McSwiggen, J. A., & Cech, T. R. (1989) *Science 244*, 679-683.
Michel, F., & Westhof, E. (1990) *J. Mol. Biol. 216*, 585-610.
Michel, F., Hanna, M., Green, R., Bartel, D. P., & Szostak, J. W. (1989) *Nature 342*, 391-395.
O'Donnell, M. E., & Kornberg, A. (1985) *J. Biol. Chem. 260*, 12875-12883.
Pace, N. R., & Marsh, T. L. (1986) *Origins Life Evol. Biosphere 16*, 97-116.
Porschke, D., & Eigen, M. (1971) *J. Mol. Biol. 62*, 361-381.
Pyle, A. M., & Cech, T. R. (1991) *Nature 350*, 718-720.
Pyle, A. M., McSwiggen, J. A., & Cech, T. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8187-8191.
Scaringe, S. A., Francklyn, C., & Ussman, N. (1990) *Nucleic Acids Res. 18*, 5433-5441.
Sugimoto, N., Sasaki, M., Kierzek, R., & Turner, D. H. (1989a) *Chem. Lett.*, 2223-2226.
Sugimoto, N., Tomka, M., Kierzek, R., Bevilacqua, P. C., & Turner, D. H. (1989b) *Nucleic Acids Res. 17*, 355-371.
Tanner, N. K., & Cech, T. R. (1985) *Nucleic Acids Res. 13*, 7759-7779.
Thompson, R. C. (1988) *Trends Biochem. Sci. 13*, 91-93.
Turner, D. H., Sugimoto, N., & Freier, S. M. (1990) in *Nucleic Acids* Landolt-Bornstein Series, Vol. 1C, (Saenger, W., Ed.) Springer-Verlag, Berlin, pp 201-227.
Wu, T., Oglivie, K. K., & Pon, R. T. (1989) *Nucleic Acids Res. 17*, 3501-3517.
Young, B., Herschlag, D., & Cech, T. R. (1991) *Cell 67*, 1007-1019.
Zaug, A. J., & Cech, T. R. (1986) *Science 231*, 470-475.
Zaug, A. J., Grabowski, P. J., & Cech, T. R. (1983) *Nature 301*, 578-583.
Zaug, A. J., Kent, J. R., & Cech, T. R. (1985) *Biochemistry 24*, 6211-6218.
Zaug, A. J., Grosshans, C. A., & Cech, T. R. (1988) *Biochemistry 27*, 8924-8931.

Recognition of Specific DNA Sequences by Mitomycin C for Alkylation†

Shiv Kumar, Roselyn Lipman, and Maria Tomasz*
Department of Chemistry, Hunter College, City University of New York, New York, New York 10021
Received August 27, 1991; Revised Manuscript Received October 31, 1991

ABSTRACT: Synthetic oligodeoxyribonucleotides were reacted with mitomycin C (MC) under conditions which restricted MC to monofunctional alkylating activity. The yields of monofunctional alkylation of oligonucleotides with variable sequence were determined by enzymatic digestion of the reaction mixture to unreacted nucleosides and the product of alkylation, a MC–deoxyguanosine adduct (**2**), followed by quantitative analysis by HPLC. The relative yields of **2** reflected relative monoalkylation reactivities. They were compared in a series of oligonucleotides having the sequence 5′-NGN′ in which the 5′-base was varied while the 3′-base was kept constant as T. Under $Na_2S_2O_4$ activation conditions a striking enhancement of the yield was observed at the 5′-CG sequence: 36%, compared to 2% at 5′-AG and 4.1% at 5′-TG. The 5′-GG sequence also showed enhanced reactivity although to a lesser extent (14.7%). The enhancements were specific to the duplex state of the oligonucleotides. Using NADPH:cytochrome *c* reductase as the reducing agent gave similar results. MC activated by acidic pH also displayed 5′-CG alkylation specificity. 10-Decarbamoyl-MC activated by $Na_2S_2O_4$ showed the same 5′-CG specificity as MC. Replacement of deoxyguanosine by deoxyinosine in the opposite strand at a 5′-CG site abolished the enhancement of alkylation. Such replacement at a 5′-GG site had a similar effect. It was found that the base 3′ to the guanine had only a relatively modest modulating effect on the enhanced reactivity of the G at the 5′-CG sequence. This 3′-base effect appeared to be independent of the 5′-base of the 5′-NGN′ triplet. The order of reactivity is 3′-(C > T > G > A). An explanation is proposed for the dominating 5′-CG (and, to a lesser extent, 5′-GG) specificity of the alkylation of DNA by MC, based primarily on the results of the inosine substitutions: At 5′-CG a H-bond is formed between the 2-amino group of guanine in the opposite strand and the 10-O atom of activated MC, facilitating alkylation at such sequence. An analogous mechanism applies at the 5′-GG site. The 5′-CG and 5′-GG sites of DNA monoalkylation by MC coincide with the two cross-linkable sites (interstrand cross-link at CG·CG and intrastrand cross-link at GG·CC). The monoalkylation specificity may be a molecular evolutionary device to guide MC preferentially to guanines located in cross-linkable sequences of DNA.

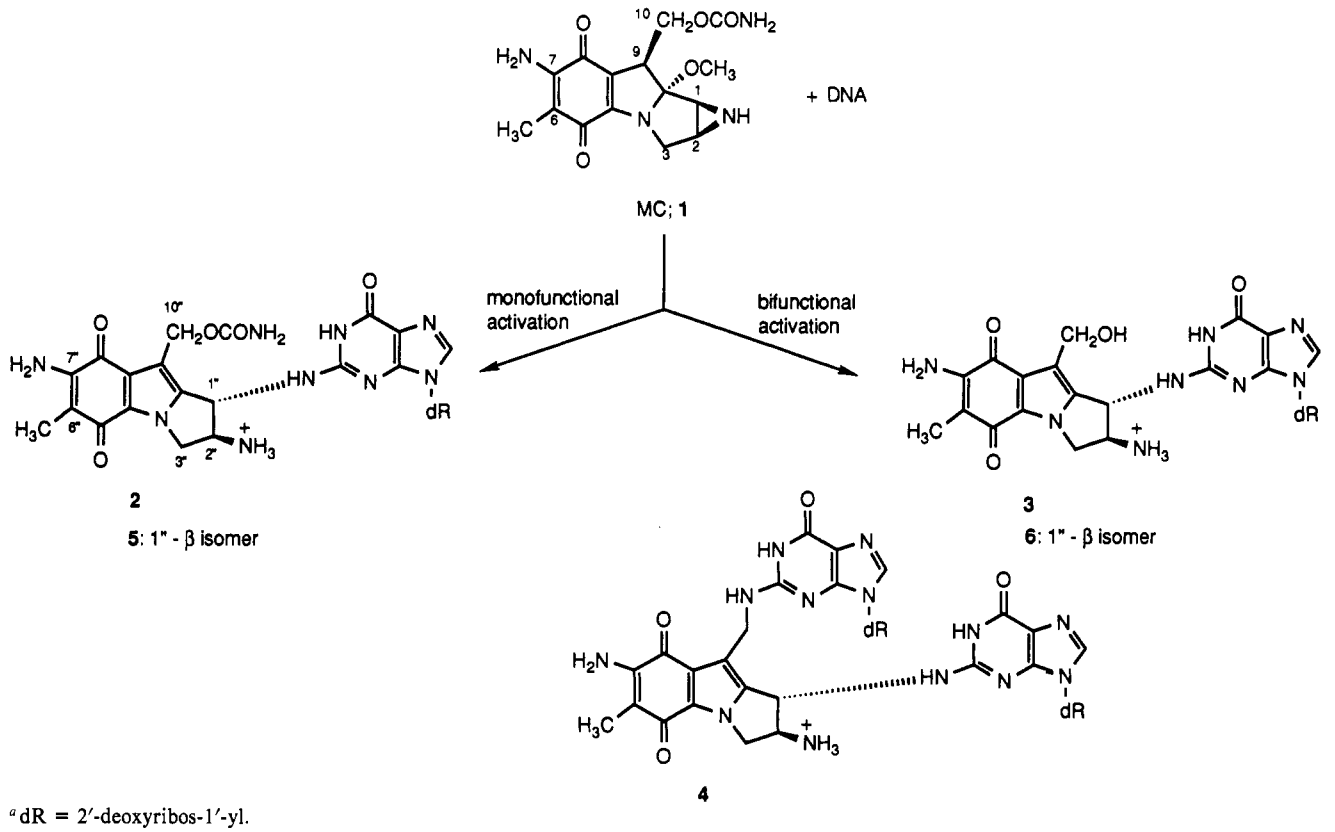
The antitumor antibiotic mitomycin C (MC;[1] **1**) is widely used in anticancer chemotherapy. Its mode of action has been a subject of great interest ever since the discovery by Iyer and Szybalski (1963) that MC cross-links the complementary strands of DNA, in vivo and in vitro. This effect is unique so far to the mitomycins among the known naturally occurring antibiotics. The cross-links result from bifunctional alkylation of DNA by MC (Iyer & Szybalski, 1964) and are accompanied by monofunctional alkylation products, i.e., drug mole-

