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Synthetic Nucleosides and Nucleotides. XVI.[1] Synthesis and Biological Evaluations of a Series of 1-β-D-Arabinofuranosylcytosine 5′-Alkyl or Arylphosphates

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Enzymatic selective dephosphorylation of 1-β-D-arabinofuranosylcytosine 3′,5′-diphosphate (**1**) with nuclease-3′-nucleotidase P_1 at 60° for 24 hr gave 1-β-D-arabinofuranosylcytosine 5′-phosphate (Ara CMP) (**2**) in good yield. The acylation of both N_4- and the 2′,3′-hydroxyl groups of **2** followed by coupling with various alcohols and phenols in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride and subsequent alkaline hydrolysis afforded the title compounds (**4**). The resulting 32 kinds of Ara CMP alkyl or aryl esters were examined to determine their biological activities, such as antiviral activity against herpes simplex type 1 in cultured human embryonic lung fibroblast cells, growth inhibitory activity against mouse leukemic L5178Y cells in culture and antileukemic activity against L-1210 in mice. Ara CMP esters were active in these assay systems.

Keywords——1-β-D-arabinofuranosylcytosine 5′-phosphate; 1-β-D-arabinofuranosylcytosine 5′-alkylphosphates; synthesis; antiviral activity; herpes simplex virus type 1; L5178Y cells, antileukemic activity; L-1210

One of the useful approaches in the design and synthesis of potentially active antitumor agents or antiviral agents is to chemically modify the compounds of known biological activity. A very minor modification of an active molecule has in some instances brought about a significant change in its bioavailability *in vivo* or an increase in biological activity with a concomitant decrease in toxicity to host. This is well illustrated by a comparison of two antitumor cytosine nucleosides, 1-β-D-arabinofuranosylcytosine (Ara C)[3] and 2,2′-anhydro-1-β-D-arabinofuranosylcytosine (Cyclo C).[4] Cyclo C is reported to be more active against leukemia L-1210 in mice[4a] and various other experimental tumor systems[4c] and less toxic than Ara C, though the only difference in their structures is the presence of a 2,2′-anhydro bond in Cyclo C.

Thus, the synthesis of derivatives of Ara C, which may liberate their 1-β-D-arabinofuranosylcytosine 5′-phosphate (Ara CMP) *in vivo*, is of great interest because Ara CMP is known to be the key intermediate in the metabolic activation of Ara C to Ara CTP *in vivo*.

Recently, chemical modification and carcinostatic evaluation of Ara C have been reported from several laboratories, including the acylation of Ara C or Ara CMP with long-chain fatty acids to provide N_4-acylamido derivatives.[5] Esterification of sugar hydroxyl groups at 3′ or

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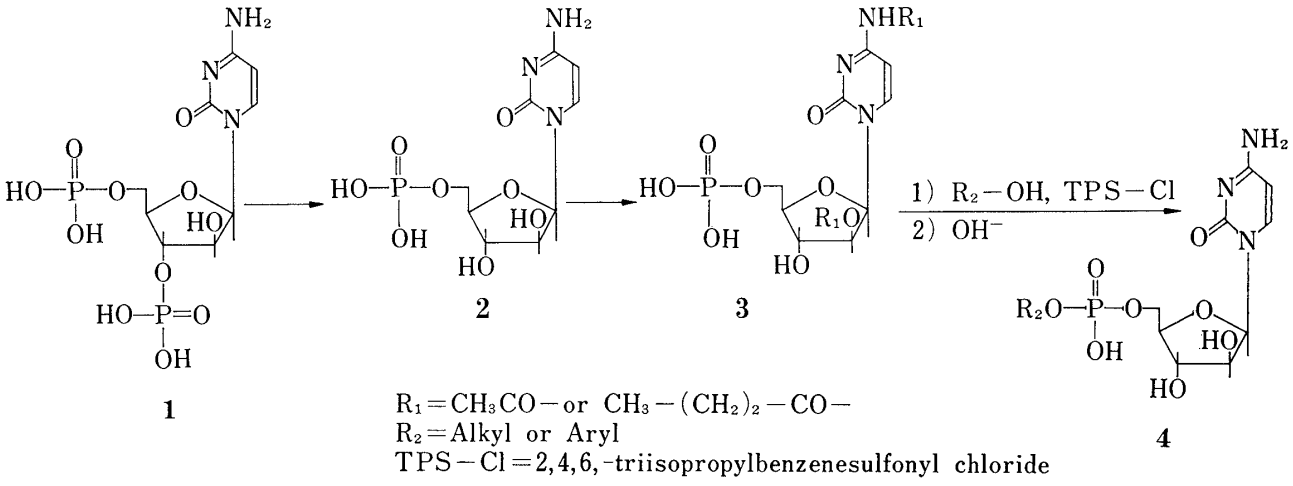
5′ or both positions of Ara C with long-chain fatty acids has also been reported.[6-7] However, a systematic study on the introduction of alkyl groups into Ara CMP with phosphodiester linkage has not been reported so far. We now report a general method for the synthesis of 1-β-D-arabinofuranosylcytosine 5′-alkyl or arylphosphates, together with the results of biological evaluations of their antiviral and antitumor activities.

