Orientation Isomers of the Mitomycin C Interstrand Cross-Link in Non-Self-Complementary DNA. Differential Effect of the Two Isomers on Restriction Endonuclease Cleavage at a Nearby Site†

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ABSTRACT: Reductively activated mitomycin C (MC) forms DNA interstrand cross-links between two guanines at CG·CG sequences. It is predictable that such cross-links should occur in two isomeric strand orientations in duplex DNA (except when located in the center of a self-complementary duplex). This was verified by the isolation and characterization of a pair of two isomeric oligonucleotides in each case of five non-self-complementary duplexes of 8-bp length, cross-linked by MC. Isomer separation was accomplished by reverse-phase HPLC. The isomers in a pair were formed in approximately 1:1 proportion. Their structures were rigorously characterized by a two-step cross-linking procedure: first, 1″-monoalkylation of each strand, followed by conversion to a cross-linked duplex by annealing the monoalkylated strand to its complement in the presence of a reducing agent. The resulting individual authentic orientation isomers were used as standards for identification of the two isomers formed in the original (one-step) cross-linking reactions. A 16-bp duplex oligonucleotide was synthesized featuring the *Alu*I cognate sequence, separated from a MC cross-link site by only 1 bp. Its two MC cross-linked isomers were prepared separately, and their rate of cleavage by *Alu*I was determined using HPLC. Cleavage of both the unmodified and cross-linked duplexes was nonsymmetrical. The isomer in which the 2″-NH_3^+ of MC is oriented toward the *Alu*I site was cleaved essentially at the same rate as the control duplex, while cleavage of the isomer with the MC indoloquinone group oriented toward the *Alu*I site was inhibited 2-fold at the faster-cleaved strand. This difference demonstrates that the two orientations of the MC cross-link can exert differential effects on protein–DNA interactions. The modest inhibition of *Alu*I cleavage relative to that by CC-1065 [Hurley, L. H., Needham-VanDevanter, D. R., & Lee, C. (1987) *Proc. Natl. Acad. Aci. U.S.A. 84*, 6412–6416] may indicate relatively low distortion of DNA by the MC cross-link. This notion is supported by a comparison with psoralen cross-links.

Mitomycin C (MC;[1] **1**) is an antitumor antibiotic, widely used in clinical cancer chemotherapy. Its mode of action is inherently related to its direct DNA-damaging activity, manifested in covalent binding and formation of interstrand cross-links in DNA in vivo and in vitro (Szybalski & Iyer, 1964). The chemistry of these covalent DNA lesions has been recently elucidated by isolation and structural determination of MC–DNA adducts formed in cell-free systems. Two monoadducts [**2a** and **2b**; Tomasz et al. (1986, 1988)] and two bisadducts [**3** and **4**; Tomasz et al. (1987) and Bizanek et al. (1992)] were identified as the major lesions (Chart I). Bisadducts **3** and **4** represent *interstrand* and *intrastrand* cross-links in duplex DNA, respectively. The same adducts have been shown to be formed in intact cells (Tomasz et al., 1987; Bizanek et al., 1992).

The structure of a duplex oligonucleotide cross-linked by MC was elucidated by a combined 2D-NMR–molecular mechanics computational study (Norman et al., 1990). The mitomycin residue cross-linking the two guanines in the self-complementary duplex hexanucleotide d(TACGTA)·d(TACGTA) was shown to fit inside the minor groove of a somewhat distorted B-DNA, spanning across the four central base pairs. The MC cross-link site is always the two guanines in the CG·CG sequence; GC·GC sites are not cross-linkable (Tomasz et al., 1987; Teng et al., 1989; Weidner et al., 1989; Borowy-Borowski et al., 1990).

Considering the nonsymmetrical nature of the cross-linking mitosene moiety **5**, it is apparent that cross-links at CG·CG should occur in two isomeric strand orientations in duplex DNA. Self-complementary duplex oligonucleotides having the CG·CG site at the center are exceptions, due to the symmetry of the entire cross-linked structure. In all other cases, two distinct cross-linked oligonucleotides may be formed with mitomycin, as shown in Chart II (**6**, **7**). As the sole precedent, this type of DNA cross-link isomerism has been recognized recently in the case of the psoralens (Shi & Hearst, 1986; Van Houten et al., 1987; Yeung et al., 1988; Haran & Crothers, 1988). Most of the other, well-studied DNA-cross-linking agents generate structurally more simple, symmetrical cross-links. Psoralen cross-linked isomers (Chart II) have been separated by gel electrophoresis by Yeung and co-workers, who demonstrated that the isomers represent *two different lesions* of DNA, in terms of both recognition and cleavage by the UvrABC repair endonuclease complex and their effects on the conformation and stability of duplex DNA (Yeung et al., 1988; Jones & Yeung, 1990; Kumaresan et al., 1992).

Similarly, we demonstrate here for the first time that the cross-linking reaction of MC with duplex oligonucleotides results in the mixture of two cross-link orientation isomers (**6**, **7**). A method is also described for generating one or the other

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[1] Abbreviations: MC, mitomycin C; M, mitosene (see definition of mitosene in footnote 2); UV, ultraviolet; TEAA, triethylammonium acetate.

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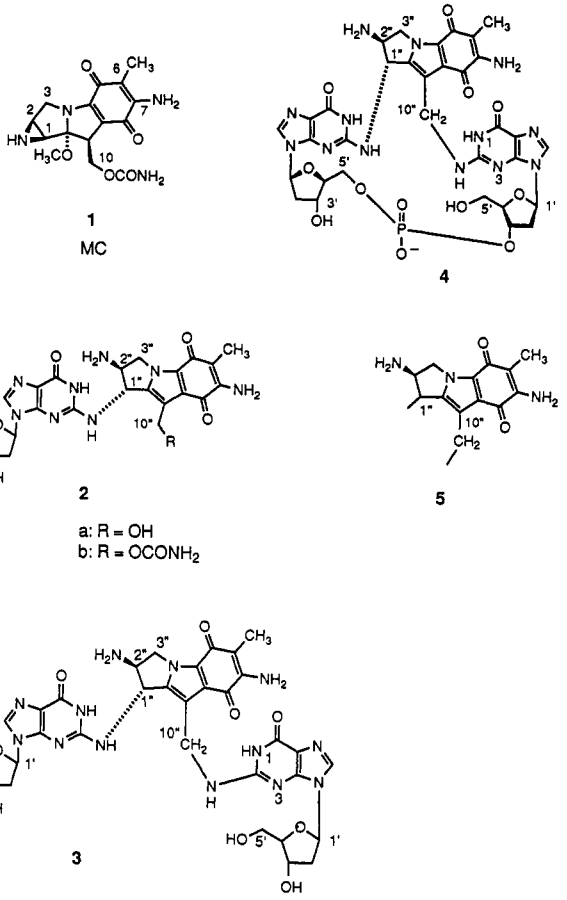
Chart I



isomer selectively. The two isomers, as in the psoralen case, display different conformational properties and, most interestingly, differential susceptibility to cleavage by *Alu*I restriction endonuclease.

