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PHOSPHOLIPASE D-CATALYZED TRANS-ALKYLPHOSPHORYLATION: A FACILE ONE-STEP SYNTHESIS OF NUCLEOSIDE 5'-ALKYLPHOSPHATES

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Phospholipase D from Streptomyces effectively catalyzed the transfer reaction of the alkylphosphoryl residue from alkylphosphorylcholines to the 5'-hydroxyl group of nucleosides regiospecifically in a two-phase system. Thus, various nucleoside 5'-alkylphosphates could be readily prepared in high yields.

KEYWORDS —— trans-alkylphosphorylation; nucleoside 5'-alkylphosphate; phospholipase D; new enzymatic reaction; phosphorylation

Some 5'-alkylphosphoryl derivatives of antitumor nucleoside analogues surpass the parent compound in their potency as an antitumor agent.[1] However, the 5'-alkylphosphoryl derivatizations of antitumor nucleoside analogues often require multi-step reactions and the overall yields were low.[1] This is due to the unusual structures of antitumor nucleoside analogues. We wish to report here a facile one-step method for preparing nucleoside 5'-alkylphosphates, in which we used a novel enzymatic reaction, namely, the transfer reaction of the alkylphosphoryl residue from alkylphosphorylcholines to primary alkanols (trans-alkylphosphorylation).

It has been known that phospholipase D from cabbage leaves catalyzes transphosphatidylation, the transfer reaction of the phosphatidyl residue from glycerophospholipids such as 3-sn-phosphatidylcholines (donor) to some primary lower alkanols (acceptor).[2] A few years ago, we found that phospholipase D from Streptomyces sp.AA 586 (PLDP)[3,4] was quite an efficient catalyst of transphosphatidylation, since this enzyme catalyzed the transphosphatidylation with a large variety of alkanols as acceptors.[5] Thus, in recent years we have been engaged in the synthesis of various phosphatidic acid esters of biological interest, especially 5'-phosphatidylnucleosides, by PLDP-catalyzed transphosphatidylation.[5] During these studies, we have recognized that PLDP is quite flexible in its recognition of phosphatidylcholines as a substrate.[6] This suggests the possibility that various types of phosphodiester compounds other than phosphatidic acid esters, might be prepared using the PLDP-catalyzed reaction. Therefore, we planned to synthesize nucleosides 5'-alkylphosphates by this enzymatic transfer reaction with alkylphosphorylcholines (**1**)[7] as donors and nucleosides as acceptors.

In the presence of PLDP, cytidine and stearylphosphorylcholine (**1a**, 3 eq) were stirred at 45°C for 6 h in a two-phase system of chloroform and sodium acetate buffer containing $CaCl_2$ (pH 5.8). This afforded the desired cytidine 5'-stearylphosphate (**2a**) in 88 %

