Reductive Alkylation of DNA by Mitomycin A, a Mitomycin with High Redox Potential†

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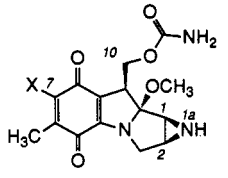
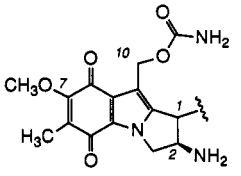
ABSTRACT: The mitomycins are a group of antitumor antibiotics that covalently bind to DNA upon reductive activation. Mitomycin A (**1b**; MA) is more toxic than its clinically useful cousin, mitomycin C (**1a**; MC). The greater toxicity of mitomycin A has been previously attributed to its higher reduction potential. In this report, the DNA alkylation products of reductively activated MA were isolated and characterized by conversion to the known 7-amino mitosene–deoxyguanosine adducts. The three major adducts formed were identified as a monoadduct, N^2-(2″β-amino-7″-methoxymitosen-1″α-yl)-2′-deoxyguanosine (**5**), a decarbamoyl monoadduct, N^2-(2″β-amino-10″-decarbamoyl-7″-methoxymitosen-1″α-yl)-2′-deoxyguanosine (**6**), and a bisadduct, N^2-(2″β-amino-10″-deoxyguanosin-N^2-yl-7-methoxymitosen-1″α-yl)-2′-deoxyguanosine (**7**). Under all reductive activation conditions employed, MA selectively alkylated the 2-amino group of guanine in DNA, like MC. In addition, both MA and MC alkylated DNA and cross-linked oligonucleotides to a similar extent. However, variations in the reductive activation conditions (H_2/PtO_2, $Na_2S_2O_4$, or enzymatic) affected the distribution of the three major MA adducts in a different manner than the distribution of MC adducts was affected. A mechanism is proposed wherein the 7-methoxy substituent of MA allows initial indiscriminate activation of either of the drugs' two electrophilic sites. While oxygen inhibited cross-linking by MC, similar aerobic conditions exhibited little influence on the cross-linking ability of MA. Hence, the greater toxicity of MA may be influenced by increased and nonselective activation and cross-link formation in both aerobic and anaerobic cells. This effect is a direct consequence of the higher redox potential of MA as compared to MC.

Mitomycin C (MC;[1] **1a**) is a clinically useful antitumor



1a (MC; X = NH_2)
1b (MA; X = OCH_3)



1c

antibiotic that has received widespread attention because of its effectiveness against the usually unresponsive solid tumors of the stomach and colon (Carter & Cooke, 1979). Early studies demonstrated that MC forms covalent monoadducts as well as interstrand cross-links with its cellular target, DNA (Iyer & Szybalski, 1963; Szybalski & Iyer, 1964a). The interstrand cross-links are believed to be the ultimate cause of tumor cell death. Furthermore, MC must first be reduced in order to complete these alkylation events (Iyer & Szybalski, 1964b; Moore, 1977; Kennedy et al., 1980). Numerous model studies have been carried out that have unraveled considerable details of the mechanism of the reductive activation and subsequent interaction of MC with nucleophiles [for example; Tomasz and Lipman (1981), Hornemann et al., (1983), Danishefsky and Ciufolini (1984), Danishefsky and Egbertson (1986), Andrews et al. (1986), Peterson and Fisher (1986), Kohn et al., (1987), and Hoey et al. (1988)]. Finally, structures of the major alkylation products between reduced MC and DNA, two monoadducts (**2** and **3**) (Tomasz et al., 1986a,b, 1988a) and one bisadduct (**4**), (Tomasz et al., 1987) have been determined. The latter was shown to constitute the interstrand cross-links of DNA by MC (Borowy-Borowski et al., 1990a). Studies into the forces governing the relative ratios of these adducts have demonstrated that the reduction conditions influence the cross-link to monoadduct ratio (Tomasz et al., 1987, 1988b).

An intense search has been underway to develop a more selective mitomycin that avoids MC's toxic side effects while maintaining its activity against tumors (Remers, 1979; Bradner et al., 1989). Design of these "second-generation" mitomycins has been based on the mechanistic studies described above. For instance, "mitosene"[2] analogues have been synthesized that attempt to substitute more effective leaving groups at the drug's known alkylation sites, its 1- and 10-positions (Leadbetter et al., 1974; Hodges et al., 1981; Orlemans et al., 1989). Perhaps a more prevalent strategy has been to change the quinone reduction potential by modifying or substituting the 7-amino group of MC. It has been a common hope that modulating the reduction potential will produce a more selective and potent antitumor agent. Remers and co-workers have analyzed an extensive series of 7-position analogues (Iyengar et al., 1983a,b, 1986), which initially suggested a correlation between reduction potential and antitumor activity

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[1] Abbreviations: MC, mitomycin C; MA, mitomycin A; UV, ultraviolet; SVD, snake venom diesterase.

[2] The term "mitosene" refers to the structure as in **1c**, without substituents at the 1-, 2-, and 7-positions (Webb et al., 1963).

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(Iyengar et al., 1981). However, a further study of a controlled set of analogues, the 7-*N*-phenyl-substituted mitosanes, demonstrated that, within the 7-amino-substitution series, other factors beside reduction potential had greater influences on antitumor activity (Sami et al., 1984).

Hence, because of the great interest in how the quinone reduction potential influences the mode of action of mitomycin analogues, we have studied in vitro the mechanism of DNA alkylation by the readily reducible mitomycin A (MA; **1b**). MA is the most toxic of the naturally occurring mitomycins (Remers, 1979). It has been postulated that a high reduction potential (due to the 7-alkoxy substituent) is the basic cause for the toxicity of MA (Remers, 1979; Hodges et al., 1981). In our studies, we hoped to discover how MA's higher reduction potential (−0.19 V for MA versus −0.40 V for that of MC; Kinoshita et al., 1971) would affect the distribution of monoadducts versus cross-links formed by MA on DNA under a series of reducing conditions. Additionally, we were interested in seeing how a change of hydrophilicity of the 7-appendage would influence the drug's binding within the minor groove of DNA. Finally, we hoped to address the speculation that the 7-position of MA may be an alternative DNA alkylation site (Iyer & Szybalski, 1964).

Mitomycin A was ideally suited for this analogue study since its mitosene derivatives can be readily converted into mitosenes harboring a 7-amino group (mitomycin C type) upon treatment with methanolic ammonia (Webb et al., 1963; Kinoshita et al., 1971). MA–DNA isolates were submitted to these conditions, and the resulting "conversion" products were then identified by comparison with the previously characterized MC–deoxyguanosine adduct standards. As is explained below, this study has shown that MA's reductive activation and subsequent alkylation of DNA does not directly reflect the previous mechanism proposed for MC and these mechanistic differences are interpreted on the basis of the structural difference between MC and MA.

Experimental Procedures

Materials. Mitomycin C was generously supplied by Dr. W. T. Doyle (Bristol-Myers Squibb Co., Wallingford, CT). MA was synthesized from MC by a published procedure (Vyas et al., 1986). Calf thymus DNA (type I; sonicated before use), DNase I, xanthine oxidase, NADH-cytochrome *c* reductase, and bacterial alkaline phosphatase (type IIIR) were obtained from Sigma (St. Louis, MO). Phosphodiesterase I (snake venom diesterase; SVD) was from Worthington Biochemicals (Malvern, PA). NADPH-cytochrome *c* reductase was a gift from Dr. Wayne Backes (Louisiana State Medical School, New Orleans, LA). Oligonucleotides were synthesized according to the phosphoramidite method on an Applied Biosystems Model 380 DNA synthesizer. All reagents for the oligonucleotide synthesis were from Applied Biosystems, Inc. (Foster City, CA).

