# Phospholipids Chiral at Phosphorus. 5. Synthesis and Configurational Analysis of Chiral [$^{17}$O,$^{18}$O]Phosphatidylethanolamine

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**Abstract:** We report the synthesis and configurational analysis of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) chirally labeled with $^{17}$O and $^{18}$O at the phosphate head group. Condensation of (*S*)-1,2-dipalmitoyl-*sn*-glycerol, $P^{17}OCl_3$, and *N*-(1-phenylethyl)-2-aminoethanol gives a diastereomeric mixture of cyclic oxazaphospholidines. The two diastereomers were separated by column chromatography. Ring opening with $H_2{}^{18}O$ followed by hydrogenolysis with $H_2$/Pd gives ($R_p$)- and ($S_p$)-[$^{17}$O,$^{18}$O]DPPE. The relative configuration and isotopic enrichments of [$^{17}$O,$^{18}$O]DPPE can be analyzed by $^{31}$P NMR following silylation of DPPE. To determine the absolute configuration, the two diastereomers of [$^{17}$O,$^{18}$O]DPPE are converted, by a combined organic and biochemical procedure, into ($R_p$)- and ($S_p$)-1-[$^{16}$O,$^{17}$O,$^{18}$O]phospho-(*R*)-propane-1,2-diol, with all P–O bonds intact. The configuration of the latter compound is then analyzed by $^{31}$P NMR as reported previously (Buchwald, S. L.; Knowles, J. R. *J. Am. Chem. Soc.* **1980**, *102*, 6601-6603).

Despite an extremely active research activity in mechanistic studies of enzyme-catalyzed reactions by use of various chirally labeled biophosphates,[2] little attention had been paid to the phospholipids, a major component of biological membranes. Recently we have initiated the synthesis and biochemical study of phospholipids chirally labeled at phosphorus, aiming at probing the mechanism of phospholipase-catalyzed reactions and the roles of the phosphate head group in protein–lipid interactions and in other membrane functions. In preliminary papers we have reported synthesis of chiral 1,2-dipalmitoyl-*sn*-glycero-3-[$^{18}$O]-phosphoethanolamine ([$^{18}$O]DPPE)[3,4] and chiral thiophospholipids[5] and use of these compounds for biochemical and biophysical study of phospholipids.[4-6] A full description of the synthesis and biochemical study of chiral thiophospholipids has also been published,[7] although absolute configurations at phosphorus have not been determined for any of the above compounds. In this paper[8] we describe in detail the synthesis and configurational analysis of chirally labeled DPPE **1-4** (Figure 1).

## Results and Discussion

**(A) Synthesis of Chiral [$^{17}$O,$^{18}$O]DPPE.** Only the synthesis of ($R_p$)- and ($S_p$)-[$^{17}$O,$^{18}$O]DPPE (**3** and **4** respectively) will be described in detail. The singly labeled compounds, ($R_p$)- and ($S_p$)-[$^{18}$O]DPPE (**1** and **2**, respectively), can be obtained by the same procedure except omission of the $^{17}$O isotope.

Table I. Summary of NMR Results of O-Silylation of DPPE

| compound | solvent | $^{31}$P δ | $^{29}$Si δ |
|---|---|---|---|
| $(EtO)_2PO_2^-K^+$ | $CDCl_3$ | +0.12 | |
| | $+Me_3SiCl$ | −8.77 | +23.33 (d, $J$ = 4.6 ± 0.4 Hz) |
| DPPE | $CDCl_3$ | +0.70 | |
| | $+(Me_3Si)_2NH$ | −8.432 | +24.847 (d, $J$ = 5.9 ± 0.4 Hz) |
| | | −8.482 (d, $J$ = 6.1 ± 0.1 Hz) | +24.893 (d, $J$ = 5.9 ± 0.4 Hz) |

Figure 2 outlines the synthesis of the two diastereomers of [$^{17}$O,$^{18}$O]DPPE. Reaction of (*R*)-(+)-1-phenylethylamine (**5**) with 2-bromoethanol gave (*R*)-2-[*N*-(1-phenylethyl)amino]ethanol (**6**).[9a] Condensation of (*S*)-1,2-dipalmitoyl-*sn*-glycerol (**7**) (synthesized as described previously[7]), $P^{17}OCl_3$, and **6** gave a diastereomeric mixture of cyclic oxazaphospholidines[9b] (**8a** plus **8b**) in 76% yield from **7**. Chromatographic separation (silica gel column, 10–40-μm particle size, using ether as an eluent) yielded separate diastereomers **8a** and **8b**. The isomer **8a** was eluted off faster, with $^{31}$P δ 19.78; the isomer **8b** was eluted off slower, with $^{31}$P δ 20.10. Hydrolysis of **8a** and **8b** separately with $H_2{}^{18}O$/$CF_3COOH$ (99 atom % $^{18}$O) in dimethoxyethane,[10] followed by hydrogenolysis with $H_2$/Pd, gave the two diastereomers of chiral [$^{17}$O,$^{18}$O]DPPE, **3** and **4**, respectively. The overall yield was ca. 20% from **7** for each isomer.

In the above synthetic procedure, use of the optically active amine **5** is of crucial importance. In our initial studies, we found that when 2-aminoethanol instead of **6** was used, the resulting diastereomeric mixture of cyclic oxazaphospholidines was very unstable and readily hydrolyzed during chromatography. Use of 2-(*N*-methylamino)ethanol gave relatively stable cyclic oxazaphospholidines, but the two isomers were not separable in $^{31}$P NMR or by chromatography.[11a]

The next task is to define the isotopic enrichments, the diastereomeric purity, and the absolute configuration (at phosphorus) of **3** and **4**, which are to be described in Sections B–D. However, it is necessary to comment on these problems at this point, on the

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(3) Abbreviations used: BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide. DPPA, 1,2-dipalmitoyl-*sn*-glycero-3-phosphate; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; HMDSA, 1,1,1,3,3,3-hexamethyldisilazane; HMPA, hexamethylphosphoramide; THF, tetrahydrofuran; TLC, thin-layer chromatography.

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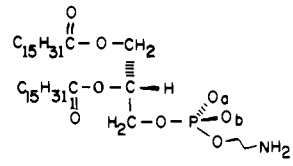

| $O_a$ | $O_b$ | Name | No. |
|---|---|---|---|
| ● | O | $(R_p)$- [$^{18}$O] DPPE | 1 |
| O | ● | $(S_p)$- [$^{18}$O] DPPE | 2 |
| ● | ∅ | $(R_p)$ - [$^{17}$O,$^{18}$O] DPPE | 3 |
| ∅ | ● | $(S_p)$ - [$^{17}$O,$^{18}$O] DPPE | 4 |

O = $^{16}$O ; ∅ = $^{17}$O ; ● = $^{18}$O

**Figure 1.** Structures of chirally labeled DPPE (**1–4**).

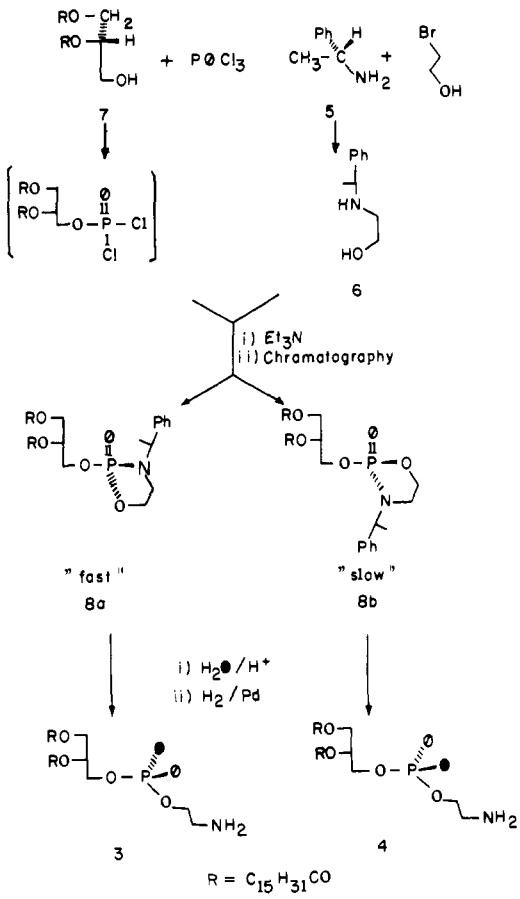


**Figure 2.** Synthesis of $(R_p)$- and $(S_p)$-[$^{17}$O,$^{18}$O]DPPE **3** and **4**.

basis of the synthetic procedure. The two diastereomers of cyclic oxazaphospholidines **8a** and **8b** have unknown configuration at phosphorus, but the diastereomeric purity is >98% based on $^{31}$P NMR. Since $^{17}$O is known to quench the $^{31}$P NMR signal of P–$^{17}$O species,[11] the $^{31}$P NMR spectra of **8a** and **8b** showed two peaks due to P–$^{16}$O and P–$^{18}$O species[12] (the magnitude of the $^{18}$O isotope shift in $^{31}$P NMR was defined as the "$S$" value;[11a] $S$ = 0.042 ppm in this case). The isotopic enrichments of **8a** and **8b** can be calculated from the ratio of the two peaks and the known $^{18}$O/$^{17}$O ratio. The atom percent $^{17}$O enrichments determined for **8a** and **8b** are 51 ± 5 and 50 ± 5, respectively.

