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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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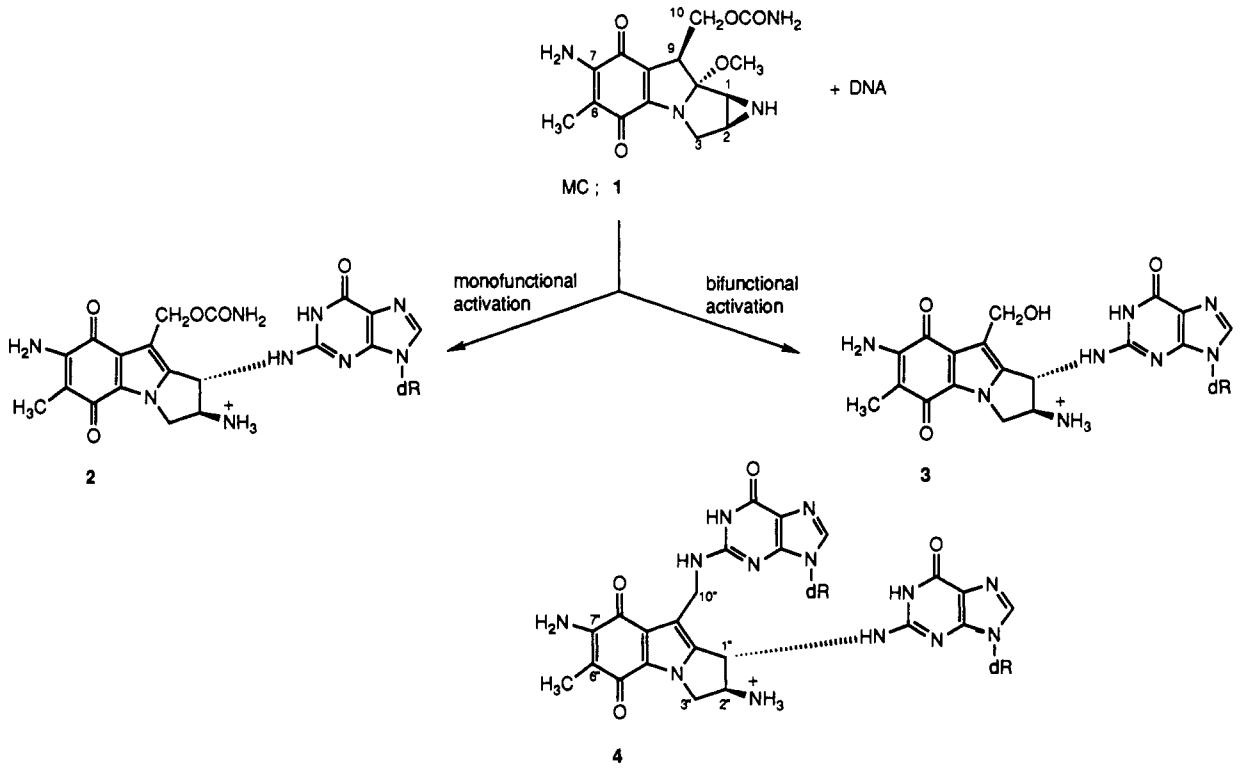
Scheme I: Formation of Adducts of Mitomycin C with DNA upon Reductive Activation[a]



[a] dR = 2′-deoxyribos-1′-yl.

activation of MC, in vitro or intracellularly, as proposed originally by Iyer and Szybalski (1964). Sartorelli and coworkers (Lin et al., 1976), who studied the enzymology of the reductive activation, introduced the term "bioreductive alkylation" for the action of MC. Moore (1977) postulated a detailed multistep mechanism, based on the original Iyer–Szybalski (Iyer & Szybalski, 1964) hypothesis which has since been largely proven experimentally, due to the contributions of numerous laboratories [Tomasz and Lipman (1981), Pan et al. (1984), Hornemann et al. (1983), Danishefsky and Ciufolini (1984), Danishefsky and Egbertson (1986), Zein and Kohn (1987), etc.].

The reductive activation of MC at both the C-1 and C-10 positions ("bifunctional activation") gives rise to the bis adduct **4** when two guanines are suitably close in duplex DNA as substrates for both active functions of one MC molecule. Adduct **3** originates from DNA sequences where this condition is not met, and the 10-position of MC reacts with H_2O instead of guanine (Scheme I). The earlier discovery of Szybalski and Iyer (1964) that exposure to MC in vivo or in vitro induces cross-links between the complementary strands of DNA is now fully explained by the isolation and identification of the bis adduct **4** from such DNA (Tomasz et al., 1987). Very recently, we reported the synthesis of oligodeoxyribonucleotides cross-linked by MC at a specific site. The cross-linked oligomers were rigorously characterized and effects of the MC cross-link on oligonucleotide properties were described, confirming the cause–effect relationship between bis adduct **4** and cross-linked DNA (Borowy-Borowski et al., 1990). The MC–DNA cross-links, by all accounts, represent the fundamental cause of the potent cytotoxicity and antitumor activity of MC [e.g., Zwelling et al. (1979) and Iyer and Szybalski (1963)].