Synthetic Method

The key intermediate in the present study, Ara CMP (**2**), is not easily synthesized by chemical means. Since Ara C has a *trans* hydroxyl group in the 2′-position, conventional phosphorylation methods for the ribonucleoside series[8,9] cannot be utilized in the arabinoside series. Phosphorylation of Ara C with phosphoryl chloride gave Ara C 2′,5′-cyclic phosphate as a main product with only a small amount of Ara CMP. In the literature, both chemical and enzymatic processes have been reported for the synthesis of phosphorylated Ara C.[10] Tener's phosphorylation using β-cyanoethylphosphate and dicyclohexylcarbodiimide[11] has been utilized for the synthesis of Ara CMP and 9-β-D-arabinofuranosyladenine 5′-phosphate (Ara AMP) by Cohen.[12] However, the yield of Ara CMP was only 6 to 22%. Thus we selected a combination of chemical and enzymatic procedures for the synthesis of Ara CMP. The starting Ara C 3′,5′-diphosphate (**1**) was prepared by the reaction of cytidine with polyphosphoric acid according to Walwick *et al.*[13] This compound was hydrolyzed with nuclease-3′-nucleotidase P_1[14] at 60° for 48 hr, followed by column chromatography on Dowex 1 (formate form) to afford Ara CMP in 70% yield. To our knowledge, this is the most convenient and economical synthetic method for Ara CMP so far reported. Esterification of Ara CMP also



1 2 3 4

$R_1 = CH_3CO-$ or $CH_3-(CH_2)_2-CO-$
R_2 = Alkyl or Aryl
TPS−Cl = 2,4,6,-triisopropylbenzenesulfonyl chloride

Chart 1

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Table I. Properties of Various Ara CMP Esters