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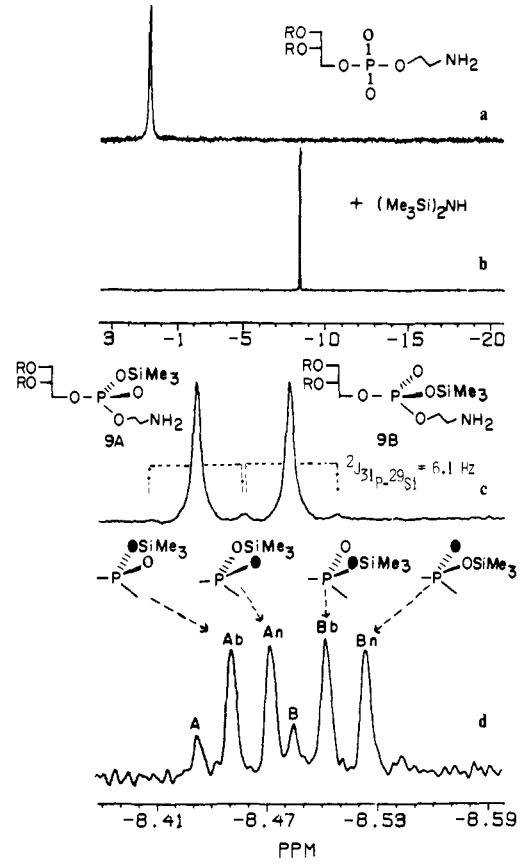


**Figure 3.** $^{31}$P NMR analysis of DPPE and [$^{18}$O]DPPE at 121.5 MHz. (a) DPPE (50 μmol) in $CDCl_3$ (2.5 mL): Spectral width 3000 Hz; acquisition time 2.7 s; 200 scans; line broadening 0.9 Hz. (b) DPPE (100 μmol) in $CDCl_3$ (2.5 mL), with addition of 50 μL HMDSA: spectral width 3000 Hz; acquisition time 2.7 s; 41 scans; line broadening –0.8 Hz; Gaussian broadening 0.07 Hz. (c) Same as (b): spectral width 500 Hz; acquisition time 16.4 s; 18 scans; line broadening –0.8 Hz; Gaussian broadening 0.07 Hz. (d) Randomly labeled [$^{18}$O]DPPE plus HMDSA: same spectral parameters as (c). All spectra were taken at 25 °C, with 16 K data points and a 90° pulse (22 s).

**(B) Silylation and $^{31}$P NMR Analysis of DPPE.** Phospholipids give broad $^{31}$P NMR signals in solution due to micelle formation. The $^{18}$O isotope shifts ($S \simeq 0.015$–$0.045$ ppm for most biophosphates)[2c,2i,12] are not resolvable in the $^{31}$P NMR of most phospholipids. Such a problem can be solved by silylation, since the O-silylated head group of DPPE is no longer amphiphilic. More importantly, O-silylation allowed us to determine not only $^{18}$O enrichments but also diastereomeric purity, of chirally labeled DPPE, by $^{31}$P NMR.

The O-silylation of DPPE was carried out by addition of a silylating agent, 1,1,1,3,3,3-hexamethyldisilazane (HMDSA), to a solution of DPPE in $CDCl_3$. The formation of the *O*-trimethylsilyl derivative was verified by both $^{31}$P NMR and $^{29}$Si NMR. As shown in Figure 3a, unlabeled DPPE in $CDCl_3$ gave a relatively broad $^{31}$P NMR signal at 0.70 ppm. Upon addition of an excess HMDSA, the signal was shifted upfield and greatly sharpened (Figure 3b). The high-resolution spectrum of the silylated DPPE (Figure 3c) showed two peaks separated by 0.050 ppm. The $^{31}$P–$^{29}$Si two-bond coupling ($^2J_{^{31}P-^{29}Si}$ = 6.1 Hz) due to the naturally abundant $^{29}$Si (4.70%) can also be observed in part of the spectrum. The $^{29}$Si NMR spectrum of the silylated DPPE showed two partially resolved doublets separated by 0.046 ppm, with $^2J_{^{31}P-^{29}Si}$ = 5.9 Hz. To ensure proper interpretation of the results, silylation of a model compound, diethylphosphate, was also investigated by $^{31}$P NMR and $^{29}$Si NMR. Table I summarizes the NMR results of silylated DPPE and silylated diethylphosphate. The results clearly established the O-silylation of DPPE. The N-silylation of DPPE by HMDSA cannot be conclusively ruled out, but it most likely did not occur under the conditions described

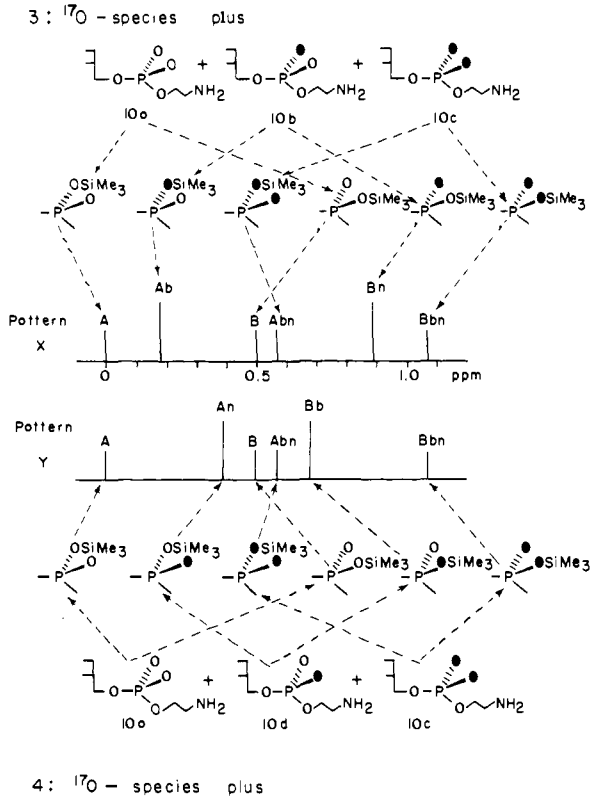


**Figure 4.** Predicted $^{31}P$ NMR for ($R_p$)- and ($S_p$)-[$^{17}O$,$^{18}O$]DPPE **3** and **4**.

above since no additional $^{29}Si$ NMR signals in the N–$SiMe_3$ region (ca. 0 ± 10 ppm) were detected (although it is not impossible in $^{29}Si$ NMR that a signal was not detectable due to long relaxation times). When a large excess HMDSA was added, or when other stronger silylating agents (such as chlorotrimethylsilane or BSTFA) were used, an additional set of $^{31}P$ NMR signals appeared at −8.5 to −8.6 ppm, possibly due to formation of N,O-disilylated DPPE.

The two predominant peaks in Figure 3c are most likely due to the two diastereomers **9A** and **9B** (abbreviated as A and B, respectively). As can be expected, the silylated product of a randomly labeled [$^{18}O$]DPPE[13] showed additional peaks Ab, An, Bb, and Bn (Figure 3d), where Ab and Bb (smaller shift, 0.018 ppm) can be assigned to species with a bridging $^{18}O$ (P–$^{18}O$–Si), whereas An and Bn (larger shift, 0.039 ppm) can be assigned to species with a nonbridging $^{18}O$ (P=$^{18}O$).[2c,2i,12] On the basis of relative peak intensities, the atom percent $^{18}O$ enrichment of the [$^{18}O$]DPPE was determined as 85 ± 5. The accuracy of the determination cannot be better than ±5% since the peaks due to $^{31}P$–O–$^{29}Si$ species were not resolved and were neglected in the calculation.

It is obvious from Figure 3d that the $^{31}P$ NMR analysis of silylated DPPE can determine not only the atom percent $^{18}O$ enrichment but also the diastereomeric purity of [$^{18}O$]DPPE. A chirally labeled [$^{18}O$]DPPE should give either predominantly Ab and Bn (separated by 0.072 ppm, defined as isomer X) or predominantly An and Bb (separated by 0.030 ppm, defined as isomer Y), depending on its configuration at phosphorus. It should be noted that up to this point the configurations of **9A**, **9B**, **3**, **4**, **8a**, and **8b** were still unknown.

**(C) Isotopic and Diastereomeric Purity of [$^{17}O$,$^{18}O$]DPPE.** In the $^{31}P$ NMR spectra of [$^{17}O$,$^{18}O$]DPPE, the species containing $^{17}O$ should not be observed due to the quadrupolar effect of $^{17}O$.[2h,2i,11,12d] If the diastereomeric purity is assumed to be 100%, isomer **3** may contain three non-$^{17}O$ species **10a**, **10b**, **10c** whereas

(13) The preparation of this particular sample of random [$^{18}O$]DPPE will be described in a later publication (R.-T. Jiang and M.-D. Tsai, unpublished results).

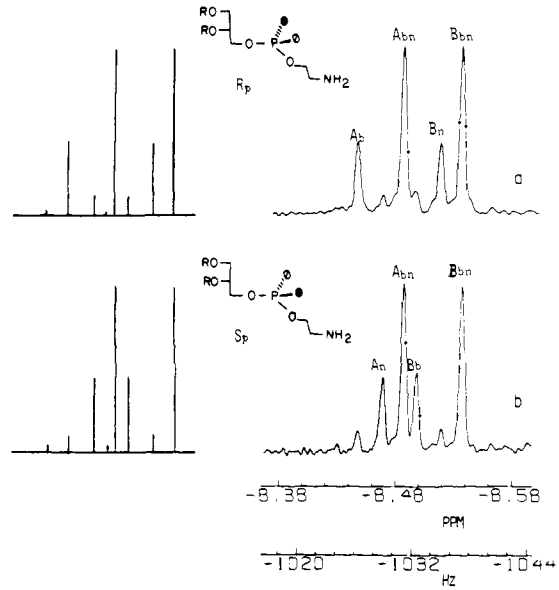


**Figure 5.** $^{31}P$ NMR spectra (at 121.47 MHz) of the silylated products of [$^{17}O$,$^{18}O$]DPPE, isomer **3** (a) and isomer **4** (b). Spectral parameters: spectral width 500 Hz; 16 K data points; $^1H$ decoupling; 90° pulse; line broadening −1.0 Hz; Gaussian broadening 0.07 Hz; chemical shift −8.5 ppm; temperature 25 °C. The calculated spectra are shown on the left side.

