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THE SYNTHESIS OF A SPIN-LABELLED GLYCERO-PHOSPHOLIPID

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0-(1,2-Distearoyl-*sn*-glycero-3-phosphoryl)-3′-hydroxymethyl-2′,2′,5′,5′-tetramethylpyrrolidine-1′-oxyl has been synthesised as a spin-labelled substrate for the study of lipid-protein interactions with particular reference to lipolytic enzymes. For this, the new spin label, 3-hydroxymethyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl was prepared and phosphorylated with 1,2-distearoyl-*sn*-glycero-3-phosphoric acid, using tri-isopropylbenzenesulphonyl chloride as the condensing agent.

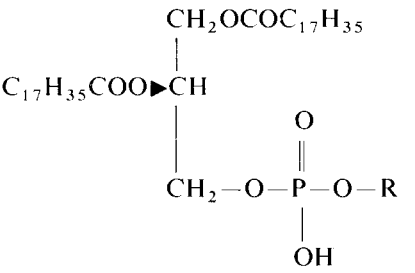
Introduction

The concept of spin labelling was introduced by McConell[1,2]) only recently, but already interesting results have been reported for investigations on several systems[3]). These include a study[4]) of the nature of the interaction between phospholipids and proteins which results in the well characterised water-insoluble, iso-octane-soluble lipoprotein complexes[5]). The systems studied[4]), even though involving the enzymes lysozyme and cytochrome c as the protein component, must be regarded as examples of lipid-protein interactions of a relatively non-specific nature. This is in contrast to the specific interactions which must occur between lipid enzymes and their substrates. A better definition and understanding of such interactions is the aim of the present study. The enzyme employed is phospholipase A (E.C. 3.1.1.4) isolated from porcine pancreas[6]).

In general, an enzyme and its substrate must come together as a complex; therefore, the formation of a lipid-protein complex must be a (vital) stage in lipolysis by phospholipase A. The use of a spin label attached to the enzyme, or a spin-labelled lipid substrate should yield valuable information about the enzyme and its interaction with the substrates. Of course, the same spin-labelled lipids could be employed in the study of the interactions in a variety of other systems.

The preparation of suitably spin-labelled substrates is an integral part of the study and this report describes our work on the design and synthesis of the glycero-phospholipids (I). The synthetic operations, especially at the phosphorylation stage, have an important bearing on the synthesis of natu-

rally occurring glycero-phospholipids, and remarks relevant to this aspect are also included.



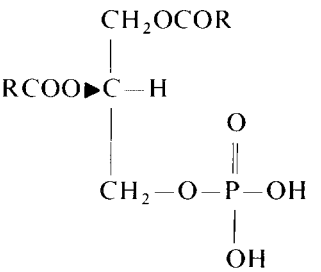
R = Spin label

I

The design of the structure of the spin-labelled lipid was guided by (i) the known relationships of substrate structure and phospholipase A specificity[7]), and (ii) the structural features essential in a stable spin-label[1,3]). A brief resumé of the relevant facts is, therefore, in order.

Substrate structure and phospholipase A specificity

The naturally occurring lipid substrates[7,8]) for this enzyme are 1,2-diacyl-*sn*-glycero-3-phosphoric acid (II)[9]) and its monoalkyl esters (III). These are very widely distributed in nature and include the



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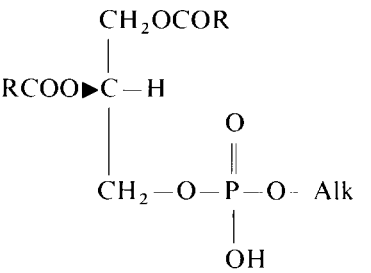


III

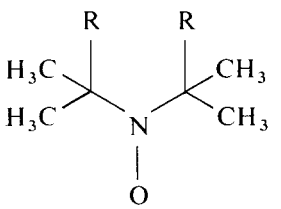
HO—Alk = e.g. choline, ethanolamine, inositol, etc.

esters of choline, ethanolamine, inositol and several more complex alcohols. They all undergo *rapid* lipolysis with the release of the (one mole) fatty acid(s) from the *sn*-2-acyloxy group. The isomeric 1,3-diacyl-*sn*-2-phosphoryl analogues have not been found in nature, but some derivatives have been synthesised; these undergo lipolysis specifically at the *sn*-1-acyloxy position but, comparatively, at a very slow rate.

