Mitomycin Dimers: Polyfunctional Cross-Linkers of DNA

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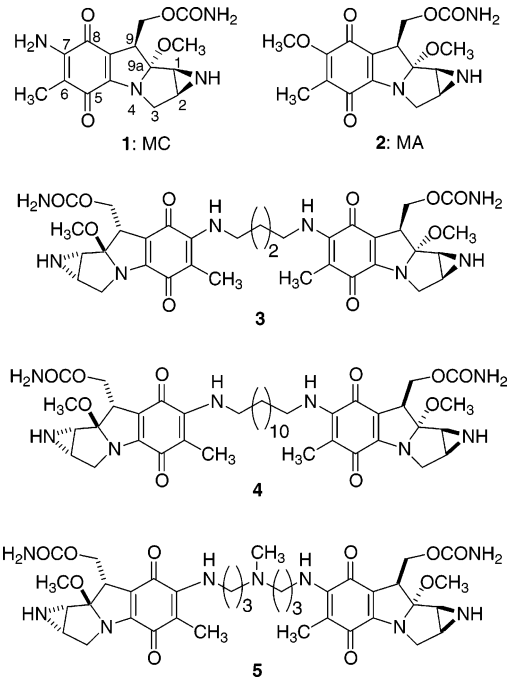
The three dimers **3**, **4**, and **5** of mitomycin C (MC), a natural antibiotic and cancer chemotherapeutic agent, were synthesized in which two MC molecules were linked with $-(CH_2)_4-$, $-(CH_2)_{12}-$, and $-(CH_2)_3N(CH_3)(CH_2)_3-$ tethers, respectively. The dimeric mitomycins were designed to react as polyfunctional DNA alkylators, generating novel types of DNA damage. To test this design, their in vitro DNA alkylating and interstrand cross-linking (ICL) activities were studied in direct comparison with MC, which is itself an ICL agent. Evidence is presented that **3**−**5** multifunctionally alkylate and cross-link extracellular DNA and form DNA ICLs more efficiently than MC. Reductive activation, required for these activities, is catalyzed by the same reductases and chemical reductants that activate MC. Dimer **5**, but not MC, cross-linked DNA under activation by low pH also. Sequence specificities of cross-linking of a 162-bp DNA fragment (*tyr*T DNA) by MC, **3**, and **5** were determined using DPAGE. The dimers and MC cross-linked DNA with the same apparent CpG sequence specificity, but **5** exhibited much greater cross-linking efficacy than MC. Greatly enhanced regioselectivity of cross-linking to G·C rich regions by **5** relative to MC was observed, for which a mechanism unique to dimeric MCs is proposed. Covalent dG adducts of **5** with DNA were isolated and characterized by their UV and mass spectra. Tri- and tetrafunctional DNA adducts of **5** were detected. Although the dimers were generally less cytotoxic than MC, dimer **5** was highly and uniformly cytotoxic to all 60 human tumor cell cultures of the NCI screen. Its cytotoxicity to EMT6 tumor cells was enhanced under hypoxic conditions. These findings together verify the expected features of the MC dimers and warrant further study of the biological effects of dimer **5**.

Mitomycin C (MC, **1**;[1] Chart 1) a natural antibiotic and cytotoxic cancer chemotherapeutic agent is used in the clinical treatment of several malignancies.[2] Its cytotoxicity to tumor tissues is attributed to its ability mainly to cross-link the complementary strands of DNA,[3] since DNA interstrand cross-links (ICLs) represent highly lethal damage to the cell.[4]

Cross-linking of DNA by MC is accomplished by sequential alkylation of the two guanines in CpG·CpG sequences by a single MC molecule. The two alkylating functions of the drug reside in the C(1) (aziridine) and C(10) (carbamate) positions. Both are unreactive until they become activated by reduction of the quinone ring (Figure 1). Reductive activation of the MC to a highly reactive bifunctional alkylating agent occurs intracellularly and can be mimicked in cell-free systems using purified reductases or chemical reducing agents. Nonreductive, acid-catalyzed activation results in monofunctional alkylation; this is likely to occur only in vitro (Figure 1). The cross-links induced by MC are formed between the exocyclic 2-amino groups of two guanines at CpG sequences, located in the minor groove of DNA.[5] Activated MC also displays monofunctional alkylation of DNA; in this case only the C(1) aziridine function

Chart 1. Structures of Mitomycin C (**1**), Mitomycin A (**2**), and Mitomycin Dimers **3**−**5**



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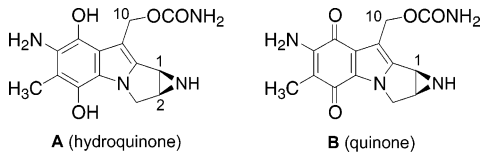


Figure 1. Activated forms of MC:[24] (A) reduction followed by loss of CH_3OH activates the C(1) and C(10) positions (bifunctional activation); (B) acidic pH catalyzes loss of CH_3OH, which activates the C(1) aziridine position only (monofunctional activation).

reacts with a guanine-N^2 nucleophile while the less reactive C(10) carbamate remains intact or is hydrolyzed. The various types of DNA adducts of MC have been observed and characterized in vitro and in vivo[6] (Scheme 1).

The DNA interstrand cross-link represents a paradigm in cancer chemotherapy.[7] This paradigm led to the design and synthesis of dimers of DNA-reactive naturally occurring monomeric antitumor agents. Remarkable examples are bizelesin, a dimeric cyclopropylpyrroloindole agent based on CC-1065,[8] pyrrolobenzodiazepine dimers, based on the anthramycin family of agents,[9] and dimers of daunorubicin.[10] The first two dimers display powerful DNA cross-linking activity; the daunorubicin dimer, which was predicted to act as a bis-intercalator, exhibits ultratight DNA binding that results in a large increase of the stability of the duplex form of DNA. In the mitomycin family, dimeric mitomycins were synthesized in which two monomeric units were dimerized by disulfide formation between their thiol substituents.[11] These disulfide-containing mitomycins were intensively studied in the past decade because of both their favorable pharmacological properties and their novel nonenzymatic mechanism of activation by thiols. The latter is probably responsible for their superior activity against MC-resistant tumor cells.[12,23] A series of simpler mitomycin dimers, lacking the disulfide function, were synthesized very recently by Kohn and co-workers.[13] Twelve dimers linked by various alkyl or aryl tethers, some of which contained ether oxygen or amino nitrogen atoms, were subjected to an SAR study with respect to in vitro DNA cross-linking efficiency and cytotoxicity toward a panel of various human tumor cell cultures. Most of the compounds were less cytotoxic than MC; only 2 of the 12 dimers possessed similar or somewhat greater cytotoxicity in most of the 7 cell lines tested. Their greater activity correlated with the presence of aryl groups in the tethers.

The present study of dimers was framed in the context of the following question: Is it possible to create a multifunctional DNA alkylating agent by dimerization of the bifunctional agent MC? It was envisioned that such an agent would form double cross-links separated by a short distance within an appropriate DNA sequence. Such complex DNA damage could present difficult challenges to DNA repair. Furthermore, such agents would in effect extend the range of DNA sequences cross-linkable by monomeric MC, which is limited to the CpG sequence. It is possible that such unconventional DNA-damaging agents would be active against drug-resistant tumors.

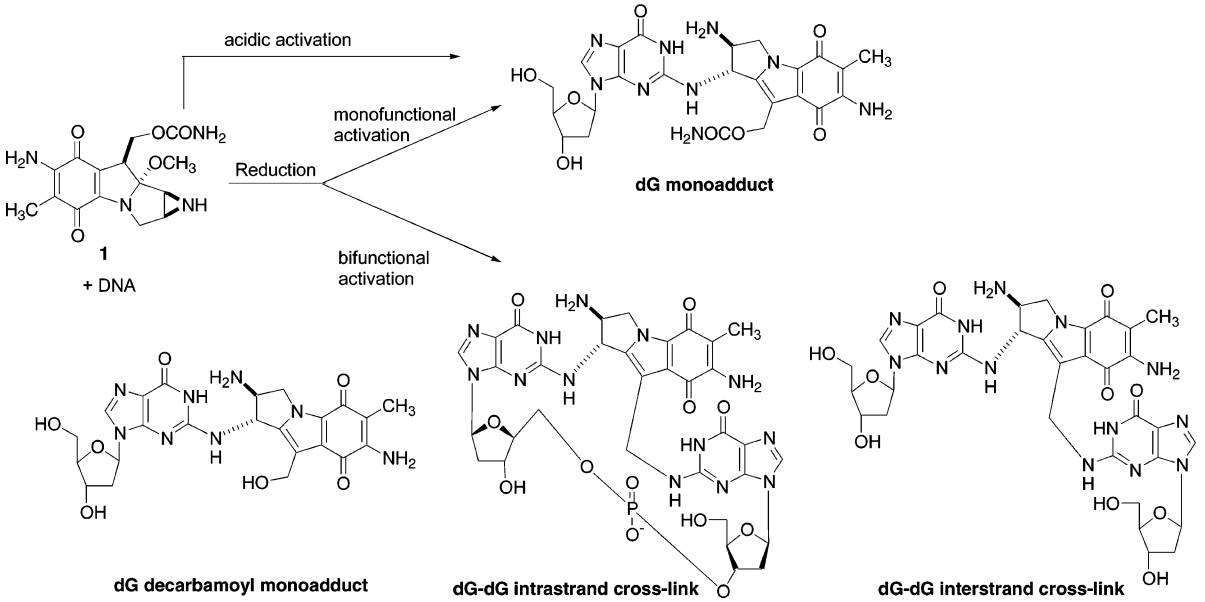
We present evidence that the MC dimers **3**−**5** alkylate and cross-link extracellular DNA multifunctionally and that they form DNA ICLs more efficiently than MC. Dimer **5** is highly and uniformly cytotoxic to all 60 human tumor cell cultures in the NCI screen. In contrast to other MC dimers studied previously by Kohn and co-workers,[13] the cytotoxicity of these three dimers correlates well with their capacity to form ICL in vitro.