Table II. Summary of Isotopic and Configurational Analysis of Chiral [$^{17}O$,$^{18}O$]DPPE

| [$^{17}O$,$^{18}O$]DPPE | 3 | 4 |
|---|---|---|
| $^{17}O$ position | | |
| atom % $^{16}O$ | 17 | 17 |
| atom % $^{17}O$ | 50 | 50 |
| atom % $^{18}O$ | 33 | 33 |
| $^{18}O$ position | | |
| atom % $^{16}O$ | 5 | 8 |
| atom % $^{18}O$ | 95 | 92 |
| diastereomeric composition | | |
| % isomer X | 85 | 15 |
| % isomer Y | 15 | 85 |
| purity, % | 47.5 | 46 |
| chirality, % | 70 | 70 |
| configuration | $R_p$ | $S_p$ |

isomer **4** may contain three non-$^{17}O$ species **10a**, **10d**, **10c**, as shown in Figure 4, and each of the non-$^{17}O$-species should give two $^{31}P$ NMR peaks upon silylation. Therefore, the $^{31}P$ NMR spectra of **3** and **4**, if diastereomerically pure, may contain as many as six peaks, as shown in Figure 4. The observed spectra of **3** and **4** are shown in Figure 5. The species A, Ab, An, B, Bb, and Bn in Figures 4 and 5 are the same as those defined in Figure 3. The species Abn and Bbn are those with both bridging and nonbridging $^{18}O$ (shifted by 0.057 ppm).

In principle, it is possible to calculate the percent $^{18}O$ enrichment (at the $^{18}O$ position) from the observed spectra in Figure 5, assuming the enrichments at the "$^{17}O$ position" are the same as those of **8a** and **8b**. With known enrichments at both positions, the diastereomeric purity can then be calculated from the relative intensities of Ab and An or Bb and Bn. However, the error in the measurement of peak intensities is ±5% for major peaks and much larger for minor peaks. In addition, major peaks should give 4.7% of "satellite peaks" (due to P–O–$^{29}Si$ species), which may overlap with minor peaks. Therefore, any calculation cannot be more accurate than ±5%. With such great errors in the isotopic enrichments, the diastereomeric purity calculated were very inaccurate and unreasonable.

It is also clear from the observed spectra that all of the three parameters (enrichments at $^{17}O$ and $^{18}O$ positions and diastereomeric purity) are within 70% of the theoretical values. Therefore, the best approach seems to simulate the spectra by varying the three parameters between 70 and 100% of the theo-

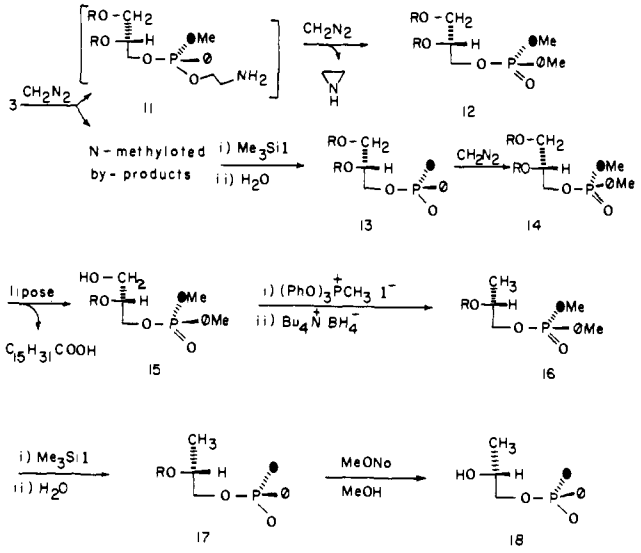

Figure 6. Conversion of ($R_p$)-[$^{17}$O,$^{18}$O]DPPE (**3**) into 1-($R_p$)-[$^{16}$O,$^{17}$O,$^{18}$O]phospho-(*R*)-propane-1,2-diol (**18**). R = $C_{15}H_{31}CO$; $^{18}$O = ●; $^{17}$O = Ø.

retical values. The isotopic enrichments and diatereomeric purity obtained by the "best fit" are summarized in Table II. The spectra calculated from the best fit parameters are also shown in Figure 5. As in our previous work on chiral inorganic thiophosphate,[14] we define the term "purity" as the percentage of the chirally labeled species (i.e., the M + 3 species) and the term "chirality" as the optical purity of the chirally labeled species. The [$^{17}$O,$^{18}$O]DPPE **3** showed 47.5% in purity and 70% in chirality, whereas the isomer **4** showed 46% in purity in 70% in chirality.

The imperfect optical purity of **3** and **4** can be attributed, in a small part, to incomplete optical purity (96%) of **5** and possibly incomplete diastereomeric purity of **8a** and **8b** (98%) and, in a large part, to partial racemization in the hydrolysis with $H_2{}^{18}O/CF_3COOH$. Although acid-catalyzed hydrolysis[15] or alcoholysis[16] of cyclic 1,3,2-oxazaphospholidines was known to proceed with inversion of configuration at phosphorus, the degree of stereospecificity may vary with different structures and different reaction conditions.[17]

**(D) Conversion of [$^{17}$O,$^{18}$O]DPPE to 1-[$^{16}$O,$^{17}$O,$^{18}$O]Phospho-(*R*)-propane-1,2-diol.** To determine the absolute configuration of chiral [$^{17}$O,$^{18}$O]DPPE, we converted the two isomers **3** and **4** separately into chiral 1-[$^{16}$O,$^{17}$O,$^{18}$O]phospho-(*R*)-propane-1,2-diol (**18**) without cleaving a P–O bond. The configuration of **18** was then established by the $^{31}$P NMR method reported previously.[18]

Figure 6 shows the procedure for the conversion of isomer **3** of [$^{17}$O,$^{18}$O]DPPE into the chiral 1-phospho-(*R*)-propane-1,2-diol (**18**). Diazomethanolysis of **3** gave the dimethyl ester of phosphatidic acid **12** in 30–60% yield.[19] The structure of **12** was characterized by $^1$H, $^{31}$P, and $^{13}$C NMR and by synthesis of unlabeled **12** from 1,2-dipalmitin (**7**), $POCl_3$, and methanol. All the P–O bonds should remain intact in this step. The phosphotriester **12** was found to be a good substrate for lipase (*Rhizopus arrhizus*),[20] which hydrolyzed the C-1 carboxylic ester of **12** in 81% yield. The product **15** was characterized by $^1$H and $^{13}$C NMR. The hydroxymethyl group in **15** was reduced to a methyl group (in **16**) by a two-step procedure: first the alcohol **15** was converted to the corresponding iodide with methyltriphenoxyphosphonium iodide, followed by reduction to **16** with tetrabutylammonium borohydride.[21] The structure of **16** was characterized by $^1$H, $^{13}$C, and $^{31}$P NMR. Demethylation of **16** with iodotrimethylsialne[22] (which does not involve cleavage of a P–O bond) gave 2-palmitoyl-1-[$^{16}$O,$^{17}$O,$^{18}$O]phospho-(*R*)-propane-1,2-diol (**17**). Methanolysis of **17** with sodium methoxide in methanol[23] gave 1-[$^{16}$O,$^{17}$O,$^{18}$O]phospho-(*R*)-propane-1,2-diol (**18**), which was characterized by $^1$H and $^{13}$C NMR.

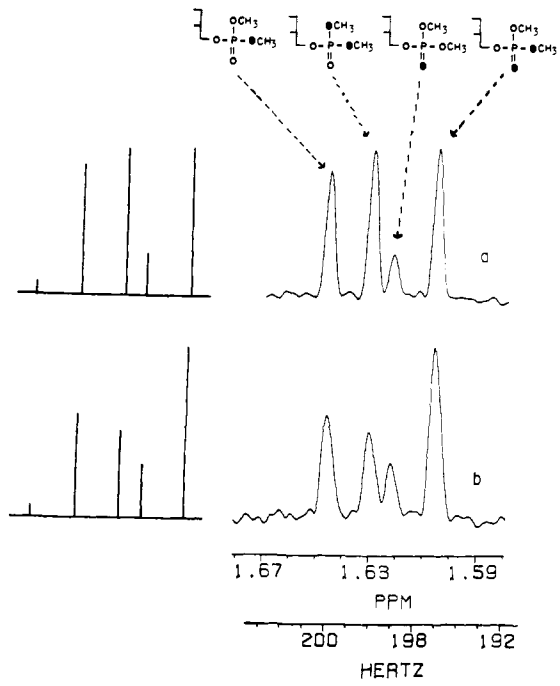


Figure 7. Calculated and observed $^{31}$P NMR spectra of **12** (a) and **14** (b) (in $CDCl_3$) obtained from [$^{17}$O,$^{18}$O]DPPE **4**. Spectral parameters are the same as in Figure 5.

The overall yield in the conversion from **3** to **18** varied from 3% to 8%. The mechanisms of some steps are commented on as follows. The diazomethanolysis of phosphatidylethanolamines was first reported by Baer and Maurukas.[19] It has been suggested that the reaction proceeds via the formation of the methyl ester of DPPE (**11**) followed by elimination of aziridine and then by a second methylation.[24] The isotopic distribution of **12**, as shown by $^{31}$P NMR (Figure 7a), is consistent with the predicted spectrum calculated on the basis of the two-step mechanism and the isotopic enrichments of the starting [$^{17}$O,$^{18}$O]DPPE.

The step with the lowest yield in the whole procedure was the diazomethanolysis of DPPE, most likely due to formation of N-methylated byproducts that are less facile to elimination of the side chain.[24] The formation of **12** was monitored by $^1$H NMR of the reaction mixture for the appearance of signals at 3.77 ppm due to $CH_3OP$. The formation of byproducts was indicated by additional peaks at 3.2–3.7 ppm. The byproducts predominated over **12** when anhydrous and ethanol-free ethereal solution of diazomethane was used. In this case, one of the byproducts isolated was identified as 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine.

