An efficient synthesis of phosphatidylcholines

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Summary This article deals with two of the major steps involved in phospholipid synthesis: the preparation of the optically pure precursors, *sn*-glycero-3-phosphocholine (GPC) and -ethanolamine, from a convenient lipid source such as egg yolk, and acylation of hydroxyl groups present in those precursors involving an acid to yield the corresponding phospholipid. Phosphatidylcholines and phosphatidylethanolamines were separated from lipids extracted from egg yolk using low-pressure column chromatography. The advantages of this method include the use of smaller volumes of solvents and silica gel and reuse of adsorbent. Acylation of GPC is aided by ultrasound from a common laboratory bath cleaner. Ultrasound-assisted base-catalyzed esterification of GPC is accomplished between 2–6 hours providing a phospholipid in more than 80% yield. This scheme is particularly valuable in the synthesis of polymerizable phospholipids. —**Singh, A.** An efficient synthesis of phosphatidylcholines. *J. Lipid Res. 1990.* **31:** 1522–1525.

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During the course of our studies on polymerizable phospholipids (1–5), we felt the need for a reliable and efficient method for phosphatidylcholine (PC) synthesis. The higher cost and the difficulty in procurement of pure chemically modified phospholipids, particularly polymerizable phospholipids, make them less attractive candidates for most technological applications. Ample reports exist in the literature describing the individual steps involved in the synthesis of phospholipids, such as isolation or preparation of isomerically pure precursors (6–8), acylation of the hydroxyl groups on precursors (8–14), the use of efficient catalysts (9, 10), and purification of the synthesized phospholipids. However, most of the schemes reported in the literature have limitations including consumption of large volumes of solvents, loss of phosphatidylethanolamine (PE) during chromatographic separation of egg PC, long *sn*-glycero-3-phosphocholine (GPC) acylation time, and a low yield of lipids. In this report, we describe a convenient, economical, and general method for phosphatidylcholine synthesis on an intermediate scale.

MATERIALS AND METHODS

Polymerizable acid anhydrides were prepared as described earlier (2, 3, 15). Both saturated and diacetylenic acid anhydrides were recrystallized from acetone. Nonpolymerizable lipids were purchased from Avanti Polar Lipids, and authentic polymerizable lipids reported here were synthesized following published procedures (3, 15). Dimethylaminopyridine was recrystallized from an ether–hexane mixture. Mixed-bed ion exchange resin, AG-501-X8 (D) was procured from Bio-Rad laboratories. The column and accessories for low pressure chromatography, including silica gel (230–400 mesh) were purchased from Aldrich Chemical Company. Chloroform for the synthesis was freshly distilled over phosphorus pentoxide. The other solvents used were HPLC grade. Thin-layer chromatographic (TLC) plates of silica gel were purchased from E. Merck. TLC plates were developed in chloroform–methanol–water 65:25:4 (v/v). The lipid spots were routinely revealed by charring and/or by molybdate, Dragendorff's reagent, or ninhydrin sprays (Sigma Chemical Company). Ultrasound was produced in an L & R T-9 (Sargent-Welch) bath type sonicator (45 KHz, 35 W). Neutral alumina (Brockman activity 1, 80–200 mesh) was procured from Fisher Scientific Co. GPC · $CdCl_2$ complex was prepared from egg PC as previously reported (4).

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; GPC, glycerophosphocholine; TLC, thin-layer chromatography; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine.

Extraction of egg yolk lipids

The egg yolk isolated from fresh eggs was washed thoroughly with acetone until the washings were colorless. The mixed lipids were extracted at room temperature from the resulting powdered egg yolk using chloroform–methanol 1:1. The solvents from the lipid extract were removed by rotary evaporation at 35°C under reduced pressure, followed by high vacuum overnight at room temperature.

Chromatography on neutral alumina

The mixed lipids thus obtained were charged on a 4.5 × 60 cm column prepared from 650 g alumina in chloroform. The column was initially eluted with chloroform to remove the colored materials present in the lipid mixture. Subsequent elution was performed by selected ratios of a chloroform–methanol solvent system. The elution rate was set at 4 ml/min. For overnight unattended runs, the rate was adjusted to 1.5 ml/min and the column was mounted on a fraction collector. Fractions at 25-ml intervals were analyzed by TLC for lipid content. The column was stopped once lecithin ceased to be eluted or upon observing the appearance of lysolecithin. Fractions containing PC were combined and the solvent was removed to collect pure PC.