The structure of the non-glyceride (polar head group) portion of the molecule (Alk in structure III) does not appear to be critical and a large number of groups are acceptable; these groups, however, do influence the rate of lipolysis. The effects observed vary with the source of phospholipase A. Similarly, the nature of the fatty groups influences the rate of lipolysis. Thus, phospholipids containing short chain water soluble fatty acids are hydrolysed at a very slow rate.

Spin labels

The structural feature (IV) is essential for stability of the nitroxide radical[10]) and is common to all the spin labels which have found practical use so far. The groups RR are commonly part of a five (V, VI, VII) or six (VIII) membered ring structure. The general advantages of the various structures have been discussed[3]).



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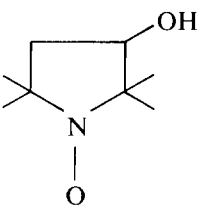

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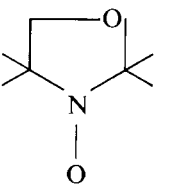

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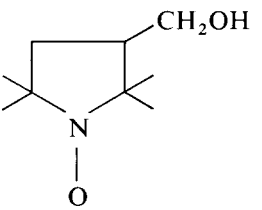

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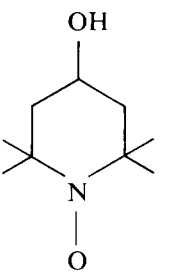
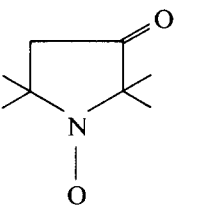


VIII



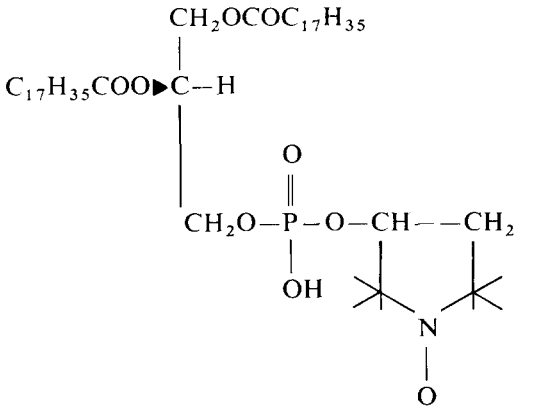
IX

The syntheses

The synthetic task thus consists in the incorporation of structures such as IV to VIII in an *sn*-glycero-3-phosphoric acid derivative (II or III) or the *sn*-2-phosphoryl isomer. Three groups of substrate are conceivable since the

spin label may be attached in III along either of the two fatty acyl residues*, or in the second esterifying alcohol (Alk in structure III). Complementary, but essential, information should emerge from studies on all three groups.

O-(1,2-Distearoyl-sn- glycero-3-phosphoryl)-3'-hydroxy-2',2',5',5'-tetra methylpyrrolidine-1'-oxyl (X)



