Comparative Stereochemistry in the Aziridine Ring Openings of N-Methylmitomycin A and 7-Methoxy-1,2-(N-methylaziridino)mitosene

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A useful method was found for the conversion of mitomycin C into N-methylmitomycin A. The latter compound gave only two products on acid hydrolysis, the *cis*- and *trans*-1-hydroxy-7-methoxy-2-methylaminomitosenes. This selectivity allowed the cis–trans ratio to be quantitatively determined as 4:1. Such a predominance of the cis isomer is unexpected in view of the trans stereochemistry obtained in the opening of simple aziridines. In order to determine if the 9a-methoxy group of mitomycins controlled the direction of aziridine ring opening 7-methoxy-1,2-(N-methylaziridino)mitosene, which lacks this substituent, was prepared and hydrolyzed in acid. It gave the same two products in a 3:1 cis–trans ratio. In the induction of λ-bacteriophage in *Escherichia coli* *cis*-1-hydroxy-7-methoxy-2-methylaminomitosene was more active than the corresponding trans isomer, but both of these compounds were less active than the aziridinomitosene or the mitomycins. Mitomycin A, mitomycin C, and N-methylmitomycin A were active against P388 leukemia in mice.

Mitomycins bind covalently to the DNA in a variety of living cells. This binding has been described as a mixture of monofunctional and bifunctional alkylations in which the true lethal effect is the formation of cross-links between complimentary DNA strands.[1] The detailed binding process is thought to involve enzymic reduction of a mitomycin to the corresponding semiquinone radical which intercalates with the DNA.[2] Subsequent reduction affords the hydroquinone, a species capable of bifunctional alkylation because of its aziridine and carbamate functions conjugated with the indolic nitrogen.[1]

In earlier studies, binding between DNA and mitomycin C (1) could not be demonstrated unless NADPH-dependent cell extracts[1] or chemical reducing agents were added.[2] However, recent studies by Lown and co-workers have shown that unreduced mitomycin C is able to alkylate DNA with cross-linking in aqueous solution at pH 4.[3] The detailed structures of the chemical products formed between mitomycin C and DNA have not been determined. Alkylation on O-6 of guanine was suggested for the reductive activation process,[1,2] but this suggestion remains to be confirmed.

One interesting feature of the binding between DNA and mitomycins is the stereochemistry of the product formed when alkylation occurs by way of the aziridine ring. In principle the alkylation could take place from either of the carbon atoms at position 1 or 2 of the mitomycin, and the entering nucleophile (possibly O-6 of guanine) could assume either a cis or trans relationship with respect to the amino group generated upon opening of the aziridine ring. The structure and stereochemistry of the product or products might be determined by the exact way in which mitomycin C fits onto the DNA prior to alkylation. Alternatively, the mitomycin molecule might have an inherent tendency to undergo ring opening in one particular direction, which could be independent of its fit onto the DNA.

We have studied the structures and stereochemistry of mitosenes formed from opening of the aziridine ring of mitomycin C (1) and mitomycin A (2) in dilute acid solutions in the absence of DNA.[4,5] These studies give an indication of the ring-opening capabilities of the mitomycins with water or other solvents as the nucleophile and without possible directing influences from preliminary noncovalent binding with DNA. They showed that, as far as isolable products were concerned, the entering nucleophile always took the 1 position and cis stereochemistry predominated over trans stereochemistry in the resulting mitosenes.[5] This stereochemical preference was unexpected since it has been reported previously that the acid-catalyzed ring opening of simple aziridines gave exclusively trans stereochemistry.[6] Unfortunately our study was limited by the fact that mitomycin C and mitomycin A decompose readily in acid solution. Typical yields of isolated mitosenes from mitomycin A were 33% of the cis isomer and 5% of the trans isomer. Even lower yields of the corresponding mitosenes were obtained from mitomycin C.[5] Thus the possibility that ring opening to give a predominant, but unstable, trans isomer or 2-substituted product could not be ruled out.

