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A Facile Enzymatic Synthesis of 5′-(3-*sn*-Phosphatidyl)nucleosides and Their Antileukemic Activities[1)]

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Phospholipase D from *Streptomyces* effectively catalyzed the transfer reaction of the phosphatidyl residue from 3-*sn*-phosphatidylcholine to the 5′-hydroxyl group of nucleosides in a two-phase system. Thus, a variety of 5′-(3-*sn*-phosphatidyl)nucleosides could be readily prepared in high yields by means of this reaction. Among them, phosphatidyl-FUR (**3b**), phosphatidyl-Ara FC (**8b**), and phoshatidyl-neplanocin A (**12b**) each produced a significant increase in the life span of mice bearing i.p.-implanted P388 leukemia, being more effective than the parent nucleosides.

Keywords——5′-(3-*sn*-phosphatidyl)nucleoside; phospholipase D; transphosphatidylation; antitumor nucleoside; antileukemic activity; P388 mouse leukemia; nucleoside 5′-phosphate

It is known that a number of nucleoside and nucleoside base analogues possess antitumor activity in various experimental tumor systems.[2)] Although some of them (*e.g.*, 5-FU, Ara C) have been used as effective chemotherapeutic agents for the clinical treatment of cancers,[2)] these antitumor agents are not always sufficient in cancer chemotherapy. There are several problems in clinical cancer chemotherapy with antitumor nucleoside or nucleoside base analogues, among which can be included rapid catabolism to biologically inactive compounds (*e.g.*, Ara C→Ara U,[3)] 5-FU→5,6-dihydro-5-fluorouracil[4)]), resistance to the drugs developed by tumor cells,[5)] and excessive toxicity of the drugs in rapidly dividing normal tissue.[2c)] In order to resolve these problems and improve the potency of antitumor nucleosides[6)] as chemotherapeutic agents, we have tried to prepare 5′-(3-*sn*-phosphatidyl) derivatives of various antitumor nucleosides (FUR,[2b,7)] FUDR,[2b,8)] FCR,[2b,7)] Ara FC,[2b,7,9)] neplanocin A,[10)] *etc.*).

5′-(3-*sn*-Phosphatidyl)nucleosides contain a phosphatidyl residue as a non-toxic carrier moiety which might protect the drug from inactivation by enzymes, and have a high affinity for cell membranes so that they can penetrate into cells. In addition, phospholipase C of cell membranes might catalyze the regiospecific cleavage of the phosphodiester linkage of a 5′-(3-*sn*-phosphatidyl)nucleoside to release the nucleoside 5′-phosphate within the cells. In some experimental tumor systems, the resistance to a cytotoxic nucleoside or a nucleoside base has been attributed to deficient activity of the nucleoside kinase[5a,b)] or the phosphoribosyltransferase,[11)] so that these cytotoxic agents are not metabolized to the 5′-triphosphates which are the actual cytotoxic metabolite. Thus, these 5′-(3-*sn*-phosphatidyl) derivatives are expected to exhibit antitumor effect against tumor systems which are resistant to the parent cytotoxic nucleoside or nucleoside base. On the other hand, it has been recognized that some chemically-prepared 5′-triphosphates of non-cytotoxic nucleoside analogues significantly

inhibit eukaryotic DNA polymerase,[12] one of the most important target enzymes in cancer chemotherapy. This fact raises the possibility that the 5′-(3-*sn*-phosphatidyl) derivatives of such non-cytotoxic nucleoside analogues might show an antitumor effect *in vivo*.

Recently, the 5′-(3-*sn*-phosphatidyl) derivatives of thymidine[13] and Ara C[14] were synthesized, and the latter (which was prepared as an analogue of CDP diacylglycerol, the biological intermediate in cellular phospholipids synthesis) showed a considerable antitumor effect in mice bearing L1210 leukemia.[14] However, other 5′-phosphatidylnucleosides have not been reported.

This paper describes a novel general method, using an enzymatic two-phase reaction, of preparing 5′-(3-*sn*-phosphatidyl)nucleosides[15] and the evaluation of their antileukemic activity against P388 leukemia in mice.

