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Dual Mechanisms of DNA Damage by $MoCH_3(\eta^3\text{-allyl})(CO)_2(phen)$ Complexes

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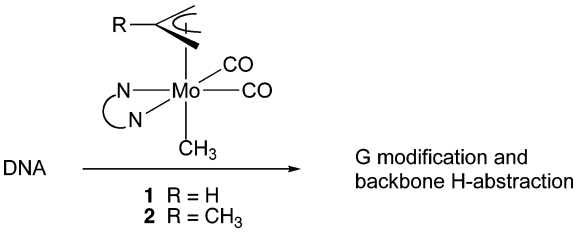

DNA damage by $MoCH_3(\eta^3\text{-allyl})(CO)_2(phen)$ complexes has been shown to occur by two mechanisms: by backbone cleavage via the abstraction of H1′ and/or H5′ from the deoxyribose moiety and by base modification, resulting in G-specific cleavage via the formation of base-labile residues methylguanine, methoxyguanine, and 8-oxo-G.

Introduction

To maintain the integrity of the genome, cells have developed mechanisms to repair damaged DNA.[1] In humans, these include reversion repair, base excision repair (BER), recombinational repair (nonhomologous end-joining or NHEJ), nucleotide excision repair (NER), and mismatch repair (MMR). As indicated by its name, each mechanism recognizes and corrects specific classes of abnormal DNA modifications. Defects in this machinery are associated with many disorders, including some hereditary cancers. For example, most familial colorectal cancer results from mutations in an MMR gene. Because MMR is responsible for responding to cisplatin 1,2-intrastrand (GpG) cross-links,[2] cells that are deficient in MMR capability would be expected to be extraordinarily sensitive to this chemotherapeutic agent;[3] ironically, however, certain mutations confer resistance to cisplatin.[2]

Nevertheless, compounds that yield more than one type of damage may be less likely to encounter such resistance problems. As such, we now report dual mechanisms of DNA damage, backbone cleavage and base modification,[4] by $MoCH_3(\eta^3\text{-allyl})(CO)_2(phen)$ complexes **1** and **2** (Scheme 1).[5] The hypothesis that these organometallic species might cleave DNA was originally suggested by the slow conversion of **1** to **3** and methane in acid-free (in the dark, over sodium carbonate) dichloromethane. This reaction was presumed to involve the production of methyl radical, which had been shown to cause strand scission in other systems.[6]

Results and Discussion

The DNA-cleaving activity of each compound under a variety of conditions was determined initially via a plasmid relaxation

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(3) For examples of chemical approaches to ameliorating cisplatin resistance: Natile, G; Marzilli, L. G. *Coord. Chem. Rev.* **2006**, *250*, 1315−1331. Guddneppanavar, R.; Saluta, G.; Kucera, G. L.; Bierbach, U. *J. Med. Chem.* **2006**, *49*, 3204−3214. van Zutphen, S.; Reedijk, J. *Coord. Chem. Rev.* **2005**, *249*, 2845−2853.

(4) Other agents have shown both backbone modification and base oxidation, although not simultaneously with a single activation method. For examples, see: Hall D. B.; Barton, J. K. *Nature* **1996**, *382*, 731−735. Boerner L. J. K.; Zaleski, J. M. *Curr. Opin. Chem. Biol.* **2005**, *9*, 135−144.

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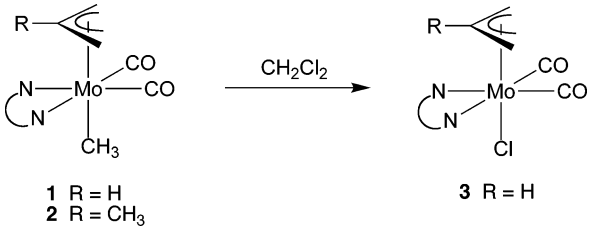
SCHEME 1



1 R = H
2 R = CH_3

3 R = H

assay, which monitors the conversion of circular supercoiled (form I) to relaxed circular (form II) or linear (form III) DNA, resulting from single- or double-strand breaks, respectively. Thus, various concentrations of each compound were incubated or irradiated with the plasmid pBR322, after which the reaction mixtures were subjected to agarose gel electrophoresis. As shown in Figure 1, irradiation of either organometallic species caused strand scission at concentrations as low as 11 μM (lane 9 for **1**) or 23 μM (lane 8 for **2**). Complex **1** gave only single-strand cleavage, while **2** also yielded form III DNA. Because no lanes showed the presence of all three forms of DNA, the linear DNA presumably arises from the form II DNA via the accumulation of random single-strand cleavage events. Although control experiments for **1** showed that both light and the complex were necessary for significant amounts of strand scission (lanes a2 and a3, respectively); interestingly, in experiments with **2**, irradiation was not required (lane b2).

