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PRODUCTION OF SPECIFICALLY DEUTERIUM LABELLED DIOLEOYL PHOSPHOLIPID SPECIES IN GRAM QUANTITIES: A CONVENIENT SYNTHESIS OF [C_{11}-$^2H_2$]OLEIC ACID

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A relatively straightforward large scale (gram quantity) synthesis of oleic acid specifically labelled with deuterium (^2H) at the 11 position ([C_{11}-$^2H_2$]oleic acid) is described. This synthesis is a modified version of the procedure of Tucker et al. (J. Labelled Compd., 7(1971)137) and involves the coupling of [C_2-$^2H_2$] nonanal with methyl 9-chlorononanoate via a Wittig reaction. The ^2H-labelled nonanal is produced in greater than 75% yield. The coupling reaction results in a 60–70% yield (based on the aldehyde) which contains 95% of the cis (oleic acid) isomer. The subsequent synthesis of 1,2-[C_{11}-$^2H_2$] dioleoyl-*sn*-glycero-3-phosphorylcholine ([C_{11}-$^2H_2$]DOPC) yields a compound which exhibits a convenient ^2H-NMR quadrupolar splitting ($\sim$ 6 KHz at 20°C). Similarly labelled dioleoyl species of phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidic acid (PA) are easily derived from the [C_{11}-$^2H_2$]DOPC. The dioleoyl phosphatidylethanolamine ([C_{11}-$^2H_2$]DOPE) shows similar bilayer to hexagonal (H_{II}) transition characteristics as unlabelled DOPE, and this transition can be conveniently monitored by ^2H-NMR techniques.

Keywords: oleic acid; deuterium label; dioleoyl phosphatidylcholine; ^2H-NMR

Introduction

^2H-NMR has proven a most useful technique for the elucidation of lipid structure and motion in model and biological membranes (for review see Ref. 16). However, relatively few laboratories have employed this technique, which is due primarily to the rather tedious nature of the synthesis of suitably ^2H-labelled lipids. Further, most specific ^2H labelling techniques have been restricted to saturated phospholipid species, whose behaviour may not be representative of the unsaturated varieties found in biological membranes [2]. With this background in mind we have developed a convenient, large scale and rapid synthesis of [C_{11}-$^2H_2$]oleic acid from which the corresponding 1,2-[C_{11}-$^2H_2$]DOPC may be obtained. From this compound similarly labelled species of [C_{11}-$^2H_2$]DOPE, dioleoylphosphatidylserine ([C_{11}-$^2H_2$]DOPS), dioleoylphosphatidylglycerol ([C_{11}-$^2H_2$]DOPG) and dioleoyl phosphatidic acid ([C_{11}-$^2H_2$]DOPA) may be easily derived.

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Materials

Methyl hydrogen nonanedioate and nonanal were obtained from Aldrich. Deuterium oxide (99.8%) and triphenylphosphine were obtained from Sigma. Sodium ethoxide was made according to standard procedures [3] and all other reagents were of reagent grade. It is of crucial importance that all solvents employed be absolutely dry. Thus tetrahydrofuran was dried by refluxing with CaH_2 (10 g/l) for 2 h and then distilled at 65–66°C onto molecular sieve type 4A. Similarly, pyridine was refluxed over KOH (20 g/kg) for 2 h and then distilled onto molecular sieve at 115°C. Methylethylketone (MEK) was dried by refluxing with $CaSO_4$ (20 g/kg) for 2 h and then distilled at 79–80°C. Finally, dimethylformamide was dried by allowing it to stand over molecular sieve and subsequent vacuum distillation. All solvents were prepared immediately before use and stored over molecular sieve. Gas chromatography column supports were obtained from Supelco Ltd.

Methods

An outline of the synthesis procedure employed is given in Fig. 1. Routine fatty acid analysis was carried out on a Hewlett Packard 7610A Hi-efficiency gas chromatograph using a glass column (1.8 m × 0.32 cm) packed with GP 10% DEGS-PS on 80/100 Supelcoport (column temperature programmed from 170°C to 200°C). Determination of the *cis* to *trans* ratio was made employing a stainless steel column (6.1 m × 0.4 cm) packed with GP 15% OV-275 on 100/120 chromosorb P AW-DMCS (column temperature 220°C).

