2 H, C_2H), 4.25 (q, 2 H, CH_2), 6.8 (s, 1 H, C_6H), 9.5 (s, 1 H, NH). Anal. ($C_7H_9NO_3S \cdot {}^1/_4H_2O$), C, H, N.

Ethyl 4-(2-Deoxy-3,5-*O*-*p*-toluoyl-β-D-*erythro*-pentofuranosyl)-3-oxo-1,4-thiazine-5-carboxylate (23). To a solution of **21** (1 g, 5.34 mmol) in dry MeCN (60 mL) was added NaH (60% in oil, 0.238 g, 5.9 mmol) in portions, and the mixture was stirred at room temperature for 30 min. 1-Chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-*erythro*-pentofuranose (2.07 g, 5.34 mmol) was added in portion with stirring. After 3 h the solution was filtered, evaporated to dryness, and passed through a silica gel column (toluene/acetone, 9:1) to give **23** (1 g, 35%): mp 120 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.26 (t, 3 H, CH_3), 2.30 (s, 6 H, 2 CH_3), 3.24 (s, 2 H, C_2H), 4.16 (q, 2 H, CH_2), 5.55 (t, 1 H, $C_1'H$), 6.58 (s, 1 H, C_6H), 7.01–7.69 (4 d, 8 H), and other sugar protons. Anal. ($C_{28}H_{29}NO_8S$) C, H, N.

Ethyl 4-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-3-oxo-1,4-thiazine-5-carboxylate (24). To **23** (0.1 g, 0.18 mmol) in MeOH (6 mL) was added a 0.81 M NaOMe solution (0.4 mL, 0.324 mmol). After 3 h at room temperature, the solution was passed through a 1 × 10 cm column of Dowex-50 (H^+) resin. The column was washed with 10 mL of MeOH/H_2O (2:1). The eluate was then evaporated, and the residue was dissolved in water. Lyophilization gave **24** (0.03 g, 52% yield): mp 121–122 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.21 (t, 3 H, CH_3), 3.26 (s, 2 H, C_2H), 4.17 (q, 2 H, CH_2), 5.54 (t, 1 H, $C_1'H$), 6.57 (s, 1 H, C_6H), and other sugar protons. Anal. ($C_{12}H_{17}NO_6S \cdot 2H_2O$) C, H, N.

4-(2-Deoxy-3,5-di-*O*-*p*-toluoyl-β-D-*erythro*-pentofuranosyl)-5-methyl-1,4-thiazin-3-one (25). To a suspension of 5-methyl-1,4-thiazin-3-one[13] (22, 0.5 g, 3.87 mmol) in dry MeCN (40 mL) was added NaH (60% in oil, 0.169 g, 4.2 mmol) in portions, and the mixture was stirred at room temperature for 30 min. 1-Chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-*erythro*-pentofuranose (1.5 g, 3.87 mmol) was added in portions with stirring. After 3 h, the solution was filtered, evaporated to dryness, and passed through a silica gel column (toluene/acetone, 9:1) to give **25** (0.7 g, 38%): mp 125–126 °C; ^{1}H NMR (Me_2SO-d_6) δ 2.15 (s, 3 H, CH_3), 2.31 (s, 6 H, 2 CH_3), 3.15 (s, 2 H, C_2H), 5.46 (t, 1 H, $C_1'H$), 5.91 (s, 1 H, C_6H), 6.98–7.65 (4 d, 8 H), and other sugar protons. Anal. ($C_{26}H_{27}NO_6S$) C, H, N.

4-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-5-methyl-1,4-thiazin-3-one (26). To **25** (0.1 g, 0.2 mmol) in MeOH was added a 0.81 M NaOMe solution (0.4 mL, 0.324 mmol). After 3 h at room temperature, the solution was passed through a 1 × 10 cm column of Dowex-50 (H^+) resin. The column was washed with approximately 10 mL of MeOH/water (2:1). The elute was evaporated. The residue was dissolved in a small amount of water/MeOH. The deblocked nucleoside **26** was obtained as a white solid on standing (28.5 mg, 57% yield): mp 114–115 °C; UV (H_2O) 298 nm; ^{1}H NMR (Me_2SO-d_6) δ 2.17 (s, 3 H, CH_3), 3.14 (s, 2 H, C_2H), 5.50 (t, 1 H, $C_1'H$), 5.93 (s, 1 H, C_6H), and other sugar protons. Anal. ($C_{10}H_{15}NO_4S \cdot 2H_2O$) C, H, N.

Cytidine Deaminase Inhibition. Mouse kidney cytidine deaminase was isolated and partially purified from mouse kidney acetone powder (prepared with acetone only) obtained from Sigma Chemical Co., St. Louis, MO. The powder was extracted as described by Liu et al.[16] and fractionated with ammonium sulfate as described by Wentworth and Wolfenden.[17] We found the K_m for cytidine with this preparation (0.10 M HEPES buffer, pH 7.0) to be 0.025 mM (lit.[16] 0.05 mM). Compound **17** gave a K_i of 0.33 mM.

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(16) Liu, P. S.; Marquez, V. E.; Driscoll, J. S.; Fuller, R. W.; McCormack, J. J. *J. Med. Chem.* **1981**, *24*, 662.
(17) Wentworth, D. F.; Wolfenden, R. *Biochemistry* **1975**, *14*, 5099.

Nucleotide Derivatives of 2,7-Diaminomitosene

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Treatment of mitomycin C with pyrimidine nucleotides in acidic media produced derivatives of 2,7-diaminomitosene in which C-1 was covalently bound to the phosphate group of the nucleotides. On reduction, these derivatives liberated the nucleotides and a mitomycin intermediate that alkylated DNA. Their reduction in the presence of 2′-deoxyguanosine produced some bifunctional alkylation as did mitomycin C. They were readily taken up by L1210 leukemia cells, in which they showed potent cytotoxicity. These properties suggest that they are acting as prodrugs capable of conversion into two active species. The uridylate derivative showed activity comparable to that of mitomycin C against P-388 leukemia in mice.

