Pyrimidine Nucleosides. 6. Syntheses and Anticancer Activities of N^4-Substituted 2,2′-Anhydronucleosides

Tadashi Kanai,* Motonobu Ichino,

Research Laboratories, Kohjin Company, Ltd., Komiyacho, Hachiouji City, Tokyo, Japan

Akio Hoshi, Fumihiko Kanzawa, and Kazuo Kuretani

National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo, Japan. *Received October 16, 1973*

Some derivatives of N^4-substituted 2,2′-*c*-araC [N^4-hydroxy- (**2a**), N^4-methoxy- (**2b**), N^4-methyl- (**2c**), and N^4-phenyl- (**2d**)] which are potential carcinostatic nucleosides were prepared by introducing the 2,2′-anhydro linkage into the corresponding ribonucleosides in one step. Compound **2a** was markedly active against leukemia L1210 in the mouse but showed only a weak inhibitory activity against L5178Y cells in culture. Compound **2a** gave 110% ILS at 300 mg/kg/day (5 days) but was toxic at 700 mg/kg/day (5 days); **2b** and **2d** were inactive against leukemia L1210 in the mouse at 100 mg/kg/day (5 days).

2,2′-*cyclo*-1-β-D-Arabinofuranosylcytosine (abbreviated to *c*-araC) has been shown to be effective in the treatment of mouse leukemia L1210[1-3] and other tumors[4,5] (C1498 leukemia, NFS sarcoma, adenocarcinoma 755, sarcoma 180, Ehrlich ascites carcinoma, lymphocytic sarcoma of Tokuzen Nakahara,[6] and NTF sarcoma[7]). The clinical usefulness of this compound has been also reported.[8] 5-Halogenated[9,10] and 3′,5′-di-*O*-acyl-*c*-araC[9] have been also found to be active against L1210 leukemia in mice.

It is known that *c*-araC is easily hydrolyzed in alkaline media to 1-β-D-arabinofuranosylcytosine; the latter undergoes rapid enzymatic deamination in man to 1-β-D-arabinofuranosyluracil (araU), an inactive metabolite.[3,11] These findings prompted us to prepare N^4-substituted derivatives of *c*-araC which might be more stable under alkaline conditions.

In view of the reported biological activity[12] of N^4-hydroxy-1-β-D-arabinofuranosylcytosine[13] (which was active *in vitro* against a line of P815 leukemia resistant to 5-fluorouracil), N^4-hydroxy- (**2a**), N^4-methoxy- (**2b**), N^4-methyl- (**2c**), and N^4-phenyl-*c*-araC (**2d**) were of interest as potential chemotherapeutic agents. This paper describes the preparation and preliminary biological testing of these compounds (**2a–d**).

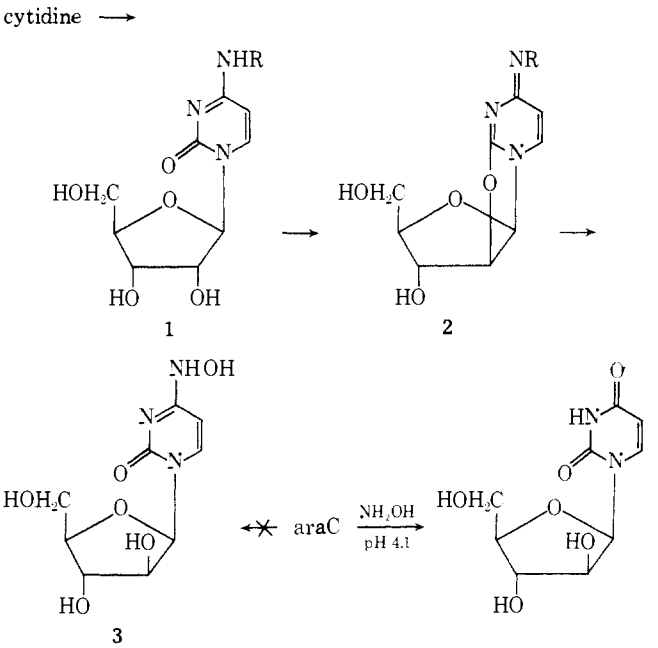
Chemistry. The direct introduction of hydroxyl or other groups into *c*-araC at the N^4 position is difficult because of the base lability of the 2,2′-anhydro linkage. It occurred to us that syntheses of **2a–d** might be feasible by introduction of the 2,2′-anhydro linkage into the preformed N^4-hydroxy- (**1a**), N^4-methoxy- (**1b**), N^4-methyl- (**1c**), and N^4-phenylcytidine (**1d**) using the procedures reported previously[9,14,15] (Scheme I).

