Unsaturated and Carbocyclic Nucleoside Analogues: Synthesis, Antitumor, and Antiviral Activity[1]

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A series of unsaturated analogues of nucleosides were prepared and their cytotoxic, antitumor, and antiviral activities were investigated. Alkylation of cytosine with (*E*)-1,4-dichloro-2-butene gave chloro derivative **2f**, which was hydrolyzed to alcohol **2h**. Cytosine, adenine, 2-amino-6-chloropurine, thymine, and (*Z*)-1,4-chloro-2-butene gave compounds **4c-f**, which, after hydrolysis, afforded alcohols **4a**, **4b**, **4g**, and **4h**. Alkenes **4d** and **4e** were cyclized to heterocycles **12** and **13**. Alkylation of 2,6-diaminopurine with 1,4-dichloro-2-butyne led to chloro derivative **6a**, which was hydrolyzed to alcohol **6b**. Allenic isomerization of **6b** gave compound **5c**. Chloro derivatives **2e-g**, **4c-f**, **5d**, and **6c-e** as well as pyrimidine oxacyclopentenes **9c** and **9d** are slow-acting inhibitors of murine leukemia L1210 of IC_{50} 10-100 μM. The most active were analogues **4c**, **4d**, **4e**, and **6e** (IC_{50} 10-20 μM). The corresponding hydroxy derivatives were less active of inactive. Inhibition of macromolecular synthesis with compounds **4c**, **4d**, **6e**, **9c**, and **9d** follows the order: DNA > RNA ≥ protein. Cytotoxic effects of **4c**, **6e**, and **9d** are not reversed with any of the four basic ribonucleosides or 2'-deoxyribonucleosides. Inhibitory activity of cytosine derivative **9c** is reversed with uridine and 2'-deoxyuridine but not with the corresponding cytosine nucleosides. Zone assays in several tumor cell lines show that active compounds are cytotoxic agents with little selectivity for tumor cells. Analogue **6c** showed 16.7% ILS in leukemia P388/o implanted ip in mice at 510 and 1020 mg/kg, respectively. Cytallene (**5b**) and 6'β-hydroxyaristeromycin (**10**) exhibited significant activity against Friend and Rauscher murine leukemia viruses. The rest of the hydroxy derivatives, with the exception of **4a**, were moderately effective or inactive as antiviral agents. None of the chloro derivatives or oxacyclopentenes exhibited an antiviral effect at noncytotoxic concentrations. *Z*-Olefin **4b** and 2-aminoadenallene (**5c**) are substrates for adenosine deaminase.

The discovery of the antibiotic neplanocin A[2] (**1a**, Chart I) provided a considerable impetus to studies of the corresponding unsaturated cyclic and acyclic analogues. Compound **1a** itself can be formally derived from adenosine by replacing the 4'-CHO grouping with the bioisosteric[3] 4'-C=CH moiety. Whereas neplanocin A (**1a**) is a selective antileukemic and antiviral agent, the cytosine analogue[4,5] **1b** is effective against a broad selection of tumors[4-7]. Removal of the 2'-CHOH function **2c** **1a** gives an acyclic analogue[8] ("nor-2'-deoxyneplanocin A", **2a**) whose guanine counterpart[8-10] **2b** can also be derived by substitution of the 3'-CHO portion of antiviral agent ganciclovir[11] ("nor-2'-deoxyguanosine", 2'-NDG, DHPG, **3a**) with C=CH. In a similar fashion, compounds[12,14] **2c** and **2d** may be related to neplanocin A (**1a**) or its guanine analogue derived by excision of the corresponding 2'-C-3'-C fragment. The relationship of antiherpetic drug acyclovir[11] (**3b**) and unsaturated compound **2d** is then similar to that of **2b** and ganciclovir (**3a**).

Adenine derivative **2a** and, particularly, 3-deazaadenine and guanine analogue **2b** exhibit antiviral activities[8-10]. Alkene **2a** was also used, along with other acyclic models of neplanocin A(**1a**), in a study relating the antiviral activity against vaccinia virus to inhibition of *S*-adenosylhomocysteine hydrolase.[15] *E*-Alkenes **2c** and **2d** exhibited little antiviral activity,[9,10,12-15] but adenine derivative **2c** is a substrate for adenosine deaminase[14]. In the *Z*-alkene series, where the distance between the nucleic acid base and hydroxymethyl group is substantially shortened relative to that in acyclovir (**3b**) or *E*-alkene **2d**, guanine analogue **4a** is an effective antiherpetic agent[9,10,16] whereas compound **4b** is inactive. Also, the chloro derivative of **2c**, compound **2e**, inhibited murine leukemia P388 in vitro[13].

Replacement of CH_2OCH_2 grouping of acyclovir (**3b**) and related compounds with a bioisosteric[3] allene system

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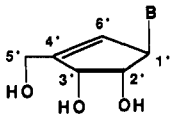
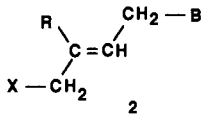
Chart I



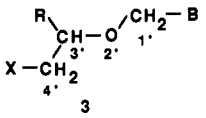
1

1a: B = Ade 1b: B = Cyt



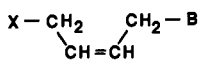
2

2a: B = Ade, X = OH, R = CH_2OH
2b: B = Gua, X = OH, R = CH_2OH
2c: B = Ade, X = OH, R = H
2d: B = Gua, X = OH, R = H
2e: B = Ade, X = Cl, R = H
2f : B = Cyt, X = Cl, R = H
2g: B = ACP, X = Cl, R = H
2h: B = Cyt, X = OH, R = H



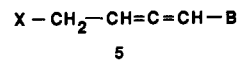
3

3a: B = Gua, X = OH, R = CH_2OH
3b: B = Gua, X = OH, R = H
3c: B = Ade, X = OH, R = CH_2Cl
3d: B = Ade, X = Cl, R = H



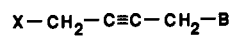
4

4a: B = Gua, X = OH
4b: B = Ade, X = OH
4c: B = Cyt, X = Cl
4d: B = Ade, X = Cl
4e: B = ACP, X = Cl
4f : B = Thy, X = Cl
4g: B = Cyt, X = OH
4h: B = Thy, X = OH



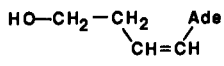
5

5a: B = Ade, X = OH
5b: B = Cyt, X = OH
5c: B = DAP, X = OH
5d: B = Ade, X = Cl
5e: B = Gua, X = OH
5f : B = Thy, X = OH
5g: B = 5-MeCyt, X = OH



6

6a: B = DAP, X = Cl
6b: B = DAP, X = OH
6c: B = Ade, X = Cl
6d: B = ACP, X = Cl
6e: B = Cyt, X = Cl
6f : B = Thy, X = Cl
6g: B = Ade, X = OH
6h: B = Gua, X = OH
6i : B = Cyt, X = OH
6j : B = Thy, X = OH



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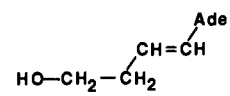

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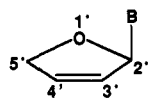
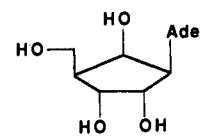


9

9a: B = Ade
9b: B = Gua
9c: B = Cyt
9d: B = Thy
9e: B = FUra



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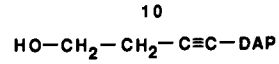

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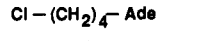


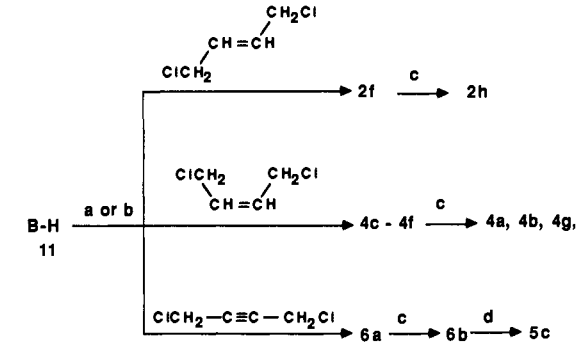
15

ACP = 2-amino-6-chloropurine DAP = 2,6-diaminopurine

FUra = 5-fluorouracil

CH=C=CH led to a new class[17-19] of antiviral agents. Thus, adenallene (**5a**) and cytallene (**5b**) are strong in-

