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Synthetic Nucleosides and Nucleotides. XXVIII.[1] Synthesis of 5-Alkylcytidines from 5-Alkylbarbituric Acids

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5-Alkylbarbituric acids (**1b—f**) were converted to 5-alkyl-2,4,6-trichloropyrimidines (**2b—f**) by using phosphoryl chloride in refluxing *n*-butyl acetate in the presence of *N*,*N*-diethylaniline hydrochloride. Treatment of **2** with sodium methoxide in dry acetonitrile followed by reaction with potassium ethyl mercaptide and desulfurization with Raney Ni afforded 5-alkyl-2,4-dimethoxypyrimidines (**5b—f**), as key intermediates in the present study. Coupling of **5** with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose in the presence of stannic chloride in acetonitrile afforded 5-alkyl-1-(2,3,5-tri-*O*-benzoyl)-β-D-ribofuranosyl-1,2-dihydro-4-methoxypyrimidin-2-ones (**6a—f**) in quantitative yields. Ammonolysis of **6** with methanolic ammonia afforded the title 5-alkylcytidines (**7a—f**). Compounds **6** were also easily converted to their uridine counterparts by treatment with hydrochloric acid. Growth-inhibitory effects of **7** on cultured mouse leukemia L5178Y cells, antiviral activity against a rhabdovirus, infectious hematopoietic necrosis virus (IHNV), in cultured CHSE-214 cells and properties as a substrate of human cytidine deaminase were also examined.

Keywords——5-alkylbarbituric acid; 5-alkylcytidine; cytidine deaminase; L5178Y cell; IHNV

In order to obtain information about the effects of the 5-substituents of pyrimidine nucleosides and nucleotides on biological activities such as antitumor and antiviral activities, and inhibition of cytidine deaminase, uridine-cytidine kinase, deoxycytidine kinase, eukaryotic and viral deoxyribonucleic acid (DNA) polymerases and ribonucleic acid (RNA) polymerases, a simplified procedure for synthesis of a series of 5-alkylcytidines was developed.

Interest in the steric and hydrophobic effects of the 5-substituents of pyrimidine nucleosides and nucleotides has been growing, and we have previously shown that the activities of thymidine phosphorylase,[2] thymidylate synthetase,[3] DNA polymerases,[4-7] and RNA polymerases,[8,9] towards substrates are greatly affected by substitution at the 5-position of the substrates with halogens or alkyl groups.

We have also reported the synthesis of various 5-alkyluridines,[10] 5-aklyl-l-β-D-arabinofuranosyluracils,[10] 5-styryluracils,[7] and 5-alkyl-l-β-D-xylofuranosyluracils.[11] Antiviral activities of these compounds were examined.[10,12-15] In contrast to these 5-alkyluracil derivatives, few synthetic and biological studies of 5-alkylcytosine nucleosides have been done. Among the 5-alkylcytidines, 5-methyl-,[16] 5-ethyl-,[17] and 5-*n*-butylcytidine,[18] were synthesized from the uridine counterparts *via* a thiation procedure. However, no simple procedure for systematic synthesis of 5-alkylcytidines has been reported so far.

Synthesis

Generally, 5-alkyluridines have been synthesized by glycosylation of a mercuri-salt[18] or bis-trimethylsilyloxy-5-alkylpyrimidines.[10] The key compounds, 5-alkyluracils, however, can only be prepared starting from α-formyl-alkyl carboxylic acid esters and urea or thiourea in very low yields.[19] Therefore, we have selected 5-alkylbarbituric acids (**1**) as starting material because they can be easily prepared from alkyl malonates and urea in good yields.[19b] Very recently, these compounds (**1**) were used as starting compounds for preparation of 5-alkyl-

uracils *via* the 6-chloro derivatives followed by hydrogenolysis.[20]

