Synthesis and Antitumor and Antiviral Activities of 1-β-D-Arabinofuranosylpyrimidine 3',5'-Cyclic Phosphates

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Ring closure of 1-β-D-arabinofuranosylcytosine 3'-phosphate was accomplished by treatment with dicyclohexylcarbodiimide in refluxing pyridine to yield 1-β-D-arabinofuranosylcytosine 3',5'-cyclic phosphate (cyclic-ara-CMP). Treatment of this product with sodium nitrite in aqueous acetic acid furnished 1-β-D-arabinofuranosyluracil 3',5'-cyclic phosphate. Conversion of cyclic-ara-CMP into 1-β-D-arabinofuranosyl-4-thiouracil 3',5'-cyclic phosphate was accomplished with hydrogen sulfide in pyridine–water. 1-β-D-Arabinofuranosyl-4-methylthio-2-pyrimidone 3',5'-cyclic phosphate was obtained by methylation of 1-β-D-arabinofuranosyl-4-thiouracil 3',5'-cyclic phosphate. Antiviral studies in cell culture reveal cyclic-ara-CMP to be as active as ara-C in inhibiting the growth of DNA viruses. Cyclic-ara-CMP shows considerable toxicity against leukemia L-1210 *in vivo*. Five daily doses of 300 mg/kg produced a 52% increase in the survival time of the mice bearing L-1210 leukemia. *In vitro* studies with partially purified cAMP phosphodiesterase show that the arabinosylpyrimidine 3',5'-cyclic phosphates are slowly hydrolyzed to the monophosphates.

The inhibitory activity of 1-β-D-arabinofuranosylcytosine (cytosine arabinoside, ara-C) against leukemias, lymphomas, and DNA viruses *in vitro* and *in vivo* is well documented.[1] The antitumor activity of ara-C against leukemia L-5178Y cells in culture was found to be due to the prevention of the conversion of cytidine nucleotides to deoxycytidine nucleotides.[2,3] Inhibition of DNA synthesis at the DNA polymerization level by ara-C has also been established.[4-7] Ara-C is converted to the triphosphate (ara-CTP), with the phosphorylation of ara-C being mediated by deoxycytidine kinase.[8] Resistance to ara-C has been found when the activity of this enzyme is significantly reduced.[8]

The use of ara-C as an antiviral agent has been essentially restricted to severe clinical infections because of the toxic effects produced by the drug at the regimens used.[9] In addition, its rapid enzymatic deamination in man to form the inactive metabolite 1-β-D-arabinofuranosyluracil makes it difficult to maintain effective chemotherapeutic blood levels.[10-13] Numerous attempts have been made to modify the chemical structure of ara-C to increase and prolong its activity. A number of analogs of ara-C, ara-FC, cytidine, and 2'-deoxycytidine were studied as substrates and inhibitors of the deaminase enzymes found in human liver and mouse kidney preparations.[14] Ara-C has been oxidized to yield 1-β-D-arabinofuranosylcytosine 3-*N*-oxide.[15] The *N*-oxide displayed antitumor activity.[15] The synthesis of a large number of nucleotides and dinucleoside phosphates containing ara-C has been reported.[16] The nucleotides and dinucleoside phosphates displayed activity against KB carcinoma cells in culture, but were cross-resistant with ara-C against an ara-C-resistant mutant (kinaseless).[17] It was suggested that the nucleotides and dinucleoside phosphates were cleaved to free ara-C, which was then converted to the metabolically active form.[18] The introduction of a fluorine atom into the 5 position of ara-C produced a new nucleoside possessing more activity[19] against transplanted mouse leukemia P-815 and P-388 and approximately equal activity[20] with ara-C against leukemia L-1210. The superiority of a 5'-ester of ara-C, 5'-adamantoyl-1-β-D-arabinofuranosylcytosine, in the treatment of L-1210 leukemic mice[21] prompted the synthesis[22] of a large number of 5'-esters of ara-C.