Scheme I



11a: B = Ade 11b: B = Cyt 11c: B = Thy
11d: B = DAP 11e: B = ACP

ACP = 2-amino-6-chloropurine DAP = 2,6-diaminopurine

a. K_2CO_3, DMSO. c. 0.1 M HCl, reflux.
b. NBu_4F, THF. d. 0.1 M NaOH, reflux.

hibitors of human immunodeficiency virus[20-22] (HIV), the etiologic agent of acquired immunodeficiency syndrome (AIDS). By contrast, butynols **6g–j**, which were also a subject of recent reports,[9,16,18,20] did not exhibit significant biological activity in any system examined to date. Lastly, a new group of unsaturated analogues, *Z*- and *E*-alkenes **7** and **8**, were found to be substrates for adenosine deaminase.[23]

The purpose of this investigation was to examine a more extensive series of unsaturated acyclic analogues of nucleosides. In view of the aforementioned results, structural types **2** and **4–6** containing hydroxy functions and chlorine atoms were of interest as potential antitumor and antiviral agents. Allenes (type **5**) were then targeted in view of an excellent anti-HIV activity[20-22] of **5a** and **5b**. Obviously, the behavior of these analogues toward other viral and tumor systems was of interest. We have also included in our study oxacyclopentenes **9a–d**, unsaturated nucleoside analogues[18,19] with an intact dihydrofuran ring lacking the hydroxymethyl group. The latter compounds relate to 2′,3′-didehydro-2′,3′-dideoxyribonucleosides, some of which have a high anti-HIV activity.[24-27] 6′β-Hydroxyaristeromycin (**10**), an analogue of neplanocin (**1a**) and mod-

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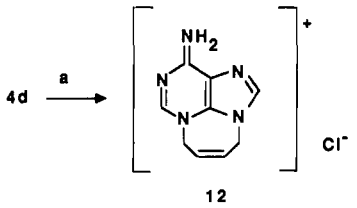
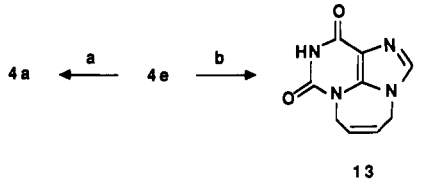
Scheme II



12

a. DMSO.

Scheme III



13

a. 0.1 M HCl, reflux, 3 h. b. 0.1 M HCl, reflux, 18 h.

erate antileukemic agent,[28] was also of interest as a potential antiviral agent.

Chemistry. All analogues of the type **2** and **4–6** were prepared by alkylation of the corresponding nucleic bases **11a–c**, 2,6-diaminopurine (**11d**), or 2-amino-6-chloropurine (**11e**) in case of guanine derivatives. A 4-fold excess of alkylating agent (1,4-dichloro-2-butene[14] or -butyne[18,19]) along with K_2CO_3 in dimethyl sulfoxide (DMSO) or tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) was employed. Subsequent hydrolysis with 0.1 M HCl at 100 °C then afforded the required hydroxy derivatives (Scheme I). Generally, a large excess (17–31 mol) of alkylating agent employed in a previous study[13] is not necessary, although amounts lower than 3 equiv decrease the yields of the respective chloro derivatives. Alkylation with 1-(benzoyloxy)-4-bromo-2-butenes or -butyne gave somewhat higher yields,[15,16] but these reagents are not commercially available. Also, chloro compounds themselves were desirable for biological investigations. At any rate, yields of alkylation with 1,4-dichloro-2-butenes or -butyne are commensurable with those obtained with use of a monochlorinated allylic reagent.[8] Thus, alkylation of cytosine (**11b**) with (*E*)-1,4-dichloro-2-butene (3-fold excess) gave the corresponding halide **2f** in 45% yield whereas a similar alkylation with (*Z*)-1,4-dichloro-2-butene afforded **4c** in 35% yield. Adenine (**11a**) was smoothly alkylated with the same reagent in the presence of TBAF in THF to afford compound **4d** (51%). The latter readily cyclized in DMSO at room temperature in quantitative yield to give tricyclic derivative **12** (Scheme II), which exhibited a half-life of 6 h as determined by ^{1}H NMR. A similar cyclization of (ω-chloroalkyl)adenines was described.[29] As indicated in our previous studies,[14,17-19] it was more advantageous to prepare guanine derivatives via 2-amino-6-chloropurine (**11e**; see also[16]) than from N^2-acetylguanine. The latter gave poor yields and low N^9/N^7 regioselectivity.[13] Thus, compound **11e** was alkylated with (*Z*)-1,4-dichloro-2-butene with K_2CO_3 in DMSO to give analogue **4e** in 55% yield (Scheme I). Likewise, thymine (**11c**) gave chloro derivative **4f** (41%). 2,6-Diaminopurine (**11d**) was converted into a sirupy and somewhat less stable **6a** in 25% yield with use of 1,4-dichloro-2-butyne. Compound **6a** in turn was hydrolyzed in refluxing 0.1 M HCl for 18 h to alcohol **6b** (72%). In a similar fashion, hydroxy derivatives **2h**, **4g**, and **4h** were readily obtained in 60–70% yields from the respective chlorides **2f**, **4c**, and **4f**. A lower (40%) yield observed in case of compound **4b** after a shorter (2 h) reflux of **4d** in 0.1 M HCl can be explained in terms of partial cyclization to tricyclic derivative **12**. Both chlorine atoms of intermediate **4e** were hydrolytically removed after a 3-h reflux to give olefin **4a** in 52% yield. The sequence **11e** → **4e** → **4a** then represents a new synthesis of this antiherpetic agent in an overall yield of 29%. Previous methods leading to **4a** gave 3% and 42% (selectively blocked alkylating agent), respectively.[9,16] Prolonged hydrolysis (18 h) of **4e** in refluxing 0.1 M HCl led to a cyclization accompanied by hydrolytic deamination to give tricyclic xanthine **13** in 37% yield (Scheme III). 2-Aminoadenallene (**5c**), a new allenic derivative prepared in this study, resulted from isomerization of 2-butynol **6b** in 0.1 M NaOH in 20% dioxane at 100 °C for 2 h (60% yield). According to ^{1}H NMR, allene **5c** was 93% pure containing 7% of contaminant which was tentatively identified[18,19] as 1-butynol **14**. Assignment of position of alkylation in purine derivatives as N^9 followed unequivocally from UV spectra. Similarly, cytosine derivatives **2h**, **4g**, and **6i** exhibited the expected UV profiles of N^1-alkylcytosines.[30] The maxima at ca. 290 nm typical for N^3-alkylcytosines in alkaline solutions were absent.

Table I. Inhibition of Murine Leukemia L1210 Cell Culture with Unsaturated Analogues of Nucleosides[a]

no.	IC_{50}[b]	no.	IC_{50}[b]	no.	IC_{50}[b]
2c	125[c]	**5a**	150	**6i**	>550
2d	200[c]	**5b**	560	**6j**	>500
2e	45	**5c**	60	**7**	200
2f	50	**5d**	45	**8**	100
2g	39	**5e**	>350	**9a**	>500
2h	>550	**5f**	100	**9b**	>350
4a	680	**5g**	620	**9c**	28
4b	490	**6b**	200	**9d**	50
4c	10	**6c**	90	**11d**	30
4d	22	**6d**	39	**12**	>1000
4e	16	**6e**	20	**13**	>1000
4f	46	**6f**	140	**15**	>440
4g	>1000	**6g**	490	araC	0.08
4h	>1000	**6h**	210		

[a] Compounds were tested against murine leukemia L1210 cells during a 24-h period, with subsequent growth measured by a clonogenic assay. The values shown are subject to an error of 10%. For details, see the Experimental Section. [b] Concentration in μM. [c] Previously reported[14] 1 mM.

