Duplex Oligodeoxyribonucleotides Cross-Linked by Mitomycin C at a Single Site: Synthesis, Properties, and Cross-Link Reversibility†

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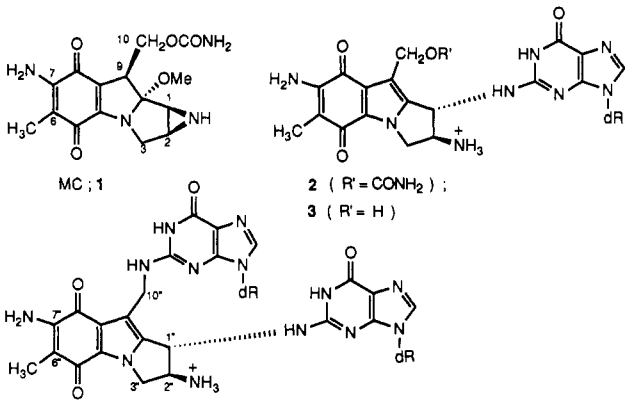
ABSTRACT: Oligodeoxyribonucleotides cross-linked by reductively activated mitomycin C (MC) were prepared and purified for the first time. The cross-linked products were structurally characterized by nucleoside and MC–nucleoside adduct analysis. Optimal conditions were established for the cross-linking reaction, resulting in high yields, typically in the 20–50% range. Nuclease digests of the cross-linked oligonucleotides yielded the same bifunctional MC–deoxyguanosine adduct as that previously isolated from DNA exposed to MC in vitro and in vivo [Tomasz et al. (1987) *Science 235*, 1204]. The cross-linked oligonucleotides displayed broad thermal melting profiles, greatly increased T_m, and complex circular dichroism spectra. Phosphodiester linkages at the cross-link were resistant to spleen exonuclease, nuclease P_1, and *Taq*I and *Cla*I restriction endonucleases; snake venom diesterase action was uninhibited. The cross-links are stable to heat at neutral pH but are removed by treatment in hot piperidine or by the reducing agents $Na_2S_2O_4$ and dithiothreitol. Mechanisms are proposed for these reactions. These studies define optimal methods for introducing mitomycin cross-links into DNA fragments at a specific site, providing a versatile tool to study the effects of the MC cross-links on DNA structure and function.

Mitomycin C (MC;[1] **1**; see Chart I), an antitumor antibiotic, has been widely used in clinical cancer chemotherapy. Its mode of action is intrinsically related to its ability to bind covalently to DNA both in monofunctional and bifunctional manners, resulting, in the latter case, in stable cross-links between the complementary strands of the genetic material (Szybalski & Iyer, 1967). Both processes require activation of the drug by reduction of its quinone system. This occurs in cells by the action of flavoreductases (Keyes et al., 1984) and can be readily mimicked in vitro in the presence of cell extracts, purified reductase, or chemical reducing agents (Iyer & Szybalski, 1964; Lown et al., 1976; Kaplan & Tomasz, 1982). We recently isolated the major covalent adducts formed between reductively activated MC and DNA in vitro as well as in vivo and determined their structures as shown (**2**–**4**) (Tomasz et al., 1986, 1987, 1988a). The bifunctional adduct **4** accounts fully for the DNA interstrand cross-links, which had been observed previously only indirectly on the basis of the altered macromolecular properties of MC-treated DNA (Szybalski & Iyer, 1967). The relative proportions of the three major adducts formed in DNA are modulated, at least in vitro, by the precise conditions of the reductive activation (Tomasz et al., 1987, 1988b).

Determination of the covalent structure of a DNA damage is fundamental but not sufficient to explain all effects of the damage on DNA function. Conformational distortions of DNA, alterations in its normal conformational dynamism, and DNA sequence specificity of the damage are additional important parameters in this respect. With regard to MC-induced conformational distortions, we proposed a snug, relatively nondistortive fit for both the monofunctional adduct (**2**) and the cross-link (**4**) in the minor groove of duplex B-DNA, based on physicochemical studies (Kaplan & Tomasz, 1982) and computer-assisted energy-minimized modeling (Tomasz et al., 1986, 1987). Similar models were proposed by Kollman's group for **2**, based on theoretical calculations (Rao et al., 1986). The stabilizing effect of bound MC on B-DNA is manifested by inhibition of the B–Z transition (Chawla et al., 1987) and spontaneous reversion from Z- to B-form, caused by the cross-linking (Chawla & Tomasz, 1988). With respect to base sequence specificity, we reported a striking bias for the CG·CG against GC·GC sequence as the site of an interstrand cross-link between the two guanines observed upon comparison of two synthetic oligonucleotide duplexes as substrates (Chawla et al., 1987). This has recently been confirmed by Crothers and co-workers (Teng et al., 1989).

The new availability of synthetic oligodeoxyribonucleotides enables one to study drug–DNA interactions at much higher structural and biological resolution than it was previously possible by using bulk DNA [for review, see Basu and Es-

Chart I[a]



[a] dR = 2′-deoxyribos-1′-yl.

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[1] Abbreviations: MC, mitomycin C; TEA, triethylammonium acetate; CD, circular dichroism; SVD, snake venom diesterase; DTT, dithiothreitol; M, mitomycin residue bound to the oligonucleotide structure.

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sigmann (1988)]. For this purpose, we describe the preparation of duplex oligonucleotides cross-linked by MC at a single site. These substances also enabled us to establish some basic chemical properties of the cross-link, such as its reversibility, as well as its action of blocking a number of nucleases.

Profound effects on nucleotide duplex stability are also described. The optimal methods of preparation of oligonucleotides cross-linked by MC site specifically, as well as a description of their analytical, chemical, and enzymological properties, should be widely applicable in diverse fields related to DNA damage.

Experimental Procedures

Materials

Oligonucleotides were synthesized by a DNA synthesizer, Model 380B, Applied Biosystems, Inc., using the phosphoramidite method. All reagents were purchased from Applied Biosystems, Inc., Foster City, CA. The crude products (1–10-μmol scale) were purified by HPLC, both at the "trityl-on" stage and after removal of the trityl group, according to the manufacturers' protocol (*Users Bulletin*, No. 13, Revised, April 1, 1987). The base composition of purified oligonucleotides was routinely checked by nucleotide analysis (as below).

