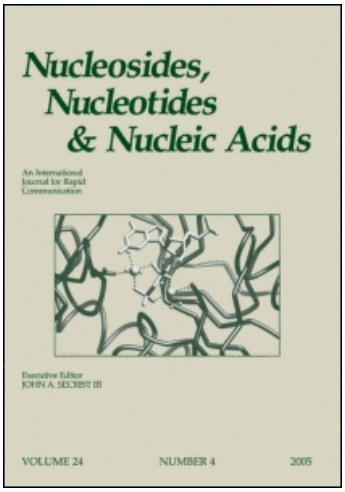

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF 6-HYDROXYGUANIDINO- AND 6-HYDROXYUREIDOPURINE AND THEIR RIBONUCLEOSIDES

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ABSTRACT

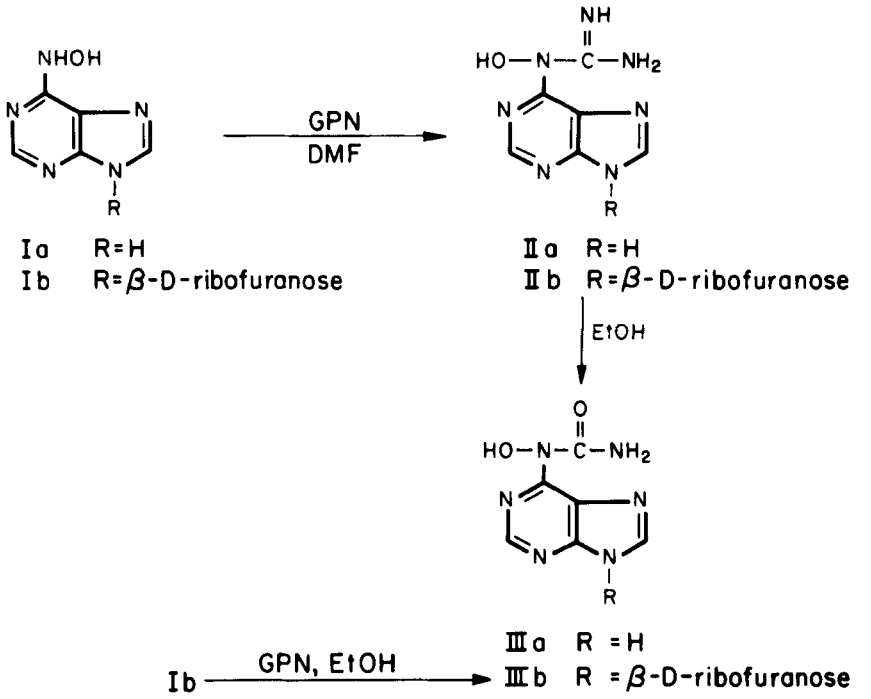
N^6-(1-hydroxyguanidino)purine IIa, and its 9-β-D-ribonucleoside derivative IIb were prepared by reacting at room temperature 6-hydroxyadenine Ia and 6-hydroxyadenosine Ib, with 1-guanyl-3,5-dimethylpyrazole nitrate in DMF. Refluxing IIa and IIb in 95% ethanol gave N^6-(1-hydroxyureido)purine and its ribonucleoside derivative respectively; the latter compound was also obtained by refluxing Ib with 1-guanyl-3,5-dimethylpyrazole nitrate in ethanol. The two base analogs were inactive against L1210 cells *in vitro*, but the nucleoside derivatives inhibited the growth of these cells by 50% at 5×10^{-6} and 6×10^{-7} M respectively. Compound IIb, at 200 mg/kg/day x 5, increased the life span of L1210-bearing DBA/2N mice by 57%. Cytofluorometric determinations showed that

IIb inhibited cell growth in the G_2 phase of the cell cycle. The compound was also found to inhibit adenosine deaminase activity with a K_i = 3.47 μM.

