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THE SYNTHESIS OF A PERDEUTERATED PHOSPHOLIPID: 1,2-DIMYRISTOYL-*sn*-GLYCERO-3-PHOSPHOCHOLINE-d_{72}

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The synthesis of a perdeuterated phospholipid – 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine-d_{72} (DMPC-d_{72}) – is described. Ordinary (protonated) DMPC was prepared by the same synthetic route and its properties were found to be the same as those of commercial DMPC. The uses of partially deuterated and perdeuterated phospholipids in NMR studies are discussed.

I. Introduction

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopic studies of compounds in phospholipid systems are limited by the intense background signals of the lipids. The background signal can be attenuated by using phospholipids with perdeuterated acyl chains, thereby facilitating the observation of small molecules such as cholesterol [1] or gramidicin A′ [2] in the bilayer. Further improvement can be achieved by preparing phospholipids with both the acyl chains and the glycerophosphocholine head group perdeuterated. Although 3-*sn*-phosphatidylcholine has been prepared with deuterium in specific places in the glycerol or choline moieties [3], the intense ^{1}H NMR background signal is not thereby eliminated. Furthermore, the synthetic route used for these compounds is not suitable for preparing 3-*sn*-phosphatidylcholines with perdeuterated head groups. Therefore we have developed a method of preparing perdeuterated 3-*sn*-phosphatidylcholines and have used it to prepare both protonated and perdeuterated 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC and DMPC-d_{72}). The reactions for the synthesis of DMPC-d_{72} (VIII) are shown in Scheme 1.

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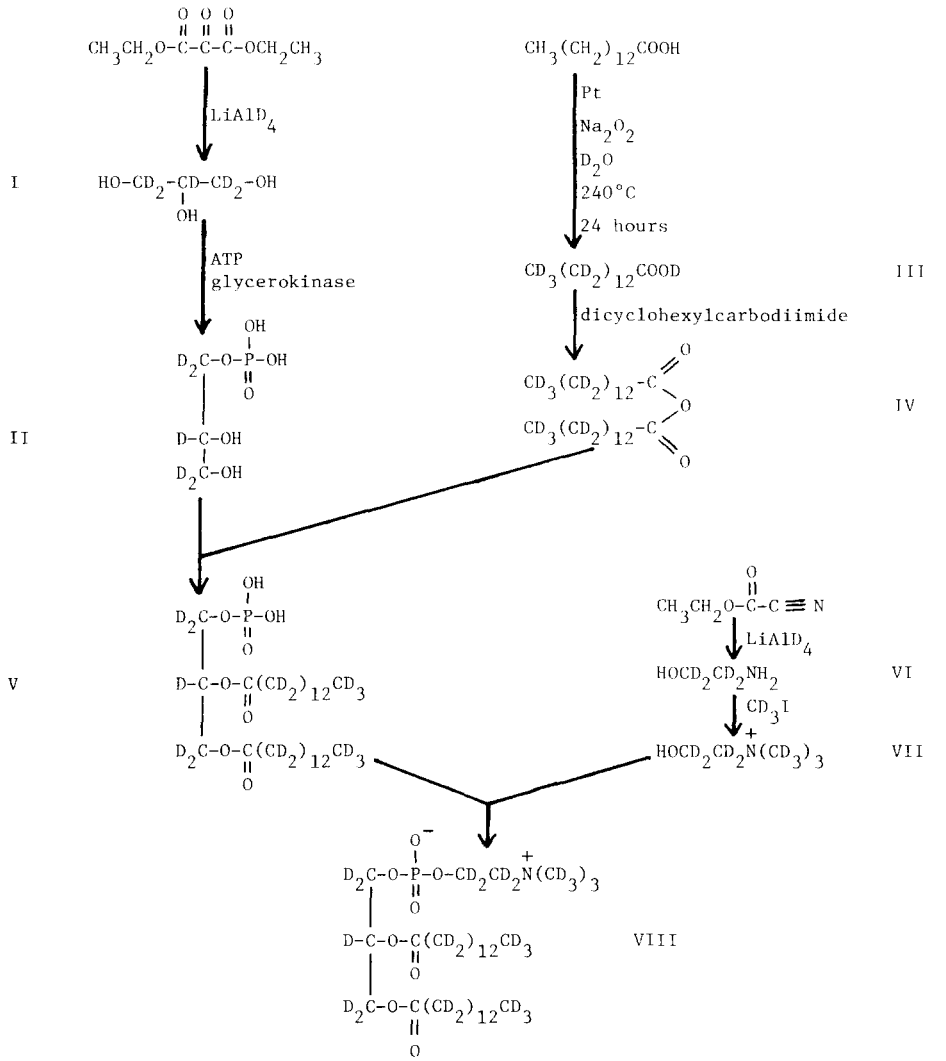
Abbreviations: DMPA, 1,2-dimyristoyl-*sn*-glycero-3-phosphoric acid; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; ^{1}H NMR, proton nuclear magnetic resonance; ^{31}P NMR, phosphorus nuclear magnetic resonance; T_1, spin-lattice relaxation time; TLC, thin layer chromatography.



