Synthesis of 1,N^6-etheno-2-aza-adenosine (2-aza-ϵ-adenosine): a new cytotoxic fluorescent nucleoside

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ABSTRACT

1,N^6-Etheno-2-aza-adenosine was synthesized by treating 1,N^6-etheno-adenosine with alkali, followed by nitrosation. The mechanism of formation of this novel nucleoside was elucidated using adenosine tritiated at C-8 and C-2, and was found to deformylate exclusively at C-2. This new 2-aza nucleoside fluoresces at 494 nm when excited at 358 nm. Toxicity study showed the compound is active in a rat mammary tumor tissue culture line, but inactive in HeLa and Glioma 26 tissue culture lines. It was also found to selectively inhibit the thymidine incorporation into DNA in a rat mammary tumor, but exhibits no ill effect on normal proliferative tissue. The reactive intermediate 3-β-D-ribofuranosyl-4-amino-5-(imidazol-2-yl) imidazole was identified and was found to be an active agent in tissue culture.

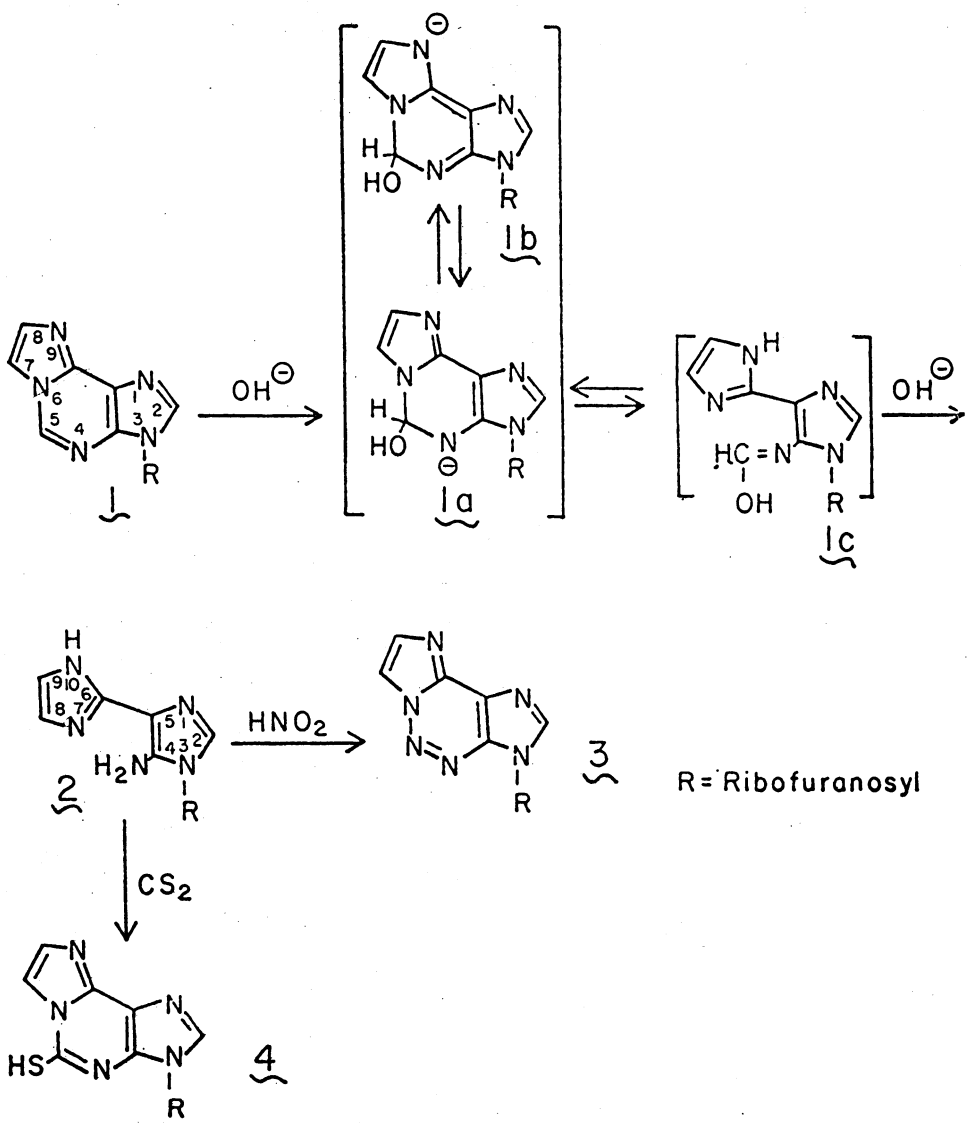
INTRODUCTION

Cytochemical methods employing fluorescent labeling compounds have been found useful to study the interaction of drugs and cells in both *in vitro* and *in vivo*. Previously, fluorescent alkylating agents[1] have been synthesized by us and used in the study of the interaction of alkylating agents and cancer cells[2]. For a similar reason, it was hoped that the interaction of nucleoside and cells could be studied with the aid of a fluorescent nucleoside. Synthetic study was therefore initiated in our laboratory to obtain such compounds, using the reaction of adenine and cytosine with chloracetaldehyde

reported by Kochetkov *et al*[3]. The synthesis of fluorescent derivatives of adenosine by the same reaction indeed yielded 1,N^6-etheno-adenosine (ε-adenosine), *1*, as reported by Secrist *et al*[4] recently.

While compound *1* was useful for biochemical fluorescent studies, at biological level the fluorescent spectrum of this compound does overlap the autofluorescence of the tissue or cell. In addition, as will be seen in the cytotoxicity study, it does not have the required cytotoxicity to be of interest in our anti-neoplastic agent study.

