Synthesis and Biochemical Properties of 8-Amino-6-fluoro-9-β-D-ribofuranosyl-9*H*-purine

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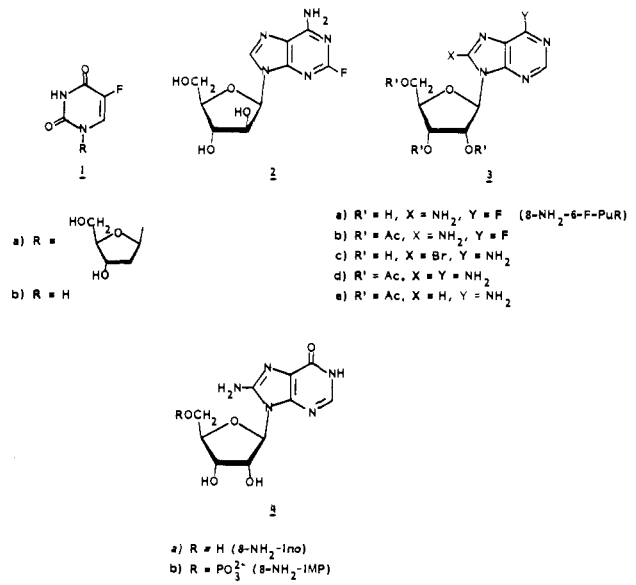
The synthesis and characterization of 8-amino-6-fluoro-9-β-D-ribofuranosyl-9*H*-purine (**3a**) are presented. This compound is a substrate for adenosine deaminase and adenosine kinase. In L1210 cells **3a** is converted to 8-aminoinosine monophosphate (**4b**), apparently by the action of AMP deaminase on the monophosphate of **3a**, as well as to the triphosphate derivative of **3a**. Pentostatin was used to inhibit adenosine deaminase, and coformycin was used to inhibit AMP deaminase in experiments designed to delineate the metabolic fate of **3a**. Pentostatin was without influence on the cytotoxicity of **3a**, but coformycin potentiated the cytotoxicity. The potentiation was associated with an increased cellular concentration of phosphates of **3a** and a decreased concentration of **4b**.

Nucleosides or nucleobases containing fluorine have proved to be interesting from a chemotherapeutic standpoint, with 2′-deoxy-5-fluorouridine (**1a**), 5-fluorouracil (**1b**), and 9-β-D-arabinofuranosyl-2-fluoroadenine (**2**) being the principal examples. The first two compounds, of course, have been important in cancer chemotherapy for a number of years, while the latter is currently in clinical trials, having demonstrated significant activity against several types of cancer.[1-3] In connection with our program involving the synthesis of nucleoside analogues with altered metabolism, we have prepared 8-amino-6-fluoro-9-β-D-ribofuranosyl-9*H*-purine (**3a**, 8-NH_2-6-F-PuR). Herein we report on its synthesis, cytotoxicity, and metabolism in L1210 cells.



Chemistry. Our synthesis of **3a** was carried out by a five-step procedure beginning with 8-bromoadenosine (**3c**). Known methods were used to convert **3c** to 2′,3′,5′-tri-*O*-acetyl-8-aminoadenosine (**3d**).[4,5] Nitrosation of **3d** with sodium nitrite or an organic nitrite in hydrogen fluoride–pyridine (60:40)[6] at low temperature resulted in the formation of 8-amino-6-fluoro-9-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-9*H*-purine (**3b**) accompanied by 2′,3′,5′-tri-*O*-acetyladenosine (**3e**) as the major nucleoside byproduct. Precedent for the process by which this byproduct is formed is found in a reduction recently reported by hydrogen abstraction from solvent.[7] After partial purification, **3b** was treated directly with ethanolic ammonia to afford **3a**. The removal of the acetyls proceeded in either ethanol or methanol, though ethanol was somewhat better. The main material loss, of course, was during the nitrosation step. The use of perfluorohexane as a heterogeneous cosolvent was of marginal value, but appeared to enhance yields slightly. Tetrahydrofuran as a cosolvent considerably increased the amount of adenosine produced, as would be expected.[7] Experimentally, it was important to extract with ethyl acetate rather than a chlorocarbon solvent to avoid the formation of 8-chloroadenosine.[8] Evidence for the desired structure was obtained by the standard methods, as reported in the Experimental Section. Of particular note was the proton-decoupled ^{13}C NMR spectrum of **3a**, which showed coupling between the fluorine and all of the purine carbons, with appropriate values for the coupling constants.[9] Additional proof of the structure of **3a** was also available. Enzymatic deamination of **3a** with adenosine deaminase produced 8-aminoinosine, which was compared to an authentic sample prepared by a different route.[23] Treatment of **3a** with 2 N HCl at room temperature also resulted in the formation of 8-aminoinosine.