Scheme 1

The synthetic routes for the preparation of glycerol-d_5 and ethanolamine-d_4 were suggested by earlier syntheses of ^{14}C-labeled compounds [4,5]. We have solved the problem of preparing the naturally-occurring optical isomer of the phospholipid by using the enzyme glycerokinase (ATP:glycerol 3-phosphotransferase, EC 2.7.1.30) [6,7] rather than by starting with D-mannitol [3]. Several reactions and separation procedures were modified from those previously reported. The *sn*-glycero-3-phosphate was separated from the adenine nucleotides by a charcoal

treatment [8] followed by precipitation as the barium salt [9,10]. Acylation of *sn*-glycero-3-phosphate was facilitated by the addition of the catalyst *N,N*-dimethyl-4-aminopyridine [11]. Problems of solubility in the reaction between phosphatidic acid and choline [12] were solved by preparing choline as the periodide salt [13]. We have also prepared protonated DMPC by the same procedure and have compared its properties with those of commercially available DMPC.

II. Materials and methods

Diethylketomalonate, methyl iodide-d_3, ethyl cyanoformate, myristic acid, deuterium oxide (99.8 atom% D), *N,N*-dimethyl-4-aminopyridine and toluene-sulfonyl chloride were from Aldrich Chemical Company, Milwaukee, WI; lithium aluminum hydride was from Alfa Ventron, Danvers, MA; lithium aluminum deuteride was from Alfa Ventron, Tridom/Fluka, Happauge, NY, and Stohler Isotope Chemicals, Waltham, MA; Dowex 50-X8 ion exchange resin, ATP (disodium salt), glycerokinase (partially purified), acid-washed activated charcoal, α-glycerophosphate dehydrogenase, phytol, and DMPC were from Sigma Chemical Company, St. Louis, MO; Dowex 1-X8, AG1-X10 and AG1-X8 ion exchange resins were from Bio-Rad Laboratories, Richmond, CA; Unisil silicic acid was from Clarkson Chemical Company, Inc., Williamsport, PA; Celite filter aid and molecular sieves were from Fisher Scientific Company, Pittsburgh, PA; platinum oxide was from Engelhard Minerals and Chemicals Corporation, Newark, NJ; Adsorbosil silicic acid products and methanolic BF_3 were from Applied Science Laboratories, Inc., State College, PA. Other reagents were of analytical grade. *N,N*-dimethyl-4-aminopyridine was recrystallized from tetrahydrofuran (4 ml/g). *p*-Toluenesulfonyl chloride was recrystallized from petroleum ether (approx. 20 ml/g). Solvents were not distilled prior to use, but were dried over 3A molecular sieves when water-free solvents were needed.

Glycerol concentration was measured by a modification of the procedure of Sardesai and Manning [14] as follows: To a dried sample were added 0.2 ml of 0.2 N sulfuric acid and 0.1 ml of 50 mM sodium metaperiodate. After 10 min 0.1 ml of 0.5 M sodium arsenite was added, and 15 min later 0.6 ml of the acetylacetone reagent was added. After a 10 min incubation at 60°C, the absorbance at 415 nm was measured. Mannitol was used as a standard.

The above procedure was used for determining ethanolamine concentrations, except that the sulfuric acid was added after the periodate treatment, immediately before adding the sodium arsenite [15].

Choline was determined by the triiodide method of Appleton et al. [13].

For total phosphate determinations, samples were ashed by a modification of the procedure of Bartlett [16] and phosphate was determined by a scaled-down modification of the method of Chen et al. [17] as follows: Dried samples were dissolved in 0.2 ml 10% sulfuric acid and heated at 200°C for 1 h. Fifty μl of 30%

hydrogen peroxide were added and the samples were heated for 40 more min. After the tubes had cooled to room temperature, 0.48 ml of water was added, followed by 0.5 ml of a freshly prepared reagent containing 0.5% ammonium molybdate and 1% ascorbic acid. After a 20 min incubation at 45°C, absorbance was measured at 820 nm.

sn-Glycero-3-phosphate was assayed by oxidizing it to dihydroxyacetone phosphate with α-glycerophosphate dehydrogenase and measuring the NADH produced [6]. The assay mixture contained 1 μmol NAD, 600 μmol hydrazine hydrochloride (pH 9.5) and 2 units of α-glycerophosphate dehydrogenase in a final volume of 1 ml. After 15 min at 37°C, the absorbance was measured at 340 nm.