The elucidation of the role of adenosine 3',5'-cyclic phosphate (cAMP) in the biology of the cell has given new emphasis to the synthesis[23] of a number of derivatives related to cAMP. The ability of exogeneous cAMP to exert the specific biological effects of cAMP upon intact cells strongly suggests that this cyclic phosphate crosses the cell membrane.[24] Recent findings have established that cyclic phosphate analogs of cAMP also enter the intact cell.[24] Since ara-C must first be phosphorylated to its 5'-mono-, di-, and triphosphates to be active as an inhibitor of DNA synthesis,[25] the synthesis of a nucleotide derivative of ara-C with the ability to cross cell membranes would be of considerable interest.

Previous attempts[26,†] to synthesize 1-β-D-arabinofuranosylcytosine 3',5'-cyclic phosphate (**2**) (cyclic-ara-CMP) by a ring closure of 1-β-D-arabinofuranosylcytosine 5'-phosphate produced 1-β-D-arabinofuranosylcytosine 2',5'-cyclic phosphate. The synthesis of cyclic-ara-CMP from the 5'-phosphate of ara-C is therefore only possible following prior blockage of the 2' position, a process that introduces numerous problems.

Examination of molecular models showed that cyclization of 1-β-D-arabinofuranosylcytosine 3'-phosphate[16,28] (**1**) could only yield the desired cyclic phosphate **2**, since bridging of the 2',3'-trans diol moiety of ara-C by a phosphate is sterically impossible. Thus ring closure of **1** to yield **2** was accomplished with dicyclohexylcarbodiimide in dilute solution without prior blockage of the 2'-hydroxyl group. The use of DMF as a cosolvent eliminated the problem caused by the low solubility of cytidine nucleotides in pyridine, as encountered by Smith, *et al.*[29]

The cyclic phosphate nature of **2** was confirmed by elemental analysis and by its electrophoretic mobility on paper in phosphate buffer, where it was compared with various cyclic and noncyclic nucleotides. The stability of **2** in acid and base negates the possibility, however remote, of the presence of a 5-membered (2',3'-trans) cyclic phosphate. Upon treatment with sodium nitrite in aqueous acetic acid at 60°, **2** was converted in almost quantitative yield to 1-β-D-arabinofuranosyluracil 3',5'-cyclic phosphate (**3**).

Utilizing the method of Ueda, *et al.*,[30] **2** was allowed to react with H_2S in pyridine–water to yield 1-β-D-arabinofuranosyl-4-thiouracil 3',5'-cyclic phosphate (**4**). Treatment of **4** with methyl iodide in dilute base furnished 1-β-D-arabinofuranosyl-4-methylthio-3-pyrimidone 3',5'-cyclic

†During the preparation of this manuscript, the synthesis of **2** was reported.[27]

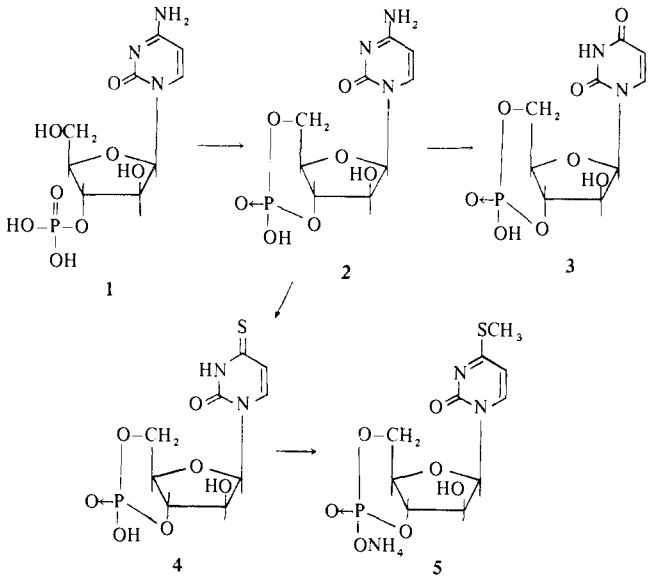
Table I. Comparative *in Vitro* Antiviral Activities[a]

