Synthesis and Biological Activity of Pyrazolo[3,4-*d*]pyrimidine Nucleosides and Nucleotides Related to Tubercidin, Toyocamycin, and Sangivamycin†

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ABSTRACT: The 6-aza analogues of toyocamycin and sangivamycin were prepared as potential cytotoxic agents. The toyocamycin analogue (4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carbonitrile) could not be obtained directly from its O-acetylated precursor but was accessible via 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-thiocarboxamide. The identity of the nitrile was verified by its ultraviolet, infrared, and mass spectra, and by its conversion to the corresponding 3-carboxamide and thiocarboxamide when treated with water or hydrogen sulfide, respectively. Bioassay of the synthetic compounds in comparison with 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (6-azatubercidin) and 4-amino-2-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine revealed that the 3-thiocarboxamido derivative was more cytotoxic to the growth of mouse fibroblasts than 6-azatubercidin, effecting killing of 3T6 cells at ≤ 1 μg/ml. 4-Amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (but not its 2-ribofuranosyl isomer) was shown to act as a substrate for adenosine deaminase from calf intestinal mucosa with an apparent K_m of 125 (vs. 20 for adenosine) and the corresponding 5′-diphosphate of 6-azatubercidin was polymerized by polynucleotide phosphorylase (*Micrococcus luteus*) in the presence of Mn^{2+} to afford a homopolymer and copolymers with adenosine. The copolymers directed the binding of [^{3}H]lysyl-tRNA to the A-site of ribosomes from *Escherichia coli*, but could not be used for the synthesis of polylysine in a cell-free system. The copolymer consisting of adenosine and 6-azatubercidin in a 2:1 ratio was found to form a 1:1 complex with poly(uridylic acid) at 4 °C.

Many synthetic and naturally occurring purine analogues have been shown to possess interesting and useful biological properties. The pyrrolo[2,3-*d*]pyrimidine tubercidin (4-amino-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine), e.g., inhibited the growth of *Mycobacterium tuberculosis* BCG, *Candida albicans*, and *Streptococcus faecalis* (8043) as well as NF mouse sarcoma, human KB cells, and human tumors (Suhadolnik, 1970). Interestingly, the corresponding 5-cyano derivative (toyocamycin) was more active against NF-sarcoma cells and certain microbes than tubercidin, while the 5-carboxamide (sangivamycin) had only very slight antibacterial or antifungal activity, but was quite active against leukemia L1210 in mice and HeLa cells grown in culture (Suhadolnik, 1970). Therefore, the observation that the 6-aza analogue of tubercidin (4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine) inhibited the growth of human epidermoid cells (Bennett et al., 1966), consistent with the general pattern of biological activity noted for other pyrazolo[3,4-*d*]pyrimidines (Robins, 1964; Montgomery and Hewson, 1967), suggested that the 6-aza analogues of toyocamycin and sangivamycin might also have potentially useful cytotoxic properties.[1]

This report is concerned with the synthesis of 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carbonitrile (6-azatoyocamycin) and 4-amino-1-(β-D-ribofuranosyl)-pyrazolo[3,4-*d*]pyrimidine-3-carboxamide (6-azasangivamycin) and with the determination of certain biochemical and biological activities of these compounds as well as 4-amino-1- and -2-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine. Also prepared for study was poly(4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine 5′-monophosphate) as well as copolymers of 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine and adenosine.

Materials and Methods

Rye grass 3′-nucleotidase, adenosine deaminase (calf intestinal mucosa, type I), snake venom (*Crotalus adamanteus*), streptomycin, and penicillin G were obtained from Sigma Chemical Company. Polynucleotide phosphorylase (*Micrococcus luteus*) was purchased from P-L Biochemi-

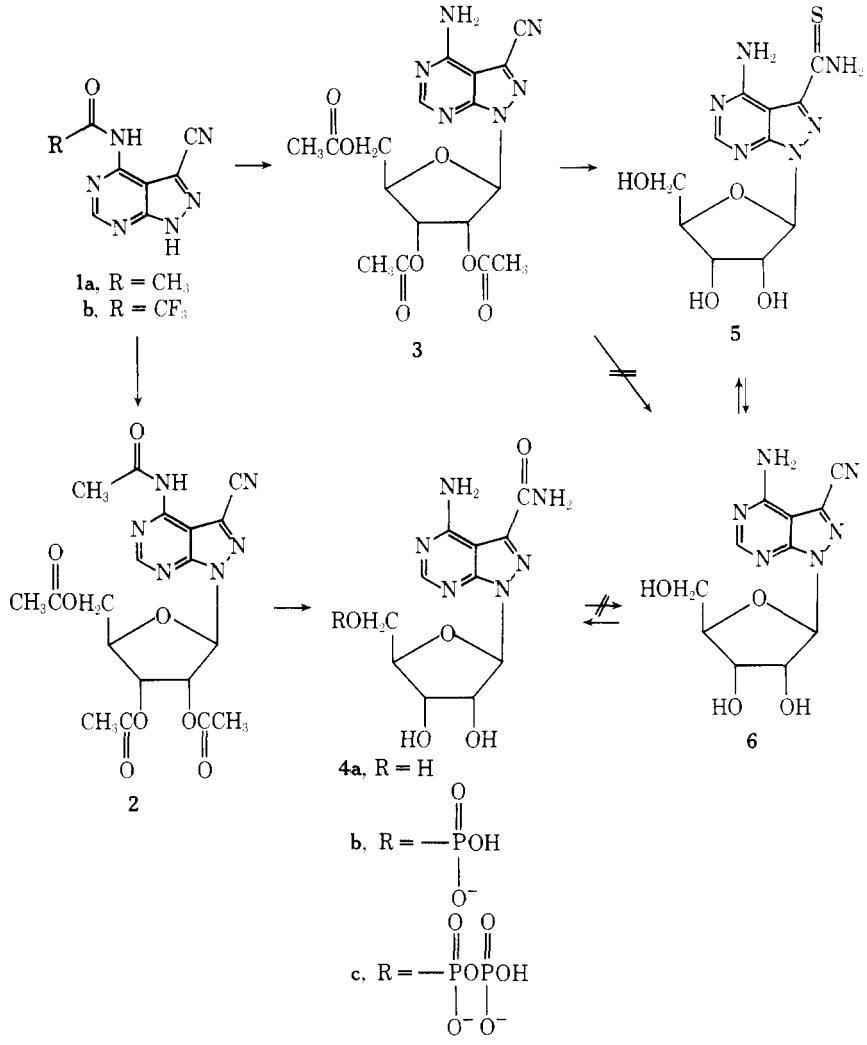
† From the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received August 22, 1975.* Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for financial support of this research.

‡ National Institutes of Health Career Development Awardee, 1975–1980; A. P. Sloan Research Fellow, 1975–1977.

§ The descriptions of the syntheses of the aza analogues of toyocamycin and sangivamycin were taken from the thesis of Dieter Werner, submitted March 1974 in partial fulfillment of the requirements for the Ph.D. degree in Chemistry. After the synthetic work was completed, a report appeared concerning the syntheses of **4a**, **5**, and **6** (Earl and Townsend, 1974).

[1] A major impetus for the extensive biochemical and biological characterization of these analogues is their confirmed activity against tumors in experimental animals. Compounds **4a** and **5**, e.g., have also been prepared independently in the laboratory of Professor Leroy Townsend (Earl and Townsend, 1974) and tested for anticancer activity at the National Cancer Institute. Compound **4a** showed significant activity against L1210 lymphoid leukemia, giving percent T/C values from 236 to 258 when administered in five doses of 50–200 mg/kg of body weight; compound **5** also had confirmed activity against the same tumor. Compound **7a** (Montgomery et al., 1964) was shown to have activity against L1210 lymphoid leukemia and P388 lymphocytic leukemia in experimental animals and against human epidermoid carcinoma in cell culture.

Scheme I



cals and both Dulbecco's modified Eagle medium and horse serum were from Grand Island Biological Company. DEAE-cellulose and GF/A glass fiber disks were obtained from Whatman, nitrocellulose filters (0.45 μm pore size) from Millipore Corporation, and L-[^{3}H]lysine, 55 Ci/mmol, from New England Nuclear Corporation.

Ultraviolet spectra were recorded on a Cary 15 UV spectrophotometer, infrared spectra on a Perkin-Elmer 457A spectrophotometer, and mass spectra on a Perkin-Elmer-Hitachi RMU-6 spectrometer using a direct inlet. Melting points were determined on a Thomas Hoover apparatus and are uncorrected. Elemental analyses were determined by Chemalytics, Inc., or by Scandinavian Microanalytical Laboratory.

