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Phospholipids Chiral at Phosphorus: Synthesis of Dioleoylthiophosphatidylcholine and Stereospecificity of Lecithin–Cholesterol Acyltransferase[1]

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Chiral 1,2-dioleoyl-*sn*-glycero-3-thiophosphocholine (DOPsC) was synthesized from chiral 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine (DPPsC) via hydrolysis to *sn*-glycero-3-thiophosphatidylcholine followed by reacylation. Both DOPsC and DPPsC were used to probe the stereochemical requirement in the enzyme–substrate recognition of lecithin–cholesterol acyltransferase (LCAT) from human plasma. In contrast to phospholipase A_2, LCAT is totally insensitive to sulfur substitution or configuration at phosphorus and gives indistinguishable K_m and V_{max} values to R_p and S_p isomers of DOPsC and DPPsC and the corresponding natural substrates. The results suggest lack of stereospecific interaction between LCAT and the phosphate group of phospholipids.

INTRODUCTION

We have previously synthesized (R_p)- and (S_p)-DPPsC[3] (Fig. 1) and shown that phospholipase A_2 (PLA2) from several different sources preferentially hydrolyzes the R_p isomer whereas phospholipase C and phospholipase D prefer the S_p isomer (*1–3*). Detailed kinetic studies of bee venom PLA2 suggested that the observed stereospecificity is induced by stereospecific coordination of Ca^{2+} to the *pro-S* oxygen of the phosphate group at the active site (*4*). This would imply that the phosphate group of phospholipids is involved in the "remote stereochemical control" as part of the enzyme–substrate recognition in the catalysis by PLA2 from bee venom.

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[3] Abbreviations used: DMPA, 4-(*N*,*N*-dimethylamino)pyridine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPsC, 1,2-dioleoyl-*sn*-glycero-3-thiophosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPsC, 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine; GPsC, *sn*-glycero-3-thiophosphocholine; LCAT, lecithin–cholesterol acyltransferase; MOPsC, 1-oleoyl-*sn*-glycero-3-thiophosphocholine; MPPsC, 1-palmitoyl-*sn*-glycero-3-thiophosphocholine; PLA2, phospholipase A_2; apo A-I, apolipoprotein A-I; POPC ether, 1-palmityl-2-oleyl-*sn*-glycero-3-phosphocholine; TLC, thin-layer chromatography.

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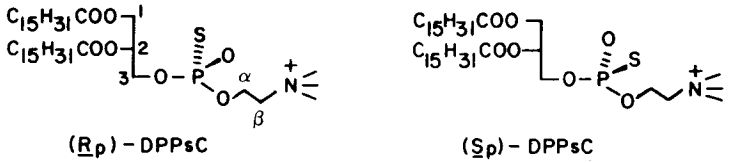

FIG. 1. Structures of (R_p)-DPPsC and (S_p)-DPPsC.

In view of the significance of these results, we have extended this study to human lecithin–cholesterol acyltransferase (LCAT, EC 2.3.1.43) (*5*). This enzyme catalyzes the formation of cholesteryl esters from cholesterol and PC in a reaction that initially involves transfer of the *sn*-2 acyl group to form an acyl-enzyme intermediate with the active site serine at residue 181 (*6, 7*). Following acyl migration to one of two equivalent serines, the acyl group is transferred to cholesterol. In the absence of cholesterol, LCAT has a PLA2 activity that does not require acyl migration (*6, 8*). Both reactions are activated by plasma apo A-I from high-density lipoproteins. Other than this functional similarity, LCAT has very different properties from PLA2. It is a glycoprotein with M_r 65,000–69,000 (*9*) and with no M^{2+} requirement. The activity of LCAT is also greatly stimulated by apolipoprotein A-I (*10*). The catalytic mechanism proposed for LCAT as described above is also significantly different from that proposed for PLA2 (*11*).

Since LCAT has been suggested to prefer unsaturated phospholipids as substrates, we synthesized unsaturated chiral thiophosphatidylcholine, (R_p)- and (S_p)-DOPsC. Both DPPsC and DOPsC were used to investigate the stereospecificity of LCAT.

EXPERIMENTAL

Materials. Apo A-I was prepared by the method of Pownall *et al.* (*12*). POPC ether was synthesized as described previously (*13*). Rexyn I-300 was purchased from Fischer. General chemicals were of reagent grade.