Chlorination of compound **1** to give 5-alkyl-2,4,6-trichloropyrimidines (**2**) was studied under several chlorination conditions. Phosphoryl chloride in refluxing *n*-butyl acetate in the presence of *N*,*N*-diethylaniline hydrochloride was found to be the best choice for the preparation of **2**. The reaction of sodium methoxide with **2** in methanol under several conditions gave 5-alkyl-2,4,6-trimethoxypyrimidines as sole products and the required 5-alkyl-6-chloro-2,4-dimethoxypyrimidines (**3**) were not obtained. After examination of solvent effects on the reaction, we found that anhydrous acetonitrile is the best solvent for this reaction. The resulting **3** were treated with potassium ethyl mercaptide in dry dimethylformamide to obtain the 6-ethylthio intermediates (**4**), followed by desulfurization with Raney Ni to afford 5-alkyl-2,4-dimethoxypyrimidines (**5**) in satisfactory yields. The coupling reaction of **5** with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose in acetonitrile in the presence of stannic chloride gave 5-alkyl-1-(2,3,5-tri-*O*-benzoyl)-β-D-ribofuranosyl-1,2-dihydro-4-methoxypyrimidin-2-ones (**6a—f**) in nearly quantitative yields. These compounds were converted to cytosine nucleosides (**7a—f**) by the reaction with methanol saturated with ammonia in a stainless steel container at 100 °C for 21 h, followed by purification by Dowex 1 (OH^-) column chromatography. Compounds **6** were also easily converted to the uridine counterparts by treatment with hydrochloric acid under conventional conditions.[21] The structure of **7** was confirmed by elemental analysis and spectrophotometries. The assignment of the β-configuration of **7a—f** was made on the basis of the positive sign of the circular dichroism (CD) band. This is in accord with the results for 1-β-D-pentofuranosyl-pyrimidines.[10,11,22] The coupling constant ($J_{1'-2'}$) of around 2.4 Hz of **7a—f** also supports the β-configuration of the compounds.

