Adenosine Deaminase Inhibitors. Synthesis and Biological Activity of Deaza Analogues of *erythro*-9-(2-Hydroxy-3-nonyl)adenine

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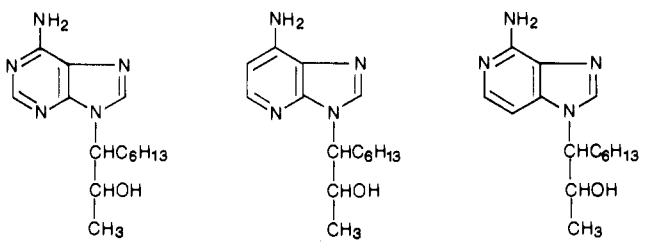
Two new deaza analogues of *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA, 1), 7-deaza-EHNA (6) and 1,3-dideaza-EHNA (11), were synthesized and evaluated for adenosine deaminase (ADA) inhibitory activity and compared with EHNA, 1-deaza-EHNA (2), and 3-deaza-EHNA (3). Substitution of a methine group for a nitrogen atom in the 7-position of the purine moiety of EHNA produces a dramatic drop in the inhibitory activity ($K_i = 4 \times 10^{-4}$ M) whereas compounds 2 and 3 are still good inhibitors ($K_i = 1.2 \times 10^{-7}$ M and 6.3×10^{-9} M respectively). EHNA and its deaza analogues so far synthesized were also tested in vitro for their antiviral and antitumor activity in a range of cellular systems. EHNA and 1-deaza-EHNA are equiactive as inhibitors of human respiratory syncytial virus (HRSV) replication (MIC = 6.25 μg/mL) while the other compounds are inactive. On the other hand, all the examined compounds displayed an antitumor activity comparable to that of the reference compound 1-β-D-arabinofuranosyladenine (ara-A), 7-deaza-EHNA being the most active of all. The results obtained showed that there is no correlation between adenosine deaminase inhibition and antiviral or antitumor activity in this series of compounds. 3-Deaza-EHNA, the most active inhibitor of ADA among the EHNA deaza analogues, greatly potentiates the antitumor activity of ara-A in vitro. In vivo activity was observed only when the two compounds were used in combination.

In the last decade, inhibitors of adenosine deaminase (ADA) have aroused interest as potential codrugs for use in combination with certain anticancer or antiviral agents which are adenosine analogues.[1] Furthermore, they may be used to mimic the effect of the genetic deficiency of the enzyme which is associated with a form of the clinical syndrome, severe combined immunodeficiency disease.[2]

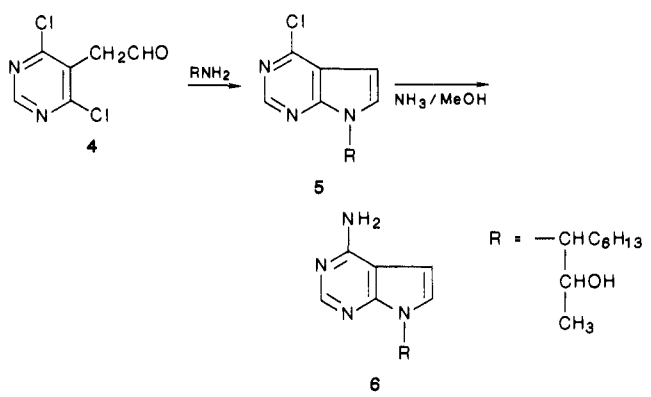
Among the ADA inhibitors, *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA, 1), a semitight inhibitor ($K_i = 1.6 \times 10^{-9}$ M), is advocated as a possible inhibitor of choice for use with such nucleosides and was preferred to pentostatin and coformycin, two extremely potent naturally occurring inhibitors ($K_i = 10^{-11}$–10^{-12} M).[3] In order to investigate which structural parameters in the purine moiety of EHNA are critical for inhibitory activity, we have undertaken a study to gain insight into the contribution of the nitrogen atoms to the binding to the enzyme.