Compounds **1a** and **1b** were easily prepared by the amine exchange between cytidine and hydroxylamine or methoxyamine.[16] However, the reaction of araC with hydroxylamine under the same conditions employed for the preparation of **1a** gave rise to araU in a fair yield. This different behavior of araC with hydroxylamine might be due to intramolecular catalysis of the 2′-hydroxyl group as proposed by Notari and coworkers[17] in the hydrolytic deamination.

It was found that the conversion of cytidine to **1c** and **1d** proceeded in good yields, using the modified procedure of Curran[18] for the conversion of 2-methylthio-4-hydroxy-6-aminopyrimidine to 2,6-bis(methylamino-4-hydroxy)pyrimidine. Replacement of the 4-amino group of cytidine with methylamino or phenylamino group was achieved by fusing cytidine with methylammonium or phenylammonium acetate at 160°.

Cyclization was effected by heating **1a–d** with partially hydrolyzed phosphorus oxychloride in a small volume of ethyl acetate to give **2a–d** in yields of 32–54%.

Scheme I



The structural assignments for **2a–d** rest upon their ultraviolet spectral properties and analyses. The 2,2′-anhydro structure of **2a** and **2b** was also confirmed by the downfield shift of the nmr signal due to H-2′. The nmr spectra of **2a** and **2b** deserve further comment. The signals due to H-5 of **2a** appeared as a multiplet, and those due to H-5 of **2b** appeared at 6.19 and 6.17 ppm as a doublet of doublets. On the other hand, there appeared well-resolved doublets due to H-6 in the nmr, *viz.*, 8.34 and 8.09 ppm for **2a** and 7.28 and 7.13 ppm for **2b**. These doublets are likely due to the presence of cis and trans isomers rather than to the coupling between H-5 and H-6. The ratio of cis–trans isomers was estimated to be 3:2 based on the intensity of the H-6 proton for **2a** or **2b** in the nmr.

As expected, compounds **2a** and **2b** were found to be more resistant to alkaline hydrolysis than *c*-araC. Nagyvary[19] found that large differences existed in the hydrolysis of *c*-araC 3′-phosphate catalyzed by bicarbonate and chloride ions, the bicarbonate ions being good catalysts for the hydrolysis of the 2,2′-anhydro linkage of *c*-araC. These authors used bicarbonate and borate buffer solutions to evaluate the stability of **2a** to hydrolysis relative to *c*-araC.

Table I. R_f Values of Some Anhydronucleosides and Related Arabinonucleosides

Compd	R_f values in Solvent A	Solvent B
AraC	0.15	0.65
AraU	0.28	0.81
c-AraC	0.05	0.64
2a	0.10	0.72
3	0.17	0.63
2c	0.16	
2c′[a]	0.30	
2d	0.26	
2d′[a]	0.63	

[a]Respective hydrolysate of **2c** and **2d** with 2 *N* KOH for 1 hr at room temperature.

Table II. Effect of Pyrimidine Anhydronucleosides against L1210 Leukemia and Cultured Cells (L5178Y)

Compd	Dose,[a] mg/kg/day × 5	% increase in life span over controls[b] (L1210 leukemia)	IC_{50} in L5178Y cells, μg/ml
c-AraC	100	76	0.041
3	100	98	5.9
2a	30	64	7.5
	100	82	
	300	110	
	500	95	
	700	<30 (toxic)	
2b	100	0	
2d	100	4	

[a]Six mice per group were used. [b]Length of survival time in control groups was 7–9 days. % = [treated − control (day)]/control (day) × 100.

Compounds **2a** and **2b** were not hydrolyzed at pH 12.0 after 5 hr at 37°, whereas *c*-araC was converted into araC to the extent of 70% at pH 7.5 for 30 min at 37°† (Figure 1).

Hydrolysis of **2a** in 0.5 *N* KOH solution at 60° for 30 min afforded **3**,[13] but prolonged hydrolysis under these conditions produced a product of unknown structure (Table I).

Biological Activity. The antitumor activity of the N^4-substituted *c*-araC prepared in this study against L1210 mouse leukemia was examined by the methods previously reported.[21] Their *in vitro* antitumor activity was also examined in cultured cells (L5178Y).