Synthesis of Compounds. 4-Acetylaminopyrazolo[3,4-d]pyrimidine-3-carbonitrile (**1a**). To a stirred solution of 842 mg (5.25 mmol) of 4-aminopyrazolo[3,4-*d*]pyrimidine-3-carbonitrile·(Taylor and Abul-Husn, 1966) in 200 ml of pyridine was added 14.5 ml of acetic anhydride. The reaction mixture was warmed to 80 °C for 5 h, concentrated under diminished pressure, and treated with chloroform to precipitate the desired product. Crystallization from ethanol gave white needles of **1a:** yield 774 mg (72%); mp >300 °C; λ_{max}^{EtOH} (pH 1) 278 nm (ϵ 10 900); λ_{min} 246 (3700); λ_{max}^{EtOH} (pH 7) 278 (10 400); λ_{min} 252 (4900); λ_{max}^{EtOH} (pH 11) 312 (10 100) and 340 (sh); λ_{min} 262 (5900); *m/e* 202, 188, 187, 175, 160, and 144; ir (nujol) 3400, 2960–2820, 2230, and 1700 cm^{-1}.

Anal. Calcd for $C_8H_6N_6O$: C, 47.52; H= 2/99/ Found: C, 47.56; H, 2.78.

4-Trifluoroacetylaminopyrazolo[3,4-d]pyrimidine-3-carbonitrile (**1b**). To a stirred suspension of 3.51 g (22.0 mmol) of 4-aminopyrazolo[3,4-*d*]pyrimidine-3-carbonitrile in 60 ml of ethyl acetate was added 5.66 ml of trifluoroacetic anhydride. The reaction mixture was stirred overnight and concentrated under diminished pressure. The brown solid residue was triturated with ethyl acetate and filtered to afford fine off-white needles of **1b:** yield 4.74 g (85%); mp 272.5–274.5 °C dec. Recrystallization from chloroform did not raise the melting point: λ_{max}^{EtOH} (pH 1) 314 nm (sh), 306 (ϵ 14 400), 254 (6600), 246 (6400), and 238 (6500); λ_{min} 263 (5900), 250 (6300), 243 (6300), and 234 (6200); λ_{max}^{EtOH} (pH 7) 315 (sh), 305 (13 900), and 255 (6700); λ_{min} 262 (5900) and 249 (6500); λ_{max}^{EtOH} (pH 10) 298 (8900); λ_{min} 263 (6600); λ_{max}^{EtOH} (pH 11) 303 (7600); λ_{min} 260 (5500); ir (nujol) 3180, 3100, 2960–2840, 2255, 1800, 1620, and 1560 cm^{-1}; *m/e* 256, 235, 187, 160, 144, and 133.

4-Acetamido-1-(β-D-tri-O-acetylribofuranosyl)pyrazolo[3,4-d]pyrimidine-3-carbonitrile (**2**). A finely ground mixture of 450 mg (2.22 mmol) of 4-acetylaminopyrazolo[3,4-*d*]pyrimidine-3-carbonitrile, 709 mg (2.22 mmol) of tetraacetylribofuranose (Zinner, 1950; Brown et al., 1955), and 51 mg (0.27 mmol) of *p*-toluenesulfonic acid was heated to 145 °C in vacuo (aspirator). When a homogeneous melt was obtained the temperature was raised to 165 °C for 10

min. The cooled melt was triturated with chloroform and the chloroform extract was purified by chromatography on silica gel, elution with ether, to afford **2** as a white foam (see Scheme I): yield 311 mg (36%); mp 72–73 °C; λ_{max}^{EtOH} (pH 2) 283 nm; λ_{min} 248; λ_{max}^{EtOH} (pH 7) 285; λ_{min} 248; λ_{max}^{EtOH} (pH 10) 325; λ_{min} 270; ir ($CHCl_3$) 3390, 3000, 2250, 1735, 1625, and 1570 cm^{-1}. Crystallization of the foam could be accomplished from methanol; the crystalline material had the same melting point and spectral properties.

4-Amino-1-(β-D-tri-O-acetylribofuranosyl)pyrazolo[3,4-d]pyrimidine-3-carbonitrile (**3**). A finely ground mixture of 6.53 g (26.1 mmol) of 4-trifluoroacetylaminopyrazolo-[3,4-*d*]pyrimidine-3-carbonitrile, 8.31 g (26.2 mmol) of tetraacetylribofuranose, and 910 mg (4.8 mmol) of *p*-toluenesulfonic acid was treated in six equal batches as follows. Each batch was submerged in vacuo (aspirator) into an oil bath which had been preheated to 140 °C. When a homogeneous melt was obtained, the temperature was raised to 160–165 °C for 5 min. The cooled melt was triturated with chloroform and the chloroform extract was adsorbed onto silica gel. The silica was treated with methanol and maintained overnight at room temperature. After evaporation of the methanol, the dry silica was poured onto a silica gel column and washed with 500 ml of ethyl acetate to afford the desired product. This material was further purified by rechromatography on silica gel, elution with ethyl acetate to afford **3** as an amorphous solid: yield 5.91 g (54%); mp 67–70 °C; λ_{max}^{EtOH} (pH 2) 281 nm; λ_{min} 248; λ_{max}^{EtOH} (pH 7) 283; λ_{min} 250; λ_{max}^{EtOH} (pH 10) 282; λ_{min} 259; ir (KBr) 3440, 3330, 2245, 1735, 1630, and 1580 cm^{-1}.

4-Amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-d]pyrimidine-3-carboxamide (**4a**). A stirred suspension of 442 mg (1.01 mmol) of 4-acetamido-1-(β-D-tri-*O*-acetylribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carbonitrile (**2**) in 10 ml of concentrated ammonium hydroxide was warmed to 60 °C for 10 min and then permitted to stand overnight at room temperature. White crystals of **4** separated and were isolated by filtration: yield 251 mg (80%); mp 261.5–263.5 °C; $\lambda_{max}^{H_2O}$ (pH 2) 270 nm (ε 9200), 216 (sh); λ_{min} 253 (7500); $\lambda_{max}^{H_2O}$ (pH 7) 282 (10 700) and 223 (sh); λ_{min} 256 (5800); $\lambda_{max}^{H_2O}$ (pH 12) 280 (11 200) and 230 (sh); λ_{min} 256 (6900); ir (nujol) 3400, 3140 (broad), 2980–2840, 1640, and 1565 cm^{-1}; *m/e* 310, 293, 292, 280, 262, 221, 207, 189, 178, and 160.

Anal. Calcd for $C_{11}H_{14}N_6O_5 \cdot H_2O$: C, 40.24; H, 4.91. Found: C, 40.35; H, 4.58.

4-Amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-d]pyrimidine-3-carboxamide 5′-Monophosphate (**4b**). This reaction was run according to the general method of Imai et al. (1969). To a stirred suspension of 461 mg (1.49 mmol) of 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carboxamide (**4a**) in 24 ml of acetonitrile at 0 °C was added 0.75 ml of pyrophosphoryl chloride. The reaction mixture was stirred for 90 min at 0 °C and then poured into an excess of ice water and neutralized with aqueous sodium hydroxide solution. The solution was treated with 45 ml of 0.5 M aqueous barium acetate solution and the resulting precipitate was removed by centrifugation. The supernatant was diluted with water and purified by chromatography on a column of DEAE-cellulose (3.2 × 60 cm), elution with a linear gradient of ammonium bicarbonate (2 l. total volume; 0–0.3 M; 16-ml fractions) at a flow rate of 170 ml/h. The appropriate fractions were pooled, concentrated under diminished pressure, and then desalted by repeated evaporations of portions of water to afford the ammonium salt of **4b** as a white, hygroscopic solid: yield 419 mg (67%); $\lambda_{max}^{H_2O}$ (pH 1) 264 nm and 218; λ_{min} 249; $\lambda_{max}^{H_2O}$ (pH 7) 281 and 225; λ_{min} 253 and 217; $\lambda_{max}^{H_2O}$ (pH 12) 280; λ_{min} 254. A portion of **4b** (40 A_{281} units) was treated with crude snake venom (*Crotalus adamanteus*) in 0.2 ml of 0.1 M Tris-HCl buffer (pH 8.7) containing 0.1 M $MgCl_2$ at 37 °C for 4 h. The product was purified by chromatography on a DEAE-cellulose column (HCO_3^- form; 0.9 × 23 cm), elution with water, and then with a linear gradient of ammonium bicarbonate (200 ml total volume; 0–0.8 M; 2-ml fractions). Most of the ultraviolet-adsorbing material eluted with the water wash and was shown to be identical in all respects with 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carboxamide.