In any case, the mixture of byproducts was dealkylated by treating with iodotrimethylsilane followed by $H_2O$ to give 1,2-dipalmitoyl-*sn*-glycero-3-[$^{16}$O,$^{17}$O,$^{18}$O]phosphate

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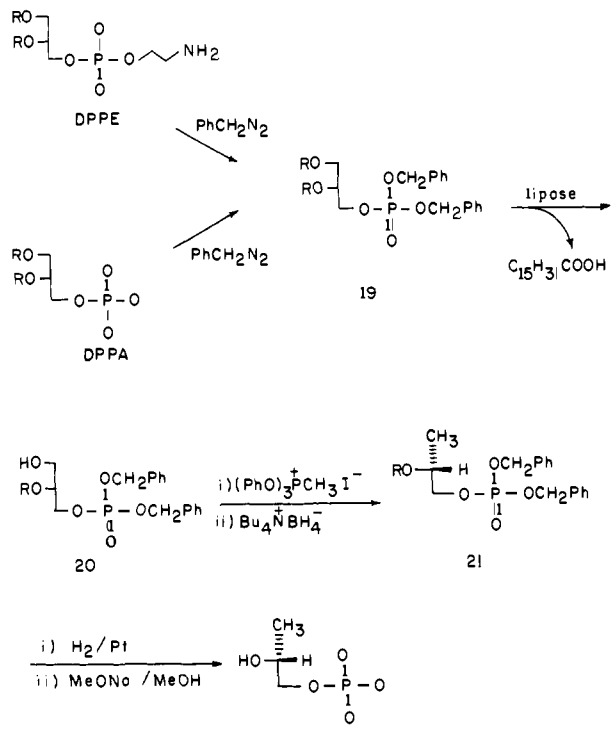


Figure 8. Alternative procedure for the conversion of DPPE into 1-phospho-(*R*)-propane-1,2-diol. R = $C_{15}H_{31}CO$.

([$^{16}O$,$^{17}O$,$^{18}O$]DPPA) (**13**), which was then converted to its dimethyl ester **14**. The $^{31}P$ NMR spectrum of **14**, in which the isotopes should be randomly distributed, is shown in Figure 7b. The observed spectrum is again consistent with the predicted one, which was calculated assuming a random isotope distribution and assuming the same isotopic enrichments as the starting [$^{17}O$,$^{18}O$]DPPE. The results ensured that no dilution of isotope occurred during diazomethanolysis and dealkylation. Therefore, **12** and **14** were combined before proceeding to the next step (lipase hydrolysis).

The lipase from *arrhizus*[25] is known to hydrolyze specifically the 1-carboxylic ester of triglycerides[26] and some phospholipids.[27] The dimethyl ester of phosphatidic acid **12** was found to be a good substrate. The hydrolysis occurred specifically at the 1-carboxylic ester, but a small degree of migration of the 2-palmitoyl group to the 1-position could occur during the column chromatography of **15**. Such an isomerization, however, could only lower the yield but not the chirality, since the resulting secondary alcohol should not interfere with subsequent reactions of **15**.

An alternative procedure for the conversion of DPPE or DPPA to 1-phospho-(*R*)-propane-1,2-diol has also been developed, as shown in Figure 8. Reaction of DPPE or DPPA with phenyldiazomethane gave the dibenzyl ester **19**. Lipase hydrolysis of **19** gave the product **20**, which was then reduced to **21** by the two-step procedure described above. Hydrogenolysis of **21** followed by methanolysis gave the 1-phospho-(*R*)-propane-1,2-diol. Such a procedure has not been applied to chirally labeled DPPE since the yield of phenyldiazomethanolysis was poor. However, the procedure may be quite useful in the conversion of chirally labeled DPPA to chiral-[$^{16}O$,$^{17}O$,$^{18}O$]phospho-(*R*)-propane-1,2-diol.

**(E) Absolute Configurations at Phosphorus.** The configuration (at phosphorus) of **18** can be analyzed by $^{31}P$ NMR following chemical cyclization (inversion) and methylation (Figure 9), as previously described for chiral 1-[$^{16}O$,$^{17}O$,$^{18}O$]phospho-(*S*)-propane-1,2-diol,[2a,18] on the basis of the quadrupolar effect of $^{17}O$[11] and the isotope shift effect of $^{18}O$[12] on $^{31}P$ NMR. In Figure 9, the species in brackets contain $^{17}O$, which are expected to quench $^{31}P$ NMR signals. Only the non-$^{17}O$-containing species of the cyclic triesters (**22**, **23**) can be observed in high-resolution $^{31}P$ NMR. Since **18** has *R* configuration at C-2, whereas the previous phosphopropanediols have *S* configuration at C-2, the $^{31}P$ NMR pattern expected for $R_p$ and $S_p$ configuration of **18** should correspond to the $S_p$ and $R_p$ configuration, respectively, of 1-[$^{16}O$,$^{17}O$,$^{18}O$]phospho-(*S*)-propane-1,2-diol. As shown in Figure 9, the $R_p$ isomer of **18** should give predominantly syn O=P-$^{18}$-$OCH_3$ (**22**) and anti $^{18}$O=P-$OCH_3$ species (**23**) of the cyclic triesters. Such an isotopic distribution pattern is what has been observed in the $^{31}P$ NMR analysis (Figure 10a) of **18** obtained from **3**. It is thus established that both **18** and **3** have $R_p$ configuration. Consistently, the other diastereomer of [$^{17}O$,$^{18}O$]DPPE, **4**, which should be $S_p$, gave 1-($S_p$)-[$^{16}O$,$^{17}O$,$^{18}O$]phospho-(*R*)-propane-1,2-diol, as shown by the $^{31}P$ NMR analysis in Figure 10b.

As in the case of [$^{17}O$,$^{18}O$]DPPE, it would be impractical and unreliable to calculate isotopic enrichments and diastereomeric purity from the relative intensity in the observed spectra in Figure 10. A more reliable approach is to simulate the spectra. If it is

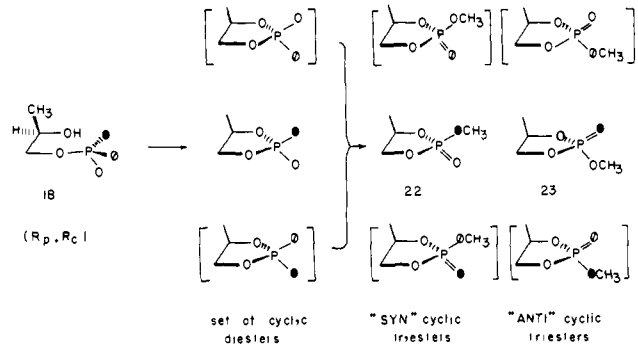


Figure 9. Ring closure and methylation of 1-($R_p$)-[$^{16}O$,$^{17}O$,$^{18}O$]-phospho-(*R*)-propane-1,2-diol (**18**), according to the procedure of Abbott et al.,[15] except that the configuration of $C_2$ is different.

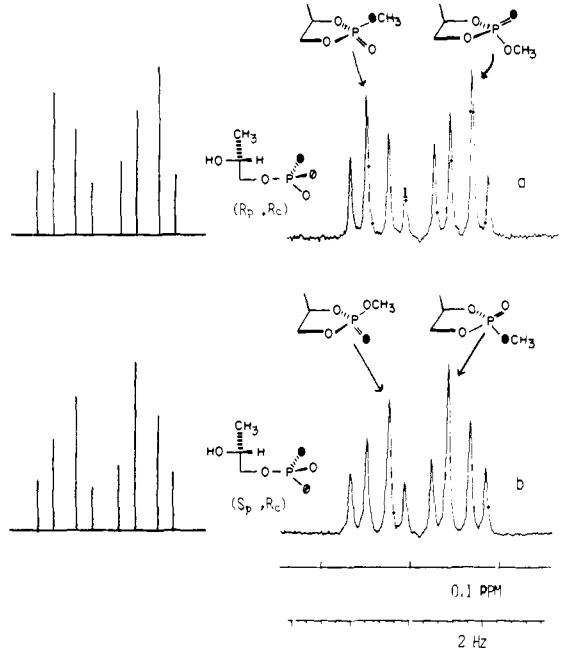


Figure 10. $^{31}P$ NMR spectra (at 121.47 MHz) of the mixtures of syn and anti cyclic triesters (in $CD_3CN$) derived from [$^{16}O$,$^{17}O$,$^{18}O$]-phospho-(*R*)-propane-1,2-diols. (a) From isomer **3** of [$^{17}O$,$^{18}O$]DPPE; (b) from isomer **4** of [$^{17}O$,$^{18}O$]DPPE. Spectral parameters: spectral width 500 Hz; 16 K data points; $^1H$ decoupling; 90° pulse; line broadening 0.2 Hz; chemical shift +19.7 ppm; temperature 25 °C. The calculated spectra are shown on the left side.

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Table III. Summary of Isotopic and Configurational Analysis of Chiral [$^{16}$O,$^{17}$O,$^{18}$O]Phospho-(*R*)-propane-1,2-diol

| | from 3 | from 4 |
|---|---|---|
| $^{17}$O position | | |
| atom % $^{16}$O | 20 | 19 |
| atom % $^{17}$O | 48 | 49 |
| atom % $^{18}$O | 32 | 32 |
| $^{18}$O position | | |
| atom % $^{16}$O | 9 | 11 |
| atom % $^{18}$O | 91 | 89 |
| diastereomeric composition | | |
| % $R_p$ isomer | 75 | 20 |
| % $S_p$ isomer | 25 | 80 |
| purity, % | 43.7 | 43.6 |
| chirality, % | 50 | 60 |
| configuration | $R_p$ | $S_p$ |

assumed that the phosphopropanediols have the same isotopic enrichments and diastereomeric purity as the starting [$^{17}$O,$^{18}$O]DPPE, that kinetic isotope effect is negligible in the ring closure, and that all isotopic species have the same nuclear Overhauser effect in $^{31}$P NMR, the calculated spectra are clearly different from the observed ones beyond possible experimental errors. However, by introducing a 2–4% dilution in isotopes and a 5–10% racemization, the simulated spectra are identical to the observed ones within experimental error, as shown in Figure 10. The data of isotopic enrichments and diastereomeric purity for the two isomers of [$^{16}$O,$^{17}$O,$^{18}$O]phospho-(*R*)-propane-1,2-diol are summarized in Table III. It should be noted that although the data in both Tables II and III have intrinsic errors, the differences are real since, if the data in Table III are used to calculate the spectra of [$^{17}$O,$^{18}$O]DPPE, the resulting spectra are different from the observed ones far beyond possible experimental errors. Such a small degree of isotope exchange and racemization most likely have occurred during the process of the cyclization reaction of **18**.