Chemistry

Although 5′-(3-*sn*-phosphatidyl)thymidine has been prepared by the phosphotriester method,[13] the synthesis of 5′-(3-*sn*-phosphatidyl)nucleosides which have ribose or arabinose as the sugar moiety and adenine or cytosine as the base moiety of the nucleoside, seems to be more complicated because of the reactivity of their functional groups. MacCoss and co-workers prepared 5′-(3-*sn*-phosphatidyl)-Ara C (**6**) in several reaction steps, but the overall yield was quite low and they pointed out the difficulty of the chemical preparation.[14]

On the other hand, it has been recognized that phospholipase D from cabbage leaves

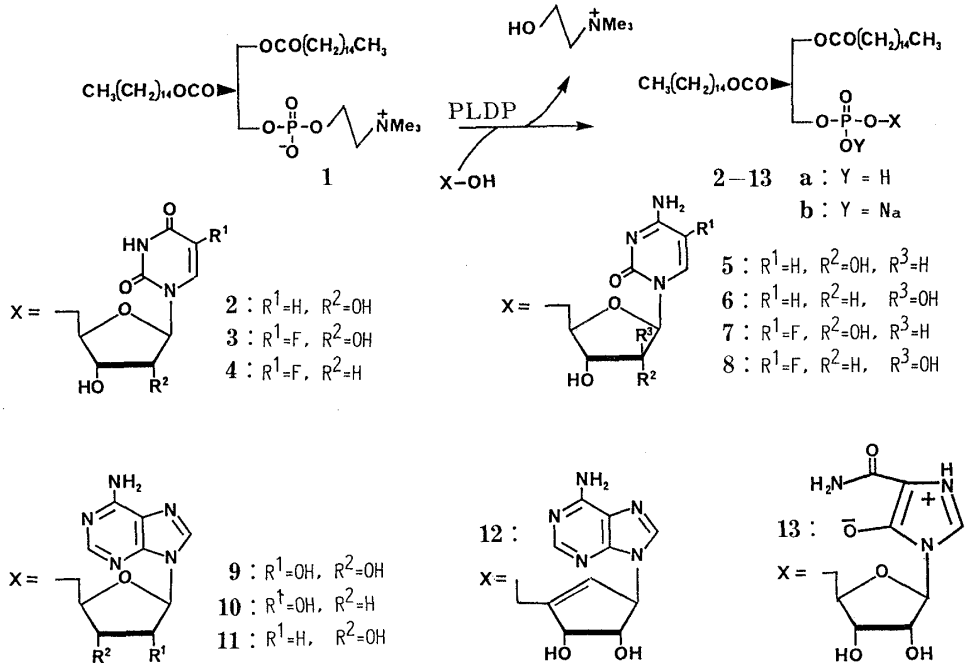

Chart 1

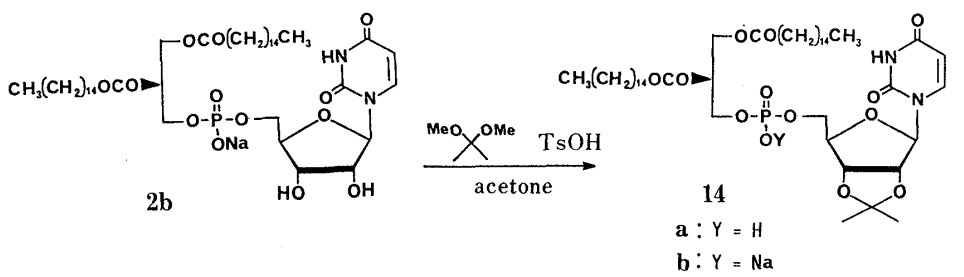

Chart 2

catalyzes transphosphatidylation, namely, the transfer reaction of the phosphatidyl residue from 3-*sn*-phosphatidylcholines to some primary lower alkanols.[16] More recently, we found that phospholipase D from *Streptomyces* sp. AA 586 phospholipase D-P, (PLDP) also catalyzed the transphosphatidylation with a large variety of alkanols as acceptors.[17] Therefore, we planned to synthesize 5′-(3-*sn*-phosphatidyl)nucleosides using this enzymatic reaction.