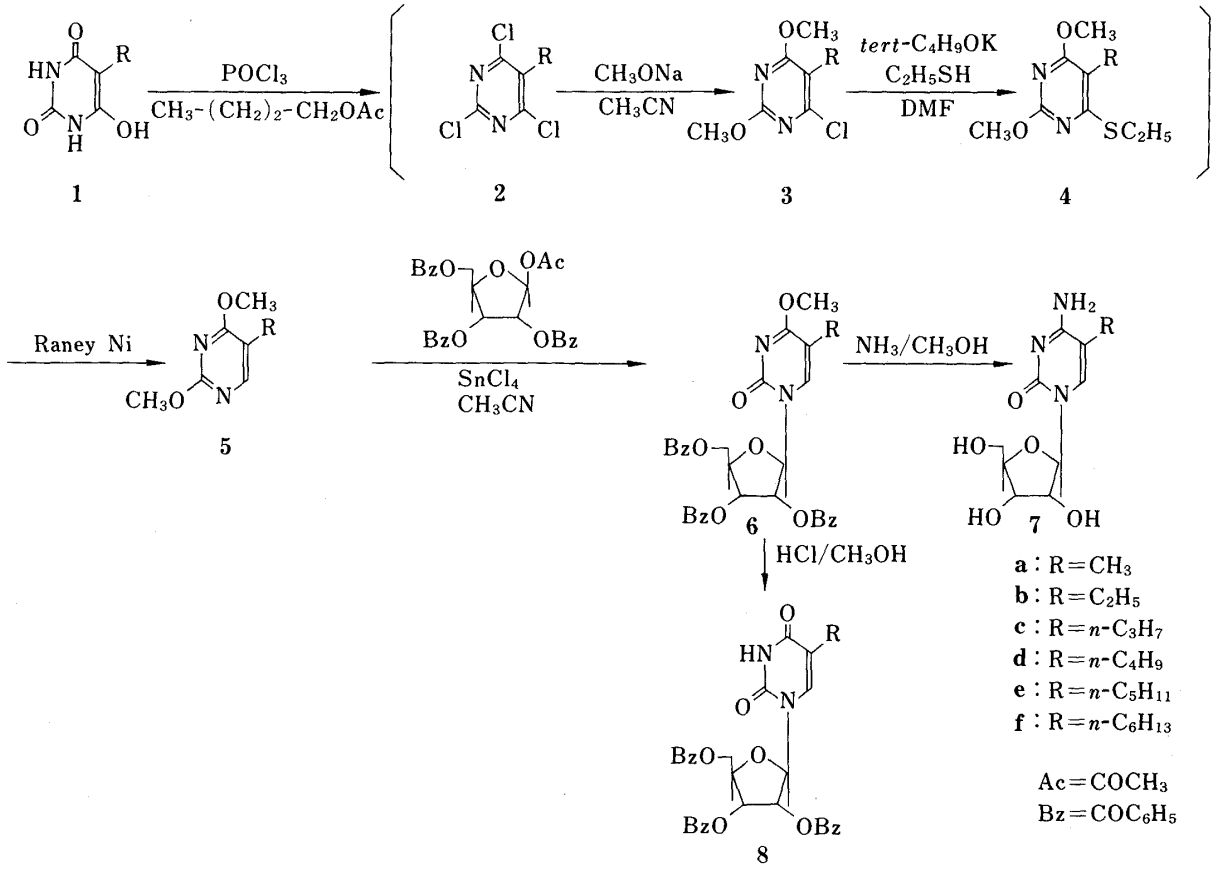

Chart 1

Biological Evaluations

Properties of 7a—f as Substrates of Human Cytidine Deaminase——Compounds **7a—f** were examined as substrates of human placental cytidine deaminase. Under conditions (see Experimental) where cytidine was completely hydrolyzed to uridine. It was found that 50% of **7a**, 12% of **7b** and 5% of **7c** were hydrolyzed to the corresponding uridine derivatives. However, **7d—f** were quite resistant to this enzyme.

Growth-Inhibitory Effects of 5-Alkylcytidines (7a—f) on Cultured Mouse Leukemia L5178Y Cells——The effects were examined according to a method described previously.[22] Among the compounds tested, **7a—c** were slightly active against these cells ($IC_{50} = 100\ \mu g/ml$) but **7d—f** were essentially inactive. This finding indicates that the affinity of a series of 5-alkylcytidines for cellular uridine-cytidine kinase is modified by the steric effect of the substituents.

Antiviral Activity——Antiviral activity of the compounds (**7a—f**) was also examined using the CPE-spot reduction method[23,24] on a rhabdovirus, infectious hematopoietic necrosis virus (IHNV), in cultured CHSE-214 cells. However, we could not find any activity at concentrations up to 100 μg/ml. The 5′-triphosphate forms of a series of 5-alkylcytidines (5-alkyl CTPs) should be substrates of DNA-dependent RNA polymerases I, II and III, and a study on this is in progress.

Experimental

Melting points were determined on a Yanaco MP-3 apparatus and are uncorrected Ultraviolet (UV) spectra were recorded on a Shimadzu UV-300 recording spectrophotometer. CD spectra were measured on a JASCO model 20 automatic recording spectro polarimeter. Mass spectra (MS) were measured on a Hitachi RMU-6E mass spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained on a Hitachi R20B high resolution NMR spectrometer. 5-Alkylbarbituric acids (**1b—f**) were prepared from diethyl alkyl malonate and urea in the presence of sodium ethoxide in dimethylformamide according to Burkhalter and Scarborough[19b] in 90—98% yields.

General Procedure for the Preparation of 5-Alkyl-2,4,6-trichloropyrimidines (2b—f)——A well-dried 5-alkylbarbituric acid (**1b—f**) (50 mmol) was added to a mixture of freshly distilled phosphoryl chloride (15 ml) and *N*,*N*-diethylaniline hydrochloride (10 g) in *n*-butyl acetate (40 ml). The mixture was refluxed under continuous stirring for 3—5 h. The solvent and excess phosphoryl chloride were then evaporated off under reduced pressure and the residue was mixed with crushed ice. After 30 min, the mixture was extracted with benzene (50 ml × 3). The benzene solution was washed with distilled water (30 ml × 2), 5% sodium bicarbonate (30 ml × 2) and distilled water (30 ml × 2), then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure. The residue was homogeneous on thin-layer chromatography on silica gel (benzene as the solvent). UV, λ_{max}^{EtOH}(nm): 267, 275 (shoulder). These compounds were used directly for the next step.

5-Alkyl-2,4-dimethoxypyrimidines (5a—f)——A solution of a 5-alkyl-2,4,6-trichloropyrimidine (**2b—f**) (*ca.* 25 mmol) in anhydrous acetonitrile (100 ml) was treated with sodium methoxide (50 mmol) and the mixture was stirred for an additional 1 h. Ethyl mercaptan (2.3 ml) and potassium *tert*-butoxide (3.52 g) were added to a solution containing a 5-alkyl-6-chloro-2,4-dimethoxypyrimidine (**3b—f**). The mixture was stirred at 75 °C for 3 h and the solvent was evaporated off. The residue was treated with cold distilled water (100 ml) and the solution was extracted with ethyl ether (50 ml × 3). The aqueous phase was discarded and the organic layer was dried over anhydrous sodium sulfate. The residual crude 5-alkyl-2,4-dimethoxy-6-ethylthiopyrimidine (**4**) was redissolved in ethanol (100 ml) and Raney Ni W-2 (3 ml) was added. The mixture was refluxed for 3 h. The catalyst was removed by filtration while hot, and the filter cake was washed with hot ethanol. The combined filtrate and washings were evaporated to dryness. The residue was dissolved in a small amount of benzene and applied to a column of silica gel (Wakogel C-200) (100 g). Elution was performed with benzene, and the eluate was evaporated to give the corresponding pure 5-alkyl-2,4-dimethoxypyrimidine (**5b—f**) (Table I). 2,4-Dimethoxy-5-methylpyrimidine (**5a**) was synthesized separately from thymine according to a known method.[25]

