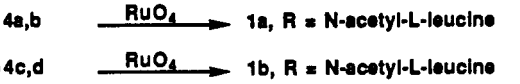
Hydrogenolysis of the benzyl ether affords four compounds, (14a–d), two sets of which could be separated chromatographically. The ^{13}C, ^{1}H, and ^{19}F NMR of the compounds obtained by chromatography indicated the more polar spot (TLC) contained a 2.5:1 mixture of diastereomers while the more rapid moving spot existed as a 1:1 mixture. Although we were unable to solve the stereochemistry through conventional NMR techniques, we were successful in assigning the relative stereochemistry of a single diastereomer by employing a phase-sensitive 2-D NOESY.[24] This experiment allows for the quantitation of interproton distances by calculating the 3-D volume integrals of the NOESY cross peaks.[25] The calculated interproton distances of the major isomer associated with the more slowly moving pair of diastereomers revealed H_2 is cis to H_3, thus establishing the cis nature of the fluorine/methyl stereochemical relationship. We were able to selectively crystallize a single diastereomer from the 14c/14d mixture and obtain confirmation of our stereochemical assignment. X-ray crystallographic analysis[26] (Figure 2) of this diastereomer (14d) revealed (Table I) a reassuring agreement in the interproton distances.

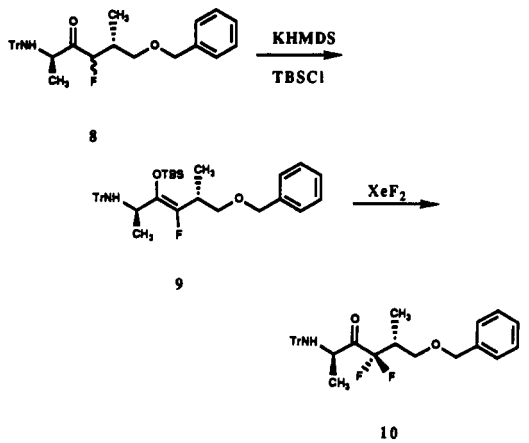
Subsequent confirmation of our successful separation of the fluorine diastereomers was provided by execution of the final step of the synthesis. Oxidation of 14a,b with RuO_4[27] afforded the target isostere 1a (56%) as a single diastereomer. Likewise, oxidation of 14c,d under identical conditions provided the diastereomeric target isostere 1b.



The synthetic blueprint outlined in this report represents the first methodology available to construct monofluoro ketone peptide isosteres with chiral centers intact and the peptide backbone spacing maintained. This method,[28] in addition to novel construction strategies for the synthesis of both difluoro ketone and trifluoromethyl ketone[29] isosteres recently developed in our laboratory, should expedite access to a variety of potent inhibitors and facilitate our understanding of the inhibitory mechanisms[1a,b,f] of this interesting class of molecules.

Acknowledgment. The authors wish to express their appreciation for the contribution of Ms. Ruth Brannon in the preparation of this manuscript. In addition, we also wish to acknowledge the technical assistance of Dr. Charles Eads.

(23) Enol ether 7b can also be fluorinated with this technique to afford the monofluoro ketone 8. However, we were unsure of the compatibility of this functionality with the required subsequent reactions and therefore opted for the reported sequence. Interestingly, we were also able to form the fluoroenol ether 9 from 8 and fluorinate once again to afford the difluoro ketone 10.



(24) We had not actually separated 14c from 14d at this point but the pertinent resonances were resolved in the ^{1}H NMR spectrum.

(25) The phase-sensitive NOESY spectrum data set was recorded into 2048 time-domain data points for 512 t_1 experiments. A mixing time of 250 ms and recycle delay of 6 s were used. A Gaussian multiplication function of 5 Hz was used in the t_1 dimension, while a Gaussian multiplication of 11 Hz and a trapezoidal function of the last 128 points of the free induction decay were applied in the t_2 dimension before Fourier transformation. The final data matrix was 1KX1K.

