Phospholipase-A_2-mediated stereoselective synthesis of (*R*)-1-*O*-alkylglycero-3-phosphate and alkyl-acyl analogues: application for synthesis of radiolabelled biosynthetic precursors of cell surface glycoconjugates of *Leishmania donovani*

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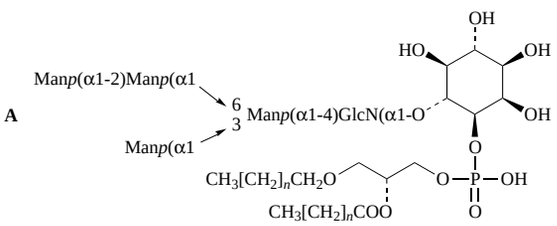
Stereoselective syntheses of (*R*)-1-*O*-alkylglycero-3-phosphate and alkyl-acyl analogues have been achieved using glycerol as starting material by an efficient application of Phospholipase-A_2 enzyme from *Naja mocambique mocambique*. **This synthetic strategy allows a high yielding preparation of radiolabelled [^{14}C] and chirally pure biosynthetic precursors of GIPLs and LPG cell surface molecules of promastigote and amastigote forms of the protozoan parasite** *Leishmania donovani*.

Introduction

The protozoan parasite *Leishmania donovani* causes visceral leishmaniasis (kala-azar)—a fatal disease widespread in India, China and other countries.[1] The parasite alternates its life cycle between sandfly midgut and human blood as promastigotes, and in macrophages as amastigotes. Recently a number of abundant and developmentally regulated non-protein glycosylated phosphatidylinositol (GPI) cell-surface molecules, *viz*. lipophosphoglycans (LPGs) and glycoinositol phospholipids (GIPLs), have been isolated from *Leishmania* and *Trypanosoma* species.[2] The LPGs of *Leishmania* contain an unusual *lyso*-alkylphosphatidylinositol (*lyso*-alkyl-PI) anchor that mimics GPI in mammalian systems. The LPG/GIPLs play a significant role in parasite virulence, transmission and intracellular survival in macrophages and its protection from host immune systems by inhibition of protein kinase C, interleukin-1 and complement-mediated lysis.[2] While Type-1 GIPLs (M2 and M3) are the major surface glycoconjugates of amastigotes, Hybrid-type GIPLs (iM3 and iM4), Type-2 GIPL (iM2), and LPG are predominant on promastigotes of *L*. *donovani*, LPG biosynthesis is reported to be down-regulated in amastigotes. Structures **A** and **B** schematically depict the structures of a major GIPL (iM4) and LPG, respectively, of *L*. *donovani*. The lipid anchor of GIPLs is (*R*)-1-*O*-alkyl$_{(18:0)}$-2-*O*-acyl$_{(16:0/18:0)}$-PI and that of LPG is of (*R*)-1-*O*-alkyl$_{(22:0/24:0/26:0)}$-PI type; these structural features are unique to *L*. *donovani* and related species, and differ from mammalian protein GPI anchors in containing a *lyso*-alkyl-PI moiety instead of a diacyl-PI. This is biologically significant in that it increases the resistance of GIPLs/LPG to enzymic/chemical degradation in vector sandfly midgut or host macrophages. The dependence of the parasite on GIPLs/LPG for survival and infectivity renders their biosynthetic pathway a potential target for development of chemotherapeutic agents, mechanism-based inhibitors and vaccine design.[2]

The biosynthesis of GIPLs/LPG of *L*. *donovani* has not been elucidated in detail and none of the enzymes have been purified to date; however, significant results have been reported, including the development of an efficient cell-free membrane preparation,[3] incorporation of the nucleotide sugar donors UDP-[^{3}H]galactose† and GDP-[^{14}C]mannose,† and the isolation of *LPG1* and *LPG2* genes[4] by functional genetic complementation from mutants defective in LPG biosynthesis. The chemical synthesis of various parts of LPG and the GPI anchor has been reported[5] by Brimacombe and Ferguson, and by Fraser-Reid.