† This research was supported by a grant (CA28681) from the National Cancer Institute and a Research Centers in Minority Institutions award (RR003037) from the Division of Research Resources, NIH.

[1] Abbreviations: MC, mitomycin C; TEAA, triethylammonium acetate; d-[G(M)pN], the dinucleoside phosphate d-(GpN) substituted at the N^2-position of G by a mitosene (M) residue identical to that in adduct **2**; SVD, snake venom diesterase.

0006-2960/92/0431-1399$03.00/0

Scheme I[a]



[a] dR = 2′-deoxyribos-1′-yl.

cules bound to one strand of DNA, by a single linkage (Tomasz et al., 1974). The damage to the structural integrity of DNA by these alkylation products most likely represents the basic cause of the cytotoxicity and antitumor activity of MC. The cross-links are generally considered to be more lethal than monofunctional alkylation products since cross-linked DNA, if caught unrepaired in a replication fork, presents an irreversible block to DNA replication (Zwelling et al., 1979).

Mitomycin C requires reductive activation to give rise to the actual alkylating agent, an unstable compound. MC activation occurs in living cells by flavoenzymes; it can be also mimicked in the test tube by chemical reducing agents or various NAD(P)H-dependent flavoreductases (Tomasz & Lipman, 1981; Pan et al., 1984; Keyes et al., 1984). The covalent reactions of the reductively activated form of MC with DNA are remarkably specific: both alkylating functions of the drug, namely, the aziridine at C-1 and the carbamate at C-10, react exclusively with 2-amino groups of guanines. This was demonstrated by isolation and characterization of monoadducts **2** and **3** and the cross-link adduct **4** from DNA exposed to MC. Two minor adducts **5** and **6** were also isolated. These are the 1″-β isomers of **2** and **3**, formed to the extent of 5% or less of **2** and **3** (Scheme I; Tomasz et al., 1986a, 1987, 1988a). The structure of a duplex oligonucleotide cross-linked at a single site by MC was directly determined by NMR combined with computation, revealing the covalent and three-dimensional alignment of the MC cross-link in the minor groove of the oligonucleotide (Norman et al., 1990).

Superimposed on the covalent reaction specificities of MC, a remarkable *base-sequence* specificity of the cross-links was demonstrated recently: cross-links were formed between the two guanines at CG·CG sequences but not at GC·GC sequences (Chawla et al., 1988; Teng et al., 1989; Weidner et al., 1989; Borowy-Borowski et al., 1990). Several different hypotheses were proposed for the molecular basis of this observed absolute discrimination between the two potential cross-link sites. Since the cross-linking process consists of *two* consecutive covalent reactions of MC (Tomasz et al., 1988b) one possibility is that sequence selection takes place at the first, monoalkylation step. If monoalkylation were absolutely specific to guanine residues preceded by a cytosine on the 5′-side, this would obviously explain the requirement for CG·CG as the DNA interstrand cross-link site: no cross-links would be formed at GC·GC since at this sequence the base 5′ to the Gs is other than C (unspecified) and therefore these guanines would be unreactive to the prerequisite monoalkylation.

Such site selectivity of DNA alkylation would not be unprecedented. Several DNA-interactive natural products are known to display remarkable sequence selectivity in their alkylation of DNA; for example the adenine alkylators CC-1065 (Reynolds et al., 1985) and duocarmycin A (Boger et al., 1990). We investigated the site selectivity of the monoalkylation process by MC. This problem was also of interest since MC may be regarded as a guanine-alkylator counterpart to the well-studied CC-1065. By use of a series of synthetic oligonucleotides as models for DNA, the relative intensities of alkylation of guanines in various sequences were quantitatively determined. It is reported that alkylation is greatly enhanced at 5′-CG sites. A hierarchy of bonding rates at 5′-NGN′ triplet sites was also established. The likely structural basis for the enhanced 5′-CG sequence recognition by MC was revealed. Remarkably, the *enhanced*, but by no means exclusive, monoalkylation at 5′-CG does not alone account for the known *absolute* selectivity of interstrand cross-link formation at such sites. The present findings allow us to propose a comprehensive theory for the structural basis of the DNA monoalkylation and cross-link site preferences of MC. While this work was in progress another report appeared on monoalkylation site specificities of MC (Li & Kohn, 1991). It lends

important qualitative support to the specific recognition of 5′-CG sequences by MC described here.

Materials and Methods

Oligonucleotides were synthesized on an automated DNA synthesizer, Model 380B, Applied Biosysems, Inc., using the phosphoramidite method. All reagents were purchased from Applied Biosystems, Inc., Foster City, CA. The crude products (1-μmol scale; trityl off), after deprotection by concentrated NH_4OH, were purified by a passage through a Sephadex G-25 (fine) column (2.5 × 56 cm; 0.02 M NH_4HCO_3 eluant). The void volume fraction containing the oligonucleotide was lyophilized. HPLC on an analytical C-3 (reverse-phase) column indicated ≥95% purity of the oligonucleotides. Their base composition was also routinely checked by enzymatic digestion to nucleosides followed by quantitative analysis using HPLC (see 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.

Mitomycin C (bulk) was supplied by Bristol Laboratories, Syracuse, NY. Decarbamoyl mitomycin C was prepared from MC by a published procedure (Kinoshita et al., 1971). NADPH:cytochrome *c* reductase was a gift from Dr. Wayne Backes, Louisiana State Medical School, New Orleans, LA.

HPLC was performed using reverse-phase columns (Beckman RPSC, C-3 Ultrapore, 4.6 × 75 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 parts of a Model 332 HPLC system. Oligonucleotides were eluted from C-3 columns using a 6–18% CH_3CN/0.1 M TEAA (pH 7.0) gradient in 24 min, at 1 mL/min flow rate. 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).