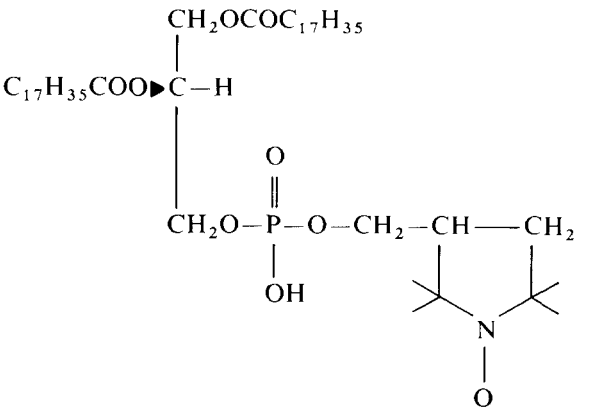
X

The syntheses of phospholipids with the spin label in the polar head group portion by the condensation of a phosphatidic acid (*e.g.* II, $R=C_{17}H_{35}$) with the known alcohol[11]) (V) was investigated first, but difficulties were encountered even in the synthesis of the starting alcohol (V). The reduction of the ketone (IX) using aluminium isopropoxide-isopropanol following the published procedure[11]) was not successful in our hands. The alcohol (V) was finally prepared by the reduction of IX using sodium borohydride[12]). Next, attempts to couple the alcohol (V) with phosphatidic acid were not successful. In the direct phosphorylation, 2,4,6-tri-isopropylbenzene sulphonyl chloride (TPS)[13]) or dicyclohexyl-carbodiimide (DCC)[14]) were tried as condensing agents (see later for further discussion). In an indirect route, phosphorylation by the Pinner-abspaltung procedure of Cramer[15]) was envisaged, but could not be carried through since the alcohol (V) (as the sodio-derivative) did not react with trichloroacetonitrile in the first stage of the reaction sequence. These difficulties arise partly from the secondary nature of the hydroxyl group and its tendency to eliminate as water and partly from the steric crowding around this hydroxyl group.

* Since the completion of our work, a mixture of glycero-phospholipids containing the spin label residue VI in the fatty acid chain, has been isolated as a metabolite of *Neurospora crassa* fed on spin-labelled fatty acid[25]).

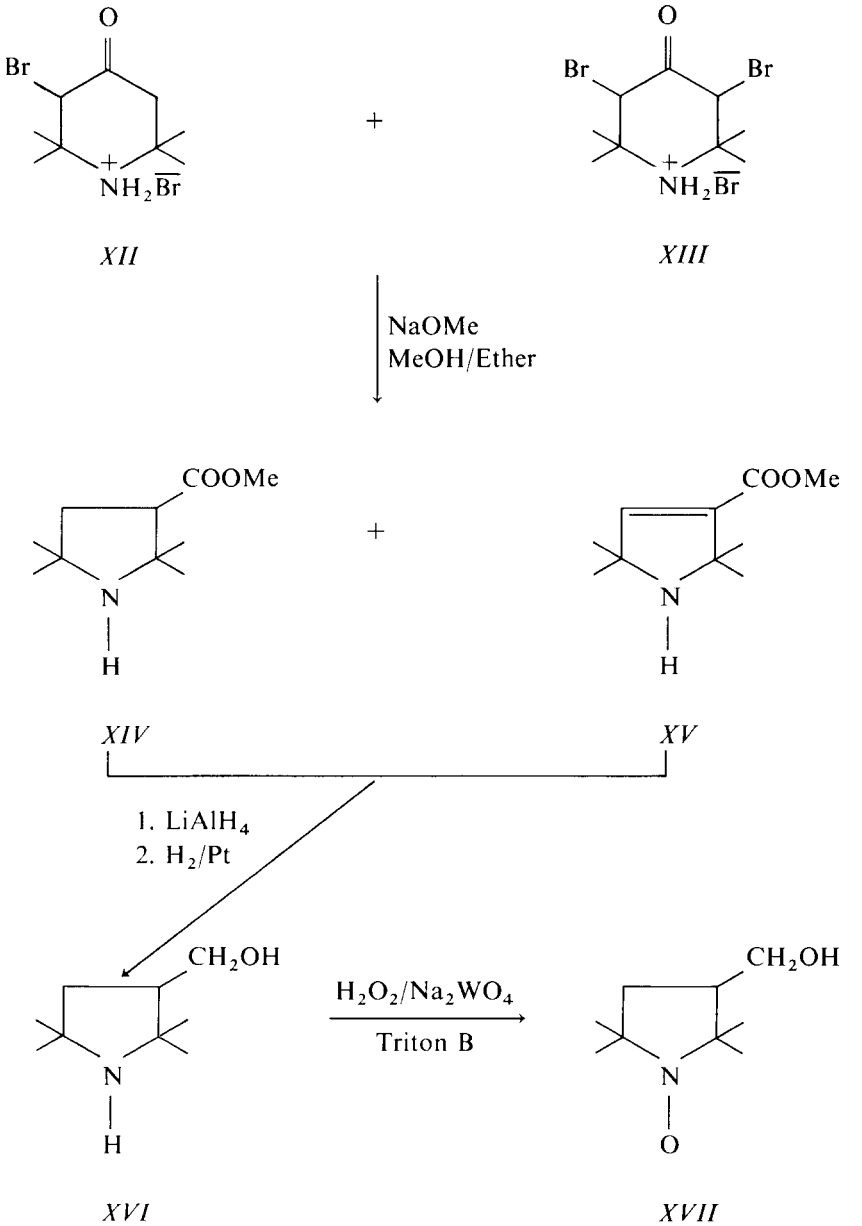
The above synthesis was attempted because the expected product (X) has desirable features. Thus, in its structure, the N-atom (even though an $>N-O$) occupies the same location relative to the phosphate group as is found in the common natural lipids, e.g. phosphatidylcholine and phosphatidylethanolamine. The freedom of rotation around the P—O and the C—O bonds ensures an intrinsically mobile spin-label. The nitroxide group has the closest possible situation relative to the phosphoric acid function and, therefore, is most likely to respond to and reflect changes in the immediate environment of this function. In the event, the next higher homologue (XI) was synthesised as a compromise.