Enzymes used and their sources were as follows: snake venom diesterase (*Crotalus adamanteus*; phosphodiesterase I), Cooper Biochemicals; *Escherichia coli* alkaline phosphatase (type III-R), Sigma; nuclease P_1, Pharmacia P-L Biochemicals; spleen exonuclease (phosphodiesterase II), Boehringer Mannheim; restriction endonucleases *Cla*I and *Taq*I, Sigma.

Mitomycin C ("bulk") was supplied by Bristol Laboratories, Syracuse, NY.

Methods

HPLC of oligonucleotides and modified oligonucleotides was performed by using reverse-phase columns (Beckman RPSC, C-3 Ultrapore, 4.6 × 75 mm for analytical and 10 × 250 mm for preparative purposes). HPLC for nucleoside and M–nucleoside adduct analysis was carried out by using reverse-phase columns of small pore size (Beckman, ODS Ultrasphere, 4.6 × 250 mm). For peak area quantitation a Beckman Model 427M integrator was used attached to a Model 265A absorbance detector (set to 254-nm wavelength), both as parts of a Model 338 HPLC system. In all cases a mixture of 0.1 M triethylammonium acetate buffer, pH 7.0, and acetonitrile was used as eluant, either isocratically or in a linear concentration gradient, as specified in each particular case.

Nucleoside and nucleoside–MC adduct analyses were carried out on a 0.02–2.00 OD_{260} scale by digestion of oligonucleotides with snake venom phosphodiesterase (6 μg) and *E. coli* alkaline phosphatase (6 μg) in 0.1 M Tris and 2 mM $MgCl_2$ buffer, pH = 8.2, at 45 °C for 4.5 h. The resulting nucleosides were analyzed by HPLC (above). Elution times of dC, dG, dT, and dA (3% acetonitrile, 97% 0.1 M TEA, pH 7.0) were 4.3, 8.7, 10.8, and 18.2 min, respectively. Elution times of **2**, **3**, and **4** (7% acetonitrile, 93% 0.03 M potassium phosphate, pH 5.5) were 15, 17, and 23 min, respectively. Peak areas were quantitated and used for calculating nucleoside molar ratios by dividing each peak area by E_{254} of the particular nucleoside (dC, 6300; dG, 13000; dT, 6600; dA, 13300).

Quantitative analysis of oligonucleotides and cross-linked oligonucleotide–MC complexes was based on absorbance measurements in 0.1 M Tris, pH 7.0 buffer. The molar extinction coefficients E_{260} of single-stranded oligonucleotides were calculated as equal to (number of purines)(14000) + (number of pyrimidines)(7000) (Zon et al., 1985).

Oligonucleotide concentration is expressed either in molarity of "strand" or in molarity of mononucleotide unit. The latter is equal to (molarity of strand) × (number of mononucleotide units per strand). Thus 1 μM d(TACGTA) (strand) is equal to 6 μM d(TACGTA) in mononucleotide units. In case the latter terminology is used, this is indicated clearly in the text.

For calculating the E_{260} value of cross-linked oligonucleotide duplexes, the E_{260} of bound MC was assumed to be 12000 (Tomasz et al., 1974) and added to E_{260}(strand 1) + E_{260} (strand 2). This formula assumes negligible hypochromicity associated with the cross-linked duplex; this is justified by the low hyperchromicity on melting, actually observed in the present work (see Figure 6).

Preparation of Cross-Linked Oligonucleotide–MC Complexes. Oligonucleotides (either self-complementary or a 1:1 molar mixture of complementary strands) were mixed with MC in 0.1 M Tris, pH 7.4, at 2 μmol/mL mononucleotide and 10 μmol/mL MC concentration, and the solution was dearated by bubbling helium gas. A dearated solution of freshly made 0.4 M $Na_2S_2O_4$ in the same buffer (1.5 mol/mol of MC) was added to the reaction mixture in five equal increments at 10-min intervals, under helium bubbling. The reaction was terminated at 60 min by exposure to air. The mixture was diluted 3-fold with warm 0.02 M NH_4HCO_3 to ensure homogeneity of the solution, followed by chromatography over a column of either Sephadex G-25 (superfine) (in the case of hexanucleotide **5**) or Sephadex G-50 (DNA grade) gel (in the case of 10-mers and higher oligomers) with 0.02 M NH_4HCO_3 as eluant. Columns of 5 × 56 cm size could be loaded with reaction mixtures containing up to 10–15 μmol of mononucleotide units. The cross-linked oligonucleotide–MC complex was eluted before the parent oligonucleotides. The fractions containing the former were pooled and lyophilized and then purified further by HPLC if necessary. The yield of the cross-linked oligonucleotide was calculated by the formula:

$$\%\ \text{yield of cross-linked oligomer} = \frac{(2)(\text{mol of cross-linked oligomer obtained})(100)}{\text{mol of parent oligomer at the start}}$$

Thermal melting transitions of parent and cross-linked duplex oligonucleotides were determined by using a Gilford 240 spectrophotometer, equipped with a heating or cooling circulation bath, automatic cuvette changer, a recorder, and a thermosensor. N_2 gas was passed through the sample compartment below ambient temperature to prevent condensation on the cuvettes. Circular dichroism spectra and circular dichroism melting curves were determined on a Jobain-Yvon Auto-Dichrograph Mark V instrument. Cooling and heating the sample compartment were accomplished via a circulating bath, and the temperature of the sample was monitored by a thermocouple immersed in the cuvette.

