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Phospholipids Chiral at Phosphorus: Synthesis and Configurational Assignment of Phosphorothioate Analogs of Phosphatidylserine[1]

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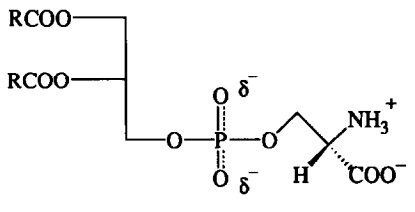
Phosphorothioate analogs of phosphatidylserine, (R_p + S_p)-1,2-dipalmitoyl-*sn*-glycero-3-thiophospho-L-serine were synthesized from 1,2-dipalmitoyl-*sn*-glycerol and *N*-trityl-L-serine methoxymethyl ester, using chloro(*N*,*N*-diisopropylamino)methoxyphosphine as a phosphitylating agent. The configuration at phosphorus of these phosphorothioate analogs was assigned on the basis of the stereospecific hydrolysis of the R_p isomer by phospholipase A_2 from bee venom.

INTRODUCTION

Phosphorothioate analogs of different types of phospholipids have been synthesized recently. These include thiophosphatidylcholine (*1–5*), thiophosphatidylethanolamine (*1–5*), thiophosphatidyl glycerol (*5*), thiosphingomyelin (*6*), platelet-activating factor (*7*), and most recently thiophosphatidylinositol (*8*). These analogs have been used to probe the mechanism of various membrane-related enzymes and the biophysical properties of phospholipid bilayers (*1–12*). These studies involve almost every major type of phospholipids, except phosphatidylserine (**1**). In this paper we report the synthesis and configurational assignment of the phosphorothioate analogs of phosphatidylserine, 1,2-dipalmitoyl-*sn*-glycero-3-thiophospho-L-serine (**2**, DPPsS).[3]



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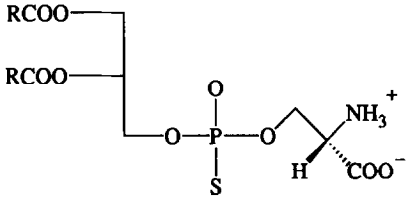

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[1] This is Paper 19 in the series "Phospholipids Chiral at Phosphorus." For Paper 18, see Ref. (*8*).

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[3] Abbreviations used: DPPsS, 1,2-dipalmitoyl-*sn*-glycero-3-thiophospho-L-serine; EDTA, ethylenediaminetetraacetate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; MPPsS, 1-palmitoyl-*sn*-glycero-3-thiophospho-L-serine; PLA2, phospholipase A_2; TLC, thin-layer chromatography.

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EXPERIMENTAL

Materials. Bee venom phospholipase A_2 (PLA2) was purchased from Boehringer-Mannheim. Other biochemicals were purchased from Sigma. All other chemicals were reagent grade. Silica gel used in liquid chromatography (Licorpre Silica 60, 200–400 mesh) and thin-layer chromatography (60 F_{254}) was obtained from EM reagent (Merck).

Chromatographic and instrumental methods. Most liquid chromatography were performed under medium pressure (ca. 20 psi) on a Licorpre Silica 60 column. ^1H NMR spectra were recorded on a Bruker AM-250 spectrometer. With Waltz-16 ^1H decoupling, ^{13}C spectra were obtained at 62.90 on a Bruker AM-250 spectrometer and ^{31}P NMR spectra were obtained at 101.25 or 121.50 MHz on a Bruker AM-250 or WM-300 spectrometer. ^{31}P chemical shifts are referenced to external 85% H_3PO_4. The ^1H and ^{13}C chemical shifts are reference to internal Me_4Si.

N-Trityl-L-serine diethylammonium salt (**4**). The procedure was adapted from Stelakatos *et al.* (*13*). In a 50-ml round-bottom flask 1.00 g (9.52 mmol) of L-serine was dissolved in 4 ml of water and 8 ml of 2-propanol. To this inhomogeneous solution was added 2.95 ml (28.56 mmol) of diethylamine. The now homogeneous mixture was stirred vigorously while 2.66 g (9.52 mmol) of trityl chloride was added in 12 equal portions over 1 h. Four minutes after the final addition of trityl chloride, 30 ml of water was added. This mixture was extracted with 2 50-ml portions of chloroform. The chloroform solution was washed with 20 ml of water and dried over magnesium sulfate for at least 4 h. The chloroform solution was then decanted and dried *in vacuo* to afford a white foamy product. This crude product was dissolved in anhydrous ether and 1 ml of diethylamine was added. The solution was cooled at 0°C overnight and the crystalline product was filtered off and washed with cold dry ether. *N*-Trityl-L-serine diethylammonium salt was produced in 21% yield and characterized by ^1H NMR (250 MHz, CD_3OD): δ 7.23–7.49 (m, 15 H, *phenyl*); 3.15 (m, 3 H, $HOCH_2CH$); 2.96 (q, J = 7 Hz, $(CH_3CH_2)_2N$); 1.25 (t, J = 7 Hz, $(CH_3CH_2)_2N$).