In the presence of an excess of uridine, DPPC was treated with PLDP in a two-phase system of chloroform and an acetate buffer (pH 6.0) to afford the desired 5′-(3-*sn*-phosphatidyl)uridine (**2a**) in 75% yield with a trace of the hydrolyzate (phosphatidic acid). However, the phospholipase D from cabbage leaves gave only the hydrolyzate under the same reaction conditions. The phosphatidyl derivative of uridine was somewhat labile in its undissociated form at the phosphate moiety (**2a**). Cleavage of palmitoyl groups probably occurred because of the acidity of the phosphate moiety. Therefore, **2a** was treated with Diaion WK-20 resin (Na^+ form) to convert it to the corresponding sodium salt (**2b**) which was stable at room temperature. Compound **2b** showed the UV absorption expected for the uracil moiety and a molecular-ion peak at *m/z* 897 (MNa) in the FAB mass spectrum. The ^{1}H-NMR spectrum of **2b** showed doublet signals of H-5, H-6, and H-1′ of the uridine moiety at 5.77, 7.86, and 5.83 ppm, respectively, and the H-2 signal of the glycerol moiety at 5.27 ppm as a multiplet. In the ^{13}C-NMR spectrum, the C-5′ signal of the uridine moiety of **2a** (66.05 or 65.46 ppm) exhibited a considerable downfield shift when compared with that of uridine (61.12 ppm), and was close to that of 5′-UMP (64.37 ppm).[18] Treatment of **2b** with 2,2-dimethoxypropane and *p*-toluenesulfonic acid in acetone gave the isopropylidene derivative (**14**). The FAB-mass spectrum of **14b** showed a molecular-ion peak at *m/z* 937 (MNa) and the ^{1}H-NMR spectrum showed the presence of the two methyl groups of the isopropylidene moiety (1.58, s and 1.37, s). 5′-Deoxyuridine[19] did not act as an acceptor of the transfer reaction of the phosphatidyl residue in this enzymatic reaction system because of its lack of the primary 5′-hydroxyl group. These results confirmed the structure of 5′-(3-*sn*-phosphatidyl)uridine (**2**).

Various 5′-(3-*sn*-phosphatidyl)nucleosides were readily obtained in high yields by the PLDP-catalyzed transphosphatidylation with the corresponding nucleosides as phosphatidyl acceptors (Table I). The structures of the 5′-(3-*sn*-phosphatidyl)nucleosides were confirmed

TABLE I. The Preparation of 5′-(3-*sn*-Phosphatidyl)nucleosides

Entry	Compound	Acceptor (eq)	Aq. phase[a] (ml)	Org. phase (ml)	Yield[b] (%)
1	**2a**	Uridine (20)	A (5)	$CHCl_3$ (20)	75
2	**3a**	FUR (10)	A (5)	$CHCl_3$ (20)	61
3	**4a**	FUDR (15)	A (5)	$CHCl_3$ (20)	76
4	**5a**	Cytidine (10)	B (5)	$CHCl_3$ (20)	84
5	**5a**	Cytidine (2.5)	B (1)	$CHCl_3$ (20)	79
6	**6a**	Ara C (10)	B (5)	$CHCl_3$ (20)	79
7	**7a**	FCR (5)	C (5)	$CHCl_3$ (20)	58
8	**8a**	Ara FC (6)	C (10)	$CHCl_3$ (20)	42
9	**9a**	Adenosine (5)	A (10)	$CHCl_3$ (20)	52
10	**10a**	Cordycepin (5)	C (5)	$CHCl_3$ (20)	78
11	**11a**	2′-Deoxyadenosine (4)	D (4)	$CHCl_3$ (20)	91
12	**12a**	Neplanocin A (5)	A (10)	$CHCl_3$ (20)	73
13	**13a**	Bredinin (20)	A (20)	$CHCl_3$ (20)	68

a) A, 250 mM $CaCl_2$, 200 mM acetate buffer (pH 6.0); B, the pH was adjusted to 4.5 by addition of 2 N HCl; C, 100 mM $CaCl_2$, 100 mM acetate buffer (pH 5.6); D, 100 mM acetate buffer (pH 4.0). *b*) Yields are based on DPPC used (367 mg, 0.5 mmol).