The results are given in Table II. From the testing data available at the present time, **2b** and **2d** were inactive at 100 mg/kg/day once daily for 5 days. Compounds **2a** and **3** were active against L1210 mouse leukemia at 100 mg/kg/day, but **2a** was toxic at 700 mg/kg/day. Compound **2a** as well as compound **3**, unlike *c*-araC, was active against L5178Y leukemia cells in culture. These findings suggest that **2a** was activated in the intact mouse and that the antileukemic activity of **3** is presumably due to its reduction to araC in the mouse.[12] Thus, it may be speculated that **2a** might be able to be converted to *c*-araC in the mouse. These studies on the mechanism of action of **2a** and **3** are now being pursued in our laboratories.

2a was also active against various tumors (ascites S180, Ehrlich ascites carcinoma, NFS sarcoma, and C1498 leukemia). Compound **2d** was inactive (83.7%, *T/C*), but **2c** possessed weak activity (53.4%, *T/C*) against ascites S180 when administered ip at 100 mg/kg/day once daily for 5 days (using ddN mice).

†Wang, *et al.*,[20] had observed that the extent of hydrolysis amounts to approximately 1% at pH 6.5 and approximately 70% at pH 9.0 after 1 hr at 37°. This difference from our results may be due to the differences in the anion used (bicarbonate and chloride ions).

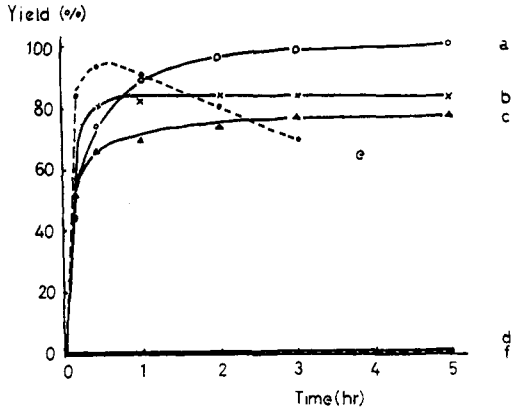


Figure 1. Hydrolysis of *c*-araC and N^4-hydroxy-*c*-araC in buffer solutions of varying pH. The yield of araC or N^4-hydroxy-1-β-D-arabinofuranosylcytosine was plotted against time. The solutions of cyclonucleosides (1%) in 0.1 *M* buffer solution (pH 4.0, 7.5, 9.0, 12.0) were kept at 37°. Aliquots were withdrawn from the solutions at different time intervals and were chromatographed on paper in solvent A to separate the products. Ultraviolet-absorbing spots were cut out and extracted with dilute HCl solution (pH 2, 5 ml) to measure optical density at 260 nm. The identification of the eluted materials as araC or **3** was by (1) examination of their uv spectra, and (2) R_f values on paper chromatography (Table I). *c*-AraC: (a) pH 7.5 (triethylammonium bicarbonate buffer); (b) pH 12 (borate); (c) pH 9.0 (borate); (d) pH 4.0 (acetate). **2a**: (e) the profile of hydrolysis with 0.5 *N* KOH at 60°; (f) pH 4.0, 7.5, 9.0, and 12.0 at 37°.

Experimental Section

Melting points (uncorrected) were determined by the capillary method. Ultraviolet spectra were obtained with a Hitachi EPS-3T or a Hitachi EPU-2A spectrophotometer. The nmr spectra in DMSO-d_6 were determined with a Varian T-60 instrument, using TMS as internal standard. Paper chromatography and tlc were carried out on Toyo Roshi No. 51A paper and Silica-Rider, respectively, using the following solvent system: (A) *n*-BuOH-H_2O (86:14); (B) *i*-PrOH-concentrated HCl-H_2O (170:41:39). R_{CR} = R_f value relative to cytidine.

Determination of Antitumor Activity against L1210 Leukemia. Groups of six male and female BDF_1 mice weighing 20 ± 2 g were implanted intraperitoneally (ip) with 1 × 10^5 cells of L1210 leukemia. All compounds to be tested were dissolved in 0.9% NaCl solution or 0.5% carboxymethylcellulose in 0.9% NaCl solution and injected ip at 100 mg/kg/day once daily for 5 days, starting 24 hr after transplantation. Antitumor activity was evaluated as the per cent increase in life span (ILS).

Determination of Antitumor Activity in Cultured Cells (L5178Y). The cells were cultured in RPMI 1640 medium supplemented with 10% calf serum in a CO_2 incubator at 37°. Antitumor activity was determined by the ratio of cell numbers in treated and control groups (*T/C*, %) after 48 hr of incubation of 5 × 10^4 cells/ml at various concentrations of test compounds. The IC_{50} (50% inhibiting concentration) was also calculated. In general, cell numbers increased 15–20 times after 48 hr of incubation.