Therefore, the reaction of **2** with DNA in the absence of light was investigated (Figure 2). In these experiments, **2** was incubated in the dark with the DNA, and the radical scavenger cysteine was added prior to exposure of the reaction mixtures to ambient light during gel loading. Under these conditions, **2** caused single-strand cleavage at concentrations above 0.7 μM, and form III DNA was produced at greater than 45 μM. The unexpected observation of all three forms of DNA in lane 4 (8.2% form I, 87.0% form 2, and 4.8% form 3) suggested the occurrence of nonrandom double-strand cleavage.[7] The fact that the ratio of single- to double-strand breaks ($n_1/n_2 = 50$)[8] in this lane is lower than the value expected (215)[9] from coincidental single-strand breaks in a plasmid of this size further supports this idea, although the mechanism by which this event occurs has not been determined.

To investigate the sequence selectivity of the DNA-cleaving behavior of one of the new compounds, compound **2** was incubated in the presence of a 3′-^{32}P end-labeled restriction fragment (Figure 3). After reaction, one sample (lane 2) was precipitated immediately, while another (lane 3) was heated with piperidine first. While both lanes show strand scission at every residue, the bands in lane 3 are more intense and exhibit increased cleavage at G residues. The relative intensity of each

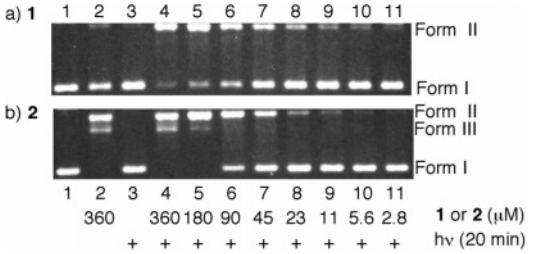


FIGURE 1. Photoinduced cleavage of pBR322 DNA (30 μM/bp in 10% DMSO/20 mM Tris buffer, pH 8) by **1** (a) and **2** (b). Mixtures in lanes 3−12 were irradiated with Pyrex-filtered light from a 450 W medium-pressure mercury arc lamp for 20 min.

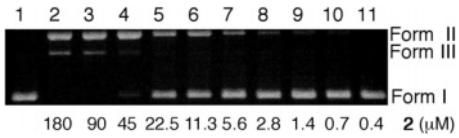


FIGURE 2. Cleavage of pBR322 DNA (30 μM/bp in distilled, deionized water) by **2**. All samples were incubated in the dark for 20 min, at which time cysteine (54 mM) was added and samples were then brought into ambient light.

band was obtained by densitometry[10] and confirms these observations. Thus, strand scission is fairly evenly spread across all residues in lane 2, with G residues accounting for 23% of the total intensity, a percentage close to the theoretical value of 26%. On the other hand, in the piperidine-treated mixture, the overall sum of intensities grew by 27%, with A, C, and T positions increasing by 20%. Cleavage at G positions grew by nearly 300%, to account for 54% of the total. The minor amount of nonselective cleavage seen in lane 2 and its intensification by subsequent base treatment is generally considered consistent with a DNA backbone modification, while the base-induced formation of lesions at G residues implies the selective modification of this base.[11]

A reasonable mechanism for the backbone cleavage involves the abstraction of a hydrogen atom by methyl radical or some species mechanistically downstream to lead to the direct strand scission observed in the plasmid assays and in lane 2 of the sequencing gel. Because such a process should be inhibited by radical trapping agents, the treatment of DNA with **1** or **2** was conducted in the presence of cysteine, a general radical scavenger,[12] or the nitroxide species 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO), which traps carbon-,[13] metal-,[14] and oxygen-centered[15] radicals (Figure 4). Only 1 equiv of cysteine was required to decrease the extent of strand scission (lane 8 vs lane 2), and 100 equiv suppressed all cleavage (lane 6). Excess TEMPO also reduced the amount of strand scission

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(7) Povirk, L. F.; Wübker, W.; Köhnlein, W.; Hutchinson, F. *Nucleic Acids Res.* **1977**, *4*, 3573−3580.