The assay for aldehydes and ketones was modified from the method of Beck [18]. Each sample was diluted to 0.4 ml with water and then 0.1 ml of 10 mM 2,4-dinitrophenylhydrazine in 2 N HCl was added. After a 60 min incubation at 38°C, 0.5 ml of 1 N NaOH was added and the samples were allowed to sit for at least 10 min before the absorbance at 540 nm was read. Dihydroxyacetone was used as a standard.

Adsorbosil-5 and Adsorbosil-5-P were used in both analytical and preparative thin layer chromatography (TLC). Zinzadze reagent for detecting phospholipid spots was prepared according to Dittmer and Lester [19]. Dragendorff spray was prepared according to Kates [20].

The following solvent systems were used (numbers represent volume ratios): A, CH_3COCH_3/CH_3OH, 19 : 1; B, $CHCl_3/CH_3OH$, 1 : 3; C, $CHCl_3/CH_3OH$, 1 : 1; D, $CHCl_3/CH_3OH/H_2O$, 10 : 10 : 1; E, $CHCl_3/CH_3OH/H_2O$, 65 : 25 : 4; F, $CHCl_3/CH_3OH$/conc. NH_4OH, 65 : 25 : 5; G, $CHCl_3/CH_3OH$/conc. NH_4OH, 90 : 104 : 11; H, $CHCl_3/CH_3OH/CH_3COOH/H_2O$, 25 : 15 : 4 : 2.

Preparative TLC plates, 1 mm thick, were prepared from an aqueous slurry of Adsorbosil-5-P. They were allowed to dry at room temperature for at least 6 h, then they were activated in a 105°C oven for several hours. Approximately 100 mg of lipid were applied to each preparative TLC plate. Analytical TLC plates (5 cm × 20 cm) were developed in the same tank as the preparative TLC plates to aid in locating the desired phospholipid. Preparative TLC plates were sprayed with a fine water mist, the desired bands were outlined, and the plate was allowed to dry. The outlined bands were scraped off and eluted 3 times with solvent system C (approx. 100 ml total), followed by solvent system D until no additional product appeared in the eluate. Solvents were removed by rotary evaporation at 40–50°C, with the addition of dry benzene and ethanol as needed to prevent foaming and to remove water.

Optical rotation was measured on a Perkin-Elmer model 141 polarimeter in 2 ml cells with a path length of 10 cm.

^{1}H NMR spectra were obtained on a Varian CFT-20 NMR spectrometer operating at 79.54 MHz or a Bruker WH-360 NMR spectrometer operating at 360 MHz. Chemical shifts in ^{1}H NMR spectra are referenced to internal tetramethylsilane (TMS) at 0 ppm in $CDCl_3$ and to internal sodium 3-trimethylsilylpropionate-

2,2,3,3-d_4 (TSP) in D_2O. Phosphorus nuclear magnetic resonance (^{31}P NMR) spectra were obtained using the Varian CFT-20 NMR spectrometer operating at 32.19 MHz.

Microanalyses were performed by Schwarzkopf Microanalytical Laboratory, Inc., Woodside, NY.

Glycerol-d_5 (I). Lithium aluminum deuteride (0.98 g, 23 mmol) was suspended in 35 ml tetrahydrofuran. Diethylketomalonate (1.83 ml, 12 mmol) in 10 ml tetrahydrofuran was added at a rate sufficient to maintain a gentle reflux. The reaction mixture was refluxed for one more hour, and then chilled on ice. Cold water (20 ml) was added with stirring. The crude product was filtered through Whatman No. 50 paper and the aluminum hydroxide residue was washed with 60 ml 0.1 N NaOH. The filtrates were combined, tetrahydrofuran was removed by rotary evaporation at 40°C, and the aqueous phase was washed with ether. Yield, by assay, was 9.0 mmol (75%). The glycerol-d_5 solution was passed through a Dowex 50-X8 cation exchange column in the H^+ form (60 ml, approx. 100 mmol). The appropriate eluate fractions were combined and passed through an AG1-X10 anion exchange column in the OH^- form (14 ml, 28 mmol). Final yield was 8.8 mmol by assay, 73%. No aldehydes or ketones were detected, indicating that their level was less than 0.05% that of glycerol. Samples of glycerol that had not been deionized were phosphorylated by glycerokinase at a somewhat slower rate than deionized glycerol, but no other problems were encountered in separating adenine nucleotides from *sn*-glycero-3-phosphate made from nondeionized glycerol.