Virus	Ara-C	2	3	4	5
Type 1 herpes simplex	++	++	±	–	±
Type 2 herpes simplex	+	+	±	+	±
Vaccinia	+	+	–	–	–
Myxoma	+	+	–	–	+
Pseudorabies	±	±	±	–	±
Type 3 adenovirus	–	–	–	–	–

[a]Studies were according to the reported[37] experimental procedure in which inhibition of virus-induced cytopathic effects (CPE) was used as the parameter for evaluation. ++, marked CPE inhibition; +, moderate CPE inhibition; ±, slight CPE inhibition; –, no CPE inhibition.

phosphate (**5**). This product was found to be unstable as the free acid and was isolated as the ammonium salt (Scheme I).

Scheme I



The formation of 1-β-D-arabinofuranosylcytosine 2′,5′-cyclic phosphate from either the 2′- or 5′-phosphate of ara-C involves small changes in the dihedral angle between H-1′ and H-2′. This was verified[26] by pmr measurements, which showed small differences between the coupling constants $J_{1',2'}$ for these three nucleotides. In contrast, formation of a 3′,5′-cyclic phosphate derivative from a ribofuranosyl or arabinofuranosyl nucleotide involves major conformational changes of the sugar moiety. Examination of molecular models shows a small dihedral angle between H-1′ and H-2′ in an arabinofuranosylpyrimidine 3′,5′-cyclic phosphate, which, according to the Karplus relationship, predicts a large coupling constant $J_{1',2'}$. This was verified by the large coupling constants ($J_{1',2'}$ = 7 Hz) found for compounds **2**–**5**, as shown in Table IV.

The *in vitro* antiviral activity of cyclic-ara-CMP, as shown in Table I, was found to be essentially equivalent to ara-C in all systems. Treatment of the eyes of rabbits infected with Type 1 herpes simplex virus using 1% cyclic-ara-CMP was markedly effective in reducing both the herpetic infectivity (opacity and fluorescein-stained lesion size) scores and the Draize (redness, swelling and discharge) scores in the eyes (Figure 1). These data have also been confirmed using 0.1% concentrations of the compound applied to both early and to established herpetic infections.[31] Cyclic-ara-CMP was also effective against both herpes simplex and vaccinia virus infections in the brains of mice when administered directly into the brain 6 hr after the virus.[31] It is interesting to note that the 2′,5′-cyclic phosphate derivative of ara-C has been reported[26] to be devoid of antiviral and antitumor activity.