5-Alkyl-1-(2,3,5-tri-*O*-benzoyl)-β-D-ribofuranosyl-1,2-dihydro-4-methoxypyrimidin-2-ones (6a—f)——Stannic chloride (2.7 g, 10 mmol) in 10 ml of acetonitrile was added to a solution of 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (5.05 g, 10 mmol) and **5** (11 mmol) in dry acetonitrile (120 ml) under stirring and cooling below 10°C. After the solution had been stirred at room temperature overnight, distilled water (30 ml) and solid sodium bicarbonate (6 g) were added to neutralize it. After vigorous evolution of carbon dioxide had ceased, the solvent was carefully removed under reduced pressure. The residue was treated with anhydrous benzene and the mixture was evaporated to dryness. This process was repeated twice. The glassy residue was extracted with boiling ethyl acetate

TABLE I. Spectral Data for 5-Alkyl-2,4-dimethoxypyrimidines (**5a—f**)

Compound	Yield from **1** (%)	Formula	MS (80 eV) (*m/z*)	^{1}H-NMR ($CDCl_3$) δ
5a	86 (from thymine)	$C_7H_{10}O_2N_2$	154 (M^+), 139 (M^+-CH_3), 124 (M^+-2CH_3)	8.05 (s, 1H, H-6), 4.01 (s, 3H, $-OCH_3$), 3.98 (s, 3H, $-OCH_3$), 2.07 (s, 3H, $-CH_3$)
5b	66.6	$C_8H_{12}O_2N_2$	168 (M^+), 153 (M^+-CH_3), 138 ($153-CH_3$), 123 ($153-2CH_3$)	8.06 (s, 1H, H-6), 4.01 (s, 3H, $-OCH_3$), 3.98 (s, 3H, $-OCH_3$), 2.52 (q, 2H, $-CH_2-$), 1.17 (t, 3H, $-CH_3$)
5c	59	$C_9H_{14}O_2N_2$	182 (M^+), 167 (M^+-CH_3), 153 ($M^+-C_2H_5$), 139 ($153-CH_3$), 123 ($153-2CH_3$)	8.04 (s, 1H, H-6), 4.01 (s, 6H, 2-OCH_3), 2.47 (t, 2H, $-CH_2-$), 1.60 (s, 2H, $-CH_2-$), 0.94 (t, 3H, $-CH_3$)
5d	48.4	$C_{10}H_{16}O_2N_2$	196 (M^+), 181 (M^+-CH_3), 167 ($M^+-C_2H_5$), 153 ($M^+-C_3H_7$), 139 ($153-CH_2$), 123 ($153-2CH_3$)	8.00 (s, 1H, H-6), 4.00 (s, 6H, 2OCH_3), 2.47 (t, 2H, $-CH_2-$), 1.45 (br, 4H, $-CH_2-CH_2-$), 0.92 (t, 3H, $-CH_3$)
5e	48.2	$C_{11}H_{18}O_2N_2$	210 (M^+), 195 (M^+-CH_3), 181 ($M^+-C_2H_5$), 167 ($M^+-C_3H_7$), 153 ($M^+-C_4H_9$), 139 ($153-CH_2$)	8.04 (s, 1H, H-6), 4.00 (s, 6H, 2-OCH_3), 2.48 (t, 2H, $-CH_2-$), 1.46 (br, 6H, C_3H_6), 0.91 (t, 3H, $-CH_3$)
5f	27	$C_{12}H_{20}O_2N_2$	224 (M^+), 209 (M^+-CH_3), 167 ($M^+-C_4H_9$), 153 ($M^+-C_5H_{11}$), 139 ($153-CH_2$), 123 ($153-2CH_3$)	8.04 (s, 1H, H-6), 4.00 (s, 6H, 2-OCH_3), 2.48 (t, 2H, $-CH_2-$), 1.46 (br, 9H, C_3H_6), 0.92 (t, 3H, $-CH_3$)

