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A FACILE ONE-STEP SYNTHESIS OF 5'-PHOSPHATIDYLNUCLEOSIDES BY AN ENZYMATIC TWO-PHASE REACTION.

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Summary: Phospholipase D from Streptomyces effectively catalyzed the transfer reaction of the phosphatidyl residue from phosphatidylcholines to the 5'-hydroxyl group of nucleosides in a two-phase system. A variety of 5'-phosphatidylnucleosides were easily prepared in high yields by this reaction.

Recently a few 5'-phosphatidylnucleosides have been prepared chemically because of their biological importance[1]. 5'-Phosphatidylthymidine was prepared by the phosphotriester method[1a], however the synthesis of 5'-phosphatidylnucleosides which have ribose or arabinose as the sugar moiety and adenine or cytosine as the base moiety of the nucleoside, are more complicated on account of the reactivity of their functional groups. MacCoss and co-workers prepared, the potent antileukemic compound, 5'-phosphatidylarabinosylcytosine in several reaction steps, and they pointed out the difficulty of its preparation[1b].

We wish to report here a novel general method of preparing 5'-phosphatidylnucleosides from phosphatidylcholines and nucleosides in a one-step reaction, in which phospholipase D-catalyzed transphosphatidylation, namely, the transfer reaction of the phosphatidyl residue from phosphatidylcholine to primary alkanols[2], was utilized.

In the presence of an excess of nucleoside, phosphatidylcholine was treated with phospholipase D from Streptomyces sp. AA 586 (phospholipase D-P, PLDP)[3] in a two-phase system of chloroform and an appropriate buffer to afford the corresponding 5'-phosphatidylnucleosides in high yields. However, the phospholipase D from cabbage leaves[2] which is known as an effective catalyst of transphosphatidylation with lower alkanols as acceptors of the phosphatidyl residue, gave only the hydrolysis product. Each of the enzymatic reaction products showed a UV absorption expected for the nucleoside base moiety and the molecular-ion peak of M + Na in a FAB-mass spectrum. ^{13}C-NMR spectra of compounds exhibited considerable down-field shifts of the C-5' signals of the nucleoside moieties, when compared with those of the parent nucleosides. Furthermore, treatment of the phosphatidylnucleosides **1a** and **1b** with 2,2-

dimethoxypropane and p-toluenesulfonic acid in acetone gave the isopropylidene derivatives, **2a** and **2b**, respectively, and 5'-deoxyuridine[4] did not act as an acceptor of the phosphatidyl residue in this enzymatic reaction system because

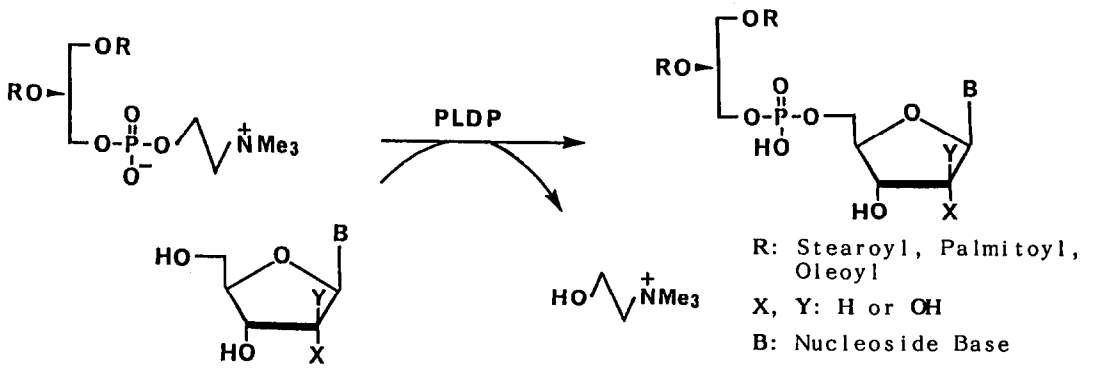
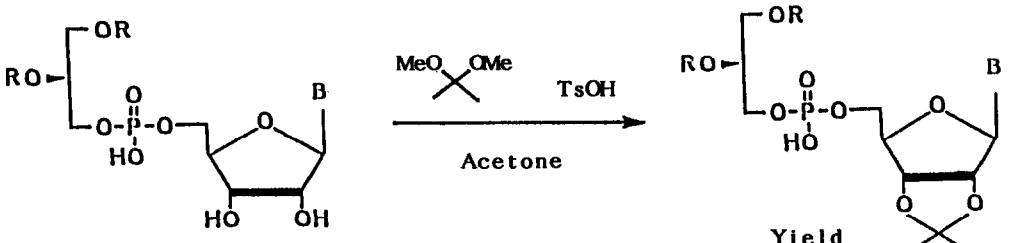


Table. The preparation of 5'-phosphatidylnucleosides[a] by PLDP-catalyzed transphosphatidylation