Materials and Methods

Mitomycin C and mitomycin A were obtained from Bristol-Myers Squibb, Wallingford, CT; mitomycin A was also received from Kyowa Hakko Kogyo Co., Ltd., Japan.

Other Materials. Organic synthesis reagents were obtained from Aldrich Chemicals, Milwaukee, WI. HPLC grade solvents were purchased from Fisher Scientific, Pittsburgh, PA. Calf thymus DNA was purchased from Sigma Chemical Co., St. Louis, MO, and was sonicated before use. NADH-cytochrome *c* reductase (NADH-FMN oxidoreductase, EC 1.6.99.3) and NADH were obtained from Sigma. Nuclease P1 (*Penicillium citrinum*, EC 3.1.30.1) and poly(dG-dC)·poly(dG-dC) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Phosphodiesterase I (snake venom diesterase, *Crotalus adamenteus* venom, EC 3.1.4.1) and alkaline

Scheme 1



phosphatase (*Escherichia coli*, EC 3.1.3.1) were obtained from Worthington Biochemical Corp. (Freehold, NJ). Coliphage T-7 DNA and the dye Hoechst 33258 were purchased from Sigma. Synthetic oligonucleotides were prepared in-house, using an automated DNA synthesizer (model 380B, Applied Biosystems, Inc.) and commercially available β-cyanoethyl phosphoramidites. *Eco*RI restriction endonuclease, pBR322 DNA, and DNA polymerase l (Klenow fragment) were purchased from New England Biolabs, Beverly, MA. [α^{32}-P]-ATP (Ci/mmol) was obtained from Perkin-Elmer Life Sciences, Boston, MA. Agarose (DNA grade) was from Fisher Scientific. The 162-bp *Escherichia coli tyr*T promoter containing *tyr*T (A93) DNA used as a PCR template was isolated from pAT plasmid by digestion with restriction enzymes *AvaI* (Promega) and *Eco*RI (Boehringer). PCR amplification, 5′-[^{32}P] end labeling of primer, purification of the [^{32}P]-PCR product, and determination of DNA concentration were as described elsewhere.[14]

General Methods. ^{1}H NMR spectra were recorded on a GE 300 MHz or a JEOL JNM GX 400 spectrometer. UV spectra were recorded in a Cary 3 UV−visible spectrophotometer. Absorbance readings were obtained in a Gilford 250 spectrophotometer. LC−MS was performed with a Hewlett-Packard series 1100 diode array HPLC system connected to a Hewlett-Packard series 1100 MSD mass spectrometer. HPLC was performed in a Beckman System Gold 125 instrument, equipped with a Beckman System Gold 168 diode array detector fitted with a Rainin Microsorb MV C18 5 mm × 250 mm column. A linear gradient of 6−50% CH_3CN in 30 mM NaH_2PO_4, pH 5.8, in 45 min at 1.0 mL/min flow rate, with no delay time, was used. Ethanol precipitations of DNA were performed by admixing the aqueous solution of DNA with 0.1 volume of 3 M NaOAc (pH 5.0) and 7 volumes of cold ethanol, followed by centrifugation (12 000 rpm, 4 °C, 15 min). The supernatant was removed, and the pellet was dried in a speed vac.

Synthesis of MC Dimers 3−5. Dimer 3. A solution of 1,4-diaminobutane (7.5 mg, 85 μmol) in CH_3OH (0.100 mL) was added to a solution of mitomycin A (60 mg, 0.172 mmol) in 2 mL of CH_3OH/DMF (1:1). The solution was stirred at room temperature for 4 h, protected from light. After 4 h, TLC (CH_3Cl/CH_3OH/Et_3N, 10:1:0.03) showed the formation of a major compound (R_f = 0.3), and two minor spots, for MA (R_f = 0.7) and the presumed product of monoaddition (R_f = 0.05). The mixture was concentrated in vacuo and purified by flash chromatography ($CHCl_3$/CH_3OH 10:1, containing 1% Et_3N) to give **3** (55 mg, 77%). ^{1}HNMR (Cl_3CD) δ (ppm): 1.65 (m, 4h), 1.98 (s, 6 H), 2.78 (m, 2H), 2.85 (m, 2H), 3.18 (s, 6H), 3.48 (d, 2H, J = 12.5 Hz), 3.49−3.58 (m, 4H), 4.25 (d, 2H, J = 12.6 Hz), 4.46 (m, 2H), 4.64 (dd, 2H, J = 4.0, 10.6 Hz), 6.63 (t, 2H, J = 5.6 Hz. ESIMS *m*/*z* (ion species), relative intensity: 598 ($M + H^+ - 2CH_3OH - HOCONH_2$), 16; 630 ($M + H^+ - CH_3OH - HOCONH_2$), 31; 745 ($M + Na^+$), 100. UV ($CH_3OH$) λ_{max} (ϵ): 227, 363 (31 000).

Dimer 4. A solution of 1,12-diaminododecane (15 mg, 75 μmol) in CH_3OH (0.100 mL) was added to a solution of mitomycin A (60 mg, 0.172 mmol) in 2 mL of CH_3OH/DMF (1:1). The solution was stirred at room temperature for 4 h, protected from light. The mixture was concentrated in vacuo and purified by flash chromatography as above to give **4** (55 mg, 88%). ^{1}HNMR (Cl_3CD) δ (ppm): 1.25−1.76 (m, 20 h), 2.02 (s, 6 H), 2.75 (m, 2H), 2.84 (m, 2H), 2.94 (m, 2H), 3.20 (s, 6 H), 3.23−3.38 (m, 4H), 3.59 (dd, 2H, J = 4.6, 10.5 Hz), 4.30 (d, 2H, J = 13.2 Hz), 4.52 (t, 2H, J = 5.9 Hz), 4.68 (dd, 2H, J = 4.4, 10.5 Hz), 6.36 (t, 2H, J = 5.7 Hz). ESIMS *m*/*z* (ion species) relative intensity: 710 ($M + H^+ - 2\ CH_3OH - HOCONH_2$), 10; 742 ($M + H^+ - CH_3OH - HOCONH_2$), 44; 857 ($M + Na^+$), 100. UV ($CH_3OH$) λ_{max} (ϵ): 227, 363 (31 000).

Dimer 5. A solution of 3,3′-diamino-*N*-methyldipropylamine (10.4 mg, 72 μmol) in CH_3OH (0.100 mL) was added to a solution of mitomycin A (60 mg, 0.172 mmol) in 2 mL of CH_3OH/DMF (1:1). The solution was stirred at room temperature for 4 h, protected from light. The mixture was concentrated in vacuo and purified by flash chromatography ($CHCl_3$/CH_3OH 4:1, containing 1% Et_3N) to give **5** (50 mg, 91%). ^{1}HNMR (Cl_3CD) δ (ppm): 1.76 (t, 4 H, J = 6.6 Hz), 1.99 (s, 6 H), 2.42 (t, 4 H, J = 6.8 Hz), 2.77 (m, 2H), 2.89 (m, 2H), 2.93 (s, 3 H), 3.15 (s, 6 H), 3.48 (d, 2 H, J = 13.0 Hz), 3.56 (dd, 2H, J = 4.4, 10.9 Hz), 3.62 (t, 4 H, J = 6.6 Hz), 4.25 (d, 2H, J = 13.9 Hz), 4.39 (m, 2 H), 4.63 (dd, 2H, J = 4.3, 10.6 Hz), 6.36 (br s, 1 H). ESIMS *m*/*z* (ion species) relative intensity: 748 ($M + H^+ - CH_3OH$), 16; 780 ($M + H^+ - CH_3OH$), 30; 802 ($M + Na^+$), 100; 818 ($M + K^+$), 33. UV (CH_3OH) λ_{max} (ϵ): 222, 371 (34 100).