Drugs, acting as covalent DNA-damaging agents, are usually selective in their reactions with DNA with respect to their precise covalent target among the four mononucleotide units of DNA. Reduced MC, for example, is highly selective to guanine N^2 positions. It is increasingly evident, however, that often a higher order of selectivity is imposed by *the DNA sequence surrounding the primary target*; that is, some target nucleotides are more reactive than others in the same DNA. A wide variety of underlying causes have been proposed, usually unique to the structure and reactivity of the particular drug [for review: Warpehoski and Hurley (1988)]. It is most likely that such phenomena contribute to the biological mode of action of a drug. In addition, they can reveal new information about the mechanism of the chemical reaction between drug and DNA.

We reported previously that MC cross-link formation in an in vitro system occurred selectively between two guanines in the CG·CG sequence of a hexanucleotide duplex, as opposed to those in the GC·GC sequence of another hexamer, having otherwise identical sequence, suggesting a general specificity of the cross-link for CG·CG sequences (Chawla et al., 1987). Recently, Crothers and co-workers (Teng et al., 1989) reported similar findings, using an extended series of duplex dodecanucleotides. In the present work, we confirm the generality of this phenomenon, showing, furthermore, that the CG·CG sequence is absolutely required for MC cross-link formation. The reason for this specificity is elucidated, on the basis of experimental evidence. Superimposed upon the CG·CG requirement, an additional four-base-pair sequence preference is also reported. These findings provide new insight into the reaction of MC with its target guanines in DNA.

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), Worthington.

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

Methods

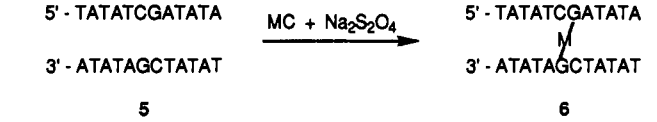
HPLC of oligonucleotides and modified oligonucleotides was performed by using reverse-phase columns (Beckman RPSC, C-3 Ultrapore, 4.6 × 75 mm for analytical and 10 × 250 mm for preparative purposes). HPLC for nucleoside and MC–nucleoside adduct analysis was carried out by using reverse-phase columns 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 dectector (set to 254-nm wavelength), both as parts of a Model 338 HPLC system. In all cases a mixture of 0.1 M TEA buffer, pH 7.0, and acetonitrile was used as eluant, either isocratically or in a linear concentration gradient, as specified.

Nucleoside and nucleoside–MC adduct analyses were carried out on 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% 0.1 M TEA, pH 7.0) were 15, 17, and 23 min, respectively. Peak areas were quantitated and used for calculation of nucleoside ratios, in comparison to a standard calibration mixture of the four nucleosides.

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)(14 000) + (number of pyrimidines)(7000) (Zon et al., 1985). For calculating the E_{260} of cross-linked oligonucleotide duplexes, the E_{260} of bound MC was assumed to be 12 000 (Tomasz et al., 1974) and added to E_{260}(strand 1) + E_{260}(strand 2).

Standard Reaction Conditions To Form Cross-Linked Oligonucleotide–MC Complexes. 12-mer oligonucleotides (either self-complementary or a 1:1 molar mixture of complementary strands) (10 A_{260} units; 330 μg; corresponding to 0.083 μmol) were mixed with 5 μmol (1.67 mg) MC in 0.1 M Tris buffer, pH 7.4 (0.45 mL), and the solution was deaerated under vacuum and then kept under helium at 5 °C. $Na_2S_2O_4$ (8 μmol; 0.05 mL of freshly made anaerobic solution in the same buffer; 0.16 M) was added in 10-μL increments at 10-min intervals. The reaction was terminated at 60 min by exposure to air. In larger than standard scale reactions, the mixture was chromatographed over a Sephadex G-50 column with 0.02 M NH_4HCO_3 buffer as eluant. The cross-linked oligonucleotide was eluted in fractions of the void volume, which were pooled and lyophilized. Final or standard-scale purification was achieved by HPLC. The products were characterized by ultraviolet spectra and nucleoside and MC–nucleotide adduct (**4**) content (Borowy-Borowski et al., 1990).

Scheme II: Cross-Linking of an Oligonucleotide by Mitomycin C[a]



[a] All nucleotide sequences given in this paper signify 2′-deoxyribonucleotides. The symbol "d" is omitted from all sequences for simplicity. The symbol M in **6** and in other formulas denotes the mitomycin cross-link between the two guanines, as in **4**.

Assay of the Extent of Cross-Link Formation in Various Oligonucleotides under Standard Conditions. After the cross-linking reaction was terminated by exposure to air (see above), aliquots of the crude mixture were immediately analyzed by HPLC. From the areas of the parent and cross-linked duplex peaks (Figure 1) the percent yield of cross-linked oligomer was calculated by the formula:

$$\%\ \text{yield of cross-linked oligomer} = (100)(0.95)(\text{area of cross-linked oligomer})/[(0.95)(\text{area of cross-linked oligomer}) + (\text{area of parent strand 1}) + (\text{area of parent strand 2})]$$

The factor 0.95 corrects for the fact that 5% of the A_{254} of the cross-linked product is due to bound MC chromophore. In the case of self-complementary oligonucleotides "parent strand 1" is identical with "parent strand 2". All cross-linking reactions were done at least twice; some of them, three to five times. The standard deviations were very low (5% or less of the measured value); see Results.