Spectroscopy. UV spectra were measured on a Perkin-Elmer λ 4B spectrophotometer in water. Proton nuclear magnetic resonance spectra were recorded on a Bruker WM-250 spectrometer, in Me_2SO-d_6 with values reported in parts per million from the internal methyl sulfoxide standard.

Analytical Scale Formation of the MA–DNA Complexes. A series of "analytical" scale complexes were formed under the following conditions. (i) *H_2/PtO_2 activation*: 3.0 μmol of calf thymus DNA (1.1 μmol/mL), 4.5 μmol of MA (1.6 μmol/mL), and 585 μg of PtO_2 (130 μg/μmol of MA) were combined in 2.8 mL of 0.015 M Tris, pH 7.4, and deaerated under bubbling argon. Hydrogenation was carried out for 6 min followed by a 10-min purge of the system with argon. Finally, air was bubbled through the reaction for 5 min to effect reoxidation. PtO_2 was removed via filtration prior to size-exclusion chromatography. (ii) *$Na_2S_2O_4$ activation*: 3.0 μmol of calf thymus DNA (1.1 μmol/mL) and 4.5 μmol of MA (1.6 μmol/mL) were dissolved in 2.8 mL of 0.015 M Tris, pH 7.4, and degassed under argon. A fresh 0.126 M solution of $Na_2S_2O_4$ (220 mg/10 mL) was made up in deaerated water. Additions of 5 × 20 μL of the $Na_2S_2O_4$ solution (12.6 μmol; 2.8 μmol/μmol of MA) were made over the course of $^1/_2$ h. Upon completion of the activation step, air was purged through the system for 5 min. (iii) *Enzymatic activation*: a solution of 4.0 μmol of calf thymus DNA, 4.0 μmol of MA, and 8.0 μmol of NADPH in 4.0 mL of 0.02 M potassium phosphate, pH 7.5, was deaerated as above, and then 6 units of NADPH-cytochrome *c* reductase was added. Incubation under deaerating conditions was continued for 20 min at 37 °C, followed by a purge of the system with air for 5 min. Activation by xanthine oxidase or NADH-cytochrome *c* reductase followed the same protocol except that NADH replaced NADPH and 2 rather than 6 units of either enzyme was employed.

Isolation and Digestion of MA–DNA Complexes. Each MA–DNA complex was separated from the hydrolysis products of MA (mitosenes) on a 2.5 × 56 cm Sephadex G-100 column as previously described for MC. Digestion was completed following the DNaseI/snake venom diesterase/alkaline phosphatase protocol previously described (Tomasz et al., 1986a). Digested complexes were analyzed by use of a Beckman ODS Ultrasphere 4.5 × 25 mm column; 13% MeCN/87% 0.03 M NH_4COOCH_3, pH 5.0; 1 mL/min flow rate.

Anaerobic versus Aerobic $Na_2S_2O_4$ Reduction. Anaerobic reduction: A mixture of DNA (3 μmol) and MA (3 μmol) in 3.0 mL of 0.015 M Tris, pH 7.4, was deaerated as above, and then a fresh 0.03 M anaerobic solution of $Na_2S_2O_4$ (4.5 μmol) was added in five portions at 5-min intervals. The reaction was completed by purging with air as above. *Aerobic reduction*: The reaction was completed following the same protocol as above except that the solution was not deaerated and the reaction was carried out in an open flask with vigorous magnetic stirring.

Formation of MA–Oligonucleotide Complexes. $Na_2S_2O_4$ activation: The oligonucleotide (20 A_{260} units; approximately 2 μmol, measured in mononucleotide units) was treated with 4 μmol of MC and 6 μmol of $Na_2S_2O_4$ in 4.0 mL of 0.1 M potassium phosphate buffer, pH 7.5, at 0 °C. Both the anaerobic and aerobic protocols were employed, exactly as described in the preceding section. *Enzymatic activation*: The enzymatic reduction conditions employed were identical with those described above for DNA except that an oligonucleotide (approximately 2.0 μmol) was the substrate.

Midscale Preparation of the MA Adducts. The pure adducts **5** and **6** were prepared by "midscale" hydrogenation activation with use of 60 μmol of MA, 30 μmol of calf thymus DNA, and 7.2 mg of PtO_2. The adducts were isolated by semipreparative HPLC: YMC-Pack A-324 S-5 120-Å ODS 10 × 320 mm column; 13% MeCN/87% 0.03 M NH_4COOCH_3, pH 5.0; 2.0 mL/min flow rate. Adduct **6** and adduct **5** were eluted at 30 and 37 min, respectively.

Samples of pure **6** as well as a mixture of **5** and **7** were prepared by midscale $Na_2S_2O_4$ activation with 60 μmol of MA, 30 μmol of calf thymus DNA, and 168 μmol of $Na_2S_2O_4$ (2.8 μmol/μmol of MA). Semipreparative isolation of the adducts by HPLC was completed as above. Under these conditions, pure adduct **6** eluted at 30 min while a mixture of adducts **5**

and **7** coeluted at 37 min. The presence of two components in the 37-min peak was confirmed with use of the analytical HPLC conditions described above.

Conversion of the MA Adducts to Their MC Analogues. Purified adduct **5**, purified adduct **6** (from the midscale H_2/PtO_2 conditions), and a mixture of adducts **5** and **7** (from the midscale $Na_2S_2O_4$ conditions) were separately converted to their 7-amino (MC-type) analogues according to the following protocol. Each sample was dissolved in 2 mL of methanolic ammonia and stirred in a sealed 4.5-mL screw-cap vial for 15 h. (A time course UV experiment on a simple mitosene analogue indicated conversion of the 288-nm band characteristic of a 7-methoxymitosene into the 310-nm band characteristic of 7-aminomitosenes in 12 h; data not shown.) Ammonia and methanol were then removed on a rotovap followed by removal of the last traces of solvent in vacuo for 2 h. The residue from each reaction was dissolved in 500 μL of HPLC buffer, and half of the sample (250 μL) was analyzed on reversed-phase HPLC with use of the semi-preparative column described above and 11% MeCN/0.03 M NH_4COOCH_3, pH 5.0, as the solvent. The analysis was carried out on a Perkin-Elmer Series 4 liquid chromatograph equipped with a Spectraflow 773 absorbance detector (254 nm) as well as a LKB 2140 rapid spectral diode array detector.

NMR of Adduct **6.** The NMR spectrum of underivatized adduct **6** (8.0 OD_{254} units collected from the midscale reactions) measured in Me_2SO-d_6 exhibited broad peaks characteristic of underivatized mitomycin–deoxyguanosine adducts (Tomasz et al., 1986a,b, 1988b). Some essential residues however were discernible: 7.94 (s), 8-H; 6.12 (t), 1′-H; 4.55 (s), 10″-H_2; 3.88 (s), 7″-OCH_3; 1.86 (s), 6″-CH_3.