The design of prodrugs that are readily converted into one or more cytotoxic species by bioactivating mechanisms is an important goal in cancer chemotherapy. This design strategy may afford reduced toxicity to patients when the bioactivating mechanism of the prodrug is primarily a property of the tumor. The reduction of quinones to hydroquinones or semiquinone radicals by cellular enzymes is one such mechanism, and it is a significant in vivo route of activation in the mitomycin family of compounds. A related approach has been taken in the investigation of nitrobenzyloxycarbonyl derivatives of 5-fluorouracil as potential conjugated bioreductive alkylating agents.[1] In vitro activation of mitomycins by acidification has also been described.[2-5] We have used this acid-catalyzed activation mechanism to form a 1-substituted mitosene co-

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(1) Lin, T. S.; Wang, L.; Antonini, I.; Cosby, L. A.; Shiba, D. A.; Kirkpatrick, D. L.; Sartorelli, A. C. *J. Med Chem.* **1986**, *29*, 84.
(2) Hashimoto, Y.; Shudo, K.; Okamoto, T. *Chem. Pharm. Bull.* **1980**, *28*, 1961.
(3) Tomasz, M.; Lipman, R. *J. Am. Chem. Soc.* **1979**, *101*, 6063.
(4) Tomasz, M.; Lipman, R. *Biochemistry* **1981**, *20*, 5056.
(5) Kowal, C. D.; Diven, W. F.; Kozikowski, A. P. *Am. Assoc. Cancer Res., 1983 National Meeting*; Abstract 983, p249.

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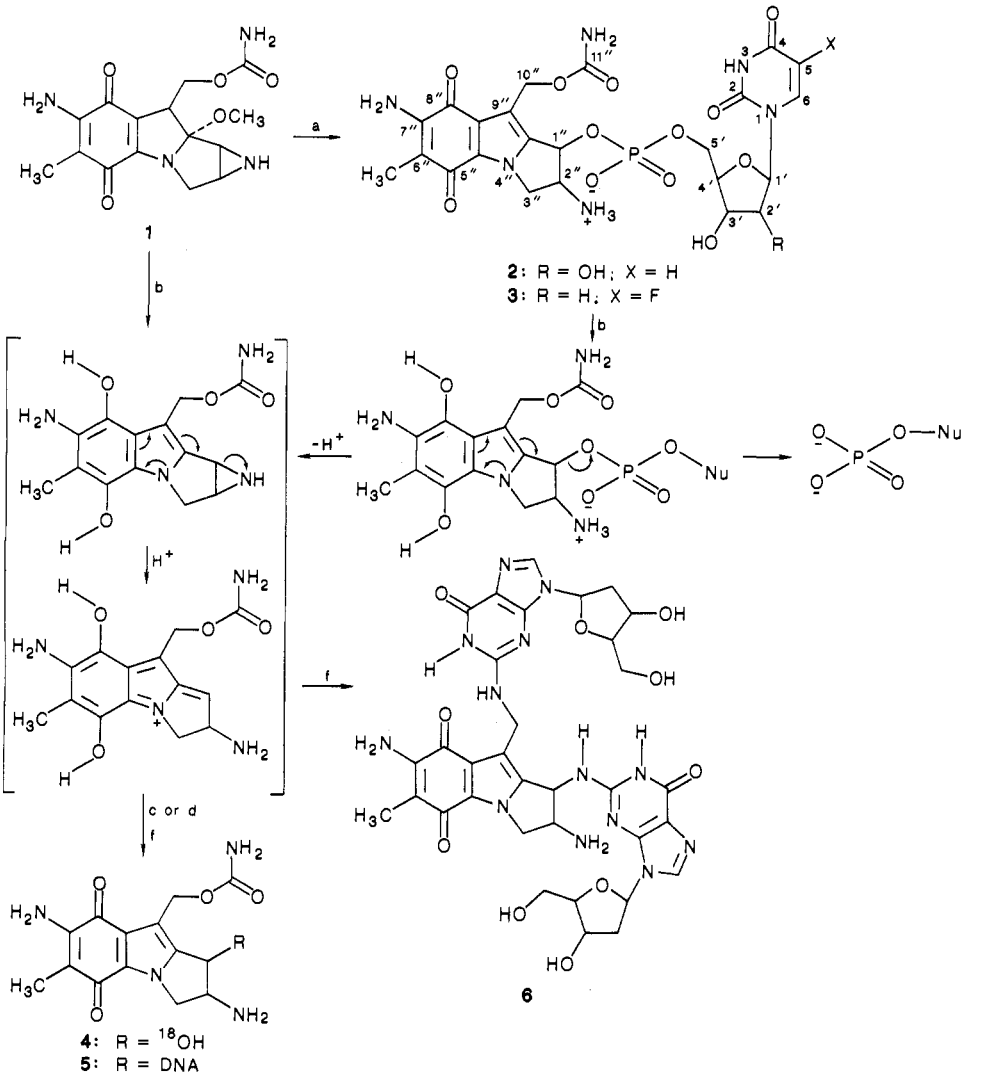
Scheme I



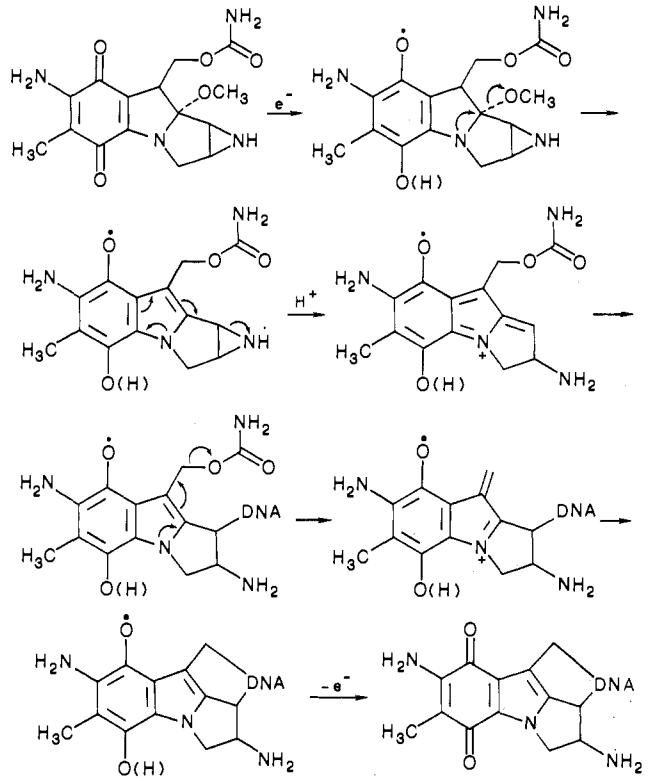
valently linked to fluorodeoxyuridylate,[5,6] an active antitumor metabolite.[7] We further hypothesized that 1-substituted mitosene nucleotide conjugates could also act as prodrugs, behaving in much the same way as the parent mitosane drug mitomycin C. Thus, reduction of a 1-substituted mitosene nucleotide conjugate should result in the liberation of the nucleotide and the formation of a reactive mitomycin intermediate.