Conversion of Mono-Linked Mitomycin to Cross-Link in Duplex Oligonucleotides. The oligonucleotide–monofunctional MC complex (**7** or **9**; M. Tomasz and R. Lipman, unpublished work) (1.3 nmol) mixed with its unsubstituted parent (3.0 nmol) in 0.48 mL of 0.1 M Tris, pH 7.4, was heated at 50 °C for 10 min and then cooled slowly to 0 °C. The mixture was deaerated by bubbling helium, and then 20 μL of 6 mM $Na_2S_2O_4$ (120 nmol), freshly prepared anaerobically, was added. The reaction was terminated after 20 min at 0 °C by exposure to air, followed by HPLC of the mixture.

Results

Formation and Separation 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 reverse-phase HPLC, eluting later than the parent oligonucleotide(s) (Figure 1). The new products were previously shown to be cross-linked duplex oligonucleotides, as illustrated in Scheme II. Their characterization included UV spectra, quantitative nucleoside– and nucleoside–MC adduct analysis, etc. (Borowy-Borowski et al., 1990). No other oligonucleotide–MC product was discernible in the HPLC pattern. All new cross-linked oligonucleotides described in this paper were analyzed in the same rigorous manner.

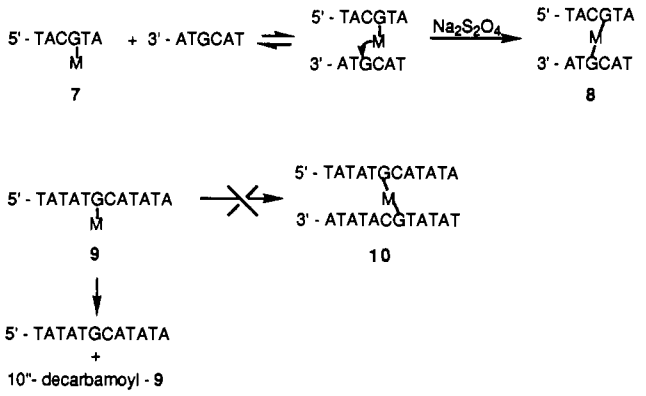
Comparison of Yields of Cross-Link at CG·CG and GC·GC Sites. As seen from the data in Table I, all oligonucleotides possessing a central CG·CG sequence gave high yields of cross-links. Simple reversal of this sequence, however, resulted in complete resistance to cross-linking in all cases. In group C, the CGC·GCG segment presents an alternative choice between CG·CG or GC·GC cross-links a priori. The actual choice is clear from the yields when one or the other of the deoxyguanosines in the –GCG– strand is replaced by deoxyinosine: the CGC·ICG analogue gave undiminished yield of cross-linking, while the CGC·GCI analogue did not react at all, indicating that the two guanines in the analogues were

Table I: Comparison of Yields of Cross-Link at CG·CG and GC·GC Sites in Oligonucleotide Duplexes

group	sequence[a]	% yield of cross-link	comment
A	5′-TATAT[CG]ATATA 3′-ATATA[GC]TATAT	19	
	5′-TATAT[GC]ATATA 3′-ATATA[CG]TATAT	0	
B	5′-ATATA[CG]TATAT 3′-TATAT[GC]ATATA	47	
	5′-ATATA[GC]ATATA 3′-TATAT[CG]TATAT	0	
C	5′-ATATA[CGC]TATA 3′-TATAT[GCG]ATAT	47	5′-CGC- formed[b] 3′-GCG-
	5′-ATATA[CGC]TATA 3′-TATAT[GCI]ATAT	50	5′-CGC- formed 3′-GCI-
	5′-ATATA[CGC]TATA 3′-TATAT[ICG]ATAT	0	5′-CGC- not formed 3′-ICG-
D	5′-ATATA[CGCG]TATAT 3′-TATAT[GCGC]ATATA	80	only one cross-link formed
	5′-ATATA[CG]TATAT 3′-TATAT[GC]ATATA	47	

[a] The boxed sites represent the crucial variable within the group. [b] The diagonal line between the G's in a duplex represents a mitomycin cross-link.

Scheme III: Cross-Linking of Mono-Linked Mitomycin to the Complementary Oligonucleotide Strand: Requirement for the CG Sequence



cross-linkable only when present in the CG·CG sequence (see "comment" in Table I, group C).

The oligonucleotide having two adjacent CG·CG sequences was cross-linked almost quantitatively (80%; Table I, group D). Nucleoside and adduct analysis of the sharp homogeneous peak from the HPLC gave dC:dG:dT:dA:**4** = 2:1:5:5:1, indicating clearly that only one cross-link adduct was present in the duplex. This result rules out cooperative formation of cross-links at directly adjacent cross-link sites. A rationale for the greatly enhanced yield at such a sequence will be presented under Discussion.