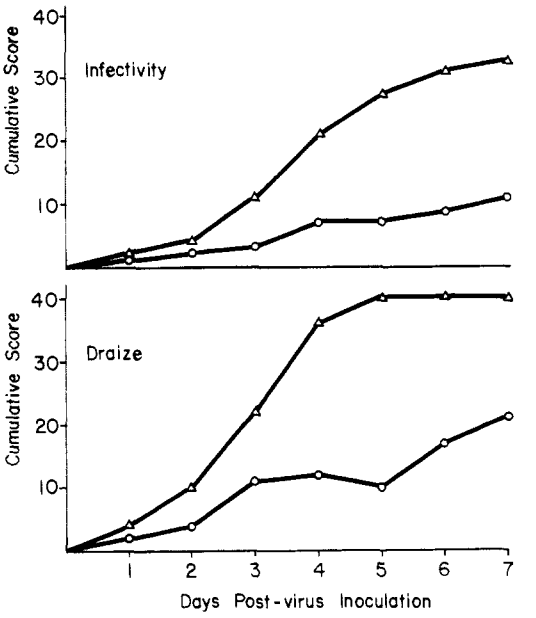


Figure 1. Effect of 1% cyclic ara-CMP (o) on herpes simplex virus-induced infectivity (opacity + fluorescein-stained lesion size) and Draize (redness + chemosis + discharge) scores in rabbit eyes. A solution of the drug dissolved in 1.4% polyvinyl alcohol (PVA) was administered 2 drops to each eye of four rabbits hourly from 8 AM to 7 PM daily for 6 days, with an 8-PM treatment daily, using drug dissolved in opthalmic ointment. Treatment begun 16-hr post-virus-inoculation. Virus control (△) rabbits received similar treatment with PVA and ointment devoid of drug. All scores were made blindly on a 0 (uninfected) to 4 (100% infection) basis daily.

The effect of cyclic-ara-CMP against mouse leukemia L-1210 (carried in DBA/$_2$ mice) was studied according to the methods described in the CCNSC protocol.[32] As can be seen in Table II, **2** at an optimum dosage (300 mgs/kg, 5 daily doses) produced a significant (52%) increase in the survival time of the leukemic mice. At this dosage no weight loss was observed among the treated animals and there were 6/6 drug control survivors. Results describing

Table II. Antileukemic Activity[a] of **2** (Ammonium Salt)

Compound	Daily dose, mg/kg × 5	30-Day survivors drug control	Mean survival, days	Extremes, days	Δ wt, 5th day	% increase in life span
Controls			8.0	7-9		
2	30	6/6	9.0	8-10	+1.4	12
2	90	6/6	9.2	8-10	+0.8	15
2	150	6/6	9.9	9-11	+0.2	24
2	300	6/6	12.2	10-13	–0.2	52
2	450	4/6	11.0	9-12	–2.1	38

[a]Groups of 6 DBA/2 female mice received 1 × 10^6 L-1210 leukemia cells each. After 24 hr, each group received daily ip injections of the test compound dissolved in isotonic saline. The controls received saline only.

Table III. Relative Rate of Enzyme Hydrolysis by a Partially Purified Rabbit Kidney cAMP Phosphodiesterase (cAMP = 1.00)

Compound	α^a
Cytidine 3',5'-cyclic phosphate	0.02
Uridine 3',5'-cyclic phosphate	0.17
Thymidine 3',5'-cyclic phosphate	0.025
1-β-D-Arabinofuranosylcytosine 3',5'-cyclic phosphate	0.05
1-β-D-Arabinofuranosyluracil 3',5'-cyclic phosphate	0.05
1-β-D-Arabinofuranosyl-4-thiouracil 3',5'-cyclic phosphate	0.03
1-β-D-Arabinofuranosyl-4-methylthio-2-pyrimidone 3',5'-cyclic phosphate	0.04

[a] α = (rate of hydrolysis of test compound/rate of hydrolysis of cAMP). The basic incubation mixture (final volume 0.7 ml) contained the following components (amounts in μmoles): Tris buffer (pH 7.50), 30; magnesium chloride, 1.0; cyclic AMP or compound, 3.5; rabbit kidney 3',5'-cAMP phosphodiesterase, 880 μg (0.10 ml). After incubation at 30° (10 min for cAMP, 45 min for the other compounds), the reaction was heated at 90° for 2.5 min and then cooled in ice. *Crotalus atrox* venom phosphodiesterase was added and incubation continued at 30° for 60 min. The reaction was terminated by the addition of 0.1 ml of cold 55% trichloroacetic acid. After addition of the trichloroacetic acid, the precipitate was removed by centrifugation and aliquots of the supernatants were analyzed for inorganic phosphate using the colorimetric method of Lowry and Lopez. Samples containing heat-inactivated kidney phosphodiesterase and the compounds were run as controls for the interference of the compounds in the phosphate assay.