This type of isomerism, inherent in site-specific cross-linking by certain nonsymmetrical DNA cross-linking agents, has been scarcely recognized in the past. It can be a very important factor, however, in determining the effects of site-specific cross-links on DNA, for example, effects on protein–DNA binding or DNA bending. Inadvertent use of a *mixture* of isomers could lead to errors in interpretation of observed results.

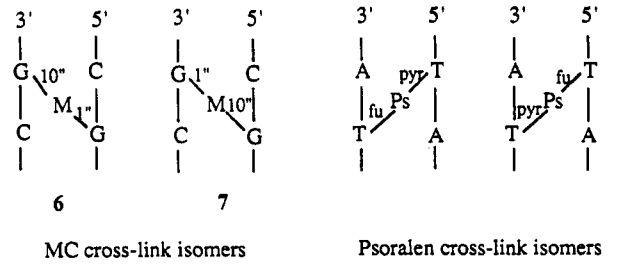
MATERIALS AND METHODS

Oligonucleotides were synthesized on an automated DNA synthesizer, Model 380B, Applied Biosystems, Inc., using the phosphoramidite method. All necessary reagents were purchased from Applied Biosystems, Inc. The crude products (1-μmol scale, "trityl-off"), after deprotection by concentrated NH_4OH overnight at 55 °C, were purified by passage through a Sephadex G-25 (fine) column (2.5 × 56 cm; 0.02 M NH_4HCO_3 eluant). The void volume fractions, containing the oligonucleotide, were lyophilized. HPLC on an analytical C-3 (reverse-phase) column (see below) indicated ≥95% purity of the oligonucleotides.

Mitomycin C (bulk) was supplied by Bristol Laboratories, Syracuse, NY. *Alu*I restriction endonuclease (type II) was obtained from Pharmacia LKB Biotechnology, Piscataway, NJ. Sources for all other materials were given in a previous publication (Kumar et al., 1992).

HPLC was performed using reverse-phase columns (Beckman RPSC, C-3 Ultrapore, 10 × 250 mm, for oligonucleotide separations and Beckman ODS C-18 Ultrasphere, 4.6 × 250 mm, for nucleoside and mitomycin–nucleoside adduct analyses). For peak area quantitation, a Beckman Model 427M integrator was attached to a Model 165A absorbance detector, set to 254-nm wavelength, both as part of a Model 332 HPLC system.

Chart II [a]



[a] M symbolizes structure **5**; the 1″- and 10″-links lead to the N^2-positions of the two guanines. Ps symbolizes a psoralen; fu and pyr indicate furan-side and pyrone-side linkages to thymidines (Shi & Hearst, 1986).

Quantitative analysis of oligonucleotides and oligonucleotide–MC complexes was based on absorbance measurements in 0.1 M Tris buffer, pH 7.0. 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). In the case of oligonucleotide–MC complexes, E_{260} of an oligonucleotide-bound mitosene[2] residue [11 000; Tomasz et al. (1974)] was added to the above.

Mitomycin Monoadduct Formation with Oligonucleotides. A mixture of two complementary oligonucleotides (1 mM in mononucleotide units each) and MC (4 mM) in 0.1 M potassium phosphate buffer, pH 7.5, were briefly heated to 50 °C and then allowed to cool to 0 °C. An anaerobic $Na_2S_2O_4$ solution (40 mM) was prepared separately in the same buffer, by purging the buffer with argon before, during, and after the addition of the solid $Na_2S_2O_4$. The $Na_2S_2O_4$ solution was added to the oligonucleotide–MC mixture to give 2 mM $Na_2S_2O_4$ concentration. The resulting mixture was stirred *exposed to air* for 1 h at 0 °C and subsequently chromatographed on a 2.5 × 56 cm Sephadex G-50 column, using 0.02 M NH_4HCO_3 as eluant. The first-eluted UV-absorbing peak contained modified and unmodified oligonucleotides together. After lyophilization of this fraction, it was separated into individual components by HPLC.

"One-Step" Mitomycin Cross-Link Formation with Oligonucleotides. Complementary oligonucleotides (1:1 molar mixture of complementary strands; 10 A_{260} units total) were mixed with 5 μmol of MC in 0.1 M Tris buffer, pH 7.4 (0.45 mL). This solution was deaerated by gently bubbling argon, via a syringe needle pierced through the rubber cap of the test tube containing the reaction solution. An additional syringe needle was inserted for gas outflow. The solution was placed in an ice bath. $Na_2S_2O_4$ (8 μmol; 0.05 mL of a 0.16 M solution made freshly under argon, in 0.1 M Tris buffer, pH 7.4) was added in 10-μL increments at 10-min intervals. The reaction was terminated at 60 min by exposure to air and immediately chromatographed over a Sephadex G-50 column, as above. The cross-linked oligonucleotides eluted as the void volume fraction, followed by the unmodified oligonucleotides as the second UV band. After the A_{260} values of each of these two fractions were determined, the two fractions (cross-linked and

[2] The term "mitosene" signifies indoloquinone derivatives of mitomycins. Mitosene itself is the indoloquinone structure as in structure **5** but without substituents in the 1″-, 2″-, and 7″-positions (Webb et al., 1962).