Sequence-Dependent Conversion of Mono-Linked Mitomycin to Cross-Link (Scheme III). The monofunctionally bound MC–oligonucleotide complex **7** was annealed with the parent d(TACGTA), and then $Na_2S_2O_4$ was added. After a brief incubation, the mixture was analyzed by HPLC (Figure 2). Approximately 98% of **7** (23.3 min elution time) was converted to the cross-linked duplex **8** (26 min elution time). The analogous experiment, in which d(TATATGCATATA) sequence was utilized (**9**), yielded no cross-linked duplex.

Table II: Structure of Oligonucleotides in Figure 3

group	subgroup	sequence[a]
I	a	5′-TATA[T]CG[A]TATA 3′-ATAT[A]CG[T]ATAT
	b	5′-ATAT[C]CG[G]ATAT 3′-TATA[G]GC[C]TATA
II	a	5′-TATA[T]CG[T]ATAT 3′-ATAT[A]GC[A]TATA
III	a	5′-ATAT[A]CG[T]ATAT 3′-TATA[T]GC[A]TATA
	b	5′-ATAT[G]CG[C]ATAT 3′-TATA[C]GC[G]TATA
	c	5′-ATAT[A]CG[C]TATA 3′-TATA[T]GC[G]ATAT
	d	5′-ATAT[A]CG[C]TATA 3′-TATA[T]GC[I]ATAT
IV	a	5′-ATAT[A]CG[C]GTATAT[b] 3′-TATA[T]GC[G]CATATA

[a] The flanking base pairs of the central CG·CG cross-link site in the duplexes are indicated by the boxes. [b] In this duplex the set of flanking base pairs is identical for each of the two adjacent CG·CGs, due to the 2-fold rotational symmetry of the sequence. Only one of the two sets is marked by the boxes.

Table III: Percent Unreacted ATATACGTATAT as a Function of Reaction Time[a]

time of reaction (min)					
10	20	30	40	50	60
75, 73[b]	67, 63, 63[b]	59[b]	50, 50, 57[b]	50, 50, 57[b]	50, 50, 57[b]

[a] For conditions of the cross-linking reaction and assay of unreacted and cross-linked oligonucleotides, see Methods. [b] $Na_2S_2O_4$ was added in one portion to the reaction mixture.

Rather, as seen in Figure 2c, a new mono-adduct derivative, eluting closer to the parent oligonucleotide, was formed. This is likely to be the 10″-decarbamoylated derivative, formed by activation of the 10″-position by $Na_2S_2O_4$ followed by reaction with H_2O (Tomasz et al., 1988b). Considerable increase of the relative amount of the parent oligomer is also apparent from the HPLC pattern, indicating that **9** lost the attached mitomycin partially upon reduction (Scheme III). Not even a trace of cross-linked oligonucleotide could be detected by HPLC (Figure 2c).

Effect of Flanking Base Pairs at the CG·CG Cross-Link Site on the Yield of Cross-Linking. A number of dodecanucleotides possessing one central CG·CG, but differing in the four flanking nucleotides, were cross-linked under the standard conditions. There was a striking, highly reproducible dependence of the yield on the flanking bases. The yields fall into four groups and are presented in Figure 3. Members of the lowest yield group (I; a, 19 ± 1%; b, 19 ± 0%) have a PyrCGPu·PyrCGPu sequence. The next group (II; a, 31 ± 2%) has PyrCGPyr·PuCGPu, and the third (III; a, 47 ± 4%; b, 37 ± 0%; c, 47 ± 0%; d, 50 ± 0%) conforms to PuCGPyr·PuCGPyr. Group IVa can be regarded as having two overlapping PuCGPyr·PuCGPyr (group III) sequences, and remarkably, the yield is nearly twice as high (80 ± 0%) as those from group III. (The full structures of groups I–IV are given in Table II.) Pseudo-first-order rate constants (k_ψ) were also calculated from the yields, justified by the fact that activated MC was added in large molar excess (approximately 120-fold) over its guanine target. Indeed, a time course of the cross-linking reaction indicated approximately pseudo-first-

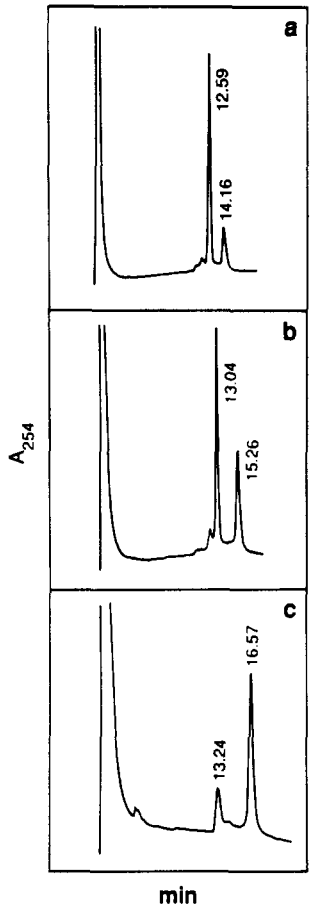


FIGURE 1: HPLC separation of parent and cross-linked oligonucleotides. (a) Oligonucleotide **5** (12.50 min) and cross-linked derivative **6** (14.6 min). (b) d(ATATACGTATAT) (13.04 min) and its cross-linked derivative (15.26 min). (c) d(ATATACGCGTATAT) (13.24 min) and its cross-linked derivative (16.57). Column: 4.6 × 75 mm, C-3, reverse phase. Solvent: 6–18% acetonitrile gradient in 0.1 M TEA, pH 7.0, in 24 min, at 1.0 mL/min flow rate.