Since N^1-alkylated adenosine rearranges by base catalysis[5-8], it is of interest to investigate the behavior of *1* under similar conditions. While *1* was stable in weak alkali, it decomposed and lost fluorescence in 0.05 N NaOH. Upon reacidification, compound *2* was isolated as the decomposition product. The presence of the etheno group apparently caused the carbon 5 of *1* to be susceptible to nucleophilic attack. This interesting reaction can be attributed in part to the stabilization of the intermediate anion (*1a*, *1b*) via resonance of the imidazole ring nitrogen. The attack on the carbon 5 by the hydroxide ion followed by ring opening and subsequent deformylation would lead to compound *2* (Scheme 1). This mechanism is supported by a study of the change in NMR of *1* during base catalysis.



SCHEME 1

When *1* was allowed to decompose in 0.5 N sodium hydroxide in D_2O, the H_7 and H_8 doublet (Fig. 1) collapsed with upfield shift to a singlet, integrating for 2 protons. This indicates that without N substitution, both imidazole protons (H_8 and H_9 of *2*), are magnetically equivalent. The H_2 shift is upfield. The H_5 underwent an upfield shift, as the formate group was formed. In the NMR spectrum of purified *2* (see Experimental Section) the signal corresponding to the formate proton is absent.

The structure of *2* was further confirmed by its mass spectrum, where both the parent ion m/e 281 as well as the (B + 1) ion, m/e 148, can be seen. To ascertain that deformylation took place exclusively at C-5 under this condition[10,11] [8-^{3}H] adenosine was used as the starting material to prepare compounds *1* and *2*, labeled at C-2. The specific radioactivity of the products remained the same throughout the reactions. However, when [2-^{3}H] adenosine was used as the starting material to prepare compound *1*, labeled at C-5, compound *2* was non-radioactive. These results confirm that the deformylation occurred only at the C-5 position and not at C-2 of *1*. Finally, cyclization of *2* with triethyl orthoformate and p-toluenesulfonic acid led to the parent compound *1*. This experiment provided additional support in that the sugar ring was not affected throughout these reactions, even though *2* can be considered as an N-glycosyl derivative.