Results

Synthesis and Purification of Cross-Linked Oligonucleotides. In the presence of MC and $Na_2S_2O_4$ at 5 °C the self-complementary or complementary oligonucleotides (as a 1:1 molar mixture in the latter case) reacted with MC to give a new band upon Sephadex G-50 chromatography, eluting at the void volume, earlier than the parent strands, indicating a product with increased molecular weight. This is illustrated in the case of **6** in Figure 1. For the separation of the shortest cross-linked duplex **5**, Sephadex G-25 instead of G-50 proved to be useful. Ultimate purification from parent material was

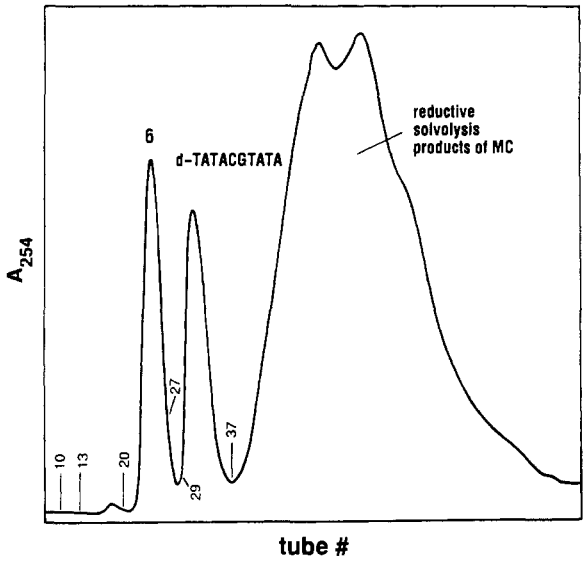
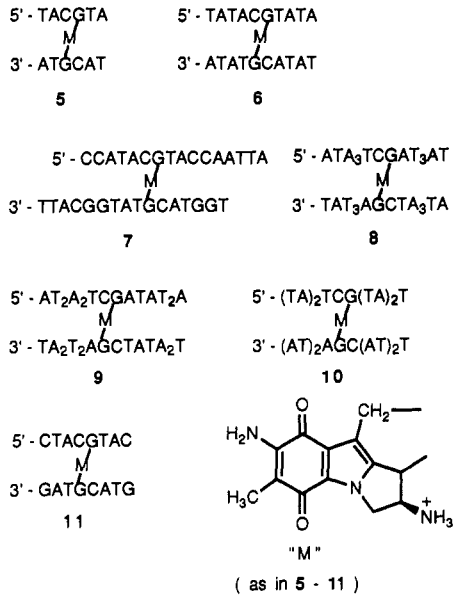


FIGURE 1: Sephadex G-50 chromatography of the crude reaction mixture in the preparation of **6**. Elution volumes: **6**, 580 mL; parent oligomer, 725 mL.

Chart II



accomplished by HPLC (Figure 2). No other oligonucleotide–MC product was usually discernible in the HPLC pattern, even when the Sephadex step was omitted. The parent and cross-linked oligomers could be also separated on denaturing 20% polyacrylamide gels, detected by ultraviolet shadowing (data not shown). The following cross-linked oligonucleotides (see Chart II) were prepared (yields of HPLC-pure products are given in parenthesis): **5** (15%); **6** (50%; see Figure 1); **7** (50%); **8** (16%); **9** (14%); **10** (31%). These yields were highly reproducible, regardless of whether the reactions were conducted on small or large scale (0.1-μmol to several-micromole range).

Proof of Structure of the Cross-Linked Oligonucleotides. The ultraviolet spectra clearly indicated the presence of the mitosene chromophore (Webb et al., 1962): shoulder at 355 nm and maximum at 312 nm (Figure 3). Nucleoside and nucleoside–MC adduct analysis by HPLC indicated in all cases (except in **7** and **11**) lack of dG and presence of the cross-link adduct **4**. This is shown for the prototype case **5** in Figure 4. Since only one guanine was originally present in a strand of parent oligonucleotide, formation of the bis adduct **4** indicates that *two* such strands were joined by the mitomycin moiety.

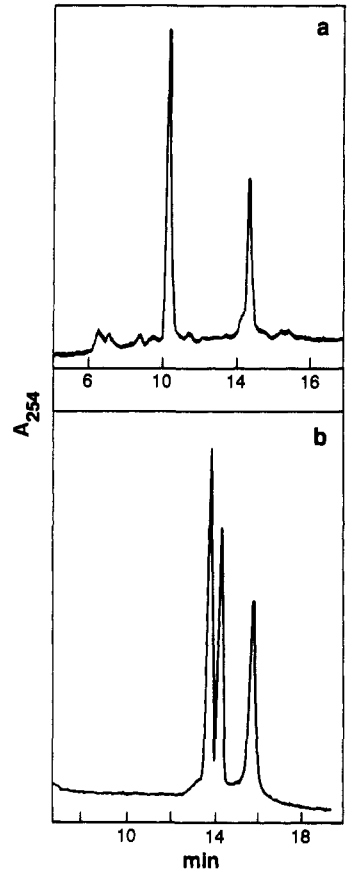


FIGURE 2: HPLC of parent and cross-linked oligonucleotides. (a) d(TACGTA) (10.2 min) and **5** (14.3 min). Gradient, 5.7–13.8% acetonitrile in 0.1 M TEA, pH 7.0, in 36 min; flow rate, 1.0 mL/min. (b) Complementary oligonucleotides d[$(TA)_2TCG(TA)_2T$] (13.4 min), d[$(AT)_2ACG(AT)_2A$] (14 min), and their cross-linked duplex (15.8 min). Gradient, 6–18% acetonitrile in 0.1 M TEA, pH 7.0, in 24 min; flow rate, 1.0 mL/min.