Compound	Formula	Analysis (%) Calcd (Found) C	H	N	P	mp (°C) Free	mp (°C) Na salt	Yield (%) from 2
Ara CMP	$C_9H_{14}N_3O_8P$	34.44 (34.32	4.33 4.46	12.99 12.67	9.58 9.40)			
Methyl	$C_{10}H_{16}N_3O_8P \cdot H_2O$	33.81 (33.79	5.11 5.11	11.83 11.70	8.72 8.57)	185—187		49
Ethyl	$C_{11}H_{18}N_3O_8P \cdot H_2O$	35.78 (35.77	5.46 5.17	11.38 11.24	8.39 8.38)	186—188	200—202	45
n-Propyl	$C_{12}H_{20}N_3O_8P$	39.46 (39.28	5.52 5.44	11.50 11.62	8.48 8.19)	187—188	203—205	52
n-Butyl	$C_{13}H_{22}N_3O_8P \cdot H_2O$	39.30 (39.11	6.09 6.01	10.58 10.29	7.80 7.66)	186—188	194—196	33
n-Amyl (Pentyl)	$C_{14}H_{24}N_3O_8P \cdot H_2O$	40.88 (40.78	6.39 6.22	10.22 10.05	7.53 7.32)	219—221	192—194	52
n-Hexyl	$C_{15}H_{26}N_3O_8P \cdot H_2O$	42.35 (42.20	6.63 6.72	9.88 9.66	7.28 7.09)	194—196	175—177	46
n-Heptyl	$C_{16}H_{28}N_3O_8P \cdot H_2O$	45.61 (45.43	6.70 6.52	9.97 9.85	7.35 7.27)	164—166	184—186	51
n-Octyl	$C_{17}H_{30}N_3O_8P$	46.85 (46.69	6.89 6.66	9.65 9.40	7.35 7.32)	162—164	179—181	64
n-Nonyl	$C_{18}H_{32}N_3O_8P$	48.10 (48.41	7.18 7.28	9.35 9.22	6.89 6.88)	209—211	212—214	41
n-Decyl	$C_{19}H_{34}N_3O_8P$	49.19 (48.99	7.34 7.42	9.06 8.87	6.68 6.41)	216—218	222—224	64
n-Undecyl	$C_{20}H_{36}N_3O_8P$	50.31 (50.02	7.60 7.72	8.80 8.93	6.49 6.37)	220—222	212—214	45
n-Lauryl	$C_{21}H_{38}N_3O_8P \cdot H_2O$	49.50 (49.23	7.91 8.04	8.25 7.99	6.08 5.89)	196—198	212—214	45
n-Tridecyl	$C_{22}H_{40}N_3O_8P$	52.27 (52.18	7.98 7.69	8.31 8.43	6.13 5.98)	202—204	194—196	47
n-Tetradecyl	$C_{23}H_{42}N_3O_8P \cdot H_2O$	51.39 (51.52	8.25 8.38	7.82 7.61	5.76 5.74)	225—227	216—218	45
n-Pentadecyl	$C_{24}H_{44}N_3O_8P$	54.02 (53.94	8.31 8.51	7.87 7.92	5.80 5.64)	221—223	197—199	60
n-Cetyl	$C_{25}H_{46}N_3O_8P \cdot H_2O$	54.83 (54.78	8.47 8.77	7.67 7.86	5.66 5.72)	226—228		81
n-Heptadecyl	$C_{26}H_{48}N_3O_8P$	55.60 (55.34	8.61 8.40	7.48 7.25	5.51 5.39)	219—221	201—203	54
n-Stearyl	$C_{27}H_{50}N_3O_8P \cdot H_2O$	54.62 (54.51	8.82 8.96	7.08 6.84	5.22 4.96)	224—226	203—205	67
n-Eicosyl	$C_{29}H_{54}N_3O_8 \cdot Na \cdot 3H_2O$	51.24 (51.36	8.75 8.49	6.18 5.88	4.56 4.35)	216—218	191—193	53
n-Tricosyl	$C_{32}H_{60}N_3O_8P$	59.46 (59.09	9.29 9.38	6.50 6.32	4.80 4.73)	217—219	204—206	42
Cyclohexyl	$C_{15}H_{24}N_3O_8P$	44.41 (44.24	5.92 5.71	10.36 10.09	7.64 7.51)	218—220	214—216	30
3β-Chlesteryl	$C_{36}H_{58}N_3O_8P$	62.50 (62.37	8.45 8.55	6.07 6.16	4.48 4.23)	222—224		60
1-Adamantyl	$C_{19}H_{28}N_3O_8P$	49.89 (49.66	6.17 5.98	9.19 9.08	6.77 6.52)	202—204	246—248	49
Phenyl	$C_{15}H_{18}N_3O_8P$	45.08 (45.31	4.51 4.39	10.52 10.28	7.76 7.71)	210—212	173—175	24
o-Chlorophenyl	$C_{15}H_{17}N_3O_8PCl$	41.54 (41.33	3.95 3.68	9.69 9.61	7.14 6.93)	232—234	198—200	33
o-Tolyl	$C_{16}H_{20}N_3O_8P$	46.45 (46.32	4.84 4.96	10.16 9.97	7.49 7.25)	224—225	203—205	34
p-Nitrophenyl	$C_{15}H_{17}N_4O_{10}P$	40.51 (40.43	3.83 3.69	12.60 12.58	6.97 6.79)	229—231	197—199	40
β-Phenoxyethyl	$C_{17}H_{22}N_3O_9P$	46.06 (45.89	5.00 4.74	9.48 9.61	6.99 6.70)	239—241	167—169	81
β-Phenylpropyl	$C_{18}H_{24}N_3O_8P$	48.98 (48.86	5.48 5.25	9.52 9.80	7.02 6.88)	231—233	172—174	60