We now report that the chemical transformations and biological properties required by this hypothesis have been verified in an in vitro system. Nucleotide derivatives of 2,7-diaminomitosene, in which the phosphate is covalently bonded to C-1 of the mitosene, have been conveniently prepared by acidification of mitomycin C in the presence of the nucleotide. Upon reduction, these derivatives are shown to liberate the intact nucleotide and a mitomycin intermediate that is capable of alkylating DNA. Importantly, they are readily taken up by tumor cells, where their bioactivation results in a potent antitumor effect. These results support the design concept of nucleotide derivatives of 2,7-diaminomitosene acting as a prodrug form. Furthermore, because cytotoxic nucleotides such as FdUMP can be used for derivatization of the mitosene, this method provides the novel possibility of delivering two antitumor species into the tumor cells.

Chemistry. Although there have been prior reports on the formation of nucleotide derivatives of mitomycin C,[1-5] none of the products have been completely characterized or prepared in sufficient quantity for chemical and biological studies. These reports have included nucleotide derivatives of 2,7-diaminomitosene with guanosine 5′-phosphate,[1] uridine 5′-phosphate[2] (UMP), uridine 5′-diphosphate,[2] and 5-fluoro-2′-deoxyuridine 5′-phosphate[5,6] (FdUMP). Additionally, all of these earlier preparations involved the activation of mitomycin C in aqueous solutions of the nucleotide. Such conditions give considerable solvolysis and mixtures of stereoisomers about the C-1–C-2 axis of the mitosene. In contrast, we have developed a simple and effective alternative preparative method, by treating mitomycin C with the nucleotide in nonhydroxylic solvents under acidic conditions, without reduction. Thus, treatment of mitomycin C (1) with 2 mol of uridylic acid in *N,N*-dimethylformamide gave the uridylate derivative **2** of 2,7-diaminomitosene as a mixture of the neutral species (zwitterion, 21%) and the uridylic acid salt trimethanolate (35%), separable by concentrating the solution and adding methanol (Scheme I). The uridylic acid salt was used for further experiments. The corresponding FdUMP derivative **3** was prepared by treating mitomycin C with 1 mol of disodium 5-fluoro-2′-deoxyuridine 5′-phosphate and 3 mol of perchloric acid in dioxane. The

(6) Kowal, C. D.; Diven, W.; Kozikowski, A.; Iyengar, B. S.; Remers, W. A., manuscript in preparation.
(7) Santi, D. V.; McHenry, C. S.; Sommer, H. *Biochemistry* **1974**, *13*, 471.

Scheme II



monoperchlorate salt crystallized with 1.5 mol of NaCl and 2.5 mol of dioxane in 35% yield. For further experiments it was converted into the neutral species (zwitterion).

Catalytic hydrogenation of **2** in [^{18}O]water gave a reduction in color of the solution from deep to faint purple. Upon workup, the deep purple color was regenerated, indicating reoxidation to the indoloquinone chromophore. The known 2,7-diamino-1-hydroxymitosene (**4**)[3,8,9] and uridylic acid were identified among the products that were separated by chromatography.[10] A ratio of peaks at m/z 320 and 322 in the EI mass spectrum of **4** obtained from this experiment showed that it had a 60% incorporation of ^{18}O. This result demonstrates that the conversion of **2** to **4** does not occur by simple phosphate hydrolysis, in which ^{18}O would be incorporated into the uridylate moiety, but by a process in which the oxygen attached to C-1 of the mitosene is replaced by ^{18}O from the solvent. The nature of this process is uncertain. A reaction in which the entire uridylate group leaves with formation of a mitosene cation is not possible at the quinone stage, because hydrolysis at neutral pH does not occur without reduction. However, this reaction might be enhanced when the quinone is reduced. Recent results from the laboratories of Tomasz,[11] Danishefsky,[12] Bachur,[13] and Kohn[14] indicate that a semiquinone radical is the reactive species formed by reduction of mitomycin C in biological and chemical systems. This species readily eliminates methanol to give a radical that undergoes opening of the aziridine ring with formation of an intermediate that can trap nucleophiles including DNA (Scheme II, after Danishefsky[12]). The same intermediate could be formed by reduction of **2**. This certainly would be the expected result with sodium dithionite, enzymes such as xanthine oxidase/NADH, or cathodic reduction. It is less clear that catalytic reduction involves radical anions; however, a hydroquinone might lead to a similar reactive intermediate. In either case, the process would involve loss of the C-1 oxygen with capture of ^{18}O from the solvent.