(8) The number of single- and double-strand cleavage events was determined via the statistical test of Povirk et al., which assumes a Poisson distribution of strand cuts; see ref 7.

(9) The expected n_1/n_2 was determined with the Freifelder−Trumbo equation [$n_2 = n_1^2(2h + 1)/4L$: Freifelder, D.; Trumbo, B. *Biopolymers* **1969**, *7*, 681], in which h is the maximum separation in base pairs between cuts on opposite strand that produces linear DNA ($h = 16$) and L is the number of phosphodiester bonds per strand of DNA (4361 for pBR322).

(10) Densitometry was accomplished with the NIH ImageJ software program. The amount of supercoiled DNA was multiplied by a factor of 1.22 to account for reduced ethidium bromide intercalation into the form I plasmid DNA.

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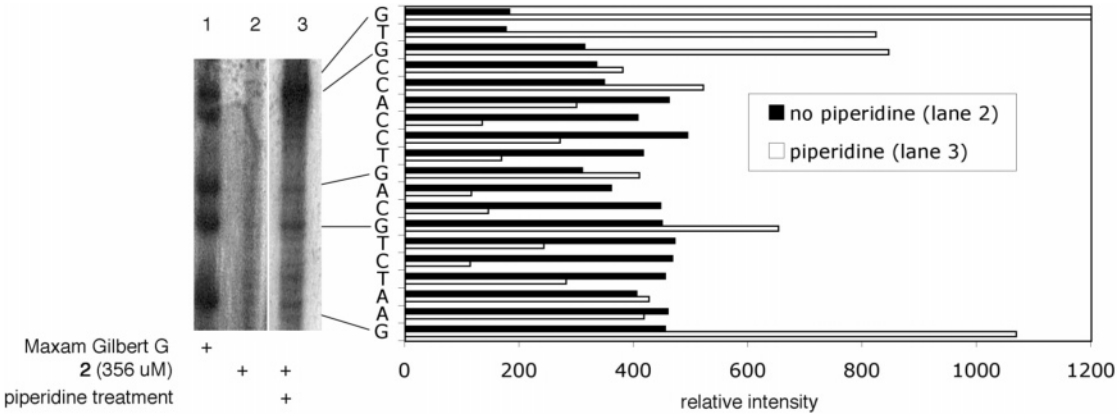


FIGURE 3. Autoradiogram and relative intensities of bands of a 10% denaturing polyacrylamide gel for cleavage of the 3′-^{32}P-end-labeled 514 bp restriction fragment (*Eco*RI/*Rsa*I) of pBR322 DNA/calf thymus DNA (118 μM/bp in 10% DMSO/10 mM Tris buffer, pH 8) by **2**. Lane 1: Maxam−Gilbert G reaction. Lanes 2 and 3: DNA + complex (536 μM). The reaction mixtures were incubated in the dark for 20 min, and the sample in lane 3 was then treated with hot piperidine. (Bar for relative intensity for 5′ G, top, is truncated; its value is 2115.)

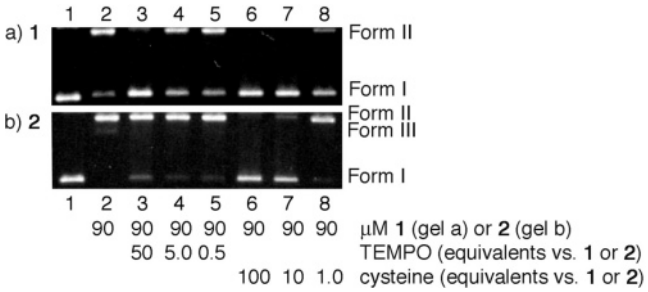
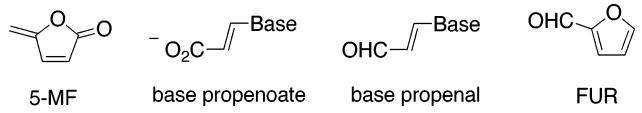


FIGURE 4. Effects of radical scavengers added prior to incubation on the cleavage of pBR322 DNA (30 μM/bp in 10% DMSO/20 mM Tris buffer, pH 8) by **1** (a) and **2** (b) without irradiation. All samples were incubated in the dark for 1 h.