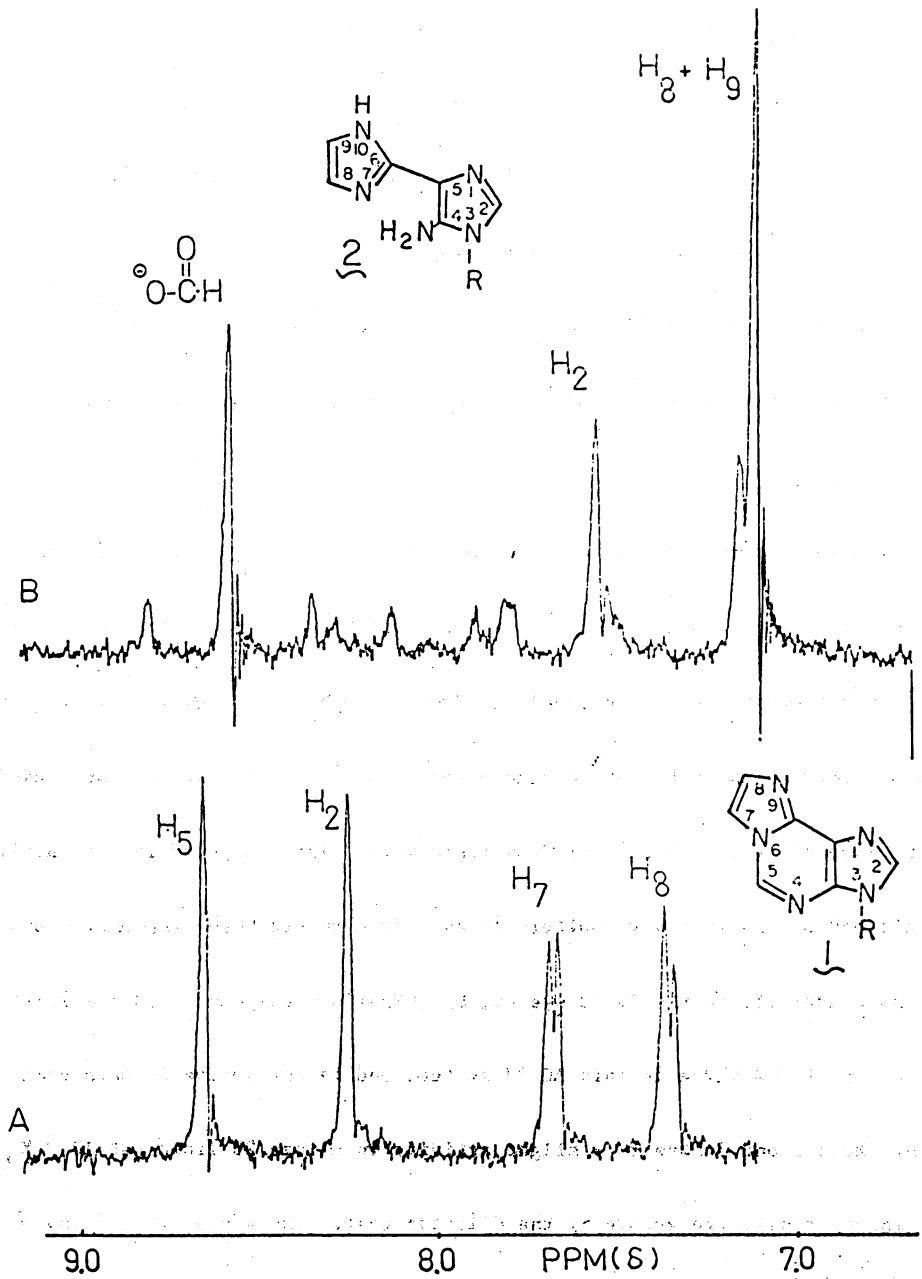


Fig. 1 NMR spectra of compound *1* in 0.5N sodium hydroxide in D_2O. A, 0 hr; B, 18 hr.

When compound *2* was allowed to react in acetic acid with sodium nitrite, 1,N^6-etheno-2-aza-adenosine (2-aza-ε-adenosine), *3*, was obtained. 2-Aza-ε-adenosine exhibited strong blue fluorescence, which can be detected at 10^{-7} M. The fluorescence emission spectrum (Fig. 2) shows a maximum at 494 nm and the excitation spectrum shows a maximum at 358 nm, (pH = 7.0 potassium citrate buffer). Therefore, it should have a useful range of fluorescence that will not be obscured by natural autofluorescence of the biological system in our cytochemical study.