N^4-Methylcytidine (1c). To 6 ml (82.5 mmol) of the oily methylammonium acetate (freshly prepared) was added 2 g (8.2 mmol) of cytidine. The mixture was heated at 160° for 50 min. Several volumes of Et_2O were added and then decanted, and this procedure was repeated. EtOAc (50 ml) was then added to the oily residue and the solvent decanted. The residue was dissolved in 20 ml of EtOH and treated with charcoal and filtered. The filtrate was evaporated and the residue was dissolved in a small volume of EtOH. Chilling the filtrate overnight afforded 0.54 g (24.5%) of N^4-methylcytidine:[22] mp 204–205°; R_{CR} (A) 0.86; λ_{max} (H_2O) 237, 271 nm; λ_{max} (1 *N* HCl) 281 nm; λ_{max} (1 *N* NaOH) 273 nm. *Anal.* ($C_{10}H_{15}O_5N_3$) C, H, N.

N^4-Phenylcytidine (1d). To the oily aniline acetate prepared by heating 5.5 g (64 mmol) of aniline and 6 ml of glacial acetic acid at 50° for 1 hr was added 2 g (8.2 mmol) of cytidine. The mixture was then heated at 160° for 4 hr. Isolation and purification of the product was carried out as described above for 1c.

Crystallization from a small volume of EtOH afforded 0.85 g (41.2%) of **1d**: mp 86° (shrinks at 48°); R_{fCR} (A) 2.14; λ_{max} (H_2O) 235 (s), 295 nm; λ_{max} (1 *N* HCl) 225 (s), 296 nm; λ_{max} (1 *N* NaOH) 296 nm. For analyses a small portion of this product was recrystallized from EtOH. *Anal.* ($C_{15}H_{17}O_5N_3 \cdot 2EtOH$) C, H, N.

N^4-Hydroxy-2,2′-*cyclo*-1-β-D-arabinofuranosylcytosine (2a). To a solution of partially hydrolyzed phosphorus oxychloride [15.1 ml (164.4 mmol) of $POCl_3$ and 2.96 ml of H_2O] in 23.9 ml of EtOAc was added 4 g (15.4 mmol) of N^4-hydroxycytidine and the mixture was heated at 70° for 2 hr. Ice water (10 ml) was added to the resulting solution. After evaporation of the EtOAc solvents the acidic aqueous solution was charged on a column of Dowex 50W (acid form, 280 ml). The column was washed with water and then with 0.1 *M* pyridinium formate buffer (pH 4.5). The initial fraction 3 l.) was discarded, and the subsequent eluates (4 l.) were evaporated to dryness *in vacuo*, followed by repeated coevaporation with water to remove the small amount of pyridine remaining. The residue was dissolved in 100 ml of water and passed through a column filled with Diaion SA-11B (chloride form, 25 ml) and the resin washed further with water. The combined effluent and washings were concentrated to dryness. The residue was triturated with EtOH when crystallization occurred. Recrystallization from MeOH afforded 1.38 g (32.2%) of **2a**: mp 184° dec; λ_{max} ($\epsilon \times 10^{-3}$) (H_2O) 253 nm (16.2); λ_{max} (1 *N* HCl) 242 (10.0), 273 nm (11.5); nmr δ 8.34, 8.09 (d of d, 1 H, C_6-H), 6.48–6.73 (m, 2 H, C_5-H, $C_{1'}$-H). 5.41 (d, 1 H. $C_{2'}$-H). *Anal.* ($C_9H_{12}O_5N_3Cl$) C, H, N.

N^4-Methoxy-2,2′-*cyclo*-1-β-D-arabinofuranosylcytosine (2b). To a stirred solution of partially hydrolyzed phosphorus oxychloride [2.93 ml (31.9 mmol) of $POCl_3$ and 0.57 ml of H_2O] in 4.63 ml of EtOAc was added 0.87 g (3.19 mmol) of N^4-methoxycytidine, and the mixture was heated at 70° for 2 hr. Ice water (2 ml) was then added, and the mixture was placed onto the column of Dowex 50W (acid form, 60 ml). After washing with water until free of ultraviolet-absorbing materials, the cyclo compound was eluted with 0.1 *M* pyridinium formate buffer (pH 4.5). The initial eluate (600 ml) was discarded, and 800 ml of the successive eluate was evaporated to give a crystalline residue. Recrystallization from MeOH gave 0.35 g (43.1%) in two crops: mp 218–220°; λ_{max} ($\epsilon \times 10^{-3}$) (H_2O) 260 nm (18.9); λ_{max} (1 *N* HCl) 242 (9.4), 272 nm (12.5); nmr δ 7.28, 7.13 (d of d, 1 H, C_6-H), 6.19, 6.17 (d of d, 1 H, C_5-H), 5.73 (d, 1 H, $C_{1'}$-H), 5.19 (d, 1 H, $C_{2'}$-H). *Anal.* ($C_{10}H_{13}O_5N_3$) C, H, N.