TLC system. The solvent system used was $CHCl_3/CH_3OH/H_2O$, 66/33/4. R_f values were 0.3 for MPPsC, 0.02 for GPsC, 0.4 for MPOsC, and 0.6 for DOPsC.

(R_p)-GPsC **(4)** *and (S_p)-GPsC* **(5).** (R_p)-MPPsC **(2)** (260 mg) was dissolved in 50 ml of methanol and mixed with 200 μl of 1 M tetrabutylammonium hydroxide in methanol. The reaction mixture was stirred at room temperature for 30 min or until the hydrolysis was complete on the basis of TLC. The mixture was then extracted according to the method of Bligh and Dyer (*14*) to remove the methyl palmitate, and the aqueous phase was treated with Rexyn I-300 to remove the base. Lyophilization of the aqueous phase gave GPsC in quantitative yield, which was characterized by ^1H, ^{13}C, and ^{31}P NMR. The two isomers showed distinct ^{31}P chemical shifts (D_2O, 121.5 MHz): (R_p)-GPsC 56.518 ppm and (S_p)-GPsC 56.540 ppm (assigned by use of a 1 : 2 mixture); ^1H NMR of (R_p)-GPsC (D_2O, 200 MHz): δ 3.16 (s, 9 H, NMe_3), 3.5-3.65 (m, 4 H), 2.8-4.0 (m, 3 H), 4.3 (m, 2 H). No

detectable difference was observed for (S_p)-GPsC. ^{13}C NMR of ($R_p + S_p$)-GPsC (CD_3OD, 50.3 MHz): δ 55.02 (NCH_3, $^1J_{CN}$ = 3.3 Hz), 60.89 (CH_2CH_2N, $^2J_{CP}$ = 5.1 Hz), 64.11 (*sn*-1 CH_2OH), 67.49 (tentatively *sn*-3 CH_2OP, $^2J_{CP}$ = 3.8 Hz), 68.20 (tentatively CH_2N, $^1J_{CN}$ = 2.8 Hz), 72.47 (*sn*-2 CHOH, $^3J_{CP}$ = 7.7 Hz).

(R_p)-DOPsC **(6)** *and (S_p)-DOPsC* **(7)**. GPsC (from 0.28 mmol of DPPsC) and DMPA (0.2 mmol) were dried *in vacuo* (<0.01 mm Hg) overnight and dissolved in 2 ml of dry pyridine. One gram of molecular sieve 3A was added to the solution under argon and stirred for 1 h. Oleoyl chloride (264 μl, 0.8 mmol) was then added to the solution. The reaction was complete after stirring at room temperature for 48 h, as monitored by TLC. The solvent was removed by rotary evaporation and replaced with chloroform. After extraction according to the method of Bligh and Dyer (*14*) (1 N HCl was added during extraction to maintain the pH at 6–6.5), the organic layer was dried and loaded to a silica gel column, eluted first with $CHCl_3$/methanol (2/3, 50 ml) to remove excess reagents. Further elution with $CHCl_3$/CH_3OH/H_2O (66/33/4) gave DOPsC (50% yield) followed with monoacylated by-products. The product was characterized by ^{31}P, ^1H, and ^{13}C NMR. The two isomers showed distinct ^{31}P chemical shifts (81.0 MHz, $CDCl_3$): (R_p)-DOPsC 56.591 ppm and (S_p)-DOPsC 56.615 ppm (assigned by use of a 1 : 2 mixture); ^1H NMR (CD_3OD, 200 MHz): δ 0.89 (t, terminal methyl), 1.3 (b, methylene groups), 1.59 (b, CH_2CH_2COO), 2.01 (bd, $CH_2CH{=}C$), 2.30 (t, J_{vic} = 7.2 Hz, *sn*-1 CH_2COO), 2.33 (t, J_{vic} = 7.2 Hz, *sn*-2 CH_2COO), 3.23 (s, NCH_3), 3.65 (t, J = 4.7 Hz, CH_2CH_2N), 4.0 (m, 3-CH_2OP), 4.2 (m, *sn*-1 CH_aH_bOCO), 4.4 (m, *sn*-1 CH_aH_bOCO), 4.37 (m, $POCH_2CH_2N$), 5.25 (m, 2-CHOCO), 5.33 (t, J_{vic} = 4.6 Hz, $CH{=}CH$); ^{13}C NMR of ($R_p + S_p$)-DOPsC (CD_3OD, 125.7 MHz): δ 14.52 (terminal methyl), 23.76 (CH_3CH_2), 26.04 and 26.06 (CH_2CH_2COO), 28.21, 30.25, 30.40, 30.48, 30.65, 30.88, 33.09, 35.01, and 35.17 (CH_2CH_2COO), 54.87 (NCH_3), 60.85 (CH_2CH_2N), 63.80 (1-CH_2O), 65.15 (3-CH_2OP, $^2J_{CP}$ = 3.8 Hz), 67.39 (CH_2N), 71.87 (2-CHO, $^3J_{CP}$ = 8.8 Hz), 130.84 and 130.99 ($CH{=}CH$), 174.61 and 174.93 (C=O). Extreme care was taken (working under an inert atmosphere and degassing of all solvents) in handling the unsaturated lecithins in order to prevent autooxidation of the double bonds. The best condition for sample storage was in degassed, purified chloroform in sealed ampules, under nitrogen, stored at −70°C.