Antitumor Activities. Dimers **3**−**5** were evaluated for antitumor activity in vitro at the NCI, using an in vitro antitumor screen. The screen consists of a panel of over 60 different human cancer cell lines derived from nine different types of tumor tissues. This panel of cell lines has been developed and operated by the NCI and has been used to test over 10 000 substances per year for growth inhibition and cytotoxicity.[28]

In an independent experiment[40] the cytotoxicity of dimers **3**−**5** to EMT6 mouse mammary tumor cells was determined using a clonogenic assay, under both aerobic and hypoxic conditions, using methods previously employed for assay of MC and its derivatives.[41]

Rate of Activation of Dimer 5 in Comparison with That of MC. (i) For low-pH activation, MC or dimer **5** (1 mM) in citrate phosphate pH 4.0 buffer was incubated at room temperature under aerobic conditions. (ii) For reductive activation by enzymes, MC or dimer **5** (1 mM) in deaerated 10 mM NaH_2PO_4/$NaHPO_4$/1 mM EDTA pH 7.5 buffer was incubated with one of three enzymes: xanthine oxidase (0.3 units/μmol drug; 2 μmol NADH/μmol drug), NADPH-cytochrome *c* reductase (0.3 units/μmol drug; 2 μmol NADPH/μmol drug), or DT-diaphorase (16 units/μmol drug; 2 μmol NADH/μmol drug) at 37 °C. (iii) For reductive activation by $Na_2S_2O_4$, MC or dimer **5** (5 μM) in 0.015 M Tris·HCL pH 7.4 buffer was deaerated with argon. $Na_2S_2O_4$ (1.5 or 5.0 μmol/μmol drug) in deaerated solution was added, and the mixture was incubated at room temperature.

All reaction mixtures included deoxythymidine (2 mM) as an inert HPLC peak intensity marker. Aliquots (25 μL) were removed at various reaction times, diluted to 200 μL, and frozen in liquid N_2 until subjeced to HPLC. The percent of remaining drug was determined by HPLC peak area integration at each time point, normalized to the marker deoxythymidine peak area. HPLC conditions were the following: 0.5−24% acetonitrile in 0.03 M K-phosphate, pH 5.0, in 60 min; flow rate of 1 mL/min; 4.6 mm × 25 mm column.

Assay of Cross-Linking of Various DNAs by MC and Dimers 3−5 under Reductive or Acidic Activation Conditions. (i) pBR322 DNA.[15] A mixture of pBR322 DNA (5 μg, 5 μL), *Eco*RI restriction endonuclease (5 μL, 50 units), *Eco*RI buffer (10 μL, supplied with the *Eco*RI enzyme), and 10 μL of sterile H_2O was incubated for 60 min at 37 °C. The linear DNA was labeled at the 3′-end by adding α-^{32}P-dATP (2 μL, specific activity of 3 mCi/nmol, 20 μCi) and Klenow polymerase (1 μL, 5 units). The mixture was incubated for 20 min at 37 °C, and the DNA was purified by ethanol precipitation. A mixture of labeled pBR322 DNA (approximately 100 ng) and MC or dimers **3**−**5** (at various concentrations) in 90 μL of 10 mM NaH_2PO_4/Na_2HPO_4/1 mM EDTA (pH 7.5) was deaerated by bubbling argon for 1 min, and 10 μL of a freshly prepared 10 mM solution of sodium dithionite in deaerated buffer was added. The samples were incubated for 45 min at room temperature, and the reactions were stopped by opening the tubes to air and gently stirring. The DNA was purified by ethanol precipitation, dissolved in strand separation buffer (30% DMSO, 1 mM EDTA, pH 9, 0.25% xylene cyanol, 0.25% bromophenol blue), heated at 90 °C for 5 min, and then immediately ice-cooled. Samples were loaded on a 1% agarose gel and run at 40 V for 16 h. The gels were dried, and the bands were visualized and quantified by phosphorimaging.

Cross-linking of pBR322 DNA following acidic activation of MC or dimers **3**−**5** was carried out using 10 mM NaH_2PO_4/Na_2HPO_4 pH 4 buffer and incubation for 2 h under aerobic conditions.

(ii) T-7 Coliphage DNA.[16] T-7 DNA (100 μM) and MC or dimer **3**, **4**, or **5** in a concentration range of 0.5−15 μM in 0.015

M Tris·HCl, pH 7.4, were deaerated followed by addition of a 10-fold molar excess of $Na_2S_2O_4$ and incubation for 60 min under purging with argon as described above. For cross-linking of T-7 DNA under acid activation, the DNA and drugs (concentration range 5−200 μM) were incubated in citrate phosphate, 10 mM Na^+, pH 4.0 buffer, for 4 h aerobically. After the reactions, 60 μL of the solution was diluted to 3 mL in 5 mM Tris·0.5 mM EDTA buffer, pH 8.0, containing 100 nM Hoechst 33258. The fluorescence of the solution was measured (excitation wavelength of 355 nm and emission wavelength of 460 nm) on a T2 Spex fluorolog spectrofluorimeter operating in the constant-wavelength mode. The solutions were transferred into glass tubes with screw caps and heated in a water bath at 95 °C for 5 min. The tubes were suddenly plunged into an ice bath for 5 min and then incubated at room temperature for 10 min. The fluorescence of the solutions was again measured under the same conditions as described earlier. Appropriate control samples in the absence of the drug were also prepared. The fraction of cross-linked DNA was calculated from the formula

$$\frac{E_A/E_B - C_A/C_B}{1 - C_A/C_B}$$

where E_A and E_B are the fluorescence intensities of the sample after and before the heat/chill step, respectively, and C_A and C_B are the fluorescence intensities of the control after and before the heat/chill step, respectively.

Cross-Linking of the 162-bp *tyr*T DNA Restriction Fragment by MC and Dimer 3 or 5 under Reductive and Acid Activation Conditions. Reductive Conditions. DNA (10 ng, approximately 5 cps) in 4 μL of TN buffer was added to a mixture of 14.5 μL of TE buffer and 2.5 μL of solutions of various concentrations of MC or dimer. This reaction mixture was vigorously deaerated by bubbling with nitrogen for 2 min with the container tightly capped. Water was deaerated for 5 min, and then sodium dithionite (sodium hydrosulfite) was added to make a final concentration of 50 mM, which was deaerated for a further 5 min. Dithionite stock (4 μL) was added to the reaction mixture to give a total volume of 25 μL, which was then deaerated for 30 s, with the container capped tightly and kept on ice in the dark for 1 h. The cross-linking reaction was stopped by adding 2 μL of 1 μg·μL^{-1} stock calf thymus DNA, 7.5 μg of tRNA in 25 μL of 0.3 M sodium acetate, and 130 μL of 100% ethanol, followed by exposure to air. After cooling in dry ice for 15 min, the samples were centrifuged at 13 000 rpm (4 °C) for 10 min and the supernatants were discarded. Pellets were dried by lyophilization and then redissolved in 10 μL of strand separation buffer.

Acidic Activating Conditions. DNA and drugs were incubated in citrate phosphate pH 4.0 buffer aerobically for 2 h. The cross-linking reaction mixtures were processed as above.

Analysis of the Cross-Linked *tyr*T DNA by Gel Electrophoresis. The samples were heated to 94 °C for 4 min to denature the DNA and then cooled on ice for 4 min. The cross-linked species were separated from the single-stranded *tyr*T DNA by running on an 8% denaturing polyacrylamide gel with 1X TBE buffer at 90 W for 1 h. The gel had been preheated to approximately 55 °C by running at 90 W for 90 min with TBE buffer. After electrophoresis the gel was soaked and fixed (10 min) in 10% acetic acid, transferred to Whatman 3 MM paper, covered in Saran wrap, and dried under suction on a Bio-Rad gel drier for 45 min at 80 °C. The dried gel was then exposed to a phosphor screen (Molecular Dynamics) at room temperature overnight.

For experiments to investigate the identity of materials left in the wells under the usual conditions of electrophoresis, the samples were loaded onto a 5% denaturing polyacrylamide gel after heating for 4 min and cooling on ice for 4 min. The gel was then run at 90 W for 13 h. Because of the fragility of the gel, it was transferred directly onto Whatman 3MM paper, omitting the acetic acid step, and then dried as above.The phosphor screen was transferred to a 425E phosphorimager (Molecular Dynamics) and scanned to reveal a radiographic image of the gel. The data were then transferred to a Macintosh, analyzed using ImageQuant, version 1.2, and the MC and dimer cross-linked bands were compared.

Chart 2. Synthetic Oligodeoxyribonucleotides

5'ATA TAC GTA TAT5'
3'TAT ATG CAT ATA3'
6
5'TAT CGA AAC GTAT5'
3'ATA GCT TTG CATA3'
7
5'TAT CGA ACG TATT5'
3'ATA GCT TGC ATAA3'
8
5'TAA ATC GAC GTA TT3'
3'ATT AGC TGC ATA AA5'
9
5'AGC GGC GCG T3'
3'TCG CCG CGC A5'
10

Noncovalent Affinity of the Dimers 3−5 for Calf Thymus DNA. The ethidium bromide displacement assay[17] was employed as described previously for other MC derivatives.[18]

Alkylation of a Series of Synthetic Oligonucleotides by MC and Dimer 5. Duplex oligonucleotides **6**−**10** (Chart 2) were each alkylated by MC or dimer **5**, using (i) an anaerobic excess of $Na_2S_2O_4$ or (ii) anaerobic NADH/xanthine oxidase for activation. Conditions were the following: (i) Oligonucleotide (5 A_{260} units), 1.25 μmol of drug, and 2 μmol of $Na_2S_2O_4$ in 0.5 mL of 0.1 M Tris, pH 7.4, were incubated at 4 °C for 60 min under anaerobic conditions (purging with argon). (ii) Oligonucleotide (5 A_{260} units), 1.25 μmol of drug, 2 mmol of NADH, and 0.4 units of xanthine oxidase in 0.5 mL of 0.1 M KH_2PO_4/0.01 M EDTA pH 5.8 buffer were incubated for 3 h at 4 °C under anaerobic conditions (purging with argon). The extent of cross-linking was assayed by passing the reaction mixture through a Sephadex G-50 column heated at 45 °C. The ratio of the first-eluted, cross-linked oligo fraction was determined by measuring UV absorbance at 260 nm of the eluates. The cross-linked fraction was enzymatically digested to nucleosides, and the digest was analyzed by HPLC, as described below for drug-treated *M. luteus* DNA.