On the basis of the results discussed above, the absolute configuration (at phosphorus) of various compounds can be summarized as follows. (1) For both [$^{18}$O]DPPE and [$^{17}$O,$^{18}$O]DPPE, the "X isomer" (that shows Ab > An and Bn > Bb in $^{31}$P NMR analysis) is $R_p$ and the "Y isomer" (An > Ab and Bb > Bn) is $S_p$, regardless of the way the compounds are synthesized. The absolute configuration of chiral DPPE can therefore be determined directly by the $^{31}$P NMR analysis following silylation. (2) Since the $R_p$ isomer shows Ab > An and Bn > Bb, the diastereomer **9A** must be $R_p$ and **9B** must be $S_p$, as shown in Figure 3. (3) If it is assumed that the ring opening catalyzed by $CF_3COOH$ proceeds with inversion of configuration,[15,16] the diastereomer **8a** ($^{31}$P $\delta$ 19.78) should be $R_p$ and **8b** ($^{31}$P $\delta$ 20.10) should be $S_p$, as shown in Figure 2. (4) Chirally labeled phosphatidylcholine with known configuration can be obtained by N-methylation of chiral DPPE **1–4**. Details in the synthesis and configurational analysis of chiral [$^{17}$O,$^{18}$O]phosphatidylcholine and their application in the stereochemical study of phospholipase D are reported in a separate paper.[28]

## Experimental Section

**Materials.** The $H_2{}^{17}O$ used (52.4 atm % $^{17}$O, 35.1 atom % $^{18}$O) was obtained from Monsanto and the $H_2{}^{18}O$ (99 atom % $^{18}$O) from Norsk Hydro. $P^{17}OCl_3$ was prepared and analyzed (by $^{31}$P NMR analysis of its trimethylphosphate) as described previously.[11a] (*S*)-1,2-Dipalmitoyl-*sn*-glycerol (**7**) was synthesized from D-mannitol as described previously.[8] (*R*)-1-Phenylethylamine (**5**) was obtained from Hoffmann-La Roche. Lipase (*R. Arrhizus*) (6600 IU/mg) was purchased from Sigma (Type XI). BSTFA and chlorotrimethylsilane were purchased from Pierce. HMDSA was obtained from Pierce or Aldrich. All other chemicals were of reagent grade or the highest purity available. Trifluoroacetic anhydride, 2-bromoethanol, and anhydrous ether were distilled before use. Methylene chloride and trichloroethylene were dried over $P_2O_5$, dimethoxyethane was dried over lithium aluminum hydride, triethylamine was dried over KOH pellets, and $CH_3CN$, $CD_3CN$, and hexamethylphosphoramide (HMPA) were deried over $CaH_2$, all of which were distilled before use. HMDSA and (*R*)-1-phenylethylamine were used without further purification.

**Chromatography.** Thin-layer chromatography (TLC) was carried out on precoated plates (0.2 mm, aluminum support, E. Merck silica gel 60 F-254, purchased from Merck). The compounds were visualized by spraying with 10% ethanolic solution of phosphomolybdic acid followed by heating. Three types of silica gel were used in column chromatography: silica gel H from Sigma, 10–40-$\mu$m particle size; silica gel 7 from Baker, <40-$\mu$m particle size, and Lichroprep Si 60 from Merck, 25–40-$\mu$m particle size. Most column chromatography was performed under moderate pressure (ca. 20 psi) and monitored by UV absorption at 280 or 254 nm. The cation-exchange resin used (Dowex AG-50-WX8, 50–100 mesh) was obtained from Bio-Rad.

**Instrumental Methods.** $^1$H, $^{13}$C, and $^{31}$P NMR spectra were obtained from both Bruker WP-200 and WM-300 spectrometers, with deuterium lock. All $^{31}$P and $^{13}$C NMR data reported were measured with broadband $^1$H decoupling. $^1$H and $^{13}$C chemical shifts are referenced to external $Me_4Si$ in the same solvent, unless otherwise specified. $^{31}$P chemical shifts are referenced to external 85% $H_3PO_4$ (determined by measuring 85% $H_3PO_4$ in an inner tube, with pure deuterated solvent in the outer tube). The positive sign represents a downfield shift in all cases. The deviation in $^{31}$P chemical shifts from some data reported in previous communications is due to a change in the external reference used in our laboratory, in order to comply with the majority of literature.

Some of the $^{31}$P NMR spectra were processed with Gaussian instead of exponential multiplication. Although Gaussian multiplication may change the relative intensity of signals with different line shapes,[29] we have extensively checked this problem and confirmed that the relative intensity of peaks due to P–$^{16}$O and P—$^{18}$O species is not affected by Gaussian multiplication, as long as the signal/noise ratio is kept high. Use of Gaussian multiplication helped resolve closely resonated signals and made the measurement of peak intensity more accurate.

$^{29}$Si NMR was obtained from Bruker WP-200 at 39.76 MHz, with deuterium lock, at ambient temperature. A relaxation agent, chromium(III) acetylacetonate, was added to reduce the relaxation time. The chemical shifts are referenced to $Me_4Si$.

Optical rotations were measured on a Perkin-Elmer 141 polarimeter in a 10-cm cell. Both melting and boiling points were uncorrected.

**2-[*N*-(1-Phenylethyl)amino]ethanol, 6.** The optically active (*R*)-(+)-1-phenylethylamine (**5**) (8.0 g, 66 mmol) ($[\alpha]^{20}_D$ 39.2° (neat), 96% optical purity) was mixed with 2-bromoethanol (5.52 g, 44 mmol) and heated at 100 °C for 2 h. The reaction mixture was neutralized with concentrated KOH. The organic layer was separated and mixed with water, and the product was extracted with chloroform. The chloroform phase was dried with anhydrous sodium carbonate and then evaporated to dryness. The residue was distilled in vacuo to give 2-[*N*-(1-phenylethyl)amino]ethanol (**6**), bp 100 °C (0.4 mmHg), in 37% yield. The pure product **6** was a colorless oil: $[\alpha]^{20}_D$ 36.4° (*c* 7.4, benzene); $^{13}$C NMR ($CDCl_3$, 50.3 MHz) $\delta$ 24.0 ($CH_3$), 49.2 ($CH_2N$), 58.1 ($CH_2O$), 61.2 (CHN), 126.5, 127.0, 128.5.

**2-(1,2-Dipalmitoyl-*sn*-glycero)-3-(1-methylbenzyl)-2-[$^{17}$O]oxo-1,3,2-oxazaphospholidines, 8a and 8b.** A solution of (*S*)-1,2-dipalmitoyl-*sn*-glycerol (**7**) (7.24 mmol) in 10 mL of trichloroethylene was added to a solution of $P^{17}OCl_3$ (11.1 mmol) and triethylamine (11.1 mmol) in 10 mL of trichloroethylene at −20 °C with stirring. The reaction mixture was allowed to warm up to room temperature in 1 h, stirred for another $^1/_2$ h, and then evaporated to dryness in vacuo. The resulting phosphorodichloridate was dissolved in fresh trichloroethylene and then mixed with a solution containing 7.84 mmol of **6** and 17.3 mmol of triethylamine in the same solvent at −20 °C. The reaction mixture was kept at room temperature for 20 h, during which some precipitate (hydrogen chloride salt of $Et_3N$) formed. All of the above steps were performed by vacuum-line techniques to avoid any moisture. The reaction mixture was then diluted with 20 mL of dry hexane and the precipitate was filtered off. The product, as a mixture of diastereomers **8a** plus **8b**, was purified by column chromatography on silica gel (Baker, <40-$\mu$m particle size, 4.0 cm × 40 cm column) by using ether as the eluent, in 76% yield (4.3 g) relative to 1,2-dipalmitin.

The presence of two diastereomers was indicated by both $^{31}$P NMR (in $CDCl_3$, $\delta$ 19.78 and 20.10) and TLC (ether, $R_f$ = 0.56 and 0.46). Separation of the two diastereomers was achieved by low-pressure liquid chromatography (<100 psi) on silica gel (Baker or Sigma) at room temperature, using anhydrous, freshly distilled ether as the eluent. A tightly packed column of 4.0 cm × 40 cm in size was sufficient to separate 1.0–1.5 g of the mixture. Separation of 4.3 g of the mixture gave 1.2 g of **8a** (eluted off faster, $R_f$ = 0.56 on TLC) (21% yield from **7**) and 1.6 g of **8b** (eluted off slower, $R_f$ = 0.46 on TLC) (29% yield from **7**).