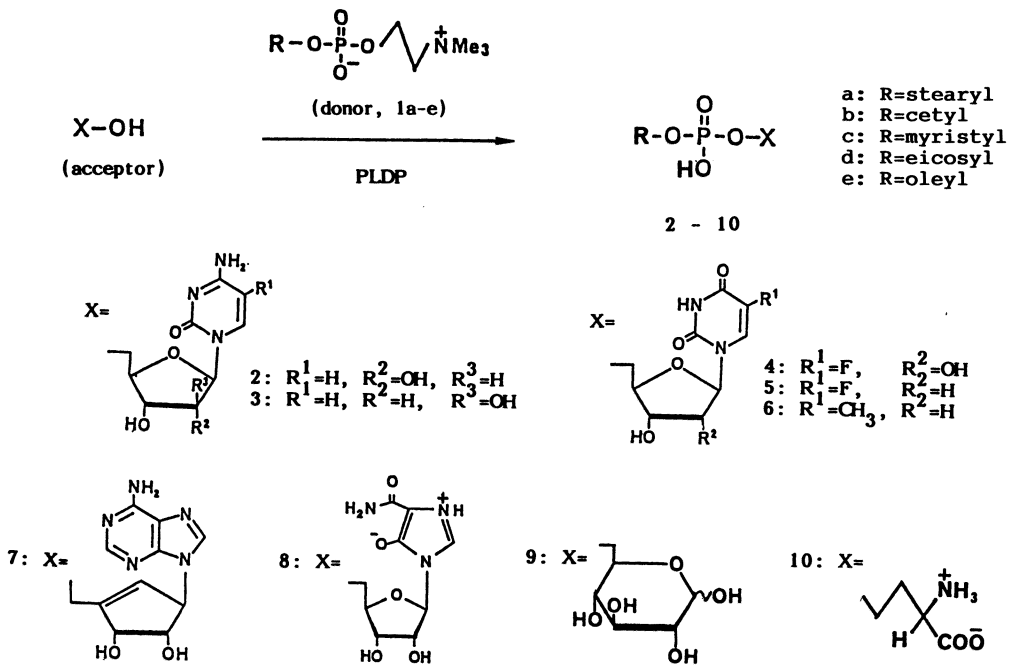

Table I. The Preparation of Nucleoside 5'-Alkylphosphates and Alkylphosphoryl Derivatives of Other Alkanols by PLDP-Catalyzed trans-Alkylphosphorylation

Entry	Acceptor	Donor (eq)	Aq/Org[a)]	Ca^{++}	Product	Yield[b)] (%)
1	Cytidine	**1a** (3)	0.025	+	**2a**	88
2	Cytidine	**1a** (3)	0.025	-	**2a**	66
3	Cytidine	**1a** (1)	0.025	+	**2a**	69
4	Cytidine	**1c** (3)	0.025	+	**2c**	77
5	Ara-C	**1a** (3)	0.025	+	**3a**	79
6	FUR	**1b** (3)	0.025	+	**4b**	72
7	FUR	**1e** (3)	0.025	+	**4e**	77
8	FUDR	**1a** (3)	0.025	+	**5a**	84
9	FUDR	**1b** (3)	0.025	+	**5b**	79
10	Thymidine	**1a** (3)	0.05	+	**6a**	81
11	Thymidine	**1d** (3)	0.05	+	**6d**	86
12	Neplanocin A	**1b** (3)	0.075	+	**7b**	76
13	Neplanocin A	**1e** (3)	0.075	+	**7e**	69
14	Bredinin	**1e** (3)	0.025	+	**8e**	66
15	D-Glucose	**1a** (3)	0.025	+	**9a**	62
16	L-Homoserine	**1a** (3)	0.025	+	**10a**	66

a) The volume ratio: aqueous phase (acetate buffer)/organic phase ($CHCl_3$).
b) The isolated yields based on the acceptor.

yield (Table I, Entry 1). The structure of this enzymatic reaction product (**2a**) was confirmed from its physical properties.[8] The absence of Ca^{++} cations (Entry 2) or a reduction in the amount of alkylphosphorylcholine (Entry 3) somewhat lowered the yield of **2a**.

The scope of this new trans-alkylphosphorylation reaction was examined by varying both the acceptors (various nucleosides and some other multi-functional primary alkanols) and the donors (alkylphosphorylcholines, **1a-e**) used, and the results are summarized in Table I.[9] Various nucleoside 5'-alkylphosphates could be readily prepared by this method (Entry 1-14). Although biologically active nucleoside analogues, such as Ara C (1-β-D-arabinofuranosylcytosine), neplanocin A ((-)-9-[trans-2',trans-3'-dihydroxy-4'-(hydroxymethyl)cyclopent-4'-enyl]adenine) and bredinin (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate), are normally resistant to the usual chemical phosphorylation because of their unique structures,[1,10] this reaction system could provide their 5'-alkylphosphoryl derivatives (Entry 5, 12, 13, 14).

In phospholipase D-catalyzed transphosphatidylation, the use of a large excess of alkanols (acceptor) to phosphatidylcholine (donor) has generally been required to promote the reaction, and the donors have been limited to glycerophospholipids.[2,5] Therefore, the method has not been practical for deriving alkanols. However, the present procedure can provide the nucleoside 5'-alkylphosphates in high yields based on the nucleoside used, in which straight-chain alkylphosphorylcholines are used as donors in the transfer reaction.

In this enzymatic reaction, a two-phase system containing a large excess of organic phase (the volume ratio , Aq/Org < 0.1) quite effectively promoted the transfer reaction and prevented the hydrolysis of the alkylphosphorylcholines; phospholipase D is better known for catalyzing the hydrolysis reaction.

Although the usefulness of enzymatic reactions is generally limited by the narrow substrate specificity of the enzyme used, the present study showed that the use of phospholipase D, which is not involved in nucleic acid metabolism, provides a general method for the preparation of a wide spectrum of nucleoside 5'-alkylphosphates.