HPLC Analysis of Nucleoside−Drug Adducts in Digests of DNA Treated with Dimer 5. DNA [*M. luteus* DNA or poly(dG-dC)·poly(dG-dC)] was treated with dimer **5** under various drug-activating conditions. (i) For acid activation, 12 mM DNA and 1 mM dimer **5** were incubated in citrate phosphate buffer, [Na^+] = 10 mM, pH 4.0, for 12 h at room temperature. (ii) For reductive activation, 12 mM DNA, 1 mM dimer **5**, and 3 mM $Na_2S_2O_4$ were incubated in 0.1 M Tris·HCl, pH 7.4, under anaerobic conditions (purging with argon) for 1 h at room temperature. After the drug treatment, DNA was either ethanol-precipitated or passed through a Sephadex-G100 column, using 0.02 M NH_4HCO_3 as eluant, and the collected DNA fraction was lyophilized. Samples were digested either with P_1 nuclease/snake venom diesterase/bacterial alkaline phosphatase or with DNAse I/snake venom diesterase/bacterial alkaline phosphatase, as previously described.[19] The digests were analyzed by HPLC using a C18 analytical column (Rainin). A linear gradient of 3−18% acetonitrile in 0.03 M K-phosphate pH 5.5 buffer in 90 min at 10 mL flow rate was used for elution.

LC−ESIMS analysis of the above digests was carried out as previously described.[20]

Results

Design and Synthesis of 3−5. We designed **3** as a dimer with a short [(−CH_2−)$_4$] tether, **4** as one with a

Table 1. In Vitro Anticancer Activity of MC and Dimers **3**−**5** against Cultured Human Tumor Cell Lines of the NCI Screen Panel[28]

compd (NCI test ref no.)	log GI_{50} [a] (mean graph midpoint), M	GI_{50} [a] (mean graph midpoint), μM
mitomycin C (S26980A)	−6.08	0.83
3 (D-702525−W/1)	−4.16	69.1
4 (D-702526−X/1)	−5.35	4.5
5 (D-702525−I/1)	−5.60	2.5

[a] GI_{50}: growth inhibitory concentration causing 50% inhibition of growth. "Mean graph midpoint" was calculated from the experimental values of GI_{50} of the approximately 60 individual cells lines constituting the test panel, and it corresponds to their mean.

long [(−CH_2−)$_{12}$] tether, and **5** with a medium-length tether [−$(CH_2)_3N(CH_3)(CH_2)_3$−]. Compound **5** was expected to be water-soluble because of the protonated amine function in its tether. We were encouraged to adopt this design by our molecular modeling[21] of an analogous MC dimer linked by the −$(CH_2)_2SS(CH_2)_2$− tether, forming a cross-link between two guanines separated by two base pairs of the duplex oligonucleotide d$(CG)_{10}$·d$(CG)_{10}$. The 2-amino groups of the guanines were linked to C(1) of each MC residue in the dimer, as in the monomeric dG monoadduct.

The facile one-step syntheses of **3**−**5** were accomplished by simultaneous displacement of the 7-methoxy group from two MA molecules[3] by the appropriate α,ω-diamines as described previously for other MC dimers of this type.[11−13,22,23] The structures of the new compounds were rigorously verified by MS and ^{1}H NMR. Serving as proof of purity, HPLC of the samples showed single peaks as detected by UV absorbance at 254 nm and the ^{1}H NMR spectra indicated no impurities.

Antitumor Activities. NCI in Vitro Antitumor Screen. The mean values of the 50% growth inhibitory values (GI_{50}) against individual cell lines observed for **3**−**5** are given in Table 1, together with those of mitomycin C determined previously in the same screen. The data show that **3**−**5** are cytotoxic; however, they are less active overall than MC. The relative order of antitumor activities of the three dimers is **5** > **4** > **3**. The same order of activity is seen when the data from individual cell lines are compared (data not shown) and also when the cytotoxicities (LC_{50}) of the three substances are compared (data not shown). It may be concluded that in general the most active dimer, **5**, exhibited only slightly lower growth inhibition and cytotoxicity than MC in the NCI tumor cell panel. It is noteworthy that its tumor selectivity profile was surprisingly uniform; no significant selectivity for any tumor cell type was observed.

Clonogenic Assay. In an independent experiment the cytotoxicity of all three dimers to EMT6 mouse mammary tumor cells was determined under hypoxic and aerobic conditions of drug treatment using a clonogenic assay. By use of 10 μM drug treatment for 4 h, dimers **3**, **4**, and **5** exhibited 50-, 5-, and 4.4-fold greater cytotoxicity to hypoxic EMT6 cells, respectively, than to EMT6 cells grown under normal aerobic conditions.[40,41] Since MC exhibits comparable oxic/hypoxic cytotoxicity differential in the same cells, this result strongly suggests that the activity of the mitomycin dimers depends on the same bioreductive activation mechanism in the cell as that of MC.

Drug Activation. Activation of the dimers **3**−**5** by low pH was observed by the gradual conversion of the dimers to unidentified mitosene hydrolysis products at pH 4.0. The conversion of the starting material was followed by HPLC, by analogy to activation of MC under these conditions.[24] The rate of activation of dimers **3**−**5** was essentially identical to that of MC by direct comparison (Figure S1a in Supporting Information).

Reductive activation of dimer **5** by three commonly employed reductases for MC activation (xanthine oxidase, NADPH-cytochrome *c* reductase and DT-diaphorase), monitored by the HPLC method, proceeded at the same rate (Figure S1b,c) as that of MC or somewhat slower (Figure S1d). Reductive activation of **5** by $Na_2S_2O_4$ was very fast, since no starting material could be detected by HPLC even at the earliest time point (2 min).

Assay of DNA Cross-Linking. pBR322 DNA. Upon reductive activation, all three dimers cross-linked linearized pBR322 DNA labeled with ^{32}P at its 3′-end as tested by the method of Hartley and co-workers.[15] According to this method, linearized DNA is end-labeled with [^{32}P]-phosphate, treated with the cross-linking agent, and subsequently denatured by heating. On cooling, the cross-linked DNA undergoes quick renaturation because of the covalent linkages(s) between the two strands, while non-cross-linked DNA remains single-stranded. The renatured and single-stranded fractions are readily separated by nondenaturing agarose gel electrophoresis and quantitated by phosphorimaging. This method was used for mitomycin and its derivatives previously.[18,23] DNA cross-linking by MC requires a reducing agent for activation of the drug; $Na_2S_2O_4$ was employed under anaerobic conditions in the present case. The results (Figure 2) show that all dimers **3**−**5** cross-link DNA under $Na_2S_2O_4$ (reductive) activation.

We also investigated whether the dimers are able to induce DNA−DNA cross-links under conditions of low pH, which activates the MC molecule monofunctionally only.[24] MC and dimers **3**−**5** were incubated with DNA in a slightly acidic buffer (pH 4.0) and analyzed for cross-links as described above. MC and dimers **3** and **4** were negative, but dimer **5** did show concentration-dependent cross-linking of pBR322.

These results were confirmed independently by a different assay;[16] T-7 coliphage DNA as substrate was submitted to cross-linking by MC, dimer **3**, and dimer **5**, activated as above. The extent of cross-linking was determined by measuring the differential fluorescence of DNA-bound dye in denatured and renatured DNA samples. Heat-denatured cross-linked molecules renature spontaneously on cooling and, as such, give rise to more intense fluorescence of the dye than control heat-denatured (non-cross-linked) DNA, which remains denatured on cooling. By use of this assay under reductive activation, both dimers **3** and **5** showed significant cross-linking activity (Table 2, Figure S2).