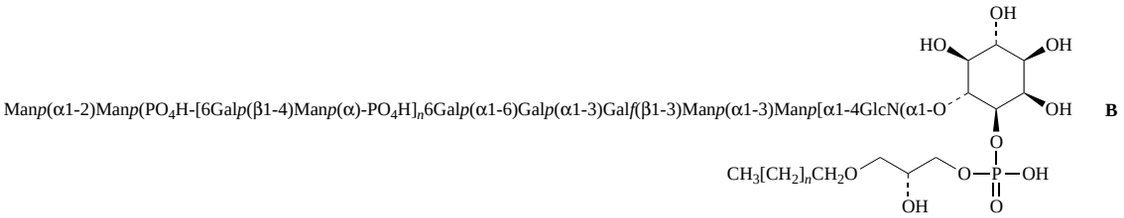


GIPL (iM$_4$) of *L*. *donovani*

In order to identify the distinct substrates and enzymes of GIPLs/LPG biosynthesis, we have initiated efforts towards investigating the mechanism of *lyso*-alkyl-PI assembly in promastigotes. From information from other eukaryotes, it has been postulated[2] that this biosynthesis is initiated by C-1 acylation of dihydroxyacetone phosphate (DHAP) by DHAP-acyltransferase, the acyl group is then replaced by an alkyl group by action of an unusual alkyl-DHAP-synthase where a long-chain alcohol nucleophile replaces the acyl moiety by a hitherto unknown mechanism. This is followed by 1,4-dihydronicotinamide adenine dinucleotide phosphate (NADPH)-mediated reduction of the carbonyl group and C-2 *sn*-acylation leading to a 1-*O*-alkyl-2-*O*-acylphosphatidic acid which is then transferred to *myo*-inositol to form 1-*O*-alkyl-2-*O*-acyl-PI. This pathway has not been established in *Leishmania* species, and other, quite different, routes for ether lipid assembly for GIPLs/LPG may exist in the parasite. The mechanism of action of the alkyl-DHAP-synthase, identification of substrates, biosynthetic sites (endoplasmic reticulum or Golgi apparatus), timing of *sn*-2 deacylation and fatty acid remodelling are of particular interest. In our biosynthetic studies we required enantiomerically pure radio- and stable-isotope labelled (*R*)-1-*O*-alkylphosphatidic acid, (*R*)-1-*O*-alkyl-2-*O*-acylphosphatidic acid and *lyso*-1-*O*-alkylphosphatidylinositol as biosynthetic precursors and substrates specifically labelled on the glycerol backbone.

The importance of phospholipids in biological processes and the use of their analogues as enzyme inhibitors and drug candidates has stimulated development of synthetic methods[6]

† UDP = uridine 5′-diphospho-, GDP = guanosine 5′-diphospho-.

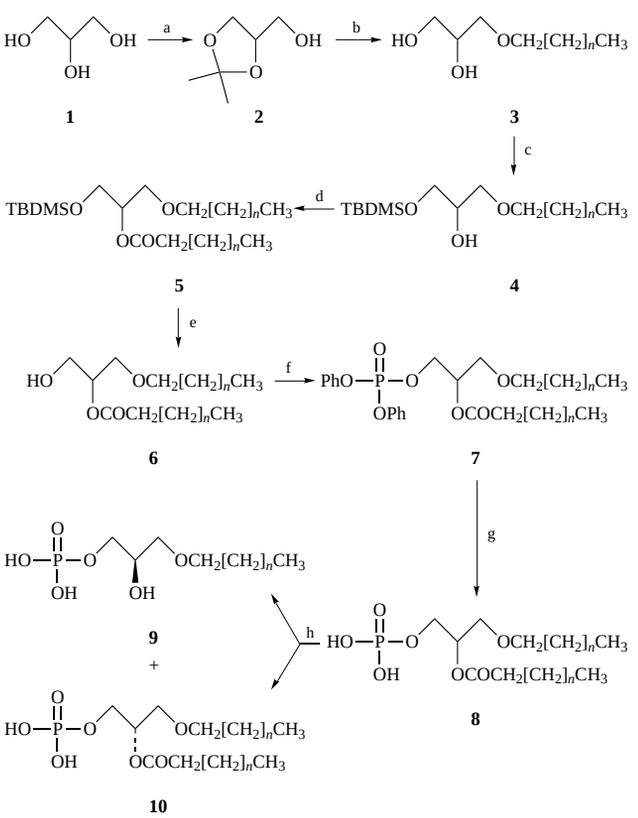


Generic structure of LPG of *Leishmania*

for structurally defined and chirally pure phospholipids. The reported methods for optically pure diacyl-, alkylacyl- and dialkyl-phospholipids use chiral starting materials such as (*R*)- and (*S*)-glycidols,[6] 2,3-*O*-isopropylidene-*sn*-glycerol[7] and 1,2-*O*-isopropylidene-*sn*-glycerol[8] (available from D-mannitol and L-arabinose respectively), epichlorohydrin,[9] substituted glycerols,[10] and tartaric acid.[11] *L. donovani* cell culture has also been used for the preparation of ethanolamine plasmalogen.[12] However, these excellent methods were not suitable for the synthesis of our required labelled compounds due to the commercial non-availability of labelled starting materials. Keeping in view the limited choice of starting material available, we designed a new general synthetic strategy starting from glycerol which is commercially available in isotopomeric forms [^{14}C, ^{3}H, ^{13}C] labelled at various positions. This scheme was also aimed at non-natural enantiomers useful in biosynthetic inhibition, mechanistic studies on phospholipase A_2 and phospholipase C and phosphatidylinositol-specific phospholipase C enzymes of the signal transduction pathway[13] and for generation of genetic mutants of the parasite strains.[4]