posed a problem, because the applicability of direct alkylation with diazoalkanes is restricted. The reaction of an alcohol or phenol and an activating reagent such as dicyclohexylcarbodiimide or arylsulfonyl chloride caused preferential cyclization of 2′- and 5′-positions and produced the 2′,5′-cyclic phosphate predominantly. Thus, we selected fully acylated Ara CMP as the starting material. Ara CMP was treated with acetic anhydride or *n*-butyric anhydride in pyridine in the presence of tri-*n*-butylamine at room temperature. After hydrolysis of the acylphosphate bond, N_4,2′,3′-tri-acylated derivatives were obtained. These fully acylated Ara CMP's (3) were dissolved in chloroform and treated with 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl), followed by reaction with various alcohols or phenols. The method used to isolate the resulting slkyl or arylester depended on the chain-length of the esters. In the case of aryl and short-chain alkyl esters, the reaction mixture was evaporated and the product was extracted with chloroform. The acyl groups were saponified with ammonia and the product was purified by column chromatography on Amberlite IRA-402 (formate form). Elution was performed with formic acid (alkyl ester) or 0.01 N HCl (aryl ester). In esters bearing long-chain alkyl groups (8 to 24 carbons), the deacylated mixture was evaporated and redissolved in distilled water. When the pH was adjusted to 1—2 by addition of hydrochloric acid, the desired esters precipitated as crystals. The structures of all compounds were confirmed by UV, NMR and elemental analysis. The compounds having a short-chain alkyl group (carbon number 1 to 9) were readily soluble in water as their sodium salts. The solubility of the compounds bearing a longer alkyl carbon chain gradually decreased as the chain length increased. The compounds were hydrolyzed with phosphodiesterase to give Ara CMP (see "Experimental").

Table II. Antiviral Activity of Various Ara CMP Esters against Herpes simplex Type 1 in Culture

Compound	Carbon number	MIC[a] (μg/ml)	VR[b]	MTC[c] (μg/ml)
Ara C		1.0	2.5	1000
Ara A		10	1.8	
IUdR		3.2	2.4	
EtUdR		3.2	2.1	
Ara CMP	0	3.2	2.4	1000
Methyl	1	3.2	2.5	1000
Ethyl	2	3.2	2.3	1000
n-Propyl	3	3.2	2.3	1000
n-Butyl	4	3.2	2.4	1000
n-Amyl	5	10.0	2.0	1000
n-Hexyl	6	10.0	2.0	1000
n-Heptyl	7	10.0	2.0	1000
n-Octyl	8	10.0	2.0	1000
n-Nonyl	9	10.0	1.9	1000
n-Decyl	10	10.0	1.6	320
n-Undecyl	11	10.0	1.2	100
n-Lauryl	12	10.0	0.7	100
n-Tridecyl	13	32.0	0.5	100
n-Tetradecyl	14	32.0	0.5	100
n-Pentadecyl	15	10.0	0.5	10
n-Cetyl	16	100.0	0.3	32
n-Heptadecyl	17	32.0	0.5	32
n-Stearyl	18	32.0	0.4	10
n-Eicosyl	20	32.0	0.5	32

a) Minimal inhibitory concentration.
b) Virus rating.
c) Maximal tolerated concentration.

Antiviral Activity

The general method for examination of antiviral activity of the compounds against herpes simplex virus in cultured human embryonic lung cells is described in "Experimental." The effectiveness of each compound was evaluated by using the modified virus rating (VR) procedure.[15,16]

As can be seen in Table II, strong antiviral activity without an inhibitory effect on non-infected host cells was found with Ara CMP and its 1- to 8-carbon alkyl esters. The minimum inhibitory concentrations (MIC) and virus rating values (VR) of Ara CMP esters bearing 0 to 4 carbon alkyl groups were almost equal to those of the known antiviral agents, 5-iodo-2′-deoxyuridine (IUdR) and 5-ethyl-2′-deoxyuridine (EtUdR). Ara CMP esters bearing 5- to 12-carbon alkyl groups showed almost the same antiviral activity (MIC) as 9-β-D-arabinofuranosyladenine (Ara A). However, the virus rating values (VR) of these derivatives were smaller than those of the short-chain alkyl esters. As the alkyl groups become longer, both MIC and VR decreased and the toxicity of the compounds towards non-infected cells (MTC) increased.

Growth Inhibitory Effects of the Compounds against Mouse Leukemic L5178Y Cells in Culture

The assay condition and the evaluation procedure are described in "Experimental."