A key question for mitomycin prodrugs such as **2** and **3** is whether they produce reactive intermediates that give bifunctional alkylation of DNA, as does mitomycin C, or merely give intermediates for monofunctional alkylation. This question was answered by experiments in which the alkylation of 2′-deoxyguanosine by **2** was compared with the effect of mitomycin C on the same nucleoside. It is known from the work of Tomasz et al. that mitomycin C alkylates the 2-amino group of guanine in DNA, affording bifunctional alkylation and interstrand crosslinking where these residues are suitably located.[11] Treatment of a solution of mitomycin C and excess 2′-deoxyguanosine with sodium dithionite resulted in rapid decolorization. The introduction of oxygen after 30 min restored the quinone color. Workup of the mixture gave a crude product that showed on TLC one major spot with R_f 0.33, a minor spot with R_f 0.49, and a number of very faint spots. Purification of the main product gave a small amount of the bis(2′-deoxyguanosine) derivative (**6**) of decarbamoyloxy-2,7-diaminomitosene, in which both C-1 and C-10 of the mitosene were covalently bound to 2-amino groups of the nucleoside residues. The elucidation of this structure is discussed below. When **2** was treated under the same conditions with 2′-deoxyguanosine and sodium dithionite, TLC on the product showed the same two spots given by mitomycin C, in about equal intensity. There also were a number of very faint spots. The spot with R_f 0.33 clearly corresponds to the bifunctional alkylation product obtained from mitomycin C. When **2** was reduced catalytically in the presence of 2′-deoxyguanosine, the TLC of the product showed no spot with R_f 0.33. It had a faint spot at R_f 0.49 and a number of very faint spots. A recent study by Tomasz et al.[15] showed that dithionite reduction of mitomycin C in the presence of DNA afforded a relatively high frequency of bisalkylation, whereas catalytic reduction under "standard" conditions gave mostly monofunctional alkylation. These results resemble ours with 2′-deoxyguanosine, and they suggest that the product with R_f 0.49 is a monofunctional 2′-deoxyguanosine derivative, possibly at C-1 of 2,7-diaminomitosene. Unfortunately, the yield of this product was so small that a structure proof could not be undertaken.

The bifunctional alkylation product **6** was confirmed by its ^{13}C and ^{1}H NMR spectra, which are given in Table I. In the ^{1}H NMR spectrum, there was an exact ratio of 3:2 for integrals corresponding to the 6-CH_3 group of the mitosene residue (δ 1.76) and H-8 of the guanine residues (δ 9.27), which showed that it was a 1:2 adduct (bifunctional alkylation). The ^{13}C NMR spectrum showed that the carbamate carbon was missing. The peak for C-10 is shifted from δ 58.8 to 45.5, which indicates that it is bonded to nitrogen rather than oxygen. As a reference, the methylene carbon of benzylamine is at δ 44.5. This result implies that alkylation by C-10 occurs on the 2-amino

(8) Stevens, C. L.; Taylor, K. G.; Monk, M. E.; Marshall, W. S.; Noll, K.; Shah, G. D.; Uzu, K. *J. Med. Chem.* **1965**, *8*, 1.
(9) Taylor, W. G.; Remers, W. A. *J. Med. Chem.* **1975**, *18*, 307.
(10) The liberation of uridylate was verified by TLC of the reaction mixture on silica gel with MeOH–$CHCl_3$ (2:8) as solvent, with authentic uridylate used for comparison.
(11) Tomasz, M.; Lipman, R.; Chowdary, D.; Nakanishi, K. *Science (Washington, D.C.)* **1987**, *235*, 1204.
(12) Danishefsky, S. J.; Egbertson, M. *J. Am. Chem. Soc.* **1986**, *108*, 4648.
(13) Bachur, N. R.; Pan, S.-S.; Andrews, P. A. *J. Am. Chem. Soc.* **1986**, *108*, 4158.
(14) Zein, N.; Kohn, H. *J. Am. Chem. Soc.* **1986**, *108*, 296.
(15) Tomasz, M.; Chawla, A. K.; Lipman, R. *Biochemistry*, in press.

Table I. ^{13}C NMR Spectral Data of Mitosenes and Their Nucleotide Derivatives[a]

carbon no.	δ values in ppm				
	2	UMP[b]	C-1 and C-10 mitosene deriv[c]	6	2′-deoxyguanosine[d]
2	150.7	149.8		158.5 or 156.5	154.0
4	163.0	162.2		151.5	151.3
5	101.9	101.0		117.5 or 118.5	116.9
6	140.5	139.7		156.5 or 158.5	157.5
8				137.5	136.2
1′	87.7	87.1		87.7	83.2
2′	73.0	72.0		3.95[e]	40.0
3′	70.0	69.0		70.5	71.5
4′	82.9	81.7		93.1	87.9
5′	64.7	64.3		64.9	62.1
1″	67.5 (br)		65.1*	55.4	
2″	53.5		57.9*	51.9	
6″	48.3		51.8*	45.5 or 45.9	
5a″	145.3		145.7	145.7	
5″	178.2		178.2	179.0	
6″	104.9		107.6	107.6	
6″-CH_3	8.2		8.4*	7.95	
7″	135.5		135.7	135.7	
8″	176.2		177.3	174.5	
8a″	115.0		115.2	117.5 or 118.5	
9″	120.6		122.4	123.1	
9a″	128.1		129.2	129.2	
10″	56.5		58.8	45.5 or 45.9	
11″	156.7		156.7		

[a] Our spectra were recorded on a JEOL FX90Q NMR spectrometer at a frequency of 22.5 MHz in DMSO-d_6 with TMS as internal standard. [b] Levy, G. C.; Nelson, G. L. *Carbon-13 NMR Spectroscopy, ACS Audio Course*; American Chemical Society: Washington, D.C. [c] Chemical shift values marked with * are for the cis isomer of 2,7-diaminomitosene and are taken from Kohn, H. L.; Bean, M. *J. Org. Chem.* **1983**, *48*, 5033. Other chemical shift values are taken from 2,7-diaminomitosenes reported by Kohn, H. L.; Zein, N.; Lin, X. Q.; Ding, J.-Q.; Kadish, K. M. *J. Am. Chem. Soc.* **1987**, *109*, 1833. [d] Chang, C.-J.; Gomez, J. D; Byrn, S. R. *J. Org. Chem.* **1983**, *48*, 5151. [e] This peak fell under the DMSO impurity in DMSO-d_6. The value given here is from another spectrum run in D_2O with CH_3CN as reference.

Table II. Uptake of the Uridylate Derivative of 2,7-Diaminomitosene by L1210 Cells

drug concn in medium, μg/mL	time, min	drug concn in cells,[a] μg/mL	drug concn in medium, μg/mL	time, min	drug concn in cells,[a] μg/mL
10	10	8.0	100	30	20
100	10	20[b]	10	60	2.5
10	30	1.0	100	60	1.0[b]

[a] Determined by washing the cells in phosphate buffered saline, lysing them by sonication in distilled water, and measuring the concentration of drug in the lysate by HPLC on a short C18 column. A standard curve of peak height against concentration was found to be linear over the range used in this experiment. [b] A second peak, eluting before **2** (more polar), was observed.

group rather than O-6 of 2′-deoxyguanosine. Other possible sites on 2′-deoxyguanosine are ruled out by the lack of change in its part of the ^{13}C NMR spectrum. Similarly, the peak for C-1 of the mitosene residue is shifted from δ 65.1 to 55.5, indicating that it also is bonded to a 2-amino group of a 2′-deoxyguanosine residue.