Since N-methylmitomycin A (3) has the aziridine ring alkylated and it has a 7-methoxy group, we thought that it might have greater stability than either mitomycin C or mitomycin A. Thus it might give an acceptable material balance in aziridine ring-opening experiments. Another important feature of N-methylmitomycin A was the fact that it had been converted into the corresponding aziridinomitosene (4).[7] As discussed below we were interested in the acid-catalyzed ring opening of the latter compound because it lacks certain modes of ring opening available to the former compound. Since mitomycin C was the only starting material available to us, it was necessary to develop a convenient procedure for its conversion into N-methylmitomycin A so that these hydrolysis experiments could be performed.

The most obvious route for the preparation of N-methylmitomycin A (3) was a combination of the literature procedures for the transformation of mitomycin C into mitomycin A (2) (alkaline hydrolysis and diazomethane O-methylation)[8] and N-methylation with methyl iodide and potassium carbonate.[9] This route was successful, but the overall yield was only 5.4%. Instability of mitomycin C to alkaline hydrolysis and partial decomposition of mitomycin A during methylation were responsible for the low overall yield. Both of these difficulties could be minimized by reversing the order of operations. Thus mitomycin C was first N-methylated to give porfiromycin,[10] which was subsequently converted into N-methylmitomycin A. In this manner an overall yield of 24–30% was obtained. The conversion of N-methylmitomycin A (3) into the corresponding aziridinomitosene 4 was accomplished by a modification (change of solvent to ethyl acetate) of the reported procedure which involves catalytic reduction followed by air oxidation.[7]

As anticipated, the hydrolysis of N-methylmitomycin A in 0.05 N hydrochloric acid went cleanly and only two

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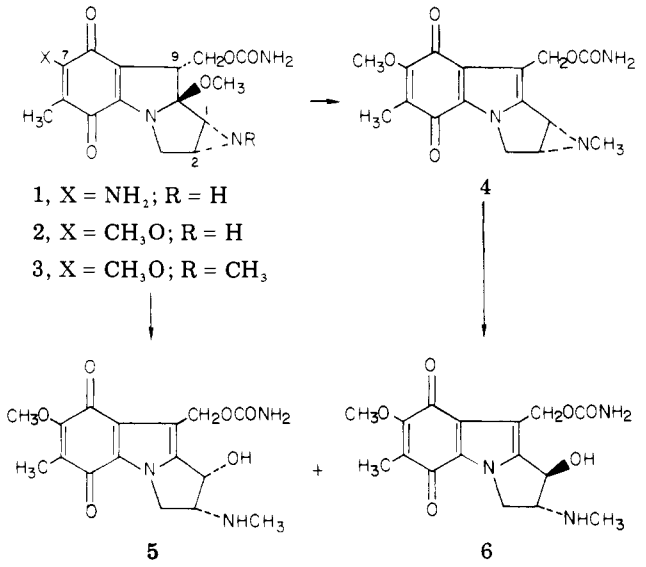
Scheme I