Procedures for LCAT studies. Human plasma LCAT was purified by a modification of the procedure of Albers *et al.* (*15*). This involved chromatography on (i) phenyl-Sepharose, (ii) DEAE-Sepharose, (iii) Cibachrome Blue, and (iv) hydroxylapatite and Sephadex G-100. The resulting protein was a single band in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The test substrates were model high-density lipoproteins composed of POPC ether, the test substrate analog, cholesterol, and apo A-I in a molar ratio of 90 : 10 : 4 : 1, respectively, and were prepared by the cholate removal technique (*16*). The POPC ether was used in excess to ensure that we were comparing the molecular species and not the microenvironmental specificity of LCAT. Substrate saturation studies were conducted at 37°C by monitoring the formation of [^{3}H]cholesterol esters (*13*).

Instrumental and spectroscopic methods. ^{31}P, ^{13}C, and ^1H NMR spectroscopies were performed on Bruker WP-200, MSL-300, and AM-500 NMR spectrometers. The chemical shifts for ^{13}C and ^1H are referenced to internal Me_4Si, whereas ^{31}P

chemical shifts are referenced to external 85% H_3PO_4 at 25°C. The + signal indicates a downfield shift in all cases. The ^{13}C and ^{31}P NMR spectra were obtained with broadband ^1H decoupling unless otherwise specified.

RESULTS AND DISCUSSION

Synthesis of chiral thiophosphatidylcholines. In our previous work DPPsC was synthesized from 1,2-diplamitin, $PSCl_3$, and choline tosylate (*17*). We have now switched to the more effective method developed by Bruzik *et al.* (*18*), which uses a key reagent $ClP(OMe)N(i\text{-}Pr)_2$. The diastereomeric mixture thus obtained was separated into R_p and S_p isomers as described earlier (*1*).