Once it was established that a mitomycin intermediate capable of trapping nucleophiles was the predominant species formed on reduction of the uridylate derivative **2**, it became important to determine if this intermediate could alkylate DNA. The DNA alkylation experiment of Hashimoto et al. was used for this purpose.[16] It involves catalytic reduction of mitomycin C in the presence of calf thymus DNA. After removal of the catalyst, the DNA is precipitated with ethanol and washed, and the noncovalently bound mitosenes are removed by dialysis. The extent of mitosene binding is determined by comparing the relative intensities, corrected for differences in molar extinction coefficients,[17] of absorptions of 310 nm (mitosene) and 260 nm (purines and pyrmidines) in the UV absorption spectrum of the modified DNA. When we repeated this experiment with mitomycin C, an incorporation of about one mitosene per 180–210 bases was found, which is in agreement with the results of Hashimoto and coworkers.[16] The uridylate derivative **2** of 2,7-diaminomitosene gave an incorporation of about one mitosene per 200–240 bases when treated in the same way. The results described above demonstrate that nucleotide derivatives of mitosenes can be efficient sources of mitomycin intermediates that alkylate DNA. This observation is consistent with mechanisms proposed by Moore[18] and by Tomasz and Lipman[4] for the role of mitomycin intermediates in DNA alkylation. However, in contrast to the proposed Moore and Tomasz mechanisms, our results show that alkylating mitomycin intermediates can arise directly from mitosenes and not just from mitosanes.

Biology. The uptake of the UMP derivative **2** into L1210 leukemia cells in culture was investigated by an HPLC method, with a short C^{18} column and methanol–water (65:35) as solvent. After a standard curve was established from different concentrations of **2**, the L1210 cells were incubated with this compound at various concentrations. At specific time points, the cells were washed and lysed by sonication in distilled water, and the concentration of **2** released from them was determined by HPLC. The results, given in Table II, show that significant

(16) Hashimoto, Y.; Shudo, K.; Okamoto, T. *Chem. Pharm. Bull.* **1983**, *31*, 861.

(17) Extinction coefficients of 6600 and 5300 were used for DNA bases and mitosene bound to DNA, respectively. The latter value was determined from a noncovalent complex of 2,7-diamino-1-hydroxymitosene with excess DNA.

(18) Moore, H. W. *Science (Washington, D.C.)* **1977**, *197*, 527.

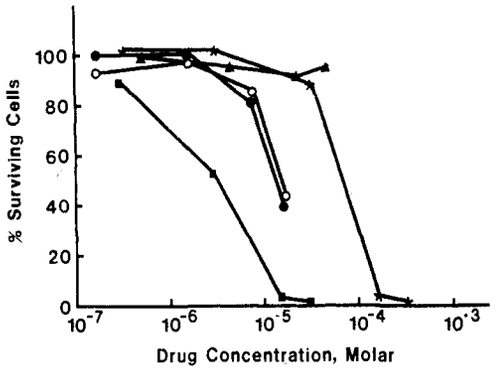


Figure 1. Effect of 1-h exposure of cloned L1210 leukemia cells to mitomycin C and nucleotide derivatives of 2,7-diaminomitosene. Mitomycin C, squares; 2,7-diamino-1-hydroxymitosene, stars; UMP derivative, closed circles; FdUMP derivative, open circles; FUdR, triangles. Vertical axis represents percent survival of colony-forming cells. Each point represents the mean of six values (three plates in each of two experiments). Coefficients of variations of 10–15% and cloning efficiencies of 2.5% were typical in these experiments.

concentrations of **2** were achieved in L1210 cells. The highest intracellular levels were produced with the shortest exposure time of 10 min, ranging from 20–80% of the extracellular concentrations following exposure to 100 or 10 mg/mL, respectively. Some of this "extracellular" drug may actually represent surface-bound material, which was resistant to the washing procedures used prior to lysis in distilled water. An exposure time of longer than 10 min substantially reduced the recovery of intact **2** in cells from 20% to only 1.0%. Overall, there was not a linear recovery from L1210 cells based on either drug concentrations or exposure times. Not withstanding this, the recovery of **2** in duplicate experiments varied very little (±12%). In addition to the chromatographic peak associated with **2** (t_R 2.5 min), a second peak (t_R 2.2 min) was observed following exposures to 100 mg/mL for 10 and 60 min (but not 30 min). This peak may represent a more polar metabolite of **2** that is variably detected by HPLC after exposure of cells to very high drug concentrations in vitro.

The effects of the nucleotide derivatives **2** and **3** on the survival of cloned L1210 cells in culture, compared with those of mitomycin C, were investigated for a 1-h exposure, and for a continuous exposure, to varying concentrations of the drugs. Also measured under the same conditions were the effects of 2,7-diamino-1-hydroxymitosene (**4**), the hydrolysis product of these three compounds, fluorodeoxyuridine (FUdR), and a combination of mitomycin C and FUdR. The parent metabolite of FUdR, fluorouracil, has been reported to be synergistic with mitomycin C against tumors in vivo in mice[19] and in clinical practice.[20] FUdR also is a potential degradation product of **3** and of the FdUMP released from **3** with reduction. Figure 1 shows the results of the 1-h exposure experiments. Nucleotide derivatives **2** and **3** are active with ID_{50} values of 1.33×10^{-5} and 1.4×10^{-5} M, respectively, about one-fifth as potent as mitomycin C (3.1×10^{-6} M, Table III), whereas 2,7-diamino-1-hydroxymitosene (**4**) was about one-tenth as potent as mitomycin C, and FUdR did not give an ID_{50}. Results of the continuous exposure experiments (8–9 days), given in Figure 2 and Table IV, show about a 6-fold enhancement in potency for mitomycin C (1.85×10^{-7} M) and considerably larger enhancements for **2** (about 80-fold, 1.62×10^{-7} M), **3** (about 40-fold, 3.50×10^{-7} M), and FUdR. In contrast, the potency of **4** was increased only 4-fold. These results indicate that the nucleotide derivatives experience a significantly greater enhancement of potency on prolonged exposure than that experienced by mitomycin species without nucleotide substituents. Two possible explanations for this difference include: (1) the requirement for FUdR metabolic conversion into FdUMP for inhibition of thymidylate synthetase, and (2) the need to maintain this enzymatic inhibition for extended periods of time to produce cytotoxicity.[21]