Sequence Specificity and Efficacy of Cross-Linking of *tyr*T DNA by MC, Dimer 3, and Dimer 5. The base sequence of the 162-bp bacterial DNA fragment *tyr*T is shown in Figure 3. Following reductive activation, the location and efficacy of cross-linking of

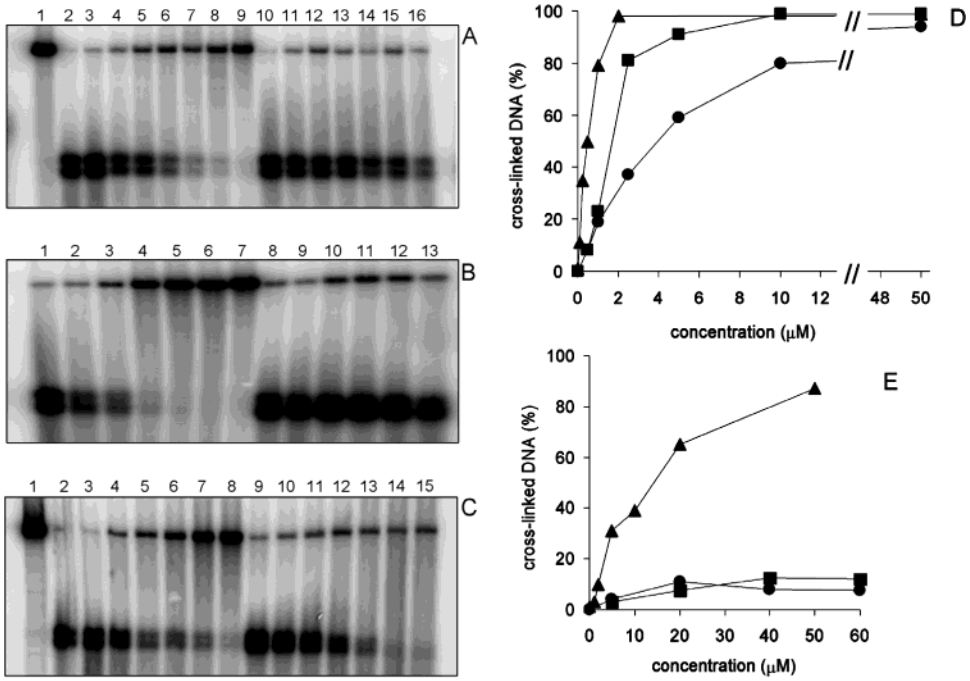


Figure 2. Comparison of the cross-linking efficiencies of pBR322 DNA by dimers **3**−**5**. Autoradiography of agarose gels showing cross-linking of 3′-^{32}P-labeled pBR322 DNA. (A) Cross-linking by compound **3**. Lanes are as follows: (1) double-strand control; (2) single-strand control; (3−9) 0, 0.5, 1, 2.5, 5, 10, 50 μM **3**, activation with 1 μM sodium dithionite; (10−16) 0, 5, 20, 40, 60, 100, 250 μM **3**, acidic activation (pH 4). (B) Cross-linking by dimer **4**. Lanes are as follows: (1−7) 0, 0.5, 1, 2.5, 5, 10, 50 μM **4**, activation with 1 μM sodium dithionite; (8−13) 0, 5, 20, 40, 60, 100, 250 μM **4**, acidic activation. (C) Cross-linking by dimer **5**. Lanes are as follows: (1) double-strand control; (2) single-strand control; (3−8) 0, 0.1, 0.25, 0.5, 1.2 μM **5**, activation with 1 μM sodium dithionite; (9−15) 0, 1, 2, 5, 10, 20, 50 μM **5**, acidic activation. (D) Comparison of the cross-linking efficiency of pBR322 DNA by **3**−**5**, with percent cross-linking as a function of the drug concentration, as measured by phosphorimaging: activation with sodium dithionite (upper); acidic activation (lower); (●) dimer **3**; (■) dimer **4**; (▲) dimer **5**.

Table 2. Cross-Linking of DNA by MC and Dimers **3**−**5**[a]

	ICL_{50}, μM[b] (ICL_5, μM[c])			
	MC	dimer **3**	dimer **5**	dimer **4**
T-7 DNA, $Na_2S_2O_4$	10	2.60	1.50	
T-7 DNA, Acid	>200	>200	20	
*tyr*T DNA, $Na_2S_2O_4$	∞ (55)	∞ (20)	150 (10)	
*tyr*T DNA, acid	undetectable	>400[d]	>400[e]	
pBR322 DNA, $Na_2S_2O_4$	12[f]	3.8	0.5	1.7
pBR322 DNA, acid		<10% at 40 μM	10% at 1 μM	10% at 40 μM

[a] ICL_{50} and/or ICL_5 values are a measure of efficiency. [b] ICL_{50}: drug concentration in the reaction mixture required for cross-linking 50% of the DNA molecule population. [c] ICL_5: drug concentration required for cross-linking 5% of the DNA molecule population. The ICL_5 values are indicated in parentheses. [d] 0.6% cross-linked at 400 μM. [e] 1.8% cross-linked at 400 μM. [f] Data from ref 18.

*tyr*T DNA by MC and dimers **3** and **5** were compared by a method based on denaturing PAGE. Using a family of DNA duplexes of identical length but in which the cross-linkable sites were progressively displaced from the center of the duplex toward the ends, Millard et al.[25] showed that DNA cross-linked by MC and other agents migrated to different positions on a denaturing polyacrylamide gel depending on the distance of the cross-link from the center of the molecule. The most centrally cross-linked species traveled the least distance from the loading wells at the top of the gel. The physical basis for this phenomenon is unknown but must presumably originate from hydrodynamic differences in the behavior of cross-linked molecules affecting their migration through the gel.

The pattern of cross-linking of *tyr*T DNA by reductively activated MC has been analyzed previously in our laboratories using denaturing PAGE.[14,26] MC cross-links are formed exclusively at the CpG sequence,[5] and this DNA contains 13 CpG steps, i.e., 13 potential cross-linkable sites, indicated by bold letters in Figure 3. Following MC treatment, five ^{32}P-labeled bands were detectable in the gel in the cross-linked (low-mobility) DNA region. All 13 DNA species, cross-linked at the different sites, were assigned to the 5 discrete radiolabeled bands. The relative distribution of two or three different cross-linked species migrating unresolved as one ^{32}P-band was not known.[14,26] This result was fully reproducible in the present work, as seen in Figure 4 in the case of MC and dimer **5**. The most striking result of this experiment is that dimer **5** cross-links DNA apparently at the same sites as MC. Treatment with dimer **3** also resulted in the same band pattern (not shown). Another equally striking finding is that dimer **5** cross-links DNA much more efficiently than MC, as determined by densitometric analysis of the gels (Figure 5 and Table 2). Densitometry of the gel illustrated in Figure 4 indicated that 100 μM MC cross-linked only 9% of the total DNA whereas dimer **5** cross-linked 51% of the DNA under identical reaction conditions.

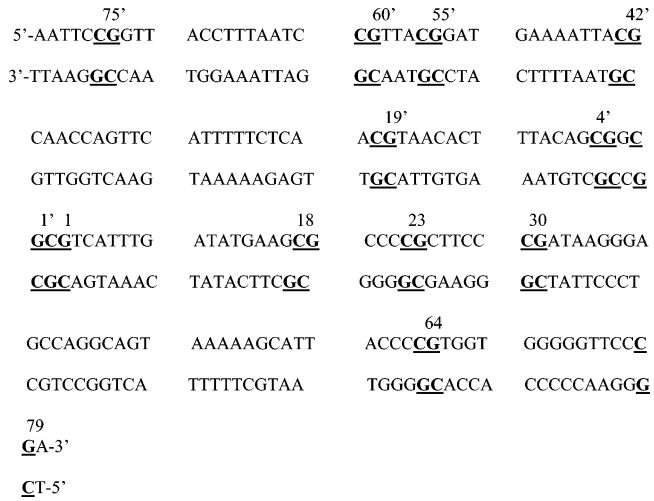


Figure 3. Base sequence of the bacterial DNA fragment *tyr*T. Shown in bold and underlined are the 13 cross-linkable CpG sites. These sites are numbered according to their distances in base pairs from the geometric center of the fragment, which is the base between 1 and 1′.