SCHEME 2



(lanes 3 and 4 vs lane 2), but to a lesser extent than cysteine, thus implicating at least the partial contribution of a radical species at some point in the mechanistic path ultimately resulting in direct DNA cleavage.

The hypothesis that the mechanism of strand scission involves hydrogen atom abstraction from the backbone of DNA is tested readily by examining cleavage reaction mixtures for the known products of these processes. Furthermore, abstraction from each sugar position gives a unique product (Scheme 2).[16] Abstraction of H1′ affords 5-methylene-2- furanone (5-MF),[17] and base propenoates result from reaction at the 3′-position.[18] Removal of H4′ leads to base propenals,[19] and abstraction of H5′ yields furfural (FUR).[17] Reaction at H2′ is not typically observed, presumably due to the low accessibility or reactivity of these hydrogens.[16]

TABLE 1. MALDI-ToF Analysis of DNA Cleavage Reaction Mixtures

obsd *m/z*			
+[a]	−[a]	assignment	calcd *m/z*
	95.35	FUR−H^+ or 5-MF− H^+	95.01
	113.38	FUR·H_2O−H^+ or 5-MF·H_2O−H^+	113.09
97.31		FUR·H^+ or 5-MF·H^+	97.03
115.45		FUR·H_3O^+ or 5-MF·H_3O^+	115.04

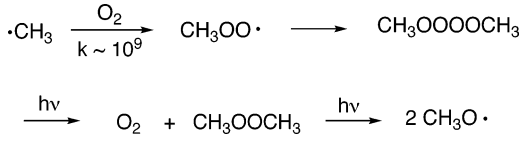
[a] Detection ion mode.

TABLE 2. MALDI-ToF Detection of Modified Bases

obsd *m/z*			
+[a]	−[a]	assignment	calcd *m/z*
	165.36	MeG−H^+	165.15
182.41		MeO-G·H^+	182.41
190.32		8-oxo-G·Na^+	190.32

[a] Detection ion mode.

SCHEME 3



To identify these DNA-derived products, mass spectrometry was employed.[18,20] After the reaction of **2** with calf thymus DNA, the small molecule products were isolated by membrane filtration. In addition to signals also observed in nontreated DNA samples, MALDI-ToF MS of this mixture in positive-ion mode (Table 1) gave new peaks at *m/z* 97.1 and 115.1. The first corresponds to 5-MF·H^+ or FUR·H^+ (exact mass 97.03), and the second is ascribed to the hydrated protonated form of one of these (calculated *m/z* 115.04). Peaks corresponding to the deprotonated form of these species were also seen in negative ion mode, further suggesting the abstraction of H1′ and/or H5′. While H5′ is much more accessible, reaction at H1′ is not unreasonable, given that copper phenanthroline complexes are minor groove intercalators that lead to H1′ abstraction.[21]