O-(1,2-Distearoyl-sn-glycero-3-phosphoryl)-3'-hydroxymethyl-2',2',5',5'-tetramethylpyrrolidine-1'-oxyl (XI)



XI

Bromination of 2,2,6,6-tetramethylpiperid-4-one[16]) at room temperature produced a mixture of the 3-bromo- and 3,5-dibromo derivatives[17]) (XII and XIII, respectively), which on further treatment with sodium methoxide in ethereal methanol gave a mixture of XIV and XV, via a Favorskii[18]) rearrangement. Reduction, first with lithium aluminium hydride and next by catalytic (Pt.) hydrogenation yielded a homogeneous product, 3-hydroxymethyl-2,2,5,5-tetramethylpyrrolidine (XVI). Oxidation of XVI with hydrogen peroxide, catalysed by sodium tungstate and triton B, afforded 3-hydroxymethyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (XVII). Finally, treatment of XVII and 1,2-distearoyl-*sn*-glycerol-3-phosphoric acid (II) in pyridine solution with TPS, effected the condensation to the spin-labelled phospholipid (XI).



Alternative procedures for the above phosphorylation were not satisfactory. Thus, the alcohol (XVII) on treatment with trichloroacetonitrile yielded its trichloro-acetimidate. However, the latter did not undergo Pinner-abspaltung on treatment with phosphatidic acid. Further, XVII and II could

be condensed in the presence of DCC, but a substantial amount of a side product accompanied the desired product (XI). This presented a serious practical difficulty, since the side product showed chromatographic and solubility behaviour identical with that of (XI), with the result that separation could not be effected.

Discussion

In the condensation of mono-alkyl phosphoric acids with alcohols to yield dialkyl phosphoric acids, self-condensation of the phosphoric acid to pyrophosphates is a competing reaction[19,20]). That this reaction is responsible for the by-product mentioned in the preceding paragraph was established by effecting a self-condensation of the phosphatidic acid (II) by DCC. The product was not isolated, but the crude material showed a new component with Rf, identical with those of (XI), in thin layer chromatography in several systems. No attempt was made, therefore, to optimise the reaction conditions. However, the formation of an intractable mixture with the by-product pyrophosphate is intrinsic only to the specific case studied above; in general, it should not present a handicap in the esterification of phosphatidic acids with other alcohols. Further, it may be possible to limit, or even prevent, the formation of pyrophosphates if a large excess of the alcohol is employed[19,20,22]). On the other hand, in the absence of a tertiary amine, the pyrophosphate, as its DCC adduct, may act as a phosphorylating agent[20]). Yet, surprisingly, the DCC mediated condensation of phosphatidic acids and appropriate alcohols has received only a limited attention for the syntheses of biologically important naturally occurring glycerophospholipids[21,22]).

As anticipated from the work of Khorana *et al.*[13]) who developed triisopropylbenzenesulphonyl chloride (TPS) as a reagent for the synthesis of internucleotide bonds, no pyrophosphate was detectable in the products from the condensation of the phosphatidic acid (II) and the alcohol (XVII) by TPS. In fact, even in the absence of the alcohol (XVII), treatment of the phosphatidic acid (II) in pyridine with TPS gave pyrophosphate just detectable by thin-layer chromatography. A large excess of the alcohol component is not required and the yields of the phospho-diester are fair. The method has promise as a general route for the syntheses of naturally occurring glycerophospholipids.