Reduction of methyl hydrogen nonanedioate

Methyl hydrogen nonanedioate (compound I, Fig. 1) (50 g, 250 mmol) was dissolved in 50 ml tetrahydrofuran (THF) and cooled to −18°C in an ice ethanol bath. The flask was flushed with a stream of dry nitrogen. Subsequently 250 ml BH_3-THF (250 mmol) [4] was added slowly (12 ml/min) to the THF solution in a fume hood. Hydrogen gas is generated on addition of the borane. The reaction mixture was well stirred and allowed to warm up to room temperature overnight. Then 150 ml ice-cold H_2O was added, followed by the careful addition of 60 g potassium carbonate. The THF phase (top phase) was separated and the aqueous phase was then extracted twice with 100 ml of ether. The ether extracts were combined, washed with 200 ml of a saturated NaCl solution and dried over $MgSO_4$. The ether was then evaporated by rotary evaporation and 47 g of crude product obtained. Subsequent high vacuum distillation yielded approx. 41 g (95% yield) of methyl-9-hydroxynonanoate

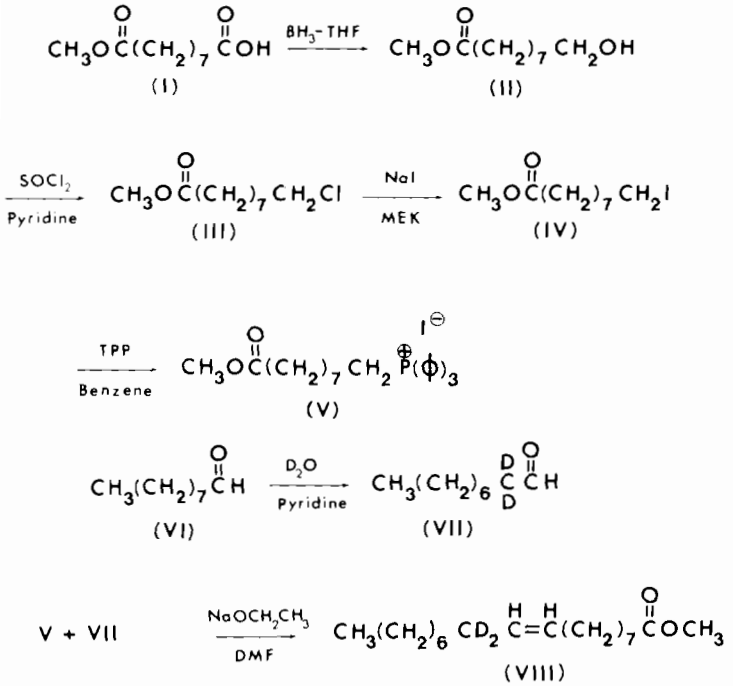

Fig. 1. Steps in the synthesis of [C_{11}-$^2H_2$]methyl oleate. (I) methyl hydrogen nonanedioate, (II) methyl 9-hydroxynonanoate, (III) methyl 9-chlorononanoate, (IV) methyl 9-iodononanoate, (V) 9-carbomethoxynonyltriphenylphosphonium iodide, (VI) nonanal, (VII) [2-$^2H_2$]-nonanal and (VII) [C_{11}-$^2H_2$]methyl oleate. $\phi = (C_6H_5)$.

(compound II of Fig. 1) which is a clear oil at 20°C (boiling point 125°C at 0.5 mmHg). A ^{1}H-NMR characterization of the product dissolved in $CDCl_3$ with TMS as reference provided the following chemical shift data: δ 1.0–2.0 [*m*, 12, $-(CH_2)_6-$]; 2.3 [*t*, 2, $-CH_2(C{=}O)O$]; 2.7 [S^{br}, 1, –OH]; 3.64 [*t*, 2, $-CH_2-OH$]; 3.67 [*s*, 3, $-OCH_3$].