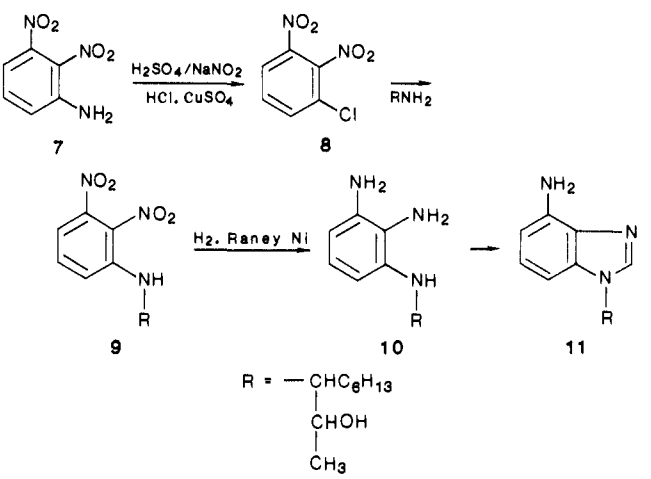
1 2 3

Recently we demonstrated that isosteric monosubstitution of the pyrimidine nitrogens by carbons can be tolerated at the enzymatic binding site.[4] In fact, 3-deaza-EHNA (3) was found to have an inhibitory activity comparable to that of EHNA itself, and 1-deaza-EHNA (2), though less potent, is a good inhibitor. Now we report the synthesis and the ADA inhibitory activity of two new deaza analogues of EHNA: the *erythro*-9-(2-hydroxy-3-nonyl)-7-deazaadenine (7-deaza-EHNA, 6) and the *erythro*-9-(2-hydroxy-3-nonyl)-1,3-dideazaadenine (1,3-dideaza-EHNA, 11). Furthermore, as it was reported that EHNA significantly inhibits replication of herpes simplex virus (HSV),[5] we have tested also the antiviral and antitumor activity of the deaza-EHNA analogues so far synthesized.

Scheme I



Scheme II



Chemistry. The synthesis of 7-deaza-EHNA (6) was performed by the method outlined in Scheme I. 4,6-Dichloropyrimidine-5-acetaldehyde (4)[6] was condensed with *erythro*-3-amino-2-nonanol[7] to obtain directly the cyclized

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Table I. Inhibition Constants of Calf Intestinal and Human Erythrocytic Adenosine Deaminase

compd[a]	calf intestine: K_i, M	human erythrocytes: K_i, M
1, EHNA	7.0×10^{-9}	4.3×10^{-9}
2, 1-deaza-EHNA	1.6×10^{-7}	1.2×10^{-7}
3, 3-deaza-EHNA	1.0×10^{-8}	6.3×10^{-9}
6, 7-deaza-EHNA	4.0×10^{-4}	1.8×10^{-4}
11, 1,3-dideaza-EHNA	7.1×10^{-5}	1.5×10^{-5}

[a] Mixture of two isomers: 2'S,3'R and 2'R,3'S.

compound **5** in high yield (83%). Treatment of **5** with methanolic ammonia at 110 °C gave the desired 7-deaza-EHNA (**6**).

The synthesis of 1,3-dideaza-EHNA (**11**) was carried out by the sequence shown in Scheme II. 1-Chloro-2,3-dinitrobenzene (**8**) was obtained from 1-amino-2,3-dinitrobenzene (**7**) by modification of the purification procedures of a method previously reported,[8] and the yield was increased from 10% to 63%. Condensation of **8** with *erythro*-3-amino-2-nonanol gave compound **9**, whose catalytic reduction afforded *erythro*-1-[(2-hydroxy-3-nonyl)-amino]-2,3-diaminobenzene (**10**). Ring closure of **10** by means of formamidine acetate gave the desired 1,3-dideaza-EHNA (**11**), whose structure was assigned on the basis of ^{1}H NMR data in deuteriochloroform, which showed the presence of an NH_2 signal at δ 4.18 exchangeable with deuteriated water and the absence of a signal corresponding to NH as in **9** (δ 6.79).