The results from an experiment in which mitomycin C and FUdR were used in equimolar combinations are given in Table IV. For each concentration, the percentages of surviving L1210 cells are given for mitomycin C and FUdR as single agents and in combination. Expected survival

Table III. ID_{50} Values for Mitosene–Nucleotide Conjugates against L1210 Leukemia Cells[a]

drug	short exposure (1 h)	prolonged exposure (8–9 h)
MMC (1)	3.1×10^{-6}	1.82×10^{-7}
FUdR	not reached[b]	4.30×10^{-6}
1-OH (4)	6.2×10^{-5}	1.58×10^{-5}
MMC–UMP (2)	1.33×10^{-5}	1.62×10^{-7}
MMC–FdUMP (3)	1.40×10^{-5}	3.50×10^{-7}

[a] ID_{50} expressed as molar concentration. [b] At 4.5×10^{-5} M, the survival was 88%.

Table IV. Expected and Observed Results of Mitomycin C and FuDR against L1210 Cells

molar concn	percent survival of L1210 cells: single agent MMC	single agent FUdR	combination: MMC + FUdR expected	combination: MMC + FUdR observed
	Short Exposure (1 h)			
1×10^{-7}	89	98	87	100
1×10^{-6}	52	94	49	48
5×10^{-5}	2	89	2	2
1×10^{-5}	1	92	1	1
	Prolonged Exposure (8–9 h)			
1×10^{-9}	98	100	98	84
1×10^{-8}	100	76	76	70
5×10^{-8}	73	68	50	42
1×10^{-7}	7	62	4	3
1×10^{-6}	1	51	0.5	1

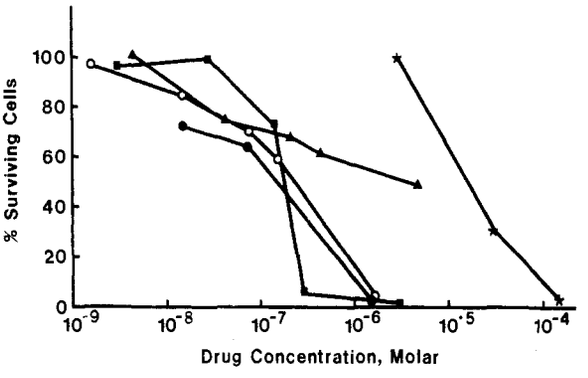


Figure 2. Effect of continuous exposure (8–9 days) of cultured L1210 leukemia cells to mitomycin C and nucleotide derivatives of 2,7-diaminomitosene. Symbols are defined in Figure 1. Each point represents the mean of six values (three plates in each of two experiments), and coefficients of variation and cloning efficiencies are as stated in Figure 1.

(19) Sartorelli, A. C.; Booth, B. A. *Cancer Res.* **1965**, *25*, 1393.
(20) MacDonald, J. S.; Schein, P. S.; Woolley, P. V. *Ann. Int. Med.* **1980**, *93*, 553.
(21) Lockshin, A.; Danenberg, P. V. *Biochem. Pharmacol.* **1981**, *30*, 247.

percentages for the combination of mitomycin C and FUdR were calculated according to the definitions and method of Valeriote and Lin.[22] The estimation of an additive effect is based on the assumption that each agent acts independently. Consequently, expected survival percentages were obtained from the product of the individual agent surviving fractions. It is clear from this table that a comparison of observed survival and expected survival shows that the combination of mitomycin C and FUdR is additive and not synergistic. This observation is in contrast to others on the effects of combinations of mitomycin C and fluoropyrimidines.[15]

In a final experiment, UMP derivative **2** was administered intraperitoneally to DBA/2J male mice bearing 10^6 P-388 lymphocytic leukemia cells according to the standard NCI protocol.[23] A range of single doses given on day 1 was studied (1.5–6.0 mg/kg). The maximal percent increase in lifespan (ILS) of 40% was obtained with a dose of 3.0 mg/kg. Higher doses produced decreased %ILS due to early (toxicity related) deaths. In comparison, mitomycin C gives ILS of 40–60% at its optimal dose of 3.2 mg/kg when tested in this laboratory. Thus the in vivo activity of **2** and mitomycin C are roughly comparable.

In summary, the nucleotide derivatives of 2,7-diaminomitosene are taken up by tumor cells and produce a significant antitumor effect in cultures and in mice. Chemical experiments suggest that they act as prodrugs that liberate two cytotoxic species on reductive activation. The resulting mitosene intermediate is capable of bifunctional alkylation. Additional nucleotide derivatives will be prepared from mitomycin C and mitomycin A.

Experimental Section

Melting points were determined with a Mel-Temp apparatus and are uncorrected. IR spectra were recorded on a Beckman IR-33 spectrophotometer, and UV–visible spectra were recorded on a Perkin-Elmer Lambda 3A spectrophotometer. NMR spectra were taken on a Brucker WM250 spectrometer or a JEOL FX90Q spectrometer, with Me_4Si as the internal reference standard. Mass spectra were measured on a Varian-MAT 311 spectrometer. Elemental analyses were performed by Mic Anal, Inc., Tucson, AZ. The results obtained were within ±0.4% of the theoretical values.