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(17) Frank, B. L.; Worth, L., Jr.; Christner, D. F.; Kozarich, J. W.; Stubbe, J.; Kappen, L. S.; Goldberg, I. H. *J. Am. Chem. Soc.* **1991**, *113*, 2271−2275; Goldberg, I. H. *Acc. Chem. Res.* **1991**, *24*, 191−198.
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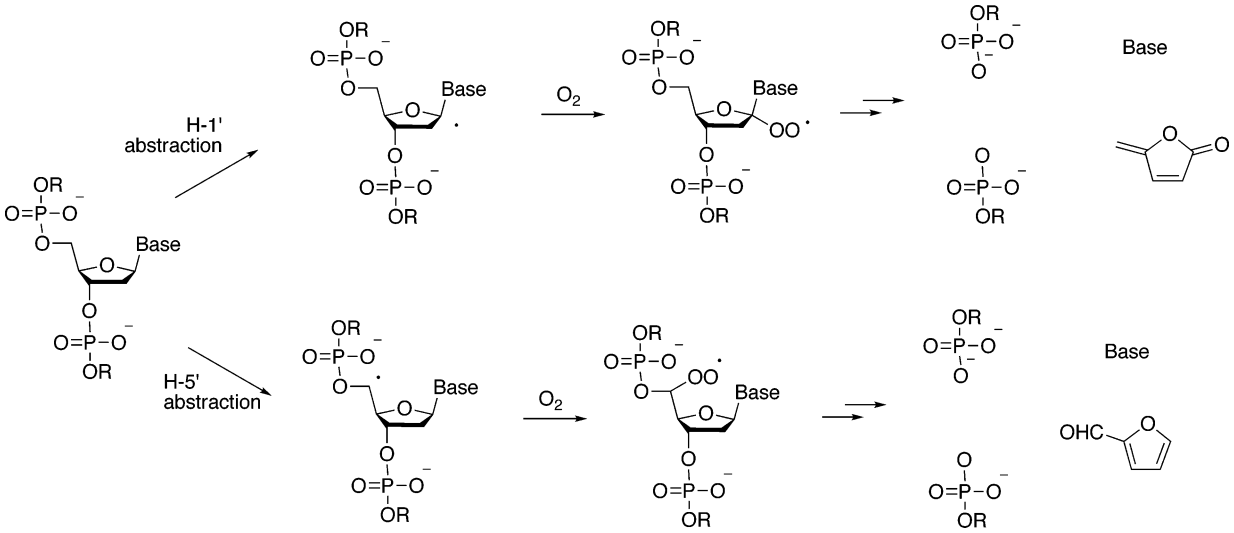
(19) Hecht, S. *Acc. Chem. Res.* **1986**, *19*, 383−391.
(20) Giloni, L.; Takeshita, M.; Johnson, F.; Iden, C.; Grollman, A. P. *J. Biol. Chem.* **1981**, *256*, 8608−8615.

SCHEME 4



The nature of the lesions at G was also investigated by MALDI-ToF MS. A solution from the reaction of **1** with calf thymus DNA was hydrolyzed to cleave both functionalized and unreacted bases from the backbone but otherwise leaving them intact[22] for analysis (Table 2). In addition to unmodified bases, a peak at *m*/*z* 165.36 was observed in negative ion mode. This signal was assigned to deprotonated methylated guanine (exact mass of 165.15), consistent with previous reports of base methylation by methyl radical.[23] In positive ion mode, peaks at *m*/*z* 182.41 and 190.32 were attributed to protonated methoxyguanine and the sodium salt of 8-oxoguanine (8-oxo-G), respectively. No other modified bases were seen. In another experiment with the mononucleoside deoxyguanosine (dG), ESI-MS of the mixture of the reaction of **1** with dG also showed the oxidation of G, with a peak at *m*/*z* 284.10 corresponding to protonated 8-oxo-dG (exact mass 284.25).

While methylated G arises from the direct reaction of methyl radical with the base, the origins of 8-oxo-G and MeO-G are less clear at this point. They may result from the direct oxidation of G by some species derived from **1**, followed by reaction with water[11] (and methyl radical for MeO-G). Alternatively, alkoxyl and/or alkylperoxyl radicals derived from the reaction of methyl radical with molecular oxygen (Scheme 3)[24] may react with G residues to produce 8-oxo-G and/or MeO-G.[11] Neither pathway can be ruled out by the experiments reported herein.

In addition, the active species responsible for backbone cleavage has not been determined, but the most likely candidates are methyl and methoxyl radical.[24] A rough calculation estimates that methyl radical reacts with oxygen about 10^6 times faster than it abstracts a hydrogen atom from DNA in this system,[24] assuming no precomplexation of **1** with DNA. However, these complexes show a strong affinity for DNA with $K_{app} = 8.55 \times 10^6$ M^{-1} for **1** and 2.15×10^6 M^{-1} for **2**. This interaction

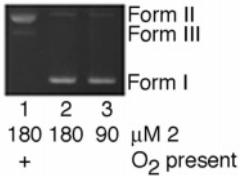


FIGURE 5. Effects of the removal of air prior to incubation on the cleavage of pBR322 DNA (30 μM/bp in 10% DMSO/20 mM Tris buffer, pH 8) by **2** without irradiation. Samples in lanes 2 and 3 were degassed by three freeze/pump/thaw cycles, and all mixtures were then incubated in the dark for 30 min.

effectively increases the rate of the reaction of methyl radical with DNA, an expectation that is confirmed qualitatively by the identification of Me-G in reaction mixtures of **1** with DNA. Nevertheless, it is difficult to predict in a quantitative manner how much the binding of **1** to DNA affects the partitioning between DNA and oxygen in the reactions of methyl radical.