Table I: Cross-Link Orientation Isomers of Oligonucleotides and Their Precursors: Structures, Yields, and Properties

precursor oligonucleotide duplex	cross-link orientation isomers		elution time (min)	yield (%)	precursor monoadduct	
5'- ACACGTCAT (8) 3'- T I TGCAI T (9)	5'- ACACGTCAT /1" M 10"/ 3'- T I TGCAI T	(18a)	68	22	5'- ACACGTCAT /1" M	(23)
	5'- ACACGTCAT / 10" M 1"/ 3'- T I TGC AIT	(18b)	70	20	M /1" 3'- TITGCTIT	(24)
5'- ACACGACAT (10) 3'- T I TGCT I T (11)	5'- ACACGACAT / 1" M 10"/ 3'- T I TGCT I T	(19a)	67	15	5'- ACACGACAT / 1" M	(25)
	5'- ACACGACAT / 10" M 1"/ 3'- T I TGCT I T	(19b)	72	11	M /1" 3'- TITGCTIT	(26)
5'- ACT CGACAT (12) 3'- T I AGCT I T (13)	5'- ACTCGACAT / 1" M 10"/ 3'- T I AGCT IT	(20a)	82	5.1	5'- ACTCGACAT / 1" M	(27)
	5'- ACTCGACAT / 10" M 1"/ 3'- T I AGCT IT	(20b)	85	4.6	M /1" 3'- TIAGCTIT	(28)
5'- ACGCGCCAT (14) 3'- T I CGCGI T (15)	5'- ACGCGCCAT / 1" M 10"/ 3'- T I CGCGIT	(21a)	56	14	5'- ACGCGCCAT / 1" M	(29)
	5'- ACGCGCCAT / 10" M 1"/ 3'- T I CGCGI T	(21b)	66	13	M /1" 3'- TICGCGIT	(30)
5'- ACCCGGCAT (16) 3'- T I GGCC IT (17)	5'- ACCCGGCAT / 1" M 10"/ 3'- T I GGCCI T	(22a)	49	12	not done	
	5'- ACCCGGCAT / 10" M 1"/ 3'- T IGGCC IT	(22b)	49		not done	

unmodified oligonucleotides) were recombined and separated into individual components by HPLC.

Conversion of MC–Monoadducted Oligonucleotides to MC–Cross-Linked Oligonucleotides. A mixture of HPLC-purified monoadducted oligonucleotide (1 A_{260} unit) and the unmodified complementary strand (1.5 A_{260} unit; ~1.5-fold molar excess) in 0.45 mL of 0.1 M Tris buffer, pH 7.4, was deaerated by argon as above and cooled to 0 °C. Excess $Na_2S_2O_4$ (20 μL of a 0.05 M solution in above buffer, freshly prepared anaerobically, as above) was added at once. The solution was stirred under argon for 40 min at 0 °C, then opened to air, and frozen until chromatographed by HPLC. A minor variation in this procedure is the use of 0.1 M potassium phosphate, pH 7.5, instead of 0.1 M Tris, pH 7.4, buffer, with no change in the results.

Proof of Structure of Monoadducted and Cross-Linked Oligonucleotides by Quantitative Analysis of Nucleoside and MC–Nucleoside Adduct Composition. The HPLC-purified product was digested with snake venom diesterase and *Escherichia coli* alkaline phosphatase, and the mixture was directly analyzed by HPLC for nucleoside and nucleoside–MC adduct composition as described in previous publications [e.g., Borowy-Borowski et al. (1990a)].

Assay of the Rate of AluI Cleavage of Unmodified Duplex Oligonucleotide 31/32 and Cross-Linked Isomer Oligonucleotides 33a and 33b. Unmodified or cross-linked oligonucleotides (26 mM in mononucleotide units) in 20 μL of 10-fold-diluted "one-for-all" buffer (as specified by the manufacturer for *Alu*I use) were briefly annealed at 55 °C and cooled slowly to 37 °C. *Alu*I (2 units) was added, and

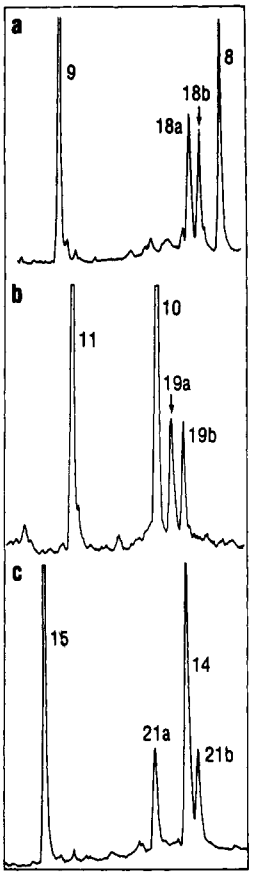
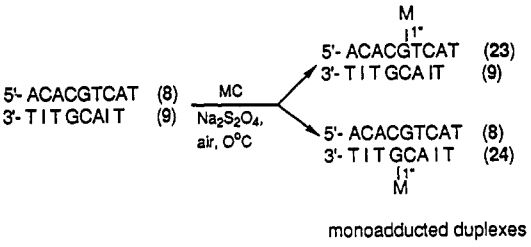


FIGURE 1: HPLC of "one-step cross-linking" reaction mixtures: separation of cross-link orientation isomers of oligonucleotides. HPLC conditions: 4.5–15% acetonitrile gradient in 0.1 M TEAA, in 300 min; 5 mL/min flow rate. (a) Reaction mixture of **8**/**9**. Elution times (min): **9**, 37; **18a**, 68; **18b**, 70; **8**, 75. (b) Reaction mixture of **10**/**11**. Elution times (min): **11**, 43; **10**, 62; **19a**, 67; **19b**, 71. (c) Reaction mixture of **14**/**15**. Elution times (min): **15**, 30; **21a**, 56; **14**, 63; **21b**, 66.

the mixture was incubated at 37 °C. Aliquots (2 μL) were removed at various time intervals (2, 5, 10, 15, 30, 60, 90, and 120 min) and added to 2 μL of 50 mM EDTA solution to quench further cleavage. The resulting mixture (4 μL) was injected quantitatively into HPLC. The HPLC peaks corresponding to the separated components were quantitated by area integration. Assignments of the first and second earliest eluting fragments as 5′-TTTAG and 5′-CTAAA, respectively, were made by nucleoside composition analysis, as described above. In each run, the numbers signifying the areas of the substrate and product peaks were added and then each individual peak area was divided by this sum. The resulting internally normalized areas were used for the calculation of rates expressed as fraction of substrate cleaved per minute under the defined standard conditions above. The initial rates were obtained under substrate-saturating conditions, as seen by linear kinetics prevailing at least up to 0.5 fractional extent of substrate cleavage in each case. Rates for the specific cleavage processes were calculated from HPLC data as follows: cleavage 1, rate of formation of fragment **34** or disappearance of **31** (the latter method was employed only in the case of the control, **31**/**32**); cleavage 2, rate of formation of fragment **35** or disappearance of **32** (the latter method was employed only in the case of the control, **31**/**32**); cleavage 1 *or* cleavage 2, rate of disappearance of the cross-linked oligonucleotide (**33a** or **33b**) or, in the case of the control (**31**/**32**), the sum of the rates of disappearance of **31** and **32**.

