nitrobenzeneacetic acid, 104-03-0; 1*H*-indole-3-carboxaldehyde, 487-89-8; 4-[2-(1*H*-indol-3-yl)ethyl]benzenamine, 96616-22-7; 2,5-dibromodibenzofuran, 10016-52-1; dibenzofuran, 132-64-9; 2,8-dibenzofurandiamine, 25295-66-3; 4-(2-pyridinylmethyl)-benzenamine dihydrochloride, 96616-23-8; 2-[(4-nitrophenyl)-methyl]pyridine, 620-87-1.

Notes

2-Fluoroformycin and 2-Aminoformycin. Synthesis and Biological Activity

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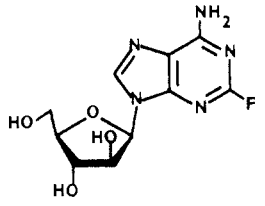
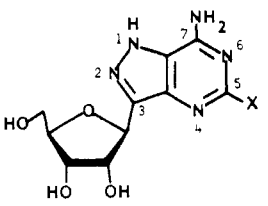
Syntheses of 2-fluoroformycin [7-amino-5-fluoro-3-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine] (**2b**) and 2-aminoformycin [5,7-diamino-3-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine] (**2c**) are described. Cytotoxicity data are given for **2b** and **2c** alone as well as with added pentostatin. Kinetic parameters for adenosine deaminase are also provided. 2-Fluoroformycin, although a much poorer substrate for adenosine deaminase than formycin A, is not nearly as cytotoxic to cells in culture.

The anticancer activity of 9-(β-D-arabinosyl)-2-fluoroadenine (2-F-araA, **1**)[1] has led us to synthesize various other ring fluorinated purine nucleosides. These have

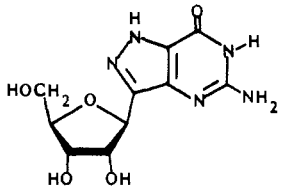


1



2

a) X = H
b) X = F
c) X = NH_2



3

included, recently, 2-fluoro-2′-deoxyadenosine,[2] 2-fluoro-8-azaadenosine,[3] and 8-amino-6-fluoro-9-(β-D-ribofuranosyl)purine.[4] A nucleoside antibiotic that has attracted considerable interest is formycin A (**2a**), a C-nucleoside closely resembling adenosine (N-9 and C-8 of adenosine are juxtaposed). Formycin A has a wide variety of biological effects, including some in vivo anticancer activity.[5-7] In terms of metabolism in mammalian cells, it is phosphorylated by adenosine kinase, carried all the way to the triphosphate level, and incorporated to some extent into nucleic acids. Formycin A is also deaminated rapidly by adenosine deaminase to formycin B, which is a modest inhibitor of purine nucleoside phosphorylase but has little toxicity to cells in culture.[8] It thus appears that any anticancer activity seen with formycin A probably can be attributed to its phosphorylation.

Insertion of a fluorine into the 2-position of a purine or purine analogue is known to greatly reduce the ability of the compound to serve as a substrate for adenosine deaminase.[9] In addition, the 2-fluoro substituent does not seriously impair phosphorylation by adenosine kinase, though bigger groups at C-2 largely prevent phosphorylation by this enzyme.[9] Consideration of the information presented above suggests 2-fluoroformycin [7-amino-5-fluoro-3-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine] as an attractive synthetic target. Herein we report the synthesis of 2-fluoroformycin as well as 2-aminoformycin, together with some biological data on these two compounds.

Functionalization at C-5 of formycin (corresponding to C-2 in the purine system) has been accomplished by ring opening of the pyrimidine after *N*-oxide formation at N-6 (purine N-1) followed by reclosure of an appropriately substituted precursor.[10] Our approach involved starting with the pyrazolo[4,3-*d*]pyrimidine nucleoside analogue (**3**) of guanosine[10] and converting it in several steps to the diamino compound **2c**, which was then converted to the desired fluoro compound **2b** by a method used successfully in other related systems.[4,11] Acetylation of crude **3** under standard conditions afforded a mixture of acetylated compounds that contained both a triacetate and a tetraacetate, as judged by mass spectral analysis. The crude