TABLE II. Analytical Data for Fully Acylated Nucleosides (**6a—f**)

Compound	Yield (%)	Formula	Analysis (%) Calcd (Found) C	H	N	^{1}H-NMR ($CDCl_3$) δ
6a	99	$C_{32}H_{28}O_9N_2$	65.75 (65.52)	4.83 (4.76)	4.79 (4.77)	8.13 (m, 6H, benzoyl *o*-), 7.49 (m, 10H, benzoyl *m*-, *p*- and H-6), 6.62 (d, 1H, H-1′), 5.85 (m, 2H, H-2′, H-3′), 4.81 (m, 3H, H-4′, H-5′), 3.98 (s, 3H, OCH_3-4), 1.65 (s, 3H, CH_3-5) $J_{1'-2'}=5.4$ Hz
6b	92	$C_{33}H_{30}O_9N_2$	66.21 (66.10)	5.05 (5.18)	4.68 (4.50)	Signals from 8.12 to 3.99 were essentially similar to those of **6a**. 2.14 (q, 2H, $-CH_2$-5), 0.90 (t, 3H, CH_3-5)
6c	90	$C_{34}H_{32}O_9N_2$	66.66 (66.74)	5.27 (5.21)	4.57 (4.40)	8.12 to 3.99 (same as **6b**), 2.05 (t, 2H, $-CH_2$-5), 1.29 (m, 2H, $-CH_2-$, 5), 0.76 (t, 3H, $-CH_3$, 5)
6d	94	$C_{35}H_{34}O_9N_2$	67.08 (66.87)	5.47 (5.49)	4.47 (4.30)	8.13 to 3.99 (same as **6c**), 2.09 (br, 2H, $-CH_2-$, 5), 1.4—1.0 (br, 4H, $-CH_2-CH_2-$, 5), (m, 3H, $-CH_3$, 5)
6e	95	$C_{36}H_{36}O_9N_2$	67.49 (67.79)	5.66 (5.76)	4.37 (4.32)	8.13 to 3.98 (same as **6d**), 2.08 (br, 2H, $-CH_2-$, 5), 1.65—1.02 (br, 6H, $-CH_2-CH_2-CH_2-$), 0.57 (m, 3H, $-CH_3$)
6f	99	$C_{37}H_{38}O_9N_2$	67.87 (67.80)	5.85 (5.90)	4.28 (4.44)	8.13 to 3.99 (same as **6e**), 2.07 (br, 2H, $-CH_2-$, 5), 1.19 (br, 8H, $-CH_2-CH_2-CH_2-CH_2-$), 0.89 (br, 3H, $-CH_3$)

(50 ml × 3). The combined extracts were evaporated *in vacuo* and the slightly yellow colored residue was dissolved in a small amount of benzene and was applied to a column of silica gel (Wakogel C-200, 150 g). The column was washed with 100 ml of a 4 : 1 mixture of benzene and ethyl acetate for removal of a small amount of sugar derivative. Elution was performed with chloroform–ethanol (9 : 1) to give the corresponding fully acylated nucleoside (**6**). The solvent was removed under reduced pressure to afford a thin-layer chromatographically homogeneous (silica gel, chloroform–ethanol, 9 : 1, v/v) colorless foam. This foam was crushed and dried in a vacuum desiccator at 45 °C for 24 h. The sample thus obtained was used for analysis. Analytical results are summarized in Table II.

5-Alkylcytidines (7a—f)——Absolute methanol saturated with ammonia at 0 °C (100 ml) was added to a solution of **6** (5 mmol) in absolute methanol (40 ml) in a sealed stainless steel vessel. The solution was heated at 100 °C for 21 h, then the solvent was removed and the residue was dissolved in distilled water (100 ml). The aqueous solution

TABLE III. Analytical Data for 5-Alkylcytidines (**7a—f**)