That **3b** was the product of the nitrosation step rather than 2′,3′,5′-tri-*O*-acetyl-8-fluoroadenosine is rather surprising in view of the literature precedents. Treatment of 8-aminoadenine with sodium nitrite in 5% hydrochloric acid results in the formation of 6-amino-8-diazopurine hydrochloride, with no diazotization at the 6-position noted.[10] Proof of the structure was achieved by conversion to 8-chloroadenine. In addition, it is known that a fluorine in the 6-position is susceptible to displacement, for example with alcoholic ammonia.[11] The stability of **3a** to this reagent is therefore unexpected; presumably the 8-amino group aids in stabilizing the 6-fluorine. If the ethanolic ammonia deblocking reaction is allowed to proceed long enough, however, some 8-aminoadenosine begins to form.

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It appears that diazotization is occurring at both the 6- and 8-amino groups, probably mainly at the 8-position, but that at the nucleoside level either the diazonium salt or the 8-fluoro compound is not stable to the reaction conditions. The 6-fluoro compound **3b**, though presumably formed in smaller amount, does survive the reaction conditions. It has been reported that 2′,3′,5′-tri-*O*-acetyl-8-fluoroadenosine is unstable toward deacetylation with base.[12]

Biological and Biochemical Results[13]

Evaluation of the cytotoxicity of 8-NH_2-6-F-PuR was carried out with both H.Ep.-2 and L1210 cells in culture. For H.Ep.-2 cells, the IC_{50} (concentration reducing colony formation by 50%) was calculated as ~1.8 μM. Considerable variation in IC_{50} values, much greater than we have found in similar assays with other nucleoside analogues, was noted. The sources of the variation are not known, but possibly may result from the variations in the relative amounts of two species of nucleotide formed intracellularly from the nucleoside (see below). For L1210 cells the IC_{50} (concentration reducing colony formation in soft agar by 50%) was about the same as the IC_{50} for H.Ep.-2 cells; the average IC_{50} for five independent experiments was 1.9 μM.

For evaluation of 8-NH_2-6-F-PuR as a substrate for adenosine deaminase (Ado-DA) we used the commercially available enzyme from calf intestine. This enzyme, rather than a preparation from H.Ep.-2 or L1210 cells, was used because of its availability in a high state of purity together with the facts that (a) it is generally similar in properties and substrate specificities to Ado-DA's isolated from other mammalian sources[14-17] and (b) it is the enzyme that has been most widely used to determine structure–activity relationships among adenosine analogues. Ado-DA is known to convert 6-halopurine nucleosides to 6-oxypurine nucleosides.[14,18] 8-NH_2-6-F-PuR was an effective substrate for this enzyme; the apparent K_m was 1000 μM (as compared to a value of 29 μM for adenosine), and the V_{max}, relative to that of adenosine as 100, was 54. One other 6-fluoropurine nucleoside, 6-fluoropurine-2′-deoxyribofuranoside, has been evaluated as a substrate for Ado-DA; the K_m was 220 μM, and the V_{max} was the same as that of adenosine.[13]

Cytotoxicity to L1210 cells was also determined in the presence of inhibitors of Ado-DA and AMP deaminase (AMP-DA). For inhibition of Ado-DA we used pentostatin, and for inhibition of AMP-DA we used coformycin; inhibition of deaminases by these nucleosides is well-documented.[19] In earlier studies with L1210 cells we have used pentostatin at a concentration of 0.35 μM to inhibit Ado-DA[20] and coformycin at a concentration of 35 μM to inhibit AMP-DA.[21] The presence of pentostatin had no effect on the cytotoxicity of 8-NH_2-6-F-PuR, whereas in the presence of coformycin a marked potentiation was observed; the IC_{50} for L1210 cells was reduced from 1.9 μM to 0.25 μM.

Assay of 8-NH_2-6-F-PuR as a substrate for adenosine kinase was carried out with enzyme isolated from L1210 cells.[22] The apparent K_m was 7 μM and the V_{max}, relative to adenosine as 100, was 120. Thus, 8-NH_2-6-F-PuR is readily metabolized to the monophosphate level by this enzyme.

The conversion of 8-NH_2-F-PuR to soluble metabolites in L1210 cells was studied by HPLC. The results of the nucleotide analyses of control cells and cells treated with 8-NH_2-6-F-PuR are shown in parts A and C, respectively, of Figure 1. The nucleotide profiles of the treated cells showed a marked decrease in ATP and GTP and the appearance of one large peak at about 8 min and one small peak between UTP and ATP at 29.7 min. A scan of the 8-min peak showed a maximum at 255 nm, the same as that of a sample of 8-NH_2-Ino (**4a**)[23] determined in the same buffer. This peak, which has a retention time about the same as that of IMP, therefore appears to be 8-NH_2-IMP (**4b**). The peak appearing at 29.7 min was too small to be scanned, but it appears to be the triphosphate of 8-NH_2-6-F-PuR (as will be shown in the discussion of Figure 1D below). To obtain additional data on the identity of the metabolites, portions of the cell extracts used in the experiments of Figure 1A,C were treated for 4 h with alkaline phosphatase, after which the resulting nucleosides were subjected to chromatography on a reversed-phase column. In the 8-NH_2-6-F-PuR-treated cells (Figure 2C) there were two peaks that were not apparent in control cells (Figure 2B), one eluting at 7–8 min and one at 23 min. These are the retention times, respectively, of 8-NH_2-Ino and 8-NH_2-6-F-PuR, determined individually and in a mixture with the natural nucleosides (Figure 2A). The small amount of 8-NH_2-6-F-PuR (Figure 2C) isolated indicates that only a small amount of the triphosphate of this nucleoside was present in the initial cell extracts, in agreement with the results of Figure 1C. Thus, the major metabolite of 8-NH_2-6-F-PuR appears to be 8-NH_2-IMP, which conceivably could be formed by three routes: (a) the action of AMP-DA on the monophosphate of 8-NH_2-6-F-PuR; (b) the action of Ado-DA on 8-NH_2-6-F-PuR to form 8-NH_2-Ino, which might then be phosphorylated; or (c) the formation of 8-NH_2-Ino as in b above, followed by its conversion, by the action of purine nucleoside phosphorylase, to 8-aminohypoxanthine, which might then be converted to the nucleotide by a phosphoribosyltransferase. That routes b and c are not operative is indicated by the observations that (a) the presence of pentostatin, which, by inhibition of Ado-DA, would block the formation of 8-NH_2-Ino, was without effect on the conversion of 8-NH_2-6-F-PuR to 8-NH_2-IMP, and (b) cells grown in the presence of 8-NH_2-Ino contained no 8-NH_2-IMP (results not shown). To obtain evidence that AMP-DA was responsible for the formation of 8-NH_2-IMP, experiments were performed with coformycin. The presence of coformycin had a marked effect on the metabolism of 8-NH_2-6-F-PuR (Figure 1D). The peak eluting at 8 min,