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Table I. Cytotoxicity (L1210 Cells)[a] Data

compd	IC_{50},[b] μM	compd	IC_{50},[b] μM
2b	12	**2c** + pentostatin	5
2b + pentostatin	22	formycin A	1.0
		formycin A + pentostatin	0.2
2c	31		

[a] Our procedure is based upon that of Thayer et al.[13] in which rapidly growing L1210 cells in suspension culture are exposed to a range of concentrations of inhibitor and the reduction in the rate of proliferation of treated cells relative to control cells is determined by means of cell counts 24 and 48 h after addition of the inhibitor. The concentration of inhibitor required to produce a 50% reduction in the rate of cell proliferation is designated IC_{50}. [b] 48 h.

mixture of acetates was treated directly with phosphorus oxychloride, tetraethylammonium chloride, and *N,N*-diethylaniline in acetonitrile at reflux[12] to produce a mixture of the corresponding 7-chloro compounds as a dark syrup. This syrup was immediately dissolved in ethanolic ammonia and heated in a pressure bomb to effect deacetylation and conversion to 5,7-diamino compound **2c** (2-aminoformycin). Diamino compound **2c** was dissolved in 3:2 HF–pyridine and treated with *tert*-butyl nitrite[4,11] to cause diazotization of the 5-amino, resulting in eventual incorporation of the fluorine at C-5 to produce **2b** (2-fluoroformycin). In other examples employing HF–pyridine,[4,11] the carbohydrate hydroxyl groups have required acylation before significant product could be obtained. With the C-nucleoside **2c** this step proved to be unnecessary. The desired product was separated from salts by the use of a Bio-Bead column. That the fluorine was at C-5, as expected, and not C-7 was proven by deamination of **2b** and **2c** with adenosine deaminase. Each compound was converted to a different deaminated material.

The cytotoxicities of 2-fluoroformycin, 2-aminoformycin, and formycin A against L1210 cells in culture (cell proliferation assay) are shown in Table I. The effects of added pentostatin, the potent adenosine deaminase inhibitor,[14] are also shown. Several statements can be made regarding this data. First, it is clear, as has been seen by others,[5] that the cytotoxicity of formycin A is enhanced by the addition of pentostatin. With 2-fluoroformycin, where the fluorine is positioned to reduce the ability of the compound to serve as a substrate for adenosine deaminase, the cytotoxicity is not improved but actually may be somewhat less. Both 2-fluoroformycin and 2-aminoformycin are at least 10-fold less cytotoxic than formycin itself. Incubation of 2-aminoformycin with pentostatin increases its cytotoxicity about 10-fold, suggesting that (1) 2-aminoformycin is readily deaminated by adenosine deaminase and (2) the deamination is not primarily responsible for the cytotoxicity of the compound.

Both 2-fluoroformycin and 2-aminoformycin have been examined as substrates for adenosine deaminase, and their K_m and V_{max} data, together with that of several other relevant compounds, are presented in Table II.

The reduced ability of 2-fluoroformycin to serve as a substrate relative to formycin A is clearly seen. Though 2-fluoroformycin is obviously a poor substrate, its V_{max}, 4% of that of adenosine, is high enough to allow some deamination to take place, though obviously not enough to affect the cytotoxicity. A comparison with adenosine and 2-fluoroadenosine shows that the related pyrazolo[4,3-*d*]pyrimidine nucleosides exhibit a similar trend. The data also show that 2-aminoformycin is an excellent substrate for adenosine deaminase, with a V_{max} about 3-fold higher than that of adenosine.

Table II. Adenosine Deaminase Kinetic Parameters[a]

compd	K_m, μM	V_{max}, mol mg^{-1} min^{-1}	V_{max}/K_m
Ado	29	435	15
2-F-Ado[b]	81	0.78	0.0096
Form A (2a)[b]	500	4000	8.0
2-F-Form (2b)	1000	17	0.017
2-NH_2Ado[b]	33	119	3.6
2-NH_2Form (2c)	290	1250	4.3

[a] Data for all compounds refer to calf intestine adenosine deaminase. See ref 15 for experimental details. [b] Bennett, L. L., Jr.; Allan, P. W., unpublished results.