Compound	Yield (%) from **6**	Recrystallization solvent	mp (°C)	Formula	Analysis (%) Calcd (Found)		
					C	H	N
7a	67	Methanol : 2-propanol (1 : 1)	215—217	$C_{10}H_{15}N_3O_5$	46.69 (46.74	5.88 5.91	16.32 16.25)
7b	52	Ethyl acetate	170—171	$C_{11}H_{17}N_3O_5$	48.70 (48.82	6.27 6.36	15.49 15.31)
7c	93	Ethanol	202—205	$C_{12}H_{19}N_3O_5$	50.52 (50.48	6.71 6.75	14.73 14.59)
7d	49	2-Propanol	114—116	$C_{13}H_{21}N_3O_5$	52.16 (52.01	7.07 7.36	14.04 13.69)
7e	37	Ethanol	111.5—113.5	$C_{14}H_{23}N_3O_5$	53.66 (53.42	7.40 7.40	13.41 13.23)
7f	21	2-Propanol	169—171	$C_{15}H_{25}N_3O_5$	55.03 (54.76	7.70 7.76	12.84 12.74)

was extracted with chloroform (30 ml × 3). The pH of the aqueous phase was adjusted to 4.5 by addition of acetic acid and this phase was reextracted for removal of a trace of benzoic acid. The aqueous solution was concentrated to a small volume (*ca.* 10 ml) and applied to a column of Dowex 1 (OH^- form) (3.2 × 33 cm). After washing of the column with distilled water (200 ml), elution with 30% aqueous methanol provided required cytosine nucleoside (elution volume, between 300—400 ml). The combined fractions were evaporated to dryness to give a colorless gum. Crystallization from an appropriate solvent afforded pure **7**. Yields and analytical results are summarized in Table III.

Conversion of 6 to 5-Alkyl-2′,3′,5′-tri-*O*-benzoyluridines (8)——Methanol (10 ml) presaturated with dry hydrogen chloride was added to a solution of one of **6b—d** (1 mmol) in 10 ml of anhydrous dichloromethane and 10 ml of absolute methanol, and the mixture was stirred at room temperature for 6 h. The solvent was removed under reduced pressure and the residue was dissolved in 50 ml of chloroform and washed with distilled water (30 ml), 5% sodium bicarbonate (30 ml × 2) and distilled water (30 ml). The solution was dried over anhydrous sodium sulfate, then the solvent was evaporated off and the residue was crystallized from ethanol to afford 2′,3′,5′-tri-*O*-benzoyl-5-ethyluridine (**8b**) (95%), 2′,3′,5′-tri-*O*-benzoyl-5-*n*-propyluridine (**8c**) (90%) or 2′, 3′, 5′-tri-*O*-benzoyl-5-*n*-butyluridine (**8d**) (87%), respectively. Their identities were confirmed by comparison with authentic specimens[10] (mixed melting point tests) and infrared spectrometry.

Assay of Cytidine Deaminase——Cytidine deaminase activity was monitored spectrophotometrically by using a UV spectrometer. In an acidic medium the UV absorption spectrum of cytosine nucleoside differs from that of the corresponding uracil nucleoside at 290 nm by $\Delta\varepsilon = -10.1 \times 10^3$, and for 5-alkylcytidines this difference is $\Delta\varepsilon = -8.7 \times 10^3$ at 290 nm and -7.0×10^3 at 300 nm. The incubation mixture contained, in a final volume of 0.2 ml, 20 μmol of Tris–HCl (pH 7.5), 0.2 μmol of $MgCl_2$, an appropriate concentration of substrate and 0.1 unit of human cytidine deaminase (specific activity, 250 units/mg protein). The reaction (at 37 °C) was terminated by addition of 0.5 N $HClO_4$ at 0 °C. The reference blanks contained all the constituents, with the exception that substrate was added to the cold mixture immediately prior to acidification. When comparing the rates of deamination of various substrates, the reactions were terminated at a time which was on the linear portion of the plot of percent substrate hydrolysis *versus* time.

Growth-Inhibitory Effect of the Compounds on Mouse Leukemia L5178Y Cells——Mouse leukemia L5178Y cells were grown in RPMI-1629 medium (Nissui) supplemented with 10% calf serum at 37 °C. One volume of the compound diluted with the same medium was added to 9 volumes of culture containing 1.2×10^5 to 1.7×10^5 L5178Y cells/ml. After incubation for 48 h at 37 °C in 5% carbon dioxide, the number of remaining cells was counted with a cell counter.

Antiviral Activity——Antiviral activity of the compounds on a rhabdovirus, IHNV, was determined by the cytopathic effect (CPE) spot reduction method[23,24] as follows. Nearly confluent CHSE-214 cells in a well of a microtiter plate were infected at a concentration forming about 100 CPE spots per well. After the addition of culture medium containing various levels of the test compound, cultures were incubated for 3 d at 15 °C. After fixing and staining, the CPE spots formed on cell monolayers were counted microscopically.

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