N^4-Methyl-2,2′-*cyclo*-1-β-D-arabinofuranosylcytosine (2c). N^4-Methylcytidine (0.50 g, 1.95 mmol) was dissolved in a mixture of partially hydrolyzed phosphorus oxychloride [1.79 ml (19.5 mmol) of $POCl_3$ and 0.35 ml of H_2O] and 2.83 ml of EtOAc and the mixture was heated with stirring at 70° for 4 hr. After the solvents were removed, the residue was dissolved in 25 ml of water. The cyclized product was isolated on a column of Diaion SK-1BS (acid form, 50 ml) by washing initially with water until free of ultraviolet-absorbing materials and then eluting stepwise with 0.1 and 0.3 *M* pyridinium formate buffer (pH 4.5). The initial eluate (1.8 l.) of 0.1 *M* buffer was discarded, and the successive eluate (4.5 l.) of 0.3 *M* buffer was evaporated to dryness. The residue was dissolved in 15 ml of water and passed through the column of Diaion SA-11B (chloride form. 5 ml), and the column was washed with water. The combined effluent and washings were concentrated to dryness. Paper chromatography of the residue showed the presence of two components [R_f (A) 0.08 and 0.16 (major)]. The major component was isolated by repeated preparative paper chromatography using solvent A. Further purification was accomplished by dissolving the product in a minimum amount of MeOH and adding a large proportion of EtOAc. A white granular solid was obtained (0.4 g, 75%); λ_{max} (H_2O) 245, 265 nm; λ_{max} (1 *N* HCl) 245, 265 nm. *Anal.* ($C_{10}H_{14}O_4N_3Cl \cdot {}^2/_3H_2O$) C, H, N.

N^4-Phenyl-2,2′-*cyclo*-1-β-D-arabinofuranosylcytosine (2d). N^4-Phenylcytidine (1.0 g, 3.1 mmol) was dissolved in a mixture of partially hydrolyzed phosphorus oxychloride [5.97 ml (65.0 mmol) of $POCl_3$ and 0.74 ml of H_2O] and 3.76 ml of EtOAc and the mixture was heated at 70° for 4 hr. The cyclized product was isolated as described above for **2c** except that the chromatography was carried out on a silica gel (Mallinckrodt, 20 g) column using solvent A. Fractions (150 ml) containing **2d** exhibited two components on thin-layer chromatography, having R_f (A) 0.73 and 0.45 (major). Removal of the volatile components and extraction of the minor component with Et_2O (50 ml × 4) under reflux gave pure **2d** as a powder (0.57 g, 54.3%): λ_{max} (H_2O) 237, 295, nm; λ_{max} (1 *N* HCl) 235, 295 nm. *Anal.* ($C_{15}H_{16}O_4N_3Cl \cdot 1.5H_2O$) C, N; H: calcd, 5.24; found, 4.71.

1-β-D-Arabinofuranosyl-4-hydroxylamino-2(1*H*)-pyrimidinone (3). Compound **2a** (80 mg, 0.29 mmol) was hydrolyzed with 5 ml of 0.5 *N* KOH at 60° for 30 min. The pH of the reaction mixture was adjusted to 4.5 with a Dowex 50W (acid form), and the resulting solution was concentrated to dryness. The residue was crystallized from EtOH to give 22 mg of **3**: mp 132° (with effervescence); $[\alpha]^{25}D$ +104° (*c* 0.3, H_2O); λ_{max} (H_2O) 237, 272 nm; λ_{max} (1 *N* HCl) 221, 282 nm. These properties were consistent with the published data.[13]

Reaction of AraC with Hydroxylamine. AraC (2.0 g, 8.2 mmol) and hydroxylamine hydrochloride (4.0 g, 57.6 mmol) were dissolved in 15 ml of water, and the mixture (pH 4.1) was stored at 55° for 1 week. The yellowish solution was concentrated to dryness and the residue was taken up in a small amount of EtOH. The insoluble material was removed by filtration, and the filtrate was concentrated to dryness *in vacuo*. The residue was dissolved in EtOH and filtered. After evaporation of the filtrate, the residue was dissolved in 2 ml of EtOH and crystallization was induced by rubbing. Recrystallization again from 50% EtOH gave prismatic crystals: 1.2 g; mp 213–214°; λ_{max} (H_2O) 262 nm; λ_{max} (1 *N* NaOH) 261 nm. The ir spectrum of the compound in KBr was identical with that of authentic araU.

