reaction was terminated by rapid filtration of samples through GF/B Whatman glass fiber filter strips pretreated with 0.1% polyethylenimine solution with a Brandel cell harvester: this was followed immediately by three rapid washes with 4-mL aliquots of ice-cold saline solution. Filters were removed and allowed to dry before assaying filter bound radioactivity by liquid scintillation spectrophotometry (43% efficiency).

The data were analyzed by using nonlinear least-squares regression analysis on the Apple II plus computer. Programs were generously written by Susan Yamamura.

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Registry No. 1, 88373-73-3; 2, 122622-48-4; 3, 122507-47-5; 4, 122622-49-5; 5, 122622-50-8; BOC-D-Pen(*p*MB)-OH, 115962-34-0; BOC-*p*-F-Phe-OH, 41153-30-4; BOC-Gly-OH, 4530-20-5; BOC-Tyr-OH, 3978-80-1; H-Tyr-D-Pen-Gly-*p*-F-Phe-D-Pen-OH, 122593-60-6; BOC-*p*-Cl-Phe-OH, 68090-88-0; H-Tyr-D-Pen-Gly-*p*-Cl-Phe-D-Pen-OH, 122593-57-1; BOC-*p*-Br-Phe-OH, 62129-39-9; H-Tyr-D-Pen-Gly-*p*-Br-Phe-D-Pen-OH, 122593-59-3; BOC-*p*-I-Phe-OH, 62129-44-6; H-Tyr-D-Pen-Gly-*p*-I-Phe-D-Pen-OH, 122593-58-2.

Alkylation of DNA by C-10 of 2,7-Diaminomitosene

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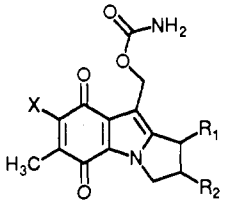
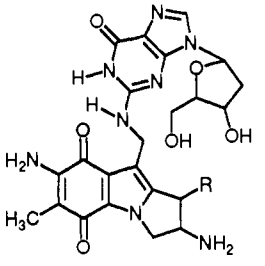
Mitomycin C and certain analogues alkylate DNA with their C-1 position and cross-link it by a second alkylation involving C-10. We now show that monoalkylation by C-10 (carbamate group) can occur for mitosene analogues that have no reactive C-1 functionality. Sodium dithionite reduction of 2,7-diaminomitosene or 2,7-diamino-1-hydroxymitosene in the presence of calf thymus DNA resulted in alkylation of the DNA to the extent of one molecule per 14 and 11 bases, respectively, although no covalent binding was observed on catalytic reduction. Reduction of each of these mitosenes by sodium dithionite in the presence of 2′-deoxyguanosine gave monoalkylation on the 2-amino group of this nucleotide. The 2,7-diaminomitosenes inhibited L-1210 leukemia cell colony formation in vitro at concentrations 3–4-fold greater (less potent) than mitomycin C. DNA single-strand breaks were also produced by each mitosene, but these lesions did not correlate with cytotoxicity and were less prominent than breaks produced by another monofunctional alkylating agent, methyl methanesulfonate. Mitosene-induced DNA strand breaks are probably due to excission–repair endonuclease activity and not from oxygen free radicals produced by redox cycling of the quinone moiety. There was no evidence of DNA–DNA cross-links by either 2,7-diaminomitosene.

The alkylation of DNA by mitomycin C has been studied by a number of investigators.[1-10] Their results have led to the definition of a process in which there is an initial alkylation on the 2-amino group of a guanine residue by C-1 of mitomycin C, following bioreductive activation and opening of the aziridine ring (Scheme I). A second alkylation by C-10 of mitomycin C, with loss of the carbamoyloxy group, is possible and results in cross-linking, providing that a second guanine residue is in an appropriate location, below the complimentary cytidine residue. If a second guanine is not in proximity, the carbamoyloxy group is simply replaced by a hydroxyl group.[10] Thus, cross-linking occurs by mitomycin C to a lesser extent than monoalkylation. Enzymatic hydrolysis of DNA bound covalently by mitomycin C has produced fragments in which the resulting mitomycin species was bound monocovalently by C-1 to the 2-amino group of 2′-deoxyguanosine or by C-1 and C-10 to the 2-amino groups of two 2′-deoxyguanosines. This confirms the chemical activation process described above.[5,6]