Experiments in which little or no oxygen was present indicated that O_2 is necessary for plasmid cleavage (Figure 5). Thus, when oxygen was removed from the samples by three freeze/pump/thaw cycles immediately upon mixing the DNA with **2**, much less strand scission was observed (lanes 2 and 3) than in mixtures incubated under air (Figure 5, lane 1 or Figure 2, lanes 2 and 3). While this result could be interpreted to mean that methoxyl radical (formed ultimately from the reaction of methyl radical with oxygen) is the most significant species in causing DNA backbone cleavage, it could also be explained by the fact that oxygen is necessary at a later point in the overall cleavage mechanism(s) initiated by abstraction of H1′ and/or H5′ (Scheme 4).[17,19]

In summary, DNA damage by $MoCH_3(\eta^3\text{-allyl})(CO)_2$(phen) complexes **1** and **2** has been shown to occur by two general mechanisms, backbone cleavage[25] and base modification. The first leads to nonspecific cleavage via the abstraction of H1′ and/or H5′ from the deoxyribose moiety and the second results in G-specific cleavage via the formation of base-labile residues methylguanine, methoxyguanine, and 8-oxo-G.

(21) Meijier, M. M.; Zelenko, O.; Sigman, D. S. *J. Am. Chem. Soc.* **1997**, *119*, 1135−1136. Bales, B. C.; Kodama, T.; Weledji, Y. N.; Pitié, M.; Meunier, B.; Greenberg, M. M. *Nucleic Acids Res.* **2005**, *33*, 5371−5379.

(22) Dizdaroglu, M. *Methods Enzymol.* **1994**, *234*, 3−16.

(23) For example: Augusto, O. *Free Rad. Biol. Med.* **1993**, *15*, 329−336. Hix, S.; Da Silva, M.; Augusto, O. *Free Rad. Biol. Med.* **1995**, *19*, 293−301. Zady, M. F.; Wong, J. L. *J. Org. Chem.* **1983**, *45*, 2373−2377. Zady, M. F.; Wong, J. L. *J. Am. Chem. Soc.* **1977**, *99*, 5096−5101.

(24) It has been asserted that methylperoxyl radical is a poor hydrogen atom abstractor, because of the slowness of this reaction. For a detailed discussion of the rates of the processes in Scheme 3, see ref 6.

(25) Interestingly, complexes **1** and **2** are some of the very few Mo complexes that have been found to cause DNA cleavage: Peng, J.; Li, W.; Zhao, X.; Han, Z.; Huang, B. *Chem. Res. Chin. Univ.* **2004**, *20*, 6−9. Lu, X.-M.; Jiang, L.; Mao, X.-A.; Ye, C.-H.; Lu, J.-F.; Cui, J.-R. *Chin. J. Chem.* **2005**, *23*, 781−785.

Experimental Procedures

Plasmid Relaxation Assays. A DMSO solution was made of the compound of interest and serial dilutions were made. The appropriate DMSO solution was added to a 1.5 mL plastic centrifuge tube containing nine times the volume of a solution containing 33.3 μM/bp DNA (pBR322) in 20 mM Tris HCl reaction buffer pH 8 (final concentration = 30.0 μM/bp). The tubes were then strapped to the outside of a water-jacketed reaction vessel for a Hanovia photolysis apparatus with a Pyrex filter and irradiated with light from a 450 W medium-pressure mercury arc lamp for 20 min. After the irradiation, 5 μL of loading buffer was added to each tube and the contents of the tube were loaded onto a 1% agarose gel and electrophoresed for 12 h at 30 V. The gel was then stained in a dilute solution of ethidium bromide ($\sim$0.5 μg/mL) for 10 min and then destained with water. The DNA was visualized with UV light.