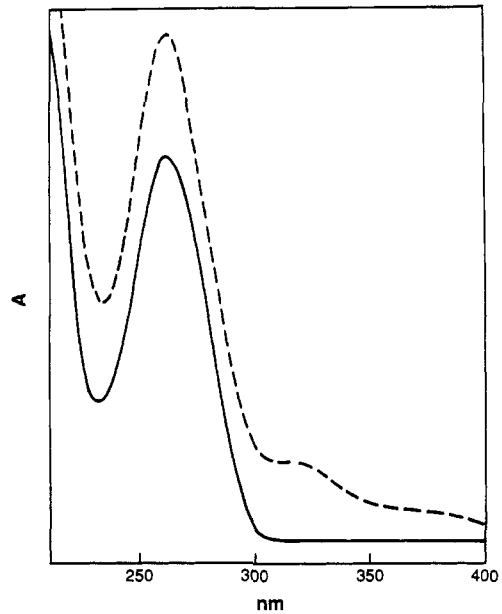


FIGURE 3: Ultraviolet spectra of TACGTA (—) and its cross-linked duplex **5** (- - -), in 0.02 M NH_4HCO_3.

Circular Dichroism Spectra (Figure 5). These were determined in the case of three cross-linked oligonucleotides, each having the same central hexanucleotide sequence around the cross-link. The spectra are drastically different from the

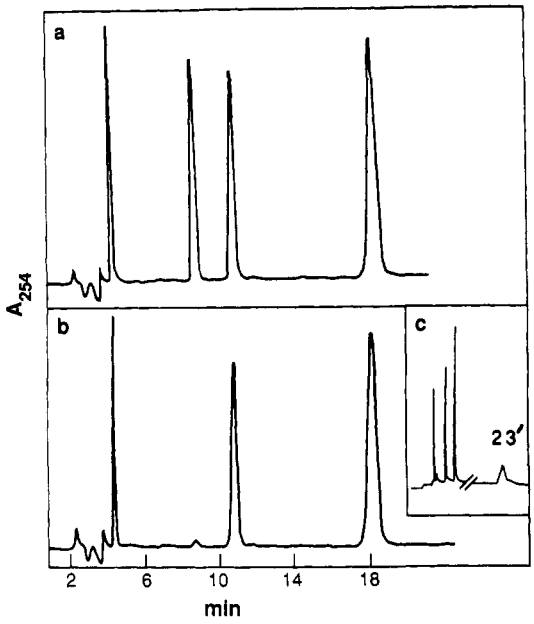


FIGURE 4: Nucleoside and nucleoside–MC adduct analysis of the cross-linked oligonucleotide duplex **5** by HPLC. (a and b) Nucleoside analysis of the parent TACGTA and **5**, respectively. Respective nucleoside ratios were dC:dG:dT:dA = 1.0:0.92:2.0:1.90 and 1.0:0.02:2.0:1.88 for parent and **5**, respectively. (c) Nucleoside–MC adduct analysis of **5**. The peak at 23 min was identical with that of authentic standard **4**.

conserved type CD spectra of the parent oligomers. A common feature of the three cross-linked products is a negative band at 290–295 nm. Complex changes in the spectra occur on heating, in contrast to that of the parent duplex, which shows only a diminution of intensity (Figure 5a).

Thermal Melting Curves (Figure 6). As measured by the increase of absorbance, the melting curves of singly cross-linked duplexes are characteristically broad and noncooperative and show much lower hyperchromism than the parent duplex. The T_m, however, is generally much higher in the cross-linked structures.

Nuclease Susceptibility of Cross-Linked Oligonucleotides. (1) Snake Venom Diesterase. All cross-linked oligonucleotides were quantitatively digested to nucleosides by snake venom diesterase, as shown by nucleoside and nucleoside–MC adduct analysis of the digests which also contained alkaline phosphatase to remove phosphate groups from the resulting mononucleotides (Figure 4). In fact, snake venom diesterase/alkaline phosphatase digestion served as a method for characterization of the cross-linked oligonucleotides; see above.

(2) Nuclease P_1. In contrast, this enzyme, which acts as both exo- and endonuclease, to give 5′-monophosphates as end products, cannot cleave the GpN linkage when G is cross-linked by MC to another G. This is shown in the case of **5**, for example: Digestion of **5** with nuclease P_1 and alkaline phosphatase yielded dC, dT, and dA, in 1:1:2 molar ratio instead of the theoretical 1:2:2 (HPLC data not shown); an unknown MC adduct fragment was also formed (peak at 16.8 min in Figure 7a). The latter fraction was collected and further digested by SVD/alkaline phosphatase, to yield cross-link adduct **4** and dT as the sole products (Figure 7b and insert in Figure 7a, respectively). This proved the structure of the "unknown fragment" as shown in Figure 7a. In contrast, when the digestion of **5** included SVD in addition to nuclease P_1 and alkaline phosphatase, the ratio of dC, dT, and dA was 1:2:2, indicating full digestion of the GpT linkage by the SVD (HPLC patterns were similar to that in Figure 4). These results are summarized in Scheme I.

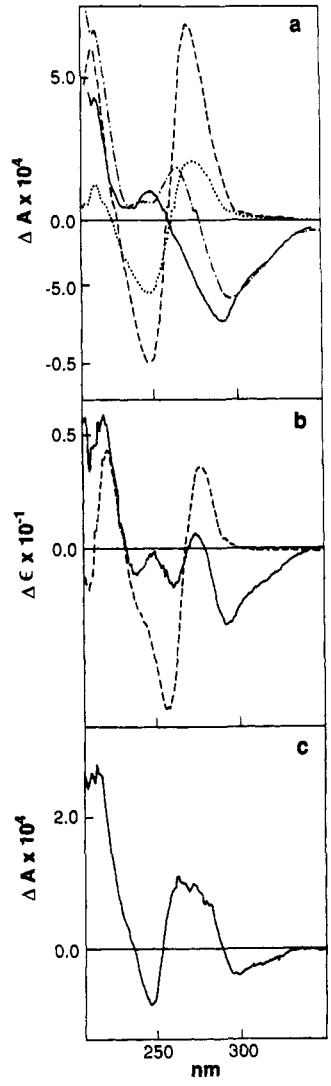
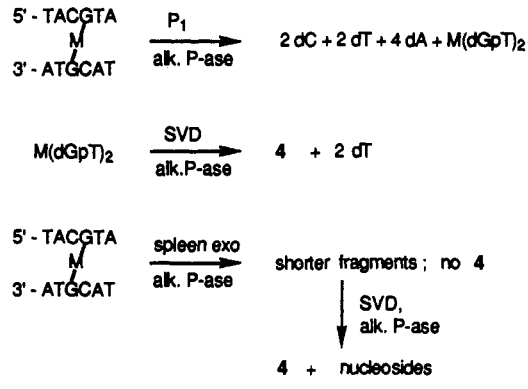


FIGURE 5: Circular dichroism spectra of parent and cross-linked oligonucleotides. Buffer: 1 M NaCl, 10 mM sodium phosphate, pH 7.2, and 0.1 mM EDTA. (a) **5** at 2 °C (—), **5** at 80 °C (- - -), d(TACGTA) at 2 °C (- - -), and d(TACGTA) at 45 °C (···), respectively. (b) **11** (—) and its parent duplex (- - -) at 2 °C. (c) **6** at 2 °C.