Products derived from monoalkylation of DNA by C-10 of mitomycin C have never been isolated. This may be explained by the observation that the aziridine ring of mitomycin C is more reactive than the carbamate group.[11] Notwithstanding this, previous biological studies on mitosenes show that compounds lacking an aziridine ring or other alkylating functionality have potent cytotoxic activity, expecially toward bacteria.[12] Thus, 1-hydroxy-7-methoxymitosene (1) which has a relatively poor leaving group at C-1, was as potent as mitomycin C against Gram-positive bacteria in a disk-plate assay.[12] It also was





1: X = CH_3O, R_1 = OH, R_2 = H
2: X = CH_3O, R_1 = R_2 = H
3: X = H_2N, R_1 = uridylate, R_2 = NH_2
4: X = H_2N, R_1 = H, R_2 = NH_2
5: X = H_2N, R_1 = OH, R_2 = NH_2

6: R = H
7: R = OH

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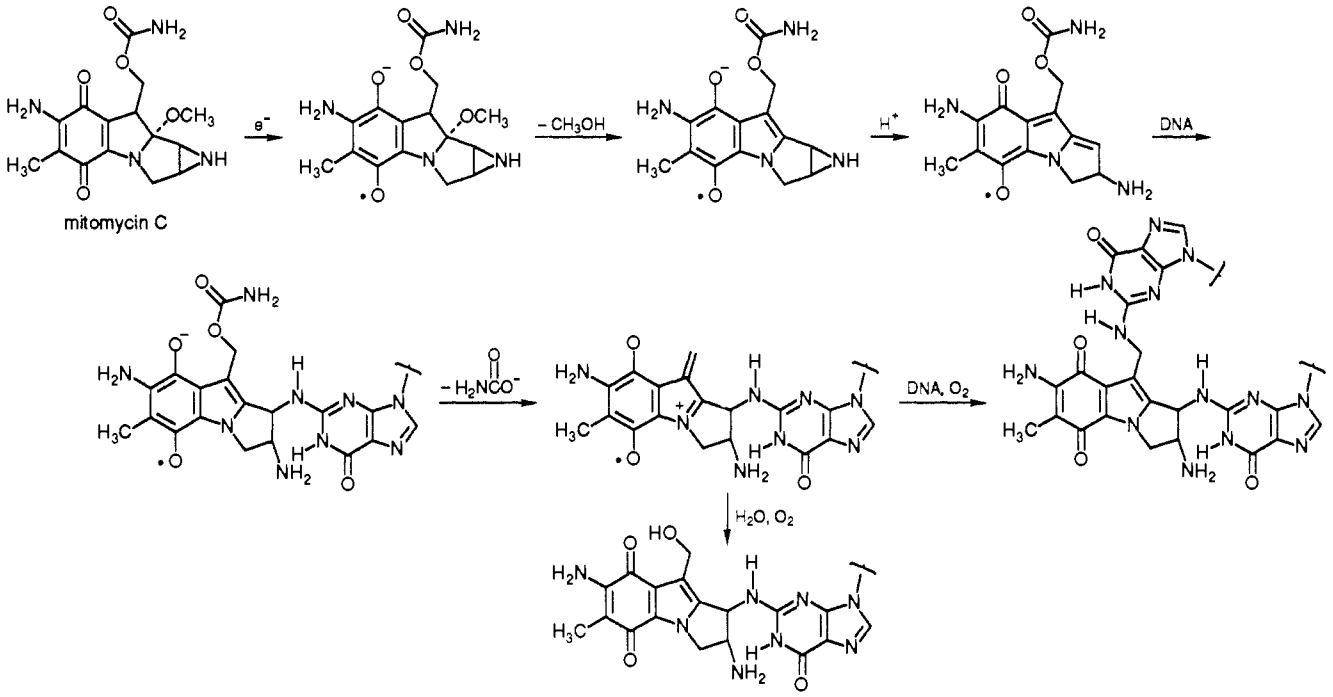
Scheme I