2,7-Diamino-1-hydroxymitosene 1-Uridylate (2). Method A. A solution of mitomycin C (50 mg, 0.15 mmol) and uridylic acid (100 mg, 0.3 mmol) in 100 mL of freshly distilled *p*-dioxane was stirred at room temperature for 5 h, during which the color turned from blue to purple. The solvent was removed by evaporation under reduced pressure, and the purple residue was extracted with 50 mL of ethyl acetate. This extract was filtered, and the solids were washed with ethyl acetate (2 × 10 mL) and methanol (2 × 10 mL) and dried under high vacuum. This procedure gave 54 mg (38% yield) of **2** as the uridylic acid salt trimethanolate, a purple solid with mp 190–200 °C dec: ^1H NMR (Me_2SO-d_6) δ 7.9 (d, 2) and 5.9 (d, 2) uracil double bond, 5.2 (s, 2, 10-CH_2 of mitosene), 1.8 (s, 3, 6-CH_3 of mitosene); IR (KBr) 3420–3100 (OH and NH), 1700 (carbamate), 1690–1670 (uracil), 1030–1060 cm^{-1}; UV (H_2O) λ_{max} 259.6, 316.7, 354.2 nm. Anal. ($C_{23}H_{27}N_6O_{13}P \cdot C_9H_{13}N_2O_9P \cdot 3CH_3OH$) C, H, N. The ^{13}C NMR peaks are listed in Table I.

Method B. A solution of mitomycin C (50 mg, 0.15 mmol) and uridylic acid (100 mg, 0.3 mmol) in 10 mL of dry DMF was stirred at 50–60 °C for 30 min, cooled to room temperature, and evaporated under reduced pressure. A methanol extract of the residue was concentrated, and the resulting purple residue was purified by preparative TLC on a silica gel plate (20 × 20 × 0.2 cm) with MeOH–$CHCl_3$ (2:8 v/v) as solvent. The major purple band was removed and extracted with MeOH–CH_2Cl_2. This extract was filtered and evaporated under reduced pressure to give 20 mg (21% yield) of **2** as the neutral species, purple solid with mp 230–237 °C dec: IR (KBr) 3500, 3420, 3320, 3180, 1700, 1670, 1040–1090 cm^{-1}; UV (MeOH) λ_{max} 249, 309, 346 nm. MS (FAB gun 1 mA at 9 kv with argon and solution matrix 1:1 glycerol–NBA in the negative ion mode), m/z 625 (M − 1).

This product was identical on TLC in the system described above with the product obtained by method A after the latter was neutralized with 0.5 M aqueous ammonium hydroxide.

2,7-Diamino-1-hydroxymitosene 1-(5-Fluoro-2′-deoxyuridylate) (3). Method A. A solution of mitomycin C (25 mg, 0.075 mmol), disodium 5-fluoro-2′-deoxyuridylate (30 mg, 0.08 mmol), and 70% perchloric acid (25 mg, 0.24 mmol) in 25 mL of *p*-dioxane was stirred at room temperature for 5 h, during which time a purple precipitate formed. The mixture was filtered, and the precipitate was washed with ethyl acetate (2 × 20 mL) and dried under high vacuum. This procedure gave 15 mg (21% yield) of **3** as the perchlorate, which contained 1.5 equiv of $NaClO_4$ and 2.5 equiv of *p*-dioxane. It had mp 175–185 °C: IR (KBr) 3200–3500, 1650–1700, 1040–1090 cm^{-1}. Anal. ($C_{23}H_{26}FN_6O_{12}P \cdot HClO_4 \cdot 1.5NaCl \cdot 2.5C_4H_8O_2$) C, H, F, N.

Method B. A solution of mitomycin C (50 mg, 0.15 mmol), sodium 5-fluoro-2′-deoxyuridylate (56 mg, 0.15 mmol), and 70% perchloric acid in 10 mL of DMF was stirred at 50–60 °C for 0.5 h, during which time the color changed from blue to purple. It was cooled to room temperature and evaporated under reduced pressure. The residue was purified by preparative TLC on a silica gel plate (20 × 20 × 0.2 cm) with MeOH–$CHCl_3$ (2:8 v/v) as solvent. The main purple band was removed and extracted with MeOH–CH_2Cl_2. This extract was filtered and evaporated, affording 28 mg (30% yield) of **3** as the neutral species (zwitterion), purple solid with mp 135–140 °C dec: NMR (Me_2SO-d_6 + D_2O) δ 7.90 (s, 1, 6-H of 5-FU), 5.07 (s, 2, 10-CH_2 of mitosene), 1.75 (s, 3, 6-CH_3 of mitosene); IR (KBr) 3420–3440, 3340–3360, 3280, 3200, 1700, 1670–1690, 1040–1090 cm^{-1}; UV (MeOH) λ_{max} 249, 308, 346 nm.

This product was identical on TLC in the system described above with the product obtained by method A after the latter was neutralized with aqueous 0.5 M ammonium hydroxide.

Catalytic Reduction of 2. A solution of the neutral species of **2** (2 mg) in 1 mL of distilled water was treated with a small amount of 10% palladium-on-charcoal. The flask containing this mixture was flushed twice with nitrogen followed by hydrogen and then a hydrogen atmosphere was maintained while stirring was made for 30 min. Reduction was indicated by a change in color from intense to faint purple. After removal of the catalyst and concentration of the filtrate, the purple residue (reoxidation to the mitosene chromophore by air) was chromatographed on a silica gel plate with MeOH–$CHCl_3$ (2:8 v/v) as solvent. The major purple spot was identical in R_f (0.33) with 2,7-diamino-1-hydroxymitosene (**4**), and a spot close to the origin (R_f 0.02) was identical with uridylic acid. Two spots with high R_f values were unknown mitomycin degradation products.

The above experiment was repeated with [^{18}O]water as the solvent. After the catalyst was removed, the solution was extracted with 5 mL of ethyl acetate, and this extract was dried and evaporated under reduced pressure. The residue was analyzed by EI mass spectrometry at 70 eV. The relative intensities of the peaks at m/z 320 (2,7-diamino-1-hydroxymitosene) and m/z 322 [^{18}O]-1-hydroxy analogue) indicated that there was about 60% incorporation of ^{18}O.