INTRODUCTION

Substitution of the exocyclic amino group of adenine or adenosine with diverse functional groups has provided compounds that are active against various experimental tumors[1,2,3]. Among these, analogs with unsaturated side chains such as N^6(Δ^2-isopentenyl)adenosine and N^6-(p-chlorophenylureido)purine have demonstrated a significant degree of antitumor selectivity. We have, therefore, prepared other such derivatives, including 6-(1-hydroxyguanidino)- and 6-(1-hydroxyureido)purine and their ribonucleosides. In this communication we report the procedures employed for the synthesis of these compounds and present some of the biological and biochemical effects they displayed. A preliminary account of these data has been given[4].

Chemistry: The synthesis of 6-(1-hydroxyguanidino)purine (IIa) and its ribonucleoside (IIb) was carried out using a reagent, 1-guanyl-3,5-dimethylpyrazole nitrate, that has, in the past, been used extensively in protein chemistry. Reaction of alkyl- or arylamines with 1-guanyl-3,5-dimethylpyrazole nitrate has been shown to lead to the formation of alkyl- or arylguanidines[5,6]. By analogy with this approach, 6-hydroxyadenine Ia[7] and 6-hydroxyadenosine Ib[8] were reacted, at room temperature with 1-guanyl-3,5-dimethylpyrazole nitrate in DMF, to give 6-(1-hydroxyguanidino)purine (IIa) and 9-β-D-ribofuranosyl-6-(1-hydroxyguanidino)purine (IIb), respectively, as their nitrate salts. Both of these compounds were obtained in about 40% yield. On refluxing in EtOH, compound IIb underwent conversion to 9-β-D-ribofuranosyl-(1-hydroxyureido)purine (IIIb), suggesting that the hydroxyguanidino compound IIb is an intermediate in the formation of the hydroxyureido derivative IIIb. On reacting 6-hydroxyadenosine with 1-guanyl-3,5-dimethylpyrazole nitrate in refluxing ethanol, IIIb was obtained as the nitrate salt. Treatment of IIb with 2 N HCl at room temperature



GPN = 1 Guanyl-3,5-dimethylpyrazole nitrate

Scheme I

for sixteen hours gave the free base IIa. Exposure of the guanidino nucleoside to 0.1 N NaOH at room temperature for 2 hours gave rise to a deeply colored solution. Catalytic hydrogenation of IIa gave adenine, whereas hydrogenation of IIb and IIIb resulted in adenosine rather than the desired 6-guanidino or 6-ureidopurine derivatives. Structure assignment of these compounds was based on their UV and pmr spectra and on elemental analysis.

Biological and Biochemical Activity. The effect of the newly synthesized compounds on the growth of various tumor cell lines *in vitro* is shown in Table *I*. Whereas the base analog IIa was found to be inactive against L1210 cells, the ribonucleoside derivatives IIb and IIIb inhibited the growth of these cells by 50% at 5×10^{-6} M and 6×10^{-7} M, respectively. By comparison, N^6-hydroxyadenosine was inhibitory to these cells at 2×10^{-6} M.

Table I
Effect of 6-(1-Hydroxyguanidino)- and 6-(1-Hydroxyureido)purine derivatives on Leukemia L-1210 Cell Growth *In Vitro*

Compound	Molar concentration for 50% inhibition of growth*
6-(1-hydroxyguanidino)purine IIa	$>10^{-4}$
6-(1-hydroxyguanidino)purine riboside IIb	5×10^{-6}
6-(1-hydroxyureido)purine IIIa	$>10^{-4}$
6-(1-hydroxyureido)purine riboside IIIb	6×10^{-7}

*The data express the mean of at least three separate determinations performed with duplicate assay samples.