Chromatography on silica gel

Silica gel, 150 g, was suspended in chloroform and the resulting slurry was poured into a low pressure chromatography column. The column was eluted with chloroform by applying pressure (10 psi) from an argon cylinder which simultaneously compressed the silica gel. This resulted in a 4.5 cm × 17 cm column. The column was charged with 60 g of lipid mixture in chloroform and initially eluted with chloroform at a rate of 10 ml/min. Subsequent elutions were made using various mixtures of chloroform and methanol. All fractions were analyzed and handled similarly as described above.

General procedure for preparation of phosphatidylcholines

Procedures similar to that used for the preparation of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were followed for all of the reactions reported in Table 1.

Into a 50-ml single-necked round-bottomed flask containing *sn*-glycero-3-phosphocholine-$CdCl_2$ complex (55 mg, 0.12 mmol) was added 148 mg (0.3 mmol) of palmitic anhydride dissolved in 10 ml of chloroform. While the resulting suspension was vigorously stirred with a Teflon-coated magnetic stirring bar, 4-dimethylamino pyridine (37 mg, 0.3 mmol) was added. The contents of the flask were then degassed with nitrogen, stoppered, protected from light, and stirred for 48 h at room temperature. The progress of the reaction was monitored by TLC on silica gel $CHCl_3$–CH_3OH–H_2O 65:25:4 and was assumed to be complete when all of the lysolecithin, formed as an intermediate, had disappeared. The chloroform was removed under reduced pressure (room temperature), and the residue was dissolved in 5 ml of $CHCl_3$–CH_3OH–H_2O 4:5:1 and passed through a column (1 cm × 17 cm) of mixed-bed resin. The column was washed with 20 ml of the same solvent. The fractions consisting of phospholipids were combined and the solvent was removed at 25°C by applying vacuum. The vacuum-dried reaction mixture was then dissolved in a minimum volume of chloroform and further purified by chromatography on a column of silica gel (1 cm × 20 cm). Elution was performed by the following solvent systems in succession: chloroform, chloroform–methanol 9:1, chloroform–methanol 1:1, and chloroform–methanol 1:9. Fractions were analyzed by TLC and those fractions containing a product with an R_f identical to that of authentic DPPC were combined and the solvent was evaporated. The phospholipid thus obtained (80 mg, 88%) had IR and ^1H NMR spectra identical to those of an authentic sample.

Ultrasound

Using procedures identical to those described above, a 50-ml thin-walled, round-bottomed flask containing the identical quantities of GPC-$CdCl_2$, chloroform, palmitic anhydride, and 4-dimethylamino pyridine was immersed in an ultrasonic cleaner and was positioned approximately one-half inch above the center of the floor of the bath. The mixture was degassed and irradiated with ultrasound for 5 h. The reaction was worked-up as described above and afforded 89 mg (98% yield) of DPPC.

RESULTS AND DISCUSSION

The synthesis of phospholipids reported here was accomplished in two steps: *a*) preparation of *sn*-glycero-3-phosphocholine from egg PC, and *b*) acylation of GPC with an acid anhydride to yield the corresponding phospholipid.

Chromatographic separation of egg PC

Classical separation of the lipid components by chromatography on an alumina column (13) results in the loss of PE, a technically useful lipid component. The only products isolated are PC and lysoPC. Alumina, while good for preparing PE-free PC, is not suitable for the separation of mixed lipids on a preparative scale. The use of silica gel allowed good separation of both PC and PE without any preferential irreversible binding to any lipid components. Low pressure chromatography (16) on a column of fine mesh silica gel (particle size 60 Å) is an attractive alternative, being faster and requiring smaller

quantities of silica gel and solvent(s). On an average run, 60 g mixed lipids afforded 12 g egg PC when chromatographed over a column made from 650 g of alumina using a total of 5.5 liters of solvents. On the other hand, similar amounts of mixed lipids afforded 15 g egg PC and 3 egg PE on a silica gel column eluted with 4 liters of solvents. In our hands, a single silica gel column could be used repeatedly up to four cycles without losing resolution efficiency.

sn-Glycero-3-phosphocholine acylation

Base-catalyzed acylation of *sn*-glycero-3-phosphocholine and lysolecithin with fatty acid anhydrides provides an efficient synthetic route to phosphatidylcholines. One of the widely used procedures developed for such conversions is that reported by Gupta, Radhakrishnan, and Khorana (9), where 4-dimethylamino pyridine is used as the catalyst. This procedure, though efficient, has one major drawback by being slow. This may cause some specialty lipids, particularly polymerizable phospholipids, to undergo undesirable side reactions. Our efforts on the synthesis of phospholipids bearing polymerizable moieties, such as methacrylate, diacetylene, or a combination of both, resulted in fair to good yields of product (1–5).