effective in activating λ-phage in *Escherichia coli*. Furthermore, 7-methoxymitosene (**2**), with no substituent at C-1, also showed in vitro antibacterial activity.[13] One plausible mode of action for these compounds is that they alkylate bacterial DNA through bioreductive activation of their C-10 position. In contrast, they do not have high antitumor potency in mice, possibly because they are rapidly metabolized or cleared from the body. The absence of host toxicity (weight loss or early deaths) at the highest tested doses supports this idea.[14-16] Thus, mitosenes appear to require cross-linking for a lethal effect against cancer cells.[1] However, mitosenes do not require an aziridine ring for antitumor activity if some other reactive functionality is present at C-1.[16] For example, the 1-uridylate derivative of 2,7-diaminomitosene (**3**) is active against P-388 lymphocytic leukemia in mice and it has been shown to alkylate calf thymus DNA and to react with two molecules of 2′-deoxyguanosine.[17]

In order to gain further insight into the cytotoxicity of mitosenes without reactive functionalities at C-1, we decided to test the ability of two model compounds to alkylate DNA. If this alkylation occurred, they would be examined further for their specific interaction with 2′-deoxyguanosine to determine if C-10 was involved. The effect on DNA also would be examined by an alkaline elution assay to determine the extent to which strand cleavage or cross-linking might occur. The two compounds chosen were 2,7-diaminomitosene (**4**) and *cis*-2,7-diamino-1-hydroxymitosene (**5**), both of which are known.[18,19]

Results and Discussion

Compounds **4** and **5** were prepared from mitomycin C by literature procedures.[18,19] Only the cis isomer of **5** is obtained when mitomycin C is treated with dilute HCl. These compounds had the same R_f values and ^{1}H NMR spectra as those of the previously reported samples. Compound **4** was characterized further by a molecular ion at m/e 304 in the EI mass spectrum. Each compound was reduced in the presence of calf thymus DNA by two different methods: catalytically with hydrogen and palladium,[3] and chemically with sodium dithionite.[20] Following filtration and air oxidation, the DNA was dialyzed to remove any noncovalently bound drug. The ratio of covalently bound drug to DNA bases was then determined by comparing the relatively intensities of ultraviolet absorption at λ 310 nm and 255 nm. For the measurement at 310 nm, the reference cell contained unbound DNA at the same concentration as DNA in the sample cell. This allowed the concentration of the drug to be determined from the difference spectrum and compensated for the small DNA absorption at that wavelength.

There was a small amount of absorption at 310 nm in the difference spectrum from a product derived from catalytic reduction of both **4** and **5**. However, the same amount of absorption was produced when calf thymus was reduced catalytically in the absence of drug. There apparently is slight DNA degradation under these conditions. Consequently, there is no evidence for the binding of **4** or **5** as a result of catalytic reduction. Mitomycin C does give a low degree of DNA binding, one molecule of drug per 180–200 bases following catalytic reduction.[3,17] Very different results were obtained when sodium dithionite was the reducing agent. Under these conditions, a ratio of one molecule of drug per 14 base pairs was obtained with **4** and

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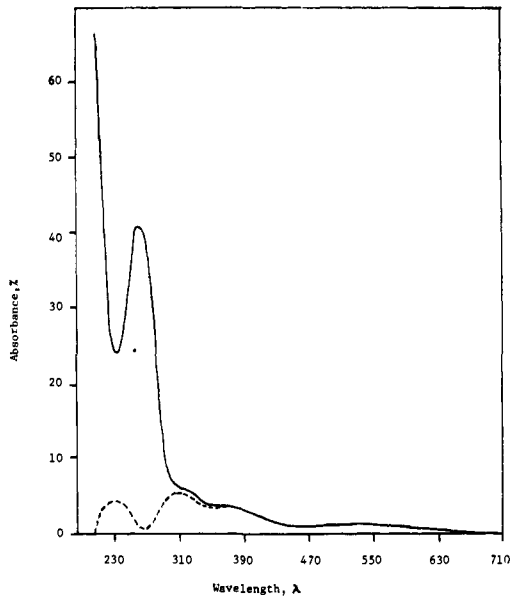

Figure 1. Ultraviolet and visible spectrum for the covalent adduct of **4** with calf thymus DNA (–); difference spectrum between the adduct and unbound calf thymus DNA (---).

the corresponding ratio was 1:11 with **5**. In comparison, reduction of mitomycin C under the same conditions gave a ratio of 1:4. Figure 1 shows the ultraviolet absorption curve for the binding of **4** to calf thymus DNA and the difference curve between this one and unbound DNA. Very similar curves were obtained for **5**.