Initial cytotoxicity of *3* is shown in Fig. 3a-c. It is of particular interest to note the selective activity of *3* which is active in the AC 33 system, a rat mammary tumor line developed from a lymphoma bearing rat after treatment and regression caused by an alkylating agent[15]. This tumor has been used in our laboratory as a model for drug resistance. This compound is less active in HeLa and Glioma 26 tissue culture lines. Even though both compounds *1* and *2* show some activity in the AC 33 system, by comparison compound *1* is the least active one of the three in this AC 33 system, and is not active in HeLa cell lines. Both *1* and *3* have only slight activity in Glioma 26 lines at 10^{-4} M; this is not considered active by the dilution test. Therefore, both *2* and *3* are potentially of interest to further cancer chemotherapy research. Wh

incubated with AC 33 tissue culture system, only *3* shows membrane fluorescence in the initial period. Further cytochemical work will be necessary to explain its activity. The mercapto derivative[4] is included in this report. Preliminary study did not show activity in the AC 33 system. Other derivatives that can be synthesized via *2* have yet to be prepared and tested.

The drug distribution study of *3* was carried out with tritium labeled *3* in AC 33 tumor bearing rats. In order to study the effect of *3* on DNA syntheses, double labeled experiments with [methyl-^{14}C]-thymidine were also performed. Three groups of rats were used, each receiving [^{3}H]-*3* , a combination of [^{3}H]-*3* and [^{14}C]-thymidine, and [^{14}C]-thymidine, respectively. The tissue distribution of *3* and [^{14}C]-thymidine was obtained from the supernatants of the homogenates (Table I). Compound *3* was taken up by all organs and, except for brain and liver, the distribution is parallel to that of thymidine (Pearson correlation coefficient, r = 0.81). The DNA's of different organs were extracted from the precipitate that was separated from the homogenate, and the radioactivities of these extracts were determined (Table II). The aza compound *3* was found not to be incorporated into DNA in all organs, except in low levels in the liver. It is not known that the radioactivity that was incorporated into the liver DNA is in the form of compound *3* or a metabolite of *3*. The most interesting result

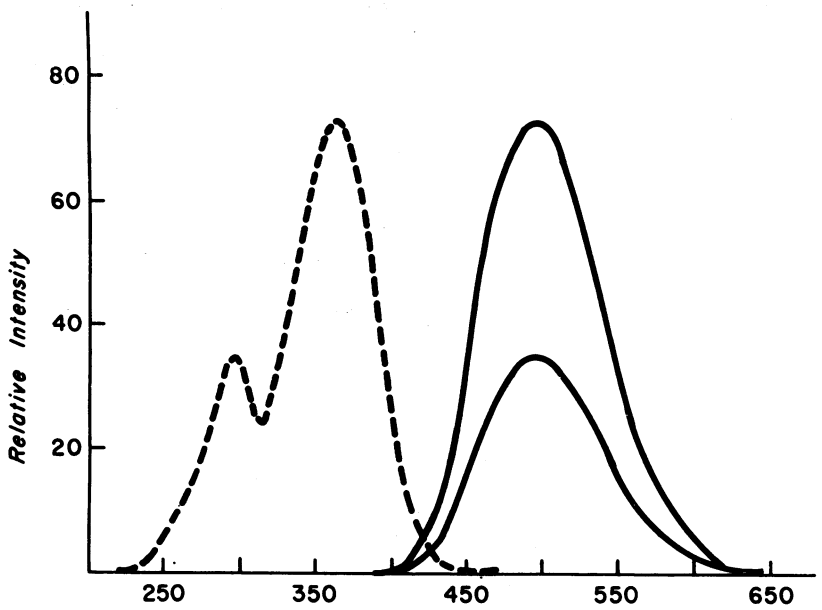


FIG. 2 Fluorescence spectra of 3 in citrate buffer (0.05 M, pH = 7.0). ___Emission (upper:excited at 358 nm; lower:excited at 295 nm); --- Excitation.