Adenosine Deaminase (ADA). *Z*-Alkene **4b** is a substrate for ADA from calf intestine albeit of lower potency than either the corresponding *E*-isomer[14] (**2c**), acetylene **6g**, or adenallene[18] (**5a**). This trend is in accord with that noted in case of isomeric 1-alkenes[23] **7** and **8**. By contrast, the antiviral activity[9,10,16] of *Z*-alkene **4a** is superior to that of *E*-isomer **2d**. Also, the cytotoxicities of *Z*-chloro analogues **4c**, **4d**, and **4e** are greater than those of the *E*-isomers **2e**, **2f**, and **2g**. Similar to adenallene[18] (**5a**), 2-aminoadenallene (**5c**) is also a substrate for adenosine deaminase. This deamination can be of preparative significance for the synthesis of guanallene (**5e**).

Antitumor Activity and Cytotoxicity. A. Structure Type 2, 4, 5, and 6. All analogues containing a reactive grouping of an allylic chloride type (compounds **2e–g**, **4c–f**, **5d**, **6c–f**) were inhibitors of murine leukemia L1210 cul-

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(30) Albert, A. In *Synthetic Procedures in Nucleic Acid Chemistry*; Zorbach, W. W., Tipson, R. S., Eds.; John Wiley & Sons: New York, 1973; Vol. 2, p 47 (Table V).

Table II. Inhibition of Macromolecular Synthesis in Murine Leukemia L1210 Cells with Selected Unsaturated Nucleoside Analogues[a]

no.	time, h	percent control		
		DNA	RNA	Protein
4c	1	80	100	100
	4	67	100	100
4d	1	80	98	99
	4	68	73	60
6e	1	60	75	85
	4	35	60	70
	8	5	18	55
9c	1	85	100	100
	4	55	85	50
9d	4	10	50	90

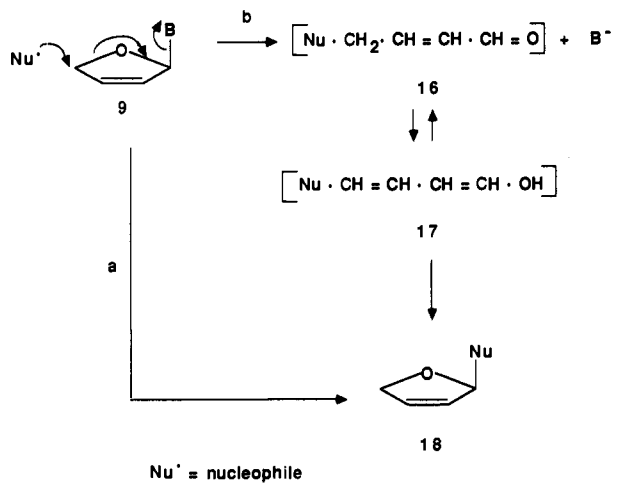
[a] Cells were incubated with IC_{50} levels of analogues for 1, 4, or 8 h and then washed. Incorporation of labeled precursors into macromolecules was determined as described in the Experimental Section. The results represent the average of three determinations with an error of 10% of the numbers shown.

ture. It should be noted that these analogues encompass a wide variety of unsaturated structures including *E*- and *Z*-alkenes, acetylenes, and allenes (**5d**). The IC_{50} values varied from 10 to 140 μM (Table I). The most active analogues (IC_{50} 10–20 μM) were *Z*-alkenes **4c–e** and acetylene **6e**. Analogue **6f** was the least active (IC_{50} 140 μM). It was reported[13] that analogue **2e** inhibited the growth of P388 murine leukemia lymphoid cell culture with IC_{50} (22 μM), half of that observed in L1210 system (45 μM, Table I). The distance between the $C_{1'}$ and $C_{4'}$ appears to be important for activity. Thus, this distance is significantly shorter (3.1 Å) in the most active analogues **4c–e** than in the remaining group of structural types **2**, **5**, and **6** (3.6–4.1 Å).[17] The reactivity of halogen also contributes to the observed inhibition. Only compound **6f** forms an exception. Thus, a lack of activity of the tricyclic product **12** as well as saturated chlorobutyl analogue **15** is in accord with this conclusion.

Studies of the inhibition of macromolecular synthesis with **4c**, **4d**, and **6e** indicated (Table II) that DNA synthesis was the most affected with a general trend of inhibition pattern being DNA > RNA ≥ protein synthesis. This inhibition is clearly time-dependent. We also found that inhibitory activity of **4c** and **6e** was not reversed with any common ribo- or 2′-deoxyribonucleoside at 2 mM concentration. One explanation of this observation is an irreversible inhibition of target enzyme or receptor. It is also obvious that activity of chlorinated analogues cannot be explained in terms of hydrolysis to the corresponding hydroxy compounds. Thus, none of the latter derivatives (Table I) exhibited an IC_{50} lower than the corresponding chloro analogue. In fact, the most active hydroxy analogue was allene **5c**. Because a direct phosphorylation of chloro analogues does not seem feasible,[31] mechanisms of action other than those involving kinase-mediated activation are likely.

Differences in the mode of action of chlorinated analogues of allylic type and 5′-deoxy-5′-halogeno nucleosides[33,35] are apparent. The latter do not contain a reactive allylic halogen and they can be considered as prodrugs of

Scheme IV



very effective therapeutic agents (*ara*-C or 5-fluorouracil). Claims[32,33] that 5′-chloro-5′-deoxy-*ara*-C acts directly as an analogue of *ara*-C appear to be questionable.[36] It is intriguing that chlorinated acyclic analogues **3c** and **3d**, which are structurally related to **2e**, were found to inhibit mammalian methylthioadenosine phosphorylase,[37] an enzyme important for salvaging methionine and adenosine. This factor could conceivably contribute to the biological activity of adenine derivatives **2e**, **4d**, **5d**, and **6c**. An exact mechanism of action of chlorinated unsaturated analogues of nucleosides is still unknown. Clearly, the latter are slow-acting, moderately active inhibitors of growth and their primary effect is on DNA synthesis.

Zone assays in several tumor systems including L1210, solid tumors and a low malignancy cell line (Table III) lend further support to such a conclusion. Generally, results from L1210 zone assays are in satisfactory accord with those listed in Table I, but some exceptions were noted. Thus, *Z*-alkene **4d** was much less active, which can perhaps be explained in terms of intramolecular cyclization under the conditions of the assay. By contrast, allenic analogue **5g** was more active, but inhibition pattern of 2-aminoadenallene (**5c**) exhibited an opposite trend. This may reflect a different phosphorylation capability in these assays. Overall, in this series of moderately active agents, little selectivity was observed between the tumor cells and low malignancy line on one hand and between L1210 and solid tumor systems on the other. Analogues **6c** and **6e** were tested in vivo in murine leukemia P388/o, implanted peritoneally, and in pancreatic ductal adenocarcinoma #03 in BDF_1 male mice. Compound **6e** gave 16.7% ILS in P388/o at 510 and 1020 mg/kg, but it was inactive in adenocarcinoma whereas both tumor systems were unresponsive toward **6c**.

B. **Oxacyclopentenes of Type 9.** Like the chloro analogues, the oxacyclopentenes[38] **9a–d** lack a phosphorylatable hydroxy group but, in addition, an obvious center

(31) A statement[32] that 5′-chloro-5′-deoxyaraC[33] can be directly phosphorylated is contradicted by a finding that it does not undergo cellular phosphorylation.[34]
(32) Birnbaum, G. I.; Budesinsky, M.; Beranek, J. *Can. J. Chem.* **1988**, *66*, 1203.
(33) Beranek, J.; Acton, E. M. *Collect. Czech. Chem. Commun.* **1984**, *49*, 2551.
(34) Fang, K. Private communication.
(35) Ajmera, S.; Danenberg, P. V. *J. Med. Chem.* **1982**, *25*, 999.
(36) Activity of 5′-chloro-5′-deoxyaraC[33] is readily explained by an assumption of minute (2%) hydrolysis to araC. Inherent risks in interpretation of results with derivatives of highly active drugs were discussed.[35]
(37) Chu, S.-H.; Chen, Z-H.; Savarese, T. M.; Nakamura, C. E.; Parks, R. E.; Jr.; Abushanab, E. *Nucleosides Nucleotides* **1989**, *8*, 829.
(38) Assignments of $H_{2'}$ and $H_{3'}$ in the ^1H NMR spectra of oxacyclopentenes[18,19] **9a–d** should be reversed in view of recent data obtained with 2′,3′-didehydro-2′,3′-dideoxyribonucleosides.[39,40] The latter confirmed the original proposal.[41] Similarly, the order of δ values of compound[42] **9e** should be $H_{2'} > H_{4'} > H_{3'}$ instead of $H_{4'} > H_{2'} > H_{3'}$.