Biological Evaluation and Discussion

Adenosine Deaminase Inhibitory Activity. The inhibition of human erythrocytic and calf intestine ADA by 7-deaza-EHNA (**6**) and 1,3-dideaza-EHNA (**11**) compared to those of EHNA (**1**), 1-deaza-EHNA (**2**), and 3-deaza-EHNA (**3**) are reported in Table I. As shown by results, the substitution of a methine group for the nitrogen atom in the 7-position of the purine moiety of EHNA produces a dramatic drop in the inhibitory activity. Also the contemporary substitution of pyrimidine nitrogens by carbons brings about a considerable reduction of activity. So it appears evident, by comparing the inhibition constants reported in Table I, that the nitrogen atoms of the EHNA purine moiety give different contributions to binding to the enzyme, the nitrogen at the 7-position being critical for the inhibitory activity of EHNA.

Antiviral Activity. EHNA and its deaza analogues were tested for their antiviral activity in solid medium against adeno, influenza type A, and mouse encephalomyocarditis viruses and were found inactive. The same compounds were also tested in fluid medium against herpes simplex virus type 1 (HSV-1), human respiratory syncytial virus (HRSV), and Coxsackie virus type B1. EHNA (**1**) and 1-deaza-EHNA (**2**) are equiactive as inhibitors of human respiratory syncytial virus replication (MIC = 6.25 μg/mL), while 3-deaza-EHNA (**3**) is completely inactive. These observations indicate that the ADA inhibitory activity of the deaza analogues of EHNA does not account for the inhibition of virus replication.

Antitumor Activity. The antitumor activity of EHNA and its deaza analogues was evaluated in vitro in two human cell lines (HeLa and KB) and in three murine leukemia cell lines (P388, P388 doxorubicin resistant, and L1210), compared to that of 1-β-D-arabinofuranosyladenine (ara-A) (Table II). All the examined compounds displayed

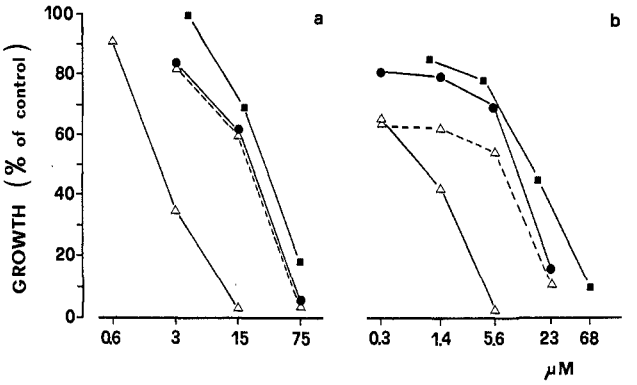

Figure 1. Cytotoxic effect of ara-A on P388 cells in the presence (△) and absence (●) of 4 μM 3-deaza-EHNA (a) and EHNA (b); (■) cytotoxic effect of 3-deaza-EHNA and EHNA alone. The dashed lines represent the theoretic additional effect on ara-A plus 3-deaza-EHNA or ara-A plus EHNA.

an activity comparable to that of the reference compound ara-A. The results obtained showed that there is no correlation between adenosine deaminase inhibition and antiviral or antitumor activity in this series of compounds.

7-Deaza-EHNA (**6**), which appeared to be the most active compound in the series, was tested also in vivo against leukemia L1210 in the mouse and found inactive.

Potentiation of Antitumor Effect of ara-A by 3-Deaza-EHNA. On the basis of the knowledge that ara-A is rapidly deaminated to arabinosyl hypoxanthine, a relatively nontoxic product, by adenosine deaminase and that EHNA, as inhibitor of the enzyme, greatly potentiates the toxicity of ara-A[9] and its antiherpes activity,[5] we tested the ability of 3-deaza-EHNA (**3**), the most active inhibitor of ADA among the EHNA deaza analogues so far synthesized, to potentiate the antitumor activity of ara-A. The results showed that ara-A had only a slight effect at 1 μM and brought about 40% of inhibition of leukemia P388 cell growth at 15 μM (Figure 1a). In the presence of 3-deaza-EHNA at 4 μM concentration, the same 15 μM ara-A produced greater than 93% inhibition. A similar potentiation of the ara-A activity was obtained in the presence of EHNA (Figure 1b). We have also tested the potentiation of the antitumor activity of ara-A by 3-deaza-EHNA in vivo against leukemia L1210 in the mouse. When given singularly, the two compounds were inactive; however, when used in combination (100 mg/kg of ara-A plus 10–30 mg/kg of 3-deaza-EHNA), the activity (% T/C = 125) was reached.