Results and discussion

Keeping in view the synthesis of (*R*)-1-*O*-alkyl-2-*O*-acyl[^{14}C]-glycero-3-phosphate and (*R*)-1-*O*-alkyl[^{14}C]glycero-3-phosphate labelled on the glycerol backbone from commercially available labelled [^{14}C]glycerol, the unlabelled synthesis was accordingly carried out from unlabelled glycerol (Scheme 1) as starting material. In the first step glycerol **1** was converted to solketal (1,2-*O*-isopropylidene-*rac*-glycerol)[14] **2** and this was purified by column chromatography instead of the previously reported distillation due to its suitability for the forthcoming labelled synthesis. The primary alcohol group of compound **2** was alkylated with 1-bromooctadecane/sodium hydride followed by deketalization in dil. HCl to give 1-*O*-octadecyl-*rac*-glycerol[15] **3**, which was crystallized from diethyl ether–methanol. The protection of the primary alcohol of monoether **3** with *tert*-butyldimethylsilyl chloride (TBDMS-Cl) in the presence of imidazole yielded 1-*O*-(*tert*-butyldimethylsilyl)-3-*O*-octadecyl-*rac*-glycerol **4**, which was acylated at its C-2 position with palmitoyl chloride–pyridine leading to 1-*O*-(*tert*-butyldimethylsilyl)-2-*O*-hexadecanoyl-3-*O*-octadecyl-*rac*-glycerol **5**, which was purified by silica gel column chromatography and crystallization. Removal of the TBDMS group from compound **5** by tetrabutylammonium fluoride (TBAF)–AcOH–tetrahydrofuran (THF) gave 1-*O*-octadecyl-2-*O*-palmitoyl-*rac*-glycerol **6**, crystallized from 95% ethanol, with minor amounts of the acyl-migrated 3-*O*-hexadecanoyl derivative. Phosphorylation of primary alcohol **6** was carried out by diphenyl phosphorochloridate–pyridine to give 3-*O*-octadecyl-2-*O*-palmitoyl-*rac*-glyceryl diphenyl phosphate **7**. This phosphorylation procedure was found to be convenient due to the good stability of the product, its easy work-up and chromatographic purification, and high yields. Catalytic hydrogenolysis of compound **7** in the presence of platinum(IV) oxide resulted in 3-*O*-octadecyl-2-*O*-palmitoyl-*rac*-glyceryl dihydrogen phosphate **8** in good yield and purity. The yields at each stage in the preparation of compound **8** were more than 70%. The choice of stearyl (octadecyl) bromide for alkylation and palmitoyl chloride for acylation was



Scheme 1 *Reagents and conditions*: a, acetone, PTSA; b, (i) 1-bromooctadecane, NaH, (ii) 10% HCl; c, TBDMS chloride and imidazole; d, palmitoyl chloride, anhydrous pyridine; e, TBAF, HOAc; f, diphenyl phosphorochloridate, pyridine; g, PtO_2, hydrogen, MeOH; h, PLA_2, calcium chloride, Palitzsch buffer, pH 7.5

based on the structure of GIPLs of *L. donovani* promastigotes. In the final step, for the enantioselective C-2 deacylation of compounds **7** and **8**, several conditions were attempted including the application of commercially available lipase enzymes in various solvents and biphasic experimental conditions without success. Hydrolysis of compound **8** was successfully carried out with high enantioselectivity with phospholipase A_2 (PLA_2) from *Naja mocambique mocambique* in the presence of Ca^{++} to provide the desired natural enantiomer (*R*)-1-*O*-octadecyl-glycero-3-phosphate **9**. The reaction was partially successful with porcine pancreas PLA_2 enzyme but with lower yields and a longer reaction time (48 h) was required whereas the reaction with *N. mocambique mocambique* PLA_2 was essentially complete within 60 min. We decided to explore the application of PLA_2 due to its high specificity that requires *sn*-3-phosphate and *sn*-2-acyl structures of glycerophospholipids, Ca^{++} ions and a micellar state for hydrolysis as elucidated by X-ray structure and stereochemical studies.[16–20] PLA_2 enzymes are reported to be highly stereospecific and only the naturally occurring L-isomer of phospholipid molecules (3-*sn*-phosphoglycerides) can be hydrolysed; the reported high specificity has been one of the reasons that PLA_2 enzymes have been exploited to only a limited extent in organic synthesis as compared to the well known lipase enzymes from various sources.