Scheme I



(3) Spleen Exonuclease. In the case of **5** a partial block at the CpG and total block at GpT phosphate linkages was observed. For example, digestion of **5** with spleen exonuclease followed by alkaline phosphatase gave dC, dI, and dT in 1:2:2 molar ratio (dI is from dA, by deamination by contaminant enzyme activity in spleen exonuclease). Nucleoside–MC adduct analysis indicated no **4** but, rather, several MC-containing oligomer fragments (Figure 7c). These fragments were collected and combined from the HPLC; further digestion by

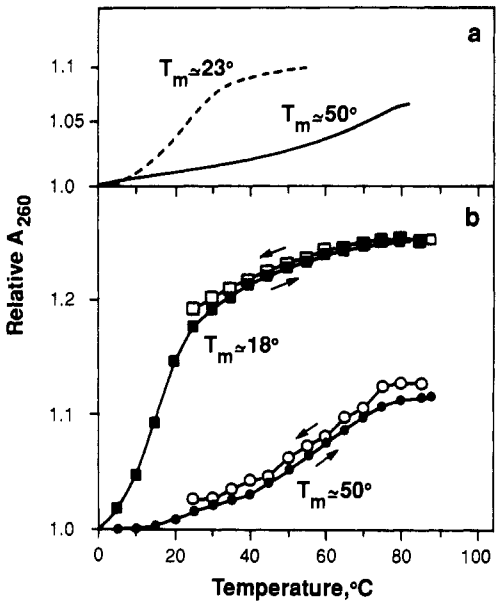


FIGURE 6: Thermal melting curves of parent and cross-linked oligonucleotides. (a) **5** (—) and its parent duplex (---); buffer, 1 M NaCl, 10 mM sodium phosphate, pH 7.2, and 0.1 mM EDTA. (b) **6** (○, ●) and its parent duplex (■, □); buffer, 0.1 M NaCl, 10 mM sodium phosphate, pH 7.2, and 0.1 mM EDTA.

SVD/alkaline phosphatase yielded **4** as the sole adduct (Figure 7d) as well as dC, dT, and dI (pattern not shown). The results are summarized in Scheme I.

Cleavage by *Taq*I or *Cla*I restriction endonuclease was completely blocked by a MC cross-link at the recognition site, as shown as follows: $[d(ATA_3TCGAT_3AT)]_2$ and its cross-linked derivative **8** were incubated with *Taq*I or *Cla*I and analyzed by HPLC at various times. The resulting patterns show that the parent oligomer was completely cleaved in 3 h into two fragments (9.9- and 10.2-min elution times) (Figure 8a–c), but the cross-linked oligomer was resistant to cleavage (Figure 8d): no diminution of intensity of the 17-min peak of **8** nor any cleavage product was observable under the same conditions even after longer digestion times.

Reversibility of the Cross-Link. (a) Hot piperidine (1 M, 90 °C) removed the cross-link from **5** partially in 2 h, and completely in 18 h, as shown by HPLC analysis (Figure 9). The intact, parent oligonucleotide was the single oligomeric product formed. The cross-link adduct **4** itself was also degraded under the same conditions, to deoxyguanosine (data not shown). The mitosene component(s) of the degradation was (were) not identified. (b) Reductive conditions were as follows: Incubation of 0.4 A_{260} unit (2 nmol) of **10** with 8 mol of $Na_2S_2O_4$ under anaerobic conditions (Methods) in 0.1 mL of 0.1 M Tris, pH 7.4 buffer at room temperature resulted in gradual loss of the cross-link as seen by HPLC analysis at various times. In this case a transient intermediate, eluting between the parent of **10** and **10** was observed. Its digestion yielded an unknown adduct (data not shown). DTT (10%) instead of $Na_2S_2O_4$ was also effective in reversing the cross-link, but slower. The kinetic course of the removal of the cross-links is plotted from the HPLC data; the half-lives in the presence of $Na_2S_2O_4$ and DTT are approximately 4.1 and 10 h, respectively (Figure 10). (c) Heating at 100 °C in 0.1 M Tris, pH 7.4, for 30 min caused no decomposition of **5** (HPLC analysis; data not shown).

Discussion

Although the cross-linked nature of MC-exposed DNA was assumed to be caused by the MC–deoxyguanosine bis adduct