N-Trityl-L-serine methoxymethyl ester (**5**). The procedure was adapted from Corey *et al.* (*14*). In a 25-ml round-bottom flask, 0.500 g (1.2 mmol) of *N*-trityl-L-serine diethylammonium salt, 365 μl (4.8 mmol) of chloromethyl methyl ether, and 2 ml (12 mmol) of triethylamine were dissolved in 5 ml of chloroform. After stirring overnight at room temperature, the reaction mixture was dried *in vacuo* and the crude product purified on silica gel using chloroform : acetone (95 : 5, v/v), R_f = 0.5, 71% yield. ^1H NMR (250 MHz, $CDCl_3$): δ 7.2 and 7.5 (m, 15 H, *phenyl*); 4.77 and 4.93 (d, J = 7 Hz, 2 H, $O–CH_2–O$); 3.73 (bs, 1 H, $CH–N$); 3.56 (bs, 2 H, $HOCH_2$); 3.32 (s, 3 H, OCH_3); 3.04 (bs, 1 H); 2.32 (bs, 1 H). ^{13}C NMR (62.90 MHz, $CDCl_3$): δ 145.59 ($CHCOOCH_2$); 128.74, 127.92, and 126.63 (*phenyl*); 91.06; 71.04; 64.83; 57.94 and 57.56.

Dipalmitoyl methylthiophosphatidyl-N-trityl-L-serine methoxymethyl ester (**9**). In a dry 100-ml round-bottom flask fitted with a septum port containing 0.500 g (0.88 mmol) of 1,2-dipalmitin was distilled in 0.750 g (0.75 mmol) of triethylamine and ca. 10 ml of anhydrous ethanol-free chloroform. To this flask was added via a