4-Amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-d]pyrimidine-3-carboxamide 5′-Diphosphate (**4c**). To 23 mg (56 μmol) of the pyridinium salt of 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carboxamide 5′-monophosphate was added 0.7 ml of water. The aqueous solution was then treated with a solution consisting of 25 μl (290 μmol) of morpholine in 0.7 ml of *tert*-butyl alcohol. To this solution was added 1.2 ml of *tert*-butyl alcohol containing 62 mg (300 μmol) of *N,N′*-dicyclohexylcarbodiimide and the combined solution was heated at reflux for 7 h. The solution was concentrated under diminished pressure and the residue was washed with water. The aqueous solution was extracted with ether and then evaporated to afford a solid residue which was dried by repeated evaporations of anhydrous pyridine under diminished pressure. This anhydrous residue was treated with 290 μmol of anhydrous mono(tri-*n*-butylammonium) phosphate in 5 ml of anhydrous pyridine. The resulting solution was concentrated to dryness, redissolved in 1 ml of anhydrous pyridine, and maintained at room temperature for 4 days. The solution was concentrated and the residue was dissolved in water, adjusted to pH 5 with dilute hydrochloric acid, and desalted by application to a column of activated charcoal (1.2 × 6 cm), elution with 1 l. of water and then 200 ml of 50% aqueous ethanol containing 2% concentrated ammonium hydroxide solution to remove the organic phosphates. Purification was effected by chromatography on a DEAE-cellulose column (2.3 × 25 cm), elution with a linear gradient of triethylammonium bicarbonate (pH 7.5) (2 l. total volume, 0–0.3 M; 17-ml fractions), at a flow rate of 135 ml/h. The appropriate fractions were combined and desalted by repeated evaporations of portions of water and ethanol to afford the triethylammonium salt of the diphosphate (**4c**) as a colorless glass: yield 220 A_{282} units (37%; 50% based on consumed **4b**); $\lambda_{max}^{H_2O}$ (pH 1) 266 nm and 223; λ_{min} 250 and 209; $\lambda_{max}^{H_2O}$ (pH 7) 283 and 236; λ_{min} 255 and 216. Phosphate analysis by modification of the method of Allen (1940) indicated the presence of 0.84 mol of acid-labile phosphate/mol of ribonucleoside (another sample of **4c** gave a value of 1.03) and 1.95 total mol of phosphate/mol of ribonucleoside.

4-Amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-d]pyrimidine-3-thiocarboxamide (**5**). To a solution of 1.55 g (3.71 mmol) of 4-amino-1-(β-D-tri-*O*-acetylribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carbonitrile (**3**) in 40 ml of methanol (distilled from sodium methoxide) was added 4.0 g (74.2 mmol) of sodium methoxide. Hydrogen sulfide was bubbled through the solution for 24 h. The precipitate was then filtered and the solid residue was triturated with water to remove inorganic salts, dried, and crystallized from methanol–water to give **5** as pale yellow crystals: yield 843

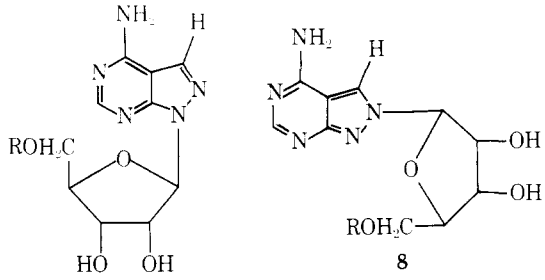
mg (70%); mp 251.5–252 °C dec; $\lambda_{max}^{H_2O}$ (pH 2) 270 nm (ϵ 10 200) and 223 (17 000), λ_{min} 261 (10 000) and 215 (16 500); $\lambda_{max}^{H_2O}$ (pH 7) 293 (11 300) and 234 (11 400); λ_{min} 253 (9300) and 224 (10 800); $\lambda_{max}^{H_2O}$ (pH 11) 279 (12 200) and 232 (13 300); λ_{min} 257 (10 200) and 226 (13 200); *m/e* 223, 203, 194, 188, 178, 160, 135, and 133.

Anal. Calcd for $C_{11}H_{14}N_6O_4S$: C, 40.48; H, 4.32. Found: C, 40.61; H, 4.07.

4-Amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-d]pyrimidine-3-carbonitrile (**6**). A sample of 117 mg of 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-thiocarboxamide was heated to its melting point. When decomposition began, the sample was removed from the oven and maintained under diminished pressure (aspirator) for 2 min. The dark-colored residue was suspended in methanol and purified by chromatography on Sephadex LH-20, elution with methanol to afford **6** as an off-white solid: yield 12 mg (12%); mp 218–223 °C; $\lambda_{max}^{H_2O}$ (pH 2) 264 nm and 222; λ_{min} 252 and 211; $\lambda_{max}^{H_2O}$ (pH 7) 280 and 231; λ_{min} 253 and 229; $\lambda_{max}^{H_2O}$ (pH 11) 281; λ_{min} 257; *m/e* 292, 274, 261, 248, 232, 203, 189, 178 (178.061), 160 (160.050), 135 (135.055), and 133 (133.039); ir (KBr) 3420, 2245, 1660, 1595, and 1570 cm^{-1}.

The same compound was obtained from **5** by the method of Earl and Townsend (1974).

4-Amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-d]pyrimidine 5′-Monophosphate (**7b**). This phosphorylation was carried out according to the method of Imai et al. (1969). To a stirred suspension of 137 mg (0.51 mmol) of 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (**7a**)



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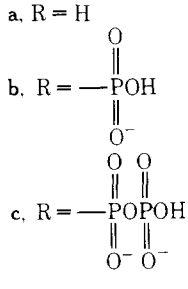
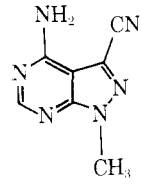
a, R = H



b, R = —PO⁻

c, R = —POPO⁻



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(Montgomery et al., 1964) in 8 ml of acetonitrile at 0 °C was added 0.25 ml of pyrophosphoryl chloride. The reaction mixture was stirred at 0 °C for 90 min and then poured into an excess of ice water, extracted with ether, and neutralized with aqueous sodium hydroxide solution. The solution was treated with 15 ml of 0.5 M aqueous barium acetate solution and the precipitate was removed by centrifugation. The supernatant was diluted with water and applied to a column of DEAE-cellulose (3 × 32 cm). The phosphate was removed by elution with a linear gradient of ammonium bicarbonate (2 l. total volume; 0–0.3 M; 13-ml fractions) at a flow rate of 175 ml/h. The appropriate fractions were pooled and concentrated and then desalted by repeated evaporations of portions of water to afford the ammonium salt of **7b** as a white solid: yield 181 mg (93%); $\lambda_{max}^{H_2O}$ (pH 1) 257 nm and 218; λ_{min} 238; $\lambda_{max}^{H_2O}$ (pH 7) 273 and 259; λ_{min} 262 and 236; $\lambda_{max}^{H_2O}$ (pH 12) 274 and 260; λ_{min} 262 and 236. Descending paper chromatography on Whatman No. 1 paper (saturated $(NH_4)_2SO_4$–water–2-propanol, 79:19:2) afforded a single ultraviolet-absorbing spot, R_f 0.45.

A portion of the product (20 A_{273} units) was treated with crude snake venom (*Crotalus adamanteus*) in 0.2 ml of 0.1 M Tris-HCl buffer solution (pH 8.7) containing 0.1 M $MgCl_2$ at 37 °C for 4 h. The product was purified by chromatography on a DEAE-cellulose column (HCO_3^- form; 0.9 × 23 cm); elution was with water and then with a linear gradient of ammonium bicarbonate (200 ml total volume; 0–0.8 M, 2-ml fractions). This product was shown to be identical in all respects with the starting ribonucleoside (**7a**).

4-Amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-d]pyrimidine 5′-Diphosphate (**7c**). To 38 mg (0.1 mmol) of diammonium 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine 5′-monophosphate (**7b**), converted to the pyridinium salt, was added 1 ml of water. The aqueous solution was treated with a solution consisting of 35 μl (0.4 mmol) of morpholine and then with a solution containing 83 mg (0.4 mmol) of *N,N′*-dicyclohexylcarbodiimide in 1.6 ml of *tert*-butyl alcohol. The combined solution was heated at reflux for 5 h. The solution was concentrated under diminished pressure and the residue was triturated with a small volume of water. The aqueous solution was extracted with ether and then evaporated to afford a solid residue which was dried by repeated evaporations of portions of anhydrous pyridine under diminished pressure. This anhydrous residue was treated with 0.44 mmol of anhydrous mono(tri-*n*-butylammonium) phosphate in 5 ml of anhydrous pyridine. The resulting solution was concentrated to dryness, redissolved in 1 ml of anhydrous pyridine, and maintained at room temperature for 4 days. The solution was concentrated and the residue was dissolved in water, adjusted to pH 5 with dilute hydrochloric acid, and desalted by application to a column of activated charcoal (1.2 × 6 cm). Elution was with 1 l. of water and then 200 ml of 50% aqueous ethanol containing 2% concentrated ammonium hydroxide solution, which effected elution of the organic phosphates. The compound was purified by chromatography on a DEAE-cellulose column (2.3 × 25 cm); elution was with a linear gradient of triethylammonium bicarbonate (pH 7.5) (3 l. total volume; 0–0.45 M; 23-ml fractions), at a flow rate of 135 ml/h. The appropriate fractions were combined and desalted by repeated evaporations of portions of water and ethanol to afford the triethylammonium salt of the diphosphate (**7c**) as a colorless glass: yield 500 A_{274} units (48%; 70% based on consumed **8b**); $\lambda_{max}^{H_2O}$ (pH 1) 257 nm; λ_{min} 237; $\lambda_{max}^{H_2O}$ (pH 7) 273 and 258; λ_{min} 262 and 235; $\lambda_{max}^{H_2O}$ 273 and 258; λ_{min} 262 and 235. Descending paper chromatography on Whatman No. 1 paper (79:19:2 saturated $(NH_4)_2SO_4$–water–2-propanol) afforded a single ultraviolet-absorbing

spot, R_f 0.52. Phosphate analysis by the method of Allen (1940) indicated the presence of 1.01 mol of acid-labile phosphate/mol of ribonucleoside (Montgomery et al., 1964) and 2.01 total mol of phosphate/mol of ribonucleoside.