Table II
Effect of 6-(1-Hydroxyguanidino)purine Ribonucleoside on the Survival of Mice Bearing Leukemia L1210*

Dose (mg/kg/day x 5)	Range (days)	Survival Parameters		
		Median	Mean	% ILS
0	6-8	7	7.0	0
50	8-9	8	8.4	14
100	9-11	10	10.0	42
200	8-13	11	10.8	57
300	6-13	13	9.5	42
400	3-3	3	3	-

*The data shown are derived from three separate determinations.

Because initial *in vivo* evaluation showed the hydroxyguanidino nucleoside IIb to be more antitumor selective than the hydroxyureido nucleoside IIIb, the former was examined in greater detail. At 200 mg/kg/day x 5 i.p., this compound increased the life-span (% ILS) of DBA/2N mice bearing leukemia L1210 by 57% (Table II). Doses above and below this concentration were less effective. In contrast, this compound was only marginally effective against B_{16} melanoma, colon 26 and Lewis lung carcinoma in mice, providing increases in life span of 24, 17 and 10%, respectively, although the tumor size decreased measurably in all treated animals.

DBA/2N female mice (19-20 g) were inoculated i.p. with 10^6 L1210 leukemia cells on day 0. Drug was administered i.p., once daily in 0.1 ml sterile water on day 1 through 5. Survival was monitored daily, and % ILS was calculated based on median survival time. All ILS values in the drug treated group were found to be significantly different from control with $p<0.01$, using Cox-Mantel analyses.

To establish the possible site of action of IIb, an inhibition analysis was carried out by adding adenosine, guanosine or inosine at graded concentrations to the L1210 culture medium containing the analog. Only adenosine, at 1×10^{-6} M, prevented growth inhibition to a limited extent (less than 2-fold). The other purines were without effect (data not shown).

To determine the effect of IIb on utilization of the precursors for RNA, DNA and protein synthesis, the incorporation of radiolabeled uridine, thymidine and leucine was examined. In L1210 cells exposed for 6 hours to 5×10^{-5} M IIb (Fig. 1A) or to 5×10^{-4} M IIb (Fig. 1B), the incorporation of thymidine, uridine and leucine was inhibited, the degree of inhibition being a function of drug concentration and length of exposure. Thymidine incorporation began to recover at a time (6 h) when uridine and leucine incorporation were still in decline.

An evaluation of the effect of IIb on cell cycle progression showed (Fig. 2) that the compound caused the accumulation of the cells in G_2. This pattern of response differs from that of the classical inhibitors of DNA synthesis and supports the notion that RNA or protein are the primary targets of the drug.

Because this compound can be viewed as a structural derivative of adenosine, its ability to serve as a substrate for adenosine deaminase from calf intestine was also assessed. The nucleoside IIb was not a substrate but it inhibited this enzyme competitively with adenosine, the Ki being 3.5 μM.

EXPERIMENTAL

Chemistry

Melting points were determined on a laboratory model Mel-Temp apparatus and are uncorrected. UV spectra were recorded on a Cary 219 spectrophotometer.