The above data indicate that 2-fluoroformycin has the properties that were expected of it when we embarked upon its synthesis. A question to be answered, then, is why it is about 12 times less cytotoxic than formycin A to L1210 cells and 60 times less toxic than formycin A plus pentostatin. In order to answer that question, we treated L1210 cells in suspension culture with 2-fluoroformycin (20 μg/mL) and formycin A (10 μg/mL) and examined the various nucleotide metabolites by HPLC analysis.[16] With formycin A, new peaks are evident in the mono-, di-, and triphosphate regions, as has been previously noted. With 2-fluoroformycin, this analysis shows the formation of a new peak in the monophosphate region but no new peaks in the di- and triphosphate regions. 2-Fluoroformycin also caused a distinct decrease in the UTP-CTP pools without significant effect on the adenine and guanine nucleotide pools. It appears, then, that 2-fluoroformycin is converted, probably by adenosine kinase, to the monophosphate level but does not proceed to any significant extent to higher phosphate derivatives. This phenomenon is rather unusual in that the enzymes that convert monophosphates to di- and triphosphates have broad specificities. Though this experiment is not definitive, it is tempting to suggest that the decreased cytotoxicity of 2-fluoroformycin is caused by its failure to be significantly metabolized past the monophosphate level in L1210 cells.

Experimental Section

Melting points were determined on a Mel-Temp apparatus and are uncorrected. NMR spectra were recorded with a Varian XL-100-15 spectrometer operating at 25.16 MHz for ^{13}C (compound **2c**) and a Nicolet NMC 300NB spectrometer operating at 75.6 MHz for ^{13}C and 300.635 MHz for ^{1}H (all other spectra). Chemical shifts are expressed in parts per million downfield from tetramethylsilane. Ultraviolet absorption spectra were determined on a Cary 17 spectrophotometer by dissolving each compound in ethanol and diluting each compound 10-fold with 0.1 N HCl, pH 7 buffer, and 0.1 N NaOH. Microanalyses were performed by Atlantic Microlab, Inc., Atlanta, GA. HPLC analyses (except for the nucleotide analysis) were carried out with an ALC-242 liquid chromatograph (Waters Associates), using a μ-Bondapak C_{18} column with UV monitoring. Mass spectra were recorded on a Varian MAT 311A mass spectrometer in the fast atom bombardment (FAB) mode. Reagent grade acetonitrile and DMF were stored over 4A molecular sieves prior to use.

5,7-Diamino-3-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine (2c). Very crude 5-amino-(3-β-D-ribofuranosyl)-pyrazolo[4,3-*d*]pyrimidin-7-one (**3**), obtained directly from the ring closure as previously described (from 7 g, 25 mmol, of formycin N^8-oxide),[10] was dissolved in dry DMF (21 mL) and the

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(16) For the HPLC system, see, for example: Bennett, L. L., Jr.; Smithers, D.; Hill, D. L.; Rose, L. M.; Alexander, J. A. *Biochem. Pharmacol.* **1978**, *27*, 233.

resultant mixture treated with pyridine (7.7 mL) and acetic anhydride (15.4 mL). After stirring at room temperature for 24 h, the solution was evaporated to dryness under reduced pressure, the residue was dissolved in $CHCl_3$ (350 mL), washed with 5% aqueous $NaHCO_3$ and water, dried ($MgSO_4$), and evaporated to dryness. This residue weighed 10 g and contained, as judged by mass spectral analysis, 5-amino-3-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidin-7-one and 5-(acetylamino)-3-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidin-7-one, along with a variety of other impurities: MS (FAB) 410 $(M + 1)^+$, 452 $(M + 1)^+$. The mixture was carried on directly.

A solution of the mixture of acetates (5 g) in dry acetonitrile (20 mL) was treated with dried tetraethylammonium chloride (3.5 g, 21 mmol), *N*,*N*-dimethylaniline (1.3 mL, 10.3 mmol), and phosphorus oxychloride (5.6 mL, 60 mmol).[12] The dark solution was immediately placed in an oil bath preheated to 100 °C and held at reflux for 25 min, and then solvent was rapidly evaporated. Ice was added to the dark residue followed by cold $CHCl_3$ (150 mL). After separation of the organic layer, the aqueous layer was extracted with cold $CHCl_3$ (3 × 50 mL). The combined organic extracts were washed with ice water, cold 5% aqueous $NaHCO_3$, and ice water, dried ($MgSO_4$), and evaporated to dryness under reduced pressure: MS (FAB) 428 $(M + 1)^+$, 470 $(M + 1)^+$. The crude, dark residue, containing 5-amino-7-chloro-3-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine and 5-(acetylamino)-7-chloro-3-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-pyrazolo[4,3-*d*]pyrimidine, as judged by mass spectral analysis, along with many impurities, was used directly in the next step.