TABLE III. Growth Inhibitory Effect of Various Ara CMR Esters on Mouse Leukemic L5178Y Cells in Culture

Compound	Carbon number	Growth Inhibition ED_{50} (μg/ml)	(μM)
Ara C		0.06	0.3
Ara CMP	0	0.1	0.3
Methyl	1	0.2	0.6
Ethyl	2	0.4	1
n-Propyl	3	0.7	2
n-Butyl	4	0.8	2
n-Amyl (Pentyl)	5	0.7	2
n-Hexyl	6	0.7	2
n-Heptyl	7	1.0	2
n-Octyl	8	1.0	2
n-Nonyl	9	2.0	4
n-Decyl	10	2.0	4
n-Undecyl	11	4.0	8
n-Lauryl	12	6.0	10
n-Tridecyl	13	10.0	20
n-Tetradecyl	14	20.0	40
n-Pentadecyl	15	10.0	20
n-Cetyl	16	50.0	90
n-Heptadecyl	17	20.0	30
n-Stearyl	18	30.0	50
n-Eicosyl	20	10.0	20
n-Tricosyl	23	100.0	150
Cyclohexyl	6	10.0	20
Phenyl	6	0.3	0.6
o-Chlorophenyl	6	0.3	0.7
o-Tolyl	7	0.3	0.6
p-Nitrophenyl	6	0.3	0.6
β-Phenoxyethyl	8	0.2	0.4
β-Phenylpropyl	9	0.3	0.6

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Table IV. Antileukemic Activity of Various Arg CMP Esters against L1210 in Mice

(% ILS, mg/kg/day × 5)

Compound	Carbon number	Dose (mg)							
		1	3	10	30	100	300	500	700
Ara C			41	43	67	90	Toxic		
Ara CMP	0	21	29	53	61	79	107	92	63
Methyl	1				26	61	100		
Ethyl	2			5	37	58	89	116	118
n-Propyl	3				42	48	85		
n-Butyl	4				65	45	84		
n-Amyl (Pentyl)	5				5	37	68		
n-Hexyl	6			0	5	49	69		
n-Heptyl	7				0	26	103		
n-Octyl	8				19	84	81		
n-Nonyl	9				23	59	89		
n-Decyl	10			20	30	33	95		
n-Undecyl	11			13	42	66			
n-Lauryl	12			47	95				
n-Tridecyl	13		10	64	122				
n-Tetradecyl	14			91	95				
n-Pentadecyl	15			66	102				
n-Cetyl	16			78	105				
n-Heptadecyl	17			53	114				
n-Stearyl	18		37	79	105				
n-Eicosyl	20		36	74	119				
n-Tricosyl	23		36	54	103				
Cyclohexyl	6				8	18	20		
Phenyl	6				36	53	123		
o-Chlorophenyl	6			36	51	74	82		
o-Tolyl	7			26	35	48	62		
p-Nitrophenyl	6				30	56	88		
β-Phenoxyethyl	8				53	84	108		
β-Phenylpropyl	9				22	65	114		
1-Adamantyl	10				0	−1			
3*β*-Cholesteryl	27				0	8	Toxic		

As can be seen in Table III, Ara CMP esters bearing relatively short alkyl groups (0 to 8 carbons) were highly cytotoxic (0.1 to 1 μg/ml or 0.3 nmol to 2 nmol/ml) and these values were satisfactory, and justified further evaluation. In the case of alkyl esters of more than 10 carbons, the cytotoxicity rapidly decreased. On the other hand, strong growth-inhibitory effects were shown by Ara CMP esters bearing aryl or aralkyl groups such as phenyl, phenylpropyl or phenoxyethyl. The ED_{50} values of these compounds were around 0.3 ng/ml or 0.6 nmol/ml. The carbon numbers of these aryl and aralkyl groups were 6 to 9.

Antileukemic Activity of Ara CMP Esters against L-1210 in Mice

The assay conditions and the evaluation method are described in "Experimental."