Chlorination of methyl 9-hydroxynonanoate

Thirty-six grams (300 mmol) of $SOCl_2$ was added dropwise, from a separating funnel, to a solution of 45 g of methyl 9-hydroxynonanoate (compound II) in 60 ml dry THF. This solution was stirred for 5 min at 20°C. Then 24 g (300 mmol) of pyridine was carefully added (dropwise) to the THF solution and the reaction mixture was stirred for an additional 10 min. (Note: There is a substantial release of SO_2 and HCl gas on addition of pyridine.) Subsequently 60 ml of H_2O was slowly added (dropwise) and the reaction mixture was extracted two times with 100 ml of ether. The combined ether extracts were carefully

neutralized (pH ~ 6.0) by addition of 3 N NaOH. The ether was then dried over $MgSO_4$ and removed by rotary evaporation, giving 46 g of crude product. High vacuum distillation (0.5 mmHg) yielded a yellowish liquid (approx. 43 g, 87% yield) with a boiling point of 100°C at 0.5 mmHg. This material was identified as methyl 9-chlorononanoate (compound IV, Fig. 1; literature boiling point (1) of 113–114°C at 1 mmHg). A ^{1}H-NMR characterization of this compound dissolved in $CDCl_3$, reference TMS gave the following data: δ 1.0–2.0 [*m*, 12, $-(CH_2)_6-$]; 2.3 [*t*, 2, $-CH_2(C=O)O$]; 3.52 [*t*, 2, $-CH_2-Cl$]; 3.67 [*s*, 3, $-OCH_3$].

^{2}H-labelling of nonanal

Forty grams (288 mmol) of freshly distilled nonanal (compound VI of Fig. 1) was added to 250 ml of dry pyridine and 61 gm (3.1 mol) of D_2O. This solution was gently heated (100°C) under a stream of N_2 for 15 h. Subsequently the reaction mixture was cooled to 20°C and 800 ml ice-cold H_2O added. This mixture was extracted twice with 100 ml hexane. The combined hexane extract was washed successively with 200 ml H_2O, 200 ml 5% HCl and 200 ml H_2O, and then dried over molecular sieve (2 h). The solvent was then removed by rotary evaporation. This process was repeated one more time. A crude yield of 39 g was obtained. Subsequent high vacuum (0.5 mmHg) distillation resulted in approx. 30 g of a colorless liquid (73% yield, b.p. 48°C at 0.5 mmHg) which was identified as [2-$^2H_2$] nonanal (compound VII of Fig. 1). ^{1}H-NMR studies indicated the absence of both α protons, indicating greater than 98% ^{2}H-labelling. A ^{1}H characterization of the product dissolved in $CDCl_3$, reference TMS was: δ 0.9 [*t*, 3, CH_3-]; 1.0–2.0 [*m*, 12, $-(CH_2)_6-$]; 9.7 [*s*, 1, CHO].

Wittig reaction

This procedure follows that due to Tucker et al. [1] with minor modifications. The iodide of methyl 9-chlorononanoate was first prepared by heating 13.5 g (90 mmol) of NaI in 70 ml dry MEK at 80°C for 30 min. Methyl 9-chlorononanoate (15 g, 72.5 mmol) was then added and the reaction mixture was refluxed for 10 h. The reaction mixture was then cooled and the MEK removed by rotary evaporation. Benzene (150 ml) was then added to the resulting slurry and this was washed with 100 ml H_2O, 100 ml 5% sodium thiosulphate and again with H_2O. The benzene solution was then dried over molecular sieve (1 h) and transferred to a 500 ml 3-neck flask. Triphenylphosphine (TPP) (23 g, 90 mmol) was added and the solution refluxed vigorously under a stream of N_2 for 10 h. The reaction mixture was cooled to 20°C and the product precipitated by addition of 300 ml diethyl ether. The supernatant was discarded and the viscous precipitate was washed three times with 200 ml ether by decantation. Residual ether was removed

under high vacuum (24 h), giving 34 g of 9-carbomethoxy nonyl triphenyl-phosphonium iodide (compound V of Fig. 1) in 84% yield. The phosphonium salt was then dissolved in 90 ml dry dimethylformamide (DMF) and stored over molecular sieve at 4°C until use.