TABLE II. ^{1}H-NMR Data for 5′-(3-*sn*-Phosphatidyl)nucleosides

Compound (Solvent)[a]	^{1}H-NMR data (δ)[b]
2b (A)	7.86 (d, H6, $J_{5,6}$=8.1 Hz), 5.83 (d, H1′, $J_{1',2'}$=4.4 Hz), 5.77 (d, H5), 5.27 (m, glycerol CH), 4.50—3.92 (m, H2′,3′,4′,5′+glycerol CH_2's), 2.32 (m, $COCH_2$), 1.64—1.26 (m, palmitoyl CH_2's), 0.88 (t, palmitoyl CH_3)
3b (A)	7.99 (d, H6, $J_{6,F}$=6.4 Hz), 5.86 (dd, H1′, $J_{1',2'}$=4.0 Hz), 5.23 (m, glycerol CH), 4.51—3.90 (m, H2′, 3′,4′,5′+glycerol CH_2's), 2.32 (m, $COCH_2$), 1.68—1.26 (m, palmitoyl CH_2's), 0.88 (t, palmitoyl CH_2)
4b (A)	8.02 (d, H6, $J_{6,F}$=6.6 Hz), 6.23 (m, H1′, $J_{1',2'a}$=$J_{1',2'b}$=5.9 Hz, $J_{F,1'}$=1.8 Hz), 5.23 (m, glycerol CH), 4.50—3.90 (m, H3′,4′,5′+glycerol CH_2's), 2.30 (m, H2′+$COCH_2$), 1.69—1.26 (m, palmitoyl CH_2's), 0.88 (t, palmitoyl CH_3)
5b (B)	7.89 (d, H6, $J_{5,6}$=7.7 Hz), 5.86 (d, H1′, $J_{1',2'}$=3.3 Hz), 5.80 (d, H5), 5.15 (m, glycerol CH), 4.42—3.88 (m, H2′,3′,4′,5′+glycerol CH_2), 2.26 (m, $COCH_2$), 1.60—1.25 (m, palmitoyl CH_2's), 0.87 (t, palmitoyl CH_2)
6b[c] (C)	7.90 (d, H6, $J_{5,6}$=7.5 Hz), 6.19 (d, H1′, $J_{1',2'}$=4.6 Hz), 5.99 (d, H5), 5.23 (m, glycerol CH_2), 4.40—3.94 (m, H2′,3′,4′,5′+glycerol CH_2), 2.35 (m, $COCH_2$), 1.68—1.29 (m, palmitoyl CH_2), 0.89 (t, palmitoyl CH_3)
7b (A)	8.02 (d, H5, $J_{6,F}$=6.4 Hz), 5.85 (dd, H1′, $J_{1'2'}$=$J_{1',F}$=*ca.* 1 Hz), 5.24 (m, glycerol CH), 4.53—3.94 (m, H2′,3′,4′,5′+glycerol CH_2's), 2.31 (m, $COCH_2$), 1.68—1.26 (m, palmitoyl CH_2's), 0.88 (t, palmitoyl CH_3's)
8b (A)	7.93 (d, H6, $J_{6,F}$=6.8 Hz), 6.06 (dd, H1′, $J_{1',2'}$=5.3 Hz, $J_{1',F}$=*ca.* 0 Hz), 5.27 (m, glycerol CH), 4.48—3.93 (m, H2′,3′,4′,5′+glycerol CH_2's), 2.32 (m, $COCH_2$), 1.67—1.26 (m, palmitoyl CH_2's), 0.88 (t, palmitoyl CH_3)
9b (A)	8.42 (s, H8), 8.21 (s, H2), 6.07 (d, H1′, $J_{1',2'}$=3.3 Hz), 5.24 (m, glycerol CH), 4.50—3.98 (m, H2′,3′,4′, 5′+glycerol CH_2's), 2.31 (t, $COCH_2$), 1.68—1.26 (m, palmitoyl CH_2's), 0.88 (t, palmitoyl CH_3)
10b (A)	8.54 (s, H8), 8.22 (s, H2), 5.99 (s, H1′), 5.23 (m, glycerol CH), 4.70 (m, H2′), 4.36—3.95 (m, H4′,5′ +glycerol CH_2's), 2.29 (m, $COCH_2$), 2.12—1.90 (m, H3′), 1.61—1.26 (m, palmitoyl CH_2's), 0.88 (t, palmitoyl CH_3)
11b (A)	8.41 (s, H2), 8.24 (s, H8), 6.47 (dd, H1′, $J_{1',2'a}$=$J_{1',2'b}$=6.3 Hz), 5.18 (m, glycerol CH), 4.61 (m, H3′), 4.49—3.89 (m, H4′,5′+glycerol CH_2's), 2.62 (m, H2′), 2.30 (t, $COCH_2$), 1.68—1.26 (m, palmitoyl CH_2), 0.88 (t, palmitoyl CH_3's)
12b (A)	8.25 (s, H8), 7.93 (s, H2), 5.99 (d, H5′, $J_{5',1'}$=1.8 Hz), 5.48 (dd, H1′, $J_{1',2'}$=3.5 Hz), 5.25 (m, glycerol CH), 4.78—3.94 (m, H2′,3′,6′+glycerol CH_2's), 2.32 (m, $COCH_2$), 1.63—1.26 (m, palmitoyl CH_2's), 0.88 (t, palmitoyl CH_3)
13a (D)	8.35 (s, H2), 5.52 (d, H1′, $J_{1',2'}$=7.3 Hz), 5.12 (m, glycerol CH), 4.41—3.71 (m, H2′,3′,4′,5′+glycerol CH_2's), 2.19 (m, $COCH_2$), 1.54—1.25 (m, palmitoyl CH_2's), 0.86 (t, palmitoyl CH_3)
14b (A)	7.77 (d, H6, $J_{5,6}$=8.1 Hz), 5.94 (d, H1′, $J_{1',2'}$=2.4 Hz), 5.78 (d, H5), 5.23 (m, glycerol CH), 4.90 (m, H2′+3′), 4.39—3.90 (m, H4′,5′+glycerol CH_2), 2.33 (m, $COCH_2$), 1.58 and 1.39 (each s, OCH_3), 1.64—1.27 (m, palmitoyl CH_2), 0.89 (t, palmitoyl CH_3)