Acetylation of Adduct **6.** Adduct **6** (from above) was acetylated following the standard protocol (Tomasz et al., 1986b) with the addition of a final C-18 Sep-Pak (Waters Associates, Milford, MA) column purification step. The column was washed sequentially with water and 10% MeCN/water, and then the orange product eluted with 30% MeCN/water. Proton NMR of this sample indicated the presence of four acetates and the 6″-CH_3 [2.06 (s), 2.01 (s), 1.86 (s), 1.84 (s), 1.79 (s), respectively] as well as the 7″-OCH_3 [3.89 (s)].

Enzymatic Treatment of Adduct **5.** To rule out the possibility that the 10″-decarbamoyl adduct **6** resulted from the hydrolysis of adduct **5** during enzymatic digestion, 0.43 $Å_{260}$ units of adduct **5** were resubmitted to enzymatic digestion as follows. The adduct **5** was dissolved in 625 μL of digestion buffer (0.005 M Tris, 0.001 M $MgCl_2$, pH 7.0). DNase I (980 units) was added (2-h incubation; 37 °C). The pH was adjusted to 8.2, and 39 units of SVD was added. After 2 h of incubation at 37 °C, another 39 units of SVD as well as 35 units of alkaline phosphatase was added. The digestion was incubated overnight.

HPLC analysis of the redigestion was carried out on a Develosil ODS-5 (Nomura Chemical) 4.5 × 280 mm column, (13% MeCN/0.03 M NH_4COOCH_3; pH 5.0; 1.0 mL/min).

Comparison of the Binding Ratio of MC versus MA. A simple UV method (Tomasz et al., 1974) for determining the binding ratio of MA adducts on DNA (moles of MA bound per moles of DNA mononucleotide unit) could not be developed because of the overlap of the 288-nm mitosene band with the 260-nm DNA band. Hence, in order to compare the alkylation efficiencies of MA with MC on DNA, a simple digestion/integration assay was employed as follows.

Three complexes were made under $Na_2S_2O_4$ activation conditions: (i) MA complex (3 μmol of calf thymus DNA, 2 μmol of MA, 6 μmol of $Na_2S_2O_4$); (ii) MC complex (3 μmol of calf thymus DNA, 2 μmol of MC, 6 μmol of $Na_2S_2O_4$); (iii) mixed MA and MC complex (6 μmol of calf thymus DNA, 2 μmol of MC, 2 μmol of MA, 12 μmol of $Na_2S_2O_4$). The complexes were isolated by use of Sephadex G-100 chromatography, and the yield of DNA was determined by UV. Each complex (2 μmol) was then digested under standard conditions as above. HPLC analysis was carried out on a Develosil ODS-5 (Nomura Chemical) analytical column (11% MeCN/0.03 M NH_4COOCH_3, pH 5.0) with a 1-μmol injection (half of the digestion volume) for each complex. The peak areas were determined with the Perkin-Elmer Series 4 liquid chromatograph software with use of normal base-line integration.

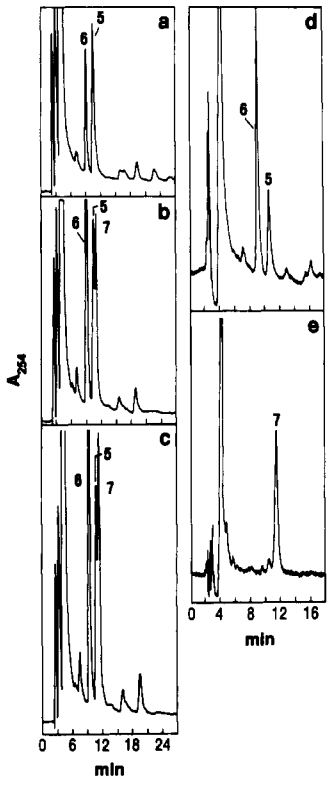
Results

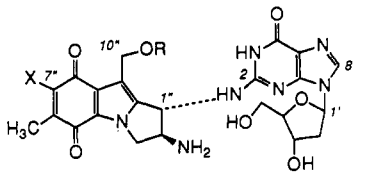
Isolation of MA–Deoxyguanosine Adducts from MA–DNA Complexes. Studies into the alkylation of DNA by MC have indicated that the reductive activation conditions influence the ratio of monoadducts to cross-links formed along the DNA strand (Tomasz et al., 1988a). Specifically, it has been noted that under autocatalytic conditions such as those employed upon chemical reduction with H_2/PtO_2 or upon enzymatic reduction (Peterson & Fisher, 1986), MC produces almost exclusively monoadducts with DNA. Presumably, the nascent hydroquinone form of the monoadduct is reoxidized more rapidly than it gives up its carbamate. However, under the fast kinetics of $Na_2S_2O_4$ reduction, the reoxidation of this intermediate does not occur and bifunctional adducts are formed (Tomasz et al., 1987, 1988a). An analogous series of reducing conditions (H_2/PtO_2, $Na_2S_2O_4$, and enzymatic) were employed in the current study of MA.

As is shown in Figure 1a, digestion of the MA–DNA complex formed under H_2/PtO_2 reducing conditions yielded two major adducts, **5** and **6**. Similarly, reduction of MA under enzymatic conditions using either NADPH-cytochrome *c* reductase, NADH-cytochrome *c* reductase, or xanthine oxidase produced a MA–DNA complex that also yielded **5** and **6** upon digestion (Figure 1d). The UV spectra of these compounds showed 257- and 273-nm bands characteristic of a deoxyguanosine adduct. Also present in the UV spectra were bands indicative of a 7-methoxymitosene at 234, 285, and 346 nm. [The 285-nm band was unveiled only after second derivatization, a technique that had proven useful for structural studies in the MC series (Verdine & Nakanishi, 1986).]

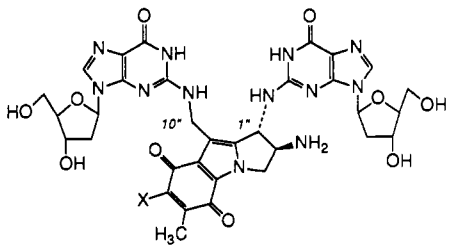
Digestion of the MA–DNA complex formed under anaerobic $Na_2S_2O_4$ conditions yielded the two adducts mentioned above plus a new adduct **7**, which eluted very closely to adduct **5** (Figure 1b). In fact, these two components did not separate on the semipreparative HPLC column used to collect enough material for the conversion experiments below.

Structural Proof for MA Adducts: Conversion to Their MC Analogues. In order to determine the structures of adducts **5**–**7**, advantage was taken of the known chemical conversion of simple MA-type mitosenes to MC-type mitosenes upon treatment with methanolic ammonia (Webb et al., 1963; Kinoshita et al., 1971). This reaction was applied to the MA–deoxyguanosine adducts. HPLC analysis of these reactions is shown in Figure 2. Although the conversions were not quantitative, all of the side products (and presumably the starting MA adduct) eluted much later than the 7-amino product. Adduct **5** was converted into an early eluted compound (21 min); this product was shown to be **2** by coinjection with the known standard (Figure 2B). Similar conversion of adduct **6** yielded a compound (19 min) that coinjected with the known adduct **3** (Figure 2C). Additionally, conversion





2: X = NH_2; R = $CONH_2$
3: X = NH_2; R = H
5: X = OCH_3; R = $CONH_2$
6: X = OCH_3; R = H



4: X = NH_2
7: X = OCH_3

d(TATATCGATATA)
8

d(ATATACGCGTATAT)
9

FIGURE 1: Analytical HPLC analysis of the digestion products of MA–DNA complexes formed under different reducing conditions: (a) MA–calf thymus DNA complex formed under catalytic H_2/PtO_2 reduction conditions. (b) MA–calf thymus DNA complex formed under strictly anaerobic $Na_2S_2O_4$ reduction conditions. (c) MA–calf thymus DNA complex formed under the aerobic $Na_2S_2O_4$ reduction conditions as described in the text. (d) MA–calf thymus DNA complex formed under enzymatic reduction conditions (NADPH-cytochrome *c* reductase). (e) MA–oligonucleotide **9** complex formed under anaerobic $Na_2S_2O_4$ reduction conditions and HPLC-purified prior to digestion. HPLC conditions were as described in the text.