High-Resolution Gel Electrophoresis of Cleaved Restriction Fragments. Reactions were carried out in 1.5 mL plastic microcentrifuge tubes. A DMSO solution (2 μL) of compound **2** was added to 18 μL of a solution containing 3′-^{32}P labeled restriction fragment (50000 cpm) and carrier calf thymus DNA (100 μM bp) in Tris acetate buffer (pH 8). The microcentrifuge tubes were strapped to the outside of a water-cooled Pyrex photolysis reactor and irradiated with light from a 450 W medium-pressure mercury arc lamp for 20 min. After the photolysis, the DNA was precipitated by adding 2 μL of NaOAc (3 M, pH 5) and 50 μL of absolute ethanol. The samples were cooled at −20 °C for 1 h and then centrifuged at 4 °C at 13000 rpm for 10 min. The supernatant was removed, and the samples were resuspended in 5 μL formamide loading buffer. Each sample was heated at 95 °C for 3 min and immediately cooled on ice for 1 min prior to loading onto a 10% denaturing polyacrylamide gel (1:19 cross-linking, 7 M urea) along with the Maxam−Gilbert G sequencing reaction. The samples were electrophoresed at 55 W and 55 °C for 1.5 h. After electrophoresis, the gel was blotted on a positively charged membrane for 20 min. After cross-linking each section of the membrane for 3 min with UV light, the membrane was exposed to X-ray film with an intensifying screen for 72 h at −40 °C.

Ethidium Bromide Displacement Assays. Fluorescence intensity was determined at an excitation wavelength of 540 nm and emission wavelength of 590 nm. Ultrapure ethidium bromide was dissolved in 20 mM Tris HCl reaction buffer (pH 8). Poly[d(AT)·(d(AT)] (ϵ_{260} = 6600 M^{-1} cm^{-1} bp^{-1}) was dissolved in 20 mM Tris HCl, 100 mM NaCl (pH 8) buffer. The exact DNA concentration was determined by UV-visible spectrophotometry.

To a 3 mL optical glass cell of 10 mm path length was added 36.8 μL of poly[d(AT)·(d(AT)] solution (0.325 mM), 30.0 μL of ethidium bromide solution (125 μM), and 2.93 mL of Tris HCl reaction buffer (pH 8). The fluorescence of this solution (4.0 μM in bp DNA, 1.26 μM ethidium bromide) was determined, and then aliquots (5−200 μL) of the appropriate molybdenum compound (76 μM in DMSO) were added. The fluorescence intensity was recorded after the addition of each aliquot. The addition of the molybdenum complex solution was repeated until the fluorescence intensity decreased to approximately 20% of its original value. A plot relating % fluorescence intensity to concentration of molybdenum compound was constructed. The apparent binding constant (K_{app}) was calculated from the following equation: $K_{EtBr}[EtBr] = K_{app}[Mo]$, where [Mo] is the concentration of molybdenum compound at 50% decrease in fluorescence and $K_{EtBr} = 9.5 \times 10^6$ M^{-1}. The graphs shown in the Supporting Information gave $K_{app} = 8.55 \times 10^6$ M^{-1} for **1** and $K_{app} = 2.15 \times 10^6$ M^{-1} for **2**.

Photolysis Reactions with 1 for MS Analysis. To a 450-μL solution of calf thymus DNA (4.4 mg/mL in distilled deionized H_2O) was added 50 μL of a solution of compound **1** (359 μM in DMSO). This mixture was incubated on the benchtop for 20 min. The solution was then transferred to Microcon-3 tubes and centrifuged at 13500 rpm for 45 min. The filtrate was then concentrated with a centrifugal concentrator and stored in the freezer for further use.

MALDI-ToF Analysis of Cleavage Products. Two reaction mixtures were combined, concentrated, and loaded on a DIOS strip. The strip was placed in a vacuum desiccator until dry. The DIOS strip was then loaded into the MALDI-ToF spectrometer for analysis. Linear negative and linear positive modes of operation were utilized. Mass acquisition range: 80−500 Da; 200 laser shots/spectrum.

Detection of Base Modifications. To a 105 μL solution of calf thymus DNA (1.1 mg/mL in distilled deionized H_2O) was added 11.7 μL of a solution of compound **1** (359 μM in DMSO). This mixture was incubated on the benchtop for 20 min. The mixture was then hydrolyzed with 0.5 mL of 60% (v/v) formic acid in a sealed tube at 140 °C for 30 min. This mixture was then analyzed by MALDI-ToF-MS as stated above. In addition, a 105 μL solution of calf thymus DNA (1.1 mg/mL in distilled deionized H_2O) was hydrolyzed with 0.5 mL of 60% (v/v) formic acid in a sealed tube at 140 °C for 30 min. The mixture was then analyzed by MALDI-ToF-MS as stated above to serve as a control.

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Supporting Information Available: General experimental methods, quantitation data for all gels, and graphs from ethidium bromide displacement assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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