(26) Single-crystal X-ray diffraction data for 14d, $C_{30}H_{54}F_2N_4O_8$, were collected on a Siemens R3m/E diffractometer with γ = 1.5418 Å (graphite monochromatized), T = 295 K, ω-scan mode (1.0° ranges, 4–30°/min speeds), $3° \leq 2\theta \leq 110°$. Cell data: a = 10.718 (4) Å, b = 17.142 (5) Å, c = 11.220 (2) Å, β = 115.88 (2)°, V = 1854.7 (9) Å^3, D_{calc} = 1.140 g/cm^3; monoclinic, space group $P2_1$, Z = 2; clear, hexagonal rod crystal 0.12 × 0.16 × 0.24 mm. A total of 4909 reflections gave 2399 unique data (R_{int} = 0.02) of which 2033 were observed ($F > 4\sigma$), absorption corrected (μ = 0.709 mm^{-1}, T_{min} = 0.87, T_{max} = 0.92), and used in the structural refinement, yielding R = 0.037 (396 refined parameters), Rw = 0.044 (weights $w = [\sigma^2(F) + 0.0008F^2]^{1/2}$), GOF = 1.15, and difference-map residuals of −0.12 to +0.16 eÅ^{-3}.

(27) Carlsen, P. H. J.; Katsuki, T.; Martin, V. S.; Sharpless, K. B. *J. Org. Chem.* 1981, *46*(19), 3936.

(28) Given the ready availability of reaction partners similar to 2 and 3 (ref 12), it is not difficult to accept the potential generality of this method for the synthesis of alternative monofluoro ketone isosteric replacements.

(29) New methods for the synthesis of difluoro ketones and trifluoromethyl ketones have been developed in our lab. The synthesis of novel inhibitors of a variety of proteolytic enzymes utilizing this methodology will be reported in due course.

Reaction of Sodium Dithionite Activated Mitomycin C with Guanine at Non-Cross-Linkable Sequences of Oligonucleotides

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Summary: The reaction of sodium dithionite activated mitomycin C with guanine at non-cross-linkable sequences in oligonucleotides (as well as DNA) yielded a mixed 1″-deoxyguanosine, 10″-sulfonate mitosene derivative, which has implications for published model activation and DNA alkylation studies of this antitumor antibiotic as well as its in vivo mode of action.

The chemotherapeutic agent mitomycin C (MC, 1) must be reduced before it alkylates its putative target, the DNA of a tumor cell.[1] The understanding of the reductive

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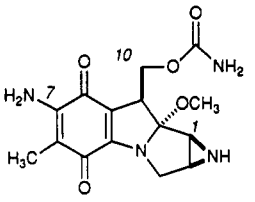
(1) Szybalski, W.; Iyer, V. N. *Fed. Proc.* 1964, *23*, 946.

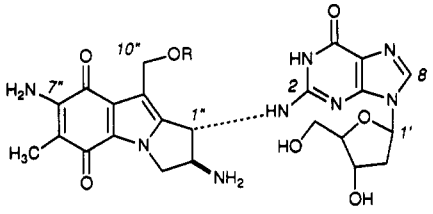
0022-3263/91/1956-4826$02.50/0



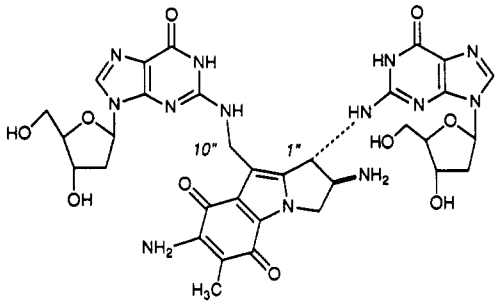
MC; 1

activation mechanism of MC has been increasing rapidly (Scheme I).[2] Quinone reduction by a variety of chemical or enzymatic methods activates MC's two electrophilic centers sequentially.[3-5] First, MC's aziridine ring cleaves, generating an electrophilic site at C-1 (7). Subsequently, the freed electrons of MC's 4-indole nitrogen facilitate the loss of its 10-carbamate group. The resultant intermediate (9) will now accept ambient nucleophiles at its highly reactive 10-position exocyclic methylene. The detailed mechanism of these steps has been investigated in many excellent model studies.[2,6]