Standard Reaction Conditions of Monoadduct Formation between Mitomycin C and Oligonucleotides: (*A*) *Reductive Activation.* (1) $Na_2S_2O_4$ as reducing agent: 12-mer duplex (complementary or self-complementary) oligonucleotides (1 μmol of mononucleotide unit/mL) were incubated with MC (4 μmol/mL) in 0.1 M potassium phosphate buffer, pH 7.5, at 0 °C in an ice bath, under stirring, open to the atmosphere. Freshly prepared $Na_2S_2O_4$ solution (50 μL; 40 mM in above buffer) was added at once, to give a concentration of 2 μmol/mL of reaction mixture. After 1 h at 0 °C, the mixture was immediately chromatographed on a 2.5 × 56 cm Sephadex G-50 column, using 0.02 M NH_4HCO_3 as eluant. (2) NADPH:cytochrome *c* reductase/NADPH as reducing agent: The procedure was the same as above, except 8 μmol/mL NADPH and 6.12 units/mL NADPH:cytochrome *c* reductase were substituted for $Na_2S_2O_4$ and the reaction was conducted anaerobically, by constant purging with helium gas.

(*B*) *Acidic Activation.* The 12-mer duplex oligonucleotides and MC were incubated in 0.1 M potassium phosphate, pH 4, at the same concentrations as in the reductive activation experiments above; no reducing agent was added. Incubation at 0 °C for 4 h was followed by room temperature incubation for 2.5 h. Only a slight pH change was observed by the end of the reaction. The reaction mixture (1 mL) was neutralized by the addition of 5 mL of 0.02 M NH_4HCO_3 and chromatographed immediately over a Sephadex G-50 column as above.

Assay of the Molar Ratios of Nucleosides and Monoadduct **2** *in Enzymatic Digests of Oligonucleotides Alkylated by MC.* Sephadex G-50 chromatography of the reaction mixtures (above) yielded quantitative recovery of the unreacted and mitomycin-substituted oligonucleotides as a single fraction, separated from all low molecular weight components. This fraction was lyophilized to a salt-free state. The material (2 A_{260} units) was digested with SVD (2 units) and *E. coli* alkaline phosphatase (1.1 units) in 0.4 mL of 0.1 M Tris–2 mM $MgCl_2$ buffer, pH 8.2, at 45 °C for 4.5 h. The digest was analyzed directly by HPLC (above). Peak areas were integrated. Molar ratios of each individual component of the HPLC pattern were calculated by dividing a peak area by E_{254} of the corresponding nucleoside or nucleoside–mitomycin adduct **2** [dC, 6300; dG, 13000; dT, 6600; dA, 13300; adduct **2**, 24000 (Tomasz et al., 1986b)].

Calculation of the Mole Percent Yield of Adduct **2** *per Mole of Duplex Oligonucleotide.* From the molar ratio of dT, above, and the known nucleoside composition of the oligonucleotide, the molar ratio of the original duplex oligonucleotide was calculated as equal to (molar ratio of dT)/(number of dT per duplex oligonucleotide). In turn, mole % yield of **2** = (molar ratio of **2**/molar ratio of duplex oligonucleotide) × 100 = (molar ratio of **2**/molar ratio of dT/number of dT per duplex oligonucleotide) × 100. This formula was used directly for yields of **2** in duplex oligonucleotides having a single G in the duplex. When two Gs were present in self-complementary sites, the calculated yield was divided by 2, in order to normalize the results to one G/duplex. When two or more Gs were present in a non-self-complementary manner, the yield of adduct **2** from each individual G site was calculated by proportioning the total yield of **2** according to the observed proportions of the individual d-[G(M)pN]s obtained in nuclease P_1 digests, as described below in detail.

Digestion of oligonucleotides alkylated by MC with nuclease P_1/alkaline phosphatase was accomplished by adding 1.6 units of nuclease P_1 to 2 A_{260} units of oligonucleotide in 0.7 mL of dilute acetic acid, pH 5.5, and incubating the mixture at 55 °C for 2 h. After adjusting the pH to 8.2 by 0.5 M Tris, pH 8.2, alkaline phosphatase (3.7 units) was added and incubation continued for 2 h at 37 °C. The digest was directly applied to HPLC. The peaks corresponding to the unmodified nucleosides and d-[G(M)pC], d-[G(M)pG], d-[G(M)pT], and d-[G(M)pA] were identified by comparison of their elution times with those of authentic standards (Tomasz et al., 1986a). Molar ratios were calculated as described above. [E_{254} values of the four d-[G(M)pN]s were calculated as the sum of E_{254} of d-(GpN) and E_{254} of the mitosene (M) residue (11000).]

Results

Assay. Since unreacted and alkylated oligonucleotides did not separate well on HPLC, the yield of formation of MC monoadduct **2** in oligonucleotides was determined by a new method: after the alkylation reaction the unreacted and alkylated oligonucleotide mixture was digested to nucleosides and nucleotide–MC adducts and these were separated and analyzed using quantitative HPLC methodology, as illustrated in Figure 1. The mol % yields of **2** determined this way were highly reproducible. Another advantage of this assay was that it was *adduct-specific*; isomeric or other minor adducts were not included.

Conditions for Selective Monofunctional Activation of MC to Form Adduct **2**. $Na_2S_2O_4$ activates MC to bifunctional

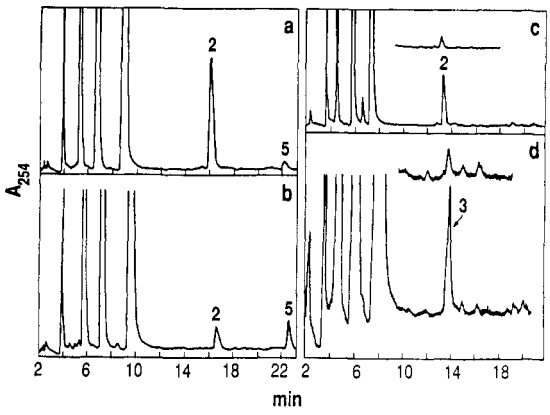


FIGURE 1: HPLC assay of monoadducts **2** and **3** in enzymatic digests of alkylated oligonucleotides. Digests of (a) oligonucleotide 3 and (b) oligonucleotide 8, both alkylated by MC, using $Na_2S_2O_4$ activation (Table I). (c) Digests of oligonucleotide 3 (bottom tracing) and oligonucleotide 8 (top, partial tracing), both alkylated by MC using cytochrome *c* reductase activation (Table I). (d) Digests of oligonucleotide 3 (bottom tracing) and oligonucleotide 8 (top, partial tracing), both alkylated by 10-decarbamoyl-MC, using $Na_2S_2O_4$ activation (Table I). Identification of the peaks of adducts (**2**, **3**, and **5**) was based on direct comparison of elution times with those of authentic standards determined just before or after the HPLC run of a digest. In (c) the elution times of **2** were lower than usual, most likely due to the use of a different HPLC column for that experiment.

reactivity under excess $Na_2S_2O_4$ and anaerobic conditions as manifested by the isolation of adducts **3** and **4** and none of monoadduct **2** from DNA when treated with MC under such conditions (Tomasz et al., 1987). However, both the presence of air and a substoichiometric amount of $Na_2S_2O_4$ have been shown to inhibit the bifunctional alkylation process. The C-10 position is not activated under such conditions and therefore only monofunctional alkylation at C-1 occurs (Tomasz et al., 1988a). Therefore, in the present work aerobic conditions and 0.5 mol of $Na_2S_2O_4$/mol of MC was employed, resulting in strictly monofunctional activation of MC, as evidenced by detection of monoadduct **2** but none of **3** or **4** in the HPLC patterns. Monoadduct **2**, however, was always accompanied by its 1″-β-isomer **5**, in low yields (Figure 1a,b). Isolation of **5** as a minor adduct from DNA (≤5% of **2**) was previously reported (Tomasz et al., 1986a). The sequence selectivity of formation of minor adduct **5** is currently under investigation. Both NADPH:cytochrome *c* reductase/NADPH and acidic pH activations resulted in the same monoalkylation patterns as the $Na_2S_2O_4$ method (e.g., Figure 1c).

