (ε 2500, 720 respectively).

1,2-*Di*-O-*stearoylphosphatidylaminoethanol*. *Route* (b).—The above phthalimido-derivative (20 mg.) and anhydrous sodium iodide (10 mg.) were heated under reflux in acetone (5 ml.) solution for 2 hr. and then cooled. The debenzylated product separated and was recrystallised twice from acetone. To the dried material methoxyethanol (5 ml.) and aqueous hydrazine (containing 5 g. of hydrazine hydrate per l.) were added and the solution was heated at 70° for 1 hr. On cooling, the product separated and was recrystallised three times from 99% ethanol. It had m. p. 180° with previous softening (Found: P, 4·1%). The material had no detectable ultraviolet light absorption in the 260 mμ range and had an infrared spectrum (KBr disc) identifying it with the material prepared by route *a*.

1,2-*Di*-O-*stearoylphosphatidylserine*.—The benzyl phosphite (from 1·67 g. of distearoylglycerol), (±)-*N*-benzyloxycarbonylserine benzyl ester (0·878 g.), 2,6-lutidine (1·0 ml.), and *N*-chlorosuccinimide (0·36 g.) were dissolved in dry benzene (50 ml.) and stirred for 22 hr. A copious precipitate of lutidine hydrochloride and succinimide was filtered off. The filtrate was evaporated, and the residue kept at 0·2 mm. for 3 hr. and then extracted with ether (3 × 10 ml.). The extract, to which was added carbon tetrachloride (30 ml.), was shaken with ice-cold 0·1N-sulphuric acid (10 ml.), and the emulsion broken by centrifugation. The lower layer was removed. Addition of carbon tetrachloride and subsequent isolation of the lower layer was twice repeated, and the combined extracts were dried (Na_2SO_4) and evaporated to give a wax. This was crystallised from light petroleum (b. p. 40—60°; 40 ml.) at 0°, and then twice from 99% ethanol (10 ml.). This material (0·31 g.) was hydrogenated over pre-reduced palladium black (0·1 g.) and platinum oxide (0·1 g.) in acetic acid (50 ml.). After addition of chloroform (30 ml.) the catalyst was removed by centrifugation and the solvent was evaporated at 25°. The product was a powder which was triturated with small amounts of acetic acid, water, acetone, and dry ether and finally dried (P_2O_5 and wax) at 0·5 mm. for 1 hr. (Found: C, 64·0; H, 10·6; N, 2·1; P, 3·8. Calc. for $C_{42}H_{82}NO_{10}P$: C, 63·7; H, 10·4; N, 1·8; P, 3·9%).

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