a) A, $CDCl_3$–CD_3OD (3:1); B, DMSO-d_6–CD_3OD–$CDCl_3$ (3:1:1); C, $CDCl_3$–CD_3OD–D_2O (2:3:1); D, DMSO-d_6–CD_3OD (20:1). *b*) The chemical shifts were indicated from tetramethylsilane as an internal standard. *c*) Our ^{1}H-NMR data for **6b** were not in accord with those reported by MacCoss *et al.*, although both preparations of 5′-(3-*sn*-phosphatidyl)-Ara C contained the same fatty acyl groups and counter cation.

by their physical properties.[13]C-NMR data of some typical 5′-(3-*sn*-phosphatidyl)nucleosides and their parent nucleosides are shown in Table IV. The C-5′ signal of the nucleoside moiety of each 5′-(3-*sn*-phosphatidyl)nucleoside exhibited a downfield shift compared with that of the parent nucleoside, and was coupled with the phosphorus atom. A coupling with the phosphorus atom was also observed in the C-4′ signal. These data clearly demonstrate that PLDP catalyzed the transfer reaction of the phosphatidyl residue to the primary hydroxyl group of various types of nucleosides (ribo-, 2′-deoxyribo-, and arabino-nucleoside) specifically from 3-*sn*-phosphatidylcholine to give the corresponding 5′-(3-*sn*-phosphatidyl)-nucleosides. Although neplanocin A and bredinin[20] are normally resistant to chemical phosphorylation because of their unique structures,[21] the PLDP-catalyzed reaction system could provide the phosphatidyl derivatives of these antitumor nucleoside antibiotics (**12**[22]

TABLE III. Physical Data for 5′-(3-*sn*-Phosphatidyl)nucleosides

Compound	UV λ_{max}[a] (nm)	FAB-mass *m/z*	Formula (*Anal.*; C, H, N)[b]	mp (°C)
2b	260	897 (MNa)	$C_{44}H_{78}N_2NaO_{13}P \cdot 2H_2O$	c)
3b	268	915 (MNa)	$C_{44}H_{77}FN_2NaO_{13} \cdot H_2O$	c)
4b	268	899 (MNa)	$C_{44}H_{77}FN_2NaO_{12}P$	196—198
5b	273	874 (MH), 896 (MNa)	$C_{44}H_{79}N_3NaO_{12}P$	257—260 (dec.)
6b	273	896 (MNa)	$C_{44}H_{79}N_3NaO_{12}P \cdot 3/2\,H_2O$	c)
7b	283	914 (MNa)	$C_{44}H_{78}FN_3NaO_{12}P \cdot H_2O$	c)
8b	284	914 (MNa)	$C_{44}H_{78}FN_3NaO_{12}P \cdot H_2O$	c)
9b	259	898 (MH), 920 (MNa)	$C_{45}H_{79}N_5NaO_{11}P \cdot 2H_2O$	c)
10b	259	882 (MH), 904 (MNa)	$C_{45}H_{79}N_5NaO_{10}P \cdot H_2O$	227—229 (dec.)
11b	259	904 (MNa)	$C_{45}H_{79}N_5NaO_{10}P \cdot H_2O$	c)
12b	260	894 (MH), 916 (MNa)	$C_{46}H_{79}N_5NaO_{10}P \cdot 3H_2O$	c)
13b	280	899 (MNa)	$C_{44}H_{79}N_3NaO_{13}P \cdot 2H_2O$	188—192 (dec.)
14b	260	937 (MNa)	$C_{47}H_{82}N_2NaO_{13}P \cdot 5H_2O$	c)

a) Measured in MeOH–$CHCl_3$ (20:1). *b*) Analyses for the elements indicated were within ±0.4% of the theoretical values. *c*) No clear melting point was observed.

TABLE IV. The ^{13}C-NMR Chemical Shifts of 5′-(3-*sn*-Phosphatidyl)nucleosides and the Parent Nucleosides[a]

Compound (Solvent)[b]	Nucleoside moiety								
	C-2	C-4	C-5	C-6	C-1′	C-2′	C-3′	C-4′	C-5′
2a (A)	150.64	164.75	101.94	140.83	89.77	74.56	69.44	82.44[c]	66.05[c]
Uridine (B)	150.94	163.40	101.96	140.97	88.04	73.74	70.11	85.05	61.12
4b (A)	148.71	157.36[d]	139.98[d]	124.32[d]	85.06	39.77	70.00	85.73[c]	64.34[c]
FUDR (B)	148.99	157.03[d]	139.97[d]	124.74[d]	84.63	39.89	70.16	87.55	61.06
6b (A)	156.26	165.07	93.91	142.42	85.79	74.24	75.63	81.91[c]	63.61[c]
Ara-C (B)	155.27	165.62	92.48	142.92	85.81	74.88	76.45	84.84	61.22