Table III. Cytotoxicities of Unsaturated Nucleoside Analogues Determined by Zone Assay[a]

			solid tumors						
			mouse			human			low-malignancy line
no.	μg/disk	leukemia L1210	PO3	C38	CO9	H125	C116	PO1	
2e	500	540–630		550–600					370–400
2f	500	420–520	550						370
2g	500	670–800		700					550–600
4b	250	150–200	200–260						150–240
4c	500	600–640			410			640	450
4d	500	150–220		240–280					200
4e	500	550		570					400
4f	500	500–610			320			550	400
5a	1000	0–160	0						
5c	1000	0–150		180			200[b]		0–40
5d	500	280	300	400		300	300[b]		270–280
5g	1000	400–500		520			500[b]		550
6c	500	630–930	520[c]	950		900			900–950
6e	1000	420–530		650–720			660[b]		500–600
6f	500	450–520		630					520–610
6h	1000	0–220	210						
9a	1000	500–520	320[d]	350–400			460[b]		500
9c	250	400–500	500				400–450	400–600	320–400
9d	1000	300–410		430			260[b]		500
NP-A[e]	1.5	370–500	80	0	40	330–400	270–340	0[b]	0–100

[a] For details, see the Experimental Section. 200 zone units = 6 mm. Compounds with activities lower than 150 units in at least one assay are not listed. [b] The CX-1 tumor culture. [c] At 250 μg/disk. [d] At 500 μg/disk. [e] Neplanocin A as a reference.

of reactivity. Therefore, it was somewhat surprising that pyrimidine analogues **9c** and **9d** inhibited the growth of murine leukemia L1210 culture. Their IC_{50} (28 and 50 μM) corresponded roughly to the activity levels observed with the chlorinated unsaturated analogues (Table I). However, the purine derivatives, **9a** and **9b**, were without effect. All of these analogues are racemic mixtures, and it is therefore conceivable that pure enantiomers of **9c** and **9d** (*R* or *S*) will show an increased potency. Compounds **9c** and **9d** inhibit macromolecular synthesis in a time-dependent fashion with DNA synthesis being affected the most (Table II). The inhibitory activity of thymine derivative **9d** is not influenced by any nucleoside or 2′-deoxynucleoside at 2 mM concentration whereas inhibition by the cytosine analogue **9c** is reversed, somewhat surprisingly, with uridine and 2′-deoxyuridine at 0.5 mM but not with cytidine or 2′-deoxycytidine. A lack of reversal of activity in case of **9d** indicates a possibility of irreversible inhibition. It is worthwhile mentioning that 5-fluorouracil derivative **9e** was more active against L1210 and sarcoma 180 in vivo[42] than 5-fluorouracil itself. Interestingly, at higher doses, an increased toxicity was noticed which may be related to that observed in our study with pyrimidines **9c** and **9d**. In principle, 1-oxa-3-cyclopentenes **9** may serve as alkylating agents reacting with a suitable nucleophile on a macromolecule (Scheme IV). Generally, two pathways are possible: an S_N2 type of attack at the C-2′ of **9** (path a) or alkylation of the C-5′ accompanied by a ring opening (intermediates **16** and **17**) to give, after recyclization, a covalently modified derivative **18** (path b). Additional studies are needed in order to clarify the mode of action of these analogues.

Again, zone assays gave a similar pattern of activity as the previous set of acyclic chloro derivatives (Table III). The activity of adenine derivative **9a**, found also in cell systems employed in antiviral studies (vide supra), is an exception. A lack of sufficient selectivity was also noted in this group of analogues.

Table IV. Antiretroviral Activity and Cytotoxicity of Nucleoside Analogues[a]

	F-MuLV		R-MuLV		cytotoxicity:[a]
compd	EC_{50}	SI	EC_{50}	SI	IC_{50}
4b	175	>1.7	124	>2.4	>300
5a	124	1.4	87.3	1.9	168
5b	8.1	60.4	11.5	42.5	489
5c	118	2.2	118	2.2	255
6g	245	1.7	156	2.6	407
7	194	>1.6	160	>1.9	>300
8	127	2.4	181	1.7	304
10	67.3	3.4	25.0	9.1	281
ddAdo	1	83.8	2.6	32.2	83.8
ddCyd	23	>4.4[b]	13.8	>7.3[b]	>100[b]
AZT	0.035	17000	0.046	12900	594
virazole	8.3	6.3	32.1	1.6	52.0
PFA	110	9.1	42.8	23.4	1000

[a] EC_{50} is the concentration in μM which reduces the virus titer by 50% and IC_{50} is the concentration which reduces cell count by 50%. Selectivity index (SI) = IC_{50}/EC_{50}. For details, see the Experimental Section. [b] SI was not calculated since 71% of cell growth occurred with 0.1 mM ddCyd, the highest concentration tested.

Antiviral Activity. As already mentioned, adenallene (**5a**) and cytallene (**5b**) are excellent inhibitors[20–22] of replication and cytopathic effect of HIV-1 and HIV-2. It was of obvious interest to compare anti-HIV activity of **5a** and **5b** with effects on other retroviruses. The Friend and Rauscher murine leukemia viruses (F-MuLV and R-MuLV) were used in our study. Although cytallene (**5b**) was the most effective agent of this series (Table IV) in all retroviral assays performed to date, important differences in the extent of antiviral activity, cytotoxicity, and mechanism of action clearly emerged. Thus, antiretroviral effect of cytallene (**5b**) against F-MuLV and R-MuLV is ca. 20–40 times lower than that found in HIV assays.[20] Nevertheless, allene **5b** was more active than 2′,3′-dideoxycytidine. Its selectivity index roughly corresponds to that of 2′,3′-dideoxyadenosine, but it surpasses other known antiretroviral agents tested for comparison with the exception of AZT. Adenallene (**5a**), also an effective anti-HIV agent,[20] had only a marginal effect in F-MuLV and R-MuLV assays. Its activity was only twice as high

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Table V. Inhibition of Incorporation of Labeled Precursors into Macromolecules of Noninfected SC-1 Cells with Selected Nucleoside Analogues

no.	concn, μM	percent precursor incorporation[a] thymidine	uridine	leucine
5a	100	80	107	94
	300	46	105	79
5b	100	89	75	91
	300	67	51	75
6g	100	60	67	88
	300	36	66	75
10	100	115	56	99
	300	110	29	53

[a] Cells were grown in the presence of analogues (0–300 μM) overnight. Four hours after addition of labeled precursor, cells were processed and extent of labeling was determined. The results are given only for two highest concentrations of analogues tested, and they are expressed as percent incorporation of precursors into cells grown in the absence of analogues. For details, see the Experimental Section.

as that of 2-butyne **6g**. The latter was inactive[20] against HIV at 100 μM. A borderline activity in F-MuLV and R-MuLV assays was also observed in case of allene **5c**, *Z*-alkene **4b**, and 1-alkenes **7**, **8**. The latter analogues were inactive or marginally active (compound **7**) in HIV assay.[20,43] The lack of anti-HIV activity of **5c** contrasts with a potent effect of the corresponding 2′,3′-dideoxyribonucleoside.[44] This can be attributed to a poor phosphorylation capability of **5c**. The rest of analogues were without effect. Somewhat surprisingly, 6′β-hydroxyaristeromycin (**10**) exhibited a significant antiretroviral effect. It was substantially more inhibitory to R-MuLV than F-MuLV. In this respect, compound **10** resembles phosphonoformate (PFA). It has to be stressed that analogue **10** as well as allenes **5a**, **5b**, and **5c** are racemic mixtures. Therefore, appropriate pure enantiomers may exhibit higher levels of antiretroviral activity.