A solution of 17.4 g (31 mmol) of the phosphonium salt in DMF was cooled to 0°C under a stream of N_2. Freshly prepared sodium ethoxide (2.0 g, 30 mmol) was added and the mixture was stirred for 30 min, resulting in the appearance of the orange-red ylid complex. Subsequently 3.9 g (27 mmol) of [2-$^2H_2$]nonanal (VII) was added. The reaction mixture was then stirred for 6 h at 0°C under a stream of N_2. Subsequently, 150 ml ice-cold H_2O was added and the mixture extracted twice with 100 ml hexane. The combined extracts were washed three times with 100 ml H_2O, then dried over $MgSO_4$. The hexane was evaporated under reduced pressure giving 7.8 g of slightly yellow oil as the crude product. Thin layer silica gel chromatography indicated that the major component was methyl oleate (compound VIII of Fig. 1). The ester was saponified by adding 100 ml 1 N KOH in 95% methanol to the crude methyl oleate and refluxing for 5 min. The solution was then cooled to 20°C and 500 ml H_2O added. The pH of the resulting mixture was lowered to 1.0 by addition of 6 N HCl. The free acid was extracted twice with 100 ml hexanes, the combined extracts were dried over $MgSO_4$ and the solvent removed under reduced pressure. This gave a crude yield of 7.4 g of yellow oil, which was purified employing a Waters Prep 500 LC apparatus using chloroform/methanol/ammonia (58%) (50 : 7.5 : 1) as the eluting solvent. Gas chromatography indicated that the product was greater than 99% 18 : 1, and the contamination with elaidic acid ($18:1_t$) as determined by high resolution gas chromatography was ~6%. The yield of Me-oleate was 4.6 g or 60%, based on the aldehyde. The yields varied from ~60–70%. The incorporation of deuterium (at the C_{11} position) into Me-oleate was >80% as determined by mass spectrometry.

Synthesis of ^2H-labelled phospholipids

The oleic acid produced by the preceding series of reactions was used to synthesize DOPC. This involves two well characterized steps. First, glycerol phosphorylcholine (GPC) is prepared from egg yolk PC [6] and subsequently the GPC is reacylated with oleic acid activated with 1, 1'-carbonyldiimidizole [7]. The reacylation is approx. 60% efficient with respect to oleic acid. The purified [C_{11}-^2H]DOPC can then be employed to obtain corresponding species of PE, PG, PA and PS employing the headgroup exchange capacity of phospholipase D [5]. DOPC and DOPE were purified as described by Tilcock and Cullis [8], DOPS as indicated by Hope and Cullis [9] whereas DOPG and DOPA were isolated according to Farren and Cullis [10] and Farren et al. [11], respectively.

Discussion

The object of the synthesis outlined here was to obtain ^{2}H-labelled varieties of dioleoyl species of phospholipid suitable for ^{2}H-NMR investigations of the structural and motional properties of lipids in membranes. It is therefore appropriate to illustrate the ^{2}H-NMR characteristics of the compounds obtained. As shown in Fig. 2, the [C_{11}-^{2}H]DOPE exhibits a bilayer to hexagonal H_{II} phase transition as detected by ^{31}P-NMR as the temperature is increased through ~5°C, in agreement with previous work for unlabelled DOPE [14]. This behaviour is paralleled by a reduction in the quadrupolar splitting ΔQ (measured as the frequency separation between the two peaks of the quadrupolar powder pattern) from 10 to 3 KHz. As has been well discussed elsewhere [15] such a reduction of ΔQ is fully consistent with the bilayer to H_{II} transition observed by ^{31}P-NMR. Representative