The dark mixture of the 7-chloro compounds obtained above was dissolved in ethanol saturated with ammonia at 0 °C (125 mL), sealed in a pressure bomb, and heated at 70 °C for 16 h. After cooling to room temperature, the pressure bomb contents were reduced in volume to remove ammonia, leaving a solid suspended in ethanol. This solid was removed by centrifugation, washed with ethanol, and then discarded. The dark red ethanol solution was evaporated to dryness, dissolved in warm methanol, and purified chromatographically on thick layer plates (Analtech GF, 2000-μm layer), eluting with CH_3CN–0.1 M NH_4HCO_3, 9:2 (v/v), to obtain 100 mg of reasonably pure material (HPLC purity 95% at 300 nm) that still contained considerable salt. This material was further chromatographed on silica gel (Analtech GF, 2000-μm layer), eluting with EtOH–H_2O, 4:1 (v/v), and on cellulose (Avicel F, 1000-μm layer), eluting with CH_3CN–1 N NH_4OH, 1:1 (v/v). Removal of the product from the cellulose plate required washing with 0.2 N NH_4OH. After evaporation of solvent, the residue was dissolved in EtOH, filtered, reduced to a small volume, and diluted with about 20 volumes of *n*-hexane to produce a white solid, which was collected by centrifugation and dried (0.1 mmHg) at room temperature to afford 60 mg of **2c**, which darkened at ca. 155 °C, with decomposition >220 °C: UV λ_{max}, nm ($\epsilon \times 10^{-3}$) [pH 1] 296 (6.5), 255 (9.4), 242 (9.4), 222 (17.8), (pH 7] 306 (5.8), 255 (sh), 220 (20.9), [pH 13] 314 (5.3), 265 (sh), 230 (sh), 220 (23.6); ^1H NMR (Me_2SO-d_6) δ 7.09 and 5.42 (2 s, 4, 2 NH_2), 4.9 and 3.4 (2 vbr s, OH, NH), 4.82 (d, 1, $J_{1',2'}$ = 7.5 Hz, H-1'), 4.43 (dd, 1, $J_{2',3'}$ = 5 Hz, H-2'), 4.05 (dd, 1, $J_{3',4'}$ = 2.5 Hz, H-3'), 3.89 (m, 1, H-4'), 3.64 and 3.48 (2 dd, 1, *J* = 2.5, 12 Hz, H-5',5''); ^{13}C NMR (Me_2SO-d_6) δ 62.55 (C-5'), 72.34, 74.72 (C-2',3'), 78.26, 85.74 (C-1',4'), 120.5 (C-7a), 139.49, 140.22 (C-3, C-3a), 152.18, 158.82 (C-5, C-7); exact mass *m*/*z* [FAB $(M + 1)^+$] calcd 283.115, found 283.116.

We were unable to obtain a proper elemental analysis on this compound even though it was homogeneous by TLC and >99.5% pure by HPLC analysis (Anal. Found: C, 41.61; H, 5.38; N, 26.16). The UV data presented above are based upon the actual molecular weight and again confirm the high degree of purity of our material. The sample apparently has a very small amount of material leached from the cellulose plate by the aqueous ammonium hydroxide.