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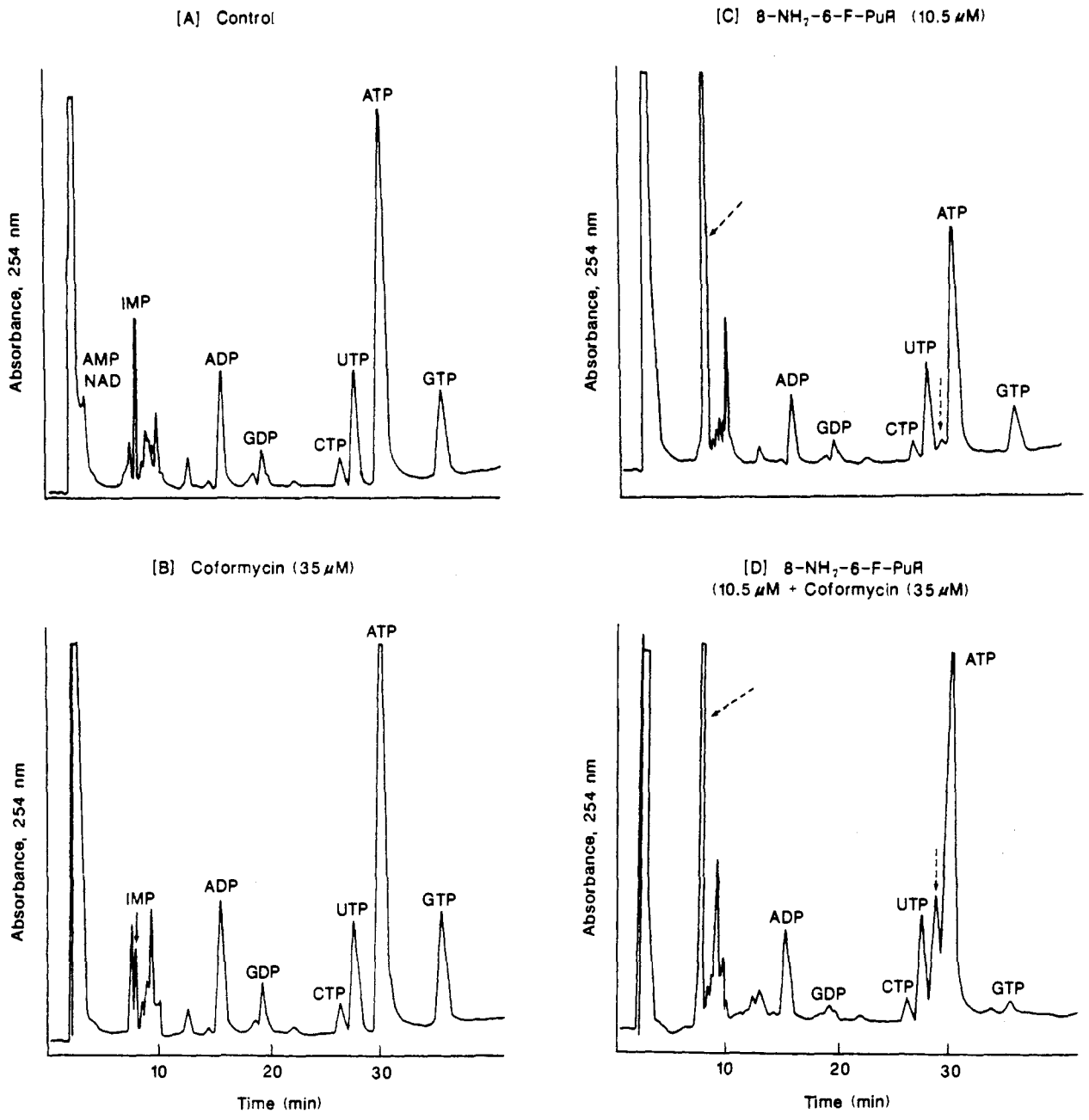