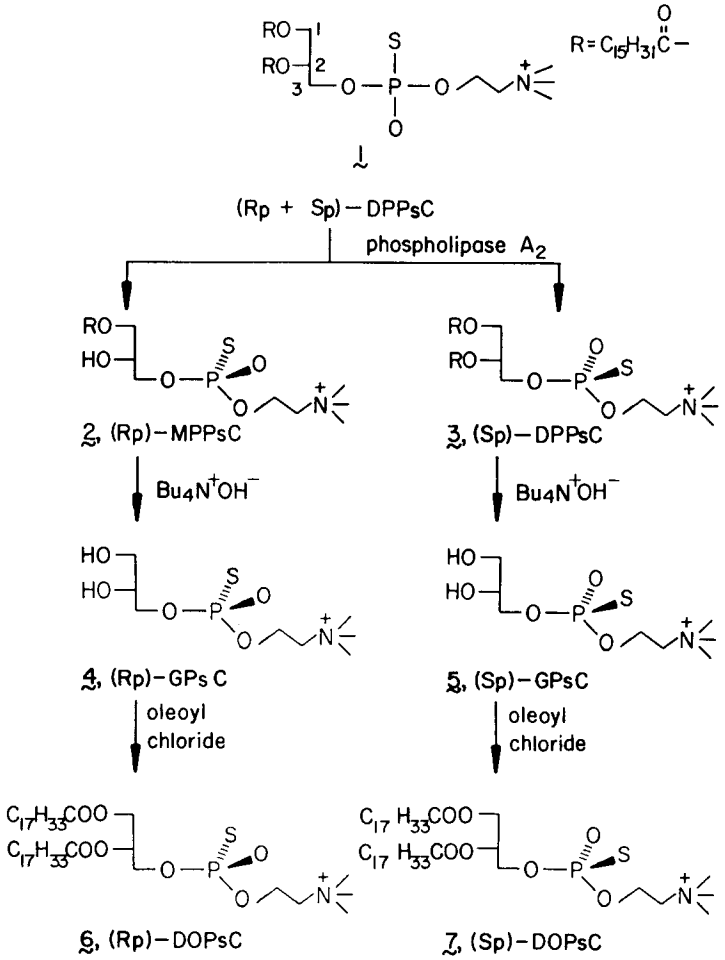

FIG. 2. Procedures for the synthesis of (R_p)-DOPsC **(6)** and (S_p)-DOPsC **(7)**.

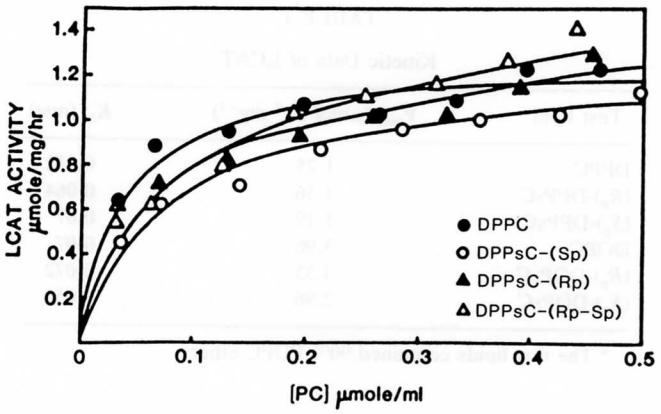

FIG. 3. Substrate saturation curves for cholesteryl ester formation from DPPC (●), (R_p)-DPPsC (▲), (S_p)-DPPsC (○), and ($R_p + S_p$)-DPPsC (△). Substrates were model high-density lipoproteins containing POPC ether, test substrate, cholesterol, and apo A-I in a molar ratio of 90 : 10 : 4 : 1.

Isomers of DOPsC can in principle be synthesized by two pathways: from 1,2-dioleoyl-*sn*-glycerol following the procedure for DPPsC, or from isomers of DPPsC. There are two problems with the former procedure: isomerization of 1,2-dioleoyl-*sn*-glycerol and oxidation of the unsaturated acyl chains. The latter pathway can avoid these problems (1,2-dipalmitin is more stable, and unsaturated acyl chains were introduced in the last step) and can also be adopted to synthesize thiophosphatidylcholines with any acyl chains. We therefore used the latter procedure as outlined in Fig. 2. Incubation of ($R_p + S_p$)-DPPsC **(1)** with bee venom PLA2 gave (R_p)-MPPsC **(2)** and unreacted (S_p)-DPPsC **(3)** (*1*). Because the stereospecificity of PLA2 toward (R_p)-DPPsC is not absolute (*4*), the digestion was carried out in two steps. The first digestion was quenched before the R_p isomer was completely hydrolyzed in order to avoid hydrolysis of the S_p isomer, while the second step was an exhaustive digestion to remove trace (R_p)-DPPsC. Hydrolysis of **2** and **3** with tetrabutylammonium hydroxide (*19*) gave (R_p)-GPsC **(4)** and (S_p)-GPsC **(5)**, respectively. The isomeric purity of **4** and **5** was judged to be >98% since no contaminating isomer was detectable by ^{31}P NMR when signal-to-noise ratios were >50. Acylation of **4** and **5** with oleoyl chloride gave (R_p)-DOPsC **(6)** and (S_p)-DOPsC **(7)**, respectively.

Lack of stereospecificity of LCAT. The substrate saturation curves of DPPsC for the production of cholesteryl esters by LCAT are similar to that of DPPC, irrespective of the chirality at phosphorus (Fig. 3). This is reflected in both V_{max} and K_m given in Table 1. Essentially the same results were obtained for the unsaturated substrate DOPsC as also shown in Table 1. The results are in sharp contrast to those of PLA2 and suggest that the interaction of LCAT with substrates does not involve a stereospecific binding with the phosphate group. This is consistent with the lack of M^{2+} requirement in the catalysis by LCAT.