sn-*Glycero-3-phosphate-d_5 (II).* To 8.8 mmol glycerol-d_5 in 150 ml water were added 6.22 g ATP (10.6 mmol) and 170 mg magnesium oxide. The pH was adjusted to 9.5–10 with NaOH and 200 units of glycerokinase were added. The reaction was allowed to proceed for 2 h at 45°C with the addition of NaOH as needed to keep the pH above 9. Charcoal (48 g) was added to remove most of the ADP and ATP, and the solution was filtered through Whatman No. 42 paper. The charcoal was rinsed with water and the combined filtrates (230 ml) were filtered through Celite to remove any remaining charcoal. The pH was adjusted to 8.2 with acetic acid, 15 ml 1 M barium acetate were added, and the mixture was chilled for 2 h and then centrifuged for 10 min at 6000 rev./min in a GSA rotor at 0–4°C. The supernatant was mixed with 4 vols. of 95% ethanol, chilled overnight at 4°C, and then centrifuged as before. The pellet was dissolved in water, giving 7.2 mmol *sn*-glycero-3-phosphate-d_5 by assay. A small amount of undissolved material was presumed to be barium salts of ATP and ADP. Sodium carbonate (8.0 mmol) was added, and the barium carbonate formed was removed by centrifugation and washed with water. The supernatants, containing 6.6 mmol *sn*-glycero-3-phosphate-d_5, were passed through a Dowex 50-X8 cation exchange column in the pyridinium form (30 ml, 50 mmol). Water was evaporated and the product was dried by repeated addition and rotary evaporation of dry pyridine. Approximately 25% of the *sn*-glycero-3-phosphate-d_5 was not soluble in dry pyridine, possibly because of incomplete conversion to the pyridinium form. Therefore it was dissolved in water, passed through a fresh

Dowex column, dried with pyridine and stored as a pyridine solution. Yield, by periodate assay, 5.8 mmol (66% from glycerol-d_5).

Myristic acid-d_{27} (III). Myristic acid was perdeuterated by a modification of the procedure of Dinh-Nguyen et al. [21]. The high temperature, high pressure deuterium-hydrogen exchange reaction was performed in a Monel alloy bomb (Parr Instrument Company, Moline, IL). After each exchange reaction, the perdeuterated myristic acid was neutralized, extracted with ether and washed with water prior to the next exchange. Freshly-reduced platinum was used for each exchange reaction. The yield after each exchange reaction was typically 80–85%. Three repetitions of the procedure gave 99.3% deuteration of the methylene and methyl groups as determined for the ^{1}H NMR spectrum of the methyl ester prepared with methanolic BF_3 [22].

Myristic anhydride-d_{54} (IV) was prepared from the perdeuterated acid by coupling with dicyclohexylcarbodiimide [23], and purity was checked by infrared spectroscopy. Yields were typically 85–90%.

1,2-Dimyristoyl-sn-*glycero-3-phosphate-d_{59} (DMPA-d_{59}, V).* To 4.23 g (8.58 mmol) myristic anhydride-d_{54} were added 630 mg (5.0 mmol) *N,N*-dimethyl-4-aminopyridine [11] and 1.15 mmol *sn*-glycero-3-phosphoric acid-d_5 in 50 ml dry pyridine. The reaction was allowed to proceed for 2 h at room temperature. Water (10 ml) was added and the anhydrides were allowed to decompose overnight. Disappearance of a peak in the ^{31}P NMR spectrum at 7.6 ppm upfield from an external 85% H_3PO_4 standard and the appearance of a new peak at –0.9 ppm indicated that the mixed anhydride had been hydrolysed to phosphatidic acid. Solvents were evaporated, and the crude product mixture was dissolved in 10 ml solvent system A and passed through a Unisil silicic acid column (15 g) equilibrated with the same solvent. Fatty acid was eluted with 100 ml solvent system A and the phosphatidic acid was then eluted with solvent system D. Solvents were evaporated and the sample was dried by repeated addition and evaporation of dry pyridine, and then dissolved in dry pyridine. Yield, by phosphate assay, 1.05 mmol (91%). ^{1}H NMR spectroscopy showed that the phosphatidic acid was in the *N,N*-dimethyl-4-aminopyridinium form.

Acylation of glycerophosphate by acid anhydrides under certain conditions has been reported to give cyclic lysophosphatidic acid as a side product [24]. Our attempts to follow the acylation procedures of Lapidot et al. [24] and Gupta et al. [11] led to low yields or numerous side reactions. Instead the catalyst *N,N*-dimethyl-4-aminopyridine was used with pyridine as the solvent, allowing a much shorter reaction time (2 h) than the reaction time of 4 days reported when chloroform was used [11]. In a preliminary experiment a small amount (2–3%) of cyclic lysophosphatidic acid was detected by ^{31}P NMR spectroscopy. The observed chemical shift of the phosphorus resonance in cyclic lysophosphatidic acid (approx. –18 ppm from an external 85% H_3PO_4 standard) is close to the observed chemical shift (–22.3 ppm) of cyclic glycerophosphate prepared according to Lapidot et al. [24] and the reported chemical shift (–20.3 ppm) of 2′,3′-cyclic cytidinemonophos-

phate [25]. Since the ^{31}P NMR spectra of both DMPC and DMPC-d_{72} had no peak in the region expected for 5-membered cyclic phosphates, any cyclic lysophosphatidic acid that was formed during the acylation step was removed by subsequent purification procedures.