In collaboration, our groups have been investigating the alkylation and cross-linking abilities of MC with DNA. Our approach has been to bind MC to DNA in vitro, isolate and characterize the MC–mononucleoside adducts formed, and then draw conclusions about the mechanism of MC's covalent binding with DNA. With this approach, three major adducts between MC and deoxyguanosine (2, 3, and 4) have been identified.[7-10] Adduct 2 represents the first



2 : R = $CONH_2$
3 : R = H



4

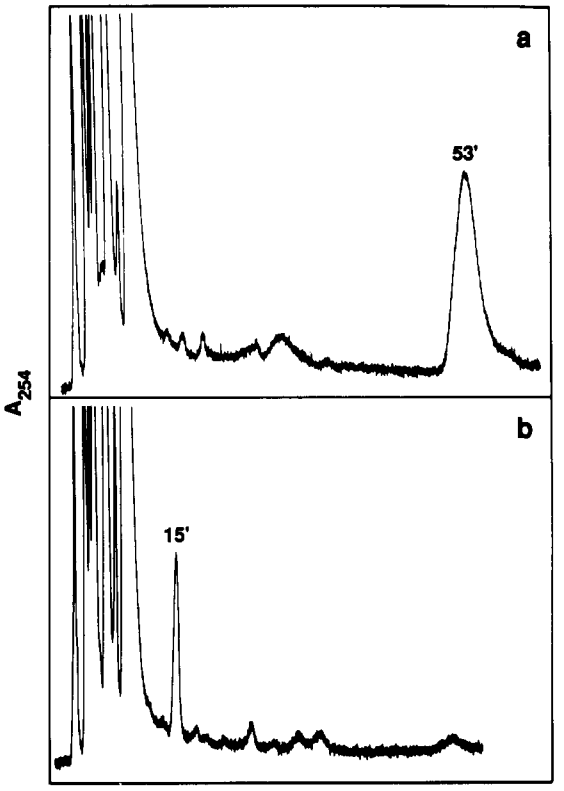


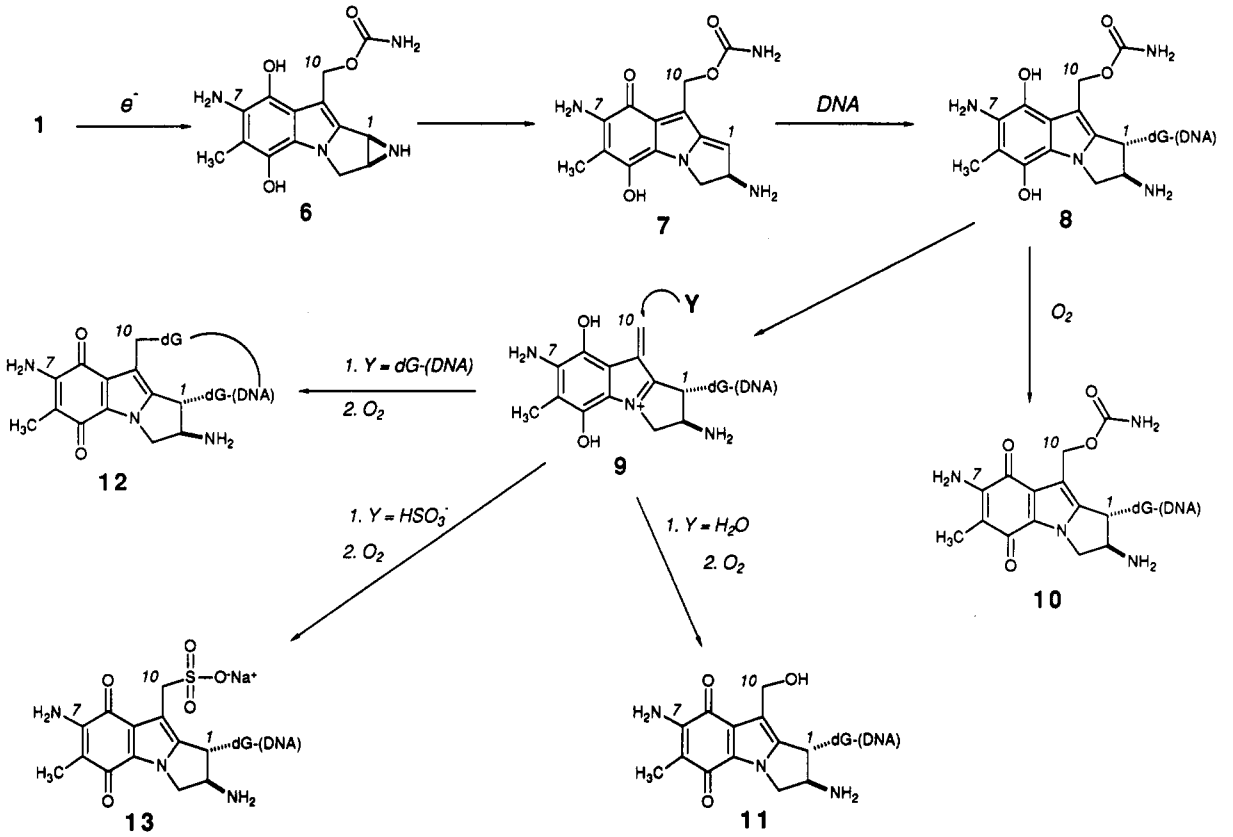
Figure 1. Isolation of MC–nucleoside adducts by HPLC from nuclease digests of two MC–oligonucleotide complexes, containing a CpG and a GpC seqeunce, respectively. (a) Self-complementary oligonucleotide d-ATATACGTATAT (CpG sequence) was treated with MC and $Na_2S_2O_4$ under anaerobic conditions at 0 °C, followed by purification of the resulting complex on a Sephadex G-50 column and digestion of the product by snake venom diesterase and alkaline phosphatase, using standard procedures described previously.