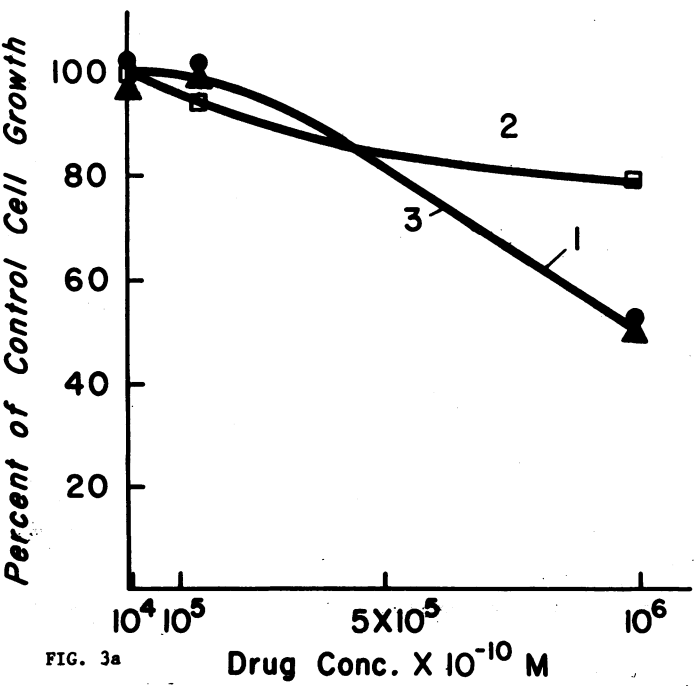
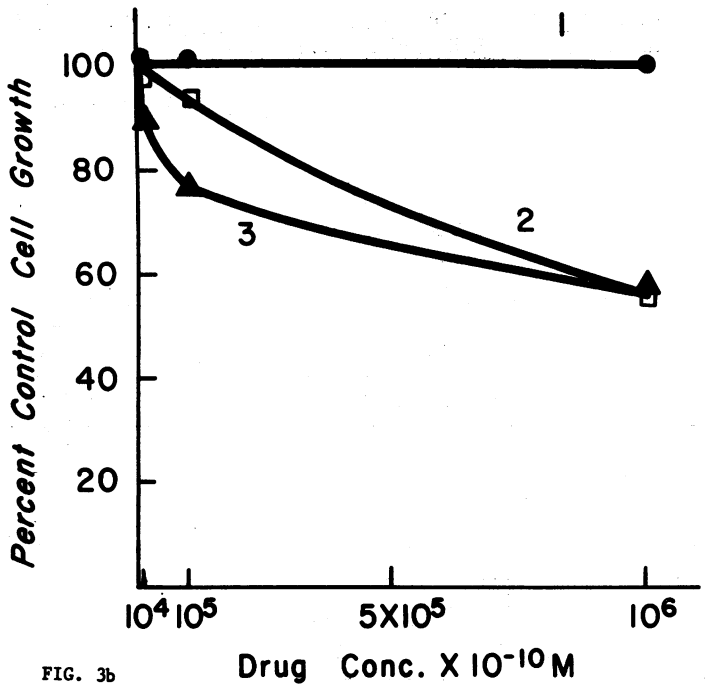


FIG. 3a


Percent Control Cell Growth
100
80
60
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1
2
3
10^4 10^5
$5X10^5$
10^6
Drug Conc. X 10^{-10} M

FIG. 3b

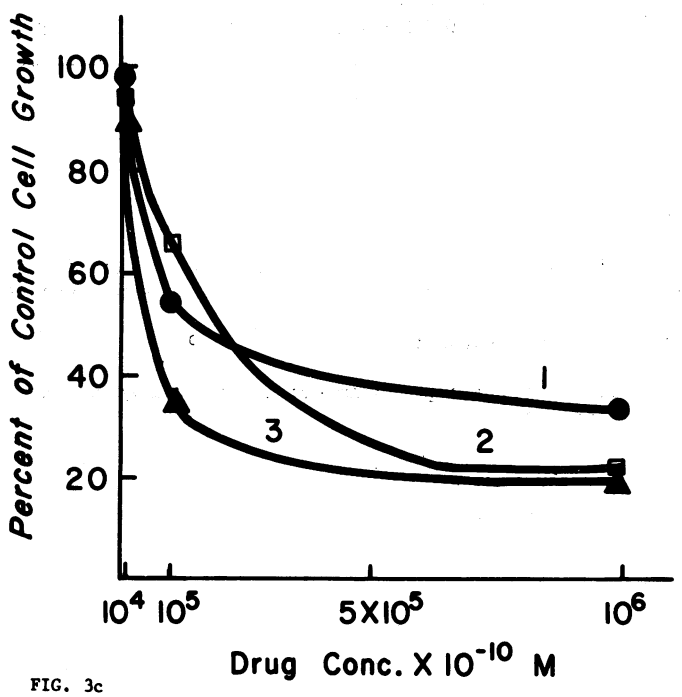
Percent of Control Cell Growth
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FIG. 3c