Table IV. Chemical Shifts[a] of Various 1-β-D-Arabinofuranosylpyrimidine Phosphates

Compound	Chemical shift, ppm (coupling constants, Hz) H-6	H-1'	H-5
1-β-D-Arabinofuranosylcytosine 3'-phosphate[b]	8.08 (8)	6.21 (3)	6.26 (8)
1-β-D-Arabinofuranosylcytosine 2',5'-cyclic phosphate[c]	8.01 (8)	6.29 (3)	6.04 (8)
1-β-D-Arabinofuranosylcytosine 3',5'-cyclic phosphate	7.62 (8)	6.39 (7)	6.06 (8)
1-β-D-Arabinofuranosyluracil 3',5'-cyclic phosphate	7.68 (8)	6.36 (7)	5.90 (8)
1-β-D-Arabinofuranosyl-4-thiouracil 3',5'-cyclic phosphate	7.50 (8)	6.32 (7)	6.58 (8)
1-β-D-Arabinofuranosyl-4-methylthio-2-pyrimidone 3',5'-cyclic phosphate	7.80 (8)	6.34 (7)	6.63 (8)

[a] Solvent used was D_2O with NaOD added to dissolve, DSS as internal reference; measurements were taken on a Hitachi R20a nmr spectrometer. [b] See ref 16. [c] See ref 26.

the effect of **2** against ara-C and 6 MP-resistant strains of leukemia L-1210 will be described elsewhere.

The marked ability of cyclic-ara-CMP (**2**) to inhibit the growth of DNA viruses *in vitro* and *in vivo* is of considerable interest. The cyclic phosphate should readily cross the cell membrane and then might well be opened by a diesterase to the monophosphate.

Cyclic nucleotide phosphodiesterases specific for uridine 3',5'-cyclic phosphate have been described by Hardman[33] and Klotz,[34] although no known phosphodiesterase specific for cCMP has been reported. The partially purified rabbit kidney phosphodiesterase showed marked activity in hydrolysis of cUMP (Table III, cUMP 17% the rate of cAMP), and thus this preparation was used as a source of the pyrimidine 3',5'-cyclic phosphate phosphodiesterase in order to compare the relative rate of hydrolysis of c-ara-CMP and cCMP.

Previous findings that cyclic nucleotide analogs of cAMP, which are resistant to cleavage by cAMP phosphodiesterase,[35] show antiviral activity[36] also suggests the possibility that **2** could exert its effects as the cyclic nucleotide. The activity of **2** might also be a result of the increased resistance to deamination. These and other possible modes of action are currently under further investigation.

Experimental Section

General. Thin-layer chromatography was carried out using the ascending technique on Analtech Uniplates coated (250 μ) with Avicel F. The solvent systems normally used were isopropyl alcohol–concentrated NH_4OH–H_2O (7:1:2 v/v) and MeOH–concentrated NH_4OH–H_2O (6:1:2 v/v). Pyridine was distilled from BaO and stored over molecular sieve (4 Å). Melting points were taken with a Thomas-Hoover Uni-Melt apparatus and are uncorrected. Uv spectra were recorded with a Cary 15 uv spectrometer. Compounds **2–5** were examined on a Savant electrophoresis apparatus at 1500 V (pH 7.2, phosphate buffer) and were found to migrate approximately the same distance as cUMP and slower than UMP.