The phosphatidic acids used in this study were prepared by synthesis. Preliminary studies to ascertain the optimum conditions for the condensation were carried out with O-1,2-distearoyl-*rac*-glycero-3-phosphoric acid which was prepared by the stearoylation of glycero-3-phosphoric acid[23]). For the final preparation, the compound having the stereochemistry of the

natural lipids, 0-1,2-distearoyl-*sn*-glycero-3-phosphoric acid (II, R=$C_{17}H_{35}$) was used; this was prepared from D-mannitol by the bromohydrin procedure[24]).

Properties of the spin-labelled lipid

The spin-labelled lipid XI was characterised as a triethylamine salt. This is a low melting (48–48.5°) solid, $[\alpha]_D^{20} + 5.7°$. It is lipolysed by phospholipase

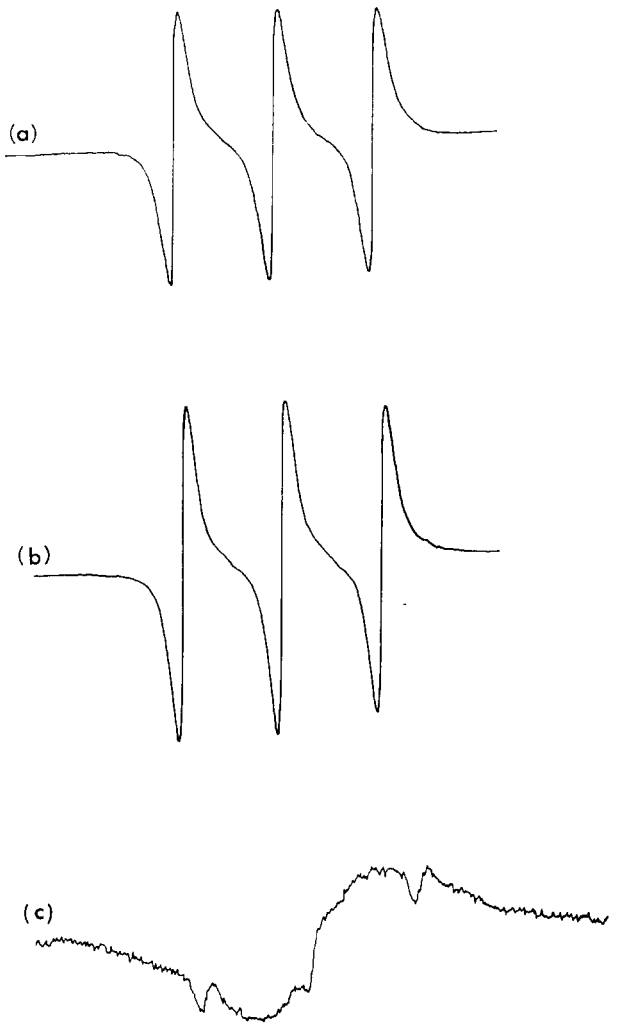


Fig. 1. ESR spectra of (a) 3-hydroxymethyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl in chloroform ($a_N = 15.8$). (b) 0-(1,2-distearoyl-*sn*-glycero-3-phosphoryl)-3′-hydroxymethyl-2′,2′,5′,5′-tetramethylpyrrolidine-1-oxyl in chloroform ($a_N = 15.8$). (c) 0-(1,2-distearoyl-*sn*-glycero-3-phosphoryl)-3′-hydroxymethyl-2′,2′,5′,5′-tetramethylpyrrolidine-1′-oxyl in water.

A (ex porcine pancreas) in an aqueous borate buffer (*p*H 7.1) of its co-dispersion with sodium deoxycholate to yield an acid (stearic) and a lysophospholipid (TLC evidence). This, of course, is to be anticipated from its synthesis from II and its formulation as XI.

The presence of the nitroxide group is indicated by the I.R. absorption at ν max. 1360 cm^{-1} and the U.V. absorption maximum at 224 mμ (ε2950). Its ESR spectrum in chloroform solution (10^{-4} molar) shows a symmetrical triplet $a_N = 15.8$ (fig. 1b). In aqueous dispersion it exhibited a hyperfine component (triplet) superimposed on a "solid-state" spectrum (fig. 1c), the former being attributed to the dissolved phospholipid and the latter presumably due to phospholipid associated in micelles or lamellae.