Figure 1. Metabolism of 8-NH_2-6-F-PuR by L1210 cells and the effect of coformycin. 8-NH_2-6-F-PuR, coformycin, or both were added to exponentially growing cultures ($\sim 5 \times 10^5$ cells/mL); cells from the same batch to which no additions were made served as controls. After 4 h the cells were harvested and washed free of medium. Perchloric extracts were prepared and assayed by HPLC on an anion exchange column (see text). New peaks appearing in the treated cells are marked by dotted arrows. The new peaks are identified (see text) as follows: the peak with retention time of 29.7 min (C and D) is the triphosphate of 8-NH_2-6-F-PuR; the peak with a retention time of about 8 min is 8-NH_2-IMP in C and the monophosphate of 8-NH_2-6-F-PuR in D. The breakthrough peak (retention time 0–2 min) consists of unidentified UV-absorbing material.

when scanned, had a λ_{max} at 265 nm, the same as that of 8-NH_2-6-F-PuR determined in the same buffer. The peak is not distinguishable by retention time from the 8-min peak appearing in Figure 1C, but the scans clearly indicate that in Figure 1C the 8-min peak is 8-NH_2-IMP and in Figure 1D the 8-min peak is the monophosphate of 8-NH_2-6-F-PuR. The 8-min peak in Figure 1D has a shoulder on the front side; this shoulder probably represents a small amount of 8-NH_2-IMP, which was not resolved in this experiment but was in others. IMP also has a retention time of about 8 min (Figure 1A,B); IMP is present in the cells of Figure 1C as a shoulder on the backside of the 8-min peak; the presence of coformycin reduced the IMP in Figure 1B and essentially eliminated it in Figure 1D. A second effect of coformycin on the metabolism of 8-NH_2-6-F-PuR is the large increase in the peak eluting at 29.7 min (Figure 1D). This peak had a λ_{max} at 268 nm, the same as that of a sample of 8-NH_2-6-F-PuR assayed in the same buffer. Reversed-phase chromatography of the phosphatase-treated cell extracts was confirmatory in showing the presence of 8-NH_2-Ino and 8-NH_2-6-F-PuR (Figure 2C,D), which were identified by retention times and by ultraviolet absorption spectra. Phosphatase-treated extracts from cells treated with 8-NH_2-6-F-PuR only had a large amount of 8-NH_2-Ino and little 8-NH_2-6-F-PuR (Figure 2C), whereas extracts from cells treated with the combination of 8-NH_2-6-F-PuR and coformycin contained little 8-NH_2-Ino and a large amount of 8-NH_2-6-F-PuR (Figure 2D). Thus, coformycin had the predicted effects: by blocking the action of AMP-DA on

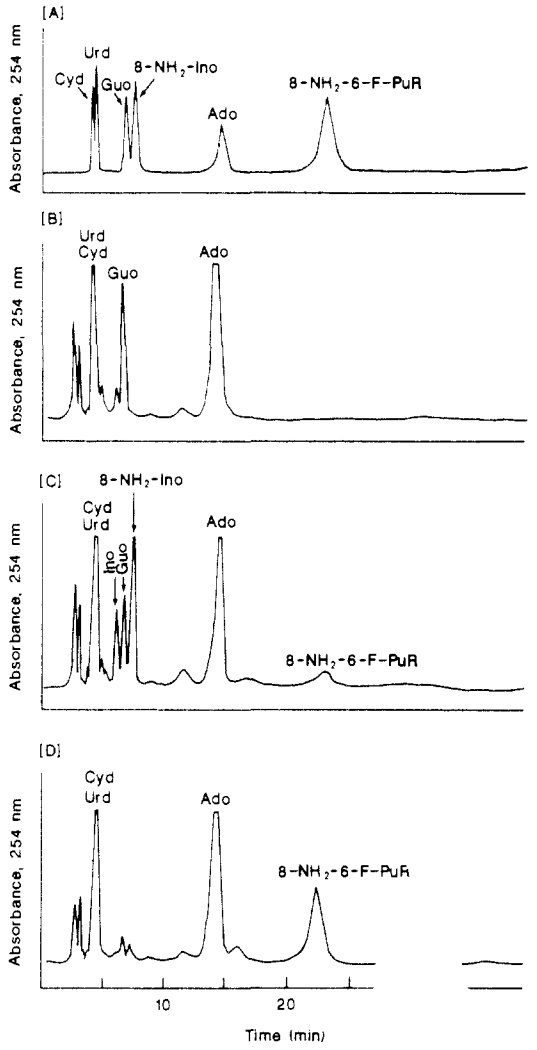

Figure 2. Reversed-phase HPLC of phosph ted extracts from control L1210 cells and from cells tre 8-NH_2-6-F-PuR or a combination of 8-NH_2-6-F-PuR mycin. The cell extracts used were the same as those use experiments of Figure 1. Portions of the lyophilized ext ere taken up in H_2O and treated with alkaline phosphata or to chromatography on a reversed-phase column (see tex (A) Standards: natural nucleosides, 8-NH_2-6-F-PuR, and 8-NH_2-Ino; (B) extract of untreated control cells; (C) extract of cells treated with 8-NH_2-6-F-PuR (10.5 μM); (D) extract of cells treated with both 8-NH_2-6-F-PuR (10.5 μM) and coformycin (35 μM). The peaks with retention times of 0–3 min in B, C, and D consist of unidentified UV-absorbing material.