7-Amino-5-fluoro-3-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]-pyrimidine (2b). Diamino compound **2c** (101 mg, 0.36 mmol) was dissolved in 3:2 HF–pyridine (3 mL) at 0 °C and then cooled to −20 °C and treated with *tert*-butyl nitrite (128 μL, 1.08 mmol).[11] After 1 h at −20 °C, HPLC analysis showed 15% starting material remaining. An additional equivalent of *tert*-butyl nitrite was added in two portions over the next hour, at which time starting material was essentially gone. The reaction mixture was added dropwise to cold aqueous 2.2 M $KHCO_3$ (40 mL) and lyophilized. The residue was dissolved in water (40 mL) and passed through a 1.5 × 30 cm column of Bio-Beads SM-4, 20–50 mesh, equilibrated with water, at a flow of ~1 mL/min. The column was eluted with water until all potassium ions were removed and a base line was obtained at 300 nm. The product was eluted with EtOH–H_2O, 1:9 (v/v). Fractions of >90% purity were combined and lyophilized. The residue was dissolved in methanol, applied to a thick-layer plate (Analtech GF, 1000-μm layer), and eluted with CH_3CN–0.1 M NH_4HCO_3, 9:2 (v/v). The residue from ethanol extraction of the product band was dissolved in water (4 mL) and applied to a water-equilibrated SM-4 Bio-Bead column (1 × 15 cm). After initial elution with water, the product was eluted with EtOH–H_2O, 1:4 (v/v). The residue from the combined, lyophilized column fractions was dissolved in a small volume of EtOH and diluted about 20-fold with *n*-hexane. The resulting white solid was collected by centrifugation, washed with *n*-hexane, and dried (0.1 mmHg) at room temperature to yield 19.7 mg (19%) of **2b**: mp 300 °C (dec); UV λ_{max}, nm ($\epsilon \times 10^{-3}$) [pH 1] 305 (sh), 295 (10.9), 284 (sh), 245 (sh), 227 (9.2), [pH 7] 305 (sh), 295 (10.5), 285 (sh), 245 (sh), 226 (8.4), [pH 13] 315 (sh), 300 (7.7), 265 (sh), 256 (5.8), 229 (17.9); ^1H NMR (Me_2SO-d_6) δ 8.2 (vbr s, NH), 5.02 (br s, 2, 2 OH), 4.93 (d, 1, *J* = 5 Hz, OH), 4.89 (d, 1, *J* = 6 Hz, H-1'), 4.44 (m, 1, H-2'), 4.04 (m, 1, H-3'), 3.61 (br dd, 1, *J* = 4 Hz, 12 Hz, H-5'), 3.48 (m, 1, H-5''); ^{13}C NMR (Me_2SO-d_6) δ 62.22 (C-5'), 71.70, 74.19 (C-2',3'), 77.1 (br), 85.31 (C-1',4'), 157.88 (C-5, *J* = 202.5 Hz), all other signals had relaxation times too long to allow sufficient data collection on our sample; MS (FAB) *m*/*z* 286 $(M + 1)^+$.

Anal. Calcd for $C_{10}H_{12}FN_5O_4 \cdot 0.75H_2O$: C, 40.20; H, 4.55; N, 23.44. Found: C, 40.38; H, 4.48; N, 23.44.

Unambiguous proof that the fluorine is at C-5 (and not C-7) can be found in the deamination of **2b** and **2c** with adenosine deaminase. On a μ-Bondapak C_{18} column at 254 nm, with a solvent mixture of A = 10 mM $NH_4H_2PO_4$, pH 5.1, and B = CH_3OH and with a linear gradient of 10% B → 50% B over 20 min, the retention times for **2b** and **2c** are 10.82 and 8.98 min, respectively. After treatment with adenosine deaminase, the product from **2b** had a retention time of 4.88 min, and the product from **2c** had a retention time of 6.45 min. The UV spectra of the two products were also distinct.

Acknowledgment. This investigation was supported by the National Cancer Institute, National Institutes of Health, DHHS, Grants R01 CA24975 and P01 CA34200. The authors are indebted to M. C. Thorpe and Dr. J. M. Riordan for the recording and interpretation of the NMR spectra, to S. Campbell and L. Rose for HPLC data, to Dr. L. L. Bennett, Jr., and P. W. Allan for carrying out the adenosine deaminase assays, and to Dr. R. W. Brockman and S. Shaddix for the metabolism study in L1210 cells.

Registry No. 2b, 97782-08-6; **2c**, 97782-03-1; **3**, 80206-18-4; 5-amino-3-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)pyrazolo[4,3-*d*]-pyrmidin-7-one, 97782-04-2; 5-(acetylamino)-3-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidin-7-one, 97782-05-3; 5-amino-7-chloro-3-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-pyrazolo[4,3-*d*]pyrimidine, 97782-06-4; 5-(acetylamino)-7-chloro-3(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine, 97782-07-5; adenosine deaminase, 9026-93-1.