As can be seen from Table IV, Ara C is active against this system at doses from 3 mg/kg to 100 mg/kg. However, at dose of 300 mg/kg, severe toxicity appeared. In contrast, Ara CMP was quite active against this system over a wide range of dosage from 1 mg/kg to 700 mg/kg. These results suggest that the toxicity of Ara C was greatly reduced by phosphorylation. Carcinostatic activity was found in the compounds 1- to 11-carbon alkyl groups on the phosphate moiety. Among these compounds, Ara CMP ethyl ester was found to be the most effective in this system because of its wide range of activity and low toxicity. The increased life span (ILS) at dose of 500 mg/kg and 700 mg/kg was approximately 120%. The Ara CMP esters having 10- to 20-carbon alkyl groups were effective at low doses. These com-

pounds showed 60—90% ILS at 10 mg/kg and 100 to 120% ILS at doses of 30 mg/kg. Ara C showed only 43% and 67% ILS at doses of 10 mg/kg and 30 mg/kg, respectively. This suggests that Ara CMP or Ara C might be released very slowly *in vivo* form Ara CMP esters bearing long chain alkyl groups. Prolonged release of such active molecules *in vivo* might provide more effective cancer chemotherapy. On the other hand, Ara CMP esters bearing an aromatic ring showed antileukemic behavior similar to that of alkyl esters with 1—11 carbons. This may be due to the sensitivity of these compounds to phosphodiesterase, which could release Ara CMP *in vivo*.

Oral administration of these drugs gave better results against L-1210 in mice. Further studies are in progress.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. UV spectra were recorded on a Beckman. Model 24 spectrophotometer. NMR spectra were recorded on a Hitachi R20B high-resolution NMR spectrometer in d_6-DMSO with tetramethylsilane as an internal standard. Thin-layer chromatography was performed on pre-coated silica gel 60 F_{254} (Merck) or cellulose plastic sheet F_{254} (Merck).

1-β-D-Arabinofuranosylcytosine 5′-Phosphate (Ara CMP) (2)——Well-dried cytidine (5 g) was treated with 50 g of polyphosphoric acid in a stoppered flask. After heating at 80° for 24 hr, the resulting clear solution was diluted with distilled water (200 ml) and the solution was heated at 100° for 60 min to hydrolyze the pyrophosphate bond. The pH was adjusted to 9.0 by the slow addition of 10% aqueous lithium hydroxide, resulting in hydrolysis of the 2,2′-anhydro intermediate to give, as the major product, the 1-β-D-arabinofuranosylcytosine 3′,5′-diphosphate (**1**). Precipitated lithium phosphate was removed by filtration and 3 volume of ethanol was added to the solution. The precipitated crude diphosphate was washed well with ethanol, dissolved in 15 ml of distilled water and applied to a column of Dowex 1 (formate form, 3×45 cm). The column was washed with distilled water (1 liter) and eluted successively with 0.01 N formic acid (500 ml) and 0.1 N formic acid (500 ml). Some monophosphate was eluted with 0.01 N and the desired diphosphate was eluted with 0.1 N formic acid. The fractions containing diphosphate were combined and concentrated. After checking the homogeneity of the residue by paper electrophoresis and thin-layer chromatography on a cellulose plate, the product was hydrolyzed with nuclease-3′-nucleotidase P_1 (50 mg) in 0.05 M sodium acetate buffer (pH 5.5, 300 ml) at 60° for 24 hr. The reaction mixture contained mainly 5′-monophosphate and a small amount of starting **1**. The solution was treated with active carbon (100 g) and filtered. The filter cake was washed with distilled water (1.5 liters) and then eluted with 50% aqueous ethanol containing 1% ammonia (1 liter). The solvent was removed under reduced pressure to give 1-β-D-arabinofuranosylcytosine 5′-phosphate (**2**) as the ammonium salt. This residue was dissolved in 20 ml of distilled water and applied to a column of Dowex 1 (formate form, 3×30 cm). Elution was performed with 0.01 N formic acid (500 ml) and the fractions containing Ara CMP were combined and evaporated *in vacuo*. The residue was co-evaporated with distilled water (5 ml×5) and then dissolved in 5 ml of water. The solution was diluted with ethanol (15 ml) and stored in a freezer overnight. The resulting precipitates were collected by filtration to give pure Ara CMP (5 g) as the free acid. The properties of this compound are summarized in Table I.