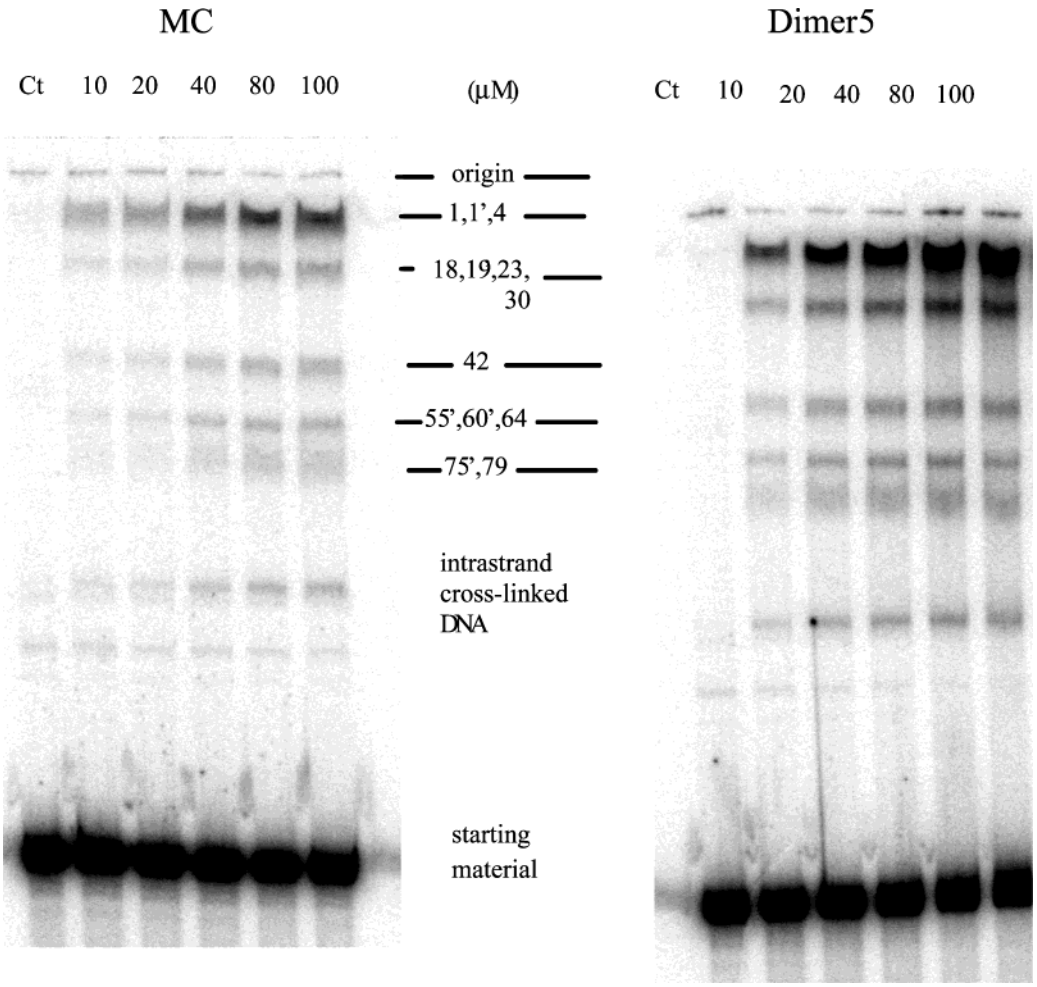


Figure 4. Resolution of the cross-linked DNA species following MC or dimer **5** treatment by denaturing PAGE. The mobility of cross-linked species is retarded with respect to the starting material. On the basis of the observation of Millard et al.,[25] those species cross-linked closest to their geometric centers are retarded to the greatest degree. The sites of the cross-linking were assigned on this basis.

Dimer **3** was less efficient than dimer **5** but more efficient than MC. The ICL_{50} value (drug concentration resulting in 50% cross-linked DNA) could only be determined for dimer **5**. Cross-linking by MC and dimer **3** did not reach the 50% level up to 200 μM drug concentration. The ICL_{50} and ICL_5 values (drug concentration resulting in 5% cross-linked DNA) are shown in Table 2. The increased cross-linking efficiency of the dimers compared with MC is fully consistent with their increased cross-linking efficiency observed in the T-7 DNA and pBR322 DNA assays (Table 2, Figure S2, Figure 2).

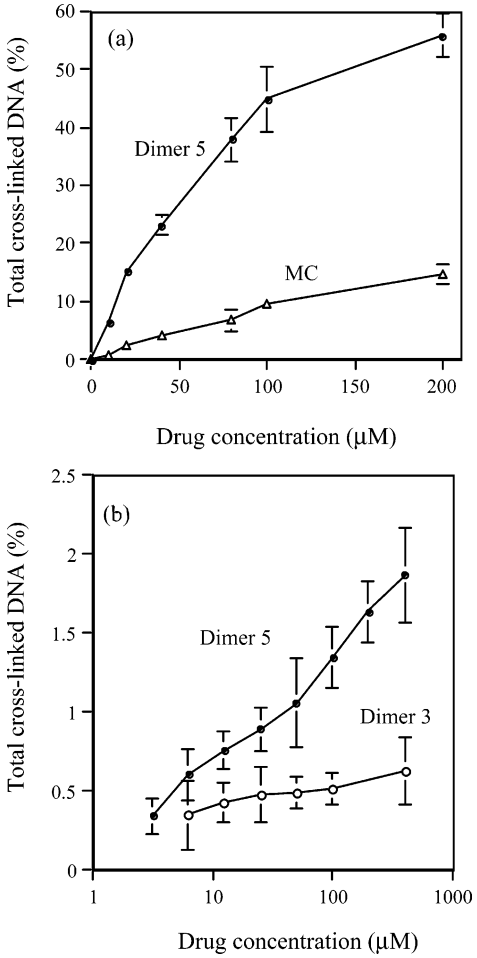


Figure 5. Dimer **5** produces enhanced total cross-linking activity per MC chromophore. The graphs were created using densitometric analysis of gels such as those shown in Figure 4. Error bars show the standard error of the mean. (a) Reductive activation. (b) Acid activation.

Enhanced Regioselectivity of Cross-Linking by the Dimers at G·C-Rich Regions of *tyr*T DNA Relative to Cross-Linking by MC. There are two G·C-rich sequences containing eight and nine contiguous G·C base pairs, respectively: one encompassing three CpGs marked 1, 1′, and 4′; another encompassing two CpGs marked 18 and 23 (Figure 3). Densitometry revealed that in the dimer-treated DNAs the two ^{32}P-bands corresponding to DNA cross-linked at these sites are much more intense than the three ^{32}P-bands corresponding to the CpG cross-links at the other sites (see Figure 4). As shown in Figure 6, with 100 μM drug treatment both bands are 8 times more intense than the corresponding MC-cross-linked bands on the same gel, while the three faster-moving bands are only approximately twice as intense as the corresponding bands in the MC lane.

Acid Catalysis of Cross-Linking of *tyr*T DNA by Mitomycins. At acidic pH, MC is monofunctionally activated at the C(1) (aziridine) position.[24] Since the dimers possess two MC units in one molecule, they could produce bifunctional alkylation overall (i.e., cross-linking) under acidic activating conditions. Cross-linking of *tyr*T DNA by dimer **5** was indeed observed under acidic drug-activating conditions using the denaturing PAGE method of assay as above. MC was negative, and dimer **3** was only weakly positive. The efficiency of acid-catalyzed cross-linking by dimer **5** was much lower than under reductive activation (Figure 5, Table 2).

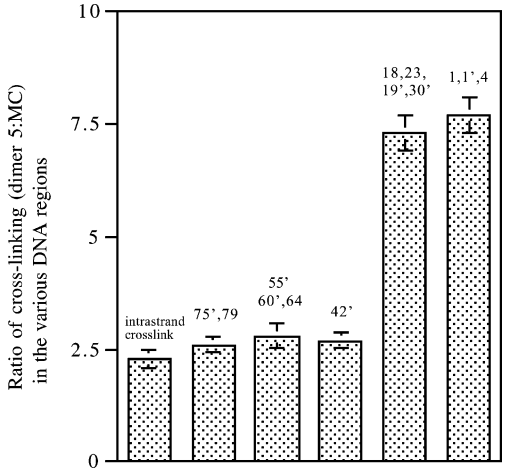


Figure 6. Relative amount of cross-linking by dimer **5** compared to that of MC at various sequences of *tyr*T DNA. The amount of cross-linking by dimer **5** is increased at every CpG site. In addition, dimer **5** shows a selective increase at the two poly(G·C) sites, (18, 23, and 4′, 1′, 1). These data are derived from the 100 μM lanes of MC and dimer **5** using densitometric analysis of gels such as those shown in Figure 4. Error bars show the standard error of the mean.

Noncovalent DNA Binding Affinity. As assayed by the ethidium fluorescence quenching method,[17] the drugs bound to calf thymus DNA 2−3 orders of magnitude more weakly than the reference drug ethidium bromide. The values of the apparent binding constants ($K_{app} \times 10^{-4}$ M^{-1}) of MC, dimer **3**, and dimer **5** were 1.7, 5.0, and 8.0, respectively. Since MC was shown earlier using other assays[27] to exhibit no significant binding to DNA, these values have only relative significance. The highest binding constant of dimer **5** is consistent with the presence of a positively charged, protonated amine function in the molecule, which also accounts for its excellent water solubility.

Adducts of Acid-Activated Dimer 5. The HPLC of digests of oligonucleotide **7**, *M. luteus* DNA, and poly(dG-dC), each treated with acid-activated dimer **5**, indicated formation of several well-separated major adducts (Figure 7a−c). The UV spectra (Figure 7d) of peaks 1 and 2 were identical and indicated a combination of mitosene[29] (λ_{max} = 320, 250 nm) and dG chromophores (λ_{max} = 250 nm). The LC−ESIMS spectra were also identical: 1001 ± 1, MH$^+$; 1039 ± 1, MK$^+$. The UV spectrum of peak 3 is a combination of mitosane[29] (λ_{max} = 370 nm), mitosene[29] (λ_{max} = 320, 250 nm), and dG (λ_{max} = 250 nm). LC−ESIMS result of peak 3 is the following: 1053 ± 1, MK$^+$. Thus, peaks 1 and 2 correspond to isomeric adducts from monofunctional alkylation by **5** (Figure 8a), having structures **11a** (α and β isomers at C(1)). Peak 3 corresponds to structure **12** (Scheme 2).