Choline-d_{13} (VII). To 0.98 g (23 mmol) lithium aluminum deuteride in 35 ml tetrahydrofuran was added 1.6 ml (16 mmol) ethyl cyanoformate in 10 ml tetrahydrofuran at a rate sufficient to maintain a gentle reflux. The reaction was refluxed for one more hour and cooled to room temperature. Water (20 ml) was added with stirring. The reaction mixture was cooled to room temperature and methyl iodide-d_3 (3.0 ml, 48 mmol) was added. The solution was stirred for 2 h and allowed to stand overnight. Aluminum hydroxide was removed by centrifugation and washed 3 times with 0.1 N NaOH (70 ml total). Tetrahydrofuran was evaporated at 40°C, and residual aluminum hydroxide was removed by centrifugation. Choline-d_{13} was precipitated by the addition of 50 ml potassium triiodide reagent [13]. After 20 min on ice, the supernatant was decanted and the choline-d_{13} precipitate was washed with 25 ml cold water. Traces of tetrahydrofuran interfere with this step. The choline-d_{13} was dried by repeated addition and evaporation of dry pyridine. Yield, by absorbance at 365 nm in 1,2-dichloroethane, 4.6 mmol (29%). Attempts to improve the yield by isolating the ethanolamine-d_4 prior to reaction with methyl iodide-d_3 or by using more methyl iodide-d_3 were unsuccessful. Use of ethyl oxamate instead of ethyl cyanoformate as the starting material also failed to increase the yield.

*1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine-d_{72} (VIII).* DMPA-d_{59}, 1.03 mmol, in the *N,N*-dimethyl- 4-aminopyridinium form was dried by repeated addition and evaporation of dry pyridine, and then dissolved in 10 ml dry pyridine. To this solution were added 2.0 mmol choline-d_{13} periodide in 10 ml dry pyridine. The reaction flask was heated to 70°C and 0.95 g (5 mmol) toluenesulfonyl chloride were added (cf. ref. 12). In preliminary experiments no significant difference was observed when toluenesulfonyl chloride was replaced with triisopropylbenzenesulfonyl chloride (TPS). After 20 min at 70°C, 1 ml water was added and the mixture was allowed to sit overnight. Solvents were removed by evaporation and the sample was dissolved in 15 ml solvent system B and passed through a mixed bed ion exchange resin [25 mmol Dowex 50-X8 (H^+) and 80 mmol Dowex 1.X8 (OH^-) or AG1-X8 (OH^-)] equilibrated with the same solvent. The phosphate-containing fractions were combined and dried by evaporating the solvents at 40°C.

The phosphatidylcholine was purified by preparative TLC in at least two different solvent systems. Since preparations differed in the level of impurities remaining after two TLC purifications, a third preparative TLC step was often necessary. Purification was conveniently followed by analytical TLC on Adsorbosil-5-P developed in solvent system F and visualized with ultraviolet light or by spraying with the Dragendorff or Zinzadze reagents (see above).

Solvent system G generally gave a good separation of the phosphatidylcholine from any remaining colored impurities. Other phosphate-containing lipids were best

separated by solvent system F. In one preparation of DMPC this sequence of two sets of TLC plates gave a colorless product that moved as a single spot, identical with commercially available DMPC, on Adsorbosil-5-P in three different solvent systems (E, F, and H) visualized by 5 different methods (ultraviolet light, iodine vapor, Dragendorff spray, Zinzadze spray, and H_2SO_4 charring). Usually one or more minor colored impurities remained after two preparative TLC purifications. These were removed by preparative TLC in a third solvent system. In one preparation of DMPC-d_{72} an impurity that was detected in the ^{1}H NMR spectrum was removed by preparative TLC in solvent system H.* Yields were generally 25–40% from DMPA.

Optical rotations in solvent system C were as follows. Synthetic DMPC-d_{72}: c = 10.5, 8.6; $[\alpha]_D$ = 7.41, 7.37; $[M]_D$ = 56.9, 56.6. Synthetic DMPC: c = 3.9, 3.0; $[\alpha]_D$ = 7.53, 6.86; $[M]_D$ = 52.5, 47.8. Commercially available DMPC; c = 5.0, 4.35; $[\alpha]_D$ = 7.60, 7.82; $[M]_D$ = 52.9, 54.4. These values agree well with the literature values [12,26] of $[\alpha]_D$ = 7.0, $[M]_D$ = 48.7 (c = 3.9). As a further criterion for enantiomeric purity, both DMPC and DMPC-d_{72} were completely hydrolysed to the corresponding lysophosphatidylcholines when subjected to phospholipase A_2 hydrolysis under the conditions given by Kates [27]. Racemic DMPC, prepared from racemic α-glycerophosphate by the reaction sequence shown in Scheme 1, was only partly hydrolysed.