N_4,2′,3′-Triacetyl Ara CMP (3a)——Ara CMP (10 g, free acid, 30.9 mmol) was treated with freshly distilled tri-*n*-butylamine (8 ml, 33.6 mmol) and acetic anhydride (20 ml) in pyridine (30 ml). The resulting mixture was stirred at room temperature for 20 hrs, then 50 ml of distilled was added to the resulting clear solution and the mixture was stirred for 2 hr at room temperature. The solvent was removed under reduced pressure and the residue was cowith distilled water several times to ensure complete removal of pyridine and pyridinium acetate. The residue was treated with anhydrous dioxane (20 ml) and evaporated to dryness to give 13.5 g of a gum which was homogeneous on thin-layer chromatography. A single ultraviolet-absorbing and phosphorus-containing spot was observed at *Rf* 0.40 (solvent A, *n*-butanol-1 M ammonium acetate, pH 8.0–ethanol=5: 3: 2, v/v/v) on a cellulose plate, and at *Rf* 0.44 (solvent B, dioxane–methanol, 20: 1, v/v) on a silica gel plate UV, $\lambda_{max}^{H_2O}$ (nm) 213, 245 and 297. This product was used directly for the next condensation step.

N_4,2′,3′-Tri-*n*-butyryl Ara CMP (3b)——Ara CMP (10 g) was treated with *n*-butyric anhydride (35 ml) in anhydrous pyridine (50 ml) in the presence of tri-*n*-butylamine (8 ml). The clear mixture was stirred for 20 hr at room temperature. Methanol (50 ml) was then added to the mixture to decompose excess *n*-butyric anhydride. Water (20 ml) was added and the mixture was stirred for 20 hr at room temperature to cleave the butyric–phosphoric anhydride bond. The solvent was removed under reduced pressure and the residue was dissolved in chloroform (200 ml). The solution was washed with water (300 ml×3). The organic layer was concentrated and the gummy residue was dissolved in 30 ml of anhydrous dioxane and evaporated to dryness. The resulting hard glassy product (13.5 g) was homogeneous on a silica gel thin-layer

chromatography. $Rf=0.60$ (Solvent B). This product was used directly for next step.

General Procedure for the Synthesis of Ara CMP alkyl (1—7 Carbons) or Arylphosphates (4)——N_4,2′,3′-tri-*n*-butyryl Ara CMP (**3b**) (from 5 mmol of **2**) in chloroform (50 ml) was treated with 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl) (3 g, 9.9 mmol). The solvent was removed and a suitable alcohol or phenol (1 to 5 ml) and anhydrous pyridine (10 ml) were added to the residue. The solution was stirred for 5 hr at room temperature. The solvent was evaporated off and the residue was treated with distilled water (50 ml) and chloroform (50 ml). After vigorous shaking, the chloroform layer was separated. Concentrated ammonia (10 ml) and ethanol (10 ml) were added, and the mixture was stirred overnight for saponification of the butyryl groups. The solvent was removed under reduced pressure and distilled water was added to the residue. The insoluble 2,4,6-triisopropylbenzenesulfonic acid was filtered off. The filtrate was diluted with distilled water to a final volume of 200 ml and the pH was adjusted to 7.0. The solution was applied to a column of Amberlite IRA-402 (formate form, 50 ml). Elution was performed with 0.01 N formic acid (in the case of alkyl esters) or 0.01 N HCl (in the case of aryl esters). The fractions containing the diester were combined and concentrated to a small volume (about 1.5 ml), then the diester was precipitated as the free acid by addition of 3 volumes of ethanol. The resulting free acid was dissolved in 5 ml of distilled water and the pH was adjusted to 7.0 with 1 N NaOH. The solution was concentrated to 1 ml. Addition of ethanol (3 ml) to the solution caused precipitation of the sodium salt. Yield and analytical results (free acid or sodium salt) are summarized in Table I.

General Procedure for the Synthesis of Ara CMP Alkyl (8 to 23 Carbons) Phosphate——N_4,2′,3′-Triacetyl Ara CMP (**3a**) (10 g) was treated with TPS-Cl (3 g) and a suitable alcohol (5 ml) in a manner similar to that described above. After the deacylation step, the aqueous solution was concentrated to 50 ml. The pH of the solution was adjusted to 1—2 by addition of hydrochloric acid, whereupon the nucleotide began to separate from the solution as the free acid. This product was collected by filtration, dissolved in water (pH 7.0) and precipitated again by addition of hydrochloric acid. The sodium salt was prepared by the procedure described above. Yields and analytical results for the series of the esters are summarized in Table I.