4-Amino-2-(β-D-ribofuranosyl)pyrazolo[3,4-d]pyrimidine 5′-Monophosphate (**8b**). This phosphorylation was carried out according to the method of Yoshikawa et al. (1967). To a cooled solution of 0.2 ml of phosphorus oxychloride in 2 ml of trimethyl phosphate was added 50 mg (0.18 mmol) of 4-amino-2-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine. The reaction mixture was maintained at 0 °C for 24 h and then added to ice water and desalted on a column of activated charcoal (1.2 × 6 cm), elution with 1 l. of water and then 200 ml of 50% aqueous ethanol containing 2% concentrated ammonium hydroxide solution to remove the organic phosphates. Purification was effected by chromatography on Dowex 1-X8, 20–50 mesh ($HCOO^-$ form; 0.9 × 21 cm), elution with water and then with a linear gradient of formic acid (2 l. total volume; 0–0.3 M; 7.5-ml fractions) at a flow rate of 50 ml/h. The appropriate fractions were combined and concentrated to afford the free acid of **9b** as a white powder: yield 42 mg (65%); $\lambda_{max}^{H_2O}$ (pH 1) 268 nm and 228; λ_{min} 242 and 226; $\lambda_{max}^{H_2O}$ (pH 7) 290 and 268; λ_{min} 272 and 243; $\lambda_{max}^{H_2O}$ (pH 10) 289 and 268; λ_{min} 272 and 244. Descending paper chromatography on Whatman No. 1 paper (saturated $(NH_4)_2SO_4$–water–2-propanol, 79:19:2) afforded a single ultraviolet-absorbing spot, R_f 0.54.

A portion of the product (60 A_{287} units) was treated with 0.7 mg of crude snake venom (*Crotalus adamanteus*) in 0.1 ml of 0.1 M Tris-HCl buffer (pH 8.7) containing 0.01 M $MgCl_2$. The reaction mixture was incubated at 37 °C for 15 h and then purified by chromatography on Dowex 1-X8, 20–50 mesh ($HCOO^-$ form; 0.5 × 6 cm), elution with water and then with a linear gradient of formic acid. All ultraviolet-absorbing material was found in the water wash. Ultraviolet spectroscopy and chromatography on Whatman No. 1 paper, development with isopropyl alcohol–concentrated ammonium hydroxide–water (7:1:2, v/v) indicated the material to be identical with the ribonucleoside (**8a**) (R_f 0.57) rather than with the ribonucleoside 5′-monophosphate (**8b**) (R_f 0.10).

4-Amino-2-(β-D-ribofuranosyl)pyrazolo[3,4-d]pyrimidine 5′-Diphosphate (**8c**). Compound **8c** was prepared from 4-amino-2-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine 5′-monophosphate (**8b**) by analogy with the synthesis of **7c**: yield 51% of **8c** as a colorless glass (60%, based on consumed **8b**); $\lambda_{max}^{H_2O}$ (pH 1) 268 nm and 229; λ_{min} 242 and 226; $\lambda_{max}^{H_2O}$ (pH 7) 290 and 268; λ_{min} 272 and 243; $\lambda_{max}^{H_2O}$ (pH 10) 290 and 268; λ_{min} 272 and 244. Descending paper chromatography on Whatman No. 1 paper (79:19:2 saturated $(NH_4)_2SO_4$–water–2-propanol) afforded a single ultraviolet-absorbing spot, R_f 0.62. Phosphate analysis by the method of Allen (1940) indicated the presence of 0.95 mol of acid-labile phosphate/mol of ribonucleoside (Montgomery et al., 1964) and 1.90 total mol of phosphate/mol of ribonucleoside.

Biochemical Preparation and Assay Procedures. Adenosine Deaminase. The deaminations were carried out on 0.3 μmol of substrate, using 0.2 μg of enzyme. The course of the reaction was monitored by thin-layer chromatography on cellulose and elution with isopropyl alcohol–concentrated ammonium hydroxide–water (7:1:2, v/v) or with *n*-butyl alcohol–glacial acetic acid–water (5:2:3, v/v). All reactions were monitored for at least 18 h, or until they had gone to completion.

Initial velocity studies were carried out at 25 °C in a final reaction volume of 3.1 ml. The reactions were initiated by the addition of the enzyme, as a concentrated solution, to the substrate which was dissolved in 0.1 M phosphate buffer (pH 7.5). The course of reaction was followed by monitoring the change in optical density at a suitable wavelength at 30-s intervals. Thus, adenosine was monitored at 265 nm, at ten substrate levels varying from 10.4×10^{-5} to 4.96×10^{-5} M, and 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine was monitored at 274 nm, at substrate concentrations varying from 10.7×10^{-5} to 3.95×10^{-5} M. The data were expressed as double-reciprocal plots of substrate vs. initial velocity and used for determination of K_m and V_{max}.

Compounds **4a, 7a,** and **8a** were used as potential inhibitors of adenosine deamination. Inhibitor concentrations up to 9×10^{-5} M gave no observable inhibition of substrate utilization, when the latter was used in the concentration range of approximately $3–8 \times 10^{-5}$ M.

Polynucleotide Phosphorylase. The polymerizations were carried out on 6 μmol of 5′-diphosphate, utilizing 150 μg of polynucleotide phosphorylase from *Micrococcus luteus* in a final reaction volume of 1.5 ml of 0.1 M Tris-HCl (pH 9.5) which was 2 mM in $MgCl_2$. The reaction mixture was incubated at 37 °C for 24 h and the extent of reaction was assayed by measuring released inorganic phosphate (Allen, 1940). Copolymerizations with adenosine 5′-diphosphate were done in the same way except that various ratios of 5′-ADP and the analogue 5′-diphosphates were utilized (e.g., 6 μmol of 5′-ADP and 1.5 μmol of **7c**) The reaction mixtures were incubated for 6 h and then extracted with an 80% liquefied phenol solution. The aqueous layer was applied to a column of Sephadex G-75 (1.4 × 81 cm) and washed with water at a flow rate of 25 ml/h (2-ml fractions). The polymeric material, present in the void volume, was hydrolyzed overnight at 37 °C with 0.3 M aqueous potassium hydroxide solution and then neutralized with Dowex 50-X8 (H^+ form). The solution was concentrated to dryness, redissolved in 0.2 ml of 0.1 M Tris-HCl (pH 7.5), and treated with 7 μg of rye grass 3′-nucleotidase. The mixture was incubated at 37 °C for 15 h, then applied to a DEAE-cellulose column and washed with water. The eluate was concentrated to a small volume and analyzed by paper chromatography on Whatman No. 1 paper, by comparison of mobilities with authentic samples. Neither **7c** nor **8c** was incorporated into polymeric material by this procedure, although the analogues were inhibitory to the polymerization of 5′-ADP itself (see, e.g., Figure 3) with **7c** being the more inhibitory.

Polymerizations in the presence of Mn^{2+} were carried out by a similar procedure. Compound **7c** (80 μmol), e.g., was polymerized with 6 mg of polynucleotide phosphorylase in a final reaction volume of 8 ml of 0.1 M Tris-HCl (pH 8.1), which was 5 mM in $MnCl_2$. The reaction mixture was incubated at 37 °C for 24 h, monitored and worked up as before, and applied to a Sephadex G-75 column (1.4 × 81 cm) and washed with water at a rate of 25 ml/h (2.3-ml fractions). The fractions (16–19) containing polymeric material were pooled and lyophilized to afford 3.1 A_{274} units of poly(4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine). Copolymerizations were run in the same way. The polymers were purified on Sphadex G-75 and analyzed as described above. The copolymerization of 5′-ADP an **7c** af-

forded much larger amounts of polymer (e.g., up to 79 A_{260} units starting from 50 μmol of a 4:1 mixture of 5′-ADP and **7c**) than were obtained by the polymerization of **7c** alone. The polymers were estimated to be at least 100–200 nucleotides in length on the basis of their behavior on Sephadex G-75 and on the ratio of nucleotide:nucleoside resulting from base hydrolysis of the polymers.