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The spectral data are summarized as follows. For **8a** (the "fast" isomer): mp 60–61 °C from methanol/$CCl_4$; $[\alpha]^{20}_D$ −6.77° (*c* 2.3, chloroform); $^{31}P$ NMR ($CDCl_3$, 81 MHz) δ 19.78 (with an additional peak due to $^{18}O$ isotope shift, *S* = 0.042 ppm); $^1H$ NMR ($CDCl_3$, 200 MHz) δ 0.87 (t, *J* = 6.3 Hz, 6 H, terminal $CH_3$), 1.25 (br s, 48 H, long-chain $CH_2$), 1.65 (d, *J* = 7.0 Hz, 3 H, $CH_3CHN$), 1.65 (m, 4 H, $CH_2CH_2C{=}O$), 2.308 (t, *J* = 7.3 Hz, 2 H, $CH_2C{=}O$), 2.321 (t, *J* = 7.3 Hz, 2 H, $CH_2C{=}O$), 3.0–4.4 (m, 9-H), 5.26 (quintet, *J* = 5.2 Hz, 1 H, $CHOC{=}O$), 7.26–7.43 (m, 5 H, phenyl); $^{13}C$ NMR ($CDCl_3$, 50.3 MHz) δ 14.14 (terminal $CH_3$), 20.28 ($CH_3CHN$), 22.70 ($CH_3CH_2$), 24.89 ($CH_3CH_2CH_2$), 29.18, 29.34, 29.39, 29.53, 29.71, 30.10, 30.44, 31.98 ($CH_2CH_2C{=}O$), 34.11 ($CH_2C{=}O$), 34.27 ($CH_2C{=}O$), 44.92 (d, $^3J_{CP}$ = 16.3 Hz, $CH_2N$), 54.94 (d, $^2J_{CP}$ = 6.0 Hz, CHN), 61.89, 63.82, 65.55 (d, $^2J_{CP}$ = 6.8 Hz, 3-$CH_2OP$), 69.78 (d, $^3J_{CP}$ = 6.8 Hz, CHO), 126.83, 127.71, 128.77, 173.0 (C=O). For **8b** (the "slow" isomer, waxy solid): $[\alpha]^{20}_D$ +20.55° (*c* 2.4, chloroform); $^{31}P$ NMR ($CDCl_3$, 81 MHz) δ 20.10 (with an additional peak due to $^{18}O$ isotope shift, *S* = 0.042 ppm); $^1H$ NMR ($CDCl_3$, 200 MHz) indistinguishable from those of **8a**, except for small differences in the unresolved multiplets (3.0–4.4 ppm) and in $CH_2C{=}O$ (2.313 ppm, *t*, *J* = 7.1 Hz, 4 H); $^{13}C$ NMR ($CDCl_3$, 50.3 MHz) indistinguishable from those of **8a** for carbons in palmitoyl groups and the phenyl group. Other carbons are δ 19.89 ($CH_3CHN$), 44.68 (d, $^3J_{CP}$ = 17.0 Hz, $CH_2N$), 54.50 (d, $^2J_{CP}$ = 7.2 Hz, CHN), 61.89, 63.92, 65.41 (d, $^2J_{CP}$ = 7.3 Hz, 3-$CH_2OP$), and 69.78 (d, $^3J_{CP}$ = 7.5 Hz, CHO). Assignments of $^1H$ and $^{13}C$ NMR spectra of **8a** and **8b** were assisted by the spectra of other phospholipids[8] an of a model compound in which the *N*-(1-methylbenzyl) group of **8** was replaced by an *N*-methyl group.[11a] However, since the two carbon nuclei in the cyclic oxazaphospholidine ring show unusually large an small couplings with $^{31}P$, the assignments should be further confirmed by model studies.

**[$^{17}O$,$^{18}O$]DPPE, 3 and 4.** The cyclic [$^{17}O$]oxazaphospholidine **8a** (1.2 mmol) was placed in a 10-mm NMR tube connected to a Teflon stopcock and dried under high vacuum for 2 h. Solvents and reagents were distilled into the tube under vacuum in the following order: 5 mL of dry dimethoxyethane, 635 μL of $H_2{}^{18}O$ (32 mmol, 99 atom % $^{18}O$), and 146 mg of trifluoroacetic anhydride (695 μmol). The reaction mixture was stirred at room temperature for $^1/_2$ h and the product precipitated. After the reactant was completely hydrolyzed as shown by $^{31}P$ NMR, the thick suspension was transferred to a flask and evaporated under reduced pressure. The residual solid was dissolved in $CHCl_3$ and washed with aqueous $NaHCO_3$. The chloroform layer was concentrated and redissolved in 10 mL of $CH_3COOH$, and 150 mg of 10% palladium catalyst on charcoal was added. Hydrogenolysis of the product was carried out at 55 °C under 1 atm of $H_2$. TLC ($CHCl_3/CH_3OH$ 9/1) indicated complete hydrogenolysis in 24 h. The catalyst was removed by filtration and the product **3** was isolated by column chromatography on silica gel ($EtOH/CHCl_3/H_2O$, 50/50/4 as the eluent) in 76% yield. The other isomer of [$^{17}O$]oxazaphospholidine **8b** was converted to the other isomer of [$^{17}O$,$^{18}O$]DPPE (**4**) according to the same procedure. The overall yield is ca. 20% from **7** for each isomer.

The [$^{17}O$,$^{18}O$]DPPEs **3** an **4** showed the same properties as commercial DPPE (Sigma) in TLC ($R_f$ = 0.33 in $EtOH/CHCl_3/H_2O$, 50/50/4) and in $^{31}P$ NMR (+0.70 ppm, br, in $CDCl_3$). The optical rotations are $[\alpha]^{20}_D$ = +6.3° (**3**) an +6.2° (**4**) (*c* 3.1, $CHCl_3/CH_3OH$, 2/1), which are consistent with that of commercial DPPE ($[\alpha]^{20}_D$ = 6.2°, same conditions) within experimental error. Therefore, **3** an **4** should be optically pure at C-2, which has *R* configuration as shown in Figure 1.

**Silylation and $^{31}P$ NMR Analysis of DPPE.** A DPPE sample of 20–40 mg was placed in a 10-mm NMR tube and dried in vacuo. Just before the NMR measurement, 2.5 mL of dry $CDCl_3$ was added and the tube was heated at ca. 50 °C until DPPE was dissolved. A $^{31}P$ NMR spectrum was taken to check the purity of DPPE. The sample was then titrated with 30–50-μL aliquots of hexamethyldisilazane (HMDSA) until the broad DPPE signal was fully converted into sharp peaks at −8.5 ppm as indicated by $^{31}P$ NMR. An anhydrous sample requires no more than 30–50 μL of HMDSA for complete silylation, but more reagent is required if the sample is not rigorously dry. If silylation is not complete with 400 μL of the reagent within 30 min, the sample is most likely impure or wet and should be further purified.

After silylation was completed by the above procedure, a high-resolution spectrum was obtained, with a spectral width of 250 or 500 Hz. It is important not to have a large excess of HMDSA; otherwise another group of peaks at −8.6 ppm may appear during prolonged accumulation, most likely due to additional silylation at the amino group. After $^{31}P$ NMR experiments, samples can be recovered by addition of $H_2O$, followed by evaporation to dryness and then by column chromatography.

**Dimethyl-1,2-dipalmitoyl-*sn*-glycero-3-[$^{16}O$,$^{17}O$,$^{18}O$]phosphate (12).** To a chloroform solution of [$^{17}O$,$^{18}O$]DPPE (**3**) (216 μmol in 2 mL) was added an ethereal solution of freshly prepared diazomethane until the solution remained yellow. The solution was kept at room temperature for 12–24 h and then evaporated to dryness. A $^1H$ NMR spectrum was taken to monitor the methylation (3.77 ppm, d, $^3J_{POCH_3}$ = 11.1 Hz). The procedure was repeated several times during 1–2 weeks, until no further increase in the methylated product can be observed. The reaction mixture was then chromatographed on a silica gel column (Lichroprep Si60, Merck) under elevated pressure (<100 psi) by using ether as the eluent. The product **12** was isolated in 30–60% yield: $^1H$ NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 6 H, terminal $CH_3$), 1.25 (br s, 48 H, methylene), 1.6 (m, 4 H, $CH_2CH_2C{=}O$), 2.31 (t, *J* = 7.4 Hz, 2 H, $CH_2C{=}O$), 2.335 (t, *J* = 7.2 Hz, 2 H, $CH_2C{=}O$), 3.770 (d, $^3J_{HP}$ = 11.1 Hz, 3 H, $CH_3OP$), 3.779 (d, $^3J_{HP}$ = 11.2 Hz, 3 H, $CH_3OP$), 4.172 (dd, $^3J_{CH_ACH}$ = 5.2 Hz, $^3J_{CH_AOP}$ = 7.25 Hz, 1 H, $CH_AH_BOP$), 4.184 (dd, $^3J_{CH_BCH}$ = 4.6 Hz, $^3J_{CH_BOP}$ = 7.0 Hz, 1 H, $CH_AH_BOP$), 4.175 (dd, $^3J_{CH_ACH}$ = 6.0 Hz, $^2J_{H_AH_B}$ = 12.0 Hz, 1 H, $CH_AH_BOC{=}O$), 4.328 (dd, $^3J_{CH_BCH}$ = 4.4 Hz, $^2J_{H_AH_B}$ = 12.0 Hz, 1 H, $CH_AH_BOC{=}O$), 5.24 (m, 1 H, CHO); the assignments were assisted by homonuclear $^1H$ decoupling; $^{13}C$ NMR ($CDCl_3$, 50.3 MHz) δ 14.09 (terminal $CH_3$), 22.72 ($CH_3CH_2$), 24.93 ($CH_3CH_2CH_2$), 29.20, 29.34, 29.40, 29.54, 29.74, 32.00 ($CH_2CH_2C{=}O$), 34.14 ($CH_2C{=}O$), 34.27 ($CH_2C{=}O$), 54.54 (d, $^2J_{CP}$ = 5.2 Hz, $CH_3OP$), 61.77 ($CH_2OC{=}O$), 65.65 (d, $^2J_{CP}$ = 5.4 Hz, $CH_2OP$), 69.63 (d, $^3J_{CP}$ = 7.4 Hz, $CHOC{=}O$), 173.06 (C=O), 173.45 (C=O). Since the $^{13}C$ NMR spectrum was actually taken from a product from unlabeled DPPE, the signal at 55.15 ppm ($CH_3OP$) did not show $^{18}O$ isotope shifts. Optical rotation: $[\alpha]^{20}_D$ +1.8° and $[\alpha]^{20}_{436}$ +3.5° (*c* 3.2, $CHCl_3$).

**Dimethyl 1,2-Dipalmitoyl-*sn*-glycero-3-phosphate (Unlabeled 12).** A solution of 1,2-dipalmitin, **7** (1.7 g, 3.0 mmol), in $CHCl_3$ was added slowly into 5 mL of $CHCl_3$ containing $POCl_3$ (691 mg, 4.5 mmol) and triethylamine (450 mg, 4.5 mmol) at 0 °C. The reaction mixture was stirred for 1 h followed by evaporation of solvent and excess reagents in vacuo. The residue was dissolved in dry THF and treated with a mixture of $Et_3N$ (1 mL) and methanol (0.5 mL). After the mixture was stirred at room temperature for 12 hours, the resulting precipitate was filtered off. The filtrate was concentrated and chromatographed as described in the previous section. The product was isolated in 58% yield and gave TLC and $^1H$ and $^{13}C$ NMR results identical with those of **12**: $^{31}P$ NMR ($CDCl_3$, 121.5 MHz) δ 1.6.