Experimental Section

Chemistry. Melting points were determined with a Büchi apparatus and are uncorrected. ^{1}H NMR spectra were obtained with a Varian EM-390 90-MHz spectrometer using tetramethylsilane as internal standard. UV spectra were recorded on a Cary 219 spectrophotometer. TLC were carried out on precoated TLC plates with silica gel 60 F-254 (Merck). For column chromatography, silica gel 60 (Merck) was used. Microanalytical results are indicated by atomic symbols and are within ±0.4% of theoretical values.

***erythro*-4-Chloro-7-(2-hydroxy-3-nonyl)pyrrolo[2,3-*d*]pyrimidine (5).** To a solution of 0.5 g (26 mmol) of 4,6-dichloropyrimidine-5-acetaldehyde (**4**)[6] in 20 mL of absolute ethanol was added 0.83 g (52 mmol) of *erythro*-3-amino-2-nonanol,[7] and the mixture was heated at 80 °C for 2 h. After concentration in vacuo, the residue was chromatographed on a silica gel column. Elution with C_6H_6–EtOAc (60:40) yielded 0.64 g (82.6%) of **5** as a chromatographically pure oil: ^{1}H NMR ($CDCl_3$) δ 3.22 (br s, 1, OH),

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Table II. In Vitro Antitumor Activity of EHNA and Its Deaza Analogues[a]

	ID_{50},[b] μM						
	human cell lines				murine cell lines		
	HeLa		KB		P388,	P388/DX,[c]	L1210,
compd	72 h	144 h	72 h	144 h	72 h	72 h	72 h
1	179.6	23.4	81.6	46.3	27.2	13.4	22.9
2	111.9	21.8	49.1	30.0	98.2	38.2	49.1
3	141.9	18.6	31.9	5.5	21.8	19.2	18.6
6	60.4	4.7	20.7	8.5	20.7	3.8	18.4
11	38.3	18.6	32.8	30.1	62.9	47.9	26.8
ara-A	46.0	27.7	89.8	25.4	12.7	10.8	23.6

[a] Exponentially growing cells (5×10^{-5}) were exposed to varying concentrations of drug for 72–144 h. The cells were then counted with a Coulter counter. [b] Inhibitory dose 50 is the concentration of the compound in the culture media that produces 50% inhibition of the tumor cell growth as compared to the untreated controls. [c] Doxorubicin-resistant cell line.

4.21 (m, 1, CH-2), 4.58 (m, 1, CH-3), 6.65 (d, 1, $J_{5,6}$ = 5 Hz, H-5), 7.40 (dd, 1, H-6), 8.62 (s, 1, H-2). Anal. ($C_{15}H_{22}ClN_3O$) C, H, N.

***erythro*-9-(2-Hydroxy-3-nonyl)-7-deazaadenine (6).** A solution of 0.55 g (1.85 mmol) of **5** in 20 mL of methanolic ammonia was sealed in a glass tube and heated at 110 °C for 30 h. The solvent was evaporated, and the residue was chromatographed on a silica gel column eluting with EtOAc–MeOH–NH_3 (95:4:1) to give 0.255 g (50%) of **6** as a chromatographically pure oil: ^{1}H NMR ($CDCl_3$) δ 0.82 (t, 3, CH_3-9), 0.94–1.50 (m, 11, CH_3-1, CH_2-5, CH_2-6, CH_2-7, CH_2-8), 2.02 (m, 2, CH_2-4), 4.27 (m, 2, CH-2 and CH-3), 5.00–6.00 (br m, 3 NH_2-6 and OH), 6.38 (d, 1, $J_{7,8}$ = 3 Hz, H-7), 7.01 (d, 1, $J_{8,7}$ = 3 Hz, H-8), 8.27 (s, 1, H-2); UV (pH 7) λ_{max} 270 nm (ε 6900). Anal. ($C_{15}H_{24}N_4O$) C, H, N.