[11] HPLC of the digested complex was carried out on a C-18 reversed phase column (Beckman; 0.46 × 25 cm; 6% acetonitrile–94% 0.03 M potassium phosphate, pH 5.4 as eluant; 1.0 mL/min flowrate). The early eluting peaks are unmodified nucleosides; the peak eluting at 53 min is adduct 4. (b) Self-complementary oligonucleotide d-TATATGCATATA (GpC sequence) was treated and analyzed as described above for (a). The peak eluted at 15 min is adduct 5.

monofunctional alkylation event between the 1-position of MC and the 2-amino group of deoxyguanosine on the floor of DNA's minor groove.[7,8] In the proposed scheme of MC binding,[9,10] monofunctionally bound MC (8) has two fates: either be reoxidized to 10 (in which case we ultimately isolate 2) or lose its carbamate first to form intermediate 9 and accept nucleophiles. Isolation of the adduct 3 from enzymatic digestion of 11 clearly indicates that water is available for attack on a monofunctionally bound, bifunctionally activated MC.[8,9] Finally, if a guanine base is available on the sister DNA strand with its 2-amino group properly aligned, a cross-link will form (12) from which we ultimately isolate 4.[10]

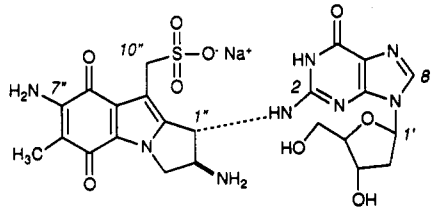
Extensive studies in several laboratories of MC's interstrand cross-linking of oligonucleotides[12-14] has indicated that cross-links will form only at the sequence CpG; GpC