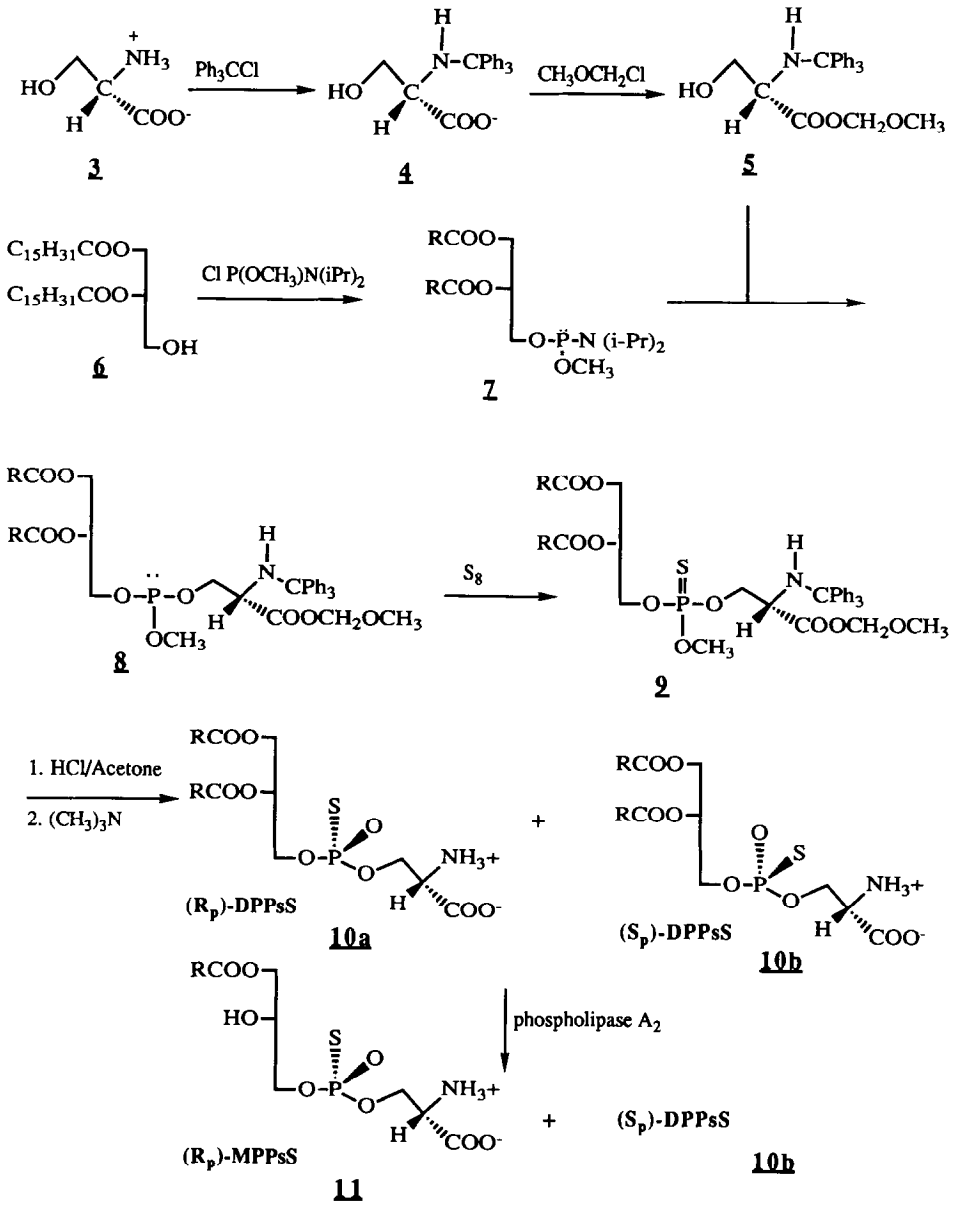
syringe 210 μl (1.05 mmol) of chloro(*N,N*-diisopropylamino)methoxyphosphine. The reaction was allowed to stir at room temperature for 2 h, after which time the solvents were removed *in vacuo*. To this crude product was added a solution containing 0.4235 g (1.05 mmol) of **5** and 0.3077 g (3.52 mmol) of tetrazole in an anhydrous mixture of dichloromethane and acetonitrile. The reaction mixture was stirred at room temperature for 12 h, after which time the solvents were removed *in vacuo* and the residue was dried under high vacuum (<0.001 Torr) for 2–3 h. To this flask was added 10 ml of anhydrous toluene followed with 0.7 g of sulfur. This reaction mixture was allowed to stir at room temperature for 46 h. The solvents were removed *in vacuo* and the resulting residue was dissolved in 2:1 (v/v) hexane:diethyl ether and applied to a silica gel column. The product **9** was isolated in 70% yield with an $R_f = 0.5$. ^{31}P NMR (101.26 MHz, $CDCl_3$): δ 69.42 and 69.33. ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.21 to 7.55 (m, 15 H, *phenyl*); 5.29 (m, 1 H, glycerol 2-C*H*); 4.85 (d, 1 H, $J = 5$ Hz, $COOCH_AH_BOCH_3$) and 4.60 (d, 1 H, $J = 5$ Hz, $COOCH_ACH_BOCH_3$); 4.15–4.45 (m, 5 H, glycerol backbone 1-CH_2, 3-CH_2, and serine –C*H*–NH); 3.74 and 3.68 (d, 3 H, $J = 13$ Hz, two diastereomers of P–O–CH_3); 3.62 (m, 2 H, serine OCH_2); 3.30 (s, 3 H, ester OCH_3); 2.81 (d, 1 H, serine N*H*); 2.30 (m, 4 H, acyl chain 2-CH_2); 1.60 (bs, 4H, acyl chain 3-CH_2); 1.27 (s, 48 H, acyl chain 4–15 CH_2); 0.89 (t, 6H, $J = 7$ Hz, acyl chain 16-CH_3).