The product was converted to the oxalate salt by mixing equimolar amounts of **6** and oxalic acid in *i*-PrOH. The solution was left overnight at 0 °C, and the solid that precipitated was recrystallized from *i*-PrOH containing 1% oxalic acid to give the analytically pure sample, mp 157–158 °C. Anal. ($C_{15}H_{24}N_4O \cdot C_2H_2O_4$) C, H, N.

1-Chloro-2,3-dinitrobenzene (8). To a stirred and ice-cooled solution of 65 mL of nitrosylsulfuric acid[8] was added portionwise 13 g (71 mmol) of 1-amino-2,3-dinitrobenzene (**7**). After the addition was complete, the mixture was heated at 60 °C for 10 min. After cooling, the mixture was carefully poured into a stirred and ice-cooled cuprous chloride solution. The reaction mixture was allowed to warm to room temperature and extracted several times with $CHCl_3$. The organic extracts were dried (Na_2SO_4) and evaporated to dryness, and the residue was chromatographed in a silica gel column. Elution with $CHCl_3$–*n*-C_6H_{14} (60:40) gave 8.94 g (63%) of **8** as a pure solid: mp 75–77 °C (lit.[8] mp 78 °C); ^{1}H NMR (DMSO-d_6) δ 7.95 (t, 1, H-5), 8.29 (d, 1, $J_{5,6}$ = 8 Hz, H-6), 8.44 (d, 1, $J_{4,5}$ = 8 Hz, H-4). Anal. ($C_6H_3ClN_2O_4$) C, H, N.

***erythro*-1-[(2-Hydroxy-3-nonyl)amino]-2,3-dinitrobenzene (9).** A mixture of 3 g (15 mmol) of 1-chloro-2,3-dinitrobenzene (**8**), 2.62 g (16.5 mmol) of *erythro*-3-amino-2-nonanol, 2.38 g of dry triethylamine, and 48 mL of nitromethane was refluxed for 3 h. The reaction mixture was concentrated in vacuo, and the residue was dissolved in water and extracted several times with chloroform. The combined extracts were dried (Na_2SO_4) and evaporated in vacuo to an oily residue, which was chromatographed on a silica gel column. Elution with *n*-C_6H_{14}–EtOAc–C_6H_6 (75:20:5) yielded 2.0 g (40%) of **9** as a pure oil: ^{1}H NMR ($CDCl_3$) δ 4.00 (m, 2, CH-2 and CH-3), 6.79 (m, 2, H-5 and NH-1), 7.52 (d, 1, $J_{5,6}$ = 8 Hz, H-6), 7.94 (d, 1, $J_{4,5}$ = 8 Hz, H-4). Anal. ($C_{15}H_{23}N_3O_5$) C, H, N.

***erythro*-1-[(2-Hydroxy-3-nonyl)amino]-2,3-diaminobenzene (10).** To a solution of 1.85 g (5.7 mmol) of **9** in 150 mL of ethanol was added 4 g of Raney nickel catalyst (washed with water), and the mixture was shaken with hydrogen at 30 psi for 4 h. After the catalyst was removed by filtration, the filtrate was evaporated and the residue was chromatographed on a silica gel column. Elution with *n*-C_6H_{14}–C_6H_6 (60:40) gave **10** as a chromatographically pure oil: yield 1 g (67%); ^{1}H NMR ($CDCl_3$) δ 3.34 (m, 1, CH-2), 3.83 (m, 1, CH-3), 6.59 (m, 1, H-5), 6.77 (d, 2, H-4 and H-6). Anal. ($C_{15}H_{27}N_3O$) C, H, N.