8-NH_2-6-F-PuR-monophosphate, it essentially eliminated the formation of 8-NH_2-IMP and thereby made possible the increased formation of the phosphates of 8-NH_2-6-F-PuR. As a practical point, it should be noted that the reactivity of the fluorine atom in 8-NH_2-6-F-PuR necessitated modification of some standard procedures. For example, we found that the fluorine atom was easily lost if the alkaline phosphatase reaction was stopped by immersion of the incubation tubes in a boiling-water bath for 2 min. The commercial phosphatase preparation contains $(NH_4)_2SO_4$, and denaturation of the phosphatase with heat generated considerable quantities of both 8-NH_2-Ino and 8-aminoadenosine. To avoid the artifactual formation of these nucleosides, the enzyme was denatured by the addition of cold 0.5 N $HClO_4$ to the ice-cold incubation mixture.

Figure 3 shows the probable pathway for the formation of the observed metabolites of 8-NH_2-6-F-PuR. This picture may not be complete, because we have not yet investigated the possibility of the conversion of 8-NH_2-6-F-PuR to deoxynucleotides or of its incorporation into polynucleotides. An unusual feature of the metabolism of this compound is the accumulation of a considerable amount of 8-NH_2-IMP. To our knowledge, accumulation of IMP analogues to the extent found in these experiments has not been observed with other nucleoside analogues, probably because other IMP analogues such as, for example, carbocyclic IMP,[21] 8-aza-IMP,[24] or 2-aza-IMP,[20] which are formed intracellularly from the corresponding analogues of hypoxanthine or adenosine, are further metabolized to the corresponding analogues of AMP or GMP. The accumulation of 8-NH_2-IMP, and the absence of phosphates of 8-aminoguanosine and 8-aminoadenosine, would indicate that 8-NH_2-IMP is not an effective substrate for the enzymes that convert IMP to AMP and GMP. With respect to mechanisms of action, the fact that coformycin, which decreases the formation of 8-NH_2-IMP, potentiated the cytotoxicity of 8-NH_2-6-F-PuR, would indicate that the phosphates of 8-NH_2-6-F-PuR are more toxic than 8-NH_2-IMP. Therefore, the mode of action of 8-NH_2-6-F-PuR would be expected to be different in cells in which AMP-DA is not inhibited and in those in which it is, because the principal metabolite is 8-NH_2-IMP in the one instance and 8-NH_2-6-F-PuR triphosphate in the other. Furthermore, the presence of two species of nucleotide metabolites, which vary in relative amounts from one experiment to another depending on the activity of AMP-DA, may explain, or be a factor in, the observed variations in cytotoxicity of 8-NH_2-6-F-PuR that has been commented upon above. We have, in fact, obtained evidence that the activity of AMP-DA may vary considerably and depends on the metabolic state of the cells.[25]

Experimental Section

Melting points were determined on a Mel-Temp apparatus and are uncorrected. NMR spectra were recorded on a Varian XL-100-15 spectrometer operating at 100.1 MHz for ^1H NMR and 25.2 MHz for ^{13}C NMR and a Nicolet NMC 300NB spectrometer operating at 300.6 MHz. Chemical shifts are expressed in parts per million downfield from tetramethylsilane. The ^{19}F NMR spectrum was recorded on a Bruker CXP-200 operated at 188.2 MHz. Ultraviolet absorption spectra were determined on a Cary 17 spectrophotometer by dissolving each compound in ethanol and diluting 10-fold with 0.1 N HCl, pH 7 buffer, and 0.1 N NaOH. Values are in nanometers, and the numbers in parentheses are extinction coefficients ($\epsilon \times 10^{-3}$). The microanalysis was performed by Atlantic Microlab, Inc., Atlanta, GA. Thin-layer and preparative-layer chromatography were carried out on Brinkman precoated silica gel plates (0.25 mm or 2.0 mm thickness). HPLC for compound characterization and purity checks was carried out with an ALC-242 liquid chromatograph (Waters Associates), using a μBondapak C_{18} column and monitoring with a UV detector at 254 nm. Mass spectra were recorded on a Varian MAT 311A mass spectrometer in the electron-impact (EI) or fast atom bombardment (FAB) mode.