Phosphodiesters and phosphomonoesters are known substrates for PLA_2 but phosphotriesters have not been reported to be substrates, which explains why PLA_2 under identical conditions could not hydrolyse 3-*O*-octadecyl-2-*O*-palmitoyl-*rac*-glyceryl diphenyl phosphate **7**. Hydrolysis of compound **8** by *N. mocambique mocambique* PLA_2 in the presence of Ca^{++} was complete in 1 h as shown by the appearance of an additional spot with lower R_f on silica TLC (spots were visualized by phosphomolybdic spray reagent). The negative-ion electrospray-mass spectrometry (ES^--MS), ^1H–^1H chemical-shift correlation (COSY), 2D heteronuclear multiple quantum-filtered correlation (HMQC) and ^{31}P NMR spectra of the product confirmed the structure of compound **9** as (*R*)-1-*O*-octadecylglycero-3-phosphate. The NMR and mass spectrometry of phosphorylated compounds such as **8** and **9** have been problematic; the problems were overcome by converting the free dihydrogen phosphate into its triethylammonium hydrogen carbonate (TEAB) salt for NMR data following a published procedure.[5] We have made use of the relatively new technique of electrospray ionization mass spectrometry (ES-MS) for mass determination of difficult-to-characterize compounds containing free phosphates. Similar compounds have recently been characterized by ES-MS application to phospholipids.[21]

For unambiguous confirmation of the absolute stereochemistry and enantioselectivity of PLA_2, we separately synthesized optically pure (*R*)-(−)-1-*O*-octadecyl-2-*O*-palmitoylglycero-3-phosphate from commercially available (*R*)-(−)-2,2-dimethyl-1,3-dioxolane-4-methanol (2,3-*O*-isopropylidene-*sn*-glycerol) following the same synthetic steps as used for the racemic material. The resultant natural enantiomer was also subjected to reaction with PLA_2 in a similar way and in this case complete hydrolysis was observed. The product of this enzymic deacylation was found to be identical in all respects (^1H, ^{13}C and ^{31}P NMR, ES-MS, optical rotation, TLC) with the chiral product **9** obtained by PLA_2 hydrolysis of *rac*-**8**.

For radiochemical synthesis, [U-^{14}C]glycerol (Amersham, UK; specific activity 147.80 mCi $mmol^{-1}$) was diluted with unlabelled glycerol to give starting material with known specific activity (7.36 μCi $mmol^{-1}$). The [U-^{14}C]glycerol was then taken through all the steps as described for the unlabelled synthesis and finally 1-*O*-octadecyl-2-*O*-palmitoyl-*rac*-[U-^{14}C]glycero-3-phosphate and (*R*)-1-*O*-octadecyl-[U-^{14}C]glycero-3-phosphate along with (*S*)-1-*O*-octadecyl-2-*O*-palmitoyl-[U-^{14}C]glycero-3-phosphate were obtained.

In the synthesis described above, reaction conditions were optimized for the micro-scale, high yielding steps to make it suitable for synthetic operation with handling of radiolabelled materials. The highly efficient application of *N. mocambique mocambique* PLA_2 for resolution of racemic alkyl-acyl-phosphatidic acids such as compound **8** by asymmetric hydrolysis in the last step ensured optical purity of the end product. The other non-natural isomer, (*S*)-1-*O*-octadecyl-2-*O*-palmitoylglycero-3-phosphate **10**, obtained in the PLA_2 reaction is also useful for biosynthetic inhibition and, by simple C-2 ester hydrolysis, (*S*)-1-*O*-octadecylglycero-3-phosphate, the chiral antipode of compound **9**, could be prepared. Therefore the synthesis allowed efficient preparation of both chirally pure *R* and *S* isomers of 1-*O*-alkylglycero-3-phosphates and also the radio- and stable isotope-labelled phospholipid precursors for biosynthetic incorporation in *L. donovani* cell and cell-free systems. The synthesis presented here has used $alkyl_{(18:0)}$ bromides and $acyl_{(16:0)}$ chlorides (present in GIPLs structure) but the strategy is equally suitable for $alkyl_{(24/26:0)}$ LPG molecules and also labelling in alkyl and acyl chains as required for biosynthetic studies on the timing and site of fatty acid remodelling that extensively occur in *Leishmania* and other parasites. Results concerning the biosynthesis of GIPLs and LPG using the various labelled precursors and inhibitors will be communicated separately.