Scheme I: Synthesis of Authentic Cross-Link Orientation Isomers

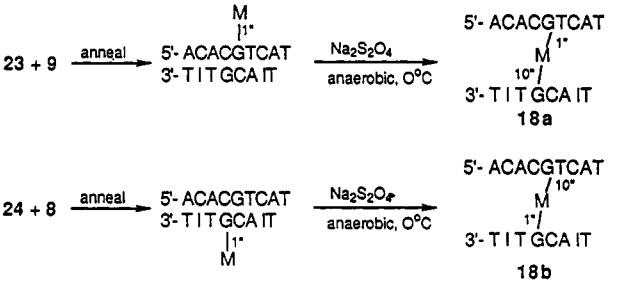
stage 1: monoalkylation



monoadducted duplexes

stage 2: separation of the monoalkylated duplexes by HPLC into individual strands (Figure **2a**). Identification of each as in Materials and Methods

stage 3: formation of authentic cross-link isomers from authentic monoalkylated precursors



The rates were calculated from the (invariably linear) data at 2 and 5 min. The entire series of the kinetic experiments was carried out twice. The maximum difference between rates calculated from the two series was 25%. The given values resulted from averaging.

RESULTS

Formation, Separation, and Yields of MC Cross-Linked Isomers in the One-Step Cross-Linking Reaction. A series of complementary pairs of oligonucleotides, listed in Table I was designed to demonstrate the existence of two cross-linked isomers based on the following considerations: (i) The oligonucleotides contain only *one* CG·CG site for cross-linking by MC (Borowy-Borowski et al., 1990). (ii) They lack self-complementarity and therefore cross-link isomerism is expected. (iii) Monoadducted single-stranded oligonucleotides are easily distinguished in a complementary pair by base composition analysis because only one of the two possible monoadducted strands contains deoxyinosine. The oligonucleotides were submitted to the one-step cross-linking reaction with MC, and the mixture was separated by HPLC into the two parent oligonucleotides and two new products as illustrated in Figure 1a–c. The latter were later identified as cross-link isomers (see below). Their structures, elution times, and percent yields are listed in Table I. All but one pair (**22**) of these isomers separated into their components. It is not clear, what factors (length, sequence, etc.) are critical for their separation by HPLC. Also, no apparent regularity was observed for relative elution times of the parent strands and their cross-linked duplexes in this series (Figure 1).

The yields of two isomers were equal within error when the flanking bases of the 5′-CpG cross-linking site were identical in both strands (**18a**, **18b**, **20a**, **20b**, **21a**, **21b**). In the one nonidentical case (**19a**, **19b**), the yields were significantly different: 15% and 11%, respectively.

No separations of two cross-linked isomers was observed by gel electrophoresis in denaturing 20% polyacrylamide (detection by UV shadowing; data not shown).

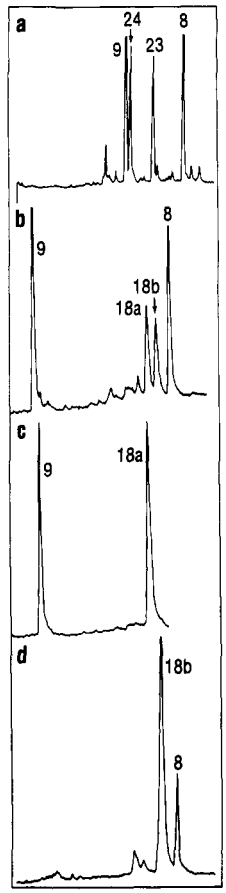
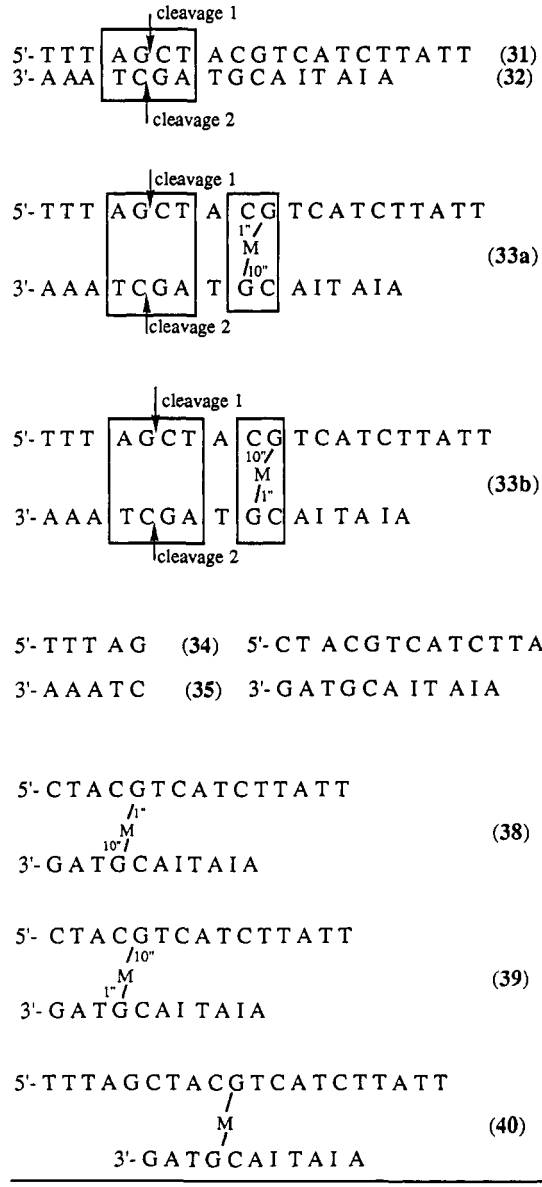


FIGURE 2: HPLC of reaction mixtures in the "two-step cross-linking" scheme of oligonucleotides. (a) Monoalkylation of **8/9**, as in Scheme I. HPLC conditions: 5–15% acetonitrile in 0.1 M TEAA, in 120 min; 5 mL/min flow rate. Elution times (min): **9**, 36; **24**, 37; **23**, 43; **8**, 52. (b) Reaction mixture of one-step cross-linking of **8/9**. HPLC conditions: 5–15% acetonitrile in 0.1 M TEAA, in 300 min; 5 mL/min flow rate. Elution times (min): **9**, 37; **18a**, 69; **18b**, 71.5; **8**, 75. (c) Reaction mixture of conversion of **9/23** to cross-linked oligonucleotide **18a**. HPLC conditions: same as in (b). Elution times (min): **9**, 37; **18a**, 67. (d) Reaction mixture of conversion of **8/24** to cross-linked oligo **18b**. HPLC conditions: same as in (b). Elution times (min): **18b**, 70; **8**, 75.