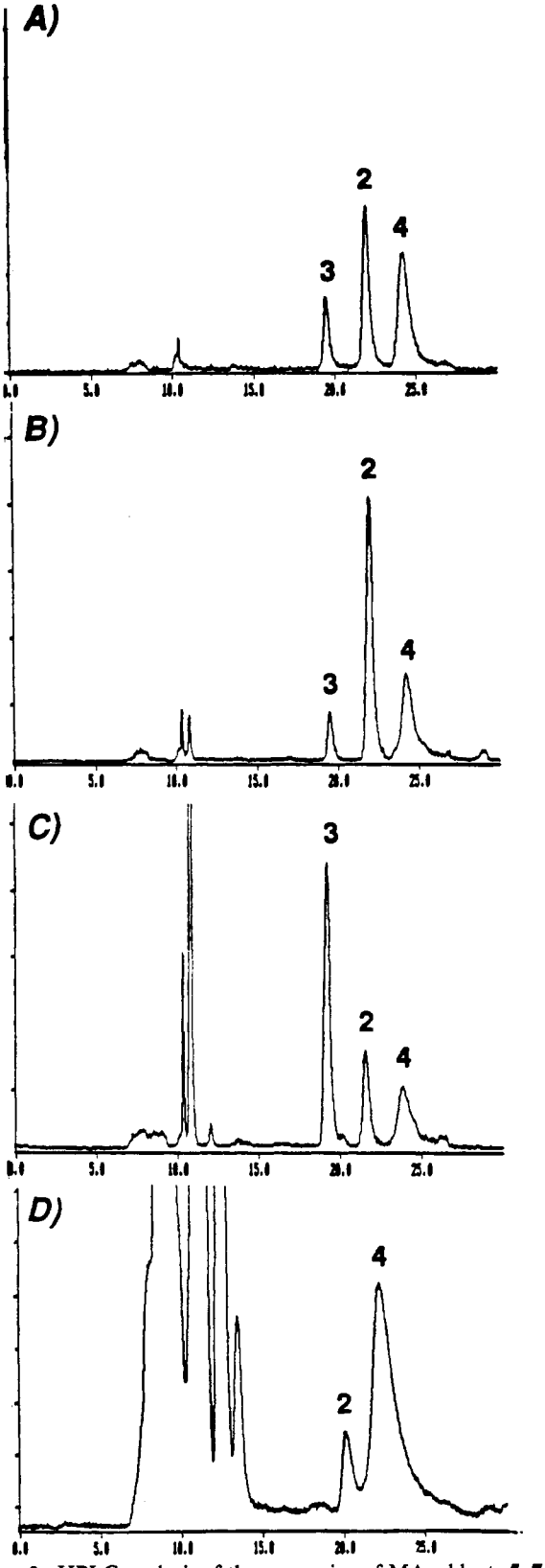


FIGURE 2: HPLC analysis of the conversion of MA adducts **5**–**7** to their respective MC analogues **2**–**4**. Reaction conditions were as described in the text. HPLC conditions were as described in the text. (A) Mixture of authentic standards **2**–**4**. (b) Coinjection of the 21-min conversion product (from reaction of **5**) with the same mixture of authentic standards **2**–**4** as in (A). (C) Coinjection of the 19-min conversion product (from the reaction of **6**) with the mixture of authentic standards **2**–**4** as in (A). (D) Coinjection of the crude reaction mixture from the reaction of the mixture **5** and **7** with the standards **2** and **4**. The HPLC trace in (D) was run on a separate day than (A–C), thus explaining the slight variation in mobility of **2** and **4**.

of the mixture of adducts **5** and **7** yielded two compounds that coeluted with adducts **2** and **4**, respectively (Figure 2D). All of the conversion products exhibited diode array UV spectra identical with those exhibited individually by the MC adduct standards **2–4**.

During the course of this work, enough material (8 A_{260} units) was collected to obtain a ^{1}H NMR spectrum of **6**. Although the peaks of this spectrum were very broad (as discussed above), two informative resonances were present. First, the appearance of the 3.88-ppm singlet confirmed that the 7-methoxy group was intact in this adduct. Furthermore, the shift of the 10″-H_2 protons (4.55 ppm) upfield from that of 10″-H_2 protons next to a 10″-carbamate substituent (5.0 ppm) was indicative of a 10″-decarbamoyl adduct.

It has been previously noted that the acetylated derivatives of the MC–deoxyguanosine adducts exhibited much sharper ^{1}H NMR spectra (Tomasz et al., 1986b, 1987, 1988b). Hence, adduct **6** was acetylated and the ^{1}H NMR of this derivative was measured. Because of the small amount of material recovered after chromatography (approximately 100 μg), only the methyl groups were discernible. Fortunately, the spectrum did confirm the presence of four acetates as expected (Tomasz et al., 1988b).

Stability of Adduct **5** *to Hydrolysis under Incubation Conditions.* The presence of the bifunctionally derivatized decarbamoyl adduct **6** in the digestion of all of the complexes was unexpected. It was expected by analogy with MC that a given reducing condition should produce either only the carbamoylated monoadduct **5** or a mixture of the decarbamoyl adduct **6** and the "cross-link" adduct **7**. Hence, the isolation of adducts **5** and **6** without the presence of **7** from some of the complexes was puzzling. In order to check the possibility that the decarbamoyl adduct **6** simply resulted from the hydrolysis of adduct **5** during enzymatic incubation, the isolated **6** was resubjected to these conditions. As is apparent in Figure 3, the carbamate group is quite stable under these conditions. The MC analogue **2** was shown previously to be similarly stable to these conditions (Tomasz et al., 1986a).

Comparison of the DNA Binding Ratio of MC versus MA. As mentioned above, the overlap of the 285-nm mitosene band with the 260-nm DNA band inhibited the development of a simple UV method for measuring the binding ratio. Hence, in order to get a qualitative picture of the comparative binding ratios between MA and MC with DNA, two complexes were formed holding all conditions identical except for the substitution of MA or MC as the alkylating agent. In addition, an experiment was completed where both MC and MA were reduced together and given an equal opportunity to alkylate DNA. The HPLC analysis of these digests (Figure 4) indicated that both drugs alkylated DNA with a similar frequency. The ratio of total MA adducts to total MC adducts was 0.93:1 in a comparison of the individaul complexes (Figure 4a,b). The reaction where both MA and MC were present (Figure 4C) exhibited a ratio of total MA adducts to total MC adducts of 1.05:1.

Cross-Linking of Oligonucleotides by MA. The self-complementary oligonucleotides [d(TATATCGATATA), **8**, and d(ATATACGCGTATAT), **9**] were previously shown to be cross-linked by MC under anaerobic $Na_2S_2O_4$ activation (Borowy-Borowski et al., 1990b). Analogous cross-linking experiments were attempted with MA. HPLC analysis of the crude reaction mixture of self-complementary oligonucleotide **9** and reduced MA indicated formation of a major new component eluting after unreacted **9** at 14.8 min (Figure 5A). Digestion of this new component yielded the single **7** (Figure 1e), proving the new 14.8-min component to be a cross-linked oligonucleotide duplex. Oligonucleotide **8** gave similar results (data not shown).