(2) For review, see: Franck, R. W.; Tomasz, M. In *Chemistry of Antitumor Agents*, Wilman, D. E. V., Ed.; Blackie and Son Ltd.: Glasgow, 1990; pp 379–393.
(3) Tomasz, M.; Lipman, R. *Biochemistry* **1981**, *20*, 5056.
(4) Pan, S.-S.; Andrews, P. A.; Glover, C. J.; Bachur, N. R. *J. Biol. Chem.* **1984**, *259*, 959.
(5) Pan, S.-S.; Iracki, T.; Bachur, N. R. *Mol. Pharmacol.* **1986**, *29*, 622.
(6) For some examples: (a) Peterson, D. M.; Fisher, J. *Biochemistry* **1986**, *25*, 4077. (b) Danishefsky, S. J.; Ciufolini, M. *J. Am. Chem. Soc.* **1984**, *106*, 6424. (c) Egbertson, M.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1987**, *109*, 2204. (d) Bean, K.; Kohn, H. *J. Org. Chem.* **1983**, *48*, 5033. (e) Bean, K.; Kohn, H. *J. Org. Chem.* **1985**, *50*, 293. (f) Kohn, H.; Zein, N. *J. Am. Chem. Soc.* **1983**, *105*, 4105.
(7) Tomasz, M.; Lipman, R.; Verdine, G.; Nakanishi, K. *Biochemistry* **1986**, *25*, 4337.
(8) Tomasz, M.; Chowdary, D.; Lipman, R.; Shimotakahara, S.; Veiro, D.; Walker, V.; Verdine, G. L. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 6702.
(9) Tomasz, M.; Lipman, R.; McGuinness, B. F.; Nakanishi, K. *J. Am. Chem. Soc.* **1988**, *110*, 5892.
(10) Tomasz, M.; Lipman, R.; Chowdary, D.; Pawlak, J.; Verdine, G. L.; Nakanishi, K. *Science (Washington, D.C.)* **1987**, *235*, 1204.
(11) Borowy-Borowski, H.; Lipman, R.; Chowdary, D.; Tomasz, M. *Biochemistry* **1990**, *29*, 2992.
(12) Borowy-Borowski, H.; Lipman, R.; Tomasz, M. *Biochemistry* **1990**, *29*, 2999.
(13) Teng, S. P.; Woodson, S. A.; Crothers, D. M. *Biochemistry* **1989**, *28*, 3901.
(14) Millard, J. T.; Weidner, M. F.; Raucher, S.; Hopkins, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 3637.

Scheme I



steps are not cross-linked. Thus, according to the observed mechanism of DNA alkylation outlined above, at GpC sequences sodium dithionite activated MC should yield decarbamoyl monoadducts (3) instead. Surprisingly, as seen in Figure 1, the reaction of an oligonucleotide containing a GpC site with sodium dithionite activated MC failed to produce the decarbamoyl adduct 3 but instead yielded a previously uncharacterized MC–deoxyguanosine adduct (5). Indeed, this unknown compound was the



5

major adduct formed from the reaction of sodium dithionite activated MC with a series of similar synthetic oligonucleotides harboring isolated "non-cross-linkable" guanines (i.e., guanines at NpG; N ≠ C) in their sequence (data not shown). Since adduct 5 was not seen in our previous studies of MC binding to calf thymus or other natural DNA,[8-10] a comparison was made of the DNA and oligonucleotide reduction conditions. The MC–DNA complexes were generally formed by using a 0.5:1 drug:nucleotide ratio[8-10] while the MC–oligonucleotide complexes were formed by using a 5:1 drug:nucleotide ratio.[11,12] Since the same amount of reductant was used per mol of MC in either case, the $Na_2S_2O_4$ concentration in the oligonucleotide reactions was approximately 10 times greater than that in the calf thymus DNA reactions. Considering the mechanism of bifunctional activation of MC (Scheme I), it was probable that the 10″-carbamate group of the initial monoadducted oligonucleotide (cf. 8) was being displaced by a nucleophilic byproduct of the sodium dithionite in the reaction mixture: most likely a bisulfite ion. To test this possibility further, *M. luteus* DNA was reacted with MC using a higher $Na_2S_2O_4$ concentration (2.1 mM instead of 0.52 mM) under the experimental protocol described previously.[10] Digestion of this MC–DNA complex now also produced adduct 5 together with 3. Calf thymus DNA gave similar results (data not shown).