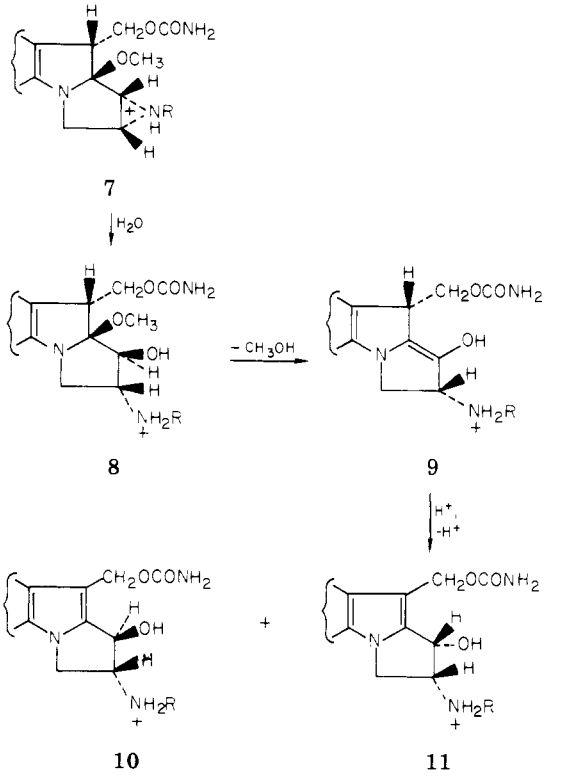


products were evident on thin-layer chromatography. They were shown to be *cis*- and *trans*-1-hydroxy-7-methoxy-2-methylaminomitosenes (**5** and **6**, Scheme I) and they could be isolated as pure crystalline solids in yields of 54.4 and 13.3%, respectively. A method based on thin-layer chromatography was developed for determining the ratios of cis and trans products formed in the hydrolysis. In this method (Experimental Section) the crude product was resolved into its isomers on a TLC plate, the separate spots were scraped off and extracted with a standard volume of methanol, and their relative amounts were determined by measurement of the optical densities at 285 nm in the ultraviolet absorption spectrum of their methanol solutions. By taking weighed samples of pure isomers through this procedure we found that 96% of the sample applied to the TLC plate could be accounted for in the UV determination.

The hydrolysis of *N*-methylmitomycin A and determination of the ratios of isomeric mitosenes were repeated two times with essentially identical results. A 4:1 ratio of cis isomer **5** to trans isomer **6** was found, which was in agreement with the isomer ratios found in our previous study on mitomycin C and mitomycin A.

The reason for such a predominance of the cis isomer from ring opening of an aziridine is not apparent, especially since it contrasts the products of simple aziridine ring openings. One possible explanation can be based on a mechanism that Stevens advanced to account for deuterium incorporation at position 1 during the solvolysis of mitomycin C in deuterioacetic acid.[11] In this mechanism (Scheme II) the first intermediate from aziridine ring opening would be the *trans*-aminohydrin **8**. However, this intermediate would undergo loss of methanol to give another intermediate **9** in which stereochemistry was lost at position 1. Protonation at position 1 followed by loss of the proton at position 9 then would give the mitosene products **10** and **11**. Since the incoming proton would prefer the side of the molecule opposite to the 2-methylammonium group, cis stereochemistry should predominate. In order to test this explanation we examined the hydrolysis of aziridinomitosene (**4**) in 0.05 N hydrochloric acid. This compound was expected to give the same two isomeric mitosenes that were obtained from *N*-methylmitomycin A; however, we anticipated a much different isomer ratio, possibly with predominance of the trans isomer, since the processes involving loss of methanol and protonation–deprotonation are not possible with **4**.

Scheme II



We found that the anticipated products were indeed formed, but once again the cis isomer predominated. Reproducible ratios of 3:1 cis to trans were obtained by the TLC separation method. The reason for cis predominance in this case is not at all apparent. There must be mechanisms in addition to the one advanced by Stevens for mitomycin hydrolysis. They might involve participation from the carbamoyloxymethyl side chain.

Since in living cells mitomycins appear to undergo aziridine ring opening after reduction to the corresponding hydroquinones,[1] we investigated the possibility of acid hydrolysis of the hydroquinone obtained by catalytic hydrogenation of *N*-methylmitomycin A. The objective was to determine if the ratio of cis to trans isomers in the mitosenes obtained in this manner (after air oxidation) differed appreciably from that obtained from hydrolysis at the quinone oxidation level. Unfortunately the hydroquinone gave a complex mixture of products. Five spots were found on TLC, of which two corresponded to mitosenes **5** and **6**.