Experimental

General methods

Melting points were determined on a Kofler hot stage apparatus and are uncorrected. Infra-red spectra were determined on a Perkin-Elmer infracord spectrophotometer model 257. 60 MHz P.M.R. spectra were recorded for deuterochloroform solutions on a Perkin-Elmer R.12 spectrometer with tetramethylsilane as the internal standard. Ultraviolet measurements, in ethanol, were made on a Unicam S.P.800 spectrophotometer. Optical rotations were measured on a Perkin-Elmer 141 automatic polarimeter, on samples dissolved in dry ethanol-free chloroform. ESR spectra were recorded on a Varian V-4502 X-band spectrometer. Elemental analyses were performed by Dr. F. Pascher, Bonn. Organic solutions were dried with magnesium sulphate and all solvents were evaporated on a rotary evaporator at a temperature $<45°$.

Thin layer chromatographic analysis was carried out on Silicagel G (Merck) with Zinzadze reagent used as the visualizing spray; phosphorus containing compounds sprayed up blue, nitroxide-white, and other compounds were detected by charring. Spin labels were normally developed in chloroform-methanol solvent mixtures, whereas acidic phospholipids were run on triethylamine impregnated Silicagel and developed with $CHCl_3$/CH_3OH/$(CH_3CH_2)_3N$, 94:5:1.

Phospholipase A, specific activity 300 Eu/mg, isolated from porcine pancreas, was kindly provided by Dr. P. C. Harries.

Synthesis of 3-hydroxymethyl-2,2,5,5-tetramethylpyrrolidine-1-oxy (XVII)

(1) *Favorskii rearrangement*

A mixture of 3-mono and 3,5-dibromo-2,2,6,6-tetramethylpiperid-4-one

hydrobromide[17]) (27 g) was suspended in a stirred mixture of methanol (34 ml) and ether (340 ml) at 0–5°. Sodium methoxide (prepared from 6 g sodium) in methanol (75 ml) and ether (500 ml) was added to the cooled suspension over a period of 1 hr, stirred an additional 2 hr, and the temperature finally allowed to rise to 25°. The mixture was filtered and the filtrate evaporated to dryness. Extraction of an aqueous solution of the residue with ether afforded, after drying and evaporation, a mixture of XIV and XV as a yellow oil (8.6 g). A portion of this mixture was used directly in the reduction stage below, but the remainder was separated into XIV and XV. Chromatography of the oil on SilicAR CC-7 (130 g) gave on eluting with 5% methanol in chloroform, pure 3-carboxymethyl-2,2,5,5-tetramethylpyrrolidine (XIV) (1.8 g), NMR τ6.3 (3H singlet, $COOCH_3$), 6.95–8.17 (3H multiplet, $-CH-CH_2$), 8.22 (1H singlet, $-NH$) 8.63, 8.73, 8.83, 8.94 (four 3H, singlets, CH_3); and 3-carboxymethyl-2,2,5,5-tetramethylpyrroline (XV) (1.4 g) NMR, τ 3.45 (1H, singlet $-CH=C-$) 6.3 (3H, singlet, $COOCH_3$), 8.18 (1H, singlet, NH), 8.6 (6H, singlet, CH_3), 8.75 (6H, singlet, CH_3).

(2) *Reduction to (XVI)*

A solution of lithium aluminium hydride (2.28 g, 0.06 mole) in dry ether (100 ml distilled from lithium aluminium hydride) was stirred under reflux for 1 hr. To this warm solution was added dropwise, the aforementioned mixture of 3-carboxymethyl-2,2,5,5-tetramethylpyrroline (XV) and 3-carboxymethyl, 2,2,5,5-tetramethylpyrrolidine (XIV) (5.4 g) at such a rate as to maintain the ether under gentle reflux. The solution was refluxed an additional 1 hr, allowed to cool and the following added cautiously: 2.3 ml water, 2.3 ml 15% sodium hydroxide solution and 6.9 ml water. The granular precipitate was removed, washed with ether and the total ethereal layers evaporated, giving a solid residue (4 g). Hydrogenation of this material (4 g) in acidified methanol (100 ml methanol + 12N hydrochloric acid to *p*H 2) over pre-reduced platinum, gave after basification (5N NaOH) and extraction with ether pure 3-hydroxymethyl-2,2,5,5-tetramethylpyrrolidine (XVI) (3.5 g) NMR τ 6.34 (2H doublet $-CH-CH_2-OH$), 7.9, (1H singlet $-NH$), 8.0–8.6 (3H multiplet), 8.77, 8.81, 8.95 (12H, 3 singlets, CH_3-).