For elemental analysis DMPC was precipitated from $CHCl_3$ solution (15 mg in approx. 0.2 ml) by the addition of 10 ml cold dry acetone and then dried under high vacuum at 70°C for 12 h. Found C, 60.12; H, 10.79; N, 1.84; P, 4.04. Calc. for DMPC · H_2O ($C_{36}H_{74}NO_9P$): C, 62.13; H, 10.72; N, 2.01; P, 4.45. Calc. for DMPC · $2H_2O$ ($C_{36}H_{76}NO_{10}P$): C, 60.56; H, 10.73; N, 1.96; P, 4.34.

III. Results and discussion

The ^{1}H NMR spectrum of DMPC-d_{72} provides an excellent criterion for its chemical and isotopic purity. The 360 MHz ^{1}H NMR spectra of DMPC and DMPC-d_{72}, both synthesized by the same reaction sequence (Scheme 1), are shown in Fig. 1. All peaks observed in the spectrum of DMPC-d_{72} (Fig. 1a) are readily assigned by comparison with previously reported assignments for dipalmitoyl phosphatidylcholine in $CDCl_3$ [28]. Note that the spin-spin splitting pattern of DMPC-d_{72} (Fig. 1a) is different from that of DMPC (Fig. 1b), as is expected from the difference in spin and magnetogyric ratio between deuterons and protons.

The levels of residual protons in DMPC-d_{72}, estimated from the ^{1}H NMR spectra of DMPC-d_{72} and the methyl ester of myristic acid-d_{27}, are as follows: acyl methyl and methylene, 0.7 ± 0.1%; α-methylene, 7 ± 2%; choline methyl, 0.35 ± 0.15%;

*With perdeuterated compounds such as DMPC-d_{72}, ^{1}H NMR is a very sensitive method for detecting impurities. Minute traces of protonated impurities, often not detectable by other means, will interfere with ^{1}H NMR studies and must therefore be removed.

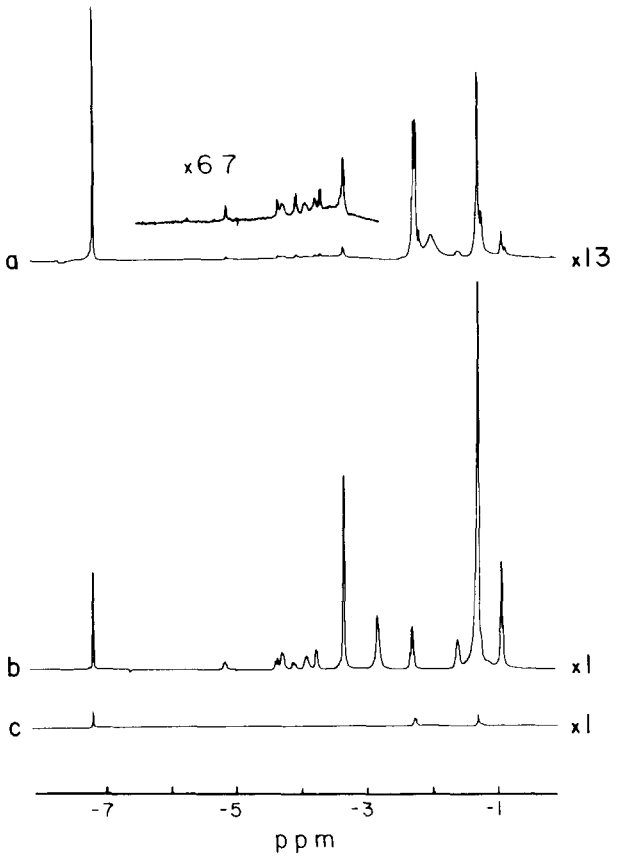


Fig. 1. 360 MHz ^{1}H NMR spectra of DMPC and DMPC-d_{72} at 24°C, 10 mg/ml: (a) DMPC-d_{72} in (99.96 atom % D) $CDCl_3$, 1000 transients, with an expanded vertical scale. The peaks at −2.00 ppm and −3.72 ppm are from traces of acetic acid and ethanol, respectively; (b) DMPC in (99.8 atom % D) $CDCl_3$, 24 transients. Peak assignments were made according to Birdsall et al. [28]: −0.89 ppm, acyl CH_3: −1.26 ppm, acyl $(CH_2)_n$; −1.59 ppm, βCH_2; −2.30 ppm, αCH_2; −2.82 ppm, residual H_2O; −3.34 ppm, choline $N(CH_3)_3$; −3.78 ppm, choline CH_2N; −3.93 ppm, glycerol CH_2OP; −4.12 and −4.38 ppm, glycerol CH_2OCO; −4.29 ppm, choline $POCH_2$; −5.19 ppm, glycerol HCOCO; −7.24 ppm, $CHCl_3$. The acyl $(CH_2)_n$ peak was cut off at half-height; (c) DMPC-d_{72} as in (a), but plotted with the same vertical scale as in (b).