Entry	Donor	Acceptor(eq)	Aq.phase[b]	Aq/Org[c]	Yield[d]
1	DPPC[e]	uridine (20 eq)	A	0.25	75 %
2	DPPC	adenosine (5 eq)	A	0.5	52 %
3	DPPC	2'-deoxyadenosine (4 eq)	B	0.5	91 %
4	DPPC	arabinosylcytosine (10 eq)	C	0.25	79 %
5	DPPC	bredinin (20 eq)	A	0.25	68 %
6	DPPC	cytidine (10 eq)	C	0.25	84 %
7	DPPC	cytidine (2.5eq)	C	0.05	79 %
8	DSPC[f]	thymidine (10 eq)	A	0.25	88 %
9	DSPC	5-fluorouridine (10 eq)	A	0.25	68 %
10	DSPC	neplanocin A[h](5 eq)	A	0.25	87 %
11	DOPC[g]	5-fluoro-2'-deoxyuridine (15 eq)	A	0.25	81 %
12	DOPC	arabinosylcytosine (10 eq)	C	0.25	78 %

a. Satisfactory elemental analyses were obtained as sodium salts.
b. A: 250 mM $CaCl_2$, 200 mM acetate buffer (pH 6.0)
   B: 100 mM acetate buffer (pH 4.0)
   C: the pH was adjusted to 4.5 by addition of 2N HCl to the nucleoside solution
c. The volume ratio: aqueous phase / organic phase
d. The isolated yields based on single experiments
e. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
f. 1,2-Distearoyl-sn-glycero-3-phosphocholine
g. 1,2-Dioleoyl-sn-glycero-3-phosphocholine
h. 6'-Phosphatidylneplanocin A was obtained (The primary hydroxyl group of neplanocin A is numbered 6')

of a lack of the 5'-hydroxyl group. Therefore, it was confirmed that PLDP catalyzed the transfer reaction of phosphatidyl residues to the primary hydroxyl groups of nucleosides specifically from phosphatidylcholines to give the corresponding 5'-phosphatidylnucleosides in this system.



R = Palmitoyl, a. B = Uracil-1-yl
b. B = Cytosine-1-yl

Yield	
a.	91 %
b.	68 %

Although bredinin[5] and neplanocin A[6] are normally resistant to chemical phosphorylation because of their unique structures[7], the PLDP-catalyzed reaction system could provide the phosphatidyl derivatives of these antitumor nucleoside antibiotics (entry 5 and 10). This enzymatic reaction system also easily provided unsaturated 5'-phosphatidylnucleosides (entry 11 and 12) although the synthesis of unsaturated phospholipid derivatives is usually difficult because of their lability.

In this enzymatic reaction system, an excess of nucleoside as an acceptor of the phosphatidyl residue and the presence of the chloroform layer are required to allow the transphosphatidylation to proceed and to prevent the hydrolysis of the phosphatidylcholines because phospholipase D is better known for catalyzing the hydrolysis reaction. Unreacted nucleosides could be easily recovered if necessary, alternatively the reduction in the volume of the aqueous phase made a saving of nucleosides possible (entry 7). Chloroform is a good solvent for phosphatidylcholines and might also protect the phosphatidyl-enzyme, the reaction intermediate, from hydrolysis.

We studied the influence of pH of the aqueous phase on the PLDP-catalyzed transphosphatidylation in this system. The optimum pH of the reaction was influenced clearly by a nucleoside used[8]. It is particularly interesting that adenine or cytosine nucleosides acted as excellent acceptors at a pH equal to the pK of a nucleoside base. While the role of the nucleoside bases, such as adenine or cytosine, is not apparent, these bases might form hydrogen bonds with aspartic acid or glutamic acid residues of PLDP at their pK during the reaction process.

This enzymatic reaction is a really novel way to provide nucleoside phosphate derivatives. From the point of view of phospholipid chemistry, this method can be applied conveniently to the synthesis of various phospholipid derivatives containing a complicated structural polar-head group.

The typical procedure: A nucleoside (5 mmol) and PLDP (3 mg, 550 units) were dissolved in an appropriate buffer (5 ml), to which a $CHCl_3$ solution (20 ml) of phosphatidylcholine (0.5 mmol) was then added and the mixture was stirred at 45°C. After 6 hr, 2 N HCl (5 ml), MeOH (20 ml), and $CHCl_3$ (20 ml) were added and the mixture was shaken. Unreacted nucleoside was recovered from the aqueous layer, the organic layer was evaporated and chromatographed on silica gel ($CHCl_3$: MeOH = 15 : 1 ⟶ 3 : 1), followed by partition ($CHCl_3$: MeOH : 0.5 N HCl = 20 ml : 10 ml : 6ml) to afford the pure product from the organic layer.

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8. The optimum pH for the reaction were investigated by HPLC analysis as follows, adenine nucleosides (adenosine, 2'-deoxyadenosine and neplanocin A): 3.5–4.0, cytosinenucleosides (cytidine, 2'-deoxy-cytidine, and arabinosylcytosine): 4.5, uracil nucleosides (uridine and 5-fluorouridine): 6.0—7.0.

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