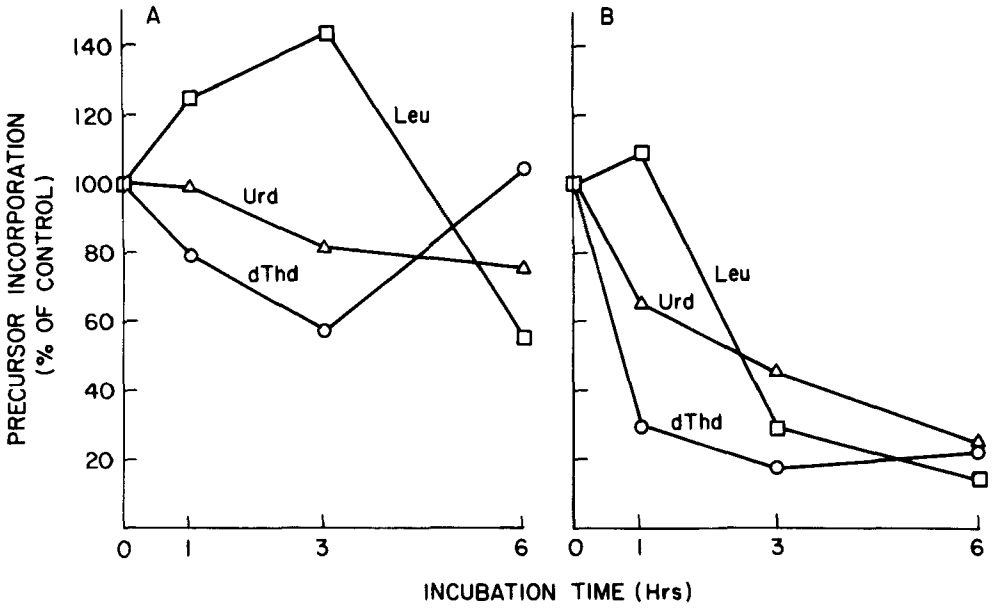


Fig. 1. Effect of (A) 5 x 10^{-5} M or (B) 5 x 10^{-4} M N^6-hydroxyguanidino-purine riboside on the incorporation of radiolabeled uridine, thymidine and leucine into RNA, DNA and protein of L1210. The data were derived from three separate determinations.

PMR spectra were determined on a Varian XL-100 spectrometer using DMSO-d_6 as the solvent and Me_4Si as internal standard. Fast atom bombardment (FAB) mass spectra were determined on a Finnigan MAT-90 mass spectrometer. The saddle field gun was operated at ~8 kV potential and 2-4 mA emission current. For FAB analysis, 2 μg of the sample was dissolved in 0.5 μl of m-nitrobenzyl alcohol. Elemental analyses were carried out by Heterocyclic Chemical Corp., Harrisonville, MO and Robertson Laboratory, Florham Park, NJ. Homogeneity of all the compounds was ascertained by TLC in three solvent systems: (a) i-ProH/conc.NH_4OH/H_2O (7:1:2 v/v); (b) EtOH/2-ethoxyethanol/16% HCOOH (4:1:2 v/v, upper phase) and (c) EtOAc/n-PrOH/H_2O (4:1:2 v/v, upper phase).

6-(1-Hydroxyguanidino)purine (IIa). To a solution of 6-hydroxyaminopurine (Ia) (377 mg; 2.50 mmol) in a mixture of DMF and H_2O (30 ml; 2:1) was added a solution of 1.00 g (5 mmol) of 1-guanyl-3,5-dimethylpyrazole nitrate in H_2O (10 ml). The reaction mixture was stirred at room temperature for 48 hours and