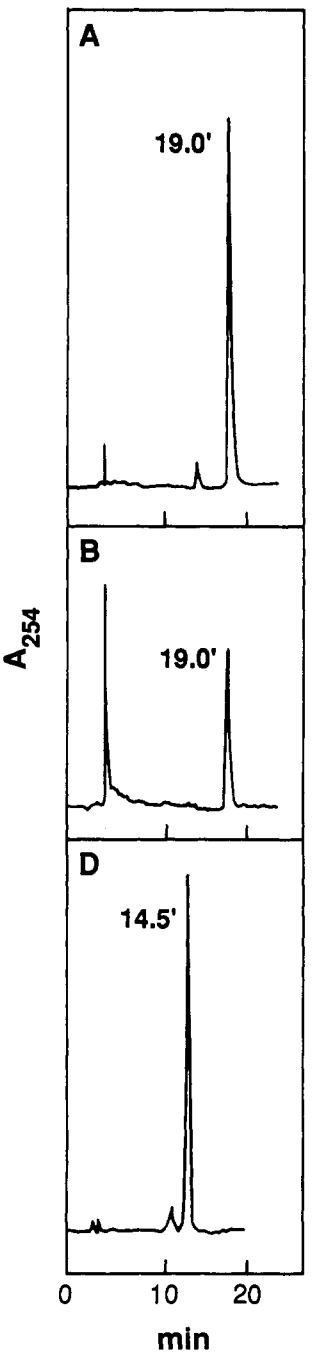


FIGURE 3: HPLC analysis of the enzymatic treatment of adduct **5**. (A) Carbamoylated monoadduct **5** prior to enzymatic treatment. (b) Monoadduct **5** after overnight treatment with DNase I, SVD, and alkaline phosphatase as described in the text. Note that the carbamate is stable under these conditions. (c) Decarbamoyl adduct **6** as reference standard. HPLC conditions were as described in the text.

Comparison of Oligonucleotide Cross-Linking by MC versus MA. In order to compare directly the efficiency of cross-linking by MA and MC, self-complementary oligonucleotide **9** was also cross-linked by MC, under identical conditions as those described for MA above, and the crude reaction mixture was analyzed by HPLC (Figure 5C). The yields of the cross-linked oligonucleotides were 53% with MA and 60% with MC as calculated from the HPLC peak areas. The yield of the cross-linking of oligonucleotide **8** by MA was 26% (data not shown) compared to the 19% cross-linking yield

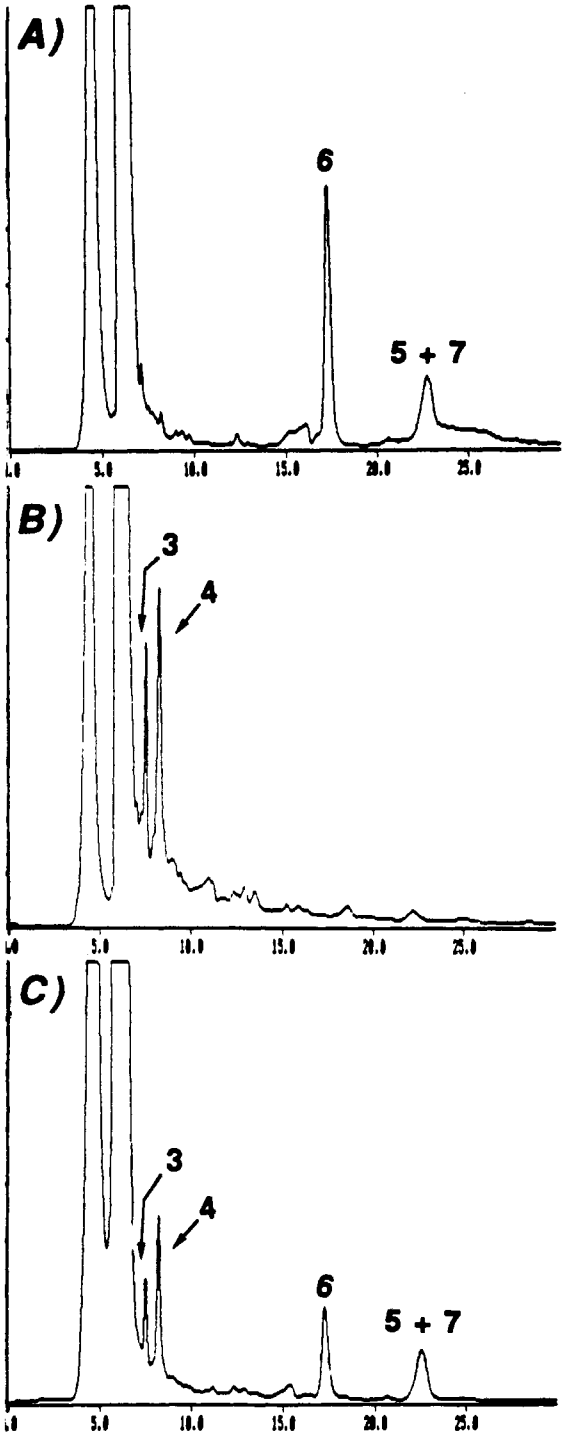


FIGURE 4: Comparison of the in vitro DNA alkylating abilities of MA versus MC by analysis of the digests of the drug–DNA complexes. (A) Digestion of a MA–$Na_2S_2O_4$–calf thymus DNA complex. (B) Digestion of a MC–$Na_2S_2O_4$–calf thymus DNA complex. Both complexes depicted in (A) and (B) were formed and digested under conditions identical with those described in the text. (C) Digestion of a calf thymus DNA complex formed between simultaneously $Na_2S_2O_4$ reduced MA and MC. HPLC conditions were as described in the text.