Experimental

General procedures

The purity of reaction intermediates and products was established by TLC on Kieselgel 60 F_{254} (Merck) glass plates, developed in 20% ethyl acetate in hexane, and spots were visualized by iodine and by Molybdenum Blue spray reagent (Sigma) for phoshorylated compounds. The UV-absorbing compounds were visualized by a UV lamp (Spectroline Model ENF-260C/F, Spectronics Corporation, USA). Purification of some of the intermediates was done on a silica gel (60–120 mesh) column. Developing solvents are described at appropriate places in this section. The intermediates were crystallized from diethyl ether or ethanol unless otherwise stated. The lipid intermediates and end products were fully characterized by Fourier transform infra-red (FT-IR), NMR, and electron-impact (EI) and ES mass spectrometry. Optical rotations were obtained using a Perkin-Elmer 241 polarimeter at ambient temperature and $[\alpha]_D$-values are given in units of 10^{-1} deg cm^2 g^{-1}. IR spectra were determined in KBr on an FT-IR spectrometer (Impact-400 D series, Nicolet Instrument Corporation, Madison, USA) using Omnic Software data system. The NMR spectra [^1H, ^{13}C, ^{31}P, and two-dimensional ^1H–^1H-correlation, HMQC and double-quantum-filter correlation (DQF-COSY)] were obtained in $CDCl_3$ on a Bruker NMR spectrometer (Avance-DRX, 300 MHz for ^1H); chemical shifts are expressed in δ (ppm) relative to $SiMe_4$ as internal standard for ^1H NMR and to 85% orthophosphoric acid as external standard for ^{31}P NMR; the coupling constant *J*-values are expressed in Hz. EI- and ES-MS data were obtained on a VG Platform-II (VG-BioTech, Fisons Instrument Co., UK) quadrupole mass spectrometer equipped with a MassLynx™ Data System and pneumatic nebulizer-assisted electrospray LC/MS interface. EI mass spectra were obtained at 70 eV. For ES-MS a 1:1 mixture of acetonitrile–water was used as carrier solvent and analyte was infused into it through the injector and introduced into the mass spectrometer at a flow rate of 10 mm^3 min^{-1}. Solvent evaporations were carried out in a Büchi Rotavapor (R-114) under reduced pressure. The entire operation of radiochemical synthesis was carried out in a hood devoted to radiochemical work.

rac-Isopropylideneglycerol 2

Glycerol (516 mg, 5.6 mmol), acetone (3 cm^3), light petroleum (40–60 °C; 3 cm^3) and toluene-*p*-sulfonic acid (PTSA) (15 mg) were allowed to react in a Dean–Stark apparatus at 54 °C for 24 h until collection of water ceased in the collecting arm of the Dean–Stark tube, during which cold water was circulated in the condenser with a chilled water circulator. The mixture was cooled to room temperature and freshly fused and powdered sodium acetate (15 mg) was added, stirring being continued for another 30 min after completion of the addition, when the mixture was filtered and the residue was washed with acetone. The filtrate and washings were pooled, the solvent was removed by evaporation, the residual liquid was purified by chromatography on a silica gel (60–120 mesh) column packed in hexane, and desired material was eluted with 40% ethyl acetate–hexane to give compound **2** as an oil (630 mg, 85%), δ_H 1.37 (3 H, s, CH_3), 1.47 (3 H, s, CH_3), 3.60, 3.75 and 4.05 (4 H, each m, 2 × CH_2) and 4.25 (1 H, quin, *J* 5.2, 2-H); EI^+ *m*/*z* 117, 115, 101, 83, 73, 72, 61, 59, 57 and 43.

rac-1-*O*-Octadecylglycerol 3

To a mixture of compound **2** (547 mg, 4.14 mmol) and 1-bromooctadecane (1.667 g, 5 mmol) in dry dimethylformamide (DMF) (10 cm^3) was added sodium hydride (250 mg, 6.25 mmol; 60% NaH in oil dispersion) in three instalments over a period of 10 min. The mixture was stirred at room temperature for 24 h after which methanol (1 cm^3) was added to destroy excess of NaH and the contents were poured into ice-cold water (15 cm^3) and extracted with hexane (10 cm^3 × 3). The