Synthesis of Authentic Individual Cross-Link Orientation Isomers via Conversion of Monoadducts to Cross-Links ("Two-Step Cross-Linking"). This was accomplished according to the sequence of reactions in Scheme I, showing the duplex pair **8/9** as specific example. The annealed duplex was submitted to monofunctional alkylation of the guanines by MC (Kumar et al., 1992), giving a mixture of the duplexes **23/9** and **8/24**.[3] This was apparent from the separation of this mixture into the four individual strands by HPLC (Figure 2a): monoadducts **23** and **24** and unmodified oligonucleotides **8** and **9**. The monoadducted oligonucleotides **23** and **24** were identified by their nucleoside and MC–nucleoside adduct compositions (**23**, dC:dG:dT:dA:**2b** = 3.0:2.0:2.9:1.1; calculated, 3:0:2:3:1; **24**, dC:dG:dI:dT:dA:**2b** = 1.2:0:1.8:2.9:1.0:1.0; calculated 1:0:2:3:1:1). In stage 3 (Scheme I), the strands were reassembled into the duplex pairs, each of which was then converted by $Na_2S_2O_4$ into the corresponding MC cross-linked isomer oligonucleotides **18a** and **18b**, respectively (Figure 2c,d). Their structures were verified by nucleoside and MC–nucleoside adduct composition analysis (**18a**, dC:dG:dI:dT:dA:**3** = 4.2:0:2.1:5.2:4.0:1.0; calculated 4:0:2:5:4:1; **18b**, dC:dG:dI:dT:dA:**3** = 4.1:0:2.0:5.1:3.9:1.0; calculated 4:0:2:5:4:1). Each of the two authentic cross-linked isomers were also coinjected with the one-step cross-linking reaction product mixture from the **8/9** duplex, resulting in unambiguous identification of each cross-linked isomer.

[3] A potential third duplex, **23/24**, in which the guanines are alkylated in both strands is unstable, as indicated by melting experiments. Its formation is therefore unlikely.

Table II: Cross-Link Orientation Isomers and Control Oligonucleotides and Their *AluI* Cleavage Fragments



Extending this scheme to the other oligonucleotide duplexes listed in Table I, with the exception of **16/17**, resulted in the synthesis of individual cross-link isomers in pure form in each case. Coinjections with the corresponding one-step cross-linking reaction mixtures led to identification of the individual cross-link isomers formed in the one-step reactions (data not shown). The percent yields of the various precursor monoadducted oligonucleotides were as follows: **23**, 19; **24**, 17.5; **25**, 24; **26–28**, not determined; **29**, 17; **30**, 21.

Synthesis of a Pair of Cross-Link Orientation Isomer Oligonucleotides Containing an AluI Cleavage Site near the CG·CG Cross-Link Site (Table II; 33a, 33b). These isomers were synthesized individually from the corresponding monoadducts, analogously to the synthesis described in the preceding section. Thus, the 20-mer **31** and 16-mer **32** were annealed,

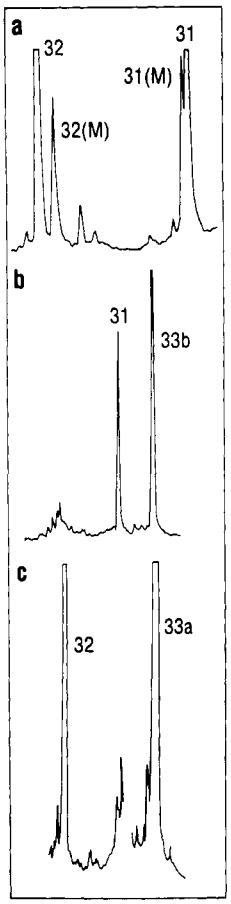


FIGURE 3: HPLC of reaction mixtures in the synthesis of two isomeric cross-linked oligonucleotide substrates for *Alu*I cleavage. (a) Monoalkylation of **31**/**32**. HPLC conditions: 4.5–15% acetonitrile in 0.1 M TEAA, in 240 min; 4.5 mL/min flow rate. Elution times (min): **32**, 137; **32**(**M**) (monoalkylation product of **32**), 142; **31**(**M**) (monoalkylation product of **31**), 186; **31**, 187. (b) Reaction mixture of conversion of **31**/**32**(**M**) to **33b**. HPLC conditions: 4.5–15% acetonitrile in 0.1 M TEAA, in 300 min; 5 mL/min flow rate. Elution times (min): **31**, 145; **33b**, 163. (c) Reaction mixture of conversion of **31**(**M**)/**32** to **33a**. HPLC conditions: same as in (b). Elutions times (min): **32**, 115; **33a**, 163.

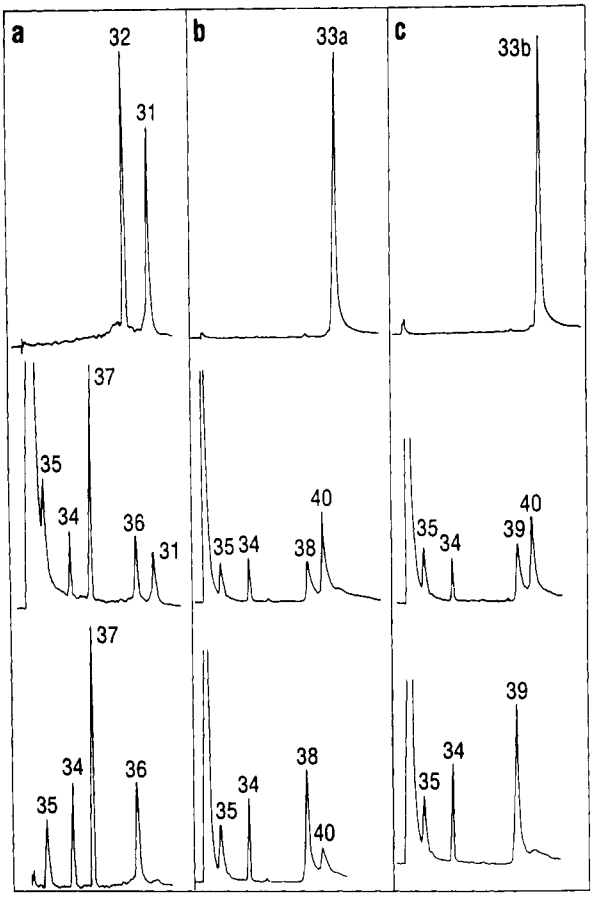


FIGURE 4: HPLC aasay of *Alu*I cleavage of the isomeric cross-linked oligonucleotides **33a** and **33b**. HPLC conditions; C-3 reverse-phase analytical column (Beckman; 4.6 × 75 mm); 6–18% acetonitrile in 0.1 M TEAA, in 30 min; 1 mL/min flow rate. (a) Cleavage of the parent duplex **31**/**32** at 0 (top), 30 (middle), and 90 min (bottom). (b) Cleavage of **33a**; same reaction times as in (a). (c) Cleavage of **33b**; same reaction time as in (a). Selected elution times (min): **35**, 2.9; **34**, 6.0; **32**, 12; **31**, 14.6; **33a**, 15.3; **33b**, 15.6.

