

Improved method for the synthesis of phosphatidylcholines

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Summary An improved method for the synthesis of phosphatidylcholines from phosphatidic acid and choline is described. The technique utilizes the tetraphenylborate salt of choline together with the condensing agent 2,4,6-triisopropylbenzenesulfonyl chloride. The yields in the reaction are consistently in the range 70–75%.—**Harbison, G. S., and R. G. Griffin.** Improved method for the synthesis of phosphatidylcholines. *J. Lipid Res.* 1984. **25**: 1140–1142.

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The synthesis of phospholipids isotopically labeled in the glycerol backbone or the head group often involves the coupling of a phosphatidic acid (PA) with an alcohol. In many cases this has been accomplished with the condensing agent 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) in pyridine, as described by Aneja, Chadha, and Davies (1). Using this approach it is possible to obtain phosphatidylethanolamines in reasonably high yields. However, the synthesis of phosphatidylcholine (PC) with this method has been difficult because of the insolubility of choline salts in solvents such as pyridine. In an attempt to circumvent this difficulty, Aneja and Chadha (2), employed choline acetate and elevated temperatures (70°C) in order to produce PC from PA. Nevertheless, the yield of this reaction is highly variable and in the best cases is generally 40–60%. Furthermore, choline acetate is a hygroscopic compound which requires laborious and extensive drying prior to use.

An alternative to the Aneja and Chadha method was recently described by Kingsley and Feigenson (3), and utilized “choline periodide”, a charge-transfer complex of choline iodide with 4 mol/mol of iodine. Using this method, we have encountered problems with the production of iodoform and its removal from the choline salt. Furthermore, choline periodide is stoichiometrically variable and tends to release free iodine on vacuum

Abbreviations: PA, phosphatidic acid; PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DMPA, dimyristoylphosphatidic acid; TPS, 2,4,6-triisopropylbenzenesulfonyl chloride; BPh₄⁻, tetraphenylborate; PMR, proton magnetic resonance; TLC, thin-layer chromatography.

drying or storage, yielding a liquid or semi-liquid product (cf. footnote on p. 806 of ref. 4). Finally, large quantities of iodine must be removed after the coupling.

Following a suggestion of Eibl (5), who noted its success in the preparation of phosphatidylcarnitine and phosphatidyl β-methylcholine (6), we have investigated the use of the tetraphenylborate (BPh₄⁻) salt of choline in the synthesis of PC. The advantages of using this derivative are threefold. Firstly, choline⁺ BPh₄⁻ is sparingly soluble in water and thus allows choline to be precipitated selectively and completely from preparative mixtures. Secondly, choline⁺ BPh₄⁻ is easily crystallized and non-hygroscopic, requiring little attention to prior drying before coupling to PA. Thirdly, it is highly soluble in organic solvents of moderate polarity such as pyridine, meaning that the coupling reaction may be carried out at room temperature or slightly above, minimizing the influence of side reactions and allowing high yields. We describe here the preparation of some deuterated choline tetraphenylborates, and their use in a relatively high-yield synthesis of dimyristoylphosphatidylcholine (DMPC).

EXPERIMENTAL PROCEDURES AND RESULTS

Materials and methods

Dimyristoylphosphatidic acid (DMPA), in the form of its 4,4'-dimethylaminopyridinium salt, was prepared by the method of Kingsley and Feigenson (3). Choline chloride was obtained from Sigma Chemical Co., St. Louis, MO. Sodium tetraphenylborate (gold label grade), ethyl cyanofornate, ethanolamine, and Merck silica gel were purchased from Aldrich Chemical Co., Milwaukee, WI, dimethylglycine ethyl ester from Pfaltz and Bauer Stamford, CT, Rexyn-300 from Fisher Chemical Co., Fairlawn, NJ, and lithium aluminum deuteride and methyl-d₃ iodide from Stohler Isotope Chemicals, Waltham, MA. Pyridine (reagent grade) was purified first by dropwise addition of chlorosulfonic acid (10 ml per liter of pyridine), then distillation, and finally redistillation over potassium hydroxide through a fractionating column. All other chemicals used were reagent grade.