Studies of incorporation of radioactive precursors into macromolecules (DNA, RNA, or proteins) of SC-1 host cells provided some evidence for the mechanism of action of active antiretroviral agents (Table V). Thus, adenallene (**5a**) inhibited DNA synthesis significantly more than protein synthesis, but it did not interfere with RNA formation at all. By contrast, order of inhibitory activity of cytallene (**5b**) was RNA > DNA > protein synthesis. A marginally effective 2-butyne **6g** followed the pattern DNA > RNA > protein synthesis. These results have indicated that there are significant differences in cellular metabolism of closely related analogues such as **5a** and **5b**. This was also recognized in reversal studies with 2′-deoxyribonucleosides in HIV-infected and uninfected ATH8 cells.[21] 6′β-Hydroxyaristeromycin (**10**) was a stronger inhibitor of RNA than protein synthesis and it did not affect DNA synthesis. Strong inhibition of RNA synthesis with analogue **10** can be probably interpreted in terms of interference with translation process (mRNA synthesis), particularly 5′-capping of mRNA. Nevertheless, compound **10** is not a broad-spectrum antiviral agent, a feature displayed[45] by agents employing such a mechanism (inhibition of *S*-adenosylhomocysteine hydrolase).

In order to investigate the spectrum of antiviral activities of unsaturated nucleoside analogues resultant from this and previous studies,[18,19] tests were undertaken in the following assays comprising both DNA and RNA viruses: Herpes simplex (HSV-1 and HSV-2), vesicular stomatitis (VSV), vaccinia (VV), cytomegalovirus (CMV), parainfluenza 3 (PIV), and measles. These studies also confirmed the antiherpetic activity[9,10,16] of *Z*-alkene **4a**. The respective EC_{50} values were 36 (HSV-1) and 72 μM (HSV-2). Neither adenallene (**5a**) nor cytallene (**5a**) exhibited any significant activity against DNA and RNA viruses tested. This is in agreement with the hypothesis that allenes **5a** and **5b** function as analogues of the corresponding 2′,3′-dideoxyribonucleosides.[17–20] The latter group of analogues also exhibits a narrow spectrum of activity limited to retroviruses[46] with the exception of 2′,3′-dideoxyadenosine, which is an antibacterial agent.[47] The only allene derivative somewhat active against HSV-1 and HSV-2 is 2-aminoadenallene (**5c**) with EC_{50} 139 and 278 μM, respectively. No activity was also observed against African swine fever virus (ASFV).[48] In accord with the aforementioned antitumor assays, chloro derivatives, particularly *Z*-alkene **4e**, and 1-oxa-3-cyclopentenes **9a**, **9c**, and **9d** exhibit significant cytotoxic effects in host cells. Thus, the latter analogues inhibited the growth of noninfected Vero cells with IC_{50} 34–45 μM.

Antiparasitic Activity. *E*-Alkenol **2c** was 100% inhibitory[49] to *Trichomonas vaginalis* at 500 μM.

Experimental Section

General Methods. See ref[18]. The following solvents were used for thin-layer chromatography (TLC): (S_1) CH_2Cl_2–MeOH (9:1), (S_2) CH_2Cl_2–MeOH (4:1), (S_3) CH_2Cl_2–ether (1:1), (S_4) CH_2Cl_2–MeOH (95:5), (S_5), ethyl acetate–MeOH (3:2), (S_6) ethyl acetate–MeOH (4:1), and (S_7) CH_2Cl_2–tetrahydrofuran (THF, 4:1). The NMR spectra were measured at 300 MHz in CD_3SOCD_3 unless stated otherwise. Electron-impact (EI-MS) and chemical ionization (CI-MS) mass spectra were determined with a Kratos MS80 RFA high-resolution instrument. For syntheses of analogues not reported here but which were used in the present biological studies, see ref 14, 18, 19, 23, and 28. Compound **15** was obtained as described.[29]

(*E*)-*N*^{1}-(4-Chloro-2-buten-1-yl)cytosine (2f). A mixture of cytosine (**11b**, 1.11 g, 10 mmol), (*E*)-1,4-dichloro-2-butene (5 g, 40 mmol), and anhydrous K_2CO_3 (2.76 g, 20 mmol) was stirred in DMSO (60 mL) for 48 h. The solvent was evaporated (oil pump, dry ice condenser), and a solid residue was washed with CH_2Cl_2–MeOH (3:2, 7 × 40 mL). The combined washings were evaporated, and the crude product was chromatographed on a silica gel column in solvent system S_1. The fractions containing the product were pooled and evaporated to give compound **2f**: 0.9 g (45%) uniform on TLC (S_2); mp 170 °C dec after crystallization from ethyl acetate–MeOH (4:1); UV (ethanol) max 275 nm (ε 7900), 209 (ε 13 100); ^{1}H NMR δ 7.50 (d, 1, H_6), 7.11 (bs, 2, NH_2), 5.88 and 5.66 (sxt and m, 2, *E*-CH=CH, *J* 15.2 Hz), 5.66 (d overlapped with *E*-CH=CH, 1, H_5), 4.26 and 4.18 (2 d, 4, CH_2); CI-MS *m/z* 202, 200 (M + H, 15.6, 47.3), 164 (M − Cl, 100.0), cytosine peaks 112, 95, 81, 69. Anal. (C, H, Cl, N).

(*Z*)-*N*^{1}-(4-Chloro-2-buten-1-yl)cytosine (4c). The procedure followed the preparation of compound **2f** (30 mL of DMSO, 18 h) using (*Z*)-1,4-dichloro-2-butene. The usual workup and chromatography gave compound **4c** (0.7 g, 35%), uniform on TLC (S_1): mp 246–250 °C after crystallization (see **2f**); UV (ethanol) max 275 nm (ε 7100), 205 (ε 16 200); ^{1}H NMR δ 7.54 (d, 1, H_6), 7.09 (2 poorly resolved s, 2, NH_2), 5.78 and 5.63 (2 m, 2, CH=CH), 5.67 (d overlapped with CH=CH, 1, H_5), 4.37 (apparent t, 4, CH_2);

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CI-MS 164 (M - Cl, 100.0), 112 (11b + H, 22.7). Anal. (C, H, Cl, N).

(*Z*)-*N*⁹-(4-Chloro-2-buten-1-yl)adenine (4d). A mixture of adenine (11a, 2.7 g, 20 mmol) and (*Z*)-1,4-dichloro-2-butene (10 g, 80 mmol) was stirred in 1 M tetrabutylammonium fluoride in THF (30 mL, 30 mmol) for 5 h at room temperature. The mixture was evaporated, and the residue was chromatographed on a silica gel column using solvent S_1. The major UV-absorbing fraction was evaporated to give a sirupy **4d**, which slowly crystallized (2.3 g, 51%): homogeneous on TLC (S_1); mp 190 °C dec after crystallization from ethyl acetate; UV (ethanol) max 261 nm (ε 15200), 212 (ε 18000); ^{1}H NMR (the spectrum scanned after 5 min showed already the presence of compound **12**, 6%) δ 8.13 and 8.09 (2 s, 2, H_2 + H_8), 7.18 (s, 2, NH_2), 5.83 (m, 2, CH=CH), 4.89 and 4.61 (2 t, 4, CH_2); EI-MS 225, 223 (M, 3.6, 10.5), 188 (M - Cl, 39.6), 174 (M - CH_2Cl, 100.0), adenine peaks 135, 108, 81, 66, 53. Anal. (C, H, Cl, N).

Cyclization of 4d. A. Isolation of Compound 12. A solution of compound **4d** (50 mg, 0.23 mmol) in DMSO (10 mL) was stirred for 48 h at room temperature. The solvent was evaporated (oil pump, dry ice condenser), and the resulting solid **12** was washed with solvent S_2 (2 × 10 mL): 50 mg (100%); mp 190 °C dec; UV (pH 7) max 271 nm (ε 13500), 207 (ε 12000); ^{1}H NMR δ 9.28 and 9.21 (2 s, 2, NH_2), 8.66 and 8.41 (2 s, 2, H_2 + H_8), 6.48 and 6.39 (2 sxt, 2, *Z*-CH=CH, *J* 10.3 Hz), 5.19 and 5.16 (2 d, 4, CH_2, *J* 6.8 and 6.4 Hz). Anal. (C, H, Cl, N) × 0.75H_2O.