order kinetics (Table III). It should be noted, however, that all of these reactions were run to the same "end point" brought about by the fast decay of the concentration of the reduced, active MC, due to hydrolysis and other competing reactions (Cera et al., 1989). Therefore, toward the end of the reaction the kinetics of the cross-linking should be deviating from strictly first order. Even so, all oligonucleotides experienced the same rate of MC decay and, therefore, the same MC concentration, for the same length of time. Due to the uncertainty of the actual length of time of the reaction of activated MC with the oligonucleotides, rate constants (k_ψ) were calculated only as *relative* to that of oligonucleotides of group I, by using the formula:

$$k_\psi(\text{rel}) = \frac{k_\psi}{k_\psi(\text{group I})} = \frac{2 - \log(\%\ \text{unreacted oligonucleotide})}{2 - \log\ (\%\ \text{unreacted oligonucleotide of group I})}$$

This gave the following k_ψ(rel) values: Ia, 1.0; IIa, 1.78; IIIa–d, 3.1, 2.2, 3.1, and 3.3, respectively; IV, 7.8.

Discussion

We reported two years ago that $[d(TACGTA)]_2$ but not $[d(TAGCTA)]_2$ could be cross-linked by reductively activated MC and suggested that cross-link formation was specific to guanines in the CG·CG sequence of duplex DNA (Chawla et al., 1987). In order to confirm this, we tested an extended series of oligonucleotides in three types of experiments: (1) simple interchange of CG·CG and GC·GC sequences in the center of AT-rich dodecamers (Table I, groups B and C); (2) alternative replacement of a guanine by inosine in the GCG·CGC sequence (Table I, group C); and (3) performing the cross-linking reaction in two separate steps. In the last experiment mono-linked intermediates **7** and **8** were prepared

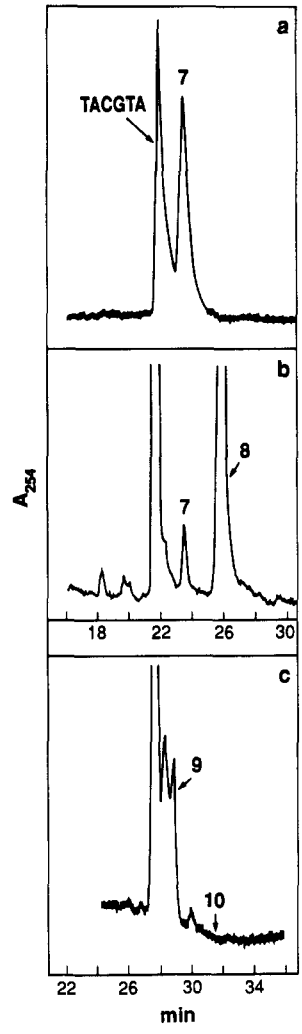


FIGURE 2: HPLC assay of the sequence-dependent conversion of mono-linked mitomycin to cross-link. (a) Standard mixture of TACGTA and its MC mono-adduct derivative **7**. (b) Products of the reaction between TACGTA and **7** upon activation by $Na_2S_2O_4$: unreacted d(TACGTA) (21.8 min), **7** (less than 2% of the original amount present as seen by peak integration), and **8**. (c) Products of d(TATATGCATATA) and its MC mono adduct derivative **9** upon activation by $Na_2S_2O_4$: d(TATATGCATATA) (27.5 min), an unknown mono-adduct derivative (28.1 min), and unchanged **9** (29.0 min). Column and solvent: as in Figure 1.

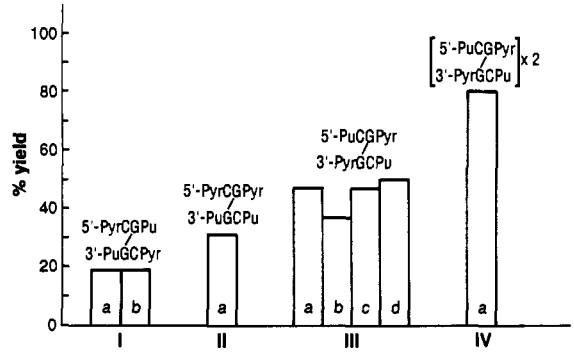
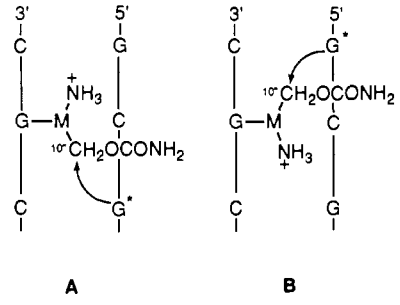


FIGURE 3: Yield of cross-linked oligonucleotides as a function of flanking base pairs at the CG·CG cross-link site. The full structures of the oligonucleotides represented by the bars in groups I–IV are given in Table II.