Synthesis of phosphatidylcholines

Choline BPh₄. Choline tetraphenylborate is prepared by adding 6.85 g (20 mmol) of sodium tetraphenylborate (Na⁺BPh₄⁻), dissolved in 100 ml of water, to 4.2 g (30 mmol) of choline chloride in the same volume of water. The gelatinous precipitate is filtered off, washed with 4 × 100 ml of water, pressed dry on the filter, and then dried azeotropically with ethanol and benzene. The yield is essentially quantitative (8.4 g). Choline tetraphenylborate crystallizes from acetonitrile as large hex-

agonal prisms, which are highly soluble in dimethylformamide and dimethylsulfoxide, slightly soluble in methanol and cold acetonitrile, and insoluble in water, chloroform, and hydrocarbon solvents.

The crystalline material appears to be indefinitely stable at -20°C ; however, lower yields in subsequent steps have been encountered using choline tetraphenylborate which had been stored for several months at room temperature, although the substance was superficially unchanged after this period. Thus, it is suggested that the material be freshly prepared before use.

Analysis ($\text{C}_{29}\text{H}_{34}\text{BNO}$): calculated: C 82.26%, H 8.09%, B 2.55%, N 3.31%; found (duplicate): C 82.28%, 82.28%, H 8.09%, 8.06%, B 2.56%, 2.59%, N 3.33%, 3.49%. Proton magnetic resonance (0.1 M in $\text{DMSO}-d_6$, 270 MHz): $\delta = 3.05$, singlet, 9H, choline methyls; $\delta = 3.34$, triplet, $J = 4.9$ Hz, 2H, choline 2,2' protons; $\delta = 3.79\text{--}3.81$, broadened multiplet, 2H, choline 1,1' protons; $\delta = 5.26$, triplet, $J = 4.1$ Hz, 1H, choline OH; $\delta = 6.76\text{--}6.81$, multiplet, 4H, tetraphenylborate 4 (para) protons; $\delta = 6.90\text{--}6.96$, multiplet, 8H, tetraphenylborate 3,5 (meta) protons; $\delta = 7.15\text{--}7.21$, broadened multiplet, 8H, tetraphenylborate 2,6 (ortho) protons.

Choline-(methyl- d_3) $_3^+$ BPh $_4^-$. Ethanolamine (1.2 ml, 20 mmol) and sodium hydroxide (4.5 g, 112 mmol) were dissolved in 100 ml of 75% aqueous ethanol and to the solution was added 11.6 g (80 mmol) of methyl- d_3 iodide. After standing at room temperature for 6 hr, the solution was concentrated to 15 ml in vacuo, diluted to 200 ml with water, and to it was added a solution of 8.0 g (23.4 mmol) of $\text{Na}^+\text{BPh}_4^-$ in 100 ml of water. The choline-(methyl- d_3) $_3^+$ BPh $_4^-$ was filtered off, dried, and purified as described above. Yield, 8.1 g (96%).

Choline-1,1'- d_2^+ BPh $_4^-$. Ten ml (8.0 g, 61 mmol) of dimethylglycine ethyl ester in 50 ml of anhydrous ether was added dropwise to 2.0 g (48 mmol) of LiAlD_4 suspended in 100 ml of absolute ether. After refluxing 1 hr, the mixture was decomposed by careful dropwise addition of 10 ml of water. After hydrogen evolution had ceased, 15 ml of CH_3I was added and the mixture was stirred overnight. It was then filtered and the precipitate was washed with four 25-ml portions of 10% NaOH in water. The combined filtrate and washings were partitioned to remove ether, washed once with 100 ml of ether, and then 34 g of $\text{Na}^+\text{BPh}_4^-$ in 350 ml of water was added. The choline-1,1'- d_2^+ BPh $_4^-$ was filtered and purified as already described. Yield, 19.9 g (77%).