the duplex was monoalkylated, and the reaction mixture was separated by HPLC into four components: unreacted **31** and **32**, monoadduct of **31**, monoadduct of **32** (Figure 3a). The two monoadducts were characterized by nucleoside and nucleoside–MC adduct (**2b**) composition analysis, which readily distinguished the two (no deoxyinosine in monoadduct of **31**) and also verified that, in each, only one of the two Gs was monoalkylated (data not shown). This G must be the one in the 5′-CpG sequence, since (a) such Gs are ~20-fold more reactive to MC than Gs in 5′-ApG (Kumar et al., 1992) and (b) both of these monoadducts were convertible *quantitatively* to cross-links (see below), which is only possible for monoadducts at a 5′-CpG sequence (Borowy-Borowski et al., 1990b).

The monoadducts were annealed with their unsubstituted complements followed by conversion to the corresponding cross-link isomers **33a** and **33b**, respectively, by $Na_2S_2O_4$ treatment. The conversion of the monoadducts to cross-links was quantitative in both cases as seen by HPLC (Figure 3b,c).

AluI Cleavage of the Cross-Link Isomers 33a and 33b and Their Corresponding Parent Duplex 31/32 (Table II; Figure 4). The restriction endonuclease *Alu*I cleaved both cross-link isomers rapidly even though the cross-link was located only one base pair away from the end of the four-base cognate sequence of *Alu*I in these substances (Table II). This was seen in the HPLC patterns of the incubation mixtures (Figure 4). The four cleavage products (**34**–**37**) of the parent duplex **31**/**32** (Figure 4a) were identified by base composition analysis. In the case of the cross-linked oligonucleotides **33a** and **33b** (Figure 4b and c, respectively), the identity of fragments **38** and **39** was obvious from the peak pattern variations with time. Kinetic experiments revealed that the cleavage of the parent duplex **31**/**32** is nonsymmetrical; cleavage 2 is faster than cleavage 1 (Figure 5a). This was also apparent in the cleavage of the cross-linked substrates **33a** and **33b**: At intermediate times, an intermediate fragment **40** is prominent in the HPLC patterns (Figure 4b,c). This phenomenon complicated the kinetic analysis somewhat. A set of kinetic data was obtained by following the time-dependent decrease of the starting materials. In the case of the cross-linked duplexes, decrease of starting material was caused obviously either by cleavage 1 or cleavage 2. In the parent duplex **31**/**32**, however, cleavages 1 and 2 are manifested *separately*, by decrease of **31** and **32**, respectively. Therefore, in order to compare "overall cleavage" kinetics of the three substrates (duplex **31**/**32**, **33a**, and **33b**) we calculated the *sum* of the individual fractional decreases of **31** and **32**. This was then compared with the fractional decreases of **33a** and **33b**. These kinetic plots designated as "cleavage 1 *or* 2" are shown in Figure 5b. The cross-link isomer **33b** is cleaved essentially at the same rate as the parent duplex, while isomer **33a** is cleaved at only half of this rate (Table III). The individual kinetics of cleavage 1 and cleavage 2 were also determined,

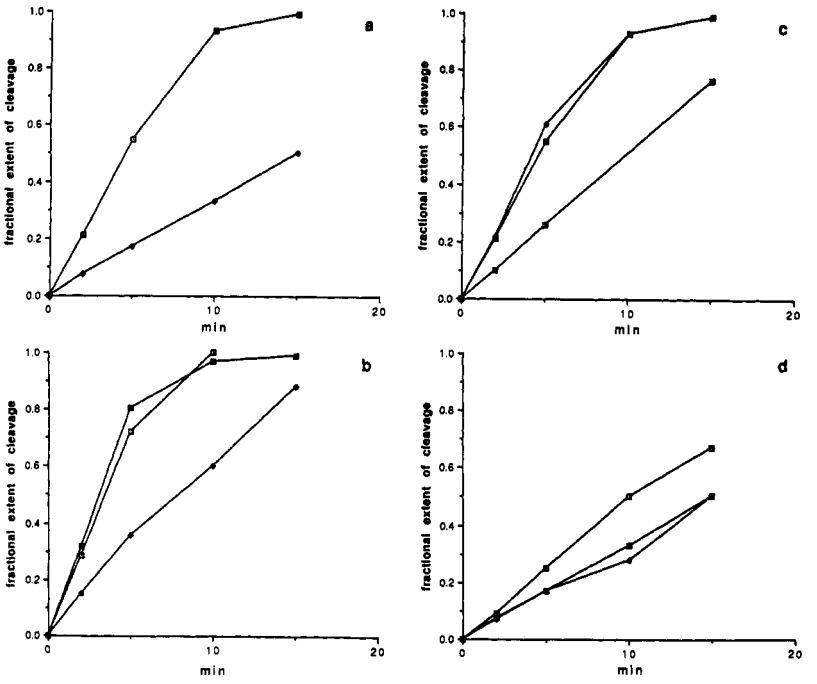


FIGURE 5: Kinetic plots of *AluI* cleavage of unmodified duplex oligonucleotide **31/32** and cross-linked isomer oligonucleotides **33a** and **33b**. (a) Cleavage 1 (◆) and cleavage 2 (□) of **31/32**. (b) Cleavage 1 *or* 2 of **31/32** (□), **33a** (◆), and **33b** (■). (c) Cleavage 2 of **31/32** (■), **33a** (□), and **33b** (◆). (d) Cleavage 1 of **31/32** (■), **33a** (□), and **33b** [same curve as **31/32** except one lower value, at 10 min (◆)].

Table III: Initial Velocities (V) of *AluI* Cleavage of MC Cross-Link Orientation Isomers of Oligonucleotides

substrate	V (fraction cleaved/min)		
	cleavage 1 *or* 2[a]	cleavage 1	cleavage 2
31/32	0.144	0.035	0.105
33a	0.072	0.045	0.051
33b	0.161	0.035	0.116

[a] As defined in the text.