B. Kinetics. A solution of compound **4d** (20 mg, 0.1 mmol) in CH_3SOCD_3 (0.5 mL) in an NMR tube was kept at room temperature, and the ^{1}H NMR spectra were periodically scanned. The half-life (6 h) was determined from the integration curves of H_2 and H_8 signals of **4d** and **12**.

(*Z*)-2-Amino-6-chloro-*N*⁹-(4-chloro-2-buten-1-yl)purine (4e). A mixture of 2-amino-6-chloropurine (11e, 1.69 g, 10 mmol), (*Z*)-1,4-dichloro-2-butene (5 g, 40 mmol), and anhydrous K_2CO_3 (1.38 g, 10 mmol) was stirred in DMSO (35 mL) for 4 h at room temperature. The solution was evaporated and residue was washed with solvent system S_2 (6 × 30 mL). The resultant solid was chromatographed on a silica gel column using solvent S_3 to give compound **4e**: uniform of TLC (S_4); 1.41 g (55%); mp 155–157 °C after crystallization from solvent S_4; UV (ethanol) max 310 nm (ε 8500), 248 (ε 6600), 223 (ε 21900); ^{1}H NMR δ 8.09 (s, 1, H_8), ca. 6.8 (bs, 2, NH_2), 5.86 and 5.80 (2 m, 2, *Z*-CH=CH, *J* 10.7 Hz), 4.81 and 4.55 (2 d, 4, CH_2, *J* 6.1 and 7.1 Hz); EI-MS 259, 257 (M, 19.7, 28.7), 210, 208 (M - Cl, 20.0, 60.2), 171, 169 (11e, 33.7, 100.0). Anal. (C, H, Cl, N).

(*Z*)-*N*¹-(4-Chloro-2-buten-1-yl)thymine (4f). The method for compound **4c** was followed with thymine (11c, 1.26 g, 10 mmol), half the amount of K_2CO_3, and reaction time 2.5 h. After evaporation of DMSO, the solid residue was washed with solvent S_1 and the crude product was chromatographed in solvent S_4 to give chloroalkene **4f** (0.88 g, 41%), homogeneous on TLC, (S_4): mp 158–160 °C after crystallization from cyclohexane–ethyl acetate (1:1); UV (ethanol) max 270 nm (ε 9800), 206 (ε 11000); ^{1}H NMR δ 11.26 (s, 1, NH), 7.46 (d, 1, H_6), 5.81 and 5.63 (2 m, 2, *Z*-CH=CH, *J* 10 Hz), 1.73 (d, 3, Me); CI-MS 217, 215 (M + H, 32.0, 82.7), 179 (M - Cl, 70.2), 43 (100.0). Anal. (C, H, Cl, N).

(*E*)-*N*¹-(4-Hydroxy-2-buten-1-yl)cytosine (2h). Compound **2f** (1 g, 5 mmol) was refluxed in 0.1 M HCl (100 mL) for 16 h. The solution was cooled in an ice–water bath and the pH was brought to 7 with 0.1 M NaOH. Evaporation gave a solid which was mixed with silica gel (5 g) and the mixture was loaded on a silica gel column. Elution with solvent S_1 afforded compound **2h**, 0.53 g (58%) uniform on TLC (S_2): mp 162–165 °C after crystallization from ethyl acetate–MeOH (9:1); UV (pH 7) max 273 nm (ε 8500), 208 (ε 11200), (pH 2) max 283 (ε 12700), 214 (ε 9900), (pH 12) max 273 nm (ε 8500), 212 (ε 10700); ^{1}H NMR δ 7.49 (d, 1, H_6), 7.05 (2 poorly resolved s, 2, NH_2), 5.65 (d + m, 3, H_5 overlapped with CH=CH), 4.74 (t, 1, OH), 4.21 and 3.89 (2 s, 4, CH_2), (CD_3OD) δ 5.87 (d, 1, H_5), 5.80 (m, 2, CH=CH); CI-MS 182 (M + H, 87.2), 164 (M - OH, 100.0), 150 (M - CH_2OH, 30.8), cytosine peaks 112, 95, 83. Anal. (C, H, N).

(*Z*)-*N*⁹-(4-Hydroxy-2-buten-1-yl)guanine (4a). Compound **4e** (2.58 g, 10 mmol) was refluxed in 0.1 M HCl (100 mL) for 3 h. TLC (S_5) showed a complete disappearance of the starting material. The mixture was worked up as in case of compound **2h**. Chromatography in solvent S_6 afforded product **4a** (1.14 g, 52%): mp 258–260 °C after two crystallizations from ethyl acetate–MeOH (1:1) (lit.[9,16] mp 264–266 °C); UV (pH 7) max 253 nm (ε 10100), 206 (ε 17600), shoulder 271 (ε 9000); ^{1}H NMR δ 10.68 (s, 1, NH), 7.63 (s, 1 H_8), 6.56 (s, 2, NH_2), 5.68 and 5.52 (2 m, *Z*-CH=CH, *J* 11.1 Hz), 4.85 (t, 1, OH), 4.60 and 4.13 (d + t, 4, CH_2); EI-MS 221 (M, 15.7), 204 (M - OH, 39.6), 190 (M - CH_2OH, 13.0), 161 (M - H_2O - HCNO, 32.1), 133 (M - H_2O - HCNO - CO, 15.8), guanine peaks 152, 109, 69, 57. Anal. (C, H, N).

(*Z*)-*N*⁹-(4-Hydroxy-2-buten-1-yl)adenine (4b). Compound **4d** (2.23 g, 10 mmol) was refluxed in 0.1 M HCl (100 mL) for 2 h. According to TLC (S_2) some starting **4d** was present but further refluxing led to decomposition. The mixture was worked up as in case of compound **2h**. Chromatography in solvent S_2 gave compound **4b**: 0.82 g (40%); uniform on TLC (S_2); mp 210–213 °C afer crystallization from ethyl acetate - MeOH (9:1) (lit.[15] mp 198–199 °C and lit.[16] 196–199 °C; UV (pH 7) max 261 nm (ε 13800), 209 (ε 16900); ^{1}H NMR δ 8.13 and 8.10 (2 s, 2, H_2 + H_8), 7.20 (s, 2, NH_2), 5.71 and 5.60 (2 m, 2, *Z*-CH=CH, *J* 10.9 Hz), 4.90 (t, 1, OH), 4.81 and 4.19 (d + t, 4, CH_2); EI-MS 205 (M, 6.3), 188 (M - OH, 3.6), 174 (M - CH_2OH, 100.0), adenine peaks 136, 119, 108, 81, 66, 54. Anal. (C, H, N).

Hydrolytic Cyclization of Chloroalkene 4e. Compound **4e** (1.29 g, 5 mmol) was refluxed in 0.1 M HCl (60 mL) for 18 h. The workup followed the procedure given for **2h**. Chromatography in solvent S_1 gave tricyclic derivative **13** (0.38 g, 37%): mp 311–314 °C after crystallization from methanol; UV (pH 7) max 267 nm (ε 10500), 242 (ε 7300), 204 (ε 15200); ^{1}H NMR δ 11.04 (s, 1, NH), 7.68 (s, 1, H_8), 6.40 and 6.28 (2 sxt, 2, *Z*-CH=CH, *J* 10.0), 4.91 and 4.69 (2 d, 4, CH_2); EI-MS 204 (M, 100.0), 161 (M - HCNO, 82.8), 133 (M - HCNO - CO, 39.2). Anal. (C, H, N).

Further elution of the column with solvent S_6 gave compound **4a** (0.2 g, 18%), mp 257–260 °C, identical with a sample prepared as described above (mixture melting point, IR, and TLC).