Choline- d_{13}^+ BPh $_4^-$. Ethyl cyanofornate (3.17 g, 32 mmol) in 50 ml of anhydrous tetrahydrofuran was added dropwise to 2.0 g of LiAlD_4 in 100 ml of anhydrous THF; the mixture was refluxed 3 hr and then cooled to room temperature. After careful decomposition with 10 ml of water, 50 ml of 10% aqueous sodium

hydroxide was added, followed by 18.6 g (129 mmol) of methyl- d_3 iodide, and stirring was continued overnight. After filtration, the mixture was evaporated to a small volume in vacuo, then diluted to 100 ml with water, and washed with 100 ml of ether. To this solution was added 13.7 g of $\text{Na}^+\text{BPh}_4^-$ in 150 ml of water. The choline- d_{13}^+ BPh $_4^-$ was filtered and purified as already described. Yield, 5.8 g (43%).

Dimyristoylphosphatidylcholine. Dimyristoylphosphatidic acid (715 mg, 1 mmol), 4,4'-dimethylaminopyridinium salt, was dried by repeated evaporation of anhydrous pyridine. It was then dissolved in 20 ml of the same pyridine, being warmed slightly to achieve full dissolution. Nine hundred mg of TPS (3 mmol, freshly recrystallized from *n*-pentane containing 1% thionyl chloride before use) was added followed by 870 mg (2 mmol) of choline $^+$ BPh $_4^-$, and the mixture was stirred for 4 hr at $30\text{--}35^{\circ}\text{C}$. Excess TPS was then decomposed with 1 ml of water, and the water and pyridine were evaporated at room temperature in vacuo. The residue was taken up in methylene chloride-methanol 1:1, passed through a 100-g column of Rexyn-300 (pre-washed with 200 ml of methanol), and again evaporated. The DMPC was then purified by single column chromatography on silica gel (50 g column, Merck grade silica gel; eluant, methylene chloride-methanol-water 10:10:1). Yield of DMPC was 515 mg (76%). The purity of the DMPC was confirmed by PMR and by TLC.

Proton magnetic resonance (10 mg/ml in CD_2Cl_2 , 270 MHz): $\delta = 0.88$, triplet, $J = 6.3$ Hz, 6H, acyl $-\text{CH}_3$; $\delta = 1.26$, broad singlet, 4OH, acyl $-\text{CH}_2-$; $\delta = 1.54\text{--}1.60$, broad multiplet, 4H, acyl $\beta\text{-CH}_2$; $\delta = 2.24\text{--}2.32$, multiplet, 4H, acyl $\alpha\text{-CH}_2$; $\delta = 3.36$, singlet, 9H, choline $-\text{CH}_3$; $\delta = 3.76\text{--}3.82$, multiplet, 2H, choline $-\text{CH}_2\text{N}$; $\delta = 3.86\text{--}3.98$, multiplet, 2H, choline $-\text{CH}_2\text{O}$; $\delta = 4.13$, doublet of doublets, $J_{\text{gem}} = -12.1$, $J_{\text{vic}} = 7.3$, 1H, glycerol H1; $\delta = 4.25\text{--}4.33$, multiplet, 2H, glycerol H3,3'; $\delta = 4.39$, doublet of doublets, $J_{\text{gem}} = -12.1$, $J_{\text{vic}} = 2.2$, 1H, glycerol H1'; $\delta = 5.16\text{--}5.24$, multiplet, 1H, glycerol H2. These parameters are essentially identical with those previously reported (7). No impurities sufficient to give a single proton resonance at 5 mol % concentration were detectable.

Thin-layer chromatography was carried out using silica-gel plates (Kieselgel 60 brand) developed in chloroform-methanol-water 65:25:4 and chloroform-methanol-28% NH_4OH 65:30:5. DMPC was dissolved in CH_2Cl_2 to a concentration of 10 mg/ml, and 0.1- μl and 5- μl volumes were applied to the plates using a micropipette. After development, products were detected using Zinzadze reagent (8), by charring, or with iodine. No additional spots were detected; it is estimated that our limit of detectability is under 1% of the main spot for spots well separated from the DMPC.

Dimyristoylphosphatidylcholine-d₇₂ was prepared from DMPA-d₅₉ (synthesized essentially according to Kingsley and Feigenson (3)), and choline-d₁₃⁺ BPh₄⁻, by the same technique, in 71% yield.■

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