***erythro*-9-(2-Hydroxy-3-nonyl)-1,3-dideazaadenine (11).** An intimate mixture of 1.02 g (3.8 mmol) of **10** and 770 mg of formamidine acetate was heated at 150 °C for 20 min. The resulting solid was chromatographed, eluting with $CHCl_3$–MeOH (95:5) to give **11** as a chromatographically homogeneous oil: yield 0.73 g (70%); ^{1}H NMR ($CDCl_3$) δ 0.85 (m, 3, CH_3-9), 1.27 (m, 11, CH_3-1, CH_2-5, CH_2-6, CH_2-7, and CH_2-8), 2.08 (m, 2, CH_2-4), 4.18 (m, 3, CH-2 and NH_2-6), 5.28 (m, 1, CH-3), 7.12 (m, 2, H-1 and H-2), 7.41 (d, 1, $J_{2,3}$ = 7 Hz, H-3), 8.01 (s, 1, H-8); UV (pH 7) λ_{max} 250 (ε 4400), 205 nm (23 100). Anal. ($C_{16}H_{25}N_3O$) C, H, N.

The base was converted to the oxalate salt, which was purified by recrystallization from *i*-PrOH containing 1% oxalic acid, mp 120–121 °C. Anal. ($C_{16}H_{25}N_3O \cdot C_2H_2O_4$) C, H, N.

Biological Studies. Enzyme Assay. The method used for the determination of activity against adenosine deaminase has been described in a preceding paper.[4]

Antitumor Activity in Vitro. Cell Lines. The two human cell lines HeLa (human cervix carcinoma) and KB (human oral epidermic carcinoma) were routinely maintained in monolayer culture in minimum essential medium (Eagle) with nonessential amino acids and Earles' BBS, supplemented with 10% fetal calf serum and antibiotics (50 units/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL kanamycin). The three murine leukemia cell lines (P388, P388/DX, and L1210) were established in vitro as suspension cultures and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 20 μM 2-mercaptoethanol, 2 μM L-glutamine, and antibiotics. The cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2. The characteristics and the in vitro cultures of these cell lines have previously been described.[10-12]

Antitumor Activity in Vivo. Animals and Tumors. Inbred DBA/2 and first generation hybrid (C57lBl/6 × DBA/2) F_1 (BDF_1) and (BA1b/c × DBA/2) F_1 (CDF_1) adult mice of both sexes were used for the evaluation of antitumor activity. All animals were obtained from Charles River Italia (Calco, Como, Italy). The animals were 2–3 months old, weighed 20–24 g, and were kept under standard laboratory conditions.

The subline of P388 leukemia resistant to doxorubicin (P388/DX) was obtained by repeated exposure to the drug in Dr. F. M. Schabel's laboratory (Southern Research Institute, Birmingham, AL) and maintained in our facilities in BDF_1 mice given weekly ip passages of 1×10^7 cells/mouse. The animals were treated 48 h after tumor inoculation with 6 mg/kg ip of doxorubicin. For experimental purposes, 1×10^6 cells/mouse were transplanted ip in the same strain of animals. Ascitic L1210 leukemia was maintained by serial ip passages in DBA/2 mice according to Geran et al.[11] For experimental purposes, 1×10^5 cells/mouse were inoculated ip in CDF_1 mice.

Antiviral Activity and Cytotoxicity in Solid Medium. Activity of adeno, influenza type A, and Columbia SK viruses has been tested respectively on DK (dog kidney cells), BHK (baby hamster kidney cells), and L929 (mouse fibroblasts) cells, in agar medium according to Herrmann.[13] Briefly: monolayers grown

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for 24–48 h have been infected with 100 PFU/mL of virus; after a 2-h adsorption, cultures are covered with the agar medium and a disk of blotting paper, embedded with 200 μg of the compound dissolved in ethanol and dried, is overlayed. After a 3–4-day incubation at 37 °C in 5% CO_2, cultures are stained with neutral red. The toxicity, as unstained halos, and the activity, as stained areas where lysis plaques produced by the virus are reduced or have disappeared, are evidenced at the same time.