(*Z*)-*N*¹-(4-Hydroxy-2-buten-1-yl)cytosine (4g). Compound **4c** was hydrolyzed according to the method described for compound **2h** on a 5-mmol scale. Chromatography afforded alkenol **4g** (0.62 g, 69%): uniform on TLC (S_2); mp 291–294 °C after crystallization from solvent S_6; UV (pH 7) max 273 nm (ε 9700), 204 (ε 15600); ^{1}H NMR δ 7.52 (d, 1, H_6), 7.02 (bs, 2, NH_2), 5.65 and 5.40 (2 m + d, 3, CH=CH + H_5), 4.77 (poorly resolved t, 1, OH), 4.29 and 4.08 (d + poorly resolved t, 4, CH_2); CI-MS 182 (M + H, 100.0), 164 (M - OH, 33.6), 150 (M - CH_2OH, 7.1), 112 (11b + H, 16.4). Anal. (C, H, N).

(*Z*)-*N*¹-(4-Hydroxy-2-buten-2-yl)thymine (4h). Chloroalkene **4f** (1.07 g, 5 mmol) was refluxed in 0.1 M HCl (60 mL) for 18 h. A subsequent workup followed the procedure for compound **2h**. Chromatography in solvent S_7 gave alkenol **4h** (0.73 g, 74%): uniform on TLC (S_1 and S_7); mp 166–169 °C after crystallization from CH_2Cl_2–THF (1:1); UV (pH 7) max 271 nm (ε 10100), 206 (ε 10000); ^{1}H NMR δ 7.43 (s, 1, H_6), 5.65 and 5.38 (2 m, 2, CH=CH), 4.75 (bs, 1, OH), 4.28 and 4.09 (2 d, 4, CH_2), 1.73 (s, 3, Me); CI-MS 197 (M + H, 100.0), 179 (M - OH, 59.2), 127 (11c + H, 53.8). Anal. (C, H, N).

2,6-Diamino-*N*⁹-(4-chloro-2-butyn-1-yl)purine (6a). The reaction was performed as in case of compound **2f** with 2,6-diaminopurine sulfate hydrate (2.66 g, 10 mmol) dried by coevaporation with DMSO (3 × 70 mL), K_2CO_3 (5.52 g, 40 mmol), and 1,4-dichloro-2-butyne (4.92 g, 40 mmol) in DMSO (70 mL) for 24 h. After the usual workup the crude material was chromatographed in solvent S_7 to afford a sirupy product **6a** (0.59 g, 25%). This product decomposes at room temperature to give several slower moving components (TLC). It was used immediately in the subsequent step (hydrolysis to alkynol **6b**).

2,6-Diamino-*N*⁹-(4-hydroxy-2-butyn-1-yl)purine (6b). Compound **6a** (0.24 g, 1 mmol) was refluxed in 0.1 M HCl (20 mL) for 18 h. The routine workup (see **2h**) and chromatography in solvent S_2 led to alkynol **6b** (0.16 g, 72%): mp 230–232 °C after crystallization from MeOH–water (3:2); UV (pH 7) max 279 nm (ε 10200), 254 (ε 8700), 217 (17600); ^{1}H NMR δ 7.75 (s, 1, H_8), 6.70 and 5.86 (2 s, 4, NH_2), 5.19 (t, 1, OH), 4.83 and 4.07 (d + m, 4, CH_2); EI-MS 218 (M, 100.0). Anal. (C, H, N) × 0.25H_2O.

(±)-2,6-Diamino-*N*⁹-(4-hydroxy-1,2-butadien-1-yl)purine (5c). Alkynol **6b** (0.22 g, 1 mmol) was refluxed in 0.1 M NaOH in 20% dioxane (20 mL) for 2 h. The solution was cooled to 5–10 °C (ice bath), and the pH was brought to 7 with 0.1 M HCl (pH

meter). The resultant mixture was evaporated and further workup followed the procedure described for compound **4b**. Chromatography afforded allenol **5c** (0.13 g, 60%): mp ca. 210 °C dec; UV (pH 7) max 276 nm (ε 11 300), 217 (ε 19 500); ^{1}H NMR δ 7.75 (s, 1, H_8), 7.12 and 6.15 (qt + q, 2, CH=C=CH), 6.80 and 5.90 (2 s, 4, NH_2), 5.14 (t, 1, OH), 4.11 (poorly resolved q, 2, CH_2); purity 93% (7% of 1-butynol[18,19] 14); ^{13}C NMR 195.93 (=C=), 106.16 and 94.28 (CH=, allene), 59.57 (CH_2), peaks of **11d** 161.16, 156.80, 151.27, 135.30 and 113.87; CI-MS 219 (M + H, 79.0), M (218, 100.0). Anal. (C, H, N) × 0.25H_2O.

Deamination with Adenosine Deaminase. A. Compound 4b. Alkenol **4b** (2 mg, 10 μmol) and adenosine deaminase from calf intestine (type[50] II, Sigma Chemical Co., St. Louis, MO, 0.2 unit) were magnetically stirred in 0.05 M Na_2HPO_4 (pH 7, 3 mL) at room temperature.[51] Aliquots were removed, and they were examined by TLC (S_1). After 30 h a partial deamination was noted; the reaction was complete in 10 days.

B. 2-Aminoadenallene (5c). The procedure A was modified as follows. Allene **5c** (0.6 mg, 2.5 μmol) was dissolved in warm buffer (pH 7.5, 0.2 mL). Adenosine deaminase (type VIII) in the same buffer (0.4 unit, 0.2 mL) was added at room temperature and the mixture was stirred. The removed aliquots were evaluated by TLC (S_2). The deamination was 95% complete in 36 h.

Murine Leukemia L1210 Assay. Analogues were dissolved in water, ethanol, or DMSO at a concentration of 10–100 mM. The solutions were stored at −20 °C. The L1210 cells were grown in sealed vessels using Fischer's medium (Gibco, Grand Island, NY) supplemented with 10‰ horse serum and gentamycin. The doubling time for this cell line is ca. 24 h. An initial estimate of drug cytotoxicity was obtained by measuring growth over several days in the presence of graded analogue levels. A Coulter ZBI cell counter and Channelyzer was used to assess cell density and size. Once an approximate IC_{50} value was obtained, a final determination was made by using a clonogenic assay. Cells were treated with analogue for 24 h, and then they were mixed with growth medium plus 0.45% agar in sterile tubes. After 7 days, the number of colonies was counted. For each experiment, ca. 1250 cells were plated. The plating efficiency for untreated cells was 80%. The results are listed in Table I.

Incorporation Studies. After exposure to IC_{50} levels of analogues for 1, 4, or 8 h, the cells were collected by centrifugation, and they were resuspended in the growth medium and incubated with 0.1 μM levels of labeled thymidine, uridine, or leucine for 30 min at 37 °C. Incorporation of label into nucleic acids or protein was then determined. Each tube contained cells (7 mg, wet weight) in the growth medium (1 mL). These studies were carried out in triplicate. The cells were collected by centrifugation and washed twice with 0.3 M $HClO_4$ to remove exogenous substrates and intracellular low molecular weight radioactive materials. The pellets were then solubilized with NCS (Amersham, England), and the resulting solutions were mixed with a liquid phosphor for determination of incorporation of substrates into macromolecules by liquid scintillation counting. The results are summarized in Table II.

Reversibility Studies. To examine the effect of nucleosides on viability, cells were exposed to IC_{50} levels of analogues in the presence of ribonucleosides and 2′-deoxyribonucleosides. The concentration of antagonist necessary to restore cell growth to control values were determined as described above.

Assay of Cell Viability by Soft Agar Colony Formation. A described procedure was followed.[52-54] All analogues were dissolved or suspended in water, ethanol, or DMF (depending on solubility) at an initial concentration of 10 mg/mL. Tumors used were as follows: murine leukemia L1210, mouse solid tumors PO3, C38, or CO9, and occassionally, human solid tumors Lung-H125, Colon #116, #8, #CX-1, and Panc-O1. For comparison, a low-malignancy cell line was included in the tests.

Preparation of Cell Suspensions. The cells were removed from their syngeneic host, 1–1.5 g was cut into 200–300-mg fragments in Hank's balanced salt solution (HBSS), and they were further disrupted with a Stomacher 80 for 20 s. This material was poured through a 100-mesh sieve, the residual material was forced through, and the sieve was rinsed twice with HBSS. The material was rapidly drawn up (10 times), and it was slowly pushed down in a glass syringe (5 mL) and poured through a 100-mesh sieve with two HBSS rinses. It was then centrifuged at 150*g* for 5 min and resuspended in HBSS (15 mL).