1-β-D-Arabinofuranosylcytosine 3',5'-Cyclic Phosphate (2). 1-β-D-Arabinofuranosylcytosine 3'-phosphate[16,28] (5.77 g, 17.9 mmoles) and 4-morpholino-*N,N'*-dicyclohexylcarboxamidine (5.24 g, 17.9 mmoles) were dissolved in anhydrous DMF (100 ml) by heating. The solvent was removed *in vacuo*. Anhydrous DMF (50 ml) was added and again removed *in vacuo*. The foam was dissolved in hot anhydrous DMF and added dropwise over a period of 105 min to a vigorously refluxing soln of DCC (21 g, 101 mmoles) in anhydrous pyridine (4.5 l). After refluxing an additional 60 min, the soln was concentrated to approx 500 ml by vacuum distn. H_2O (400 ml) was added to the cooled soln and then Et_2O (600 ml). The insoluble dicyclohexylurea was removed by filtration. The aqueous layer of the filtrate was reduced to dryness *in vacuo*, the foam dissolved in H_2O, and the soln acidified to pH 1 with 12 *N* HCl. After 5 min, the pH was adjusted to 6 with concentrated NaOH. The soln was applied to a column (50 cm × 6 cm diameter) of S & S standard grade DEAE-cellulose (HCO_3^- form). Elution was carried out using a linear salt gradient of 0.075 *M* triethylammonium bicarbonate (pH 7.5) (4 l.) in the reservoir and H_2O (4 l.) in the mixing chamber. Fractions (20 ml) were collected on a uv-recording (254 nm) collector and **2** appeared in fractions 160–323. The combined fractions were evapd *in vacuo*, and H_2O (50 ml) was added and removed *in vacuo*. The solid was dissolved in H_2O (25 ml) and applied to a column (30 cm × 4 cm diameter) of Dowex 50W-X8 (H^+ form), and the column developed with H_2O. The eluent was reduced to dryness *in vacuo*, and the solid recrystallized from H_2O to yield 0.645 g of analytical **2**. Slightly less pure compd was obtained by freeze-drying the filtrate: total yield 1.902 g, 34.8%; mp 294–298° dec; $\lambda_{max}^{pH\ 1}$ 277 (ε 14,200), $\lambda_{max}^{pH\ 7}$ 268 (10,400), $\lambda_{max}^{pH\ 11}$ 268 (10,400), 236 nm (8550). *Anal.* ($C_9H_{12}N_3O_7P$) C, H, N.

1-β-D-Arabinofuranosyluracil 3',5'-Cyclic Phosphate (3). A soln of **2** (0.500 g, 1.64 mmoles) in AcOH–H_2O (50 ml, 5:1) was heated at 60°, and $NaNO_2$ in portions of 60 mg was added every hour until the uv of the soln had a maximum at 262 in pH 1. The soln was concentrated to dryness *in vacuo*, followed by addition and evaporation of EtOH (50 ml). The residue was dissolved in H_2O (20 ml) and applied to a column of Dowex 50W X8 resin (H^+ form). H_2O eluted the uracil nucleotides first, followed by a small amount of cytosine nucleotide. The fractions containing the uracil nucleotides were combined, reduced in vol, and applied to a DEAE-cellulose column. Following elution and conversion as above, **3** was obtained: 0.464 g, 92.7%. A portion was recrystallized from EtOH for analysis: mp decomp slowly >230°; $\lambda_{max}^{pH\ 1}$ 262 (ε 7260), $\lambda_{max}^{pH\ 11}$ 260 nm (6000). *Anal.* ($C_9H_{11}N_2O_8P$) Calcd: C, 35.30; H, 3.62; N, 9.15. Found: C, 34.38; H, 3.30; N, 9.02. (Combustion analyses were repeated and the carbon was consistently low, a problem often encountered with phosphates–all other analytical data were consistent with this structure.)

1-β-D-Arabinofuranosyl-4-thiouracil 3',5'-Cyclic Phosphate (4). A soln of **2** (0.915 g, 3 mmoles) in 9 ml of H_2O was placed in a steel bomb and cooled in Dry Ice–acetone. A mixt of pyridine (12 ml) and liquid H_2S (24 ml) was added, and the bomb was heated at 60° for 48 hr. After cooling in Dry Ice–acetone, the bomb was opened and allowed to warm to room temp, and the contents were then evaporated to dryness *in vacuo*. EtOH (25 ml) was added and removed *in vacuo*. The residue was dissolved in H_2O (80 ml), and the soln filtered and applied to a column (50 cm × 6 cm diameter) of S & S DEAE-cellulose (HCO_3^- form). Elution was carried out as described for **2** with triethylammonium bicarbonate. The major uv-absorbing fractions were reduced to dryness and the compound was converted to the acid form on a column of Dowex 50W-X8. Removal of the solvent *in vacuo* followed by addition and removal of EtOH provided 0.837 g, 86.5%, of **4**. Crystallization from EtOH furnished an

analytical sample: mp 220° dec; $\lambda_{max}^{pH\,1}$ 330 (ε 21,800), $\lambda_{max}^{pH\,11}$ 317 (21,600), $\lambda_{max}^{pH\,7}$ 330 nm (23,200). *Anal.* ($C_9H_{11}N_2O_7PS$) C, H, N.