The acid hydrolysis of *N*-methylmitomycin A had not been reported previously. There had been two reports on the hydrolysis of mitomycin B,[8,12] which should give the same mitosene products (or their enantiomers if the recently published[13] absolute stereochemistry of mitomycin B is correct). However, these reports indicated only one product of unspecified stereochemistry and its melting (decomposition) point, given in one of these reports,[8] was not identical with either of our compounds. Therefore it was necessary for us to undertake the characterization of both isomers. In addition to establishing the empirical formulas and functional groups of these molecules, we were able to differentiate between cis and trans stereochemistry by examining the ^1H NMR coupling constants between protons on carbons 1 and 2 in the diacetates prepared from isomers **5** and **6**. The *cis*-diacetate showed $J = 7$ Hz and the *trans*-diacetate showed 4 Hz. These values are in good agreement with those obtained for the corresponding diacetates of the isomeric mitosenes obtained from mi-

Table I. Induction of λ-Bacteriophage in *E. coli*[a]

Compd	Ratio of plaques induced by test compound at μg/mL to those induced in control					
	3.1	0.8	0.2	0.05	0.0125	0.003
1			ta, T[b]	17.6, T	6.6	2.7
2				ta, T	31.1, T	7.4
3		ta, T	21.2	8.2	3.7	1.8
4	35.4, T	38.8		3.4	3.0	0.8
5	22.4, T	4.4	2.0			
6	7.0	2.4				
12[c]	6.9	2.9				

[a] For a complete description of the assay procedure, see K. E. Price, R. E. Buck, and J. Lein, *Appl. Microbiol.*, 12, 428 (1964). [b] ta means too active to measure; T means toxic to culture. [c] This number refers to 1-hydroxy-7-methoxymitosene, the analogue of 5 and 6 without the 2-methylamino group.

tomycin A, whose stereochemistries were determined unambiguously.

Biological Activity. Induction of lytic activity by λ-bacteriophage inhabiting *Escherichia coli* is a useful preliminary screen for compounds with potential antitumor activity. The effects in this assay shown by the compounds described above are listed in Table I. This table shows that mitomycin C (1) and mitomycin A (2) are highly effective at very low doses. *N*-Methylmitomycin A (3) and 7-methoxy-1,2-(*N*-methylaziridino)mitosene (4) also are active but at somewhat higher doses. Of the two mitosenes without aziridine rings, the cis isomer 5 has higher activity than the trans isomer 6. Both of these compounds are less active than 4 or the mitomycins. Comparative testing data on cis- and trans-1,2-disubstituted mitosenes have been difficult to obtain. Only one other result is available and it also indicates greater potency for the cis isomer.[12] In this case the antibacterial activities of the *N*-demethyl analogues of 5 and 6 were compared. Compound 12, a synthetic analogue of 5 and 6 that lacks the 2-methylamino group and is racemic (at the 1 position), has activity comparable to that of 6.

Table II shows the relative activities of mitomycin C, mitomycin A, and *N*-methylmitomycin A against P388 leukemia in mice. Of these compounds, only mitomycin C has been reported to be active against leukemia in mice and this was against L1210 leukemia.[14] It is apparent from the table that *N*-methylmitomycin A is as active as mitomycin A against P388 leukemia, but mitomycin C is significantly more active than either of them. We were unable to prepare enough of compound 4 for this assay, but it has been shown to have some effect against L1210 leukemia.[14]

Experimental Section

Melting points were determined on a Thomas-Hoover capillary apparatus. Infrared spectra were determined in KBr disks on a Beckman IR-33 spectrophotometer. Nuclear magnetic resonance spectra were recorded on a Varian EM-360 spectrometer using tetramethylsilane as a standard and ultraviolet spectra were recorded on a Cary-17 spectrophotometer. Elemental analyses were performed by the Microanalytical Laboratory, Department of Chemistry, Purdue University. Analytical results were within ±0.4% of theoretical values. Each compound had IR and NMR spectra compatible with its structure.