Cytotoxicity. Stock cultures of H.Ep.-2 cells were maintained in SRI-14 medium[26] and stock cultures of murine leukemia L1210 cells in Fischer's medium.[27]

For determination of cytotoxicity to H.Ep.-2 cells, 100 cells were incubated in 8-oz plastic prescription bottles in 10 mL of medium to which 8-NH_2-6-F-PuR had been added at various concentrations; bottles to which no additions were made served as controls. After a 10-day incubation the colonies that were formed were

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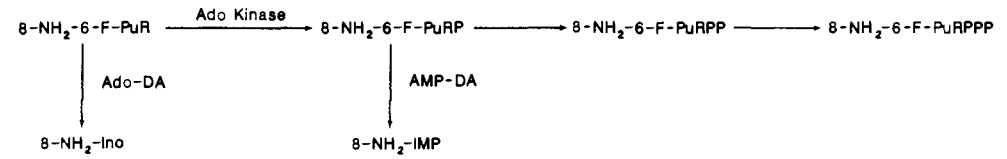

Figure 3. Pathways of metabolism of 8-NH_2-6-F-PuR in L1210 cells: Ado-DA, adenosine deaminase; AMP-DA, AMP deaminase.

stained and were counted visually. These procedures are described in detail elsewhere.[28] Each concentration of nucleoside was assayed in triplicate in each experiment, and eight individual experiments were performed. Toxicity to L1210 cells was determined similarly by colony counts except that the colony formation was accomplished in soft agar.

Substrate Activity for Adenosine Deaminase (Ado-DA). Calf intestinal adenosine deaminase (Sigma Chemical Co., St. Louis, MO) was used for the assay. Preliminary to the assay, 8-NH_2-6-F-PuR was incubated with a large excess of the enzyme and the reaction was followed spectrophotometrically (270 nm) until no further change in absorbance occurred. During the course of the reaction, the UV absorbance maximum shifted from 270 nm to 263 nm and the molar absorbance increased from 11 700 to 13 600 M^{-1} cm^{-1}. The spectrum of the product was the same as that of an authentic sample of 8-NH_2-Ino.[23] From these data, 295 nm (ΔA = 4400) was chosen as the wavelength at which the reaction could best be followed. The assay of 8-NH_2-6-F-PuR as substrate was performed in 0.05 M phosphate buffer, pH 7.5. Kinetic constants were determined from double reciprocal plots of data from experiments in which 10 concentrations of substrate were used (not shown).

Substrate Activity for Adenosine Kinase. Adenosine kinase purified from L1210 cells[22] was used for the assay. The assay was performed by incubating the substrate and enzyme in 50 mM Tris buffer containing Mg^{2+} and ATP-γ-^{32}P, after which the ^{32}P-labeled monophosphate formed was isolated by paper chromatography and assayed in a liquid scintillation spectrometer.[22] Kinetic constants were determined by double reciprocal plots (not shown).

Metabolism of 8-NH_2-6-F-PuR. The conversion of 8-NH_2-6-F-PuR to soluble metabolites in L1210 cells was studied by HPLC; the methods have been described in detail elsewhere in studies with another nucleoside analogue.[29] To cultures of L1210 cells that were in logarithmic growth (~5 × 10^5 cells/mL), 8-NH_2-6-F-PuR was added at various concentrations and cells were harvested 4 h thereafter. The cells were washed free of medium and extracted with cold 0.5 N $HClO_4$. The extract was neutralized with $KHCO_3$; the $KClO_4$ was removed by centrifugation, and the supernatant was decanted and lyophilized to dryness. The residue was taken up in H_2O and portions, corresponding to 2.5 × 10^7 cells, were subjected to HPLC (Series 4 Liquid Chromatograph, Perkin-Elmer, Norwalk, CT) on a Partisil-10 SAX anion exchange column (Whatman, Inc., Clifton, NJ). Elution was achieved with a 40-min linear gradient from 5 mM $NH_4H_2PO_4$, pH 2.8, to 750 mM $NH_4H_2PO_4$, pH 3.7, at a flow rate of 2 mL/min. Nucleosides, resulting from the action of alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) on the cell extract, were analyzed on a Spherisorb ODS 5-μm column (Phase-Sep., Norwalk, CT); the eluant was 25 mM $NH_4H_2PO_4$ (pH 5.5)–acetonitrile (95:5, v/v), and the flow rate was 1 mL/min. The ultraviolet absorption spectra of selected peaks were determined by means of a Model 2140 rapid spectral detector (LKB Instruments, Gaithersburg, MD).

2′,3′,5′-Tri-*O*-acetyl-8-aminoadenosine (3d). This compound was prepared by the literature procedures[4,5] starting from 8-bromoadenosine and proceeding through 8-azidoadenosine and 2′,3′,5′-tri-*O*-acetyl-8-azidoadenosine to **3d** without purification. Previously **3d** has only been obtained as a syrup or foam, but we were able to obtain it as an amorphous solid in one large batch (ca. 50 g) by taking up the syrup in ethyl acetate and scratching, resulting in precipitation of an off-white solid. Addition of ether to the filtrate after removal of the first solid gave even more material. Either this solid or a free flowing foam obtained from the syrup, though neither is completely homogeneous, is suitable for proceeding on to the next steps. Treatment of pure **3d** (obtained chromatographically) as described above will afford the same amorphous solid, which shrinks with slight discoloration from 89 to 104 °C, with some gas evolution at 104 °C.