Acylation of GPC is a biphasic reaction. The acylation rates are dependent not only on the efficiency of the agitation of the reactants but also on the availability of clean reactive surfaces for the reactant GPC · $CdCl_2$. During the course of these studies we discovered that simple replacement of magnetic stirring with ultrasound, derived from a common laboratory cleaner, significantly increased the rates of acylation and improved the yields (**Table 1**). Treatment of a suspension of GPC · $CdCl_2$ in chloroform with palmitic anhydride using a procedure similar to that described by Gupta et al. (9) afforded an 88% isolated yield of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) after 48 h stirring at room temperature. In contrast, a similar preparation using ultrasound as the means of agitation produced an almost quantitative yield of DPPC after only 5 h. Similar results were found for the synthesis of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), DMaPC (5), DMPC (2), 1-palmitoyl,2-MaPC (7), and DAPC (4) (see Table 1). Whereas acylation of 1-(12-methacryloyloxy)dodecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine with palmitic acid anhydride gave erratic yields (33–68%) of 6 when magnetic stirring was used, ultrasound-assisted reactions consistently afforded isolated yields in excess of 80% after 2 h. In the case of the diacetylenic lipid, 4, the high yields were not obtained every time. In our hands, one out of our batches produced a lower yield of lipid. During ultrasound agitation the bath temperature rises up to 50°C. We have taken care to maintain the bath temperature at or below 30°C. To demonstrate that the increase in the rate of acylation

TABLE 1. Phosphatidylcholine synthesis

Reactants	Product	Method of Agitation	Time	Yield
			h	%
GPC · $CdCl_1$				
+ Palmitic anhydride	1, DPPC	Magnetic	48	88
		Ultrasound	5	98
+ Myristic anhydride	2, DMPC	Magnetic	41	55
		Ultrasound	5	69
+ Oleic anhydride	3, DOPC	Magnetic	41	37
		Ultrasound	5	48
+ Diacetylenic anhydride[a]	4, DAPC[b] (ref. 20)	Magnetic	48	60
		Ultrasound	8	30–65
+ Methacrylate anhydride[c]	5, DMaPC[d] (ref. 5)	Magnetic	48	36
		Ultrasound	10	63
Lyso MaPC[e]				
+ Palmitic anhydride	6, 1 Ma, 2 Palmitoyl PC (ref. 5)	Magnetic	24	33–68
		Ultrasound	2	85
Lyso PPC				
+ Methacrylate anhydride	7, 1 Palmitoyl, 2 Ma PC (ref. 5)	Magnetic	72	83
		Ultrasound	5	85

[a]Tricosa-10,12-diynoic acid anhydride.
[b]1,2 Bis(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine.
[c]12-Methacryloyloxy dodecanoate anhydride.
[d]1,2-Bis(12-methacryloyloxy) dodecanoyl-*sn*-glycero-3-phosphocholine.
[e]1-(12-Methacryloyloxy) dodecanoyl,2-hydroxy-*sn*-glycero-3-phosphocholine.

is not due to a rise in bath temperature, we placed the reaction flask in the bath in a position unsuitable for ultrasound agitation. We did not observe any appreciable appearance of phospholipid in the reaction mixture up to a period of 5 h. The ultrasound has been frequently used as tool in organic synthesis for achieving higher yields (17–19) of the products, particularly when the reactants are in heterogeneous phase.

In order to confirm that no significant acyl migration from C-1 to C-2 occurred during the acylation of the OH group on C-2, 1-palmitoyl, 2-(12-methacryloyloxydodecanoyl)-*sn*-glycero-3-phosphocholine was hydrolyzed enzymatically with phospholipase A_2 (crude rattlesnake venom, *Crotalus adamanteus*) (20). The ^1H NMR spectrum of the recovered fatty acid indicated purity of 12-methacryloyloxydodecanoic acid greater than 95%. Analysis by gas chromatography and TLC confirmed the absence of palmitic acid.

The modified procedures described herein for the synthesis of phosphatidylcholines are simple and allow for significantly improved rates and isolated yields.

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