Binding of Lysyl-tRNA to E. coli Ribosomes. This assay was carried out according to the method of Leder (1968). To a solution of 0.05 M Tris-HCl (pH 7.2, total reaction volume 50 μl) containing 25 mM ammonium chloride and 5 mM magnesium chloride was added 4.9 A_{260} units of low salt washed *E. coli* ribosomes (Ravel and Shorey, 1971) and then 0.13 A_{260} unit of the appropriate polyribonucleotide message. The reaction was initiated by the addition of 0.14 A_{260} unit of [^{3}H]lysyl-tRNA (32 900 cpm). After a predetermined time interval, 1 ml of cold Tris-HCl buffer was added and the mixture was filtered through a nitrocellulose filter. The filter was dried and radioactivity was determined in solution (Bray, 1960).

Polylysine Synthesis. This assay was carried out by modification of the method of Gottesman (1971). To a solution of 0.04 M Tris-HCl (pH 7.8, total reaction volume 400 μl) containing 0.015 M magnesium acetate, 0.03 M potassium chloride, 8 mM β-mercaptoethanol, 6 mM phosphoenolpyruvate, 0.25 mM GTP, and 1.5 mM ATP was added 31.3 μM L-[^{3}H]lysine, 539 Ci/mol, 20 μg of pyruvate kinase, 27 A_{260} units of *E. coli* tRNA, and 2.75 A_{260} units of the appropriate polyribonucleotide message. The solution was equilibrated at 37 °C and the reaction was initiated by the addition of 3.4 A_{260} units of low salt washed *E. coli* ribosomes (Ravel and Shorey, 1971) and 50 μl of crude *E. coli* supernatant factors. At predetermined time intervals, 50-μl aliquots of solution were applied to glass fiber disks which had been pretreated with 200 μl of 0.4 M aqueous sodium hydroxide. The dried disks were washed successively with three portions of 5% aqueous trichloroacetic acid (containing 0.25% sodium tungstate), ethanol, and ether. The disks were dried and radioactivity was determined.

Mouse Fibroblast Assay Procedures. Mouse fibroblasts (3T6) were cultured in Dulbecco's modified medium, supplemented with penicillin-G (6 mg/100 ml), streptomycin (10 mg/100 ml), and horse serum. Tissue culture dishes containing 4 ml of medium were inoculated with 2×10^4 cells. After 24 h of incubation, the medium was replaced with fresh medium containing **4a, 5, 7a, 8a,** or adenosine at one of the six concentrations tested in duplicate for each compound. The dishes were incubated at 37 °C and each was observed for growth relative to drug-free controls at 3 and 5 days. Growth was recorded on an arbitrary scale of A (confluent monolayer) to F (all cells dead). Inhibition was regarded as fewer cells than in the drug-free controls, and toxicity was indicated by cell detachment from the monolayers.

Results

Preparation of 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carbonitrile (**6**) was attempted initially by acetylation of 4-aminopyrazolo[3,4-*d*]pyrimidine-3-carbonitrile, fusion of the acetylated product (**1a**) with tetraacetylribofuranose in the presence of *p*-toluenesulfonic acid to give 4-acetamido-1-(β-D-tri-*O*-acetylribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carbonitrile (**2**), and deblocking of **2** to afford **6.** Although deblocking with concentrated ammonium hydroxide at 70 °C for 15 min did remove the four *O*- and *N*-acetyl protecting groups, the resulting product had no absorption near 2250 cm^{-1} corresponding to the 3-cyano moiety and was subsequently identified as 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carboxamide (**4a**) by virtue of its mass spectrum and elemental analysis. Several additional attempts were made to effect deblocking of **2** without concomitant addition to the cyano moiety. These attempts involved the utilization of diazomethane (Bredereck et al., 1956; Haines et al., 1962), anhydrous propanolic and *tert*-butanolic ammonia, and methylamine (Rosenthal and Baker, 1973), but all were unsuccessful. Attempted dehydration of carboxamide **4a** utilizing a variety of methods (Kaiser and Hauser, 1966; Kaiser et al., 1967; Ellzey et al., 1967; Ressler and Ratzkin, 1961; Krynitsky and Carhart, 1963; Herbst and Wilson, 1957; Yamato and Sugasawa, 1970; Trippett and Walker, 1960; Reisner and Horning, 1963) was also unsuccessful.

In an effort to circumvent the difficulties encountered in the deblocking of **2,** 4-trifluoroacetamidopyrazolo[3,4-*d*]pyrimidine-3-carbonitrile was prepared and fused with tetraacetylribofuranose. The crude product was adsorbed on silica gel, treated with methanol, and maintained at room temperature overnight. This afforded 4-amino-1-(β-D-tri-*O*-acetylribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carbonitrile (**3**) in 54% yield after additional purification on silica gel. Once again, however, the cyanide moiety was too reactive to permit removal of the *O*-acetyl groups to afford compound **6.** Therefore, compound **3** was treated with hydrogen sulfide in anhydrous methanol containing 20 equiv of sodium methoxide. 4-Amino-1-(β-D-ribofuranosyl)-pyrazolo[3,4-*d*]pyrimidine-3-thiocarboxamide (**5**) precipitated from solution during the reaction and was recrystallized from methanol–water as pale yellow crystals in 70% overall yield. When a sample of the thiocarboxamide was heated to its melting point and then maintained under diminished pressure, 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carbonitrile (**6**) was obtained. Treatment of this material with hydrogen sulfide and sodium methoxide in methanol effected its conversion back to thiocarboxamide **5,** and solution of **6** in water or aqueous ammonia at room temperature hydrated readily to afford carboxamide **4a,** as judged by thin-layer chromatography on silica gel and ultraviolet spectroscopy (Figure 1). Compound **6** was also characterized by its mass spectrum (*m/e* 292 (M^+), 203, 189, and 160) and infrared spectrum, the latter of which had an absorption at 2245 cm^{-1}. The position of ribosylation in compounds **2–6** was established by comparison of the ultraviolet spectrum of **6** with those of the corresponding *N*-methyl analogues 4-amino-1-methylpyrazolo[3,4-*d*]-pyrimidine-3-carbonitrile (**9**) and 4-amino-2-methylpyrazolo[3,4-*d*]pyrimidine-3-carbonitrile (**10**), the structures of which have been firmly established (Hecht et al., 1975; Earl et al., 1975) (Figure 2).

Five compounds were assayed for cytotoxity in cultures of exponentially growing mouse fibroblast cell line 3T6 at doses of 1–200 μg/ml. Tested compounds included **4a** and **5,** as well as 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (**7a**) and 4-amino-2-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (**8a**) (Montgomery et al., 1964). The cells were grown in Dulbecco's modified Eagle minimum essential medium supplemented with horse serum to exclude extracellular adenosine deaminase (Ishii and Green, 1973). After incubation for 3 days the three ribonucleosides related structurally to adenosine (**4a, 5,** and **7a**) all caused inhibition of growth at 1 μg/ml (Table I). 4-Amino-1-(β-