Scheme IV: Opposite Orientations of Mono-Linked Mitomycin



first and then reactivated by $Na_2S_2O_4$ in the presence of the parent strand for completion of the cross-link (Scheme III). Such a two-step cross-linking sequence was shown previously to occur in DNA (Tomasz et al., 1988b). The results of each of the three types of experiments showed unequivocally that the mitomycin cross-links were formed exclusively at CG·CG sites. The last experiment showed, in addition, that it is the second step of the two-step process that accounts for this stringent sequence requirement.

After completion of this work, Crothers and co-workers (Teng et al., 1989) reported the results of a study based on essentially the same experimental design as our type 1 experiments above, confirming our earlier (Chawla et al., 1987) and present conclusions on CG·CG specificity of the MC cross-links. Gel electrophoretic mobility served as the assay for the yield of cross-linked oligonucleotides, which were otherwise not characterized. In another recent study, applying DNA sequencing techniques to a model synthetic oligonucleotide, having both a single GC and a CG site, led to the same conclusion (Weidner et al., 1989). Thus the unique orientation of the MC cross-link now seems firmly established and is formally analogous to the preferred orientation of the light-induced cross-links by psoralen between T's at the AT·AT sequence (Gamper et al., 1984).

In search for an explanation for this specificity, Teng et al. (1989) computed the energies of oligonucleotides cross-linked by MC at CG·CG and GC·GC sequences and found essentially no difference between the two classes in total energy changes upon formation of cross-linked structures from parent duplex; therefore, preferential cross-linking could not be rationalized by energy minimization of the final product. Calculation of relative electrostatic potentials at the reactive N^2 atom of G in GpC and CpG did not yield a correlation either. They hypothesized that a kinetic factor was responsible: At GC·GC a larger distance has to be bridged over by the mono adduct in the second step of the cross-linking reaction (cf. our Scheme III) than at CG·CG. This may slow down the rate of the second step in the case of GC·GC, and the activated monofunctionally attached MC decays instead in some way. This hypothesis is not easily testable, however. We now present evidence that an alternative mechanism is responsible for the CG·CG cross-link specificity as follows:

It is seen from space-filling models (Tomasz et al., 1986) that there are two alternative orientations of the monofunctionally bound MC for fitting in the minor groove (Scheme IV, A and B), differing by approximately 180° rotation around the MC–guanine bond. In A, the C-10″ position, still bearing the carbamate, points in the 3′ direction; therefore, it is lined up for cross-link formation at CG·CG. In B, the C-10″ points toward 5′, lining up for a GC·GC cross-link. Since a 180° rotation of the covalently bound bulky mono adduct in the minor groove is impossible, the orientation of the mono adduct is fixed, and the cross-link should reflect this fixed orientation. Whether this is A or B is determined by factors of the first, mono-adduct-forming bonding step. What are these factors? Kollmann, Remers, and co-workers (Rao et al., 1986; Remers et al., 1988) presented a large number of theoretical, energy-minimized MC mono adduct–oligonucleotide model structures, all with drug in the A orientation; in these, the C-10″ carbamate binds to DNA by two H-bonds, one of them to the 2-NH_2 of the guanine in the opposite strand. Verdine (1986) arrived at similar conclusions, by similar methodology. Also, our space-filling modeling indicated that A produced a better fit than B and also showed the H-bonds (Tomasz et al., 1986). All this is suggestive of greater stability of A than B; this factor could determine the orientation of MC in the transition state of the first bonding step (mono adduct formation). The present results of the two-step cross-linking experiments (Scheme III) show that this is indeed the case: Since conversion of **7** to **8** was essentially quantitative, the orientation of the drug in **7** must have been exclusively the A type; the cross-linking step "trapped" the *existing* A orientation. The quick and quantitative conversion from mono adduct to cross-link rules out the possibility that a reorientation from B to A in **7** took place during the brief activation period. This reorientation could have occurred only by dissociation/reassociation of the mono-linked MC from the oligomer or by dissociation/reassociation of the duplex itself; such processes, however, are not only unlikely, but they would have resulted in losses of the cross-link-active mono adduct since rapid reactions of solvent with any free or mono-linked drug (in single strands) competes with cross-link formation (Tomasz et al., 1988a; Cera et al., 1989). In conclusion, it is apparent that the basis for the CG·CG specificity of MC cross-links is the fixed A-type orientation of its precursor, the mono adduct. In turn, this orientation is determined by its greater stability over that of the alternative one (B). The bulky C-10″ carbamate is likely to be responsible for the preference in orientation. It is then not surprising that the energy calculations of Crothers and co-workers of the cross-linked oligonucleotides yielded no clue for the observed sequence discrimination by the cross-links: the carbamate is no longer present in the final structures. Determining the structure of the mono adduct–oligonucleotide complex by physical methods, e.g., NMR, should constitute a critical test of this conclusion.