head group methine and methylene, 0.6 ± 0.2%. The different levels of residual protons reflect the different methods of incorporating deuterium into each position. The relatively high level of α-methylene protons in phosphatidylcholine with perdeuterated acyl chains has been observed previously [2] in DMPC-d_{54} prepared by the acylation of *sn*-glycero-3-phosphocholine with myristic anhydride-d_{54}. A similarly high level of α-methylene protons was also observed in DMPA-d_{59} but, in contrast to the report of Petersen et al. [29], the α-methylene position was as

extensively deuterated as the rest of the acyl chain in both myristic acid-d_{27} and its methyl ester. Thus it appears that protons are incorporated into the α position during or shortly after the acylation and are not present in the original perdeuterated acid.

The reduction in intensity of the proton resonances of DMPC-d_{72} compared to DMPC can best be seen by comparing Figs. 1b (DMPC) and 1c (DMPC-d_{72}), where the spectra are plotted with the same vertical scale.

The ^{31}P NMR spectra of DMPC and DMPC-d_{72} dispersed in detergent [30] are shown in Fig. 2. The chemical shifts measured relative to external 85% H_3PO_4 are +0.65 and +0.56 ppm for DMPC and DMPC-d_{72} respectively, in good agreement with the value of +0.65 ± 0.08 ppm reported for other phosphatidylcholines [30]. In the absence of broadband proton decoupling the DMPC spectrum reveals a

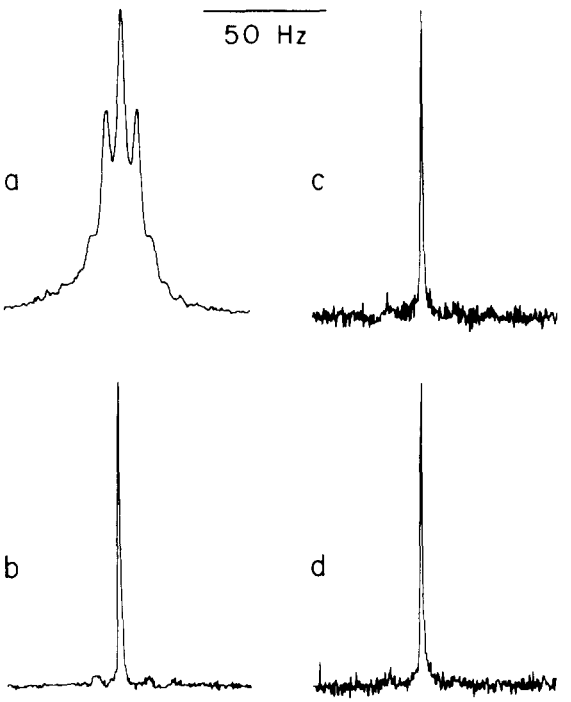


Fig. 2. ^{31}P NMR spectra of DMPC and DMPC-d_{72} at 32°C. Each sample contained 0.8 ml of 10% (w/v) potassium deoxycholate (pH 8) plus 0.05 ml of 0.5 M ethylenediamine tetraacetate (EDTA), sodium salt (pH 8) and approx. 50 mg phospholipid: (a) DMPC, 6000 transients without broadband proton decoupling, with a sensitivity enhancement giving 0.3 Hz line broadening. Acquisition time 8 s; (b) DMPC, 100 transients with decoupling. Acquisition time 4 s; (c) DMPC-d_{72}, 100 transients without decoupling. Acquisition time 4 s; (d) DMPC-d_{72}, 100 transients with decoupling. Acquisition time 4 s.

quintet (Fig. 2a) with a $^{31}P-^{1}H$ coupling constant of 6.4 ± 0.2 Hz, in agreement with the reported value of 6.1 Hz [31]. Decoupling causes the quintet to collapse to a singlet (Fig. 2b). In contrast, broadband proton decoupling has no observable effect on the singlet in the spectrum of DMPC-d_{72} (Figs. 2c and 2d). The observed