extracts were pooled and evaporated and to the residue was added 10% HCl (10 cm^3) and the mixture was refluxed at 120 °C for 30 min and left overnight, the resulting pale brown lumps then being filtered and washed with methanol (30 cm^3). The filtrate was extracted with hexane, which was concentrated and mixed with the filtered material; pooled material was then dried in a vacuum desiccator over anhydrous $CaCl_2$ and the product was recrystallized from boiling diethyl ether–methanol (9:1) to give compound **3** (1.039 g, 73%), δ_H 0.88 (3 H, t, *J* 6, CH_3), 1.26 (30 H, s, 15 × CH_2), 1.56 (2 H, m, OCH_2CH_2), 3.44–3.53 (6 H, m, CH_2OCH_2 and 3-H_2) and 3.85 (1 H, m, 2-H); EI^+ *m*/*z* 345.2, 313.2, 283.2, 269.2 and 253.2 and fragments of the aliphatic chain with regular mass difference of 14 amu, equivalent to sequential loss of CH_2; ν_{max}/cm^{-1} 3375, 2927 and 2855.

rac-1-*O*-(*tert*-Butyldimethylsilyl)-3-*O*-octadecylglycerol 4

Compound **3** (500 mg, 1.45 mmol) was dissolved by warming in dry DMF and imidazole (226 mg, 3.32 mmol) and TBDMS-Cl (251 mg, 1.66 mmol) were added. The mixture was stirred at room temperature for 24 h and solvent was evaporated off under vacuum at 75 °C. The residue was dissolved in diethyl ether and washed with water, and the ether layer was dried with sodium sulfate and concentrated. The oily granular material was further dried under high vacuum at 70 °C. Compound **3** (660 mg, 100%) was homogeneous as shown by TLC and NMR data; δ_H 0.06 [9 H, s, $SiC(CH_3)_3$], 0.88 [9 H, overlapping s and t, $Si(CH_3)_2$, CH_3], 1.26 (30 H, s, 15 × CH_2), 1.56 (2 H, m, OCH_2CH_2), 3.42–3.51 (4 H, m, CH_2OCH_2 and 3-H_2), 3.64 (2 H, m, CH_2OCH_2) and 3.85 (1 H, m, 2-H); EI^+ *m*/*z* 459.3, 401.2, 383.2, 343.2 and 247, and fragments of the aliphatic chain with regular mass difference of 14 amu, equivalent to sequential loss of CH_2; ν_{max}/cm^{-1} 3374, 2927 and 2854.

rac-1-*O*-(*tert*-Butyldimethylsilyl)-2-*O*-hexadecanoyl-3-*O*-octadecylglycerol 5

To a mixture of compound **4** (500 mg, 1.09 mmol), anhydrous pyridine (300 mm^3) and toluene (10 cm^3) was added palmitoyl chloride (330 mg, 1.2 mmol) dropwise under dry nitrogen over a period of 20 min. The mixture was stirred at room temperature for 2 days and was then partitioned between diethyl ether and water (10 cm^3 each). The organic layer was washed successively with 0.25 M sulfuric acid, saturated aq. sodium hydrogen carbonate and water. The organic layer was evaporated and the residue was purified on a silica gel column. The desired material was crystallized from boiling 95% ethanol and the desired oily granular compound **5** was obtained (614 mg, 81%), δ_H 0.06 [9 H, s, $SiC(CH_3)_3$], 0.88 [12 H, overlapping s and t, $Si(CH_3)_2$, 2 × CH_3], 1.25 (56 H, br s, 15 × CH_2 and 13 × CH_2), 1.56 (2 H, m, OCH_2CH_2), 2.31 (2 H, t, *J* 7.45, CH_2CH_2CO), 3.41–3.50 (2 H, m, 3-H_2), 3.56 (2 H, m, CH_2OCH_2), 3.72 (2 H, t, *J* 4.5, CH_2OCH_2) and 4.99 (1 H, quin, *J* 5, 2-H); EI^+ *m*/*z* 639.5, 383.3, 313.2 and 239.2, and fragments of the aliphatic chain with regular mass difference of 14 amu, equivalent to sequential loss of CH_2; ν_{max}/cm^{-1} 2920, 2854, 1744 and 1179.