Plating Conditions for Tumors Used in the Assay. A hard-agar bottom layer (0.8% noble agar) containing media and serum (1:1 Fishers–CMRL-1066 plus 12% horse serum plus 0.6% tryptic soy broth) was poured into 60 mm plastic dishes and it was allowed to solidify. A soft agar top layer (0.44% noble agar) containing enriched media, serum (1:1 Fishers–CMRL-1066 plus 12% horse serum), and titered cells was poured on top and allowed to solidify. The cells are plated at 1 × 10^5 or less per 60 mm plate to obtain 300–1000 colonies per plate.

Zone Assay Methodology. Each analogue dilution (0.05 mL) was applied on a disk (6.5 mm, standard hole punch of Whatman No. 1 filter paper). The disks were allowed to dry, and then they were placed midway between the center and the edge of the dish containing both leukemia and solid tumor cell type. The plates were incubated for 6–10 days, and they were examined on an inverted microscope (10 × eyepiece with a micrometer scale, 4 × objective) for measurement of the zone of inhibition. A zone of inhibition (measured from the edge of the disk to the first colony) of less than 150 units (1 unit = 32 μm) for either the leukemia or solid tumor cells indicates an agent of insufficient cytotoxic activity. A difference of at least 250 units between the zone for any solid tumor and leukemia is indicative of a significant differential effect. The results are summarized in Table III.

Antiviral Assays. The analogues were dissolved in DMSO (10 mg/mL), and they were diluted in cell culture medium to 1 mg/mL. For antiretroviral assays compounds were solubilized in DMSO at 30 mM. They were diluted with EMEM-5 (Eagle's minimal essential medium) before refeeding the cell cultures. The reference compounds were as follows: acyclovir, virazole, 2′,3′-dideoxyadenosine (ddAdo), 2′,3′-dideoxycytidine (ddCyd), 3′-azido-3′-deoxythymidine (AZT), and phosphonoformate (PFA). They were solubilized in saline and diluted as stated above.

Host Cells. Vero, L929, and human foreskin fibroblast cells were grown in stationary culture with MEM containing 10% fetal bovine serum, glutamine, and antibiotics. The cells were transferred by trypsinization weekly. Primary rabbit kidney cells were prepared from the trypsinized kidneys of baby rabbits. The SC-1 and XC cells were obtained from the American Type Culture Collection. They were propagated in EMEM-10 containing glutamine, gentamycin, and 10% fetal bovine serum. Trypsinized cells were passaged biweekly.

Viruses. The antiviral effect of the analogues was assayed in the following systems: Friend murine leukemia virus (F-MuLV), Rauscher murine leukemia virus (R-MuLV), herpes simplex virus (HSV-1, McIntyre and HSV-2, MS), vesicular stomatitis virus (VSV, Indiana strain), parainfluenza-3 virus (PIV, HA-1 strain), vaccinia virus (VV, vaccine strain), cytomegalovirus (CMV, strains AD169 and Davis), and measles virus. HSV-1 and HSV-2 were originally obtained from the American Type Culture Collection (ATCC), and they have undergone numerous passages in primary rabbit kidney cells or Vero cells. The F-MuLV was purchased from the ATCC. The spleens from the third serial passage in mice (intraperitoneal inoculation) were homogenized (10% weight/volume) in tissue grinders in basal medium of Eagle (BME). After clarification by centrifugation, the supernatant was stored in

(50) Two recent lots of this enzyme (#107F-8015 and 128F-8010) exhibited an unusually low activity (below 0.2 unit/mg of solid).

(51) The same procedure was used for deamination of alkenes[23] 7 and 8.

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aliquots at −70 °C. The R-MuLV, obtained from Electro-Nucleonics, Inc., was inoculated intraperitoneally into Balb/c mice, and the spleens were collected 28 days after inoculation. Spleen homogenates following the second passage in mice were prepared as described for F-MuLV. Assay conditions are given only for those viruses where an antiviral activity was observed with any analogue.

Assay of F-MuLV and R-MuLV. The assays followed a described procedure[55] with a slight modification. The Costar trays (24 wells) were seeded with 0.9 mL of SC-1 cells (1×10^4 cells/well). After overnight incubation, polybrene (Sigma Chemical Co.) was added to each well (0.1 mL, giving the final concentration 8 mL/mL). The cultures were incubated for 1 h and then drained. The virus (0.2 mL/well) diluted with EMEM-5 containing 8 μg/mL of polybrene was added to each well, and the mixture was incubated for 2 h at 37 °C. The virus was removed, the cultures were washed with EMEM-5 (1 mL), and they were refed with EMEM-5 (0.9 mL). Diluted analogue was added to each well to give the final concentration indicated for each experiment. After 72 h at 37 °C, the medium was removed, and the cultures were irradiated for 20 s with UV light with two 15-W G15T8, GE germicidal tubes (860–870 $\mu W/cm^2$) at a distance of 31 cm. The XC cells (1 mL containing 2×10^5 cells) in EMEM-10 were added to the irradiated cultures and incubated for additional 48 h at 37 °C. The medium was replaced after 24 h. The cells were fixed with 10% formalin in 2% sodium acetate, and they were stained with crystal violet 48 h after seeding the XC cells. Multinucleated cells were counted with the aid of dissecting microscope. The means of the counts of multinucleated cells in the analogue-treated cultures were compared with those in nontreated cultures. The EC_{50} were determined by a linear regression analysis. The results are summarized in Table IV.

Cytotoxicity. The SC-1 cells (1×10^4 cells/mL) were seeded into each well of Costar 24-well tray. After overnight incubation at 37 °C, the drained cultures were refed with EMEM-10 (0.9 mL). Appropriately diluted analogues were added to each well (0.1 mL) in triplicate and the incubation at 37 °C continued for additional 72 h. The medium was aspirated, the cell monolayers were washed with phosphate-buffered saline (1 mL/well), and, finally, the cells were removed by trypsinization (EDTA trypsin, 0.5 mL/well). The cells (0.3 mL was added to 9.7 mL of 0.9% saline) were counted in a Coulter counter. The mean cell counts from three wells for each drug dilution were used to determine the percent of control cell counts. Linear regression analysis was used to calculate the IC_{50} values. For results, see Table IV.

Inhibition of Macromolecular Synthesis with Nucleoside Analogues. The SC-1 cells were incubated with nucleoside analogues as described above. Thymidine (C^3H_3, 2.5 μCi), uridine (^{3}H-5,6), or leucine (^{3}H-4,5) was added to appropriate wells. Four hours later the medium was aspirated and the wells were washed with ice-cold polybrene sulfate (PBS, 3 × 0.5 mL). The cells were then lysed by adding lysis buffer: 1% sodium dodecyl sulfate (SDS), 0.01 M EDTA, and 0.01 M Tris, pH 7.7. Aliquots (100 μL) of the lysates were placed on Whatman 2.1 GF/B filter and dried. The filters were successively washed with 5% CCl_3COOH, 95% EtOH, absolute EtOH, and ether. After drying, the filters were placed in scintillation vials with 0.3 M KCl (0.8 mL) and gently swirled. Prior to counting, insta-gel (10 mL) was added and the samples were throughly mixed by vortexing. The results are listed in Table V.

Assay of HSV-1 and HSV-2. Vero cell monolayers in 96-well Costar trays formed 24 h after seeding trypsinized cells (0.2 mL, 1.2×10^6 cells/mL). The growth medium (EMEM + 10% fetal bovine serum and antibiotics) was replaced with BME-3 (50 μL). Triplicate wells across the top row of the tray received the analogue (50 μL). The analogue was serially diluted (2-fold) with use of a multichannel micropipet. Duplicate wells of each analogue dilution were infected by the addition of appropriately diluted stock virus (50 μL), while the third row of wells containing diluted analogue was fed with BME-3 (50 μL) to serve as a cytotoxicity control. The cultures were incubated at 37 °C until viral cytopathology was distinct in control cultures (usually 24–48 h after infection). The cultures were stained with crystal violet, and the analogue concentration which reduced the viral cytopathology by 50% or was cytotoxic for 50% of cells was recorded.

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