Once the alkylation of DNA was established, it became important to examine our supposition that **4** and **5** were binding to the 2-amino group of a guanine residue by their C-10 atoms. This was done by reducing each compound with sodium dithionite in the presence of excess 2′-deoxyguanosine. We had shown previously that this reducing agent was more effective than catalytic hydrogenation for the alkylation of 2′-deoxyguanosine by mitomycin C or **3**.[17] With **4** and **5** a single product was obtained in each case. As discussed below, these products, **6** and **7**, were identified by their ^1H and ^{13}C NMR spectra, together with IR and UV spectra, as 2′-deoxyguanosine alkylated on the 2-amino group by C-10 of the mitosene.

The mitosene derivatives of 2′-deoxyguanosine were characterized by their UV–visible absorption spectra, which were essentially unchanged from those of the starting mitosenes. This result showed that the chromophore was intact. The loss of carbonyl absorption at 1700 cm^{-1} in the IR spectra demonstrated loss of the carbamate substituent, whereas a new carbonyl group at 1690 cm^{-1} was attributed to the guanosine moiety. Peaks clearly distinguishable in the ^1H NMR spectrum of **7** included H-8 of guanine at δ 7.90, the anomeric carbon of 2′-deoxyribose at δ 6.2, the 5-methylene of this sugar at δ 3.6, and the mitosene methyl group at δ 1.75. Most importantly, the presence of a doubled doublet at δ 4.3 indicated that the C-10 methylene was attached to nitrogen, rather than oxygen (which would have given a doublet at δ 5.3). Similar peaks were found in the ^1H NMR spectrum of **6**. Table I lists the ^{13}C NMR peaks for **6** and **7**, along with 2′-deoxyguanosine and **5** as reference compounds. There are close correspondences for every peak in **6** and **7** with those in the reference compounds, with the following exceptions: (1) the 1″ carbon peak of **6** is considerably upfield from the others because it is a methylene rather than an oxygen-substituted methine carbon; (2) the 10″ carbon peaks of **6** and **7** are upfield from that of reference compounds **5** because they are substituted by nitrogen, whereas **5** has

Table I. ^{13}C NMR Spectral Data of Mitosene Derivatives of 2′-Deoxyguanosine[a]

carbon no.	δ values in ppm			
	7	6	2′-deoxyguanosine[b]	5[c]
2	155.3	157.0	154.0	
4	153.5	150.0	151.3	
5	117.5	117.0	116.9	
6	156.3	158.0	157.5	
8	134.63	137.0	136.2	
1′	88.8	84.0	83.2	
2′	~40.0[d]	~40.0[d]	40.0	
3′	70.5	73.0	71.5	
4′	96.5	90.0	87.9	
5′	60.95	62.5	62.1	
1″	63.3	31.0[e]		65.1
2″	55.97	55.0		57.9
3″	53.91	53.0		51.8
5a″	149.91	145.0		145.7
5″	178.0	172.1		178.2
6″	108.0	106.0		107.6
6a″CH_3	8.5	8.5		8.4
7″	135.5	136.0		135.7
8″	177.5	180.0		177.3
8a″	114.0	115.0		115.2
9″	121.5	121.5		122.4
9a″	128.8	128.5		129.2
10″	44.5	46.5		58.8

[a] Spectra were recorded on a JEOL FX90Q NMR spectrometer at a frequency of 22.5 Hz in DMSO-d_6 with TMS as internal standard. [b] Chang, C.-J.; Gomez, J. D.; Byrn, S. R. *J. Org. Chem.* **1983**, *48*, 5151. [c] Kohn, H. L.; Zein, N.; Lin, X. Q.; Ding, J.-Q.; Kadish, K. M. *J. Am. Chem. Soc.* **1987**, *109*, 1833. [d] This peak fell under the DMSO impurity in DMSO-d_6. [e] 1″C is a methylene group in **6**.

the carbamate oxygen as a substituent. These results confirm the structures of **6** and **7** and support the idea that mitosenes can alkylate the 2-amino groups of guanine residues in DNA through their C-10 atoms.