Kinetics. It was shown previously that the alkylation of guanine by $Na_2S_2O_4$-activated MC in large excess proceeds by approximately first-order kinetics, until it is abruptly terminated, due to the exponential decay of activated MC (Borowy-Borowski et al., 1990; Cera et al., 1989). The observed alkylation yields obtained with the various oligonucleotides under identical reaction conditions can therefore be regarded as a measure of relative reactivities of the oligonucleotides, provided that the standard time allowed for the reactions is past their common "termination time". The present conditions satisfied this provision since it was determined that termination of the reaction (no further increase in yield) occurred at approximately 5 min for $Na_2S_2O_4$-activated reactions and 30 min for the cytochrome *c* reductase activated process (data not shown); i.e., well before the allotted 60-min standard reaction time in both cases.

Irreversibility of monoadduct formation under the actual reaction conditions was tested by the following experiment: The reaction products of oligonucleotide 2 and MC under $Na_2S_2O_4$ activation (Table I) were isolated directly (not after enzymatic digestion) by HPLC, under conditions previously described (Borowy-Borowski et al., 1990). The four components present in this mixture (the two unsubstituted starting strands and two M-substituted strands) were well separated in the system and each was identified by nucleoside analysis (Borowy-Borowski et al., 1990; data not shown). One of the isolated substituted strands, 5′-ICACG(M)TCIT, was annealed with the complementary unsubstituted strand 5′-CIACGTIC (3 A_{260} units each) in the usual reaction buffer and then incubated under the standard conditions of monoadduct formation, and the mixture was analyzed by HPLC. No change as compared with the starting composition of the oligonucleotide mixture was observed, indicating that no mitosene dissociated from the adducted strand during the incubation. Had it done so, two additional components would have appeared in the HPLC pattern: *unsubstituted* 5′-ICACGTCIT and *substituted* 5′-CIACG(M)TIC, resulting from realkylation of the duplex after the dissociation. Additionally, 5′-CIACG(M)TIC was incubated with excess $Na_2S_2O_4$ anaerobically at 0 °C; no loss of M was detectable. It is concluded from these results that the monoadduct was not reversible under these conditions.

Differentiation between Monoadducts Formed at Different G Sites in the Same Oligonucleotide: The Nuclease P_1 Method. Nuclease P_1 acts both as exo- and endonuclease, producing 5′-monophosphates. It cannot, however, cleave the GpN phosphodiester linkage when G is monoalkylated by MC, as seen from the resulting end products[2] d-[G(M)pN], dinucleoside phosphate derivatives of **2** (Tomasz et al., 1986a). Therefore, the 3′-nucleoside N serves as a marker for the location of the adduct within the oligonucleotide. Analysis by HPLC of nuclease P_1/alkaline phosphatase digests enabled us to determine the relative molar ratios of d-[G(M)pN] adducts originating from the same oligonucleotide. Typical

Table I: Mole Percent Yields[a] of Adduct **2** in Various Oligonucleotides Alkylated by MC

	oligonucleotide	mol % yield of **2** per G with activation by		
no.	structure[b]	$Na_2S_2O_4$	cytochrome reductase	acidic pH
1	5′-CIACGTIC	3		
2	5′-CIACGTIC 3′-TICTGCACI	33		
3	5′-ATATACGTATAT 3′-TATATGCATATA	31.4 (±1.2)[c]	21.6 (±0.9)[c]	9.9 (±0.3)[c]
4	5′-ATTAACGTTAAT 3′-TAATTGCAATTA	36		
5	5′-ATTATGG*TTATT 3′-TAATACCAATAA	14.7[d]	8.4	
6	5′-ATTATTGTTATT 3′-TAATAACAATAA	4.1	2.4	
7	5′-ATTATAGTTATT 3′-TAATATCAATAA	2	0.3	
8	5′-TATATGCATATA 3′-ATATACGTATAT	6.9 (±0.0)[c]	2	3.0 (±0.2)[c]
9	5′-ATTAAGCTTAAT 3′-TAATTCGAATTA	4.0		
10	5′-ATTATGGTTATT	1.3		

[a] See Materials and Methods for calculation. [b] The underlined sequences represent 5′-NGN′ triplets in which G is the alkylation site to give adduct **2**. [c] At least three determinations. [d] Yield of **2** at G*; see *a*.

[2] After alkaline phosphatase treatment.

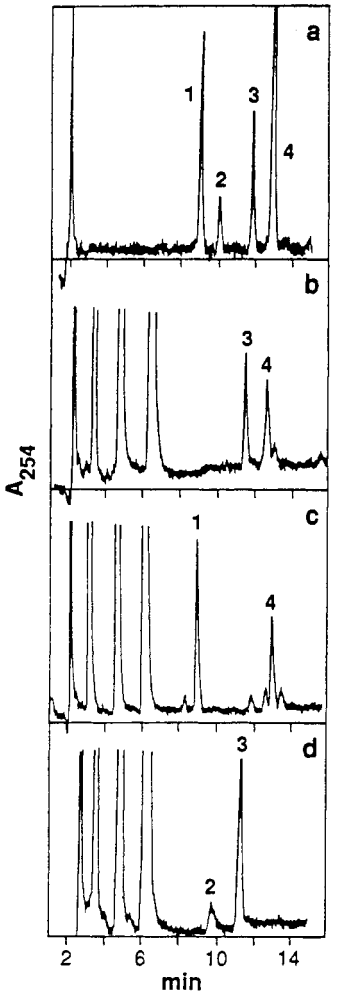


FIGURE 2: HPLC assay of d-[G(M)pN] (dinucleoside phosphate derivatives of adduct **2**) in nuclease P_1/alkaline phosphatase digests of alkylated oligonucleotides. (a) Mixture of the four authentic standard d-[G(M)pN]s: 1, d-[G(M)pC]; 2, d-[G(M)pG]; 3, d-[G(M)pT]; 4, d-[G(M)pA]. (b) Digest of oligonucleotide duplex 3 (Table III). (c) Digest of oligonucleotide duplex 5 (Table III). (d) Digest of oligonucleotide duplex 5 (Table I).

HPLC patterns are shown in Figure 2.

Effect of the 5′-Base on the Yield of Monoadduct Formation. Table I (oligonucleotides 2–7) presents the mol % yields of monoadduct **2** (normalized for one G; see Materials and Methods for calculations) in a series of oligonucleotides having the sequence 5′-NGN′, in which the 5′-base N was varied while the 3′-base N′ was kept constant as T. The results are also illustrated in Figure 3a. They indicate a striking enhancement of the yield at 5′-CG sequences: 33–36%, compared to 2% at 5′-AG and 4.1% at 5′-TG. The 5′-GG sequence also showed enhanced reactivity, although to a lesser extent (14.7% yield). The relative reactivities of the Gs in the four triplets may then be expressed as follows: 5′-CGT/5′-GGT/5′-TGT/5′-AGT = 16.5:9:2:1. The observed enhancements at 5′-CG and 5′-GG (from here on referred to as 5′-base effect) are specific to the duplex state of the oligonucleotides, as seen from the low yields of **2** in the case of oligonucleotides 1 and 10: 3% and 1.3% compared to 33% and 14.7% of the corresponding duplex oligonucleotides 2 and 5, respectively. As another indication of duplex requirement, alkylation of oligonucleotide 2 at room temperature instead of the standard 0 °C resulted in a decreased yield, from 33 to 15%.