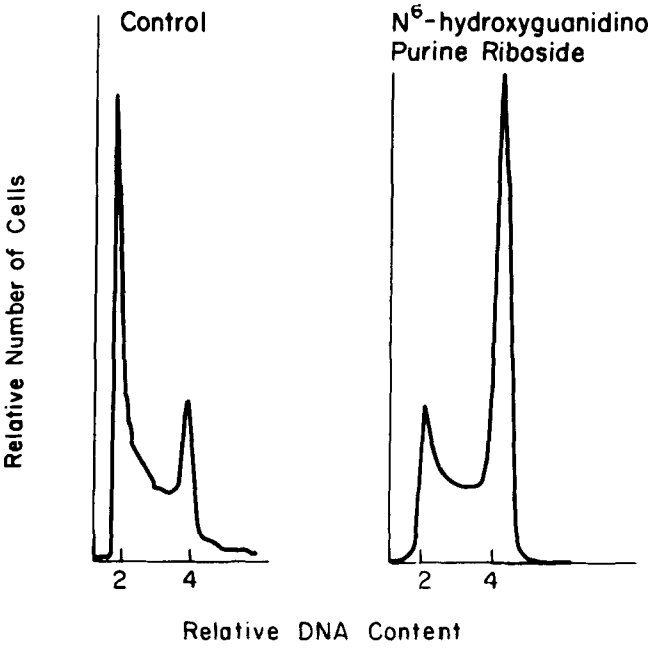


Fig. 2. Effect of N^6-hydroxyguanidinopurine riboside on the DNA-content distribution in a population of L1210 cells. The cells were incubated in the absence (control) or presence of 5×10^{-6} M drug, and cell cycle distribution was determined by flow cytometry, after staining the cells with the DNA-specific fluorochrome 4',6-diamino-2-phenylindole, in the presence of 0.2% Triton X-100[11]. Fluorescent intensity was measured on an ICP-22 flow cytometer, using UV excitation (peak transmission at 360 nm) with an emission filter of 450-490 nm.

evaporated to dryness at 40° under vacuum. Residual DMF was azeotroped three times with anhydrous toluene and the gummy residue triturated three times with 30 ml of boiling ether. The resulting gum was dissolved in 100 ml of absolute EtOH, decolorized with activated charcoal, concentrated to 15 ml and kept at 4° overnight. The crystalline material was collected on a filter, and recrystallized twice from absolute EtOH to yield 291 mg (39.9%) of 6-(1-hydroxyguanidino)purine nitrate (IIa), m.p. >350°, UV λ_{max} 267 (ϵ 15,600) (H_2O) 269 (ϵ 13,800) (0.1 N HCl) and 282 nm (ϵ 14,000) (0.1 N NaOH); pmr δ8.51 (broad s, 2H, C=NH and N-OH) 8.13 (s, 1H, 2-H), 7.97 (s, 1H, 8-H) and 3.70 (broad, 2H, $-NH_2$) ppm. Anal. calc. for $C_6H_7N_7O \cdot HNO_3 \cdot 2H_2O$ (292.2): C, 24.66; H, 4.10; N, 38.36. Found: C, 24.45; H, 3.85; N, 38.51.

6-(1-Hydroxyguanidino)purine Riboside (IIb) Nitrate Salt. To a solution of N^6-hydroxyadenosine (Ib) (283 mg; 1 mmol) in anhydrous DMF (10 ml) was added 1-guanyl-3,5-dimethylpyrazole nitrate (201 mg; 1 mmol). The mixture was stirred at room temperature under anhydrous condition for 7 days and the resulting clear yellow solution was evaporated to dryness in vacuo at 40°. Residual DMF was azeotroped three times with 10 ml anhydrous toluene and the gummy residue triturated four times with 20 ml boiling ether. The gum remaining was dissolved by warming in anhydrous 100 ml MeOH and after decolorization with activated charcoal, was concentrated to a small volume and kept at 4° overnight. The crystalline product was collected on a filter, washed with MeOH and recrystallized twice from MeOH to give 155 mg (39.9%) of the pure product as the nitrate salt, m.p. >300° (decomp.); UV λ_{max} 266 (water), 267 (0.1 N HCl) and 278 nm (0.1 N NaOH); pmr δ8.70 and 8.61 (2, 1H each, C=NH and N-OH), 8.41 (s, 1H, 8-H), 8.06 (s, 1H, 2-H), 5.90 (d, 1H, J = 7 Hz, 1'-H), number of peaks in the region 5.66-3.50 (8H, H and OH protons of ribose) and 3.36 ppm (s, 2H-NH_2). In the positive ion FAB, the compound gave ions at 326 (M+H) supporting the molecular weight 325 for the compound. Other ions were at 364 (M+K), 194 (B+2H) and 135 (adenine). Anal. calc. for $C_{11}H_{15}N_7O_5 \cdot HNO_3$ (338.31): C, 34.02; H, 4.12, N, 28.86. Found: C, 33.99; H, 4.38; N, 28.72.