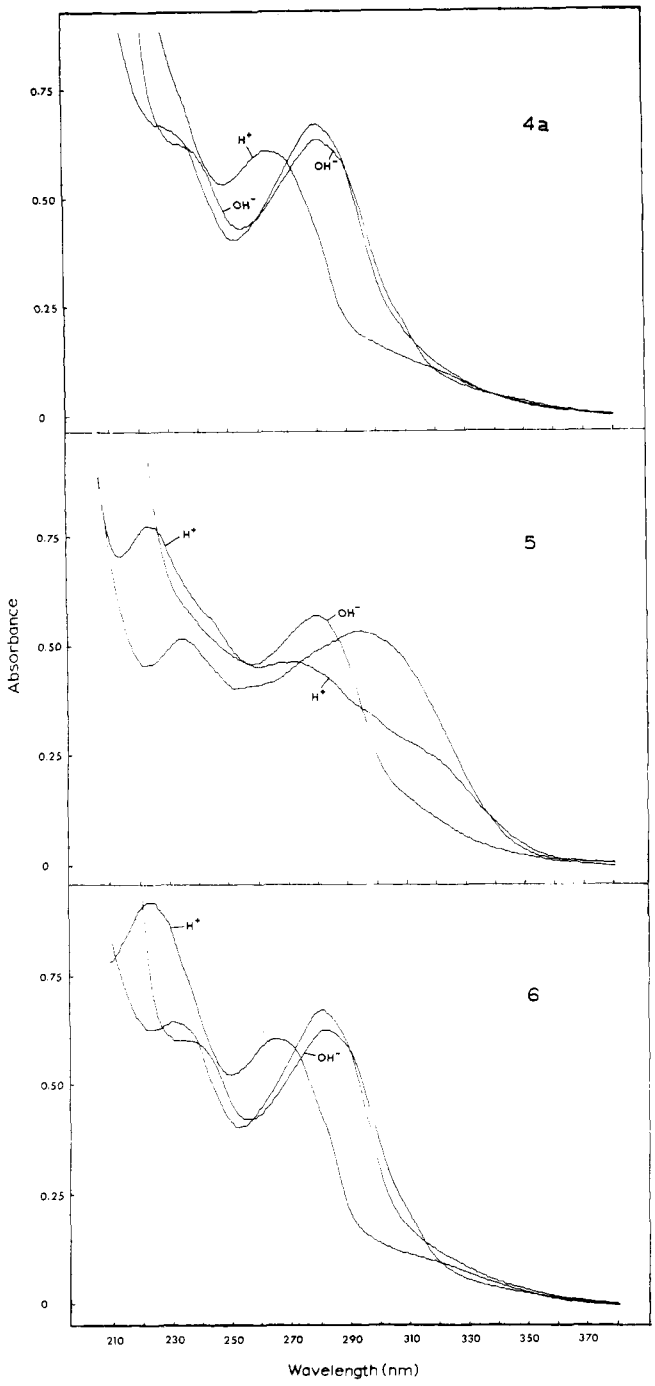

FIGURE 1: Comparison of the ultraviolet spectra of 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carboxamide (**4a**), 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-thiocarboxamide (**5**), and 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carbonitrile (**6**) in water at pH 1, 7, and 12.

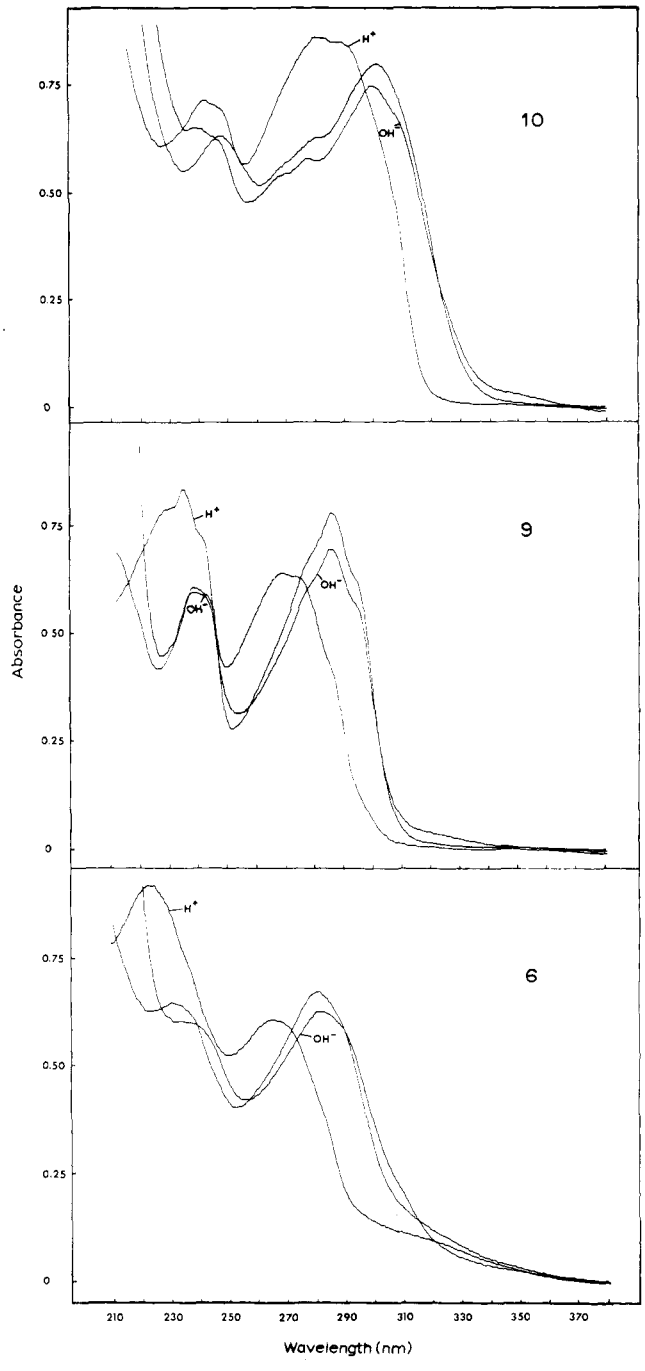

FIGURE 2: Comparison of the ultraviolet spectra of 4-amino-2-methylpyrazolo[3,4-*d*]pyrimidine-3-carbonitrile (**10**), 4-amino-1-methylpyrazolo[3,4-*d*]pyrimidine-3-carbonitrile (**9**), and 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carbonitrile (**6**) in water at pH 1, 7, and 12.

D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carboxamide (**4a**) was lethal at 100 μg/ml, while the corresponding thiocarboxamide (**5**) was lethal at 10 μg/ml. The unsubstituted analogue, 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (**7a**), exhibited toxicity after 3 days at a concentration of 25 μg/ml. In contrast to this, the isomeric ribofuranoside **8a** was not toxic after 5 days of incubation at concentrations up to 200 μg/ml.

Compounds **7a** and **8a** were tested as potential substrates and inhibitors of adenosine deaminase from calf intestinal mucosa. Only compound **7a** was utilized as a substrate by the enzyme, affording a new product after several hours of incubation, as judged by cellulose TLC. Compound **7a** afforded a single spot on cellulose TLC (R_f 0.73; λ_{max} 274 nm) when developed with isopropyl alcohol–concentrated ammonium hydroxide–water (7:1:2, v/v). The presumed deaminated product had R_f 0.68 (λ_{max} 251 nm) in this system. Development of the TLC plate with *n*-butyl alcohol–acetic acid–water (5:2:3, v/v) afforded R_f values of 0.73 and 0.62 for substrate and product, respectively. Initial velocity measurements (Table II) indicated an apparent K_m of 125 μM for **7a** (vs. 20 for adenosine) and a V_{max} 6.4% that of adenosine. Bennett et al. (1969) reported the same relative V_{max} for **7a** and adenosine, but slightly higher K_m values (**7a**, 250 μM; adenosine, 33 μM). Neither **7a** nor **8a**

Table I: Inhibition of Mouse Fibroblasts (3T6) by Adenosine Analogues.[a]

Compound	3 Days		5 Days	
	Inhibits (μg/ml)	Kills (μg/ml)	Inhibits (μg/ml)	Kills (μg/ml)
Adenosine	1	5	≤1	2.5
4-Amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carboxamide (**4a**)	50	100	1	50
4-Amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-thiocarboxamide (**5**)	≤1	10	≤1	≤1
4-Amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (**7a**)	≤1	25	≤1	≤1
4-Amino-2-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (**8a**)	b	b	b	b

[a] Cultured in horse serum (Ishii and Green, 1973). [b] Inactive at concentrations up to 200 μg/ml.

Table II: Utilization of Analogues by Adenosine Deaminase.

Compound	Apparent K_m (μM)	V_{max}[a]
Adenosine	20	1
4-Amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (**7a**)	125	0.064
4-Amino-2-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (**8a**)	b	b

[a] Relative to adenosine. [b] Not utilized by the enzyme.

was found to be inhibitory to the utilization of adenosine by the enzyme.

Compound **4a** was converted to 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carboxamide 5′-monophosphate (**4b**) in 67% yield by treatment with pyrophosphoryl chloride in acetonitrile. Also converted to their respective ribonucleoside 5′-monophosphates were compounds **7a** and **8a.** That each of the compounds was phosphorylated specifically on the 5′ position was shown by digestion of the ribonucleotides with snake venom, which reafforded the parent ribonucleoside analogue in each case. The ribonucleoside 5′-monophosphate analogues were then converted to their respective 5′-phosphoromorpholidates by treatment with dicyclohexylcarbodiimide and morpholine in *tert*-butyl alcohol and finally to the ribonucleoside 5′-diphosphate analogues by the action of mono(tri-*n*-butylammonium) phosphate on the phosphoromorpholidates. The 5′-diphosphates were characterized by their ultraviolet spectra, chromatographic mobilities relative to the corresponding monophosphates, and by analysis for acid-labile and total phosphate content.

Attempted polymerizations of compounds **7c** and **8c** using polynucleotide phosphorylase from *Micrococcus luteus* in the presence of Mg^{2+} did not afford any polymeric material. In addition, neither of the two compounds would copolymerize with adenosine 5′-diphosphate, although both inhibited the polymerization of 5′-ADP itself, with **7c** being the more inhibitory (Figure 3). In the presence of Mn^{2+}, however, compound **7c** formed a homopolymer and copolymers when incubated with adenosine 5′-diphosphate. The

Table III: Polymerization of 4-Amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine 5′-Diphosphate and Adenosine 5′-Diphosphate by Polynucleotide Phosphorylase.