Cytotoxicity gross evaluation firstly is assessed on the basis of toxicity halos in tests with agar medium, and as maximum tolerated dose (MxTD) in liquid medium, at the same time with the test of antiviral activity previously described. A more accurate evaluation of cytotoxicity is obtained by incubating Hep cells with serial doses of the compound for 3 days and by calculating the 50% inhibiting dose ($TCID_{50}$) after determination of the protein contents.[14]

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Antiviral Activity and Cytotoxicity in Fluid Medium. Determination of cytotoxicity and of herpes simplex, HRSV, and Coxsackie viruses inhibition in human carcinoma cells was performed as previously described.[15]

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Registry No. 4, 16019-33-3; **5**, 111209-66-6; **6**, 103298-51-7; **6** (oxalate), 111209-67-7; **7**, 602-03-9; **8**, 602-02-8; **9**, 111209-68-8; **10**, 111209-69-9; **11**, 103298-52-8; **11** (oxalate), 111209-70-2; *erythro*-3-amino-2-nonanol, 51714-10-4; adenosine deaminase, 9026-93-1.

Supplementary Material Available: Antiviral activity and cytotoxicity of EHNA deaza analogues in fluid medium (Table III) and antitumor activity of ara-A and 3-deaza-EHNA (**3**) alone and in combination on murine leukemia L1210 (Table IV) (1 page). Ordering information is given on any current masthead page.

Synthesis and Biological Activities of 4-*O*-(Difluoromethyl)-5-substituted-uracil Nucleoside Analogues

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Various 4-*O*-difluoromethyl analogues of 5-substituted uridine (Urd), 2′-deoxyuridine (dUrd), and arabinofuranosyluracil (araU) nucleosides were prepared via a CF_2-insertion reaction into 4-*O*-silylated nucleosides and evaluated for activity against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) and cytotoxicity in human embryonic lung fibroblast (HELF) cell cultures. The introduction of the 4-substituent led to a strong reduction of antiviral activity for dUrd but not for araU analogues. Three of the 4,5-disubstituted uracil nucleoside derivatives, 4-*O*-(difluoromethyl)-5-bromo-araU (**5c**), -5-methyl-araU (**5e**), and -(*E*)-5-(2-bromovinyl)-araU (**5g**), displayed a high and selective inhibitory effect against HSV-1, but only **5e** was effective against both HSV-1 and HSV-2 comparably with the antiherpes potential of the reference compounds 9-[(2-hydroxyethoxy)methyl]guanine (acyclovir) and 1-β-D-arabinofuranosylthymine (araT).

Analogues of thymidine (dThd; 5-methyl-2′-deoxyuridine), a natural precursor of deoxyribonucleic acid (DNA), were among the first compounds discovered to exert antiviral activity against DNA viruses.[1] During the past 10 years we have been engaged in the synthesis and biological evaluation of 5-substituted pyrimidine nucleoside analogues with antiviral potential.[2-4] In the search for compounds that surpass the antiviral effect of 5-iodo-2′-deoxyuridine (IdUrd)[1] with simultaneously reduced toxicity to uninfected host cells, a large number of 5-substituted dUrd and 2′-deoxycytidine (dCyd) derivatives have been synthesized.[3,5,6] In our pursuit of this approach, (*E*)-5-(2-bromovinyl)-dUrd (BrV-dUrd) emerged as a highly potent and selective antiherpes agent that inhibits the replication of herpes simplex virus type 1 (HSV-1), varicella zoster virus (VZV), and Epstein–Barr virus (EBV), but not of herpes simplex virus type 2 (HSV-2), at levels far lower than the cytotoxic concentrations.[5,7-10] Much effort has been invested in elucidating the ideal structural requirements of the 5-vinyl substituent for maximal antiherpes viral activity and low cytotoxicity;[11] but exchange of the olefinic hydrogen atoms by other halogens or by nonhalogen substituents as well as comparison of *E* and *Z* isomers did not improve the low anti-HSV-2 potential of BrV-dUrd.[12,13]

The newly synthesized 5-substituted 1-β-D-arabinofuranosyluracil (araU) analogues[14,15] have attracted con-

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