As expected from the DNA binding experiment, no alkylation of 2′-deoxyguanosine occurred when mitosene **5** was reduced catalytically in its presence. There was some hydrolysis of the carbamoyl group of **5**.

The cytotoxicity assays with the 2,7-diaminomitosenes show that relatively high concentrations of each agent are required to significantly reduce L-1210 leukemia cell colony formation in vitro (Table II). The 1-h drug concentrations which reduced colony formation to 50% of controls (IC_{50} value) were 36 μg/mL for **4** and 18 μg/mL for **5**. This is similar to the IC_{50} of 32 μg/mL for methyl methanesulfonate, a classic monofunctional DNA alkylator (Table II).[21] By comparison, the IC_{50} for the bifunctional alkylator mitomycin C in this cell line was 5.0 μg/mL.

In the alkaline elution studies, none of the compounds reversed DNA double-strand breaks induced by ionizing radiation. This confirms that these agents do not produce DNA–DNA cross-links (data not shown). Each of the three monofunctional alkylators did produce DNA single-strand breaks following a range of 1-h drug exposures (Table II). However, the 2,7-diaminomitosenes produced substantially fewer strand breaks than did methyl methanesulfonate. Of interest, strand-break frequencies were proportional to cytotoxicity only with compound **4**. With **5** no strand breaks could be detected unless supralethal drug exposures were used. In contrast, methyl methanesulfonate produced substantial strand breaks even at noncytotoxic concentrations. These breaks increased further when cytotoxic drug concentrations were used. The single-strand breaks produced by the 2,7-diaminomitosenes were not consistently reversed by coincubation with the oxygen free radical

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Table II. Relationship between Cytotoxicity and DNA Single-Strand Breaks for Monofunctional Mitosene Alkylating Agents

compound	exposure concentration, μg/mL × 1 h	% survival colony-forming units[a]	DNA single-strand breaks (rad equiv)[b]
2,7-diaminomitosene (4)	10	100	0
	50	43	110 (18)
	100	8	320 (65)
	250	0.9	470[c]
	500	0	535 (47)
2,7-diamino-1-hydroxymitosene (5)	1.0	100	NT[d]
	10	81	0
	50	2	0
	100	0	50 (18)
	500	0	395 (20)
methyl methane sulfonate	10	100	583[c]
	50	32	935[c]
	100	0.2	2150[c]

[a] Mean of three determinations of L-1210 murine leukemia cell colony formation in soft agar (≥60-μm size). [b] Rad equivalents relate to the dose of ionizing radiation required to produce comparable elution patterns [mean (standard error) of three determinations]. [c] Mean of duplicate determinations. [d] NT, not tested.

scavenger mannitol[17] (data not shown).

These findings suggest that 2,7-diaminomitosenes produce DNA single-strand breaks as is observed with other classical monofunctional alkylating agents.[22] However, these breaks do not directly correlate with cytotoxicity and are not blocked by an oxygen free radical scavenger. Thus, the breaks do not appear to be due to redox reactions of the quinone moiety. Rather, the DNA damage from the 2,7-diaminomitosenes probably relates to the production of alkali-labile DNA sites following cellular excission–repair endonuclease activity.[22] This enzymatic activity is known to mediate the removal of monoadducts from DNA strands, as has been previously demonstrated with methyl methanesulfonate.[22]

Conclusions

2,7-Diaminomitosenes without good leaving groups at C-1 were able to alkylate isolated DNA following sodium dithionite reduction, but not catalytic reduction. The covalent bond probably was formed between the 2-amino group of a guanine and C-10 of the mitosenes, based on the results of a separate experiment in which the mitosenes were reduced in the presence of 2′-deoxyguanosine. There was no evidence for DNA cross-links according to alkaline elution studies following incubation of the mitosenes with L-1210 leukemia cells. These results are consistent with a mode of action in which bioreductive activation of C-10 is followed by monoalkylation of DNA. They demonstrate that covalent binding between C-1 of mitosenes and DNA is not required to position the molecules for the C-10 alkylation. This result supports a computer modeling study that showed favorable noncovalent and monocovalent binding for 2,7-diaminomitosene in the putative mitomycin receptor on DNA.[23]