**Dimethyl 1,2-Dipalmitoyl-*sn*-glycero-3-[$^{16}O$,$^{17}O$,$^{18}O$]phosphate (14).** The N-methylated byproducts (450 mg) from the diazomethanolysis of **3** was dried under vacuum. Five milliliters of dry $CH_2Cl_2$ was added through rubber septum, followed by 253 mg of iodotrimethylsilane (180 μL, 1.26 mmol), at −20 °C. The reaction mixture was allowed to warm up to room temperature in 1.5 h, left for an additional hour, and then evaporated to dryness. The residue was refluxed with 5 mL of 95% ethanol. The resulting brown solution was evaporated to dryness and treated with an ethereal solution of $CH_2N_2$. Chromatographic separation on silica gel gave 231 mg of **14**. TLC, $^1H$ NMR, and $^{13}C$ NMR of **14** were identical with those of **12**. The $^{31}P$ NMR analyses of **12** and **14** are described in the text.

**Dimethyl 2-Palmitoyl-*sn*-glycero-3-[$^{16}O$,$^{17}O$,$^{18}O$]phosphate (15).** A combined sample of **12** and **14** (514 μmol) was dissolved in a mixture of ether (1 mL) and methanol (0.2 mL) and then mixed with an aqueous solution (0.8 mL) containing 50 mM $Ca(NO_3)_2$, 0.4 M $(NH_4)_2SO_4$, and 7500 IU of lipase (*R. arrhizus*, 0.9 mg of protein). The resulting heterogeneous reaction mixture was stirred vigorously at room temperature. The progress of the reaction was followed by TLC (ether, $R_f$ = 0.29 and 0.15 for substrate and product, respectively). After the reaction was complete (ca. 2 h), the water phase was washed thoroughly with ether (3 × 5 mL) and ether layers were pooled and evaporated. The oily residue was chromatographed on silica gel (Lichroprep, Merck, 1.0 × 25 cm column size). The fatty acid was first eluted off with ether/methanol (50/1) (flow rate 1.0 mL/min). The product **15** was then eluted off with ether/methanol (50/2) (flow rate 2.0 mL/min) in 81% yield, with $R_f$ = 0.43 in benzene/acetone/chloroform, 4/3/3. The product was contaminated with trace amount of a byproduct with a slightly higher $R_f$ (0.49). The byproduct is most likely the 1-palmitoyl isomer of **15** due to isomerization, which can be minimized by minimizing the duration of chromatography (ca. 1 h). No attempt was made to remove the trace byproduct since it should not interfere with the reaction in the following step: $^1H$ NMR of **15** ($CDCl_3$, 200 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3 H, terminal $CH_3$), 1.26 (br s, 24 H, methylene), 1.6 (m, 2 H, $CH_2CH_2C{=}O$), 2.35 (t, *J* = 7.5 Hz, 2 H, $CH_2C{=}O$), 3.792 (d, $^3J_{HP}$ = 11.2 Hz, 3 H, $CH_3OP$), 3.798 (d, $^3J_{HP}$ = 11.1 Hz, 3 H, $CH_3OP$), 3.79 (2 H, $CH_2OH$, partially overlapped with $CH_3OP$ signals), 4.25 (dd, $^3J_{HH}$ = 4.7 Hz, $^3J_{HP}$ = 8.8 Hz, 2 H, $CH_2OP$), 5.04 (quintet, *J* = 5.0 Hz, 1 H, $CHOC{=}O$); $^{13}C$ NMR ($CDCl_3$, 50.3 MHz) δ 14.12 (terminal $CH_3$), 22.71 ($CH_3CH_2$), 24.93 ($CH_3CH_2CH_2$), 29.15, 29.30, 29.38, 29.50, 29.70, 31.96 ($CH_2CH_2C{=}O$), 34.29 ($CH_2C{=}O$), 54.60 (d, $^2J_{CP}$ = 6.0 Hz, $CH_3OP$), 60.37 ($CH_2OH$), 65.23 (d, $^2J_{CP}$ = 5.3 Hz, $CH_2OP$), 72.36 (d, $^3J_{CP}$ = 6.4 Hz, $CHOC{=}O$), 173.30 (C=O). Since the $^{13}C$ NMR

spectrum was actually measured on a product obtained from unlabeled DPPE, the signal at 55.21 ppm ($CH_3OP$) did not show $^{18}O$ isotope shifts.

**1-[$^{16}O$,$^{17}O$,$^{18}O$]Phospho-2-palmitoyl-(*R*)-propane-1,2-diol Dimethyl Ester (16).** Methyltriphenoxyphosphonium iodide (0.5 mmol in 1 mL of dry $CH_2Cl_2$) was added with a dry syringe to an anhydrous solution of **15** (416 μmol) in $CH_2Cl_2$ at room temperature. The reaction was followed by TLC (benzene/acetone/chloroform, 4/3/3). Additional reagent (0.25 mmol) was added after 1 h and the reaction mixture was kept until the reactant disappeared. Methanol (2 mL) was then added to quench the excess reagent and the solvents were evaporated. The residue was chromatographed on silica gel (Lichroprep). All UV-absorbing compounds were first eluted off with ether/hexane (1/1). Further elution with ether gave the iodo derivative (72–81% yield) as an oil, which was not characterized. The product was dissolved in anhydrous HMPA (300 μmol in 1 mL), an tetrabutylammonium borohydride (105 mg, 408 μmol) was added in small portions at room temperature. The reaction was monitored by TLC (ether, $R_f$ = 0.25 for reactant an 0.185 for product) and was quenched with 0.1 M borate buffer (pH 6.6, 10 mL) when complete. The resulting emulsion was extracted with ether (3 × 5 mL), and the ether extract was washed with water and evaporated. Chromatography of the oily residue on silica gel (Lichroprep, ether as the eluent) gave the product **16** (as an oil, which solidified upon storing at room temperature) in 62% yield from the iodo derivative: $^1H$ NMR ($CDCl_3$, 200 MHz) δ 0.88 (t, $J$ = 6.7 Hz, 3 H, terminal $CH_3$), 1.26 (br s), 1.62 (m, 2 H, $CH_2CH_2C$=O), 2.30 (t, $J$ = 7.6 Hz, 2 H, $CH_2C$=O), 3.770 (d, $^3J_{HP}$ = 11.1 Hz, 3 H, $CH_3OP$), 3.774 (d, $^3J_{HP}$ = 11.1 Hz, 3 H, $CH_3OP$), 3.9–4.2 (m, 2 H, $CH_2OP$), 5.1 (m, CHOC=O); $^{13}C$ NMR ($CDCl_3$, 50.3 MHz) δ 14.08 (terminal $CH_3$), 16.09 ($CH_3CH$), 22.71 ($CH_3CH_2$), 24.97 ($CH_3CH_2CH_2$), 28.73, 29.34, 29.40, 29.52, 29.71, 31.99 ($CH_2CH_2C$=O), 34.52 ($CH_2C$=O), 54.47 (d, $^2J_{CP}$ = 5.6 Hz, $CH_3OP$, br, $^{18}O$ shifts not resolved), 68.71 (d, $^3J_{CP}$ = 7.3 Hz, CHOC=O), 69.43 (d, $^2J_{CP}$ = 5.7 Hz, $CH_2OP$), 173.75 (C=O); $^{31}P$ NMR ($CDCl_3$, 81.0 MHz) δ 1.637 (25%, P–$^{18}O$–C), 1.621 (27%, C–$^{18}O$–P–$^{18}O$–C), 1.612 (16%, P=$^{18}O$), 1.597 (32%, $^{18}O$=P–$^{18}O$–C). Since some signals overlap partially, the measurement of peak intensities could have an error of ±10%.

**1-[$^{16}O$,$^{17}O$,$^{18}O$]Phospho-2-palmitoyl-(*R*)-propane-1,2-diol (17).** Thoroughly dried **16** (186 μmol, 5 h under high vacuum) was dissolved in 2 mL of anhydrous methylene chloride (distilled under vacuum) and the solution (capped with a rubber septum) was cooled down to −20 °C. Iodotrimethylsilane (55 μL, 385 μmol) was added with a dry syringe. The reaction mixture was brought up to room temperature during 3 h and the solvent removed under reduced pressure. TLC indicated that all of the reactant had disappeared. The solid residue was dissolved in 95% methanol (5 mL) and refluxed for 1 h. The resulting light-brown solution was evaporated to dryness and the resulting waxy solid chromatographed on silica gel, eluted first with chloroform/methanol (20/1) followed with chloroform/ethanol/water (50/50/4). The product **17** was obtained in quantitative yield as a slightly impure, yellowish solid, which was used in the next reaction directly without further purification or characterization.