Reduction of Mitomycin C in the Presence of 2′-Deoxyguanosine. A solution of mitomycin C (50 mg, 0.15 mmol) and 2′-deoxyguanosine monohydrate (300 mg, 1.05 mmole) in 50 mL of water (warmed to 40 °C to dissolve the solids) was deaerated by bubbling N_2 through it for 1 h. Addition of sodium dithionite (50 mg, 0.29 mmol) in 0.5 mL of water caused decolorization of the solution within 15 s. After being stirred under N_2 for 30 min, the solution was treated with O_2 (bubbled in), which produced a purple color. Concentration of the resulting solution by rotary evaporation under reduced pressure gave a solid, which showed on TLC (silica gel, 1% NH_4OH/2-propanol, 3:20 v/v) a major purple spot at R_f 0.33, a minor spot at R_f 0.49, and a number of very faint spots. This crude product was difficult to separate from

(22) Valeriote, F.; Lin, H. *Cancer Chemother. Rep. Part 1* **1975**, *59*, 895.
(23) Geran, R. I.; Greenberg, N. H.; MacDonald, M. M.; Schumacher, A. M.; Abbott, B. J. *Cancer Chemother. Rep. Part 3* **1972**, *3*, 1.

the excess of 2′-deoxyguanosine. However, repeated column chromatography on silica gel (70–270 mesh) with the solvent system described above, followed by preparative TLC on a precoated silica gel plate (20 × 20 × 0.2 cm) with the same solvent gave a small amount of product that showed no traces of other compounds according to this TLC system. This product (**6**) showed λ_{max} (MeOH) 248, 266, 276, 308, 341, and 510–530 nm. The ^{1}H NMR spectrum (DMSO-d_6 containing 5% CF_3CO_2D; TMS standard) showed δ 1.76 (s, 3, 6″-CH_3 of mitosene), 2.2–2.5 (m, 2′-H's of 2-deoxyriboses), 3.6–3.7 (br s, 5′-Hs of 2-deoxyriboses), 3.8–4.1 (m, 3′-H of 2-deoxyriboses), 4.2–4.7 (m, 1″-H of mitosene, 10″-CH_2 of mitosene, and 4′-H of 2′-deoxyriboses), 5.8 (br s, 2″-H of mitosene), 6.1–6.4 (m, 3″-H of mitosene and 1′-H of 2-deoxyriboses), and 9.27 (s, 2, 8 H of guanines). It was difficult to obtain precise integrals for most of the signals, but the mitosene peak at δ 1.76 and the guanine peak at δ 9.27 were sharp and well-separated from other signals. Their 3:2 ratio indicated that there was one mitosene per two 2′-deoxyguanosines in the product. The ^{13}C NMR data of this product is given in Table I.

Reduction of 2 in the Presence of 2′-Deoxyguanosine. Method A. Sodium Dithionite. A mixture of **2** (5 mg), 2′-deoxyguanosine (30 mg), and water (5 mL) was warmed at 40 °C to make a solution and then deaerated by bubbling in N_2 for 1 h. Sodium dithionite (5 mg) in water (0.5 mL) was added, and the resulting solution, which decolorized rapidly, was stirred under N_2 for 30 min. Oxygen was then bubbled in and the purple solution was concentrated to 1 mL in a rotary evaporator. Thin-layer chromatography of this residue on silica gel with the system described above gave two faint spots at R_f 0.33 and R_f 0.49, which were identical with those from the mitomycin C reaction by parallel and overspot TLC, plus a number of very faint spots.

Method B. Catalytic Method. A mixture of **2** (5 mg), 2′-deoxyguanosine (30 mg), 10% palladium-on-carbon catalyst (5 mg), and water (5 mL) was warmed at 40 °C, flushed twice with hydrogen, and then stirred under hydrogen for 20 min, during which time it became colorless. After being flushed twice with nitrogen, the mixture was stirred in open air for 15 min, which resulted in a purple color. The catalyst was removed by filtration, and the filtrate was concentrated on a rotary evaporator to 0.5 mL. Thin-layer chromatography of this residue with the system described above gave a spot at R_f 0.49 that was identical with one from the mitomycin C reduction. There were a number of other very faint spots but none at R_f 0.33.

DNA Binding Studies. These studies were conducted according to the procedure of Hashimoto et al.[2] A solution of **2** or the mitomycin C control (10 mg) in 10 mL of water was mixed with a solution of calf thymus DNA (20 mg, from Sigma) in 10 mL of water. Approximately 5 mg of palladium-on-charcoal was added, and a hydrogen atmosphere was introduced (flushed twice) and maintained for 15 min. The reaction vessel was evacuated and flushed with N_2, and then the purple solution was stirred in air for 10 min. After filtration, the filtrate was diluted with 200 mL of ice-cold ethanol. The purple DNA strands that separated were collected, washed with 50% aqueous ethanol (10 mL), and dried under vacuum. They were then dissolved in 10 mL of water and dialyzed, with a dialysis sack (Sigma 250-9U) and 150 mL of water in the external chamber. After 72 h no further change was observed in the two chambers. The concentration of mitosene tightly bound to the DNA was calculated from the relative intensities of absorbances at λ_{max} 259 nm (nucleotides) and 310 nm (mitosene) in the solution in the dialysis sack. For these calculations, extinction coefficients of 6600 and 5300, respectively, were used. The extinction coefficient for the mitosene unit was based on an independent determination of 2,7-diamino-1-hydroxymitosene (**4**) in the presence of DNA (no covalent binding). The results indicated a ratio of one mitosene per 180–210 bases in the case of mitomycin C and one mitosene per 200–240 bases in the case of **2**.

Clonogenic Antitumor Assay. The antitumor activity of the nucleotide derivatives was evaluated by using in vitro colony-forming assays in soft agar. L1210 murine lymphocytic leukemia cells (4×10^4/mL) were exposed to drug for either 1 h or continuously (drug added to final plating medium consisting of RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin and streptomycin, and 0.3% agar (all vol/vol)). Plates were innoculated at 37 °C, 10% CO_2, 90% air for 8–9 days and colonies > 60 μm in size were counted by a computerized image analyzer.[24]

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Registry No. **1**, 50-07-7; **2**, 114612-47-4; **3**·$HClO_4$, 114580-92-6; **4**, 1096-49-7; **4** ([^{18}O]-1-hydroxy analog), 114563-68-7; **6**, 114563-69-8; uridylic acid, 58-97-9; disodium 5-fluoro-2′-deoxyuridylate, 103226-10-4; 2′-deoxyguanosine, 961-07-9.

(24) Salmon, S. E.; Young, L.; Lebowitz, J.; Thomson, S.; Einspahr, J.; Tong, T.; Moon, T. E. *Int. J. Cell Cloning* **1984**, *2*, 142.