The superiority of sodium dithionite reduction over catalytic reduction for promoting the alkylation of DNA and other nuclophiles by both C-1 and C-10 of mitomycin C has been reported.[20] These results have been explained by a difference in mechanism: catalytic reduction is a two-electron process that gives a relatively stable hydroquinone, whereas dithionite reduction is a one-electron process that gives an unstable radical anion.[11,20] The radical anion readily loses the elements of methanol.[11,24] Subsequent protonation and aziridine ring opening affords a reactive intermediate capable of alkylation by C-1 (Scheme I). The resulting compound still is a radical and it can readily lose the carbamoyloxy substituent, which results in a species that can give C-10 alkylation.[20] When mitosenes such as **4** and **5** are reduced in the presence of nucleophiles, a process closely related to the second stage of alkylation by mitomycin C probably occurs. Thus, sodium dithionite reduction would give radical anions that readily lose the carbamyloxy substituent substituent to afford species that alkylate by C-10. In contrast, catalytic reduction would give hydroquinones that are stable. They simply would revert to starting materials **4** and **5** or the corresponding decarbamoyl derivatives following air oxidation. The hydroquinones from **4** or **5** must be more stable than the one formed from mitomycin C, because the former give no alkylation, whereas the latter gives a low level of monoalkylation.

Although these mitosenes do not have substantial antitumor activity in mice, they are cytotoxic to L-1210 at concentrations only slightly higher than those of mitomycin C (IC_{50} of 18–30 vs 5 μg/mL). They produce concentration-dependent DNA single-strand breaks that are (1) not consistently associated with cytotoxicity, (2) not blocked by an oxygen-free radical scavenger, (3) not as numerous as those caused by methyl methanesulfonate, and (4) probably related to DNA excision–repair endonuclease activity. The smaller amount of strand cleavage by **4** and **5** compared with methyl methanesulfonate, a potent mutagen, suggests greater selectivity and the possibility of lower mutagenicity for the mitosenes.

Experimental Section

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 JEOL FX90Q spectrometer, with Me_4Si as the internal reference standard. Mass spectra were measured on a Varian 311 spectrometer.

DNA Binding Studies. A. Dithionite Reduction. A mixture of **4**, **5**, or mitomycin C(10 mg) and calf thymus DNA (20 mg, from Sigma) in water (10 mL) was deaerated by bubbling nitrogen through it for 30 min. It was then treated with a solution of sodium dithionite (5 mg) in 0.2 mL of water with vigorous stirring. The resulting solution was decolorized instantly from purple to pale pink. The nitrogen was stopped and the solution was stirred in air for 15 min, during which time the purple color of the quinone returned. It was transferred to a Sigma 250-9U dialysis sack, which was suspended in 175 mL of distilled water. Dialysis was conducted for 7 days at 5–10 °C with the medium being replaced every 24 h. At the end of this time, the solution in the dialysis sack was diluted 100-fold and the concentration of mitosene bound tightly to DNA was calculated by the following method. The nucleotide concentration was determined first by

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measuring the percent absorbance at 255 nm, with an extinction coefficient of 6600 and water in the reference cell. A solution of unbound DNA was then placed in the reference cell and its concentration was adjusted until it had exactly the same absorbance at 255 nm as the bound DNA. The resulting difference spectrum (exemplified for 4 in Figure 1) then gave the absorbance at 310 nm for the mitosene, corrected for the small tailing absorbance of DNA at this wavelength. Coefficients of 12500 and 5300, respectively, were used for calculating the concentrations of adducts from 4 and 5 at 310 nm. These coefficients were determined independently from 4 and 5 in the presence of DNA without covalent binding. The results indicated the following ratios of mitosene to DNA bases in the adducts: 4, 1:14; 5, 1:11; mitomycin C, 1:4.