Hydrochloride Salt of IIb: The nitrate salt of IIb (100 mg) was dissolved in 5 ml water and placed on a 11.5 x 1.5 cm column of Bio-Rad AG-1-X2 anion exchange resin (Cl^- form). The material was eluted with 50 ml water, evaporated to dryness and crystallized from anhydrous EtOH to yield the hydrochloride salt of 6-(1-hydroxyguanidino)purine riboside (85 mg; 90.9%); m.p. 145-6° (dec.); UV λ_{max} 266 (ϵ 22,500) (water), 267 (ϵ 20,800) (0.1 N HCl) and 277 nm (ϵ 17,700) (0.1 N NaOH). Anal. calc. for $C_{11}H_{15}N_7O_5 \cdot HCl \cdot H_2O$ (379.78): C, 34.78; H, 4.74; N, 25.82. Found: C, 34.11; H, 4.58; N, 25.69.

6-(1-Hydroxyureido)purine Riboside (IIIb) Nitrate Salt: To a solution of N^6-hydroxyadenosine (Ib) 283 mg; 1 mmol) in 100 ml absolute EtOH was added 1-guanyl-3,5-dimethylpyrazole nitrate (201 mg; 1 mmol). The mixture was refluxed

for 16 hours and the clear brown solution was evaporated to dryness. The residue was triturated three times with 30 ml each of boiling ether and the gum remaining was dissolved in 50 ml absolute EtOH, decolorized with activated charcoal, concentrated to a small volume and kept at room temperature. The crystalline product was collected on a filter, washed with EtOH and recrystallized four times from absolute EtOH to yield 173 mg (44.5%) of 6-(1-hydroxyureido)purine riboside nitrate, m.p. >300° (decomp.); UV λ_{max} 267 and 275 (water), 273 (0.1 N HCl) and 294 nm (0.1 N NaOH); pmr δ9.46 (s, 1H, N-O<u>H</u>), 8.72 (s, 1H, 8-<u>H</u>), 8.62 (s, 1H, 2-<u>H</u>), 6.03 (d, 1H, J = 6 Hz, 1'-<u>H</u>), number of peaks in the region 5.60-3.66 (8H, H and OH protons of ribose) and 3.36 ppm (broad s, 2H, -N<u>H</u>$_2$). <u>Anal</u>. calc. for $C_{11}H_{14}N_6O_6 \cdot HNO_3$ (389.3): C, 33.93; H, 3.86; N, 25.19. Found: C, 34.10; H, 4.12; N, 25.47.

<u>Free Base-IIIb:</u> The nitrate salt of IIIb (100 mg) was dissolved in 5 ml of water and placed on a 10.5 x 1.5 cm column of Bio-Rad AG-1-X2 (Cl^- form) anion exchange resin. The product was eluted with 100 ml of water, the eluate evaporated to dryness and crystallized twice from EtOH to yield 62 mg (69.1%) of 6-(1-hydroxyureido)purine riboside IIIb as the free base, m.p. 142-4° effervescence). UV λ_{max} 267 (ε 23,000) and 274 (ε 19,200) (water), 274 (ε 22,700) (0.1 N HCl) and 293 (ε 21,800) and 277 nm (ε 16,200) (0.1 N NaOH). Negative and positive ion FAB mass spectra gave m/z at 325 (M-H) and 327 (M+H respectively for the expected molecular weight of 326. <u>Anal</u>. calc. for $C_{11}H_{14}N_6O_6 \cdot 0.5C_2H_4OH$ (349.32): C, 41.26; H, 4.90; N, 24.07. Found: C, 40.84; H, 5.02; N, 24.10.

<u>Catalytic Hydrogenation of IIa</u>. To a solution of 100 mg of IIa in 75 ml of anhydrous MeOH was added 10% 50 mg Pd/C. The mixture was hydrogenated at room temperature and atmospheric pressure for 16 hours, the catalyst removed by filtration, and the solution concentrated to a small volume and kept at 4° overnight. The crystalline product was filtered off and washed with MeOH to yield 67 mg of the product (98.8%), m.p. 222° (effervescence). The product was identified as adenine nitrate on the basis of UV spectra and TLC comparison.