8-Amino-6-fluoro-9-β-D-ribofuranosyl-9*H*-purine (3a). A solution of **3d** (4.88 g, 12.19 mmol) in hydrogen fluoride–pyridine (60:40, 15 mL)[6] was prepared by chilling the HF–pyridine to −25 °C to −30 °C in a polypropylene vessel equipped with a drying tube and adding **3d** to it with magnetic stirring. It was desirable to obtain complete solution as rapidly as possible (≤20 min).[30] To this solution was added 5 mL of perfluorohexane followed by solid $NaNO_2$ (1.26 g, 18.29 mmol), which was added in portions over 10–15 min. Both $NaNO_2$ and *tert*-butyl nitrite were acceptable, but $NaNO_2$ appeared to afford slightly better yields in most runs. The mixture was stirred vigorously for 1.5 h at −25 °C to −30 °C and for 10–15 min at 0 °C and was then poured into 150 mL of ice water. The aqueous solution was immediately extracted with ethyl acetate (8 × 50 mL). If perfluorohexane has been used, it will form a third, lower layer that can be removed. The organic extracts were combined and washed with saturated $NaHCO_3$ solution (2 × 100 mL) and water (100 mL) and dried ($MgSO_4$). Evaporation to dryness left 3.2 g of a red syrup. This material, which contained **3b** and **3e** as the two major components plus assorted minor products (TLC, 95:5 $CHCl_3$–CH_3OH), was partially purified by chromatography. This purification can be run with thick-layer plates, gravity chromatography, or flash chromatography, depending upon the amount of crude material isolated in the run. In all cases the elution solvent is 95:5 $CHCl_3$–CH_3OH. In the run above, column chromatography was used, and fractions containing **3b** were combined and concentrated, giving a total of 1.68 g of a red-orange syrup. At this point pure **3b** can be obtained by one additional chromatography after decolorization, but the partially purified material was routinely used to carry onto the final product: MS (FAB, M + 1), calcd *m/z* 412.127, found *m/z* 412.126; UV (qualitative, EtOH) λ_{max} pH 1, 262 nm; pH 7, 267; pH 13, 271;[31] ^1H NMR (300 MHz, $CDCl_3$) δ 2.04, 2.15, 2.18 (3 s, 9, 3 $COCH_3$), 4.36 and 4.63 (2 dd, 2, H-5′,5″, $J_{4',5'}$ = 3.5 Hz, $J_{4',5''}$ = 2.5 Hz, $J_{5',5''}$ = 12 Hz), 4.46 (m, 1 H-4′, $J_{3',4'}$ = 4 Hz), 5.56 (dd, 1, H-3′, $J_{2',3'}$ = 6 Hz), 5.88 (apparent t, 1, H-2′), 6.32 (d, 1, H-1′, $J_{1',2'}$ = 6.5 Hz), 6.74 (s, 2, NH_2), 8.37 (s, 1, H-2).

The partially purified **3b** was taken up in 25 mL of ethanolic ammonia (saturated at 0 °C), stoppered, and allowed to stand at room temperature overnight. After evaporation of the solvent, the residue was purified by thick-layer chromatography (elution 2 times with 85:15 $CHCl_3$–CH_3OH) to afford 474 mg of reasonably pure **3a**. Again larger batches can be purified by flash chromatography. Although a yield calculation was difficult because the starting **3d** was not pure, the yield at this point was generally 10–15%. Final purification was accomplished by crystallization (and recrystallization, if necessary) from methanol, yielding a white solid that can come out in several forms, including an amorphous solid and needles. No decomposition or fluorine replacement was seen by boiling in methanol. Typically, the melting behavior (regardless of form) was slow, decomposition beginning at ca. 200 °C, with the sample becoming dark brown by ca. 225 °C. One

(28) Bennett, L. L., Jr.; Schnebli, H. P.; Vail, M. H.; Allan, P. W.; Montgomery, J. A. *Mol. Pharmacol.* **1966**, *2*, 432.
(29) Bennett, L. L., Jr.; Smithers, D.; Hill, D. L.; Rose, L. M.; Alexander, J. A. *Biochem. Pharmacol.* **1978**, *27*, 233.
(30) This observation appears true for the conditions that are described. An obvious experiment would be to dissolve the material initially in pyridine, cool the solution, and dilute with cold 70:30 hydrogen fluoride–pyridine to get the proper volume and concentration. This procedure, unfortunately, did not improve yields. In fact, it gave a lower than average yield, although one that was in the normal range obtained.
(31) These values differ significantly from those reported in ref 12 for 2′,3′,5′-tri-*O*-acetyl-8-fluoroadenosine.