***N*-Methylmitomycin A (3).** A mixture of mitomycin C (1.0 g, 3 mmol), methyl iodide (8.51 g, 60 mmol), and anhydrous potassium carbonate (4.14 g, 30 mmol) in 150 mL of acetone was refluxed for 12.5 h. The purple reaction mixture was filtered through Celite and the filtrate was concentrated to give a dark residue, which was hydrolyzed in 0.1 N sodium hydroxide (100 mL) for 4 h at room temperature. The dark purple solution was cooled briefly and acidified to pH 4 with 1 N sulfuric acid. The aqueous phase was extracted with ethyl acetate and evaporated to give a purple residue. Treatment of this residue with diazomethane (generated from 2 g of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 2.14 g of potassium hydroxide pellets, 6 mL of water, and 20 mL of diethyl ether) in diethyl ether at 0 °C overnight followed by column chromatography on silica gel (ethyl acetate as eluent) afforded 320 mg (30%) of dark purple crystals, mp 141–142 °C dec (lit.[15] mp 145 °C).

Hydrolysis of *N*-Methylmitomycin A. A solution of *N*-methylmitomycin A (203 mg, 0.56 mmol) in 46 mL of 0.05 N HCl was stirred at room temperature. At the end of 1.5 h the orange solution was cooled in ice and its pH was adjusted to 8.2 with 5% aqueous sodium bicarbonate solution. The resulting aqueous phase was extracted with ethyl acetate. The organic extract was washed with water, dried (Na_2SO_4), and removed under reduced pressure to give 165 mg of orange crystals. A very small amount (5 mg) was saved for UV determination while the bulk of the reaction product was applied to thick-layer chromatography on silica gel plates eluting with methanol. Two components were observed and separated. The more mobile component, isolated as an orange powder (26 mg, 13.3%), mp 166 °C dec, was

Table II. Activities of Mitomycins against P388 Murine Leukemia[a]

Compd	Dose, mg/kg/day	MST,[b] days	Effect, MST, % T/C	Av wt change, g	Survivors	
					Day 5	Day 30
1 (mitomycin C)	3.2	27.0	270	−2.3	6/6	2/6
	1.6	17.5	175	+0.1	6/6	0/6
	0.8	17.5	175	−0.8	6/6	1/6
	0.4	13.0	130	0	6/6	0/6
	0.2	12.5	125	+0.3	6/6	0/6
	0.1	12.0	120	0	6/6	0/6
2 (mitomycin A)	3.2	18.0	180	−2.1	6/6	0/6
	1.6	16.5	165	−3.1	6/6	0/6
	0.8	15.5	155	−2.6	6/6	0/6
	0.4	14.0	140	−0.4	6/6	0/6
	0.2	13.0	130	+0.1	6/6	0/6
	0.1	12.0	120	+0.3	6/6	0/6
3 (*N*-Methylmitomycin A)	6.4	16.5	165	−2.4	6/6	0/6
	3.2	18.0	180	−3.1	6/6	1/6
	1.6	14.5	145	−1.6	6/6	0/6
	0.8	14.5	145	−0.3	6/6	0/6
	0.4	13.0	130	+0.9	6/6	0/6
	0.2	11.5	115	+0.3	6/6	0/6
Control	Saline	1.0		+3.4	10/10	0/10

[a] A tumor inoculum of 10^6 ascites cells was implanted intraperitoneally in CDF_1 male mice. The treatment was once daily on days 1 and 5 only. For a detailed description of the test protocol, see *Cancer Chemother. Rep., Part 3*, 3, 9 (1972). [b] MST is the median survival time for the group of six mice.

trans-1-hydroxy-7-methoxy-2-methylaminomitosene (**6**): IR (KBr) 2.90–3.10 μ (OH, NH).

Approximately half of the orange powder was treated with 0.2 mL of acetic anhydride in 0.5 mL of pyridine for 2 h at room temperature. Removal of pyridine gave an orange solid. The NMR spectrum (Me_2SO-d_6) showed a broad doublet at δ 6.19 (J = 4 Hz, C_1H), indicative of trans stereochemistry.[5]

The less mobile component, *cis*-1-hydroxy-7-methoxy-2-methylaminomitosene (**5**), was isolated as orange crystals (106 mg, 54.4%): mp 155 °C dec; IR (KBr) 2.90–3.10 μ (OH, NH).