B. Catalytic Reduction. A solution of 4 or 5 (10 mg) in 20 mL of water was prepared by warming the mixture to 35 °C. Calf thymus DNA (20 mg) was added and the mixture was shaken vigorously until the DNA dissolved. Palladium-on-carbon catalyst (10%, 5 mg) was added, and the mixture was deaerated under vacuum, flushed twice with hydrogen, and then stirred under a hydrogen atmosphere for 20 min. During this time, the purple color of the quinone became faint. The flask was evacuated, flushed with N_2, and then O_2 was bubbled through the solution for 2 min. The resulting purple mixture was filtered and the filtrate was dialyzed as described above. Determination of the concentrations of the mitosenes in the adducts from 4 and 5 with DNA by the difference spectrum method described above showed very small absorbance at 310 nm, corresponding to one mitosene per 580–700 bases. In order to determine if this absorbance really came from adducts, we conducted an experiment in which calf thymus DNA was reduced, filtered, and dialyzed under the conditions described above, but without any mitosene present. The same small degree of absorbance at 310 nm was observed. Consequently, there is no evidence for formation of adducts.

Reduction of 4 and 5 in the Presence of 2′-Deoxyguanosine. A mixture of 5 (40 mg, 0.125 mmol) and 2′-deoxyguanosine monohydrate (250 mg, 0.88 mmol) in 25 mL of water was warmed to 40 °C to make a solution, which was deaerated by bubbling N_2 for 60 min. To this warmed solution was added sodium dithionite (50 mg, 0.29 mmol) in 0.5 mL of water, and the resulting solution was stirred under N_2 for 30 min. The purple color changed to pale yellow in the first 5 min. Bubbling O_2 through the solution for 2–3 min restored the purple color. The solution was concentrated to dryness at 50 °C on a rotary evaporator, and the crude product was purified by preparative TLC using a precoated silica gel plate (20 × 20 × 0.2 cm) and 1% NH_4OH/2-propanol, 3:20 v/v as developing solvent. Two purple bands formed. The faster moving one was starting material and the slower moving one was product. The latter was scraped from the plate, extracted with CH_3OH/CH_2Cl_2 (50:50 v/v), and filtered. Concentration of the filtrate gave a small amount of 7 as a purple solid that had R_f 0.386 on silica gel TLC with 1% NH_4OH/2-propanol (2:8 v/v) as solvent. It showed the following data: λ_{max} (MeOH) 244, 273, 304, 344, 502–532 nm; ν_{max} (KBr) 3200–3400 (OH and NH_2), 1690 (CO of guanine) cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.90 (s, 1, 8 H), 6.5 (br, 2, 7″ NH_2), 6.1 (br s, 1, 1′H), 4.8 (m, 1, 1″H), 4.4–4.6 (m, 2, 10″CH_2), 3.6 (br s, 2, 5′CH_2), 1.7 (s, 3, 6a″CH_3) ppm. Other peaks overlapped and it was not possible to derive precise δ values for them. The ^{13}C NMR spectrum is given in Table I.

Compound 6 was prepared by the procedure described for 7, except that the workup of the reaction mixture was modified. The mixture obtained from 4 (20 mg) and sodium dithionite (125 mg) in H_2O (12.5 mL) was concentrated under reduced pressure and the residual solid was extracted with EtOAc (10 mL). The insoluble part was washed with EtOAc (5 mL × 2) followed by MeOH (10 mL × 2) and then dried under high vacuum. This procedure gave 5 mg of 6 as a purple solid: IR (KBr) 3200–3400 (OH and NH_2), 1690 (CO of guanine) cm^{-1}; UV (MeOH) 245, 260, 307, 343, 520–535 nm; NMR (DMSO-d_6) δ 1.48 (s, 6a″CH_3), 2.1–2.4 (m, 2′CH_2), 3.54 (s, 5′CH_2), 4.3–4.6 (br s, 10″CH_2), 6.11 (t, 1′H), 6.47 (br s, 7″NH_2), 7.90 (s, 8 H) ppm. The ^{13}C NMR spectrum is given in Table I. Compound 6 had R_f 0.08 on silica gel TLC with 1% NH_4OH/2-propanol (2:8 v/v) as solvent.