Substrate (μmol)		$MnCl_2$ (μmol)	Incubation Time (h)	Polymer (A_{260} Units)	Ratio of Nucleosides (Adenosine/**7a**)
ADP	**7c**				
40	10	25	8	79	80/20
21	10	15	24	46	65.5/34.5
45	5	25	7	60	88/12
	80	40	24	3.1	

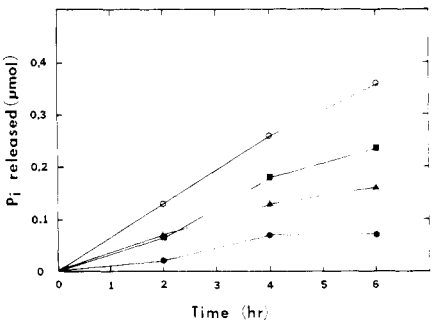

FIGURE 3: Inhibition by 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine 5′-diphosphate (**7c**) of the polynucleotide phosphorylase-promoted polymerization of 5′-ADP. Incubations were carried out at 37 °C with 40 μg of polynucleotide phosphorylase in 0.1 M Tris-HCl (pH 8.1), containing 2 mM $MgCl_2$, 4 mM 5′-ADP, and **7c** at concentration levels of 0 (○), 1 (■), 2 (▲), and 4 (●) mM.

copolymerizations were carried out with several ratios of **7c** and 5′-ADP; the resulting copolymers all contained the two heterocycles in the same ratios in which they were introduced into the individual incubation mixtures (Table III). The 5′-diphosphate of the "unnatural" isomer (**8c**) would not form a homopolymer, but did form a copolymer with adenosine 5′-diphosphate when the two compounds were incubated as a 1:2 mixture (of **8c**:5′-ADP) in the presence of the enzyme. Analysis of polymer content revealed that no more than 5–10% of the polymer consisted of **8;** the analogous experiment with **7c** afforded polymer containing 34% of the analogue. Also attempted was the copolymerization of adenosine 5′-diphosphate (10.8 μmol) and 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carboxamide 5′-diphosphate (**4c;** 5.4 μmol) in the presence of Mn^{2+}. The resulting copolymer (35 A_{260} units) was hydrolyzed by treatment with 0.3 M potassium hydroxide at 37 °C for 19 h and shown to contain approximately 95% adenosine and no more than 5% 4-amino-1-(β-D-ribofuranosyl)pyrazolo-[3,4-*d*]pyrimidine-3-carboxamide (**4a**). As with species **7c** and **8c,** no polymerization or copolymerization with 5′-ADP was observed in the presence of Mg^{2+}.

The copolymers of adenosine and 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (**7a;** Table III) were assayed as potential mRNA analogues in the nonenzymatic A-site binding of *E. coli* lysyl-tRNA to ribosomes from *E. coli.* As shown in Figure 4, the copolymers stimulated the binding of lysyl-tRNA ~60–70% as well as poly(adenylic acid), while the binding obtained in response to the homopolymer of **7a** was only slightly better than that obtained in response to poly(uridylic acid). When the same homopolymer and copolymers were utilized as mRNA analogues for the cell-free synthesis of polylysine, no protein synthesis was observed (Figure 5).

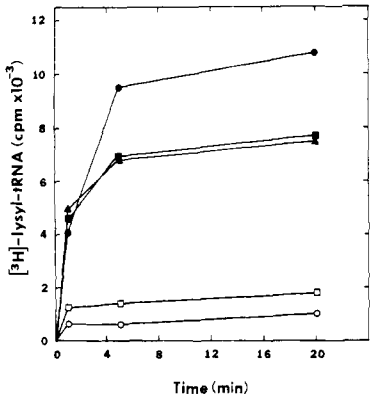

FIGURE 4: A-site binding of [^{3}H]lysyl-tRNA to low salt washed ribosomes from *E. coli* in response to poly(A) (●), 2:1 poly(A:**7a**) (■), 9:1 poly(A:**7a**) (▲), poly(4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine) (□), and poly(U) (○). Experimental details are given under Materials and Methods.

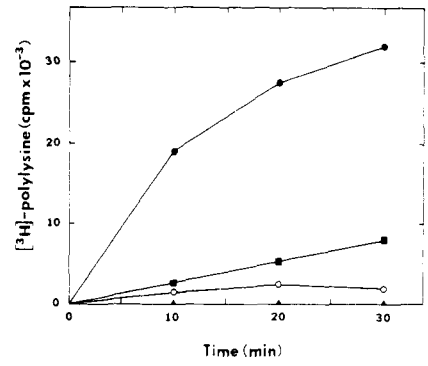

FIGURE 5: Synthesis of [^{3}H]polylysine in a cell-free system containing tRNA, low salt washed ribosomes, and supernatant factors from *E. coli*, and poly(A) (●), poly(U) (■), 2:1 poly(A:**7a**) (○), or 9:1 poly(A:**7a**) (▲). The reaction mixtures were incubated at 37 °C and monitored for the incubation of [^{3}H]lysine into base-stable polymers. Experimentals details are given under Materials and Methods.

The copolymers were assayed for interaction with poly(U) at room temperature and at 4 °C. Although the polymers containing adenosine and **7a** in a ratio of 9:1 and 4:1 (Table III) were not observed to form a complex at room temperature or at 4 °C, the 2:1 polymer was found to form a complex with poly(U) at 4 °C (Figure 6).

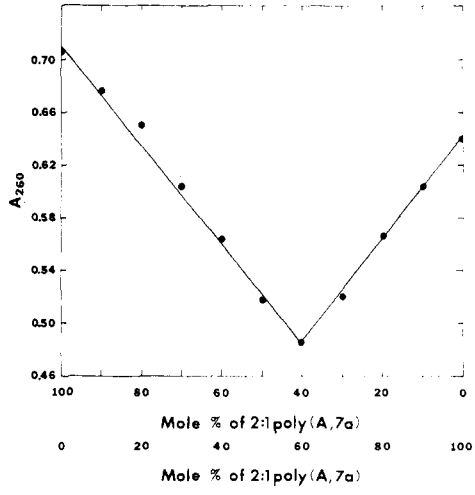

FIGURE 6: Mixing profile of poly(U) and 2:1 poly(A:**7a**). The complex was formed in 0.05 M sodium cacodylate buffer (pH 7.0), containing 0.10 M sodium chloride. Equimolar stock solutions of poly(U) and 2:1 poly(A:**7a**) (ε (p) 9.9 × 10^3) were combined in different ratios and maintained at 4 °C for 24 h. The solutions were then allowed to warm to room temperature for 30 min and the A_{260} value of each was determined.

Discussion

The synthesis of the 6-aza analogue of tubercidin, 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (**7a**), was first reported by Davoll and Kerridge (1961) and later by Montgomery et al. (1964), who prepared an authentic sample by the fusion of 4-benzamidopyrazolo[3,4-*d*]pyrimidine with tetraacetylribofuranose, followed by deblocking with methanolic sodium methoxide. Both the 1- and 2-β-D-ribofuranosyl isomers (**7a** and **8a**) were obtained by this procedure and the position of the ribose moiety in each compound was established by comparison of the ultraviolet spectra of **7a** and **8a** with those reported (Davoll and Kerridge, 1961) for the known (Cheng and Robins, 1956; Schmidt et al., 1959) 4-amino-1- and -2-methylpyrazolo[3,4-*d*]pyrimidines. Preparation of the 6-aza analogues of sangivamycin and toyocamycin (**4a** and **6**) has been attempted by an analogous procedure, by the fusion of 4-acetamidopyrazolo[3,4-*d*]pyrimidine-3-carbonitrile (**1a**) and tetra-*O*-acetyl-β-D-ribofuranose to afford predominantly one of the two possible ribosylated pyrazolo[3,4-*d*]pyrimidines (**2**). Deblocking of compound **2** could be accomplished with aqueous ammonium hydroxide, but this procedure effected concomitant hydration of the cyano moiety to afford 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carboxamide (**4a**). Several attempts were made to convert **2** to **6** under milder conditions but these were all unsuccessful, as were experiments involving the dehydration of **4a.** Also prepared for attempted deblocking under milder conditions was 4-amino-1-(β-D-tri-*O*-acetylribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-carbonitrile (**3**), which was accessible by methanolysis of the corresponding 4-trifluoroacetyl derivative on silica gel. Although several methods were employed in an effort to convert **3** to **6,** none was successful. Compound **6** was eventually obtained by conversion of **3** to 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine-3-thiocarboxamide (**5**), the latter of which lost elements of hydrogen sulfide when heated to its melting point and then maintained under diminished pressure. Compound **6** was converted to carboxamide **4a** and thiocarboxamide **5** by treatment with water (or aqueous ammonia) and hydrogen sulfide, respectively, and was also characterized by its ultraviolet, infrared, and mass spectra (Figure 1). The same compound was obtained from **5** by treatment with mercuric chloride in dimethylformamide, according to the method of Earl and Townsend (1974).