TABLE I: RADIOACTIVITY DISTRIBUTION OF [^{3}H]-3 AND [^{14}C]-THYMIDINE IN TUMOR (AC 33) BEARING RATS[a]

Tissue	Drug[b]			
	[^{3}H]-3	[^{3}H]-3 + [^{14}C]-Thymidine		[^{14}C]-Thymidine
	^{3}H	^{3}H	^{14}C	^{14}C
Brain	0.160 ± 0.024[c]	0.209 ± 0.063	0.457 ± 0.097	0.360 ± 0.114
Heart	0.247 ± 0.010	0.314 ± 0.014	0.240 ± 0.075	0.288 ± 0.010
Intestines	0.311 ± 0.062	0.478 ± 0.115	0.244 ± 0.017	0.194 ± 0.053
Kidney	0.387 ± 0.024	0.651 ± 0.107	0.318 ± 0.008	0.255 ± 0.056
Liver	0.468 ± 0.195	0.435 ± 0.081	0.463 ± 0.040	0.342 ± 0.047
Mammary Gland	0.136 ± 0.059	0.162 ± 0.074	0.118 ± 0.037	0.083 ± 0.014
Ovary	0.285 ± 0.091	0.283 ± 0.128	0.174 ± 0.024	0.201 ± 0.014
Pancreas[d]	0.327	0.322	0.184	0.205
Tumor	0.136 ± 0.037	0.167 ± 0.065	0.225 ± 0.097	0.181 ± 0.067
Serum[e]	2.30 ± 0.19	6.00 ± 0.33	4.10 ± 0.30	5.12 ± 0.90

a Lewis-Wistar rats (female) are 10 days after transplantation with (AC 33) tumor cells. Radioactivities of tissues were obtained from supernatants of tissue homogenates.

b Drug concentrations (see Experimental Section).

c Mean ± S.E. of percentage activities per gram wet tissue (based on the radioactivities injected in the rats).

d Single determination.

e Calculations based on 10 ml serum.

TABLE II: RADIOACTIVITY DISTRIBUTION OF [^{3}H]-*3* AND [^{14}C]-THYMIDINE IN DNA OF TUMOR (AC 33) BEARING RATS[a]

	Drug[b]			
Tissue	[^{3}H]-*3* / [^{3}H]	[^{3}H]-*3* + [^{14}C]-Thymidine / [^{3}H]	[^{3}H]-*3* + [^{14}C]-Thymidine / [^{14}C]	[^{14}C]-Thymidine / [^{14}C]
Brain	0	0	0.003 ± 0.001[c]	0.003 ± 0.001
Heart	0	0	0.004 ± 0.000	0.009 ± 0.001
Intestines	0	0	0.327 ± 0.099	0.121 ± 0.090
Kidney	0	0	0.018 ± 0.006	0.014 ± 0.007
Liver	0[d]	0[d]	0.043 ± 0.017	0.035 ± 0.010
Mammary Gland	0	0	0.002 ± 0.001	0.023 ± 0.008
Ovary	0	0	0.073 ± 0.047	0.021 ± 0.004
Pancreas	0	0	0.032[e]	0.038[e]
Tumor	0	0	0.085 ± 0.031	0.444 ± 0.293

a Lewis-Wistar rats (female) are 10 days after transplantation with (AC 33) tumor cells.

b Drug concentrations (see Experimental Section).

c Mean ± S.E. of percentage activities per gram wet tissue (based on the radioactivities injected in the rats).

d This organ has detectable counts of about 0.01%.

e Single determination.

is that *3* does inhibit (ca. 80% inhibition) the thymidine incorporation into DNA in the tumor ($p < .01$) and yet stimulates the uptake in intestine ($p < .01$). These data would suggest that the drug does not have deleterious effects on normal proliferative tissue, which is an important aspect to be considered in the use of chemotherapeutic agents for neoplasm.