Sequence Selectivity under Enzymatic Reduction Conditions. Activation of MC by NADPH:cytochrome *c* reductase resulted in enhancements of alkylation at the same 5′-CG and

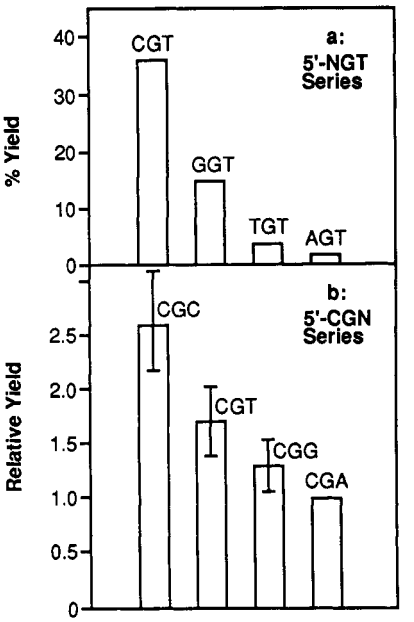


FIGURE 3: Yield of monoadduct **2** as a function of the 5′-base (a; 5′-NGT series) and the 3′-base (b; 5′-CGN series). (a) The complete sequence of the oligonucleotides and the % yield data are given in Table I (oligonucleotides 4–7, $Na_2S_2O_4$ activation column). (b) The relative yields were calculated and averaged from data obtained with seven oligonucleotide duplexes, given in Table III, last column, as described in the text.

5′-GG sequences as $Na_2S_2O_4$ activation (Table I; compare oligonucleotides 3 and 5–7; see also Figure 1c). Although the yields were somewhat lower under these conditions, the observed relative specificities were in good agreement with those of the more efficient $Na_2S_2O_4$-activated system.

Sequence Selectivity under Acid Activation Conditions. Oligonucleotides 3 and 8 in Table I served as the benchmark pair to compare the selectivities of acid-activated MC with those of the $Na_2S_2O_4$-activated and cytochrome *c* reductase-activated drug. Acid-activated MC showed the same selectivity of alkylation in this test as its two reductively activated counterparts: as seen from the data in Table I, the yield of **2** at the 5′-CGT sequence was enhanced 3.3-fold over that at the 5′-TGC sequence, in qualitative agreement with the 4.5- and 5.4-fold enhancements observed in the two reductive activation systems.

The alkylation selectivity of 10-decarbamoyl-MC was compared to that of MC, under $Na_2S_2O_4$ activating conditions. Again, in the test using the benchmark pair, i.e., oligonucleotides 3 and 8 (Table I), similar selectivity to that of MC was observed, as illustrated in Figure 1d: 5% yield of adduct **3** at the 5′-CGT sequence and 0.8% yield at 5′-TGC, indicating approximately 6-fold selectivity for the former.

Replacement of deoxyguanosine by deoxyinosine at 5′-CG duplex sites dramatically diminished the alkylation yields. As seen in Table II, change from oligonucleotide 11 to oligonucleotide 12 completely abolished the enhanced, 36% yield of alkylation; the yield reverted to the base value of 2–4%, seen with low-reactivity-type oligonucleotides (cf. Table I). Similarly, diminished yields were obtained with oligonucleotides 14 and 15, compared to the parent 13. Inosine replacement at the 5′-GG site also abolished the yield enhancement at that site, as seen upon comparison of oligonucleotides 16 and 17 in Table II.

Effect of the 3′-Base on the Yield of the Monoadduct. Direct comparison of the yields of **2** from oligonucleotides 6 and 8 (Table I) indicated 6.9/4.1 = 1.7-fold alkylation preference of 5′-TGC over 5′-TGT; similar comparison of

Table II: Effect of Inosine Substitutions on the Yield of **2** in Oligonucleotides Alkylated by MC under $Na_2S_2O_4$ Activation Conditions

oligonucleotide no.	oligonucleotide structure	mol % yield of **2** per G[a]	5′-CG-3′-GC-
11	5′-ATTAACGTTAAT 3′-TAATTGCAATTA	36	yes
12	5′-AATTACGTTTAA 3′-TTAATICAAATT	2.6	no
13	5′-ATATACGCGTATAT 3′-TATATGCGCATATA	13	yes
14	5′-ATATACICGTATAT 3′-TATATGCICATATA	2	no
15	5′-ATATACGCIATATA 3′-TATATICGCTATAT	1.8	no
16	5′-TATAAGG*CATTA 3′-ATATTCCGTAAT	13.2[b]	
17	5′-TATAAIGCATTA 3′-ATATTCCGTAAT	3.2	

[a] See Materials and Methods for calculation. [b] Yield of **2** at G*; see *a*.

oligonucleotides 7 and 9 gave 4/2 = 2-fold preference of 5′-AGC over 5′-AGT. This good agreement between the 3′-base effects in the two sets suggested that the 3′-base exerted its effect independently of the 5′-base and that this effect is relatively modest, compared to that of the 5′-base (see above). A systematic study of the 5′-CGN series confirmed these notions. Duplex oligonucleotides having two different 5′-CGN sites in the same or opposite strands were alkylated. Using the nuclease P_1 method, the relative molar yields of alkylation at these sites were determined by assaying d-[G(M)pN]; the results are shown in Table III. The experimentally obtained 5′-CGN/5′-CGN′ yield ratios listed in the last column were all normalized to 5′-CGN/5′-CGA yield ratios in which the value of the 5′-CGA yield was fixed as 1.0. This gave the following relative reactivities of the Gs in the four triplets: 5′-CGC/5′-CGT/5′-CGG/5′-CGA = 2.6:1.7:1.3:1.0 (±18%). These results are illustrated in Figure 3b.

The 3′-base effect seems to be independent from the nature of the 5′-base. The ratio of reactivities of the Gs in the 5′-CGC/5′-CGT triplet pair is 1.5 as calculated from the above relationship; in the 5′-TGC/5′-TGT triplet pair it is 1.7 and in the 5′-AGC/5′-AGT pair it is approximately 2, as discussed above. This constitutes excellent agreement among the three triplet pairs, having different 5′-bases from one pair to another. In another test of this phenomenon, the 5′-GGT/5′-GGA adduct ratio (for the internal Gs) was calculated from the data in Table III as 1.4; this is again in good agreement with the 1.7 ratio observed in the case of 5′-CGT/5′-CGA, above.

Discussion

Computer-generated models of the MC monoadduct **2** incorporated in duplex oligonucleotides indicated a 3 base pair span by the bound drug molecule, extending by 1 base pair above and 1 base pair below the covalently linked guanine in the minor groove, in a fixed orientation (Figure 4) (Remers et al., 1988; Verdine, 1986). This model suggested that monoadduct formation may be influenced by the base sequence of the triplet 5′-NGN′ in which G is the covalent reaction site. The results of the present study fulfilled this expectation: Both the 5′-base and the 3′-base of the triplet were shown to have an effect on the reactivity of G toward MC. The 5′- and 3′-base effects were assessed separately, by examining the reactivity of Gs in two series of triplets: 5′-NGT and 5′-CGN, respectively, with N being variable in each case. A large enhancement was observed at 5′-CGT relative to other 5′-NGT triplets (Figure 3a). In the 5′-CGN series N = C was also the most enhancing as the 3′-base but the effect was relatively modest: 1 order of magnitude lower than that exerted by C in the 5′-base position (Figure 3b).