Catalytic Reduction of 5 in the Presence of 2′-Deoxyguanosine. A mixture of 5 (10 mg, 0.03 mmol) and 2′-deoxyguanosine monohydrate (20 mg, 0.07 mmol) in 20 mL of water was treated with 5 mg of 10% palladium-on-carbon catalyst and reduced with hydrogen as described above. The filtrate was analyzed by TLC on silica gel using methanol/chloroform (2:8) as solvent. There was no spot corresponding to 7, with the sample prepared above. The major product was identical in R_f value with an authentic sample of *cis*-10-decarbamoyl-2,7-diamino-1-hydroxymitosene[18] in the same system.

Cytotoxicity Studies. Murine L-1210 leukemia cells were grown in RPMI-1640 medium (Grand Island Biologicals) containing 5% (v/v) heat-inactivated fetal bovine serum. Cells were exposed to the mitosenes dissolved in pH 7.4 phosphate-buffered saline for 1 h at 37 °C and then centrifuged twice in growth medium to remove unbound drug. The cells (5×10^4) were then plated in 1.0 mL of medium (containing 0.3% (v/v) agar) onto a growth medium/agar underlayer in 35-mm plastic Petrie dishes (Falcon Plastics).[25] The plates were incubated for 7–10 days at 37 °C in a humidified incubator and tumor cell colonies ≥60 μM in size were counted by inverted-phase microscopy using an automated image-analysis system.[26] Survival of drug-treated, colony-forming cells was compared to untreated control plates in which approximately 600–700 colonies developed over the incubation period.

DNA Single Strand Break Studies. Murine L-1210 leukemia cells were grown for 4 doublings (48 h) in RPMI 1640 growth medium containing 2.0 μCi of [2-^{14}C]thymidine (sp act. 50 mCi/mmol, Research Products International Corp.). The cells were then placed in nonradioactive medium for 24 h to allow for full incorporation of [^{14}C]thymidine into high molecular weight DNA. Following this, the cells were exposed to a compound for 1 h at 37 °C and then centrifuged twice in RPMI 1640 medium at 4 °C to remove unbound drug. The iced cells (10^6/sample) were then lysed on 2-μM pore size, 25-mm poly(vinyl chloride) filters (Millipore Corp.) with 5 mL of a solution containing 2% SDS (w/v) 0.1 M glycine, and 0.02 M sodium EDTA, pH 10.0 To dissolve any DNA–protein cross-linkes, a proteinase K solution (0.5 mg/mL, E Merck and Co., Darmstadt, Germany) was eluted over the cell lysate for 1 h. This solution was then rinsed out using phosphate-buffered saline, pH 7.4. For the next 15 h, an alkaline DNA-unwinding (elution) solution was pumped over the filters [0.02 M EDTA (acid form) 1% sodium dodecylsulfate, pH 12.1, with tetrapropylammonium hydroxide (RSA Corp., Ardsley, NY)]. Hourly fractions of 2.5 mL (containing single-stranded, ^{14}C-labeled DNA) were collected. DNA retained on the filter was then measured by scintillation counting and single strand DNA elution rates were calculated for each treatment. The first-order rate constant for [^{14}C]DNA elution was taken to represent the frequency of DNA single-strand scission as described in detail previously by Kohn et al.[27] DNA single-strand damage was converted to rad equivalents by comparing drug-induced DNA elution rates to elution rates produced by different doses of ionizing radiation delivered to untreated L1210 cells on ice using a 4 MeV linear accelator (Linac-4^R, Varian Associates, Palo Alto, CA). In some studies, L-1210 cells were irradiated after a 1-h treatment with a mitosene and then treated as above. In this way, any mitosene-induced DNA–DNA cross-linking could be detected as a reversal of the rapid DNA-elution patterns produced by the X-ray treatments.[27] All conditions were maintained at 4 °C to prevent enzymatic reversal of DNA lesions following the drug and/or radiation treatments.

Registry No. 4, 78598-43-3; 5, 123148-61-8; 6, 123126-67-0; 7, 123126-68-1; 2′-deoxyguanosine, 961-07-9.

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