Acknowledgment. The authors wish to acknowledge the capable laboratory assistance of Mr. Takashi Kojima and Mr. Osamu Maruyama. We also thank Mr. Tokuro Nakamura, the manager of the laboratories, for his interest throughout this study and Professor Y. Mizuno of the Faculty of Pharmaceutical Sciences, University of Hokkaido, for reviewing the manuscript.

References

(1) A. Hoshi, F. Kanzawa, K. Kuretani, M. Saneyoshi, and Y. Arai, *Gann*, **62**, 145 (1971).
(2) D. J. Gish, G. L. Neil, and W. J. Wechter, *J. Med. Chem.*, 14, 882 (1971).
(3) M. C. Wang, R. A. Sharma, and A. Bloch, Abstracts of Papers, 164th National Meeting of the American Chemical Society, New York, N. Y., Aug 27, 1972, MEDI 67.
(4) K. Kuretani, *Igaku No Ayumi*, **80**, 795 (1972).
(5) W. Nakahara and R. Tokuzen, *Gann*, **63**, 379 (1972).
(6) R. Tokuzen and W. Nakahara, *Cancer Res.*, **29**, 730 (1969).
(7) W. Nakahara, F. Fukuoka, R. Tokuzen, and K. Aoki, *Gann*, **56**, 309 (1965).
(8) Y. Sakai, C. Konda, M. Shimoyama, T. Kitahara, T. Sakano, and K. Kimura, *Jap. J. Clin. Oncol.*, **2**, 57 (1972).
(9) T. Kanai, M. Ichino, A. Hoshi, F. Kanzawa, and K. Kuretani, *J. Med. Chem.*, **15**, 1218 (1972).
(10) J. J. Fox, E. A. Falco, I. Wempen, D. Pomeroy, M. D. Dowling, and J. H. Burchenal, *Cancer Res.*, **32**, 2269 (1972).
(11) A. Hoshi, M. Yoshida, F. Kanzawa, K. Kuretani, T. Kanai, and M. Ichino, *Chem. Pharm. Bull.*, **21**, 1446 (1973).
(12) M. R. Dollinger, J. H. Burchenal, W. Kreis, and J. J. Fox, *Biochem. Pharmacol.*, **16**, 689 (1967).
(13) I. Wempen, N. Miller, E. A. Falco, and J. J. Fox, *J. Med. Chem.*, 11, 144 (1968).
(14) T. Kanai, T. Kojima, O. Maruyama, and M. Ichino, *Chem. Pharm. Bull.*, 18, 2569 (1970).
(15) T. Kanai, M. Ichino, A. Hoshi, and K. Kuretani, *Tetrahedron Lett.*, 1965 (1971).
(16) (a) N. K. Kochetokov, E. I. Budowsky, E. D. Sverdlov, R. P. Shibaeva, V. N. Shibaev, and G. S. Monastirskaya, *Tetrahedron Lett.*, 3253 (1967); (b) N. K. Kochetokov and E. I. Budowsky, *Progr. Nucl. Acid Res. Mol. Biol.*, **9**, 403 (1969).
(17) R. E. Notari, M. L. Chin, and A. Cardini, *Tetrahedron Lett.*, 5103 (1967).
(18) W. V. Curran and R. B. Angier, *J. Org. Chem.*, **28**, 2672 (1963).
(19) C. M. Tapiero and J. Nagyvary, *Nature (London)*, **231**, 42 (1971).
(20) M. C. Wang, *et al.*, *Cancer Res.*, **33**, 1265 (1973).
(21) A. Hoshi, F. Kanzawa, K. Kumagai, and K. Kuretani, *Cancer Chemother. Rep.*, **53** (1), 165 (1969).
(22) J. J. Fox, D. Van Praag, I. Wempen, I. L. Doerr, L. Cheong, J. E. Knoll, M. L. Eidinoff, A. Bendich, and G. B. Brown, *J. Amer. Chem. Soc.*, **81**, 178 (1959).