Inspection of the yields of CG·CG cross-links in a series of oligonucleotides, as summarized in Figure 3, reveals an order of preference among *four-base-pair sequences*, PuCGPyr·PuCGPyr being the most preferred. Examination of the possible causes of this preference requires an analysis of the origin of the guanine specificity of alkylation of DNA by MC. In the ensuing discussion we adapt the "binding" vs "bonding" terminology, specifying noncovalent and covalent interaction, respectively (Warpehoski & Hurley, 1988).

Upon review of previous work, it becomes apparent that the guanine specificity of MC is determined solely by covalent reactivity factors, intrinsic to the structure of the guanine nucleoside. This conclusion is based on the following observations: Deoxyguanosine itself is readily alkylated by reduced MC, to yield adduct **3** and its 1″-stereoisomer (Tomasz et al., 1986b). Other deoxynucleosides give no adducts (unpublished work). Dinucleoside phosphates d(GpN) and d(NpG) (N = A, T, C) are also alkylated, exclusively at the dG residue (Tomasz et al., 1983, 1986a,b). On the polynucleotide level, the observed guanine specificity is independent of secondary structure, since both single- and double-stranded synthetic polynucleotides are alkylated, strictly depending on their guanine content (Tomasz et al., 1974; Lipman et al, 1978).

Native DNA is alkylated in linear proportion to its GC content (Lipman et al., 1978), giving guanine adducts only. Denatured calf thymus DNA and M13 phage single-stranded DNA yield the same set of adducts as native DNA (Tomasz et al., 1986a).

Although guanine specificity is not dependent on binding, binding does occur, nevertheless. We reported reversible formation of electrostatic complexes of MC with DNA, RNA, and even with poly(vinyl sulfate) when the drug was reduced and then reoxidized, at high concentration. The structure of the binding drug species was not determined. Nonactivated MC showed no binding (Lipman et al., 1978). Kollman, Remers, and their co-workers (Rao et al., 1986; Remers et al., 1988) modeled noncovalent complexes of both MC and its presumed activated form, the quinone methide (Moore, 1977), by molecular mechanics simulation; the models indicated numerous noncovalent interactions between charged as well as H-bond donor/acceptor groups between drug and DNA. Recently, equilibrium dialysis studies in our laboratory confirmed binding (weak, electrostatic) by active-form analogues of reduced mitosenes but showed no binding by MC itself (Tomasz & Behr-Ventura, 1988). This difference is readily explained by the different basicities of MC (pK_a 3.1–3.5) and the mitosenes (pK_a 7–8). Importantly, Teng et al. (1989) showed by competition experiments that reduced mitomycin binds to A·T and C·G base pairs of oligonucleotides equally well (binding affinity approximately 600 M^{-1}), and as such, it is the first step in the cross-linking process at the covalently reactive CG·CG sequence. They concluded, however, that it is not responsible for this sequence specificity. In view of this apparent lack of *sequence-specific binding* of MC to DNA, the observed *sequence-specific bonding* at PuCGPyr·PuCGPyr should be explained by other factors. We propose a rationale as follows: The four Pu or Pyr bases flanking the required CG·CG site form only three independent flanking base-pair combinations due to the 2-fold rotational symmetry of the central CG·CG sequence; the cross-link yields fit into the same three groups: PuCGPyr·PuCGPyr, PuCGPu·PyrCGPyr, and PyrCGPu·PyrCGPu (Figure 3). What is the difference among these groups of sequences that could be relevant to the rate of cross-link formation? We should look first at the bases directly flanking the reactive (guanine) bases. The 5′ neighbor of both guanines is fixed as cytosine in all sequences. What is varied, however, is the 3′ neighbor! The observed yield or rate order is 3′-Pyr at both guanines > one 3′-Pyr and one 3′-Pu at the two guanines > 3′-Pu at both guanines. This may indicate that the approach by the drug to the guanine along the *bonding* reaction coordinate is sterically more favorable at GpPyr. This is unlikely, however, since theoretical energy-minimized models show no difference in ease of accommodating either 3′-Pu or 3′-Pyr in mono adduct–oligonucleotide complexes (Remers et al., 1988). The other possibility is that the guanine N^2 atom of GpPyr is more reactive toward the drug. Models of the GpPu and GpPyr steps in DNA show that the guanine $N^{(2)}H_2$ group is completely stacked over a 3′-Pu ring; on the other hand, it is directly above O^2 of a 3′-Pyr. This oxygen, with its partial negative charge, presents a negative electrostatic field which should enhance the nucleophilic reactivity of the N^2 of the guanine. This may then be a new example of alkylation rate acceleration by DNA by molecular electrostatic potential effects (Pullman & Pullman, 1981). In their outstanding review, Hurley and Warpehoski (1988) point out that DNA may assist in its own alkylation in some cases. Since the nucleophilic attack of the G-N^2 on the activated form of the drug necessarily involves development of a positive charge and ultimate loss of a proton from G-N^2 (cf. **4**), we suggest that the O^2 of the 3′-Pyr catalyzes this path by acting as the proton acceptor due to its favorable dipole moment and steric alignment. Such an effect is not possible in the GpPu step.[2]