**1-[$^{16}O$,$^{17}O$,$^{18}O$]Phospho-(*R*)-propane-1,2-diol (18).** Five milliliters of 0.2 M sodium methoxide in methanol was added to 185 μmol (73.5 mg) of **17**. The reaction mixture was warmed gently until the reactant was completely dissolved and then kept at room temperature for 3 h. After the reaction was complete as indicated by TLC (methanol/chloroform/water, 66/33/4; $R_f$ = 0.25 for reactant and 0.0 for product), the solution was neutralized by acetic acid (5 mL) and evaporated to dryness. The residual solid was treated with chloroform (10 mL) and extracted with water (3 × 10 mL). The water extract was freeze-dried to give a mixture of **18** (as a sodium salt) and sodium acetate, as indicated by $^1H$ NMR in $D_2O$. The mixture was then converted into their pyridinium salts by passing it through a cation-exchange column (Dowex AG-50WX8, 50–100 mesh, pyridinium form, 1.5 × 25 cm). The UV-absorbing fractions were collected and freeze-dried. The more volatile acetate was removed in this process as indicated by $^1H$ NMR, and 34.4 mg of pure **18** was obtained (78% yield): $^1H$ NMR (pyridinium salt, $D_2O$, 300 MHz, referenced to DSS) δ 1.10 (d, $J$ = 6.5 Hz, 3 H, $CH_3CH$), 3.676 (ddd, $^2J_{H_AH_B}$ = 10.7 Hz, $^3J_{HH}$ = 6.6 Hz, $^3J_{HP}$ = 6.6 Hz, 1 H, $CH_AH_BOP$), 3.795 (ddd, $^2J_{H_AH_B}$ = 10.7 Hz, $^3J_{HH}$ = 3.5 Hz, $^3J_{HP}$ = 6.0 Hz, 1 H, $CH_AH_BOP$), 3.963 (qdd, $^3J_{CHCH_3} \approx$ 6.6 Hz, $^3J_{CHCH_A}$ = 6.6 Hz, $^3J_{CHCH_B}$ = 3.5 Hz, 1 H, $CH_3CH$); $^{13}C$ NMR (sodium salt, $D_2O$, 50.3 MHz, referenced to DSS) δ 20.44 ($CH_3$), 69.78 (d, $^3J_{CP}$ = 7.1 Hz, CH), 72.17 (d, $^2J_{CP}$ = 4.8 Hz, $CH_2OP$).

**Dibenzyl 1,2-Dipalmitoyl-*sn*-glycero-3-phosphate (19).** A suspension of DPPE (72 μmol) in chloroform (2 mL) was treated with an ethereal solution of phenyldiazomethane (prepared freshly from benzaldehyde and hydrazine[30]) at room temperature for 4 days, during which slow evolution of gas bubbles was observed. After decomposition of the excess reagent by adding acetic acid, the reaction mixture was evaporated to dryness and then evaporated in vacuo at elevated temperature to remove benzyl acetate. Column chromatography on silica gel (Baker, hexane/ether, 1/1) gave **19** in 10% yield.

A larger quantity of **19** was prepared from (*S*)-1,2-dipalmitin (**7**), $POCl_3$, and benzyl alcohol by a procedure analogous to the preparation of unlabeled **12** described above. Direct benzylation of DPPA in dry chloroform with phenyldiazomethane also gave **19** in 40% yield: $^1H$ NMR ($CDCl_3$, 200 MHz) δ 0.88 (t, $J$ = 6.7 Hz, 6 H, terminal $CH_3$), 1.26 (br s, 48 H, methylene), 1.52 (br s, 4 H, $CH_2CH_2C$=O), 2.26 (t, $J$ = 7.8 Hz, 2 H, $CH_2C$=O), 2.27 (t, $J$ = 7.7 Hz, 2 H, $CH_2C$=O), 4.03–4.30 (m, 4 H, $CH_2OC$=O and $CH_2OP$), 5.03 (d, $^3J_{HP}$ = 8.3 Hz, 2 H, $CH_2Ph$), 5.04 (d, $^3J_{HP}$ = 8.3 Hz, 2 H, $CH_2Ph$), 5.16 (quintet, $J$ = 5.2 Hz, 1 H, CHO), 7.35 (br s, 10 H, $C_6H_5$); $^{13}C$ NMR ($CDCl_3$, 50.3 MHz) δ 14.1 (terminal $CH_3$), 22.80 ($CH_3CH_2$), 24.97 ($CH_3CH_2CH_2$), 29.28, 29.42, 29.64, 29.82, 30.27, 30.36, 32.07 ($CH_2CH_2C$=O), 34.18 ($CH_2C$=O), 34.31 ($CH_2C$=O), 61.87 ($CH_2OC$=O), 65.71 (d, $^2J_{CP}$ = 5.3 Hz, $CH_2OP$), 69.66 (d, $^3J_{CP}$ = 9.1 Hz, CHO), 69.81 (d, $^2J_{CP}$ = 5.9 Hz, $CH_2Ph$), 128.38, 129.05, 173.31 (C=O), 173.69 (C=O); $^{31}P$ NMR ($CDCl_3$, 81.0 MHz) δ 0.58 (septet in undecoupled spectrum, $^3J_{HP}$ = 7.9 Hz).

**Dibenzyl 2-Palmitoyl-*sn*-glycero-3-phosphate (20).** One gram of **19** was hydrolyzed by 7500 IU of lipase as described above for the hydrolysis of **14**. The product **20** was isolated in 64% yield. No byproduct due to migration of the acyl group was detectable: $^1H$ NMR ($CDCl_3$, 200 MHz) δ 0.88 (t, $J$ = 6.7 Hz, 3 H, terminal $CH_3$), 1.25 (br s, 24 H, methylene), 1.59 (m, 2 H, $CH_2CH_2C$=O), 2.29 (t, $J$ = 7.6 Hz, 2 H, $CH_2C$=O), 3.69 (d, $J$ = 5.2 Hz, 2 H, $CH_2OH$), 4.14 (dd, $^3J_{HH}$ = 4.7 Hz, $^3J_{HP}$ = 8.6 Hz, 2 H, $CH_2OP$), 4.96 (quintet, $J$ = 4.9 Hz, 1 H, CHO), 5.05 (d, $^3J_{HP}$ = 8.4 Hz, 4 H, $CH_2Ph$), 7.35 (br s, 10 H, $C_6H_5$); $^{13}C$ NMR ($CDCl_3$, 81.0 MHz) δ 14.20 (terminal $CH_3$), 22.82 ($CH_3CH_2$), 25.02 ($CH_3CH_2CH_2$), 29.26, 29.41, 29.50, 29.61, 29.83, 30.20, 32.09 ($CH_2CH_2C$=O), 34.39 ($CH_2C$=O), 60.55 ($CH_2OH$), 65.17 (d, $^2J_{CP}$ = 5.4 Hz, $CH_2OP$), 69.98 (d, $^2J_{CP}$ = 5.5 Hz, $CH_2Ph$), 72.46 (d, $^3J_{CP}$ = 6.0 Hz, CHO) 128.43, 129.08, 129.12, 135.99 (d, $^3J_{CP}$ = 6.8 Hz, phenyl $CCH_2OP$), 173.77 (C=O); the assignments of $^{13}C$ NMR signals were assisted by comparing with the spectrum of the corresponding dimethyl ester **15**; $^{31}P$ NMR ($CDCl_3$, 81.0 MHz) δ 0.44.

**1-Phospho-2-palmitoyl-(*R*)-propane-1,2-diol Dibenzyl Ester (21).** Treatment of **20** with methyltriphenoxyphosphonium iodide followed by reduction with tetrabutylammonium borohydride as described above gave **21** in 45% overall yield: $^1H$ NMR ($CDCl_3$, 200 MHz) δ 0.88 (t, $J$ = 6.5 Hz, 3 H, terminal $CH_3$), 1.19 (d, $J$ = 6.5 Hz, 3 H, $CH_3CH$), 1.25 (br s), 1.57 (m, 2 H, $CH_2CH_2C$=O), 2.24 (t, $J$ = 7.5 Hz, 2 H, $CH_2C$=O), 3.98 (m, 2 H, $CH_2OP$), 5.040 (d, $^3J_{HP}$ = 8.2 Hz, 2 H, $CH_2Ph$), 5.046 (d, $^3J_{HP}$ = 8.2 Hz, 2 H, $CH_2Ph$), 5.04 (m, 1 H, CHO), 7.35 (br s, $C_6H_5$); $^{13}C$ NMR ($CDCl_3$, 81.0 MHz) δ 14.14 (terminal $CH_3$), 16.09 ($CH_3CH$), 22.77 ($CH_3CH_2$), 25.01 ($CH_3CH_2CH_2$), 29.23, 29.36, 29.57, 29.78, 30.27, 32.03 ($CH_2CH_2C$=O), 34.53 ($CH_2C$=O), 68.62 (d, $^3J_{CP}$ = 8.0 Hz, CHO), 69.34 (d, $^2J_{CP}$ = 5.5 Hz), 69.59 (d, $^2J_{CP}$ = 6.1 Hz), 128.18, 128.84, 136.09 (d, $^3J_{CP}$ = 7.0 Hz, phenyl $CCH_2OP$), 173.40 (C=O); $^{31}P$ NMR ($CDCl_3$, 81.0 MHz) δ −0.56.

Hydrogenolysis of **21** (78.5 mg in 5 mL of ether, with 50 mg of 10% Pd/C) followed by methanolysis in 10 mL of 0.1 M MeONa/MeOH as described above gave 1-phospho-(*R*)-propane-1,2-diol in 50% yield.

**Acknowledgment.** We are indebted to Ru-Tai Jiang of this laboratory for synthesis of 1,2-dipalmitoyl-*sn*-glycerol and randomly labeled [$^{18}O$]DPPE and to the National Institutes of Health for financial support (Research Grant GM 30327). The NMR facilities used were funded in part by Grants GM 27431 from the National Institutes of Health and CHE 7910019 from the National Science Foundation.

**Registry No. 1**, 80548-28-3; **2**, 88035-45-4; **3**, 88035-46-5; **4**, 88035-47-6; **5**, 3886-69-9; **6**, 80548-31-8; **7**, 30334-71-5; **8a**, 88035-48-7; **8b**, 88154-70-5; **9a**, 88035-49-8; **9b**, 88035-59-0; **10a**, 923-61-5; **10c**, 88035-60-3; **12**, 88035-50-1; **12** (unlabeled), 88082-31-9; **13**, 88035-51-2; **15**, 88035-52-3; **16**, 88035-53-4; **17**, 88035-54-5; ($R_p$,$R_c$)-**18**, 88035-55-6; ($S_p$,$R_c$)-**18**, 88035-58-9; **19**, 80795-00-2; **20**, 88035-56-7; **21**, 88035-57-8; ($S_p$,$R_c$)-**22**, 88082-32-0; ($R_p$,$R_c$)-**22**, 88082-35-3; ($S_p$,$R_c$)-**23**, 88082-33-1; ($R_p$,$R_c$)-**23**, 88082-34-2; 1-phospho-(*R*)-propane-1,2-diol, 52730-47-9.

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