Further indication of the structure of 5 came from the reaction of the monoadduct 2 with $Na_2S_2O_4$ as follows. A pure sample of the known adduct 2[7] (0.14 μmol; 3.8 A_{260} units, 1.56 A_{310} units) was dissolved in 1.0 mL of water and degassed for 15 min under bubbling argon. Reduction was carried out by the addition of 5 × 20 μL of a deaerated 6 mM $Na_2S_2O_4$ solution over the course of one-half hour (0.6 μmol; 4.3 μmol $Na_2S_2O_4$/μmol 2). Upon completion, air was bubbled through the mixture for 5 min to effect reoxidation. The resultant pink solution was chromatographed on a Sephadex G-25 column (fine; 2.5 × 56 cm; 0.02 M NH_4HCO_3 eluant.) The one main peak from this column (200 mL elution volume) was collected and lyophilized. HPLC analysis of this fraction indicated that dithionite treatment of 2 yielded adduct 5 along with some unknown mitosenes and deoxyguanosine.[15] Compound 5 was identified by its diode array UV spectra (very similar to that of 2) and co-injection with the standard 5 collected from the enzyme digests of the oligonucleotide complexes. In addition, the circular dichroism spectrum of 5 displayed the expected 520-nm negative Cotton effect indicative of 1″-α stereochemistry.[16]

A more convenient source of the unknown adduct 5 was now available. Hence, repetition of this reaction from a larger amount of 2 yielded enough 5 (~175 μg) for proton

(15) The formation of deoxyguanosine is significant since it adds support for the recent proposal that the cross-linking event is reversible under reductive conditions.[11]

(16) Tomasz, M.; Jung, M.; Verdine, G. L.; Nakanishi, K. *J. Am. Chem. Soc.* **1984**, *106*, 7367.

NMR analysis in Me_2SO-d_6. The resonances exhibited by **5** were quite broad as has been seen for all of the underivatized MC–deoxyguanosine adducts so far isolated.[7-10,17] Of particular note in the NMR spectrum of **5**, however, was the upfield shift of the typical AB quartet of the mitosene 10″-CH_2 protons to 4.0 ppm. Decarbamoyl mitosenes exhibit similar upfield shifts.[9] In addition, assignment of this upfield shift to the presence of a C-10″–sulfur linkage is in agreement with the NMR data published for a series of 10-(ethyl xanthyl)mitosenes.[6d,18]

The structure of compound **5** was confirmed by FAB negative mass spectroscopy. The molecular weight of **5** is 589, in agreement with the observed ions at m/e 588 $[M - H]^-$ and 611 $[M + Na - H]^-$.

Formation of the bisulfite adduct **5** is a consequence of the redox chemistry of sodium dithionite.[19,20] In aqueous media, with the presence of an appropriate oxidant, the dithionite ion undergoes the following half redox reaction:

$$S_2O_4^{2-} + 2H_2O \rightarrow 2HSO_3^{2-} + 2H^+ + 2e^-$$

Hence, the byproduct from the production of $2e^-$ in this reaction is 2 mol of bisulfite ion. Bisulfite ion is, therefore, quite abundant in the reactions with high dithionite concentrations. Reactions of $Na_2S_2O_4$ with reduced MC itself have been studied previously.[21] Several major mitosene–bisulfite adducts were formed and partially characterized. The most abundant product was shown to be a bisulfite–mitosene monoadduct substituted at the 10-position of the mitosene. This further strengthens the case for the formation of **5** in the present system. Such adducts were also shown to be the only products in the absence of DNA under similar conditions[3] and therefore they most likely represent the material balance for the mitomycin that did not react with the DNA in the reactions described here. The reaction of bisulfite ion with activated MC is an inactivating process, which may interfere with MC's ability to alkylate DNA in our in vitro reactions.[12,22]