EXPERIMENTAL SECTION

UV absorption spectra were measured on a Beckman model DB-G recording spectrophotometer (Table III); fluorescent spectra were determined with an Aminco-Bowman spectrofluorometer (Fig. 2); NMR spectra were measured with a Varian spectrometer A-60. Chemical shifts are given in ppm on a δ scale; coupling constants are expressed in Hz; TMS was used as internal standard. Thin layer chromatography was carried out by the ascending method with Eastman Chromagram Sheets 6065 (cellulose with fluorescent indicators). Solvent systems are S_1, 2-propanol; ammonium hydroxide:water (7:1:2, v); S_2, ethanol:ammonium acetate 1M (7:3, v). Radioactivity was measured in an Intertechnique SL30 liquid scintillation spectrometer. Mass spectra were obtained with an MS 9 instrument at 70 volts. [Methyl-^{14}C]- thymidine and [8-^{3}H]-adenosine were obtained from Schwarz-Mann and [2-^{3}H]-adenosine was a product of New England Nuclear.

Table III: QUANTITATIVE ULTRAVIOLET ABSORBTION DATA

Compound	pH	λ max (nm)	(10^{-3} ε)	λ max (nm)	(10^{-3} ε)	λ min (nm)
2	1.5	281	(11.9)			228
	7.0	274	(13.3)			226
	14.0	266	(15.7)			228
3	1.5	265	(4.6)	241	(21.6)	---
	7.0	290	(5.0)	242	(21.5)	256
	14.0	290	(4.5)	242	(22.4)	259
4	1.5	312	(18.0)	239	(13.9)	260
	7.0	317	(15.2)	246	(13.0)	266
	14.0	318	(14.4)	248	(13.9)	266

1,N^6-Etheno-adenosine (ε-adenosine), 3-β-D-ribofuranosyl imidazol, (2,1-*i*) Purine Hydrochloride, *1*. This compound was prepared according to the procedure of Barrio *et al*[12]. It was found to be stable at pH 9.0 in tris buffer, but loss of fluorescence was observed when treated with dilute NaOH.

3-β-D-Ribofuranosyl-4-amino-5-(imidazol-2-yl) imidazole, *2*. A solution of *1* (2 g, 6.1 mmole) in 40 ml of 0.5 N NaOH was allowed to stand at room temperature overnight. The pH of the solution was adjusted to 7.5 by concentrated HCl. The aqueous solution was evaporated to dryness with a rotary evaporator, and the residue was triturated with 10 ml cold water; the insoluble product was then collected by filtration. Recrystallization from 15 ml hot water yielded pure *2* as white needles, (1.13 g, 66%), m.p. 225-227° [dec.] after air drying; NMR (DMSO-d_6 + D_2O) δ 7.55 (s, 1, H_2), 7.10 (S, 2, H_8 and H_9), 5.65 (d, J = 6.0, 1, H_1'). R_f's:S_1 (0.68);

S_2 (0.69). The UV spectra of *2* and its pH shift is consistent with the unsubstituted imidazole structure (Table III).

Anal. Calcd. for $C_{11}H_{15}N_5O_4$: C, 46.97; H, 5.38; N, 24.90.

Found: C, 46.89; H, 5.60; N, 24.75.

1,N^6-Etheno-2-aza-adenosine, *3*. An acetic acid solution, (30 ml), of *2*, (2 g, 7.1 mmole) was treated with an aqueous solution of sodium nitrite (0.5 g, 7.1 mmole, in 2 ml). The reaction mixture was allowed to stand at room temperature for 15 min. The solvent was removed by rotary evaporator. Water and ethanol were used to co-evaporate with the residue to remove the acetic acid. The solidified residue was recrystallized from 80% ethanol after it was treated with charcoal to give 1.75 g (83%) needles; m.p. 179-181° [dec.]; NMR (DMSO-d_6) δ 8.86 (d, J = 1.8, 1, H_7), 8.01 (d, J, = 1.8, H_8), 9.13 (S, 1, H_2), 6.36 (d, J = 4.5, 1, H_1'). R_f's: S_1 (0.70); S_2 (0.73). Mass spectrum: m/e 292 (parent ion) and m/e 160 (B + 1). Its UV spectra shift only at low pH (Table I), suggesting that protonation of N cannot take place above pH 7.