Adducts of Reductively Activated Dimer 5 with Oligonucleotides. In an attempt to observe tri- or tetrafunctional alkylation by dimer **5** (Figure 8e,f), a series of duplex oligonucleotides were designed in which two CpG sites were separated by one, two, or three A·T base pairs (**7**−**9**). The series also included a G·C-rich oligonucleotide sequence[14] of *tyr*T DNA (**10**) corresponding to the central region (Figure 3). Reductive activation

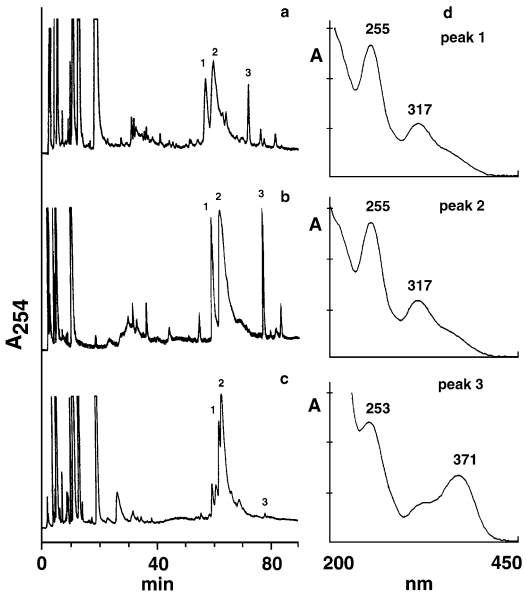
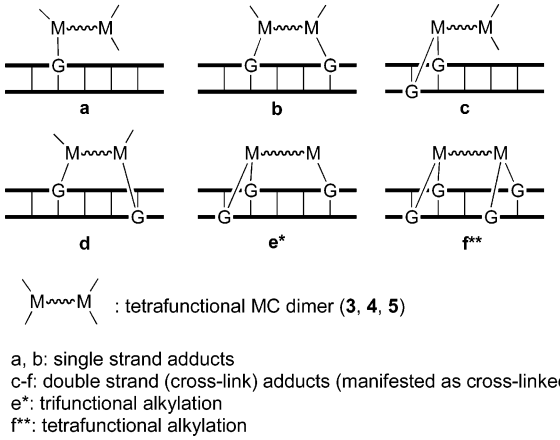


Figure 7. HPLC and UV spectra of adducts from acid-activated alkylation of various DNAs by dimer **5**. HPLC: (a) digest of oligonucleotide **7**; (b) digest of poly(dG-dC)·poly(dG-dC); (c) digest of *M. luteus* DNA. The time scale in (c) is different from that in (a) and (b). UV spectra: (d) shows the UV of the three major HPLC peaks marked 1, 2, and 3 in the HPLC tracings.

by $Na_2S_2O_4$ or xanthine oxidase led to cross-linked oligonucleotides that were isolated by Sephadex G-50 chromatography (data not shown). The cross-linked oligos were digested to mixtures of nucleosides and nucleoside−drug adducts, and the digests were analyzed by HPLC. A highly complex mixture of adducts was observed by monitoring the UV absorbance of the eluate. There was no significant difference among the elution patterns of digests of the various oligonucleotides, and therefore no evidence was obtained for sequence-specific tri- or tetrafunctional alkylation in these experiments (data not shown).

Adducts of Reductively Activated Dimer 5 with *M. luteus* DNA. Under $Na_2S_2O_4$ activation, extremely complex HPLC patterns were obtained indicating formation of practically intractable arrays of adducts.



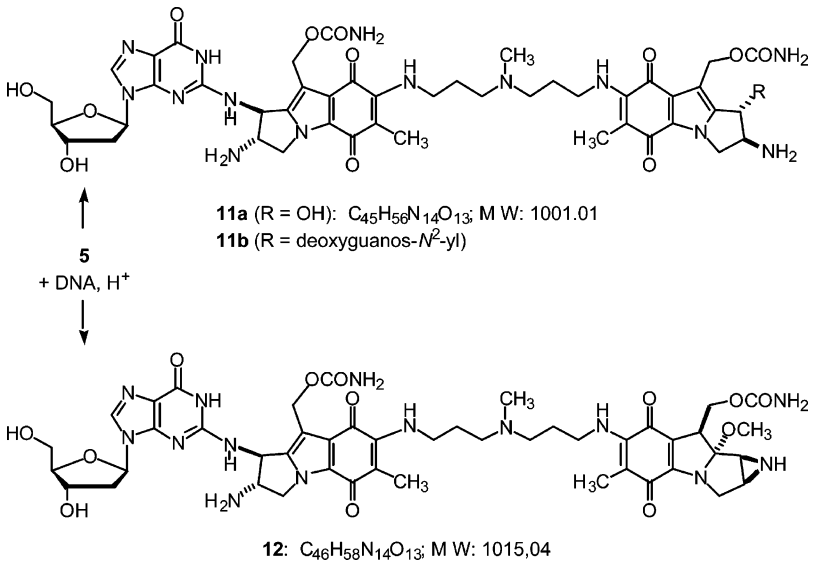
a, b: single strand adducts
c-f: double strand (cross-link) adducts (manifested as cross-linked DNA)
e*: trifunctional alkylation
f**: tetrafunctional alkylation

Figure 8. Schematic diagrams of potential adducts of MC dimers with DNA.

Reactions with H_2/PtO_2 and NADH/NADH cytochrome *c* activated dimer **5** appeared to be simpler (Figure 9a−c). UV spectra of individual components were similar to those of adducts of acid activation (Figure 7d) with two important exceptions: several peaks indicated [2 mitosene]/[4 dG] and [2 mitosene]/[3 dG] stoichiometries, as determined from the 250:320 nm:nm spectral ratios in the UV spectrum (3.6 and 2.7, respectively; see Figure 9e,d). The UV adducts **11a** and **11b** served as authentic references for the [2 mitosene]/[1 dG] stoichiometry (2.3 spectral ratio). This convenient empirical method of estimating MC/guanine chromophore ratios from UV spectra was applied previously to distinguish MC−guanine monoadducts from MC−guanine bis-adducts.[30,31] The present results serve as evidence for trifunctional and tetrafunctional alkylation of DNA by MC−dimer **5** (Figure 8e and Figure 8f type adducts, respectively).

The complex mixture of adducts in the case of $Na_2S_2O_4$ activation of dimer **5** is in sharp contrast with the DNA adduct pattern of $Na_2S_2O_4$ activated monomeric MC, usually consisting of two or three major components only.[31,32] Although the simplified diagram of potential dimer−DNA adducts (Figure 8) does not indicate the multiplicity of structures possible because of different substitution in the activated C(1) and C(10)

Scheme 2. Formation of Adducts of Dimer **5** and Deoxyguanosine under Acidic Activation



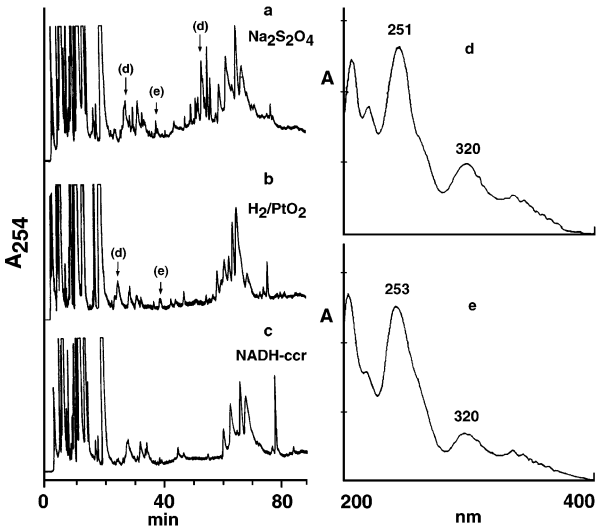


Figure 9. HPLC and UV spectra of adducts from reductively activated alkylation of *M. luteus* DNA by dimer **5**. HPLC: (a) digest of $Na_2S_2O_4$ activated alkylation; (b) digest of H_2/PtO_2 activated alkylation; (c) digest of NADH-cytochrome *c* activated alkylation. Conditions are as follows: 3−18% acetonitrile in 0.03 M KH_2PO_4, pH 5.5, in 90 min; flow rate of 1 mL/min; UV detection at 254 nm. UV spectra: (d) spectra of peaks indicated by (d) in the HPLC tracings on the left; (e) spectra of peaks indicated by (e) in the HPLC tracings on the left.

positions by small molecules (H_2O, bisulfite ion), such products are likely to be formed.[33] Accordingly, several bisulfite-containing adducts were detected by LC−ESIMS (data not shown). Another factor contributing to the unique complexity of dimeric mitomycin adducts is formation of adducts in which one-half of the dimer is unaltered mitosane.[29] This is seen clearly in the UV spectra of some adducts because the spectra show the presence of both the mitosene (λ_{max} = 320 nm) and mitosane (λ_{max} = 370 nm) chromophores[29] (e.g., Figure 7d, peak 3).

Discussion

Synthetic dimers of the bifunctional DNA alkylator MC have a potential for multifunctional alkylation of DNA, resulting in novel types of DNA lesions. This potential is illustrated in Figure 8, where parts e and f depict covalent tri- and tetrafunctional attachments of an MC dimer to duplex DNA segments. As a consequence of such new multifunctional interactions with DNA, the cytotoxic activity of MC may be altered when it is incorporated into a covalent dimer. MC dimers can be readily synthesized by the reaction of MA with α,ω-diamines, and a study of structure−activity relationships of 16 such dimers was recently reported.[13] The primary objective of the present study of the three new dimers **3**−**5** was to probe the complex chemistry of the interaction of the drugs with DNA using two different approaches: characterization of their interstrand cross-linking (ICL) of DNA and analysis of covalent nucleoside−drug adducts.