Thus, although a polar headgroup on the diacylglycerol is required for substrates

TABLE 1

Kinetic Data of LCAT

Test lipid[a]	V_{max} (μmol h^{-1} mg^{-1})	K_m (mM)
DPPC	1.25	0.032
(R_p)-DPPsC	1.36	0.064
(S_p)-DPPsC	1.19	0.07
DOPC	3.96	0.07
(R_p)-DOPsC	3.35	0.072
(S_p)-DOPsC	2.96	0.056

[a] The test lipids contained 90% POPC ether.

of LCAT, the structure of the polar headgroup of a phosphoglyceride appears to be a relatively weak determinant of the acyl donor activity. There was also no great preference for unsaturation for the substrate, as reflected in the kinetic data in Table 1. Although earlier work suggested that unsaturated fatty acyl chains were transferred selectively, recent studies indicated that this is largely a microenvironmentally derived specificity. In fact, most of the common phospholipids are LCAT substrates if they are placed in a fluid environment such as high-density lipoprotein (*20*) or POPC (*13*).

REFERENCES

1. Bruzik, K., Jiang, R.-T., and Tsai, M.-D. (1983) *Biochemistry* **22,** 2478–2486.
2. Jiang, R.-T., Shyy, Y.-J., and Tsai, M.-D. (1984) *Biochemistry* **23,** 1661–1667.
3. Bruzik, K., and Tsai, M.-D. (1990) *in* Methods in Enzymology, in press.
4. Tsai, T.-C., Hart, J., Jiang, R.-T., Bruzik, K., and Tsai, M.-D. (1985) *Biochemistry* **24,** 3180–3188.
5. Jonas, A. (1987) *in* Plasma Lipoproteins (Gotto, A. M., Jr., Ed.), Vol. 14, pp. 299–329, Elsevier, Amsterdam.
6. Jauhiainen, M., and Dolphin, P. J. (1986) *J. Biol. Chem.* **261,** 7032–7043.
7. Yang, C.-Y., Manoogian, D., Pao, Q., Lee, F.-S., Knapp, R. D., Gotto, A. M., Jr., and Pownall, H. J. (1987) *J. Biol. Chem.* **262,** 3085–3091.
8. Aron, L., Jones, S., and Fielding, C. J. (1978) *J. Biol. Chem.* **253,** 7220–7226.
9. Marcel, Y. L. (1982) *Adv. Lipid Res.* **19,** 85–136.
10. Smith, L. C., Pownall, H. J., and Gotto, A. M., Jr. (1978) *Annu. Rev. Biochem.* **47,** 751–777.
11. de Haas, G. H., and Volwerk, J. J. (1982) *in* Protein-Lipid Interactions (Jost, P. C., and Griffith, O. H., Eds.), Vol. 1, pp. 69–149. Wiley, New York.
12. Pownall, H. J., Massey, J. B., Kusserow, S. K., and Gotto, A. M., Jr. (1978) *Biochemistry* **17,** 1183–1188.
13. Pownall, H. J., Pao, Q., and Massey, J. B. (1985) *J. Biol. Chem.* **260,** 2146–2152.
14. Bligh, C. B., and Dyer, W. (1959) *Canad. J. Biochem. Physiol.* **37,** 911–918.
15. Albers, J. J., Lin, J. T., and Robert, G. P. (1979) *Artery* **5,** 61–75.
16. Matz, C. E., and Jonas, A. (1982) *J. Biol. Chem.* **257,** 4535–4540.
17. Vasilenko, I., DeKruijff, B., and Verkleij, A. J. (1982) *Biochim. Biophys. Acta* **685,** 144–152.
18. Bruzik, K., Salamonczk, G., and Stec, J. (1986). *J. Org. Chem.* **51,** 2368–2370.
19. Brockerhoff, H., and Yurkowski, M. (1965) *Canad. J. Biochem.* **43,** 1777.
20. Ueno, K., Sakuma, N., Kawaguchi, M., Fujinami, T., and Okuyama, H. (1986) *J. Biochem.* **99,** 541–547.