The main conclusions regarding the sequence specificity of the monoalkylation of guanine by MC are summarized as follows: (i) 5′-CGN triplets show greatly enhanced reactivity; 5′-CGC is the most reactive of all four. (ii) 5′-GGN triplets show moderately enhanced reactivity, approximately half that of the 5′-CGNs. (iii) The 3′-base of the triplet has a relatively modest modulating effect; the order of reactivity is 3′-(C > T > G > A). (iv) Reactions are not absolutely specific to 5′-CG and 5′-GG: Adduct **2** was well detectable in all oligonucleotide reactions (Table I; see Figure 1b for a salient example). (v) The 5′- and 3′-base effects appear to be independent of each other. Therefore, relative yields (reactivities) of experimentally untested 5′-NGN′ triplets may be estimated from those of others, determined by experiment. For example, the ratio of the yield of 5′-CGT to the yield of 5′-CGA, found

Table III: Relative Molar Yields of d-[G(M)pN][a] in Oligonucleotides Alkylated by MC under $Na_2S_2O_4$ Activation Conditions

oligonucleotide no.	oligonucleotide structure[b]	relative yields of d-[G(M)pC]	d-[G(M)pG]	d-[G(M)pT]	d[G(M)pA]	relative yields of alkylation at various NGN's
1	5′-ATTATCGCTATT 3′-TAATAGCGATAA	1			0.54	CGC[c]/CGA=1:0.54
2	5′-ATTAGCGTTATT 3′-TAATCGCAATAA	1		0.68		CGC[c]/CGT=1:0.68
3	5′-ATTATCGTTATT 3′-TAATAGCAATAA			1	0.57	CGT/CGA=1:0.57
4	5′-ATTATCGGTATT 3′-TAATAGCCATAA		1	0.51	0.75	CGG/CGA/GGT = 1:0.75:0.51
5	5′-ATATCGCGATAT 3′-TATAGCGCTATA	1			0.32	CGC/CGA=1:0.32
6	5′-TATCCGCGGATA 3′-ATAGGCGCCTAT	1	0.67		0.25	CGC/CGG/GGA = 1:0.67:0.25
7	5′-ATATACGCGTATAT 3′-TATATGCGCATATA	1		0.55		CGC/CGT = 1:0.55

[a] Dinucleoside phosphate derivatives of adduct **2**. [b] The guanines marked with a dot are potential sites for d-[G(M)pN] products. [c] d-[G(M)pC] was assumed to arise exclusively from alkylation at CGC. The other potential triplet from which d-[G(M)pC] could have originated is AGC; it is relatively unreactive, however (Table I, oligonucleotide 9), and was considered to have negligible contribution to the observed yield of d-[G(M)pC].

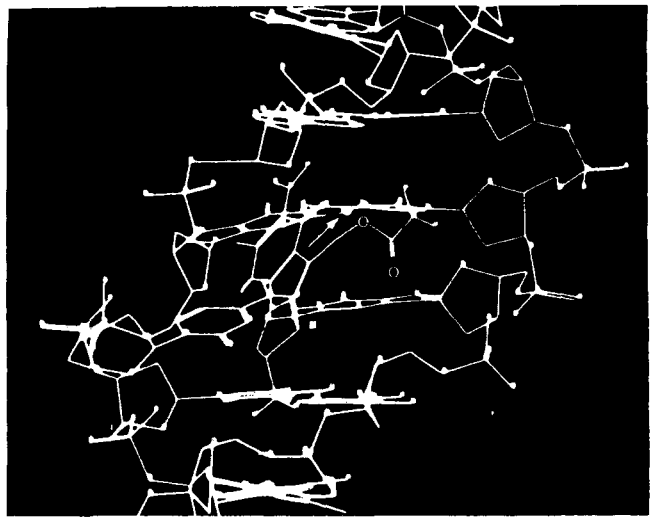


FIGURE 4: Computer-simulated energy-minimized molecular model of MC monoadduct **2** incorporated into the central 5′-CG sequence of the self-complementary oligonucleotide d-GCATCGATGC in its duplex B-DNA form. An approximately 6 base pair long segment from the middle of the structure is shown. With the MacroModel program, monoadduct **2** itself was first energy-minimized to a root-mean-square gradient (RMSG) value of 0.1 kJ/(mol Å), and then the C-1″/N^2 bond was severed and the remaining mitosene structure was docked and connected to the N^2 atom of the guanine in the central 5′-CG of the oligonucleotide duplex. Subsequent energy minimization was terminated at RMSG = 0.4 kJ/(mol Å). The white square indicates the location of the covalent linkage between the mitosene (C-1″) and guanine (N^2). The arrow points to the putative H-bond between the N^2-H of the guanine in the opposite strand and the C-10″-O of the mitosene (cf. Figure 5, structure A). Reproduced with permission from data of Verdine (1986).

to be 1.7:1.0 (Figure 3b), applies also to the yield ratio of 5′-AGT/5′-AGA. Since 5′-AGT gave 2% yield experimentally (Figure 3a), the yield of 5′-AGA is estimated as 2/1.7 = 1.2%.

A highlight of the present findings, i.e., enhanced recognition at 5′-CG and, to a lesser extent, 5′-GG sequences, receives important support by a recent study of Li and Kohn (1991). In their work, two DNA restriction fragments were monoalkylated by MC under $Na_2S_2O_4$ activation conditions. Applying the λ-exonuclease assay, exonuclease stop sites were observed at all 5′-CG and 5′-GG sequences upon gel electrophoresis of the λ-exonuclease digests, indicating 5′-CG and 5′-GG specificity of the alkylation sites. No quantitative data were derivable from the electrophoretic pattern, however, and no information was obtained regarding 3′-base specificity. The powerful new type of assay used in the present work (isolating the adduct **2** directly) provides a quantitative and detailed picture of MC alkylation specificity encompassing both the 5′- and 3′-bases adjacent to the alkylated guanine. It also shows that this specificity is not absolute for 5′-CG and 5′-GG, as indicated by the above authors' results. Nevertheless, the qualitative agreement obtained between the restriction fragment and synthetic oligonucleotide systems lends considerable mutual validity to these findings.

Another study of the sequence specificity of MC monoalkylation was carried out theoretically by Remers, Kollmann, and co-workers (1988). Four decanucleotides having adduct **2** substituted in the central G in each of 5′-CGC, 5′-GGC, 5′-AGC, and 5′-TGC sequences were compared with respect to their "net binding energies". It was concluded that 5′-CGC was thermodynamically most favored to bond MC as monoadduct, and 5′-GGC was second, in remarkable agreement with the present results.

Demonstration of the same sequence selectivity by NADPH:cytochrome *c* reductase-activated MC as that in the

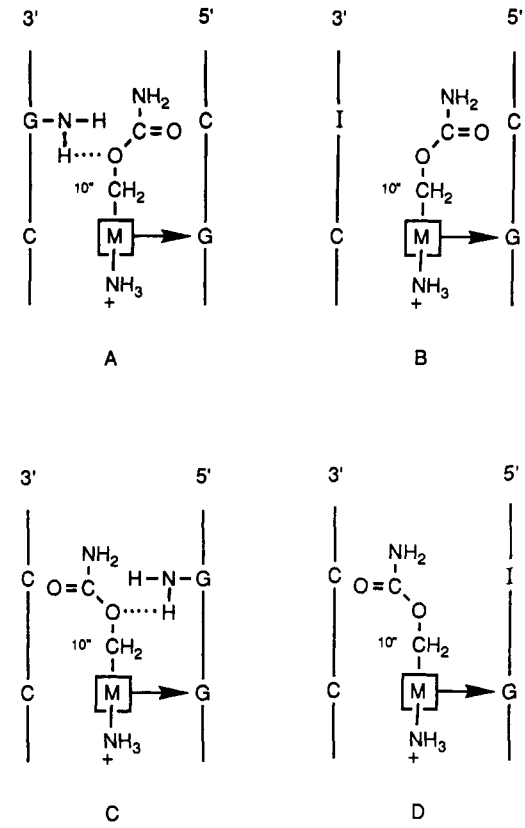


FIGURE 5: Sequence-specific H-bond between C-10″-O of the activated form of MC and DNA. The boxed symbol M represents MC in its covalently reactive form(s), generated by reduction or protonation; two of its functional groups (C-10″-carbamate and 2″-NH_3^+) are indicated specifically. These diagrams are simplified pictures of the alignment of the drug in the minor groove, drawn after the molecular model in Figure 4. (A) Specific H-bond at 5′-CG sequence. (B) Lack of H-bond at 5-CG when the opposite strand is inosine-substituted. (C) Specific H-bond at 5′-GG sequence. (D) Lack of H-bond at 5′-IG sequence.