Compound (Solvent)[b]	Dipalmitoylglycerol moiety					
	CH_2OP	CH_2O	CH	CO	CH_3	Others
2a (A)	65.46[c]	62.13	69.62[c]	173.01, 172.66	14.40	34.42—22.96
4b (A)	63.31[c]	62.35	70.25[c]	173.24, 172.91	13.87	34.13—22.57
6b (A)	63.36[c]	62.41	70.24[c]	173.24, 172.89	13.90	34.14—22.58

a) The chemical shifts are indicated from tetramethylsilane as an internal standard (δ). *b*) A, $CDCl_3$–CD_3OD (3:1); B, DMSO-d_6. *c*) A coupling with phosphorus was observed. *d*) A coupling with fluorine was observed.

and **13**).

In this system, adenine or cytosine nucleosides acted as excellent acceptors of the phosphatidyl residue at a pH equal to the p*K* of the nucleoside base.[23] 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 p*K* during the reaction process.

The kinetics of the PLDP-catalyzed transphosphatidylation was studied by preparing Lineweaver–Burk plots. It is noteworthy that the kinetic behavior showed good agreement

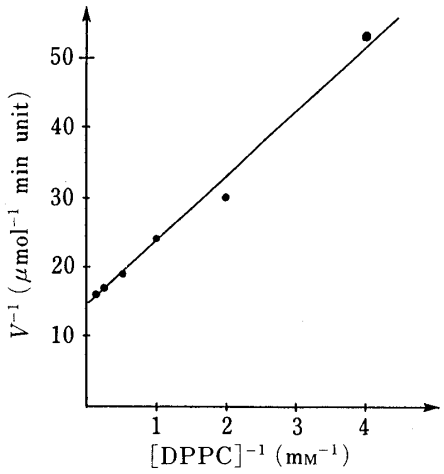

Fig. 1. Lineweaver–Burk Plot of the Transphosphatidylation

Reaction conditions: aqueous phase, 100 μl of acetate buffer (pH 6.0, 200 mM) containing 250 μmol of uridine; organic phase, 1000 μl of $CHCl_3$ containing DPPC (0.25—8 μmol); PLDP, 0.6 unit; temperature, 25 °C; time, 10 min. Donor, DPPC. Acceptor, uridine.

with that expected for Michaelis–Menten kinetics in spite of the use of a heterogeneous reaction system (Fig. 1).

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 hydrolysis of the phosphatidylcholine, because phospholipase D is better known for catalyzing the hydroysis reaction. Unreacted nucleosides could be easily recovered if necessary; alternatively a reduction in the volume of the aqueous phase would make a saving of nucleosides possible (Table I, entry 5). Chloroform is a good solvent for phosphatidylcholines and possibly also protects the phosphatidyl-enzyme, the reaction intermediate, from hydrolysis. Other organic solvents such as diethyl ether, hexane, or acetonitrile, may be used for PLDP-catalyzed transphosphatidylation, but these solvents were not suitable for the preparation of phosphatidyl derivatives because of the poor solubility of DPPC in these solvents.

In conclusion, the present enzymatic method is a novel approach to the preparation of nucleoside phosphate derivatives. From the point of view of phospholipid chemistry, this method can be widely used to synthesize various types of phospholipid derivatives containing a complicated polarhead group.

Biological Activity and Discussion

Antileukemic activities of 5′-(3-*sn*-phosphatidyl)nucleosides and their parent nucleosides against i.p.-implanted P388 leukemia in mice are summarized in Table V. As expected, the 5′-(3-*sn*-phosphatidyl) derivatives of the usual nucleosides (**2b**, **5b**, **9b**, and **11b**) had no antitumor effect. The 5′-(3-*sn*-phosphatidyl) derivatives of cordycepin (3′-deoxyadenosine)[24] and bredinin (**10b** and **13b**, respectively) did not exhibit any significant effect in this system. However, treatment with 5′-(3-*sn*-phosphatidyl)-FUR (**3b**) at the optimum dose of 30 mg/kg per day for 5 successive days showed a remarkable ILS value of 206% with a slight weight loss in mice; the result was clearly superior to that obtained with FUR, the parent nucleoside. Under our experimental conditions, the optimum dose found for FUR was 3 mg/kg per day, and this dose given for 5 d showed an ILS value of 100%. Similar effects were observed with 5′-(3-*sn*-phosphatidyl)-Ara FC (**8b**) and 6′-(3-*sn*-phosphatidyl)neplanocin A (**12b**), which exhibited higher activities (ILS 167% and 180%, respectively) than their parent nucleosides at the optimum doses. 5′-(3-*sn*-phosphatidyl)-Ara C (**6b**), which was previously reported by MacCoss *et al.*,[14] also showed a significant activity (ILS > 185% with one 35-day survivor). 5′-(3-*sn*-Phosphatidyl)-FUDR (**4b**) and -FCR (**7b**) possessed moderate activities (ILS 30% and 56%, respectively) at their optimum doses.