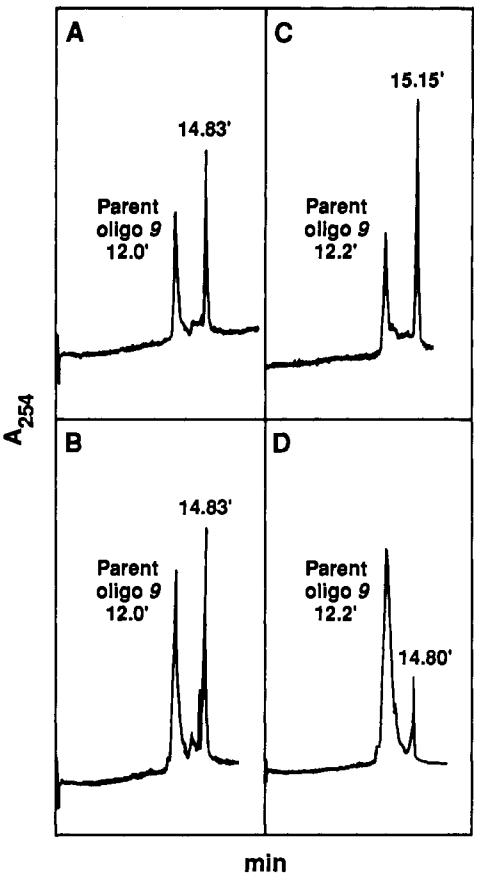


FIGURE 5: Comparison of the oligonucleotide cross-linking efficiencies of MA versus MC under anaerobic and aerobic $Na_2S_2O_4$ conditions. The sequence of the self-complementary oligonucleotide **9** employed in all of the reactions was d(ATATACGCGTATAT). (A) HPLC analysis of the crude reaction mixture obtained upon *anaerobic* treatment of oligonucleotide **9** with MA. The parent **9** elutes at 12.0 min while the MA-cross-linked oligonucleotide elutes at 14.83 min. (B) HPLC analysis of the crude reaction mixture obtained upon *aerobic* treatment of oligonucleotide **9** with MA. Elution times of the parent **9** and cross-linked product are the same as above. (C) HPLC analysis of the crude reaction mixture obtained upon *anaerobic* treatment of oligonucleotide **9** with MC. The parent **9** elutes at 12.2 min while the MC-cross-linked oligonucleotide elutes at 15.15 min. (D) HPLC analysis of the crude reaction mixture obtained upon *aerobic* treatment of oligonucleotide **9** with MC. Note the lack of cross-linked oligonucleotide at 15.15 min. The 14.80-min peak is thought to be a monoadducted oligonucleotide as described in the text. HPLC conditions were as follows: 4.6 × 75 mm C-3 reversed-phase column using a 6–18% acetonitrile gradient in 0.1 M TEA, pH 7.0, in 24 min, at a 1.0 mL/min flow rate.

of **8** by MC reported previously (Borowy-Borowski et al., 1990b).

Effects of Air on Formation of Adducts with DNA. Aerobic $Na_2S_2O_4$ treatment produced a MA–DNA complex (Figure 1c) that upon digestion yielded a virtually identical HPLC pattern of adducts as that of the complex formed under anaerobic treatment (Figure 1b). This result is in stark contrast to the results obtained for MC where the presence of oxygen influenced the product ratios dramatically (Tomasz et al., 1988a). Comparison of the patterns of parts b and c of Figure 1 also indicates an approximately 20% decrease in the total adduct yield in the presence of air. In contrast, MC exhibited a 60% decrease in the total adduct formation in a comparative experiment (data not shown).

Effect of Air on Oligonucleotide Cross-Linking. Self-complementary oligonucleotide **9** was separately cross-linked by MA and MC under anaerobic and aerobic $Na_2S_2O_4$ conditions. The HPLC analysis of each crude reaction mixture is illustrated by Figure 5A–D. The HPLC patterns in the case of MA indicate a moderate decrease of the ratio of cross-linked oligonucleotide (14.83-min peak) to parent oligonucleotide upon addition of air to the reaction as judged by relative peak heights (compare parts A and B of Figure 5). In contrast, MC does not cross-link the oligonucleotide under aerobic activation conditions at all, as seen by the lack of the MC–oligonucleotide cross-link peak at a 15.2-min elution time in Figure 5D. The

Table I: Distribution of Mitomycin–DNA Adducts Formed under Various Activating Conditions[a]

drug	activating conditions			
	H_2/PtO_2	flavoreductase	$Na_2S_2O_4$ (anaerobic)	$Na_2S_2O_4$ (aerobic)
MC	**2**	**2**	**3, 4**	**2, 3, 4**
MA	**5 < 6**	**5 < 6**	**5, 6, 7**	**5, 6, 7**

[a] Data for MC adapted from Tomasz et al. (1988a).

small peaks eluting between the parent and the cross-linked oligonucleotide in all of the traces were not identified but most likely represent monoadducted oligonucleotides.

Discussion

MA is more toxic and therefore less effective as a chemotherapeutic agent than its clinically useful cousin MC (Remers, 1979; Sami et al., 1987, 1989; Bradner, et al., 1989). The reason(s) for this variation in activity between the two closely related antibiotics is currently unknown. In this report, the DNA alkylation chemistry of MA was studied in hope that some factor would be uncovered (base site selection, overall alkylation ability, reductive activation mechanism, etc.) that would explain the differential effectiveness of MC over MA.

First, it was shown unambiguously that MA, just like MC, selectively alkylates the 2-amino group of deoxyguanosine. Presumably the 7-methoxy is far enough removed from the reactive aziridine ring that it exerts no influence on the drug's selectivity. It is also apparent that, at least in these in vitro reactions, the 7-methoxy group does not influence the amount of DNA binding of this drug. The yield of total adducts was the same from the MC or MA reactions with DNA (Figure 4A,B). Simultaneous reduction of both compounds, followed by digestion of this mixed DNA complex, also produced a similar final yield of adducts (Figure 4C). Hence, within the limits of this assay, it appears that both drugs have an equal probability to covalently bind DNA. A previous molecular modeling study had predicted stronger binding to DNA of MC over MA (Rao et al., 1986). The major difference noted between these two complexes was the ability of the 7-amino group of MC to hydrogen bond to a phosphate of the DNA backbone (Rao et al., 1986). In our present experiments no preference in adduct formation was noticed.

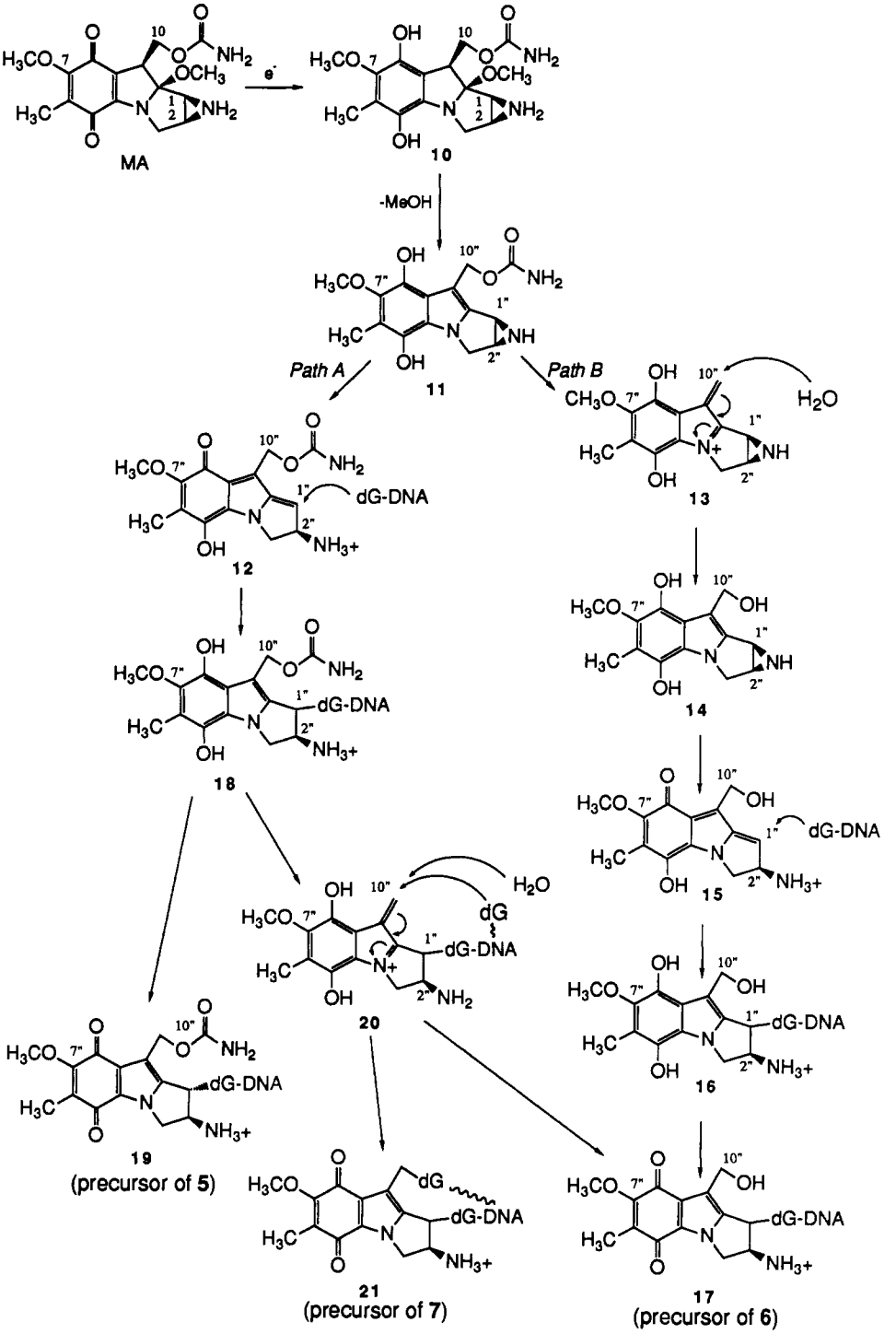
Similarly, it has been shown earlier that both MA and MC *cross-link* DNA to an equal extent (Szybalski & Iyer, 1967). In confirmation, it is now demonstrated that synthetic oligonucleotides are cross-linked to a similar extent by MA and MC: oligonucleotide **8** gave 26% (MA) and 19% (MC) cross-linking yields while oligonucleotide **9** gave 53% (MA) and 60% (MC) cross-linking yields under the same anaerobic reductive activation conditions as those used by Szybalski and Iyer. These results are apparently in contrast to those of Crothers and co-workers who found the N^{1a}-methyl derivative of MA to be twice as effective for cross-linking synthetic oligonucleotides as MC (Teng et al., 1989). This suggests that methylation of the aziridine enhances DNA binding. If so, this phenomenon warrants further investigation.