Ammonium 1-β-D-Arabinofuranosyl-4-methylthio-2-pyrimidone 3',5'-Cyclic Phosphate (5). To a soln of 4 (0.322 g, 1 mmole) in MeOH–H_2O (5 ml, 8:2) was added concentrated NH_4OH dropwise until pH 11. MeI (1 ml) was added, and the soln stirred at room temp for 5 hr, then kept at 5° for 12 hr. Crystalline NH_4I was removed by filtration and the filtrate concentrated to dryness *in vacuo*. The residue was dissolved in H_2O (25 ml) and applied to a column of S & S DEAE-cellulose. Following elution with triethylammonium bicarbonate as described above, the appropriate fractions were combined and concentrated to dryness *in vacuo*. H_2O (50 ml) was added and removed *in vacuo*. This process was repeated. The resulting solid was dissolved in H_2O (20 ml) and applied to a column of Dowex 50W-X8 (NH_4^+) and the column developed with H_2O. The eluate was concentrated to dryness and the residue crystallized from EtOH for analysis: yield, 0.165 g, 47%; mp 245° dec; $\lambda_{max}^{pH\,11}$ 303 (ε 14,900), $\lambda_{max}^{pH\,7}$ 303 nm (15,150). *Anal.* ($C_{10}H_{16}N_3O_7PS$) C, H, N.

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Pyrimidine Nucleosides. 5. Syntheses of Carcinostatic Halogenocyclonucleosides

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Some derivatives of 2,2'-anhydro-ara-C (III), carcinostatic nucleosides, were prepared: 5-bromo (IIa), 5-iodo (IIb), and 5-fluoro (IIc) derivatives were prepared by heating the corresponding ribonucleosides with a partially hydrolyzed phosphorus oxychloride in ethyl acetate. Acyl derivatives (IVa,b) of IIa were obtained directly by bromination of 2,2'-anhydro-ara-C with bromine and an acid anhydride (Ac_2O, $(PhCO)_2O$), which were converted to 1-β-D-arabinofuranosyl-2-amino-5-bromo-4-imino-1,4(2*H*)-dihydropyrimidine (VII) with ammonia in MeOH. Hydrogenation of anhydronucleosides was also reported. All of 2,2'-anhydro-ara-C derivatives were markedly active against leukemia L1210 in mice, but 6-hydroxy-2',6-anhydro-ara-C (IX) prepared from 5-iodo-ara-C displayed only a weak activity.

1-β-D-Arabinofuranosylcytosine has been used clinically in the treatment of acute leukemias and lymphomas.[1] However, the compound is deaminated very rapidly to inactive 1-β-D-arabinofuranosyluracil.[2] In a short communication,[3] the present authors reported that 2,2'-cyclo-arabinofuranosylcytosine (abbreviated to cyclo-ara-C) (III), which was resistant to cytidine deaminase of mouse kidney, was the most active (against L1210) and the least toxic among the antitumor agents tested.

This finding prompted us to study other pyrimidine cyclonucleosides. In view of the reported biological activities of some halogenated pyrimidine deoxyribonucleosides,[4] 5-halogenated derivatives of III were of interest. This paper deals with the preparation and preliminary biological testing of these compounds.

By conventional halogenation of nucleosides, *viz.*, bromination by bromine water, iodination by iodine monochloride,[5] iodine with iodic acid,[6] or iodine in the pres-