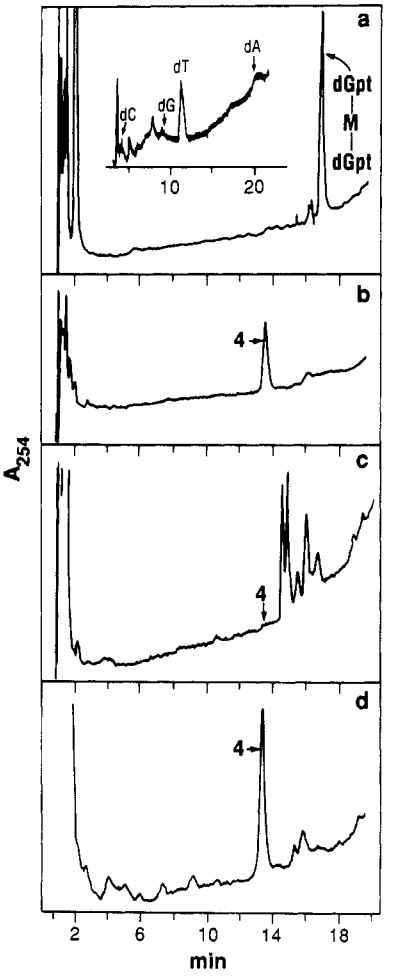


FIGURE 7: HPLC patterns of cross-linked oligonucleotides digested by nuclease P_1/alkaline phosphatase of spleen exonuclease/alkaline phosphatase. (a) Cross-linked oligonucleotide **5** (3 A_{260} units/mL) in dilute acetic acid (pH 5.5) was incubated with nuclease P_1 (0.5 unit/A_{260} unit) at 55 °C for 2 h. The pH was brought to 8.2 by addition of 0.5 M Tris, pH 8.2. Alkaline phosphatase (1.6 units/A_{260} unit) was added and incubation continued at 37 °C for 2 h. The digest was directly applied to HPLC. Column, 4.6 × 75 mm, C-3 reverse phase; solvent, 3–12% acetonitrile in 0.1 M TEA, pH 7.0, in 18 min; flow rate, 1 mL/min. (b) The 16.8-min peak $M(dGpT)_2$ from (a) was digested by SVD/alkaline phosphatase as under Methods, and then the digest was directly applied to HPLC. Column and solvent were the same as in (a). Insert in (a): Same as (b) except column was C-18 reverse phase; solvent was 3% acetonitrile and 97% 0.1 M TEA, pH 7.0. (c) Cross-linked oligonucleotide **5** in 0.015 M Tris, pH 6.5 (1–3 A_{260} units/mL) was incubated with spleen exonuclease (0.5 unit/A_{260} unit) for 2 h at 37 °C. The pH was changed to 8.2 by addition of 0.5 M Tris, pH 8.2, and alkaline phosphatase (1.6 units/A_{260} unit) was added; then incubation continued for 2 h. The digest was directly applied to HPLC. Column and solvent were the same as in (a). (d) The 14–17-min peaks from (c) were pooled and digested by SVD/alkaline phosphatase, as under Methods, and then the digest was directly applied to HPLC. Column and solvent were the same as in (a).

4, isolated from such DNA (Tomasz et al., 1987), the present work demonstrates rigorously by direct chemical synthesis and product analysis that **4** indeed constitutes the cross-linked site, since the two guanines linked to the mitomycin must originate from two individual strands of our oligonucleotides. The results also show that the cross-link is directly responsible for the typically altered behavior of DNA usually associated with the action of cross-linking agents (Fujiwara, 1983): (i) increased molecular weight, as seen here by gel filtration, HPLC, and gel electrophoresis of the oligonucleotides, and (ii) characteristically altered melting and renaturation behavior as seen in Figure 6.

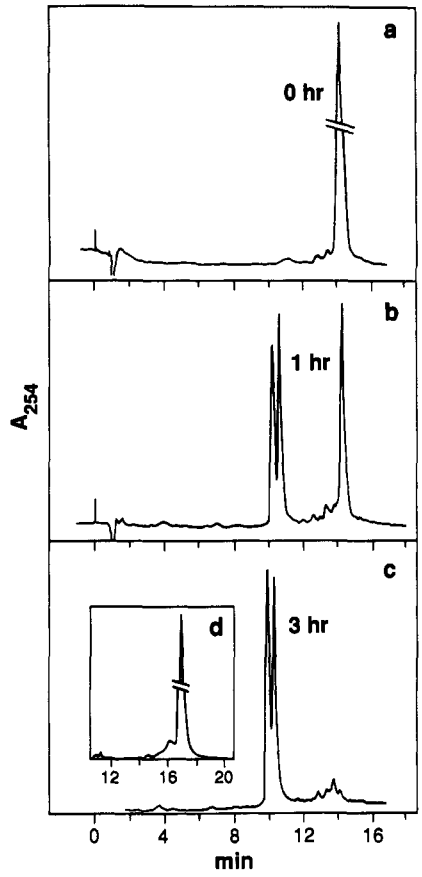


FIGURE 8: HPLC assay for cleavage of **8** and its parent duplex by *ClaI* restriction endonuclease. Cleavage conditions: 0.157 A_{260} unit (approximately 5 μg) of substrate was digested with *ClaI* (Pharmacia LKB), according to the manufacturer's protocol, at 37 °C. Before the onset of the digestion at 37 °C, the mixture was cooled to 16 °C for 20 min, to promote duplex formation. The cooling procedure was repeated after each hour of 37 °C incubation. Cleavage was tested by direct injection of digest portions into HPLC. Column, 4.6 × 75 mm, C-3 reverse phase; solvent, 6–20% acetonitrile gradient in 0.1 M TEA, in 28 min; flow rate, 1.0 mL/min. (a–c) Parent duplex as substrate; (d) **8** as substrate, after prolonged digestion.

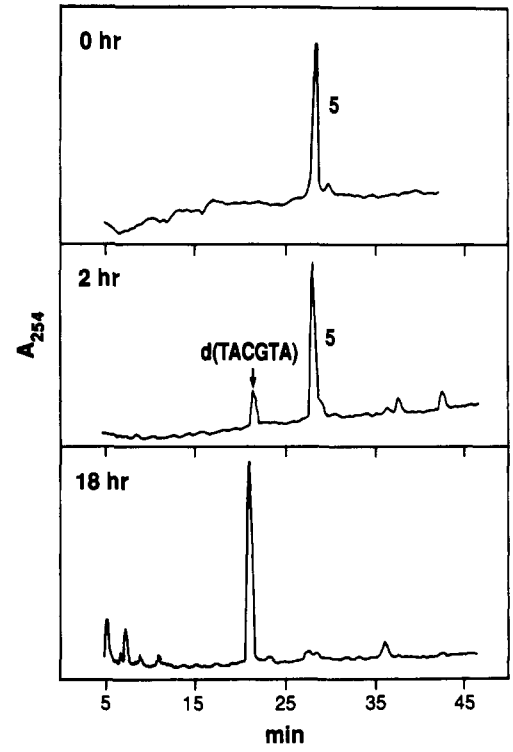


FIGURE 9: HPLC assay for reversal of the cross-link by hot piperidine. Column, 10 × 150 mm, C-3 (reverse phase); solvent, 5–11.2% acetonitrile in 0.1 M TEA, pH 7.0, in 50 min; flow rate, 5 mL/min.