$Na_2S_2O_4$ activation system is significant, since it indicates that this selection prevails under bioreductive alkylation conditions; therefore the results may be extrapolated with more confidence to intracellular alkylation of DNA by MC.

One of the most interesting questions posed by the results is what factors determine the enhanced reactivity of MC with guanines in the 5′-CG sequence. A decisive clue was provided by the alkylation behavior of inosine-substituted oligonucleotides (Table II): When inosine replaced guanine in the strand opposite from the CG alkylation site, the enhanced reactivity of the guanine in the 5′-CG sequence was abolished. Thus, the 2-NH_2 group of the guanine in the opposite strand must be essential for the enhancement.[3] We propose, as shown in Figure 5, that it forms a specific H-bond to the C-10″ O-atom of activated MC and this connection facilitates the alkylation step, either by simply concentrating the precovalent species at such sites or, more likely, by also enhancing the rate constant for the covalent reaction. This is in accordance with current understanding of the sources of reactivity of bound complexes (Menger, 1985). (A third alternative, stabilization

[3] The possibility that the inosine-containing duplexes dissociated under the reaction conditions (0 °C, 0.1 M phosphate, pH 7.4), and therefore the lower yield was the consequence of the single-stranded state, was ruled out unequivocally by oligonucleotide melting curve determinations. For example, the T_m of oligonucleotides 11 and 12 was 32.1 and 25.6 °C, respectively (0.1 M NaCl–0.015 M Tris–0.001 M Na_4EDTA, pH 7.5) and the melting profiles indicated that both were present fully in the duplex form at 0 °C, the temperature employed in the reactions with MC (unpublished observation).

of the *product* by this H-bond, may not play a part in this case, since the reaction is irreversible under the conditions used in the present work.) Previous modeling studies support the proposed mechanism: both CPK and energy-minimized modeling of the monoadduct indicate close proximity of the C-10″ oxygen to the G-N^2 in the opposite strand (Tomasz et al., 1986; Remers et al., 1988; Verdine, 1986; Figure 4). The results of inosine substitution in the 5′-GG series (Table II; oligonucleotides 16 and 17) suggest that an analogous model applies to the enhanced reactivity of the guanine at 5′-GG, also shown in Figure 5.

This mechanism, explaining the enhanced reactivity at 5′-CG and 5′-GG sites, is different from a previous model, proposed by Li and Kohn (1991). These authors also predicted a critical H-bond between the drug and the nonbonding guanine in the opposite strand or in the same strand in the adjacent position, respectively. However, it was assigned between the 8″-OH of the hydroquinone ring of the reduced form of MC and the N-3 atom of the opposite guanine, in the 5′-CG case, or N-3 of the adjacent guanine, in the 5′-GG case. This model is not compatible with the results of the present study. Specifically, the observed inosine substitution effects and the sequence specificity of the alkylation by *acid-activated MC*, which is obviously in the quinone form lacking an 8″-OH group, provide strong evidence for our model, as in Figure 5.

The effect of the 3′-base on the reactivity of G in 5′-NGN′ triplets is more moderate than that of the 5′-base: only a 2.6-fold difference was observed between the least reactive (N′ = A) and most reactive (N′ = C) members of the series (see Figure 3b). A similar, 2.5-fold increased reactivity was reported for the cross-linking reaction of MC at 5′-CGPyr, compared to that at 5′-CGPu (Borowy-Borowski et al., 1990; Millard et al., 1991). It was proposed to be due to the effect of the 3′-base on the rate of the monoalkylation step. Since such an effect was directly observed now for the monoalkylation alone, this indicates that our proposal was correct. A possible mechanism, as suggested in detail earlier, is that 3′-Pyr assists in the removal of the proton from the 2-amino group of the guanine during the monoalkylation bonding process (Borowy-Borowski et al., 1990).

Relationship between Sequence Specificity of Cross-Link Formation and Monoalkylation. A remarkable result emerging from these studies is that the monoalkylation by MC is specific to 5′-CG, that is, the same sequence as required for MC cross-link formation. Is it then simply sequence selection at the monoalkylation step that explains the sequence specificity of cross-links to CG·CG sites, as opposed to GC·GC sites? The present results show that it is not. Cross-link specificity for CG·CG was found earlier to be virtually absolute, since cross-linking of oligonucleotides having the GC·GC sequence (e.g., oligonucleotide 8 in Table I) was consistently undetectable (Teng et al., 1989; Weidner et al., 1989; Borowy-Borowski et al., 1990). In contrast, the degree of selectivity for 5′-CG at the monoalkylation step, as observed here, is far from absolute. The prototype 5′-GC-containing oligonucleotide 8 (Table I) is monoalkylated at 6.9% yield, as compared to 31% yield of alkylation of oligonucleotide 3; this corresponds to only 4.5-fold selectivity of monoalkylation for the 5′-CG sequence. Figure 1 (a and b) illustrates these results clearly. Oligonucleotide 9, another 5′-GC-containing one, was also monoalkylated at a well-detectable level. There is apparently an additional, independent factor which prevents closure of the cross-link after the monoalkylating step has taken place. This factor is most likely the specific orientation of the monoadduct, in which the C-10″ position of the drug is extending in the 5′-direction from the covalently linked guanine (Figures 4 and 5). In the 5′-CG sequence this places C-10″ in juxtaposition to the N^2 of the guanine in the opposite strand (arrow in Figure 4), ready for bond formation, i.e., cross-link closure. In the 5′-GC sequence, however, the same drug orientation places the C-10″ of MC 2 base pairs away from the guanine in the opposite strand, thereby excluding any chance for cross-link closure. Consequently, guanines at the GC·GC sequence are not cross-linkable. Evidence for the specificity of this orientation of the monoadduct has come from several modeling studies (Tomasz et al., 1986; Verdine, 1986; Rao et al., 1986; Remers et al., 1988; Arora et al., 1990; Millard et al., 1990), as well as certain experimental indications (Borowy-Borowski et al., 1990). Hopkins and co-workers (Millard et al., 1990) attributed it to the chirality of the C1″–N^2 (guanine) bond, which renders the opposite orientation sterically impossible in the minor groove.

From the above analysis a remarkable conclusion emerges: In its initial, monofunctional attack on DNA, MC utilizes a special mechanism for recognizing 5′-CG and 5′-GG sites selectively (proposed as in Figure 5), which guides the drug preferentially to its potential cross-link producing sites, at CG·CG and GG·CC (interstrand and intrastrand[4] cross-links, respectively). As a result, relatively few of the available drug molecules will attack DNA at the "nonproductive" 5′-TG and 5′-AG sites, i.e., where subsequent cross-link formation is not possible. This mutual reinforcement among the covalent reactivity, molecular geometry, and specific, noncovalent sensor group (proposed to be the C-10″ side chain) of the MC molecule to induce the lethal DNA cross-linking action at maximum efficiency may be a remarkable example of molecular evolutionary design of natural toxic agents.

Acknowledgments

We thank Dr. D. M. Vyas of Bristol-Myers Squibb Co., Wallingford, CT, for a generous gift of mitomycin C. We also thank Dr. G. L. Verdine, Harvard University, for his permission to reproduce the model of the MC monoadduct for Figure 4 from his Ph.D. Thesis.

References

Arora, S. K., Cox, M. B., & Arjunan, P. (1990) *J. Med. Chem. 33*, 3000–3008.