Although the DNA adducts of MA and MC are similar, a difference was noted: the *distribution* of the two monoadducts, **5** and **6**, does not follow the same pattern as that of the corresponding monoadducts **2** and **3** in the MC series (Table I). In the latter, **2** and **3** are formed in a mutually exclusive manner, reflecting either monofunctional or bifunctional activation of MC, respectively, depending on the activation conditions (Tomasz et al., 1988a). Thus, H_2/PtO_2 and various flavoreductases activate MC monofunctionally, at C-1″, yielding **2** as the sole product, while anaerobic $Na_2S_2O_4$ induces purely bifunctional activation, at C-1″ and C-10″, yielding **3** and **4** and none of **2**. In contrast, under H_2/PtO_2 or flavoreductase activation MA gives *both* **5** and **6** (Figure 1a,d) and under anaerobic $Na_2S_2O_4$ *all three* MA adducts **5**–**7** are observed (Figure 1b). It is also apparent that under all conditions the 10″-decarbamoylated MA adduct **6** is predominant (Figure 1). This has not been observed in the case of MC: the analogous MC adduct **3** is either not formed or formed in approximately equal proportion of **4**, as described above (Tomasz et al., 1987, 1988a). It appears, then, that the mechanism of the reductive activation cascade and subsequent reactions of MA with DNA differ from those of MC in some respect. Specifically, a new pathway may exist in the MA series for formation of the 10″-decarbamoylated monoadduct **6**, which enhances its relative yield in comparison to that in the MC series. We propose the mechanism depicted in Scheme I to account for these findings. This mechanism begins like the accepted pathway of MC activation. Namely, reduction of the quinone frees the electron density of the 4-nitrogen, which aids in the expulsion of methanol from the 9/9a positions of MA's backbone. The two-electron-reduced intermediate (hydroquinone), rather than the one-electron-reduced intermediate (semiquinone), is indicated for this reaction in view of recent evidence (Hoey et al., 1988). The resulting intermediate **11**, unlike its MC cousin, can be activated at *either* its 1-position (opening of the aziridine, path A) *or* its 10-position (expulsion of the carbamate, path B). In other words, intermediate **11** alternatively opens its aziridine to produce the quinone methide **12** or loses its carbamate group to produce the exocyclic methylene intermediate **13**.

Path A is the same mechanism as that operating in the reductive alkylation of DNA by MC (Tomasz et al., 1988a), accounting qualitatively for the formation of all three adducts **5**–**7**. Path B results from competition of carbamate elimination with the aziridine ring opening of **11**. Such competition is not observed in the case of MC; aziridine opening and 1″-substitution proceeds faster than carbamate elimination, as shown by numerous studies of the reductive activation of MC, cited in the introduction. According to path B, the activated 10″-position of adduct **13** traps water to produce **14**. A decarbamoyl version (**15**) of the quinone methide intermediate **12** can now be generated via delocalization of the quinone electrons. It is this decarbamoylquinone methide intermediate **15** that goes on to produce the final decarbamoyl MA–DNA lesion **17**. In fact, path B from intermediate **14** on is analogous to the alkylation of DNA by 10″-decarbamoyl-MC (**3**) observed earlier (Tomasz et al., 1988b). We believe that it is partitioning at this early point in the MA activation cascade (intermediate **11**) that ultimately produces the different product ratios between MC and MA.

What enables MA (and not MC) to undergo this facile decarbamoylation? Possibly, the less-electron-donating 7-methoxy group (as compared to the 7-amino group of MC) decreases delocalization of the quinone electrons into the aziridine ring. This enables activation of the 10″-carbamate via the indole electrons to compete with aziridine ring opening for the fate of intermediate **11**. In an alternative explanation, the proton of the 8-hydroxyl of MC's hydroquinone may be more acidic than the 8-hydroxy proton of MA's hydroquinone due to the well-documented hydrogen bonding between the 7-amino group (donor) and the 8-oxygen (acceptor) in MC. Loss of the 8-hydroxy proton from reduced MC would therefore be more facile than from MA, which lacks this hydrogen bond. Subsequently, electron delocalization required

Scheme I



for the **11** to **12** tautomerization step would be a more favored pathway in the case of MC.

Both quinone methide intermediates, carbamoylated **12** and decarbamoylated **15**, are considered by this mechanism to be the minor groove binders that ultimately alkylate the 2-amino group of deoxyguanosine. Since products with sole 10″-alkylation of DNA are not isolated from the MA–DNA complex digestions, we believe that intermediat **13** either has no intrinsic ability to bind DNA or is not given the opportunity to alkylate DNA. A steric argument can be made for the differential DNA binding ability of **12** and **15** versus intermediate **13**. The delocalization of electrons in **12** and **15** is likely to make these intermediates planar and hence more capable to slip into the minor groove of DNA. The aziridine carrying intermediate **13** may more reflect the cup-shaped configuration of the parent compound, MA (Hirayama & Shirahata, 1989), and therefore not fit well into DNA's minor groove.

Perhaps a simpler explanation of the proposed differential reactivity lies in the kinetics. Intermediate **13** may be much less stable then the quinone methides **12** or **15**. Hence, **13** is readily attacked by water before binding to DNA, while the longer lived **12** and **15** have time to locate their minor groove target.