Catalytic Hydrogenation of IIb. To a solution of 100 mg of 6-(1-hydroxyguanidino)-purine riboside monohydrochloride (IIb) in 25 ml anhydrous MeOH was added 50 mg of 10% Pd/C. The mixture was hydrogenolyzed at atmospheric pressure and room temperature for 16 hours and the catalyst removed by filtration. The filtrate was concentrated to a small volume (~7 ml) and kept at -2° overnight. The crystalline product was collected on filter, and identified as adenosine hydrochloride by UV spectra and TLC comparisons.

Catalytic Hydrogenation of IIIb. 6-(1-Hydroxyureido)purine riboside was hydrogenolyzed in the same manner as IIa and the product identified as adenosine by UV spectra and TLC.

Conversion of 6-(1-hydroxyguanidino)purine riboside (IIb) to 6-(1-hydroxyureido)purine riboside (IIIb). The nitrate salt of IIb (50 mg) was dissolved in 95% EtOH and refluxed overnight. Paper chromatography of aliquots of the solution showed three major spots; one corresponding to unchanged starting material IIb and the others to IIIb and adenosine. The total sample was streaked on Whatman 3MM paper, and developed in solvent B. The three bands were eluted with water, the eluates lyophilized and the residue subjected to TLC comparison with authentic samples and to UV spectral measurements. By UV quantitation, it was estimated that IIIb was formed in 43.8% yield.

Conversion of 6-(1-hydroxyguanidino)purine (IIa) to 6-(1-hydroxyureido)-purine (IIIa). Nitrate salt of IIa (10 mg) was dissolved in an EtOH-water mixture (85:15; 2.0 ml) and refluxed for 8 hrs. Paper chromatographic examination of the solution showed three spots; one corresponding to unchanged IIa, another to a new product IIIa and the third to adenine. The three bands were separated by preparative paper chromatography in solvent B. The band corresponding to compound IIIa was eluted with water and subjected to UV spectral analysis. By UV quantitation this compound was formed in about 29% yield. This material was then placed on a 2 cm x 1.0 cm column of AG1-X2 (Cl^-) anion exchange resin and eluted with 10 ml water. The eluate was evaporated to dryness and the residue crystallized from MeOH as the hydrochloride, UV λ_{max} 265 (water), 272

(0.1 N HCl) and 271 nm (0.1 N NaOH), m.p. 250° (decomp.); pmr δ (D_2O) 8.92 (s, 1H, 2-H) and 8.86 ppm (s, 1H, 8-H). Anal. calc. for $C_6H_6N_6O_2 \cdot HCl$: C, 31.24; H, 3.04; N, 36.44. Found: C, 30.86; H, 3.16; N, 36.78.

Acid hydrolysis of 6-(1-hydroxyguanidino)purine riboside (IIb). Compound IIb (10 mg) was dissolved in 10 ml of 2 N HCl and maintained at room temperature for 16 hours and then evaporated to dryness. TLC of the residue in solvents b and c showed a single spot corresponding to IIa. The residue displayed UV absorption maxima at 266 nm (H_2O), 269 nm (0.1 N HCl) and 282 nm (0.1 N NaOH) and, on the basis of TLC comparisons and UV spectra, the material was identified as IIa.

Biology

The in vitro cytotoxicity and in vivo antitumor activity of the compounds and the effect of IIb on precursor incorporation was assayed as previously described[9]. Incorporation was studied using [5-^{3}H]uridine (38.9 ci/mmol), [methyl-^{3}H]thymidine (20 ci/mmol) and [4,5-^{3}H(N)]-L-leucine (50 ci/mmol), all obtained from New England Nuclear. The procedures employed for determining adenosine deaminase inhibition have also been published[10]. The inhibition analysis was performed by adding the purine nucleosides at concentrations ranging from 1×10^{-4} to 1×10^{-7} M to the growth medium containing graded concentrations of IIb.

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