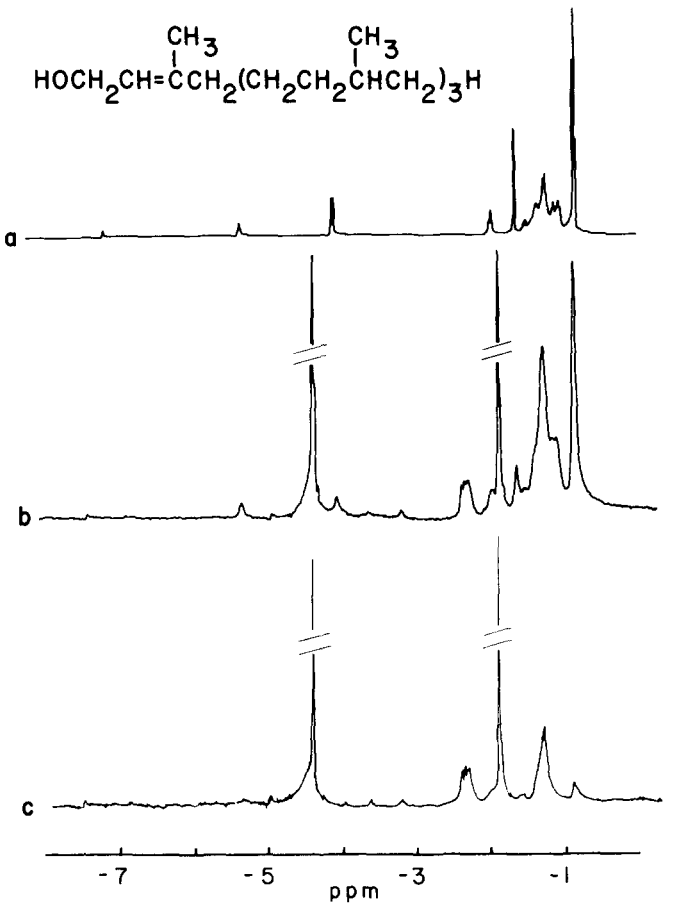


Fig. 3. 360 MHz ^{1}H NMR spectra: (a) Phytol, 10 mg/ml in $CDCl_3$ at 24°C, 24 transients with an acquisition time of 2 s and a sensitivity enhancement giving 0.5 Hz line broadening. Assignment of peaks: −0.86 ppm, isoprenoid CH_3; −1.05 to −1.60 ppm, isoprenoid CH_2 and CH; −1.68 ppm, $=C-CH_3$; −2.00 ppm, $=C-CH_2-CH_2$; −4.15 ppm, CH_2OH; −5.40 ppm, CH=C; −7.24 ppm, $CHCl_3$; (b) Phytol (5 wt%, 13 mol%) in DMPC-d_{72} vesicles (10 mg/ml), 50°C, 400 transients with an acquisition time of 1 s and a sensitivity enhancement giving 1 Hz line broadening; (c) Sonicated DMPC-d_{72} vesicles, 10 mg/ml in (99.96 atom% D) D_2O, 50°C, 400 transients with an acquisition time of 1 s and a sensitivity enhancement giving 2 Hz line broadening. The large peaks at −4.40 ppm and −1.89 ppm are due to residual HDO and a trace of residual acetate, respectively.

reduction of the spin-spin splitting of the ^{31}P resonance and the expected reduction in the dipolar contribution to the ^{31}P linewidth in phospholipids with perdeuterated headgroups may prove to be useful in studies involving anisotropic, restricted, or slow molecular motion.

^{31}P spin-lattice relaxation times (T_1) were measured by the inversion-recovery ($180° - \tau - 90°$) method on the same detergent-dispersed samples after they were deoxygenated by three cycles of vortexing under an argon atmosphere. The observed T_1 of 5.2 ± 0.2 s for DMPC-d_{72} is significantly longer than the observed T_1 of 2.7 ± 0.2 s for DMPC, as is expected from the lower magnetogyric ratio of deuterons compared to protons.

When DMPC-d_{72} is sonicated into bilayer vesicles together with small molecules of biological interest, a number of resonances from the incorporated compounds are visible in the ^1H NMR spectrum [32]. The 360 MHz ^1H NMR spectrum of phytol in DMPC-d_{72} vesicles at 50°C is shown in Fig. 3b. Several peaks can be assigned to specific phytol protons by comparison with the spectrum of phytol in $CDCl_3$ (Fig. 3a) and the spectrum of DMPC-d_{72} vesicles (Fig. 3c). This confirms the expectation that perdeuteration of the head group of phosphatidylcholines would greatly attenuate the background phospholipid signal in the ^1H NMR spectrum.

The synthetic procedure presented here, when used in conjunction with previously reported reactions for selective hydrogen incorporation [3], provides methods for preparing phosphatidylcholines with protons in one or a few selected positions in the glycerophosphocholine head group. Such compounds should be especially useful in studies with shift reagents, since overlap of shifted peaks often prevents accurate determination of peak positions (P.R. Meers, pers. comm.). In addition, this procedure can easily be adapted for the preparation of other perdeuterated phospholipids since a number of different alcohols have been reacted with phosphatidic acids to give the corresponding phospholipids [33–35].

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