1,2-Dipalmitoyl-sn-glycero-3-thiophospho-L-serine (*(R_p + S_p)-DPPsS* (**10a** + **10b**)). The *N*-trityl and methoxymethyl ester groups were removed by treatment of **9** with concd HCl in dry acetone (*15*). Specifically, 0.203 g (0.194 mmol) of **9** was dissolved in 10 ml of a solution containing 3 ml of concd HCl in 180 ml of dry acetone. The reaction mixture was allowed to stir at room temperature for 2.5 h. The reaction was assessed of its completion by the disappearance of the reactant using TLC in 2:1 (v/v) hexane:diethyl ether, $R_f = 0.5$. This reaction mixture was dried under high vacuum (<0.001 Torr) overnight. To this flask was added ca. 5 ml of anhydrous toluene and 20 ml of trimethylamine. The reaction mixture was stirred at room temperature overnight and the product was visualized using TLC in 5:1 (v/v) chloroform:methanol, $R_f = 0.4$, 70% yield. ^{31}P NMR (101.26 MHz, $CDCl_3$): δ 57.85 ppm. The two diastereomers could not be resolved in $CDCl_3$ or a mixture of $CDCl_3$ and CD_3OD. Resolution of the two diastereomers is described in the text. ^{1}H NMR (250 MHz, $CDCl_3$): δ 5.3 (m, 1 H, glycero-*sn*-2 C*H*); 4.40 – 4.00 (m, 5 H); 3.60 (dd, 2 H, $^3J_{HP} = 12.5$ Hz, $^3J_{HH} = 5.0$ Hz, serine OCH_2); 3.48 (s, 2 H, serine NH_2); 2.33 (m, 4 H, α-CH_2 of acyl chains); 1.60 (bs, 4 H, β-CH_2 of acyl chains); 1.26 (bs, 48 H, acyl chain 4-15 CH_2); 0.88 (t, 6 H, $J = 7$ Hz, acyl chain 16-CH_3). The two doublets collapsed to one with a coupling constant of 5 Hz upon irradiation of the phosphorus.

The hydrolysis of DPPsS by bee venom PLA2 was followed by ^{31}P NMR (121.5 MHz) at room temperature in a 10-mm NMR tube, using 32 mg of substrate and 0.3 mg of enzyme in 2 ml of a buffer containing 50% (v/v) D_2O, 5% (w/v) Triton X-100, 50 mM Hepes buffer (pH 7.2), 2.5 mM Ca^{2+}, and 0.25 mM disodium EDTA.

RESULTS

(R_p + S_p)-DPPsS was synthesized as shown in Scheme I. L-Serine (**3**) was first treated with trityl chloride to give *N*-trityl-L-serine (**4**), in the form of diethylam-



SCHEME I. Synthesis of (R_p + S_p)-DPPsS and hydrolysis by PLA2.

monium salt (*13*). The carboxyl group of **4** was then protected with chloromethyl methyl ether to give *N*-trityl-L-serine methoxymethyl ester (**5**) in 71% yield. 1,2-Dipalmitoyl-*sn*-glycerol (**6**) was phosphitylated with chloro(*N*,*N*-diisopropylamino)methoxyphosphine to give a phosphoramidite intermediate (**7**), and this phosphoramidite intermediate was condensed with the protected serine **5** to give a phosphite triester adduct (**8**) (*5*). The resulting phosphite triester was sulfurized with an excess of S_8 to give a phosphorothioate triester, **9**. The overall yield from

1,2-dipalmitoyl-*sn*-glycerol to **9** was 70%. (R_p + S_p)-DPPsS (**10**) was obtained in 70% yield from **9** by deprotection of the *N*-trityl group and the methoxymethyl group by acid hydrolysis followed with demethylation with trimethylamine.

Unlike other thiophospholipids, the two diastereomers of DPPsS were not readily distinguishable by proton, ^{13}C, or ^{31}P NMR. This is partly due to the fact that phosphatidylserine has a stronger tendency to aggregate and give broader NMR signals, as shown by the broad resonance in ^{31}P NMR at 58.78 ppm (Fig. 1, top spectrum). However, by treating the mixture with PLA2 from bee venom, the two isomers can be resolved due to stereospecific hydrolysis of one of the diastereomers, as shown in the remaining spectra in Fig. 1. Since PLA2 has been shown to be specific to the R_p isomer of thiophosphatidylcholine and thiophosphatidylethanolamine (*1–4*), the isomer hydrolyzed by PLA2 (δ ^{31}P 58.73 ppm) should be