It is interesting to note that the decarbamoyl adduct **3** was not isolated in appreciable amounts from these oligonucleotide reactions. In fact, the difficulty in generating an oligonucleotide containing this decarbamoyl adduct by re-reduction of monoadducted oligonucleotides by $Na_2S_2O_4$ has been previously noted.[23] It is now obvious that bisulfite ion competes with water for the activated C-10″ position of the monofunctionally bound drug. Hence, re-reduction of monoadducted oligonucleotides[12] most likely generates the bisulfite adduct rather than the decarbamoyl adduct on the oligonucleotide strand.

Isolation of the bisulfite adduct **5** from GpC sequences is in stark contrast to the isolation of the cross-link adduct **4** from CpG sequences under the same reaction conditions (Figure 1). Clearly, the exocyclic amino group of the second alkylatable guanine residue successfully competes against the abundant bisulfite ion only in CpG sequences. In GpC sequences this amino group must be poorly positioned for attack,[24] thus allowing the bisulfite adduct to form (cf. **9** in Scheme I). It is also notable that the yield of the bisulfite adduct (Figure 1b) is only 3%, while the yield of the cross-link adduct **4** in the CpG-containing oligonucleotide (Figure 1a) is 46% (calculated on the basis of oligonucleotide, as described previously[12]). This large difference in adduct yields may reflect the modulating effect of the DNA sequence surrounding the target guanine.[25]

Finally, isolation of **5** has implications for the in vivo mode of action of MC. Namely, if the activated monoadduct **9** is free to accept a bisulfite ion, then it is also free to accept a host of biological nucleophiles. This suggests that MC will form cross-links between DNA and the nucleophilic residues of a nearby DNA-binding protein. Such DNA–protein cross-links could in part be responsible for MC's ability to inhibit DNA synthesis.[1]

Acknowledgment. This research was supported by grants GM34509 (to K.N.) and CA28681 (to M.T.), a Research Centers in Minority Institutions award (RR003037) from the Division of Research Resources, NIH (to Hunter College), and a PSC-CUNY Faculty Research Award (to M.T.). We are grateful to Dr. Dario Gargiulo for helpful discussions.

(17) The following resonances were assigned: 6.4 (bs), 7″-NH_2; 6.2 (t), 1′-H; 4.0 (dd), 10″-H_2; 2.7 (m), 2′-H_a; 2.2 (m), 2′-H_b; 1.7 (s), 6″-CH_3.

(18) Hornemann, U.; Iguchi, K.; Keller, P. J.; Vu, H. M.; Kozlowski, J. F.; Kohn, H. *J. Org. Chem.* **1983**, *48*, 5026.
(19) Watt, G. D.; Burns, A. *Biochem. J.* **1975**, *152*, 33.
(20) Lambeth, D. O.; Palmer, G. *J. Biol. Chem.* **1973**, *248*, 6095.
(21) Hornemann, U.; Ho, Y.-H.; Mackey, J. K.; Srivastava, S. C. *J. Am. Chem. Soc.* **1976**, *98*, 7069.

(22) Hence, it is significant that it was recently discovered that Cr-$(ClO_4)_2$ bifunctionally activates MC cleanly (Hong, Y. P.; Kohn, H. *J. Am. Chem. Soc.* **1990**, *112*, 4596). Application of this reagent to reactions of MC with DNA may thus increase the yield of cross-linking by avoiding the formation of adduct **5**.

(23) Chawla, A. Ph.D. Thesis, City University of New York; 1987. Borowy-Borowski, H. and Tomasz, M., unpublished results.
(24) Models for this "poor positioning" have been proposed.[12-14]
(25) Li, V.; Kohn, H. *J. Am. Chem. Soc.* **1991**, *113*, 283.