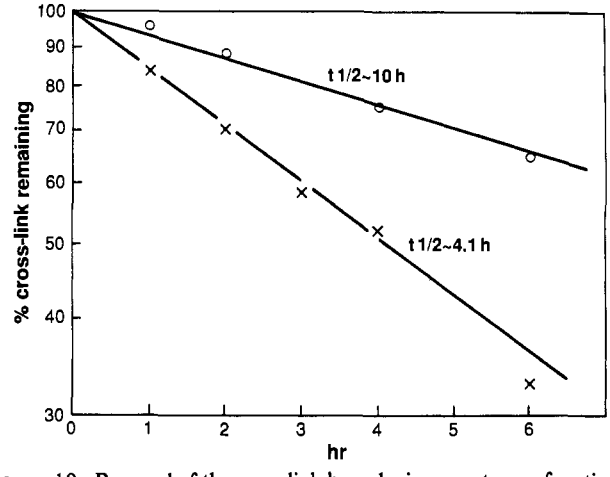


FIGURE 10: Reversal of the cross-link by reducing agents as a function of time. (×) $Na_2S_2O_4$; (○) DTT.

Our method of preparation of the cross-linked oligonucleotides, resulting in high yields (up to 50%) on either small or preparative scale, in the HPLC-pure state, should prove of great general use for obtaining site-specifically cross-linked DNA fragments for structural, biochemical, and biological studies. Since MC is used in clinical therapy of cancer, such studies, relating to its mode of action, are of great interest, especially in the field of antitumor drug design. With respect to the yields, it should be noted that cross-link formation absolutely requires the CG·CG sequence (Chawla et al., 1987; Teng et al., 1989; Weidner et al., 1989; Borowy-Borowski et al., 1990). Additional structural features of the oligonucleotide, such as its length, also play a role: the yield of **5** is only 15–20%, compared to a 50% yield of **6**, which differs by having four additional, nonreactive nucleotides in each strand. This may be explained by lower duplex stability of the parent oligomer of **5** under the cross-linking reaction conditions. Base sequence also influences the yield (Borowy-Borowski et al., 1990). Considerably lower yields of cross-linked oligonucleotides were reported by Teng et al. (1989), using a MC activation procedure very similar to the present one. For example, $[d(ATATATCGATATAT)]_2$ and $[d(AAAATCGATTT)]_2$ gave 1.6 and 2.5% yields, respectively, compared to our approximately 10-fold higher yields of the same products, using the neutral pH activating conditions. The reason for the large difference between the results from the two laboratories is not known.

The broad, noncooperative thermal melting profiles of two cross-linked oligonucleotides (Figure 6) indicate very little hyperchromicity at room temperature over that at 0 °C; thus it appears that the cross-link stabilizes the duplex structure. They are strikingly similar to melting curves of oligonucleotides cross-linked by psoralen at their central position (Shi & Hearst, 1986). These broad curves, with low hyperchromicity, reflect closely the broadened melting profiles, increased T_m, and lower hyperchromicity previously observed with DNA and poly(dG-dC)·poly(dG-dC) upon modification by activated MC (Cohen & Crothers, 1970; Tomasz et al., 1974; Chawla et al., 1987).

The circular dichroism spectra (Figure 5) of the cross-linked oligonucleotides are vastly altered as compared to that of the parent duplexes, with a qualitatively similar feature: each has a broad negative band between 290 and 295 nm. The thermal stability of the cross-linked oligonucleotides, noted above, is also reflected in the lack of change in the CD at temperatures

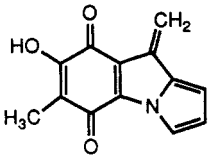
ranging from 2 to 25 °C (not shown). The 290–295-nm negative bands have also been observed with MC-modified DNA and poly(dG-dC) and were attributed to CD of the chiral covalent adduct **4** superimposed on the normal CD of B-DNA (Chawla et al., 1987); the present findings are consistent with this notion.

Nuclease Susceptibility. The resistance of **8** and **9** to cleavage by the restriction endonucleases *Taq*I and *Cla*I may not be too surprising since the modification is right at the restriction site as marked by the arrows. Since the factors of *Taq*I or *Cla*I/DNA recognition are not known and the effect of an alkyl group in the minor groove (in this case, the N^2 position of guanine) on restriction enzyme cleavage has not been tested to our knowledge, the resistance was not entirely predictable, especially since MC is presumed to be relatively nondistortive to DNA structure (Tomasz et al., 1987; Chawla et al., 1987).

Nuclease P_1 action is completely blocked at the 3′-phosphodiester of both guanines of the cross-link; the end products are cross-linked dinucleotides $[d(GpN)]_2M$ (Figure 7; Scheme I). The same block was observed previously at monofunctional mitomycin adducts in DNA, resulting in modified dinucleotides [d(GpN)]M (Tomasz et al., 1986). MC appears to be unique among covalent DNA modifiers in inhibiting nuclease P_1, since this enzyme has been used routinely for digestion of DNA adducted with various carcinogens. Such application of P_1 to MC-treated DNA should be avoided in light of these findings.

The observed inhibition of the action of spleen exonuclease by the cross-link is somewhat different from that of nuclease P_1, in that it extends beyond the 3′-phosphodiester of the cross-linked guanine: several partially digested fragments were isolated (Figure 7c), which were not converted further unless SVD was added (Figure 7d). Again, this enzyme is clearly not suitable for MC–mononucleotide adduct isolation although it has been routinely used in covalent carcinogen–DNA adduct studies according to the ^{32}P-postlabeling methodology of Randerath et al. (1981). The only reliable exonuclease for digesting through MC-modified sites, according to our present results, is snake venom diesterase. This should be kept in mind if one intends to apply the ^{32}P-postlabeling procedure for analysis of adducts of MC formed in vivo, and previous reports (Reddy & Randerath, 1987; Kato et al., 1988) should be reevaluated in the light of these findings.