These results indicate that the 5′-(3-*sn*-phosphatidyl) derivatives of cytotoxic nucleosides

TABLE V. Antitumor Activity against i.p.-Implanted P388 Leukemia in Mice[a]

Compound	Dose (mg/kg)		ILS[b] (%)	35-day survivor
	Daily	Total		
2b	50	250	11	0
3b	3	15	31	0
	10	50	130	0
	30	150	206	0
4b	10	50	30	0
5b	50	250	−3	0
6b	50	250	>185	1
7b	5	25	39	0
	15	75	56	0
8b	3	15	32	0
	10	50	78	0
	30	150	167	0
9b	50	250	10	0
10b	50	250	−3	0
11b	50	250	5	0
12b	3	15	53	0
	10	50	73	0
	30	150	180	0
13b	50	250	−6	0
FUR	3[c]	15	100	0
Ara FC	30[c]	150	87	0
Neplanocin A	1[c]	5	51	0

a) Each group of five B6D2F$_1$ mice (wt: 17—22 g) received i.p. inoculation of 1×10^6 cells on day 0. Treatments (i.p.) were initiated 24 h after tumor inoculation. Animals were observed daily until death or for 35 d. *b*) Percent increase in life span: $(T/C-1) \times 100$. The control mice died in 7—10 d. *c*) A maximum ILS value was observed at this dose.

possess more potent antitumor effects than their parent nucleosides, the activity being somewhat influenced by the properties of the parent nucleosides.

All the 5′-(3-*sn*-phosphatidyl)nucleosides synthesized were tested for antiproliferative activity against L5178Y mouse leukemia cell line. Although the parent nucleosides such as FUR, FUDR, neplanocin A, *etc.*, have a potent cytotoxicity, none of these 5′-(3-*sn*-phosphatidyl) derivatives showed a definite activity in this system at less than 20 μg/ml (data not shown). The much lower activities of 5′-(3-*sn*-phosphatidyl) derivatives when compared to the corresponding parent nucleosides in this *in vitro* system can be explained by a much slower release of the active nucleotide from the 5′-(3-*sn*-phosphatidyl)nucleoside.

Further evaluation of these 5′-(3-*sn*-phosphatidyl)nucleosides against various experimental tumor systems is being carried out.

Experimental

Melting points were determined on a Yanagimoto micromelting point apparatus (MP-3) and are uncorrected. The NMR spectra were recorded with a JEOL FX-90, FX-100, or FX-400 spectrometer with tetramethylsilane as an internal standard. UV spectra were recorded with a Shimadzu UV-240 spectrophotometer and mass spectra (FAB) were measured on a JEOL JMS D-300 spectrometer in the presence of NaCl. HPLC was carried out on a Shimadzu system consisting of an LC-6A solvent pump, an SCL-6A system controller, a CTO-6A column oven, an SPD-6A UV spectrophotometric detector, and a C-R3A chromatopack. Thin layer chromatography was carried out on Merck precoated plate 60F$_{254}$ and the compounds were detected by visualization under a UV lamp (254 nm) or by using Dittmer–Lester spray.[25] Silica gel flash chromatography was conducted with Merck Silica gel Art. 9385. PLDP was supplied by the diagnostic division of Toyo Jozo Co. DPPC and nucleosides were purchased from Nihon Seika

Co. and Yamasa Shoyu Co., respectively. Chloroform for the enzymatic reaction was purchased from Merck (for spectroscopy, stabilized with amylene).

General Procedure for the Preparation of 5′-(3-*sn*-Phosphatidyl)nucleosides——PLDP (3 mg, 550 units) was dissolved in an appropriate buffer containing nucleoside (see Table I), to which a $CHCl_3$ solution (20 ml) of DPPC (367 mg, 0.5 mmol) was added. The mixture was stirred vigorously at 45 °C for 6 h, then 2 N HCl (5 ml), MeOH (20 ml), and $CHCl_3$ (20 ml) were added and the whole was shaken. Unreacted nucleoside was recovered from the aqueous layer. The organic layer was washed twice with 10 ml of water and evaporated to dryness, followed by silica gel flash chromatography ($CHCl_3$: MeOH = 15 : 1 → 3 : 1). Fractions containing the desired product were evaporated, $CHCl_3$ (20 ml), MeOH (10 ml), and 0.5 N HCl (5 ml) were added to the residue, and the mixture was shaken. The organic layer was washed twice with water (5 ml) and evaporated to give the pure product as a white powder (the free acid).