Thus, this enzymatic reaction is really a novel way to provide nucleoside phosphate derivatives. Furthermore, the alkylphosphoryl derivatives of glucose or homoserine (**9a** and **10a**, respectively) could also be prepared by this means (Entries 14 and 15). This result shows that PLDP would catalyze the regiospecific trans-alkylphosphorylation to the primary hydroxyl group of various types of multi-functional compounds.

Typical Procedure: A nucleoside (0.5 mmol) and PLDP (15 mg, 2780 units) were dissolved in 1 ml of 200 mM sodium acetate buffer containing 250 mM of $CaCl_2$ (pH 5.8). A $CHCl_3$ solution (40 ml) of alkylphosphorylcholine (1.5 mmol) was then added, and the mixture was stirred at 45°C for 6 h. Then aqueous HCl (0.5 N, 6 ml) and MeOH (20 ml) were added and the mixture was shaken. The organic layer was washed twice with water and evaporated to dryness, followed by silica gel flash chromatography ($CHCl_3$:MeOH:AcOH=200:20:1 → 200:40:1 → 160:80:1). Fractions containing the desired product were evaporated, and the residue was partitioned ($CHCl_3$:MeOH:0.3 N HCl = 20 ml:10 ml:6 ml). The organic layer was washed twice with water and then concentrated to afford the desired product.

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4) Abbreviations used are: PLDP, phospholipase D from Streptomyces sp. AA586; Ara C, 1-β-D-arabinofuranosylcytosine; FUR, 5-fluorouridine; FUDR, 5-fluoro-2'-deoxyuridine.

5) S.Shuto, S.Ueda, S.Imamura, K.Fukukawa, A.Matsuda, and T.Ueda, Tetrahedron Lett., **28**, 199 (1987); S.Shuto, H.Itoh, S.Ueda, S.Imamura, K.Fukukawa, M.Tsujino, A.Matsuda, and T.Ueda, Chem.Pharm.Bull., **36**, 209 (1988); S.Shuto, S.Imamura, K.Fukukawa, H.Sakakibara, and J.Murase, ibid.,**35**, 447 (1987).

6) S.Shuto, Ph.D. Thesis, Hokkaido University, Sapporo, Japan (1988); for example, acyl carbonyl groups, a hydrophobic group at the 2-position, or the asymmetric carbon center of phosphatidylcholines was not essential in the substrate recognition as a phosphatidyl donor by PLDP.

7) Alkylphosphorylcholines were readily obtained from corresponding alcohols; M.Ozaki, I.Hara, Yukagaku, **30**, 15 (1980); R.L.Magolda and P.R.Jhonson, Tetrahedron Lett., **26**, 1167 (1985).

8) Physical data of **2a**: mp, 167-171°C (204-207°C, dec.). Anal.Calcd for $C_{27}H_{50}N_3O_8P$; C,56.33; H,8.75; N,7.30. Found; C,56.17; H,8.98; N,7.19. MS (FAB); m/e 576 (MH). UV, λ_{max}^{MeOH}277 nm. ^{1}H-NMR ($CDCl_3$:DMSO-d_6 = 5:1, DCl-added, 90 MHz); δ, 8.09 (d,1H,H-6, J=7.3 Hz), 6.39 (d,1H,H-5), 5.91 (bs,1H,H-1'), 4.31-3.89 (m,7H,H-2',3',4',5' and Stearyl CH_2), 1.66-1.26 (m,32H,Stearyl CH_2's), 0.88 (t,3H,Stearyl CH_3). ^{13}C-NMR ($CDCl_3$:DMSO-d_6 = 5:1, DCl-added, 100 MHz); δ, 158.77 (C-4), 145.98 (C-2), 143.50 (C-6), 93.82 (C-5), 89.27 (C-1'), 82.25 (C-4'), 73.66 (C-2'), 68.51 (C-2'), 66.66 (C-5'), 64.63 (Stearyl CH_2O), 31.01-21.78 (Stearyl CH_2's), 13.33 (Stearyl CH_3). Treatment of **2a** with 2,2-dimethoxypropane and p-toluenesulfonic acid in acetone gave the corresponding isopropylidene derivative, which clearly demonstrated that the alkylphosphoryl group was attached to the 5'-position of cytidine.

9) Structures of all the enzymatic products were confirmed from their instrumental analyses (^{1}H-NMR, ^{13}C-NMR, MS, UV spectra, and elemental analysis).

10) The chemical phosphorylation of the primary hydroxyl group of neplanocin A by the phosphodiester method was unsuccessful; S.Shuto, K.Fukukawa and T.Ueda, unpublished work.

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