If all this is true, then GpT may be expected to increase the rate of cross-linking more than GpC does, since the O^2 of C in GpC is H-bonded to the opposite guanine and, therefore, its negative dipole is attenuated. Remarkably enough, this appears to be the case; IIIb in which *both GpPyr* sequences are GpCs, gives the lowest (almost anomalous) yield in this group, in which all the other oligonucleotides have at least one GpT sequence. In addition, IIId, in which the C of the GpC is paired with I and therefore its O^2 is not H-bonded, gives a better yield than IIIc. These findings support the proposed mechanism. It is not known whether it applies to both steps of the cross-linking reaction or only to one, in which case the observed greater enhancement by *two* 3′-Pyr's rather than one (group III vs group II) is merely statistical, due to a double chance for reaction at a GPyr site.

The remarkably high yield (80%) and rate (8-fold over that of group I) of cross-linking of the CGCG·CGCG-containing oligonucleotide (Table I, D; Figure 2, IVa) represents a special case. The data indicate slightly over 2-fold increase of rate over that of IIIc, as a result of adding a second CG·CG to the existing CG·CG in IIIc. Only one cross-link/molecule was formed, ruling out a cooperativity effect at two adjacent CG·CG sites. Both CG·CG's of IVa are flanked by the same base pairs as the single CG·CG in IIIc. Thus the same cross-link target site is present twice in IVa. The doubled rate could thus be readily explained by the statistical factor. Teng et al. (1989), however, observed sigmoidal yield increases upon adding tandem CG·CG sequences in an extensive series of oligonucleotides, indicating special reactivity of the drug with such tracts. Whichever is the case, it is apparent that $(CG)_n$·$(CG)_n$ tracts represent "hot spots" for cross-linking of DNA by MC.

In summary, the observed PuCGPyr·PuCGPyr four-base-pair sequence preference by the MC cross-link is the composite result of two component sequence specificities: absolute specificity for CG·CG and moderate specificity for a GPyr step in both strands. (The latter automatically specifies the PuC step in the opposite strand.) The first component is rationalized by the favorable orientation of the mono adduct precursor and the second by enhancement of the reactivity of the N^2 of guanine by the proximity of the O^2 of a 3′-Pyr, which helps to remove the N^2 proton. GpT appears to be more enhancing than GpC. It is noteworthy that Ueda et al. (1984) observed a modest specificity for GpT for reactions of unspecified nature of MC with DNA.

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[2] Even if the 3′-Pu could also act as a H^+ acceptor, this effect would be limited by steric hindrance of the 3′-Pu to rotation of the G–N^2 bond. Such rotation is necessary for proton transfer and simultaneous covalent bond formation between the G N^2 atom and the drug.

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MgADP-Induced Changes in the Structure of Myosin S1 near the ATPase-Related Thiol SH1 Probed by Cross-Linking†

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ABSTRACT: The structural consequences of MgADP binding at the vicinity of the ATPase-related thiol SH1 (Cys-707) have been examined by subjecting myosin subfragment 1, premodified at SH2 (Cys-697) with *N*-ethylmaleimide (NEM), to reaction with the bifunctional reagent *p*-phenylenedimaleimide (pPDM) in the presence and absence of MgADP. By monitoring the changes in the Ca^{2+}-ATPase activity as a function of reaction time, it appears that the reagent rapidly modifies SH1 irrespective of whether MgADP is present or not. In the absence of nucleotide, only extremely low levels of cross-linking to the 50-kDa middle segment of S1 can be detected, while in the presence of MgADP substantial cross-linking to this segment is observed. A similar cross-link is also formed if MgADP is added subsequent to the reaction of the SH2-NEM-premodified S1 with pPDM in the absence of nucleotide. Isolation of the labeled tryptic peptide from the cross-linked adduct formed with [^{14}C]pPDM, and subsequent partial sequence analyses, indicates that the cross-link is made from SH1 to Cys-522. Moreover, it appears that this cross-link results in the trapping of MgADP in this S1 species. These data suggest that the binding of MgADP results in a change in the structure of S1 in the vicinity of the SH1 thiol relative to the 50-kDa "domain" which enables Cys-522 to adopt the appropriate configuration to enable it to be cross-linked to SH1 by pPDM.

The central problem in the mechanism of muscle contraction at the molecular level is the nature of the force-generating structural change occurring in the myosin subfragment 1 (S1)[1] crossbridge while it is hydrolyzing MgATP and interacting with actin. Although the kinetics of the ATPase cycle in the

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[1] Abbreviations: S1, myosin subfragment 1; pPDM, *p*-phenylenedimaleimide; NEM, *N*-ethylmaleimide; HPLC, high-performance liquid chromatography; SH2-NEM-S1, S1 premodified at the SH2 thiol with *N*-ethylmaleimide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, dinitrophenyl; TFA, trifluoroacetic acid; DTT, dithiothreitol.

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