So far we have been able to explain the predominant formation of adduct **6** under all reductive activation conditions. We have yet to explain why only under $Na_2S_2O_4$ reduction (and not H_2/PtO_2 or enzymes) the cross-link is formed. Analogous differential product ratios have been observed upon

reduction of MC (Tomasz et al., 1987, 1988a). Thus, this part of the cascade reflects the mechanism proposed for MC reduction. Namely, under autocatalytic conditions (H_2/PtO_2 or enzymes) excess MA reoxidizes compound **18** to the final product **19** (Peterson & Fisher, 1986; Egbertson & Danishefsky, 1987). However, the kinetically fast reduction by $Na_2S_2O_4$ yields intermediate **20** before such reoxidation can occur. The exocyclic methylene of **20** can be attacked by an amino group of deoxyguanosine on the sister strand (to yield cross-link **21**) or can be quenched by water to yield **17**.

The relatively subtle difference in the mechanism of DNA adduct formation, uncovered in the present work, does not offer any simple explanation for the greater potency of MA and other 7-alkoxymitomycins versus MC and its 7-nitrogen congeners. A more significant difference, however, may be the differential response of the reductive activation steps of MA and MC *to aerobic conditions*. Specifically, selective inhibition of the formation of MC cross-links by air under dithionite activation was previously demonstrated, as manifested by a dramatic decrease of the bisadduct **4** relative to the other adducts under aerobic conditions (Tomasz et al., 1988a). Similarly, complete inhibition of the cross-link adduct was observed in the analogous reaction of porfiromycin, the aziridine-methylated derivative of MC (Tomasz et al., submitted for publication). This inhibition was interpreted as due to premature oxidation of the activated monoadduct by O_2 back to the quinone form, thereby preventing further steps in the cross-linking process. The analogous experiment with MA as the alkylating agent produced different results (Figure 1). The DNA product profile of dithionite reduction with and without air was identical. In addition, the yield of cross-linking of the oligonucleotide **9** by MA was essentially unaffected by air while cross-linking was completely abolished in the analogous reaction with MC (Figure 5). Because of the lower electron donating nature of its 7-methoxy group, the intermediate **18** is less prone to reoxidation than its 7-amino cousin. Specifically, the midpoint 2-electron redox potential of 7-methoxymitosenes (−0.39 V) is 0.13 V higher than that of the 7-nitrogen series (−0.53 V) (Remers, 1979), and, therefore, the thermodynamic driving force for the reaction of reduced 7-methoxymitosenes with oxygen is lower. This difference may be reflected also by slower *kinetics* of the oxidation in the MA series, explaining the diminished inhibition of the activated state of MA by air compared to that of MC. Such a correlation between redox potential and rate of oxygen reactivity is well-known as demonstrated recently in a mitomycin analogue series (Islam & Skibo, 1990).

In the MC series the presence of air influences not only the distribution of the three major DNA adducts but also *the total amount of DNA adducts formed* (Tomasz et al., 1988a). This decrease of the total adduct yield under aerobic conditions is presumably caused by quick reoxidation of the *first* reduced intermediate of MC's reduction cascade (i.e., the hydroquinone or semiquinone forms of MC). This results in a slower rate of the overall alkylation process as demonstrated earlier by Sartorelli and co-workers (Kennedy et al., 1980). It is shown here that the total amount of MA adducts formed with DNA is decreased only slightly (approximately 20%) under aerobic reaction conditions, in contrast to the 60% decrease observed in a similar reaction with MC. Thus, it may be concluded that oxygen is not causing appreciable redox cycling between the hydroquinone (**10**) (or its precursor semiquinone $MA^{\bullet-}$) and quinone (MA) forms of the mitomycin (Scheme I). Interestingly, this is expected also on thermodynamic grounds: MA is so electropositive that there is little redox potential difference between the $O_2/O_2^{\bullet-}$ and $MA/MA^{\bullet-}$ couples at the actual concentrations of O_2 and MA in the system (Svingen & Powis, 1981).

Hence, the in vitro mechanism of binding of MA to DNA appears to follow a different pathway than that of MC. It will be interesting to study the DNA binding of other potent mitomycins to see if a general pattern develops that could correlate the distribution of adducts formed on DNA with some property of the analogues, especially their reduction potential. The MA–deoxyguanosine adducts isolated in this study (**5**, **6**, and **7**) could be key to such a future analogue study. As mentioned earlier, many semisynthetic mitomycins are derived from MA by a simple displacement of the 7-methoxy group with an amine. In a similar way, a set of standards for the DNA adducts of these analogues could be obtained by reaction of the adducts **5**, **6**, and **7** with the amine of interest. Thus, expensive, time-consuming full spectroscopic analyses of future analogue–DNA adducts could be avoided.

Significance

The influence of the 7-substituent on the alkylation reaction between the mitomycins and DNA has been studied in detail by comparing the reactions of MA with the well-studied MC. It has been shown that at least in vitro MA and MC alkylate DNA similarly. Both drugs react preferentially with the 2-amino of deoxyguanosine, alkylate DNA with a similar frequency, and cross-link oligonucleotides to the same extent. Hence, the differential toxicity of these two agents does not appear to lie in the type of lesion each drug ultimately leaves on the DNA strand. Rather, a prior event regulates toxicity and effectiveness. Although differential cellular uptake may be influential, it is also probable that both the relative ease (i.e., extent) of reduction and the stability of the reduced species in air are significant factors in the modulation of toxicity. Thus, Pan and Gonzales (1990) found that within a series of seven mitomycins, the higher its redox potential the *faster* the mitomycin was reduced enzymatically, with MA displaying an approximately 6-fold rate of reduction over that of MC in their system, and that a significant correlation of redox potential existed also with the cytotoxicity, with MA being the most cytotoxic in the series to human colon cancer cells. The facile reduction of MA by thiols and enzymes was also suggested as a cause of more random toxic effects, since the toxic reduced species generated in high concentration could react indiscriminately with cellular constituents instead of reaching its target DNA. This is not the case for MC and its 7-*N*-alkyl analogues since they are not reducible by thiols (Senter et al., 1988; Vyas et al., 1989). Our finding that the alkylation and cross-linking of DNA by MA are not significantly inhibited by air is also attributed to the higher redox potential of MA, and therefore it provides a direct link between redox potential, DNA damage, and cytotoxicity by the mitomycins. It also suggests that MA may not be a hypoxia-selective agent in vivo, in contrast to MC and porfiromycin (Kennedy et al., 1980; Keyes et al., 1985).[3] All of these differential effects of MA indicate that the higher redox potential lowers the target selectivity of this mitomycin, accounting for the greater toxicity and the generally less favorable therapeutic properties as compared to the 7-nitrogen substituted analogues, such as MC. These conclusions may generally apply to 7-alkoxy-substituted mitomycins as a class (Sami et al., 1987, 1989; Vyas et al., 1989).

[3] Experimental evidence in support of this proposal, utilizing EMT6 mouse mammary tumor cells under hypoxic and aerobic conditions as a test system, was obtained recently (A. C. Sartorelli and S. Rockwell, personal communication).

Finally, we have found no indication that the 7-methoxy of MA is a third alkylation site as initially proposed (Iyer & Szybalski, 1964). In fact, this work indicates that the 7-substituent has little influence on DNA binding. Presumably, this appendage protrudes from the minor groove of DNA as has been noted in computer modeling studies (Rao et al., 1986; Remers et al., 1986, 1988; Tomasz et al., 1987). Hence, the 7-position may serve as an ideal point for attachment of delivery agents (for example, antibodies or oligonucleotides) when designing a more tumor-selective mitomycin.

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