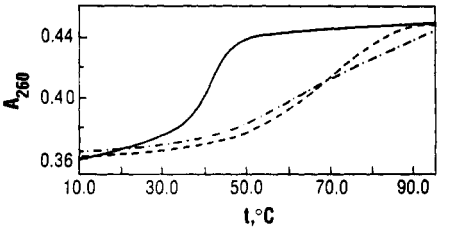


FIGURE 6: Thermal melting curves of isomeric cross-linked oligonucleotides **33a** and **33b**. —, Melting curve of control duplex **31/32**; - - -, melting curve of **33a**; – · –, melting curve of **33b**. Each substrate was at 1.1 μM concentration, in 0.1 M NaCl–0.015 M Tris–0.001 M EDTA, pH 7.5 buffer. A Varian Cary 3 spectrophotometer, equipped with "thermo program" accessories was utilized; the heating rate was set to 0.5 °C/min. The T_ms calculated from the curves by the "average" method of the computer data processing software supplied by Varian were 40.0, 64.4, and 61.3 °C, for **31/32**, **33a**, and **33b**, respectively.

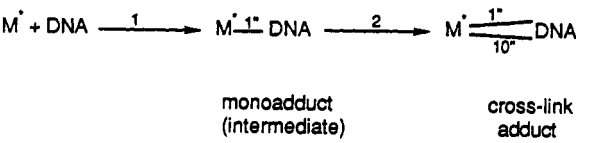
for all three substrates (see Materials and Methods). The results (Table III; Figure 5c) indicate that cleavage 2, which is the faster of the two cleavages in the parent duplex, is *selectively* inhibited in **33a**, while not inhibited in **33b**. This must be the main factor responsible for the observed overall inhibition of cleavage 1 *or* cleavage 2 in **33a** (Table III). Neither cleavage 1 nor cleavage 2 is inhibited in the other cross-link isomer **33b**.

Duplex heat stabilities of the cross-linked isomers **33a** and **33b** and their parent duplex precursor (**31/32**) were determined by thermal melting. The melting curves are shown in Figure 6. The cross-linked isomers melt at a much higher T_m and with less cooperativity than the parent duplex. The isomer **33a** had a higher T_m (64.4 °C) than **33b** (61.3 °C).

DISCUSSION

It is demonstrated here that cross-linking of oligonucleotides by reductively activated MC leads to two cross-link isomers, differing in the orientation of the cross-linking mitosene moiety **5** with respect to the duplex sequence. These isomers arise by attack of the activated MC on the guanine in either strand at the requisite CG·CG cross-link site, giving rise to a 1″-linked monoadduct intermediate in either strand. These transient intermediates are then rapidly converted to the isomeric cross-links by forming the second linkage at the 10″-position, to the guanine in the other strand [Scheme II; Tomasz et al. (1988b)]. The ratio of formation of the isomeric cross-

Scheme II



links (Table I) is influenced slightly by the flanking bases of CG·CG: It is approximately 1.1:1.0 in the three cases in which the flanking bases are identical in the two strands (**8/9**, **12/13**, **14/15**) and 1.4:1.0 when they are not (**10/11**). This latter ratio reflects a much smaller influence of the flanking bases than that in the case of a psoralen in which the ratio of the two cross-linked isomers was 10:1 in an oligonucleotide (Yeung et al., 1988).

The total yield of the two isomeric cross-links taken together decreases in the order 5′-PuCGPyr (42%) > 5′-PuCGPu (26%, 27%) > 5′-PyrCGPu (9.7%, 12%), exactly as we observed previously in a different series of oligonucleotides (Borowy-Borowski et al., 1990). This variation reflects the influence on the yield of the four flanking bases at the CG·CG cross-link site and it is likely to be related to the known influence

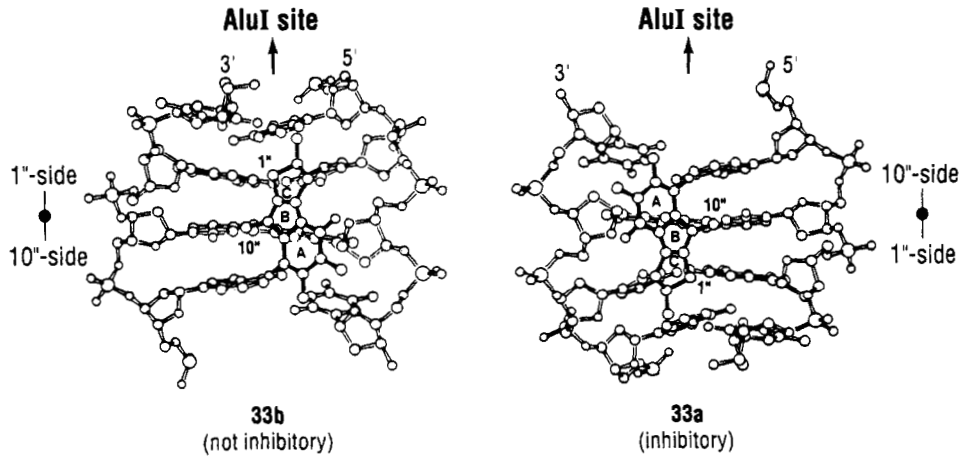


FIGURE 7: Models of the 4-bp ACGT·ACGT segment of **33a** and **33b**, cross-linked at the central CG·CG in two opposite orientations of the mitomycin residue. These models are reproductions of the NMR-derived model of the central 4-bp segment of the cross-linked hexanucleotide d(TACGTA)·d(TACGTA) (Norman et al., 1990). In that self-complementary hexanucleotide, no isomerism exists. Attaching the *Alu*I site to the symmetrical 4-bp segment at either the 1″-side or the 10″-side of the mitosene (as well as the appropriate sequences to the opposite end of this segment) creates the two isomers **33a** and **33b**.

of the 3′-base on *monoadduct* formation at the 5′-CGN sequence (Kumar et al., 1992; Kohn et al., 1992). Another significant conclusion from the results in Table I is that the two MC cross-link isomers are formed in similar proportion under the in vitro cross-linking conditions, regardless of the DNA sequence; it is always a mixture that is obtained. This is likely to be the case in vivo as well, since the cross-linking reaction itself is not enzyme-mediated.