sample, however, had mp 231-233 °C dec. HPLC (elution solvent 9:1 H_2O-acetonitrile): the retention time of **3a** varied between 7.5 and 9 min, with adenosine varying between 5.5 and 7 min. For a given run, the ratio of retention times was close to 1.3 (8-NH_2-6-F-PuR/Ado). The stability of **3a** in buffered solution at pH 4, 7, and 10 was monitored by HPLC. At pH 7 and 10, **3a** was stable indefinitely at room temperature, while at pH 4 **3a** was slowly converted to 8-aminoinosine: ^{1}H NMR (100 MHz, Me_2SO-d_6) δ 3.66 (m, 2, 2 H-5'), 4.01 (m, 1, H-4'), 4.16 (m, 1, H-3'), 5.20, 5.37, 5.63 (3 br s, 3, 2',3',5'-OH), 6.03 (d, 1, $J_{1',2'}$ = 7 Hz, H-1'), 7.48 (br s, 2, NH_2), 4.67 (m, 1, H-2'), 8.25 (s, 1, H-2). Addition of D_2O caused the disappearance of all exchangeable protons and also allowed assignment of $J_{2',3'}$ = 5 Hz, and $J_{3',4'}$ = 2 Hz; ^{13}C NMR (100 MHz, Me_2SO-d_6) δ 61.21 (C-5'), 70.44, 70.67 (C-2',3'), 85.80, 86.90 (C-1',4'), 118.93 (C-5), 146.23 (C-4), 154.44 (C-6), 155.31 (C-8), 156.84 (C-2), $^1J_{C_6F}$ = 247.8 Hz, $^3J_{C_4F}$ = 13.4 Hz, $^2J_{C_5F}$ = 28.7 Hz, $^3J_{C_2F}$ = 13.3 Hz, $^4J_{C_8F}$ = 1.3 Hz; ^{19}F NMR (Me_2SO-d_6) δ 84.0 relative to $CFCl_3$ (δ 0), using $C_6H_5CF_3$ (δ -63.7) as external standard; MS (EI), m/z 285 (M^+), 254 ($M - CH_2OH)^+$, 182 ($B + 30)^+$, 153 ($B + H)^+$; UV (H_2O) λ_{max} pH 1, 263.5 nm (13.3) and 237 (5.0); pH 7, 269 (12.4), 253 (sh); pH 13, 271 (12.1), 253 (sh). Anal. Calcd for $C_{10}H_{12}FN_5O_4$: C, 42.11; H, 4.24; N, 24.55. Found: C, 42.15; H, 4.45; N, 24.52.

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N-(Cyanomethyl)- and *N*-(2-Methoxy-1-cyanoethyl)anthracyclines and Related Carboxyl Derivatives

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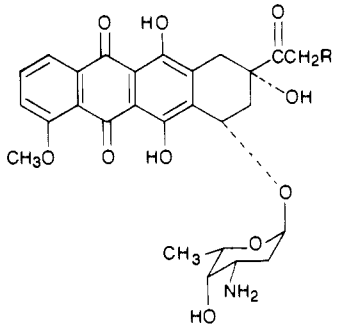
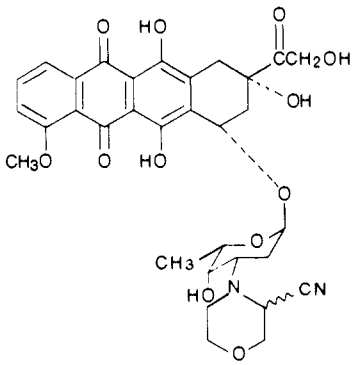
Treatment of doxorubicin with formaldehyde and NaCN afforded the *N*-(cyanomethyl) derivative as a stable α-cyano amine with but moderate antitumor activity in mice, although it was prototypal to the intensely potent α-cyanomorpholine derivative. 2-Methoxyacetaldehyde and NaCN afforded the *N*-(2-methoxy-1-cyanoethyl) derivative as an open-chain analogue of the cyanomorpholine. This analogue underwent rapid hydrolysis to doxorubicin and appeared to act as a prodrug, giving increased antitumor efficacy although with decreased potency. *N*-(Carboxymethyl)daunorubicin was a highly water-soluble but inactive analogue, synthesized by N-alkylation with ethyl iodoacetate and saponification. The similar N-alkylation of *N*-(cyanomethyl)daunorubicin demonstrated the combining of *N*-alkyl chains having different functional substituents.

3'-Deamino-3'-(3-cyano-4-morpholinyl)doxorubicin (**3**) was recently described[1-3] as the most potent of the antitumor anthracyclines, with 100- to 1000-fold decreases in dose requirement relative to the parent (doxorubicin, **2**). Several findings suggest that the added α-cyanomorpholine function in **3** is the critical feature for not only increases in potency but for apparent changes in mechanism of action. These factors include the high structural specificity for potency that is being seen in comparisons of **3** with related analogues;[4] absence in **3** of the cross resistance with **2** seen in previous analogues;[2,4] and developing evidence that **3** unlike other anthracyclines is irreversibly bound to intracellular constituents.[5-7] The α-cyano amine is a function that occurs in a few other antitumor agents such as saframycin A and cyanonaphthyridinomycin, which are postulated to bind to DNA.[8-10] An α-cyano substituent renders an amino N nonbasic. As a nonbasic analogue with intense potency, **3** is unlike other types of nonbasic anthracyclines (*N*-acyl and deamino derivatives, some with interesting antitumor efficacy in mouse screens)[11-13] that show decreased potency. Attention is thus focused on the α-CN amine of **3** as a site of interest.

We now describe the synthesis and evaluation of the *N*-cyanomethyl derivatives (**4**, **5**) as the simplest α-cyano amines from daunorubicin (**1**) and doxorubicin (**2**). The *N*-(2-methoxy-1-cyanoethyl) derivatives (**6**, **7**) are open-chain cyanomorpholine analogues derived by deletion of one ring carbon (C5''). Several *N*-(carboxyalkyl)-



1, R = H
2, R = OH



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