Stability of the Mitomycin Cross-Link. The cross-links are stable to heat in neutral buffer. In hot aqueous 1 M piperidine, however, both the C-1″ and C-10″ mitosene–guanine linkages are eliminated slowly, but cleanly, to regenerate the parent oligonucleotides. No intermediate was detected in the reaction. No attempt was made to characterize the degraded mitosenes; it was noted, however, that the purple mitosene color changed to intense yellow during the reaction, suggesting that the yellow "enepyrrole" (**12**) or a similar derivative shown to be formed



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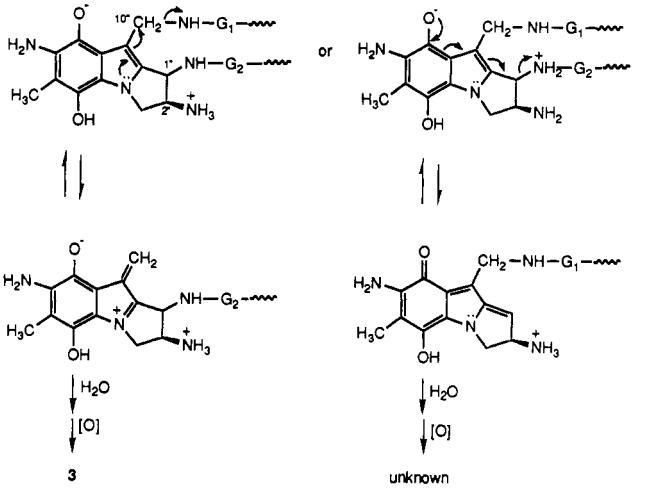
from reduced mitosenes in aqueous pyridine (Egbertson & Danishefsky, 1987) may be the eliminated species. The lack of damage to the attached nucleosides in this treatment was confirmed by high recovery of deoxyguanosine when **4** was treated with hot piperidine. This method of removal of the cross-link will be useful in DNA sequence specificity methodologies applied to MC, since gel electrophoretic analysis of DNA fragments requires prior removal of bound drug.

Scheme II



The dissociation of the MC cross-link from oligonucleotides by the reducing agents $Na_2S_2O_4$ and DTT is not entirely unexpected, since the reversibility of nucleophilic addition of H_2O to the C-1″ position of the active (quinone methide) form of reduced MC was noted by Peterson and Fisher (1986) in their studies of MC activation. The reversibility of attack on the C-10″ position is observed here for the first time. For complete removal of the cross-link, both events have to occur, in sequence. Scheme II indicates mechanisms for each of the two alternative first events. The actual sequence of events is not known, since the transient intermediate detectable in the HPLC of the reaction mixtures was not characterized. Preliminary results indicate that it contains an unknown adduct, rather than **3**, suggesting that the C-1″ bond breaks first. It should be noted that reversibility of the MC cross-link is not significant at the low temperature of its synthesis (0–5 °C) used in the present work. It may operate, however, in vivo, in a reductive cellular environment.

Acknowledgments

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Recognition between Mitomycin C and Specific DNA Sequences for Cross-Link Formation†

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ABSTRACT: An extensive series of oligodeoxyribonucleotides was reacted with reductively activated mitomycin C (MC), and the resulting cross-linked drug–oligonucleotide complexes were isolated by reverse-phase HPLC and characterized by nucleoside and MC–nucleoside adduct analysis. HPLC also served for assay of the yield of cross-linked oligonucleotides. AT-rich duplex oligonucleotides, containing a single central CG·CG, gave high yields of cross-links between the two guanines while those having GC·GC, instead, gave none. In another series, the central sequences CGC·GCG and CGC·ICG both yielded 50% cross-link while CGC·GCI was completely resistant. Cross-linking was conducted also in two steps: Oligonucleotides substituted monofunctionally by MC at guanine at either a CG or GC sequence were annealed with their complementary strands followed by reductive reactivation of the bound MC to form a cross-link. The CG oligomers were cross-linked quantitatively while the GC ones were again resistant. These results show unambiguously that the MC cross-link is absolutely specific to the CG·CG duplex sequence, confirming our previous finding [Chawla, A. K., Lipman, R., & Tomasz, M. (1987) in *Structure and Expression, Volume 2: DNA and Its Drug Complexes* (Sarma, R. H., & Sarma, M. H., Eds.) Adenine Press, Guilderland, NY]. Evidence is presented that this specificity is due to the specific orientation of the monofunctionally attached MC in the minor groove. Superimposed on the CG·CG requirement, a four-base-pair sequence preference was observed at PuCGPyr·PuCGPyr sequences. This suggests that the guanine N^2 atom of GpPyr is more reactive toward the drug than that of GpPu, due to the favorable effect of the negative dipole of the O^2 of the Pyr on the reaction; in accordance, GpT was more reactive than GpC. The CGCG·CGCG sequence exhibited a 2-fold rate of cross-linking; only one cross-link was formed per such sequence, however. $(CG)_n \cdot (CG)_n$ tracts probably represent "hot spots" for MC cross-links in DNA.

The antitumor antibiotic mitomycin C (MC;[1] **1**) reacts covalently with DNA in a remarkably specific manner: both of its alkylating functions, namely, the aziridine at C-1 and the carbamate at C-10, react exclusively with N^2-positions of guanines. This was demonstrated recently in our and Nakanishi's laboratories, in collaboration, primarily by isolation and characterization of mono adducts **2** and **3** and bis adduct **4** from DNA exposed to MC (Scheme I; Tomasz et al., 1986a, 1987, 1988a). These reactions occur only upon reductive

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[1] Abbreviations: MC, mitomycin C; TEA, triethylammonium acetate; UV, ultraviolet.

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