Boger, D. L., Ishizaki, T., Zarrinmayeh, H., Munk, S. A., Kitos, P. A., & Suntornwat, O. (1990) *J. Am. Chem. Soc. 112*, 8961–8971.

Borowy-Borowski, H., Lipman, R., & Tomasz, M. (1990) *Biochemistry 29*, 2999–3004.

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.) pp 305–316, Adenine Press, Guilderland, NY.

Cera, C., Egbertson, M., Teng, S. P., Crothers, D. M., & Danishefsky, S. J. (1989) *Biochemistry 28*, 5665–5669.

Iver, V. N., & Szybalski, W. (1963) *Proc. Natl. Acad. Sci. U.S.A. 50*, 355–365.

Iyer, V. N., & Szybalski, W. (1964) *Science 145*, 55–68.

Keyes, S. R., Rockwell, S., & Sartorelli, A. C. (1985) *Cancer Res. 45*, 3642.

Kinoshita, S., Uzu, K., Nakano, K., & Takahashi, T. (1971) *J. Med. Chem. 14*, 109–112.

[4] It was shown recently that MC intrastrand cross-links are formed at 5′-GG sequences (Tomasz et al., 1990; also R. Bizanek et al., manuscript submitted).

Li, V., & Kohn, H. (1991) *J. Am. Chem. Soc. 113*, 275–283.
Menger, F. M. (1985) *Acc. Chem. Res. 18*, 128–134.
Millard, J. T., Weidner, M. F., Raucher, S., & Hopkins, P. B. (1990) *J. Am. Chem. Soc. 112*, 3637–3641.
Millard, J. T., Weidner, M. F., Kirchner, J. J., Ribeiro, S., & Hopkins, P. B. (1991) *Nucleic Acids Res. 19*, 1885–1891.
Norman, D., Live, D., Sastry, M., Lipman, R., Hingerty, B. E., Tomasz, M., Broyde, S., & Patel, D. J. (1990) *Biochemistry 29*, 2861–2876.
Pan, S.-S., Andrews, P. A., Glover, C. J., & Bachur, N. R. (1984) *J. Biol. Chem. 259*, 959–966.
Rao, N. S., Singh, U. C., & Kollman, P. A. (1986) *J. Am. Chem. Soc. 108*, 2058–2068.
Remers, W. A., Rao, S. N., Wunz, T. P., & Kollman, P. A. (1988) *J. Med. Chem. 31*, 1612–1620.
Reynolds, V. L., Molineux, I. J., Kaplan, D. J., Swenson, D. H., & Hurley, L. H. (1985) *Biochemistry 24*, 6228–6237.
Teng, S. P., Woodson, S. A., & Crothers, D. M. (1989) *Biochemistry 28*, 3901–3907.
Tomasz, M., & Lipman, R. (1981) *Biochemistry 20*, 5056–5061.
Tomasz, M., Mercado, C. M., Olson, J., & Chatterjie, N. (1974) *Biochemistry 13*, 4878–4887.
Tomasz, M., Chowdary, C., Lipman, R., Shimotakahara, S., Veiro, D., Walker, V., & Verdine, G. L. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6702–6706.
Tomasz, M., Lipman, R., Verdine, G. L., & Nakanishi, K. (1986b) *Biochemistry 25*, 4337–4343.
Tomasz, M., Lipman, R., Chowdary, D., Pawlak, J., Verdine, G. L., & Nakanishi, K. (1987) *Science (Washington, D.C.) 235*, 1204–1208.
Tomasz, M., Lipman, R., McGuinness, B. F., & Nakanishi, K. (1988a) *J. Am. Chem. Soc. 110*, 5892–5896.
Tomasz, M., Chawla, A. K., & Lipman, R. (1988b) *Biochemistry 27*, 3182–3187.
Tomasz, M., Borowy-Borowski, H., & McGuinness, B. F. (1990) in *Molecular Basis of Specificity in Nucleic Acid–Drug Interactions (The Jerusalem Symposia on Quantum Chemistry and Biochemistry)* (Pullman, B., and Jortner, J., Eds.) Vol. 23, pp 551–564, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Verdine, G. L. (1986) Ph.D. Thesis, Columbia University, New York, NY.
Weidner, M. F., Millard, J. T., & Hopkins, P. B. (1989) *J. Am. Chem. Soc. 111*, 9270–9272.
Zon, G., Gallo, K. A., Samson, C. J., Shao, K.-L., Summers, M. F., & Byrd, R. A. (1985) *Nucleic Acids Res. 13*, 8181–8196.
Zwelling, L. A., Anderson, T., & Kohn, K. W. (1979) *Cancer Res. 39*, 365–369.

Proton Exchange in DNA–Luzopeptin and DNA–Echinomycin Bisintercalation Complexes: Rates and Processes of Base-Pair Opening†

Jean Louis Leroy,*,‡ Xiaolian Gao,§,∥ Vinod Misra,§ Maurice Guéron,‡ and Dinshaw J. Patel§

Groupe de Biophysique, Ecole Polytechnique, 91128 Palaiseau, France, and Department of Biochemistry and Molecular Biophysics, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Received July 31, 1991; Revised Manuscript Received October 23, 1991

ABSTRACT: Imino proton exchange studies are reported on the complexes formed by bisintercalation of luzopeptin around the two central A·T pairs of the d(CCCATGGG) and d(AGCATGCT) duplexes and of echinomycin around the two central C·G pairs of the d(AAACGTTT) and d(CCAAACGTTTGG) duplexes. The depsipeptide backbone of the drugs occupies the minor groove of the complexes at the bisintercalation site. The exchange time of the amide protons of the depsipeptide rings provides a lower estimate of the complex lifetime: 20 min at 15 °C for the echinomycin complexes and 4 days at 45 °C for the luzopeptin complexes. The exchange time of imino protons is always shorter than the complex lifetime. Hence, base pairs open even within the complexed oligomers. For the two base pairs sandwiched between the aromatic rings of the drug, the base-pair lifetime is strongly increased, and the dissociation constant is correspondingly reduced. Hence, the lifetime of the open state is unchanged. This suggests similar open states in the free duplex and in the complex. In contrast to the sandwiched base pairs, the base pairs flanking the intercalation site are not stabilized in the complex. Thus, the action of the bisintercalating drug may be compared to a vise clamping the inner base pairs. Analysis suggests that base-pair opening may require prior unwinding or bending of the DNA duplex.

Luzopeptin and echinomycin are bifunctional intercalating agents of related structure which possess antimicrobial and antitumor activities (Rose et al., 1983; Ward et al., 1965). They consist of two aromatic rings attached by a cyclic (luzopeptin) or a bicyclic (echinomycin) peptidic linker (Chart I). Both drugs bisintercalate in DNA, with the cyclic peptidic linker in the minor groove.

The quinoline rings of luzopeptin bisintercalate at the d(C–A) d(G–T) step of the d(CATG) (Zhang & Patel, 1991) and d(GCATGC) (Searle et al., 1989) sequences, spanning the two central A·T pairs. All the base pairs of the d(-CATG-) sequence retain the Watson–Crick alignment.

The quinoxaline rings of echinomycin span the two C·G pairs of the d(-ACGT-) and d(-TCGA-) sequences. The A·T pairs flanking the intercalation sites may adopt the Hoogsteen

† This research was supported in part by NIH Grant CA 46778 to D.P. and by the Association pour la Recherche sur le Cancer to M.G.; X.G. was a fellow of the Leukemia Society of America.

‡ Ecole Polytechnique.
§ Columbia University.
∥ Present address: Glaxo Research Labs, 5 Moore Drive, Research Triangle Park, NC 27709.

0006-2960/92/0431-1407$03.00/0