rac-2-*O*-Hexadecanoyl-1-*O*-octadecylglycerol 6

To a mixture of compound **5** (595 mg, 0.85 mmol) and acetic acid (200 mm^3) was added 1 M TBAF in THF (3 cm^3) over a period of 15 min at 5–10 °C, the mixture was then stirred at room temperature for 2 days and, after cooling to 0–5 °C overnight, the resulting granular solid was filtered off and washed with ice-cold 95% ethanol. This was dissolved in hot ethanol and the solution was cooled to subzero temperature for crystallization, crystals were filtered off and traces of ethanol were removed under high vacuum to give compound **6** (483 mg, 97% total crystalline material), TLC (R_f) 0.48 in ethyl acetate–hexane (20:80); δ_H 0.88 (6 H, t, *J* 6, 2 × CH_3), 1.25 (56 H, br s, 15 × CH_2 and 13 × CH_2), 1.56 (2 H, m, OCH_2CH_2), 2.33 (2 H, t, *J* 7.5, CH_2CH_2CO), 3.41–3.50 (2 H, m, 3-H_2), 3.56 (2 H, m, CH_2OCH_2), 3.72 (2 H, t, *J* 4.5, CH_2OCH_2) and 4.99 (1 H, quin, *J* 5.0, 2-H); EI^+ *m*/*z* 582.4, 564.4, 383.2, 345.2, 330.2, 313.2, 283.2 and 239.2, and fragments of the aliphatic chain with regular mass difference of 14 amu, equivalent to sequential loss of CH_2; ν_{max}/cm^{-1} 3364, 2927, 2855, 1749 and 1174.

rac-2-*O*-Hexadecanoyl-3-*O*-octadecylglyceryldiphenyl phosphate 7

To compound **6** (110 mg, 0.19 mmol) in ice-cold anhydrous pyridine (2.6 cm^3) was added diphenyl phosphorochloridate (100 mg, 80 mm^3, 0.38 mmol) dropwise over a period of 5 min. The mixture was stirred at room temperature overnight, and water (200 mm^3) and benzene (5 cm^3) were added. The resulting solution was washed successively with 10 ml each of water, saturated aq. sodium hydrogen carbonate, and brine. The benzene layer was dried over sodium sulfate and concentrated, the residue was purified on a silica gel column, and the desired material was eluted with 5% MeOH in chloroform. The purity of this material was checked on TLC on a silica gel plate developed with chloroform–methanol (95:5), visualized by iodine, UV and Molybdenum Blue spray reagent (121 mg, 75%), δ_H 0.88 (6 H, t, *J* 6, 2 × CH_3), 1.25 (56 H, br s, 15 × CH_2 and 13 × CH_2), 1.56 (2 H, m, OCH_2CH_2), 2.24 (2 H, t, *J* 7.5, CH_2CH_2CO), 3.39 (2 H, t, *J* 6.6, CH_2OCH_2), 3.53 (2 H, d, *J* 5.4, CH_2OCH_2), 4.43–4.48 (2 H, m, CH_2OP), 5.17 (1 H, quin, *J* 5, 2-H) and 7.1–7.5 (10 H, m, 2 × Ph); δ_C 14.28, 22.87, 24.91, 26.19, 29.30, 29.87, 32.10, 34.14, 63.59, 63.63, 69.72, 69.78, 72.06, 76.78, 120.26–129.85, 150.70, 150.78 and 173.53; δ_P 13.15; EI^+ *m*/*z* 721.8, 564.6, 521.5, 483.3, 469.2, 437.2, 381.3, 325.3, 251.1 and 231.1. The ^1H and ^{13}C assignments were confirmed by ^1H–^1H COSY and HMQC NMR experiments.

rac-2-*O*-Hexadecanoyl-1-*O*-octadecylglycero-3-phosphate 8

To compound **7** (20 mg, 23 mmol) were added methanol (2 cm^3), chloroform (0.5 cm^3) and platinum(IV) oxide (3 mg), and hydrogen gas was bubbled through this mixture while it was stirred for 12 h. The resulting mixture was filtered and the filtrate was evaporated to dryness. The material was then purified on a silica gel column with desired material being eluted with chloroform–methanol (65:25) to give compound **8** (15 mg, 92%), purity was checked on a TLC plate developed in chloroform–methanol–water (65:25:4) and visualized by Molybdenum Blue spray reagent.

Alternatively, to a stirred solution of glacial acetic acid (1 cm^3) and platinum(IV) oxide (10 mg, 0.044 mmol) was bubbled hydrogen gas for 1 h and a solution of compound **7** (8.3 mg, 10.2 μmol) in cyclohexane–glacial acetic acid (1:1; 2 cm^3) was quickly added. The mixture was stirred at room temperature for 6 h, filtered through Celite and the filtrate was evaporated to dryness to give compound **8** (6.5 mg, 98%). The NMR data of compound **8** was obtained on its TEAB salt form. δ_H 0.88 (6 H, t, *J* 6, 2 × CH_3), 1.25 (56 H, br s, 15 × CH_2 and 13 × CH_2), 1.56 (2 H, m, OCH_2CH_2), 2.29 (2 H, t, *J* 7.5, CH_2CO), 3.42 (2 H, t, *J* 6.8, CH_2OCH_2), 3.60 (2 H, br s, CH_2OCH_2), 4.45 (2 H, br s, CH_2OP), 5.34 (1 H, m, 2-H) and additional signals of TEA; δ_C 14.27, 22.86, 26.26, 29.41, 29.88, 32.08, 34.06, 63.56 and 173.5; δ_P 1.21; ES^--MS *m*/*z* 661.6 $[M - H]^-$.