The structural assignments of **7a** and **8a** rest ultimately on the correctness of the structures of the model compounds 4-amino-1-methylpyrazolo[3,4-*d*]pyrimidine (Cheng and Robins, 1956) and 4-amino-2-methylpyrazolo[3,4-*d*]pyrimidine (Schmidt et al., 1959). Likewise, the identification of the position of ribosylation in **2–6** was made by comparison of the ultraviolet spectrum of **6** with those of 4-amino-1-methylpyrazolo[3,4-*d*]pyrimidine-3-carbonitrile (**9**) and 4-amino-2-methylpyrazolo[3,4-*d*]-3-carbonitrile (**10**) (Figure 2), the latter of which was assigned a structure based in part on its chemical conversion to a compound identical with 4-amino-2-methylpyrazolo[3,4-*d*]pyrimidine (Hecht et al., 1975; Earl et al., 1975). To obtain independent verification of the position of ribosylation (Montgomery et al., 1964) as well as the anomeric configuration (Montgomery

et al., 1964; Revankar and Townsend, 1971; Earl et al., 1972) of these ribonucleoside analogues, **7a** and **8a** were compared for activity in certain bioassays in the belief that the compound structurally related to the isomeric adenosine molecule would more nearly resemble that species in biological activity.[1,2] Thus, 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (**7a**) and 4-amino-2-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (**8a**) were tested as substrates and inhibitors of adenosine deaminase. Neither of the compounds inhibited utilization of adenosine by the enzyme and only compound **7a** was utilized by the enzyme as a substrate, affording a new product after several hours of incubation which was presumably the deaminated analogue 4-hydroxy-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine. The apparent K_m for **7a** (125 μM) was higher than the value of 20 μM measured for adenosine and the analogue was utilized with a V_{max} 6.4% that of adenosine (Table II).

As has been shown for many cytotoxic nucleoside analogues, including the naturally occurring pyrrolo[2,3-*d*]pyrimidine ribofuranosides (Suhadolnik, 1970), the observed killing of mouse fibroblasts (3T6) by adenosine occurs after conversion of the ribonucleoside to 5′-AMP (Ishii and Green, 1973). That the effect is mediated through the initial formation of adenosine 5′-monophosphate may be inferred from the lesser sensitivity to adenosine killing of a mutant cell line (3T6-TM) deficient in adenosine kinase. As a further test of the assignments of the position of ribosylation in compounds **7a** and **8a,** the compounds were utilized as potential analogues of adenosine in promoting inhibition of the growth of a mouse fibroblast cell line (3T6). Because **7a** was found to be a substrate for adenosine deaminase, an enzyme which is relatively abundant in calf serum, the cells were cultured in Dulbecco's modified medium, containing no purines or pyrimidines and supplemented with 10% horse serum. As indicated in Table I, compound **7a** was observed to be inhibitory to 3T6 after 3 days and lethal after 5 days at the lowest tested concentration (1 μg/ml). Its activity is thus similar to that of adenosine itself (Ishii and Green, 1973). Compound **8a** was not inhibitory after 5 days at any tested concentration (up to 200 μg/ml), consistent with the belief that it was the 2-β-D-ribofuranosyl isomer of 4-aminopyrazolo[3,4-*d*]pyrimidine. Also tested for cytotoxicity were compounds **4a** and **5.** As shown in Table I, carboxamide **4a** was inhibitory to the cells at a concentration of 1 μg/ml (5-day observation) and toxic at about 50 μg/ml. The thiocarboxamide was found to be more toxic, resulting in cell detachment at concentrations of 10 μg/ml after 3 days and 1 μg/ml after 5 days. Thus, compounds **5** and **7a** are very potent cytotoxic agents, although not as cytotoxic as species like tubercidin (Acs et al., 1964; Chan et al., 1973).

The 5′-triphosphates of tubercidin, toyocamycin, and sangivamycin have been utilized as substrates for the CTP(ATP)-tRNA nucleotidyltransferase from rabbit liver (Uretsky et al., 1968). All three were incorporated into rabbit liver tRNA, but only the transfer RNA terminating in tubercidin functioned in similar fashion to unmodified tRNA. The 5′-diphosphate of tubercidin was polymerized by polynucleotide phosphorylase (Ikehara and Fukui, 1968), affording homopolymers at least 100 nucleotides in length which gave 25% hypochromicity at 270 nm in 0.05 M cacodylate buffer. In analogy with this work, **7a** and **8a** were converted to their respective 5′-diphosphates (**7c** and **8c**) and then utilized as potential substrates for polymerization in the presence of polynucleotide phosphorylase from *Micrococcus luteus* and Mg^{2+}. Neither of the compounds afforded polymeric material, nor would either copolymerize in the presence of adenosine 5-diphosphate. However, the substitution of Mn^{2+} for Mg^{2+} in the incubation mixture resulted in the formation of polymers from **7c.** This compound gave both homopolymers, at least 100–200 nucleotides in length as judged by Sephadex chromatography and by the ratio of 5′-monophosphates:ribonucleosides after base hydrolysis, and mixed polymers with adenosine when polymerized in the presence of 5′-ADP. When the incubation mixtures contained 5′-ADP:**7c** ratios of 9, 4, and 2, the resulting polymers were found to contain 12, 20, and 34% **7a,** i.e., in the same approximate proportion in which it was incubated relative to 5′-ADP. Compound **8c,** on the other hand, would not give homopolymers in the presence of Mn^{2+}. When incubated in the presence of an additional two parts of 5′-ADP, **8c** was incorporated into the resulting polymer, but only to the extent of 5–10%, as compared with 34% for **7c** under the same conditions (Table III). The polymerization of **4c** by polynucleotide phosphorylase was also attempted. Although no polymerization occurred in the presence of Mg^{2+}, the substitution of Mn^{2+} permitted the formation of copolymers of **4a** and adenosine. The content of **4a** in the polymers, however, was low relative to its abundance in the incubation mixtures.

Trinucleotides containing tubercidin in place of adenosine have been shown (Ikehara and Ohtsuka, 1965) to stimulate the A-site binding of lysyl and threonyl tRNAs, and homopolymers of tubercidin have been reported to function as templates for the synthesis of polylysine in a cell-free system (Suhadolnik, 1970). Therefore, it seemed of interest to utilize the polymers containing 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (**7a**) in similar experiments. The nonenzymatic A-site binding of *E. coli* lysyl-tRNA in response to the polymers was assayed by the method of Leder (1968), using low salt washed ribosomes from *E. coli.* As shown in Figure 4, the 9:1 and 2:1 copolymers of **7a** and adenosine stimulated A-site binding only about 60–70% as well as poly(adenylic acid) itself and poly(4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine) was only slightly more stimulatory than poly(uridylic acid). Since the copolymers did exhibit some activity in promoting A-site binding of lysyl-tRNA, the same polymeric species were tested for their ability to mediate the formation of polylysine. This assay was carried out by modification of the method of Gottesman (1971) and utilized low salt washed ribosomes and crude supernatant factors from *E. coli.* Neither the copolymers of **7a** and adenosine nor the homopolymer of **7a** gave significant stimulation of polylysine synthesis, relative to controls which contained no exogenous mRNA or which utilized poly(U) (Figure 5).

Ikehara and Fukui (1968) have shown that a polymer of tubercidin can form a 1:1 complex with poly(uridylic acid) and it was of interest to determine whether the copolymers

[2] The compound believed to be 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine (**7a**) has been shown to act as a substrate for adenosine deaminase (Bennett et al., 1969) and adenosine kinase (Schnebli et al., 1967), but **8a** was not tested in comparison. Since at least one other isomer of adenosine which differs in the position of the ribofuranosyl moiety (isoadenosine, 6-amino-3-(β-D-ribofuranosyl)purine) has been shown to act as a weak substrate for these two enzymes (Schnebli et al., 1967; Wolfenden et al., 1967), it seemed judicious to measure the activities of both **7a** and **8a** before using the results of the assays to corroborate the structural assignments.

of adenosine and **7a** could also form complexes with poly(U). Experiments were therefore carried out with the three available copolymers (Table III) at room temperature and at 4 °C, in the presence or absence of Mg^{2+}. The polymer containing adenosine and **7a** in a ratio of 2:1 was found to form a 1:1 complex with poly(uridylic acid) at 4 °C in a 0.05 M sodium cacodylate buffer solution (pH 7.0), containing 0.1 M sodium chloride (Figure 6). Neither of the other copolymers was observed to form a complex with poly(U).

Acknowledgments

We thank Dr. Robert Engle, National Cancer Institute, and Dr. Leroy Townsend, University of Utah, for permitting us to cite the testing results for compounds **4a, 5,** and **7a** as antitumor agents in experimental animals. We thank Professor Howard Green for the mouse fibroblast cell line used in this study and Professor Dietmar Seyferth for the use of his infrared spectrometer.

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