(3) *Oxidation to Nitroxide (XVII)*

A mixture of 3-hydroxymethyl-2,2,5,5-tetramethylpyrrolidine (XVI) (2 g), triton B (200 mg, 40% w/v), sodium tungstate (100 mg) and hydrogen peroxide (3 ml, 100 vol) in water (20 ml) was left at room temperature for 24 hr. The aqueous solution was extracted with chloroform (3 × 50 ml) and the organic layer dried and evaporated. Crystallisation of the residue from hexane-ether gave yellow needles (1.5 g), m.p. 111–114°, ν max. 3380 ($-OH$),

1360 cm^{-1} (N–O); λ max. 234 mμ (ε 2,600); ESR, Fig. 1(a) (10^{-4} molar in chloroform, triplet, $a_N = 15.8$).

Found: C, 62.92; H, 10.50; N, 8.03%

$C_9H_{18}NO_2$ requires: C, 62.75; H, 10.53; N, 8.13%

O-(1,2-Distearoyl-sn-glycero-3-phosphoryl)-3'-hydroxymethyl-2',2',5',5'-tetramethylpyrrolidine-1'-oxyl (XI)

1,2-Distearoyl-*sn*-glycero-3-phosphoric acid (380 mg, 1 mol equiv.) and 3-hydroxymethyl-2,2,5,5-tetramethyl-1-oxyl (190 mg, 2 mol equiv.) were dissolved in a mixture of chloroform and pyridine and the solvent removed under reduced pressure. The procedure was repeated twice and the residue finally evacuated over phosphorus pentoxide. The residue was taken up in dry pyridine (40 ml), 2,4,6-tri-isopropyl-benzenesulphonyl chloride (360 mg, 2.1 mol equiv.) added and the mixture shaken vigorously at room temperature for 7.5 hr. After removing the insoluble material, water (80 ml) was added – with cooling – to the filtrate and left for 20 hr at 23° before evaporating to dryness. Trituration of the dry solid residue with hexane and evaporation of the filtrate effected partial purification of the required product. Final purification was accomplished by chromatography on triethylamine impregnated SilicAR (12 g), the pure product (120 mg) being eluted with chloroform-triethylamine (99:1). Repeated crystallisation from cold acetone gave a yellow amorphous solid, m.p. 48–48.5°, $[\alpha]_D + 5.7°$ (c, 1.00); ν max. ($CHCl_3$) 3300(OH), 2500–2100 [$(CH_3CH_2)_3\overset{+}{N}H-$)], 1730 (ester), 1360 (>N–O), 1230 and 1160 cm^{-1} (ester); λ max. 224 (ε 2950), 209 mμ (ε 2950); NMR τ 8.75 ($-CH_2$) ESR spectrum (10^{-4} molar solution in $CHCl_3$) triplet, $a_N = 15.8$.

Found C, 66.48 H, 11.27 N, 2.77%

$C_{48}H_{93}NO_9P$, $(C_2H_5)_3N$, H_2O requires C, 66.29 H, 11.33 N, 2.86%

Lipolysis of labelled lipid (XI) with phospholipase A

Labelled lipid (XI) (10 mg) sodium deoxycholate (3 mg), calcium chloride (5N, 1 ml) and phospholipase A (2 mg) were dispersed in borate buffer (1 ml, *p*H 7.1), at 40° for 3 hr. The mixture was extracted with ether, dried and evaporated yielding a white solid (7.7 mg). TLC analysis (CH_3OH/$CHCl_3$/Et_3N, 6:98:1) revealed that the residue contained only a single phosphorus containing compound, having an Rf value < Rf value of the starting lipid. Methylation with diazomethane and analysis by GLPC (gas liquid partition chromatography; 20% D.E.G.S./Temp. 165°) revealed the presence of pure methyl stearate.

A parallel experiment carried out in the absence of phospholipase A gave unchanged starting material only.

Acknowledgements

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