(*R*)-1-*O*-Octadecylglycero-3-phosphate 9

To a solution of alkyl-acylphosphatidic acid **8** (6.6 mg, 10 μmol) in diethyl ether (150 mm^3) and water (150 mm^3) containing $CaCl_2 \cdot 2H_2O$ (1.15 mg, 7.8 μmol) were added 50 mM Palitzsch[22] buffer A (borax, 145 mm^3), 200 mM Palitzsch buffer B (boric acid, 8 mm^3) and phospholipase A_2 (10 μg, 14 U; E.C. 3.1.1.4; *N. mocambique mocambique*). This biphasic mixture was stirred at 35 °C for 1 h. In parallel a mock experiment was also run which contained everything except substrate. To the upper organic phase containing the product **9**, as shown by TLC, was added toluene (50 mm^3) and the mixture was then evaporated to dryness. To the residue was added acetone (200 mm^3) to yield a solid (1.4 mg, 66%). TLC of this material in chloroform–

methanol–water (65:25:4) shown by Molybdenum Blue spray reagent showed a spot with lower R_f as compared with starting material. Similar experiments were also conducted using PLA_2 from porcine pancreas (Sigma, P-6534) with or without Ca^{++} ions. $[\alpha]_D^{20}$ −1.42 (*c* 0.14, $CHCl_3$); δ_H 0.88 (3 H, t, *J* 6, CH_3), 1.25 (30 H, br s, 15 × CH_2), 1.53 (2 H, m, OCH_2CH_2), 3.42 (2 H, s, CH_2OCH_2), 3.53 (2 H, br s, CH_2OCH_2), 3.93 (2 H, br s, CH_2OP) and 4.34 (1 H, br s, 2-H); δ_C 14.30, 22.87, 26.22, 29.89, 29.42, 32.08, 34.30, 63.41, 69.55 and 71.89; δ_P 2.58; ES^--MS *m*/*z* 423.1 $[M - H]^-$. From preparative TLC compound (*S*)-(+)-1-*O*-octadecyl-2-*O*-palmitoylglycero-3-phosphate **10** was obtained. $[\alpha]_D^{20}$ +0.83 (*c* 0.12, $CHCl_3$–MeOH, 65:25); δ_H 0.88 (6 H, t, *J* 6, 2 × CH_3), 1.25 (56 H, br s, 15 × CH_2 and 13 × CH_2), 1.56 (2 H, m, OCH_2CH_2), 2.29 (2 H, t, *J* 7.5, CH_2CO), 3.42 (2 H, t, *J* 6.8, CH_2OCH_2), 3.60 (2 H, br s, CH_2OCH_2), 4.45 (2 H, br s, CH_2OP), 5.34 (1 H, m, 2-H) and additional signals of TEA; ES^--MS *m*/*z* 661.6 $[M-H]^-$.

In order to establish the absolute stereochemistry of (*R*)-1-*O*-octadecylglycero-3-phosphate **9** obtained from PLA_2 hydrolysis of racemic material **8**, enantiomerically pure (*R*)-2-*O*-hexadecanoyl-1-*O*-octadecylglycero-3-phosphate and (*R*)-1-*O*-octadecylglycero-3-phosphate were also independently synthesized starting from (*R*)-(−)-2,2-dimethyl-1,3-dioxolane-4-methanol (2,3-*O*-isopropylidene-*sn*-glycerol, Aldrich) following the same steps as for racemic starting material. A comparison of products **9** from both the routes established their absolute stereochemistry and enantioselectivity for PLA_2 enzyme-catalysed hydrolysis.

(*R*)-1-*O*-Octadecyl-[U-^{14}C]glycero-3-phosphate

For radiochemical synthesis, 40 μCi stock [U-^{14}C]glycerol (Amersham, UK, specific activity 147.80 mCi mmol^{-1}) was diluted with 500 mg of unlabelled glycerol to give specific activity of 7.36 μCi mmol^{-1}. This was taken through all the steps described under unlabelled synthesis and finally *rac*-2-*O*-hexadecanoyl-1-*O*-octadecyl-[U-^{14}C]glycero-3-phosphate and (*R*)-1-*O*-octadecyl-[U-^{14}C]glycero-3-phosphate were obtained. This entire operation was carried out in a fume-hood devoted to radiochemical work.

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