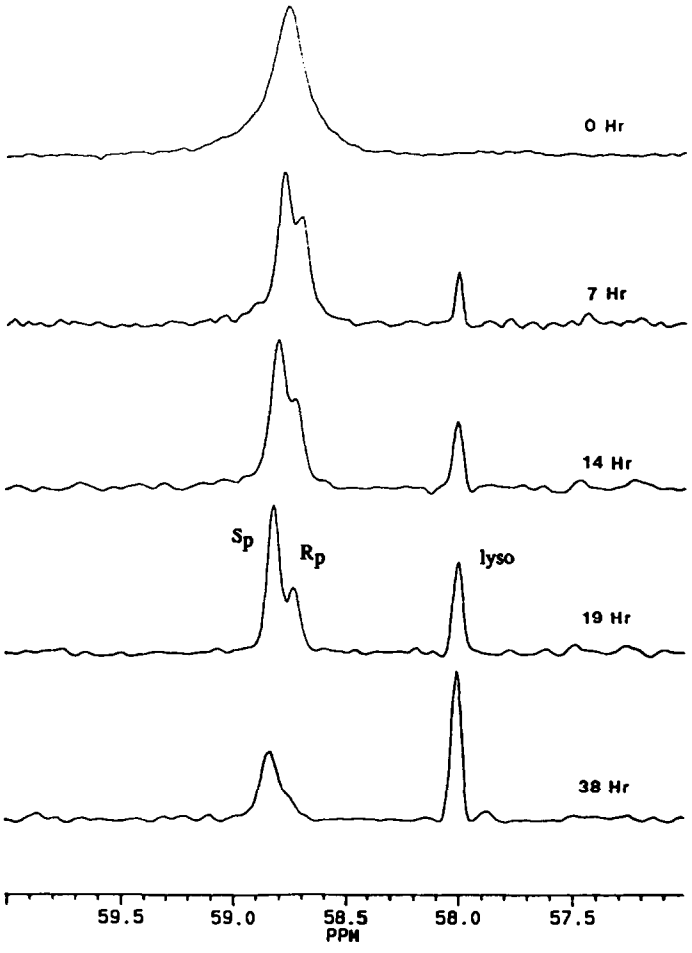
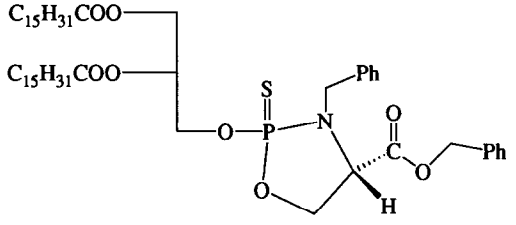


FIG. 1. Configurational assignment of R_p and S_p DPPsS. Top spectrum: ^{31}P NMR (101.26 MHz) of (R_p + S_p)-DPPsS in 50% (v/v) D_2O, 5% (w/v) Triton X-100, 50 mM Hepes buffer (pH 7.2), 2.5 mM Ca^{2+}, and 0.25 mM disodium EDTA. Remaining spectra: after partial hydrolysis by bee venom PLA2.

the R_p isomer and the other isomer (δ ^{31}P 58.84 ppm) should be the S_p isomer. The new peak at 58.01 ppm can be attributed to the lyso-product of the reaction, (R_p)-MPPsS (**11**). Such a configurational assignment should be very reliable since PLA2 shows a very stringent stereochemical requirement at the chiral phosphorus center but is relatively nonspecific toward the type of headgroup (*16*). The same approach has been used to assign the configuration of chiral thiophosphatidylinositol recently (*8*).

DISCUSSION

One of the reasons synthesis of chiral DPPsS was not achieved earlier is the limitation in choosing suitable protection for the amino and carboxyl groups. The commonly used benzyl protective group could not be used since debenzylation is a problem in the presence of both the phosphorothioate and the carboxylic ester functions. Indeed we have attempted to synthesize pure isomers (R_p and S_p) of DPPsS via the cyclic 2-thiono-1,3,2-oxazaphosphilidine intermediate (**12**). This intermediate has been synthesized and characterized, and the R_p and S_p isomers



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have been separated by column chromatography. However, debenzylation of the ring-opened product of **12** was unsuccessful by hydrogenolysis over 10% Pd–C, by trimethylsilyl iodide (*17*), by aluminum trichloride (*18*), by potassium carbonate or copper(II) sulfate in water–tetrahydrofuran (*19, 20*), by boron tribromide (*21, 22*), or by boron trifluoride and a thiol (*23, 24*). Details of these experiments have been described by Loffredo (*25*). Attempts have also been made to produce DPPsS by incubating thiophosphatidylethanolamine (S_p isomer), L-serine, and phospholipase D from cabbage or spinach. This was unsuccessful because thiophosphatidylethanolamine is a already very poor substrate for the hydrolysis reaction catalyzed by phospholipase D (*4*), and it appeared to be worse for transphosphatidylation. The final procedure presented in this paper for the synthesis of DPPsS appears to be simple and efficient.

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