General Method of Conversion to the Sodium Salts——The 5′-(3-*sn*-phosphatidyl)nucleoside (from 0.5 mmol of DPPC) prepared by the above method was dissolved in a small volume of $CHCl_3$–MeOH–H_2O (10 : 5 : 1), and the solution applied to a column of Diaion WK-20 resin (2 × 5 cm, Na^+ form) packed in the same solvent. The column was developed in the same solvent and the eluate was concentrated to afford a white precipitate of the required product as the sodium salt. The yield was almost quantitative.

2′,3′-*O*-Isopropylidene-5′-(3-*sn*-phosphatidyl)uridine (14)——*p*-Toluenesulfonic acid · H_2O (19 mg, 0.1 mmol) was added to a suspension of 5′-phosphatidyluridine (**2b**, 90 mg, 0.1 mmol) in 2,2-dimethoxypropane (3 ml) and acetone (3 ml), and the mixture was stirred at room temperature for 4 h. The reaction mixture was neutralized with 1 N $NaHCO_3$, then evaporated to dryness, and the residue was partitioned between 25 ml of $CHCl_3$–MeOH (2 : 1) and 5 ml of 0.25 N HCl. The organic layer was washed twice with 5 ml of water and evaporated to give the desired product as a white powder (free acid form, 84 mg, yield 91%). The white powder was converted to the Na^+ salt by dissolution in $CHCl_3$–MeOH–H_2O (10 : 5 : 1) and passage through a column of Diaion WK-20 resin (1 × 3 cm, Na^+ form), packed and developed in the same solvent. The eluate was concentrated to afford **14b** as a white precipitate (79 mg).

Kinetic Study of the Transphosphatidylation——A $CHCl_3$ solution (1000 μl) of DPPC (0.25, 0.5, 1.0, 2.0, 4.0, or 8.0 mM) was added to a 100 μl of acetate buffer (200 mM, pH 6.0) containing PLDP (0.6 unit) and uridine (2.5 M), and the mixture was stirred vigorously at 25 °C for 10 min. The reaction was stopped by addition of 100 μl of 3 N HCl. The organic layer contained the enzymatic product, and the yield of 5′-(3-*sn*-phosphatidyl)uridine was measured by HPLC (column, Lichrosorb RP-8, 0.4 × 15 cm; eluate, 0.2 M NH_4OAc : MeOH = 7 : 1; flow rate, 1 ml/min; temperature, 35 °C; UV detector, 260 nm; retention time, 8.8 min).

Antitumor Assay in Mice——P 388 lymphocytic leukemia cells were maintained by serial transplantation in C57BL/6 × DBA/2 F_1 ($B6D2F_1$) male mice (purchased from Charles River Japan, Inc.). P 388 lymphocytic leukemia cells (1×10^6) were inoculated i.p. into $B6D2F_1$ male mice (average weight = 20 g). After 24 h, animals were randomized into groups of five and housed in shoebox cages. For administration to animals, 5′-(3-*sn*-phosphatidyl)nucleoside was sonicated in Tris buffered saline, and this preparation was administrated i.p. on day 1 through day 5 (*i.e.*, treatment was initiated 24 h after tumor inoculation).

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References and Notes

1) Abbreviations used are: 5-FU, 5-fluorouracil; Ara C, 1-β-D-arabinofuranosylcytosine; Ara U, 1-β-D-arabinofuranosyluracil; FUR, 5-fluorouridine; FUDR, 5-fluoro-2′-deoxyuridine; Ara FC, 1-β-D-arabinofuranosyl-5-fluorocytosine; DNA, deoxyribonucleic acid; CDP, cytidine-5′-diphosphate; PLDP, phospholipase D-P; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; 5′-UMP, uridine-5′-monophosphate; UV, ultraviolet; FAB, fast atom bombardment; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography.
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23) The optimum pH for the reaction were investigated by HPLC analysis and the results were as follows: adenine nucleosides (adenosine, 2′-deoxyadenosine, and neplanocin A), 3.5—4.0; cytosine nucleosides (cytidine, 2′-deoxycytidine, and Ara C), 4.5; uracil nucleosides (uridine and FUR), 6.0—7.0.
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