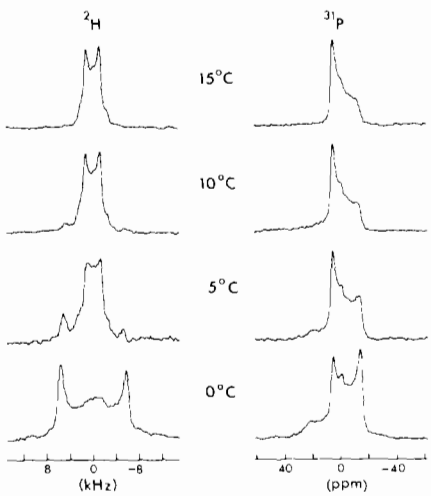

Fig. 2. ^{31}P- and ^{2}H-NMR spectra as a function of temperature of fully hydrated [C_{11}-$^2H_2$]DOPE. The ^{31}P-NMR spectra were obtained on a Bruker WP 200 spectrometer operating at 81.0 MHz for ^{31}P in the presence of proton decoupling. Free induction decays were accumulated from up to 1000 transients by employing a 15-μs 90° radio-frequency pulse, 20 KHz sweep width, and a 0.8-s interpulse delay, in the presence of broad band proton decoupling. An exponential multiplication corresponding to a 50-Hz line broadening was applied to the free induction decay prior to Fourier transformation. The ^{2}H-NMR spectra were obtained by using a Bruker WP 200 spectrometer operating at 30.7 MHz. Free induction decays were accumulated for up to 100 000 transients by employing the quadrupolar solid echo pulse sequence [16], employing a 200-μs interpulse delay and a 15-μs 90° radio-frequency pulse, 100 KHz sweep width, and a 0.01-s inter-pulsetrain delay. An exponential multiplication corresponding to a 100-Hz line broadening was applied prior to Fourier transformation. All spectra were recorded at 20°C. The lipid samples (40 μmol phospholipid) were dispersed in 1 ml 10 mM Tris–HCl (pH = 7.0), 100 mM NaCl employing deuterium depleted water.

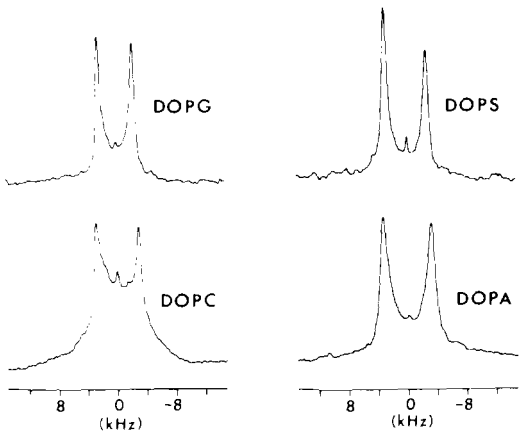

Fig. 3. 30.7 MHz ^{2}H-NMR spectra of fully hydrated [C_{11}-$^2H_2$]DOPC, [C_{11}-$^2H_2$]DOPG, [C_{11}-$^2H_2$]DOPA, [C_{11}-$^2H_2$]DOPS. Sample preparation and the conditions for obtaining the ^{2}H-NMR spectra are identical to those described in the legend of Fig. 2.

^{2}H-NMR spectra for [C_{11}-$^2H_2$]DOPC, DOPG, DOPS and DOPA are illustrated in Fig. 3. It may be noted that the quadrupolar splittings observed for these liquid crystalline C_{11}-$^2H_2$-labelled phospholipids are relatively convenient in that the signals are not excessively broad. This leads to good signal to noise characteristics, where 30 μmol of labelled phospholipid gives rise to an adequate ^{2}H-NMR spectra for accumulation times of 15 min or less.

With regard to the synthesis itself, the procedure detailed here allows the rapid production of gram quantities of C_{11}-$^2H_2$-labelled dioleoyl phospholipids. In our experience 10 g of [C_{11}-^{2}H]DOPC can be obtained within 3–4 weeks. These compounds are extremely useful, both for investigating order and motion in the hydrocarbon region as well as for indicating the polymorphic phase structure adopted. This potential has already been demonstrated for DOPA systems [11] where ^{31}P-NMR assignments of phase structure are not necessarily unambiguous. A more important example concerns mixed lipid systems, in which the behaviour of individual (labelled) components can be monitored [12, 13], providing information that is not available through alternative techniques for determining lipid structure such as freeze-fracture, X-ray or ^{31}P-NMR.

Acknowledgements

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