Since interstrand cross-linking of DNA is considered to be the most biologically relevant chemical function of the mitomycins, we first characterized qualitatively and quantitatively the cross-linking activities of the three dimers in parallel with MC using DNA substrates of four different sizes: T-7 phage DNA (∼7000 bp), pBR322 DNA (4363 bp), *tyr*T-DNA (162 bp), and oligonucleotides (10−12 bp). In the earlier study of cross-linking activities of other MC dimers,[13] pBR322 DNA alone was employed and no quantitative data were reported. Here, the use of four different DNAs and several different methods of assay led uniformly to the conclusion that all three dimeric mitomycins in this study are more active DNA cross-linkers than monomeric MC and that **5** is the most active of the three. Both T-7 DNA and *tyr*T DNA were cross-linked approximately 6-fold more efficiently by dimer **5** than by MC (Table 2).

The relative cross-linking activities of the three dimers correlated with their overall relative growth inhibitory and cytotoxic potencies (Table 1). The magnitudes of both the cross-linking activity and biological potency fell in the order **5** > **4** > **3**. No such correlation was observed in the previous dimer study cited above.[13] It is tempting to suggest that the superior activity of dimer **5**, compared to the other two dimers **3** and **4**, is due to its weak but pronounced noncovalent affinity for DNA determined in the present work, which must originate from the positively charged protonated amino group in the tether of **5**.

An explanation for the strikingly higher yields of cross-linking by the dimers (calculated per MC unit) than by the parent compound (Table 2) is suggested as follows: It is known that MC cross-links are specific to the CpG sequence.[5] Alkylation of guanines by MC in non-CpG sequences is unproductive of interstrand cross-links, leading to monofunctional DNA adducts instead[34] (Scheme 1). Alkylation of a non-CpG guanine by one unit of the dimer, however, may be followed by alkylation of a second (CpG or non-CpG) guanine by the dimer's other mitomycin unit in the opposite strand of DNA, resulting in an ICL (Figure 8d). This cross-linking path is not available to monomeric MC. We obtained supportive evidence for this scenario by observing that acidic (nonreductive) activation of MC dimers yielded ICLs in all three DNAs tested, although at relatively low efficiency (Table 2). Since only the aziridine function of MC is activated by acids at pH 4.0,[24] these experiments prove that the dimers are capable of forming ICLs by linking both of their two C(1) atoms to DNA (Figure 8d). The same mechanism was proposed earlier by Kohn and co-workers[13] who demonstrated ICL formation by a dimer of 10-decarbamoyl MC under $Na_2S_2O_4$ mediated activation. 10-Decarbamoyl MC is known to alkylate DNA mostly monofunctionally by its C(1) function, even under reductive activation, although a relatively weak C(1), C(10) cross-linking activity has been detected recently in our laboratory.[19]

Sequence specificity associated with MC-mediated cross-linking of a 162 bp restriction fragment, the bacterial *tyr*T DNA, has been characterized previously in our laboratories.[14,26] It has been possible to map the cross-links to specific CpG sites within the DNA sequence (Figure 4) based on the method of Millard and co-workers.[25] The intensity of cross-linking per CpG step was greater in the central G·C-rich region numbered 1, 1′, 4′ than elsewhere, and this regioselectivity of MC was attributed to its exceptionally high reactivity toward guanines in successive CpG steps, reported previously by Teng et al.[36] The *tyr*T DNA contains only

one such site (5′-GCGCGT), located in the 1, 1′, 4′ central region.

The mitomycin dimers map to the same sites as MC, indicating that the same CpG-specific cross-link chemistry dominates the reactivity of the dimers (Figure 4). A striking difference, however, is the much greater (3- to 4-fold) regioselectivity of dimer **5** for both the 1, 1′, 4′ and 18, 23 regions compared to MC (Figure 6). These two regions (Figure 3) are G·C-rich, containing eight and nine contiguous G·C base pairs, respectively. The greater frequency of dimer cross-links in these regions compared to the frequency of MC cross-links formed under analogous drug treatment may be due to the same mechanism as that suggested above for the observed greater *global* cross-linking efficiency of dimers: monofunctional cross-linking of two non-CpG guanines located in opposite strands (Figure 8d). This mode of cross-linking, unique to the dimers, is expected to be selectively enhanced at G·C-rich sequences, explaining the observed enhanced regioselectivity of dimer cross-linking in *tyr*T DNA (cf. Figure 6). We propose that the regioselectivity of the mitomycin dimers has the same origin as that of the observed regiospecificity of the dimer bizelesin[37,38] and the (predicted) regiospecificity of the PBD dimers.[39]

Covalent Adducts upon Acid Activation. Covalent deoxyguanosine adducts of dimer **5** with various DNAs were unambiguously detected by HPLC (Figure 7). The structures of the three major adducts were established by the UV spectra and LC−ESIMS, as **11a** (2 stereoisomers) and **12** (Scheme 2), corresponding to monofunctional adducts as in the general diagram in Figure 8a. Attempts at identification of the other adducts seen in the HPLC were unsuccessful. However, unambiguous demonstration of cross-link formation in the same DNAs (Figures 2, 5b, 6b) constitutes proof of bifunctional alkylation products as diagrammed in Figure 8d.

Covalent Adducts upon Reductive Activation. HPLC−UV spectra proved that dithionite-activated dimer **5** reacts covalently with DNA. The complexity of the adduct mixtures precluded identification of individual components by LC−ESIMS partly because the adducts produced intractable fragmentation patterns in the LC−ESIMS. Very significantly, however, two adducts from the cross-linked fraction of oligonucleotide **6** were identified on the basis of their UV spectra as trifunctional and tetrafunctional adducts of DNA (Figure 8e and Figure 8f, respectively).

Reductive Activation, Cytotoxicity, and Cross-Linking Efficiency. A hallmark of the mode of action of MC as an antitumor agent is its enhanced cytotoxicity to hypoxic tumors and hypoxic tumor cells in culture.[2] By analogy to MC, reductive activation is required for cytotoxicity of dimer **5** as judged from the large oxic/hypoxic differential of this property seen in EMT6 cells treated with dimer **5**.[40] In cell-free systems the dimers were substrates for the same purified reductases as MC (Supporting Information, Figure S1). While cytotoxicity and DNA cross-linking potency correlate among the three dimers, MC did not fit this correlation because MC exhibits higher growth inhibitory and cytotoxic activities than the dimers in the in vitro tumor cell panel of the NCI but exhibits relatively low cross-linking potential in the cell-free cross-link assays. Since MC is structurally different from the dimers, its cytotoxicity may be modulated differently by in vivo factors such as cellular uptake or reductive metabolism within the cell. In contrast, cross-linking potency, as measured in vitro, is a measure of intrinsic chemical reactivity of the drug, regardless of such factors, explaining the disparate results above.

Conclusions

We synthesized and studied the mode of action of three dimeric mitomycins **3**−**5**. These substances proved to be highly reactive DNA alkylating and cross-linking agents in vitro, although they were in general less cytotoxic than MC, as tested in tumor cell cultures. Similarly, lower cytotoxicities were reported in a study of other synthetic MC dimers.[13]

The covalent interaction of dimers **3**−**5** with DNA is analogous to that of MC. Reductive activation is required for their alkylation and cross-linking of DNA, and the activation is catalyzed by the same reductases and chemical reducing agents as those that activate MC. It is notable, however, that in contrast to MC, acidic pH also catalyzes cross-link formation by the dimers. In further analogy to MC, cytotoxicity of the dimers to tumor cells is enhanced under conditions of hypoxia.

The superior DNA cross-linking activity of the dimers relative to MC indicates operation of a mechanism inherent in DNA alkylation by dimeric agents in general: one *monofunctional* alkylation event by each reactive unit of a dimer constituting a DNA cross-link. The enhanced regioselectivity of cross-linking by dimer **5** relative to MC in the two G·C-rich sequences of *tyr*T DNA resembles the enhanced regioselectivity of bizelesin at A·T-rich regions of SV40 DNA relative to its monofunctional parent compound CC-1065.[37,38] Enhanced DNA regioselectivity as a general property of dimers has been proposed to have pharmacological significance.[39]

As another unique feature of the action of mitomycin dimers, we present evidence for tri- and tetrafunctional alkylation of DNA by dimer **5** in the cell-free system, resulting in novel types of DNA lesions. Closer structural investigation of these adducts is warranted, considering that they may represent a form of DNA damage resistant to DNA repair processes. Furthermore, as an extension of this study, potential activity of dimer **5** against drug-resistant tumor cells seems to be worth exploring.

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Supporting Information Available: Rates of reductive and acidic activation of dimers and MC and assay of dimer cross-linking of T-7 DNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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(1) Abbreviations: MC, mitomycin C.; MA, mitomycin A.; ICL, interstrand cross-link; SAR, structure−activity relationship; bp, base pair; LC−MS, liquid chromatography−mass spectroscopy; ESIMS, electrospray mass spectroscopy; TE buffer, 1 mM Tris, 1 mM EDTA (pH 7.4); TN buffer, 10 mM Tris, 10 mM NaCl; TBE buffer, 89 mM orthoboric acid, 2 mM EDTA; dG, deoxyguanosine.

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