Spectroscopic Characterization of the Ara CMP Esters by UV and NMR——The ultraviolet absorption spectra of Ara CMP and its alkyl and aryl esters have an absorption maximum at 272 nm (water) in all cases.

In the NMR spectra, the 6-H and 5-H protons of the cytosine nucleus were observed at δ 7.6—8.0 (d) and δ 5.7—6.0 (d), respectively. The anomeric proton of the sugar moiety (1′-H) was observed at δ 6.0 (t). In Ara CMP alkyl esters, the methylene protons appeared at around 1.20—1.70, depending on the alkyl carbon number. Ara CMP esters bearing an aromatic ring such as the phenyl ester showed aromatic proton chemical shifts at around δ 6.6—7.5.

Enzymatic Hydrolysis of Ara CMP Esters with Phosphodiesterase——Ara CMP ester (2 mg) in 0.05 M Tris-HCl (pH 8.5) (0.4 ml) containing 0.01 mM $MgCl_2$ and snake venom phosphodiesterase (0.03 unit) was incubated at 37° for 2 hr. The product was detected by paper electrophoresis (in 0.05 M triethylammonium bicarbonate, pH 8.0, 700 V for 40 min) and UV absorption measurement. Ara CMP alkyl esters (1—8 alkylcarbons) were hydrolyzed completely to Ara CMP, whereas Ara CMP alkyl esters (more than 9 carbons) were hydrolyzed very slowly. On the other hand, aryl esters including phenylpropyl and phenoxyethyl ester were hydrolyzed more easily than alkyl esters bearing a short carbon chain (almost complete hydrolysis after 1 hr).

Assay and Evaluation of Antiviral Activity——Monolayer culture of human embryonic lung fibroblast cells was carried out in plastic microplates (Linbro Scientific Co. Ltd., model FA 16-24 TC) using Eagle's basal medium (BME-Diploid, Grand Island Biological Co. Ltd.) supplemented with 10% bovine fetal serum (Flow Laboratories), penicillin (100 unit/ml) and streptomycin sulfate (100 μg/ml). The cells were infected with 30 $TCDI_{50}$ of type 1 herpes simplex virus (HSV/1), strain VR-3. Approximately 30 min after infection, the virus solution was removed and 0.5 ml of test compound in the same medium was added to the appropriate well. Test compounds were diluted in one-half $\log_{10}$ dilutions; 1000 μg/ml was the maximum concentration used. The plates were incubated for three days and examined microscopically for virus-induced cytopathogenic effect (CPE). The effectiveness of each compound against HSV/1 was evaluated numerically, using the modified virus rating (VR) procedure.[15,16] Antiviral activity was also expressed in terms of minimal virus inhibitory concentration (MIC) of the compound. Each experiment included a cell control (cells with the test compound alone), a virus control (cells with virus in the test medium) and a toxicity control (cells with the compound in the test medium).

Assay Method for Growth Inhibitory Effect of the Compounds against Mouse Leukemic L5178Y Cells in Culture——Mouse leukemic L5178Y cells were grown in RPMI-1649 medium[17] (Nissui Seiyaku Co., Ltd.) supplemented with 10% calf serum (Flow Laboratory, Md, U.S.A.) at 37°. One volume of the test compound diluted with the same medium was added to 9 volumes of the culture containing between 1.2×10^5 and 1.7×10^5 L5178Y cells per ml. After incubation for 48 hr at 37° in 5% carbon dioxide, the number of remaining cells was counted with a cell counter (Toa micro cell counter, model 1002).

17) G. Moore, D. Mount, G. Tara, and N. Schwartz, *Cancer Res.*, **23**, 1735 (1963).

Assay of the Effect of the Compounds against Leukemia L-1210 in Mice——Male (18—22 g) or female (16—20 g) BDF_1 mice were inoculated intraperitoneally with 1×10^5 cells of L-1210 and divided into groups of 5 mice. Compound to be tested were administered intraperitoneally once a day for 5 days starting 24 hr after tumor implantation. Then mean survival time (MST) of each group was examine. Antileukemic activity was evaluated in terms of the percentage increased of life span (%ILS) over the control group. %ILS=(treated MST/control MST-1)$\times$100. The results of this study are shown in Table IV.