A portion (36 mg) of the orange crystals was acylated with 0.3 mL of acetic anhydride in 1 mL of pyridine. Removal of pyridine gave 28 mg of orange crystals whose NMR (Me_2SO-d_6) showed a doublet at δ 6.24 (J = 7 Hz, C_1H), indicative of cis stereochemistry.[5]

For UV determination, 5 mg of the above crude reaction product was dissolved in 5 mL of methanol. A 100-μL sample was removed and applied onto a 5 × 20 cm silica gel plate (no indicator). After elution with methanol the two components were separated and each component was dissolved in 25 mL of methanol. The UV spectrum of each showed absorption maxima at 285 and 233 nm. Comparison of peak heights at 285 nm gave a cis (less mobile)–trans (more mobile) ratio of 81.9:18.1.

This acid hydrolysis was repeated twice using 5 mg of *N*-methylmitomycin A and 1.2 mL of 0.05 N HCl. Determinations of UV absorption showed a cis–trans ratio of 82.8:17.2 and 78.7:21.3.

Hydrolysis of *N*-Methylmitomycin A after Reduction. A pressure-equalized addition funnel which contained 10 mg of *N*-methylmitomycin A, 5 mg of PtO_2, and 5 mL of ethyl acetate was fitted with an Erlenmeyer flask with a ground glass joint. Hydrogen was bubbled through until the solution turned colorless. The PtO_2 was removed by filtering through a pad of Celite into the Erlenmeyer flask under a positive pressure of nitrogen. The colorless filtrate was concentrated under reduced pressure to a pale yellow residue. Deoxygenated hydrochloric acid (0.05 N, 1.2 mL) was then added at room temperature. At the end of 1.5 h, the pH of the reaction solution was adjusted to 8 with 5% sodium bicarbonate. The resulting solution was stirred in air for 45 min. Extraction with ethyl acetate gave an orange residue. TLC analysis indicated that two of the five spots corresponded to the *cis*- and *trans*-1-hydroxy-7-methoxy-2-methylaminomitosenes (**5** and **6**).

7-Methoxy-1,2-(*N*-methylaziridino)mitosene (4). Hydrogen was bubbled through a solution of *N*-methylmitomycin A (**3**) (141 mg, 0.388 mmol) in ethyl acetate (30 mL) in the presence of platinum oxide (30 mg). Within 15 min the initially red-purple solution turned almost colorless. Hydrogen was terminated at the end of 25 min and the platinum oxide was filtered through a pad of Celite. The pale yellow filtrate was oxidized by aeration to a red solution. The solvent was evaporated under reduced pressure and the orange residue was chromatographed on silica gel to give 42 mg (32%) of orange crystals. The infrared spectrum was identical with that of an authentic sample.[7,16]

Hydrolysis of 7-Methoxy-1,2-(*N*-methylaziridino)mitosene. A solution of 7-methoxy-1,2-(*N*-methylaziridino)mitosene (**4**) (10 mg, 0.03 mmol) in 2.4 mL of 0.05 N HCl was stirred at room temperature. At the end of 1.5 h the orange solution was diluted with water and its pH was adjusted to 8 with 5% aqueous sodium bicarbonate solution. Extraction with ethyl acetate, drying over Na_2SO_4, and removal of organic solvent yielded an orange residue. On TLC this residue showed two main spots which had R_f values identical with those of **5** and **6**. The methanol extracts of these spots had the appropriate UV absorption at 285 and 233 nm. Comparison of the peak heights at 285 nm gave a cis–trans ratio of 73.2:26.8. A repeat of this experiment gave a cis–trans ratio of 76.5:23.5. Concentration of the methanol solutions gave orange solids which had IR absorption spectra identical with those of **5** and **6** prepared directly from *N*-methylmitomycin A.

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(16) We thank Dr. J. S. Webb of Lederle Laboratories for a copy of the infrared absorption spectrum of 7-methoxy-1,2-(*N*-methylaziridino)mitosene.