Anal. Calcd. for $C_{11}H_{12}N_6O_4 \cdot \frac{1}{2} H_2O$: C, 43.85; H, 4.36; N, 27.89.

Found: C, 43.73; H, 4.29; N, 27.50.

[^{3}H]-*3* was made by the same procedure using [8-^{3}H]-adenosine as the starting material.

1,N^6-Etheno-2-mercapto-adenosine, *4*. A suspension of *3* (0.56 g, 2 mmole) in 15 ml

dry pyridine and 7.5 ml carbon disulfide were refluxed in a hot water bath until all the solid was dissolved. Judging by the TLC, all the starting material had reacted. The heating was continued for another hour, and the product started to precipitate from the solution. The suspension was allowed to cool to room temperature and the solvent was removed by rotary evaporator. The residue was allowed to co-evaporate with water and ethanol under reduced pressure to remove the pyridine. The solid was then recrystallized from 12 ml water to give 0.60 g (92%) after air drying.

m.p. 225-227° [dec.]; NMR (DMSO-d_6) δ 7.83 (d, J = 2.0 Hz, H_8); 8.40 (d, J = 2.0 H_7); 8.43 (S, 1, H_2); 6.04 (d, J = 5.0 H_1'). R_f's: S_1 (0.61); S_2 (0.55). Its UV spectra (Table III) suggested that the compound is stable at alkaline pH.

Anal. Calcd. for $C_{12}H_{13}N_5O_4S \cdot H_2O$: C, 42.22; H, 4.42.

Found: C, 42.29; H, 4.26.

Reaction of *2* with Triethyl Orthoformate. To the suspension of *2* (112 mg, 0.4 mmole) in 4 ml triethyl orthoformate, p-toluenesulfonic acid (20 mg, 0.024 mmole) was added. The mixture was allowed to stir at room temperature for 4 hr to give a clear solution. The solvent was removed by evaporation and the residue was treated with 5 ml 0.5 N of hydrochloric acid. Judging by the TLC, the product was transformed to *1* after 15 min at room temperature. The solution was then adjusted to pH 4 by sodium hydroxide, and evaporated to dryness. The residue was

extracted with absolute ethanol and recrystallized from methanol and ether to give 25 mg *1* (20%).

Cytotoxicity Studies. The cytotoxicity of the compounds was tested on Glioma 26, HeLa and rat mammary tumor (AC 33) tissue culture lines, using our previously described technique[13]. Membrane fluorescence of cells incubated on coverslips could be seen, but no nuclear fluorescence was observed.

Drug Distribution Studies. Three groups of three rats (Lewis-Wistar strain, weighing ≈ 250 g) each were used in drug distribution and [^{14}C]-thymidine incorporation studies. They were injected i.p. with 0.80 ml of [^{3}H]-*3* (1.0 μc, 8.0 μmole); [^{3}H]-*3* (0.9 μc, 7.2 μmole) plus [^{14}C]-thymidine (1.68 μc, 0.17 μ mole); and [^{14}C]-thymidine (1.53 μc, 0.15 μmole), in 0.9% NaCl, respectively. All rats were sacrificed exactly 2 hr after injection by exsanguination under light ether anesthesia. The tumors and tissues were homogenized with 10-20 volumes of 0.60 M $HClO_4$ with a Polytron Homogenizer (Brinkman). The homogenates were centrifuged at 27,000 g for 30 min. Aliquots (∿ 0.05 g of tissue) of the supernatants were counted for radioactivity to determine the drug distribution, and DNA was extracted from the precipitate according to the method of O'Connor[14].

Scintillation Counting. Radioactivity was measured with an SL30 liquid Scintillation Spectrometer (Intertechnique) at an efficiency of 46% for ^{3}H and 90%

for ^{14}C. The average counting errors are 2.9% and 2.1% for ^{14}C and ^{3}H, respectively. The scintillation liquid was of the following composition: 900 ml toluene, 100 ml Biosolv (Beckman) and 4 g Omnifluor (New England Nuclear).

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