A second major objective of this work was to see whether the two newly discovered isomeric cross-links have differential effects on properties of DNA. In particular, effects on DNA cleavage by the *Alu*I restriction endonuclease were investigated. Previously, a similar system was used for probing the effect on the cleavage of DNA modified by the monofunctional alkylator CC-1065 (Hurley et al., 1987). In our system the cross-linked site was placed at 1-bp distance away from the end of the 4-bp cognate site of *Alu*I, 5′-TGCA (Table II). Asymmetry in the rates of cleavage of the two strands was observed with both the parent and cross-linked duplexes (Figure 5a; Table III). This is not uncommon for a variety of type II restriction enzymes, with either DNA or oligonucleotides as substrate, and is generally attributed to a difference in the sequences flanking the cognate site on the two sides [e.g., Rubin and Modrich (1978) and Van Cleve and Gumport (1992)]. We find here that superimposed upon this asymmetry of the cleavage of the control duplex oligonucleotide by *Alu*I, a selective inhibition of cleavage 2 (the faster of the two in the control) is observed for cross-linked isomer **33a**. The other isomer **33b** shows no inhibition of either cleavage 1 or cleavage 2 with respect to the control duplex. This is also reflected in the overall cleavage of the three substrates ("cleavage 1 *or* cleavage 2"; see the Results section for definition): **33a** is cleaved slower than the other two (Table III; Figure 5b). It is possible that the selective inhibition of cleavage 2 in **33a** is due to selective inhibition of the subunit of the dimeric enzyme which carries out cleavage 2. Most likely this subunit responds to structural perturbation by the cross-link on the right-hand side of the cognate site while the other subunit in 2-fold symmetrical juxtaposition on the left side of cognate site encounters no perturbation. Since both cross-linked isomers are located at the same base pair distance with respect to the *Alu*I cognate site, the differential inhibition of cleavage 2 by **33a** but not by **33b** must be inherent in the opposite orientation of the cross-link in the two isomers.

What could be the basis for this difference? As seen in the models [adapted from the NMR-derived model of Norman et al. (1990); Figure 7], the 1″-side and the 10″-side of the mitosene cross-link interact differently with DNA: The 10″-side, comprised of the bulky aromatic chromophore (rings A and B), is asymmetrically disposed inside the minor groove, flat against the backbone of the 10″-linked DNA strand, extending one base pair beyond the cross-link. The 1″-side, comprised of ring C and its 2″-NH_3^+ appendage situated in the center of the groove, is pointing in the other direction in the DNA. The 2″-NH_3^+ group extends similarly one base pair beyond the cross-link, in the direction opposite from that of the 10″-side of the mitosene. In the two isomeric cross-links, either the 10″-side or the 1″-side of the mitosene is the one closer to the *Alu*I site (**33a** and **33b**, respectively). Inhibition is observed when the 10″-side (bulky aromatic portion) but not when the 1″-side is in proximity. Unfortunately, the mechanism of recognition and cleavage of DNA by *Alu*I is unknown, and therefore it is not possible to correlate these structural features of the mitomycin–DNA cross-link isomers with a precise mechanism of their interference with *Alu*I action. One can speculate that the cross-linking mitosene moiety is so close to the scissile bonds, that it may directly block the binding of the protein. It is equally possible that there is normally no contact between the enzyme and DNA at the cross-link site (minor groove); rather, the presence of the cross-link exerts a conformational distortion of the DNA, which inhibits binding and/or catalysis by *Alu*I even if these activities are located in the major groove. Continuing the speculation, the distortion may even have a dynamic component, such as change in DNA flexibility, further inhibiting enzymatic action. The thermal melting profiles of the isomers (Figure 7) indicate that the cross-link stabilizes the duplex structure of both oligonucleotides, reflected in their much higher T_m as compared with that of the unmodified parent duplex. This effect has been observed previously with other oligonucleotides cross-linked by MC (Borowy-Borowski et al., 1990a). Psoralen cross-links have the same effect (Shi & Hearst, 1986). Although the two MC isomers differ somewhat in their T_m (**33a** melts ~3 °C higher), it would be very difficult to assign a specific structural difference between the isomers on this basis alone.

Regardless of the exact basis of this inhibition, it is specific to **33a** and therefore the two isomers of the MC cross-link

clearly present two different DNA substrates to *Alu*I endonuclease. It would be interesting to test other enzymes and DNA binding proteins in the MC–DNA isomeric cross-link system. In the case of the psoralen cross-link isomers, Yeung and co-workers observed a 10-fold differential rate of excision by UvrABC endonuclease (Jones & Yeung, 1990).

Finally, it is notable that the MC cross-links exerted a much lower inhibitory effect on *Alu*I cleavage than the monofunctional DNA minor-groove alkylator CC-1065, reported by Hurley et al. (1987). In the latter case, *complete* inhibition of *Alu*I cleavage was observed for one of the DNA strands, at 11-bp distance from the alkylated site, in contrast to the mere 2-fold inhibition of cleavage we observe here for cleavage 2 (Figure 5c, Table III). Since the DNA model systems in the two investigations are not identical (117-bp restriction fragment vs 16-bp oligonucleotide), the conclusion that the MC cross-links are less distortive than the CC-1065 adduct is made herein with some caution. Nevertheless, the fact that even at this close proximity to the enzyme recognition site one of the isomers **33b** caused no inhibition at all while the other, **33a**, inhibited the cleavage only 2-fold is remarkable. The generality of this low distortion profile of the MC–DNA cross-links remains to be tested.

In order to enhance our understanding of the effects of the covalent MC cross-links in DNA, it is useful to draw comparisons with the much studied covalent cross-links by psoralens (cf. Chart II). Thus, it is significant that the MC cross-link isomers used in this study could not be separated on denaturing PAGE, in contrast to the good separations in the case of the 4,5′,8-trimethylpsoralen cross-link isomers of oligonucleotides (Kumaresan et al., 1992). The structural basis of the different electrophoretic mobility of the two psoralen cross-link isomers was examined in a recent study by Yeung and co-workers (Kumaresan et al., 1992) and was attributed to a selective, localized base pair disruption in both "arms" of the cross-linked oligonucleotide. (The other base pairs remained intact during migration in the denaturing gel.) The location and extent of helix destabilization were *different* in the two isomers, accounting for the differential retardation on the denaturing gel. The origin of these "bubbles" was proposed to be DNA distortion at the cross-link site itself, where one of the cross-linked T·A base pairs is not H-bonded (Tomic et al., 1987; Kumaresan et al., 1992). The MC cross-link is entirely different, however: The cross-link site (CG·CG) is fully base paired (Norman et al., 1990) and no separation of the orientation isomers is observed by gel electrophoresis. Thus, there is no reason to assume local base pair disruptions, at or near the MC cross-link site. This is again consistent with the remarkably low extent of inhibition of *Alu*I cleavage as discussed above.

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