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# Metallobleomycin-Mediated Cleavage of DNA Not Involving a Threading-Intercalation Mechanism

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**Abstract:** The DNA cleavage properties of metallobleomycins conjugated to three solid supports were investigated using plasmid DNA, relaxed covalently closed circular DNA, and linear duplex DNA as substrates. Cleavage of pBR322 and pSP64 plasmid DNAs by Fe(II)·BLM $A_5$-CPG-$C_2$ was observed with efficiencies not dissimilar to that obtained using free Fe(II)·BLM $A_5$. Similar results were observed following Fe(II)·BLM $A_5$-CPG-$C_2$-mediated cleavage of a relaxed plasmid, a substrate that lacks ends or negative supercoiling capable of facilitating strand separation. BLMs covalently tethered to solid supports, including Fe(II)·BLM $A_5$-Sepharose 4B, Fe(II)·BLM $A_5$-CPG-$C_6$, and Fe(II)·BLM $A_5$-CPG-$C_2$, cleaved a 5′-$^{32}$P end labeled linear DNA duplex with a sequence selectivity identical to that of free Fe(II)·BLM $A_5$; cleavage predominated at 5′-$G_{82}T_{83}$-3′ and 5′-$G_{84}T_{85}$-3′. To verify that these results could also be obtained using other metallobleomycins, supercoiled plasmid DNA and a linear DNA duplex were employed as substrates for Co(III)·BLM $A_5$-CPG-$C_2$. Free green Co(III)·BLM $A_5$ was only about 2-fold more efficient than green Co(III)·BLM $A_5$-CPG-$C_2$ in effecting DNA cleavage. A similar result was obtained using Cu(II)·BLM $A_5$-CPG-$C_2$ + dithiothreitol. In addition, the conjugated Co·BLM $A_5$ and Cu·BLM $A_5$ cleaved the linear duplex DNA with a sequence selectivity identical to that of the respective free metalloBLMs. Interestingly, when supercoiled plasmid DNA was used as a substrate, conjugated Fe·BLM $A_5$ and Co·BLM $A_5$ were both found to produce Form III DNA in addition to Form II DNA. The formation of Form III DNA by conjugated Fe·BLM $A_5$ was assessed quantitatively. When corrected for differences in the intrinsic efficiencies of DNA cleavage by conjugated vs free BLMs, conjugated Fe·BLM $A_5$ was found to produce Form III DNA to about the same extent as the respective free Fe·BLM $A_5$, arguing that this conjugated BLM can also effect double-strand cleavage of DNA. Although previous evidence supporting DNA intercalation by some metallobleomycins is convincing, the present evidence indicates that threading intercalation is not a requirement for DNA cleavage by Fe(II)·BLM $A_5$, Co(III)·BLM $A_5$, or Cu(I)·BLM $A_5$.

Bleomycins (BLMs) are glycopeptide-derived antitumor antibiotics originally isolated from *Streptomyces verticillus* by Umezawa and co-workers.[1] These antitumor agents are believed to mediate their therapeutic effects by binding and oxidatively cleaving DNA in the presence of a metal ion cofactor.[2]

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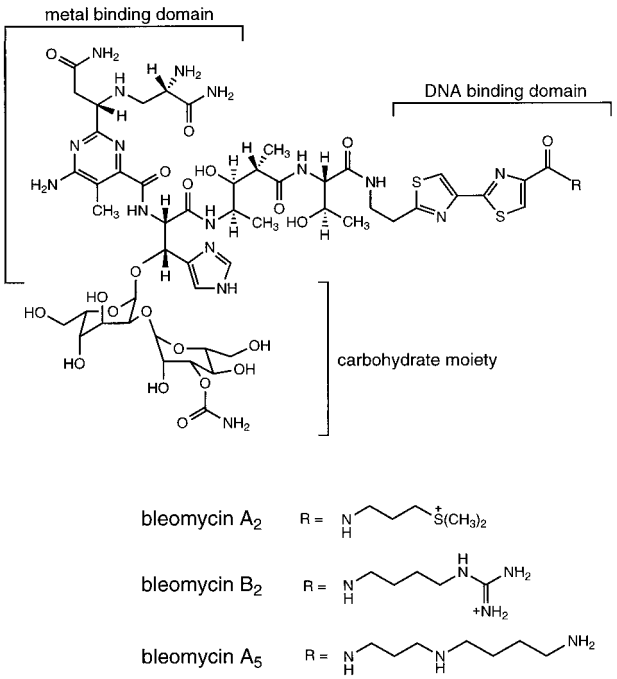


**Figure 1.** Structures of bleomycin congeners. The functional domains are indicated.

Metallobleomycins also degrade RNA in an analogous fashion, and this may well constitute an additional theapeutic locus for BLM.[3] Several metals have been shown to support BLM-mediated DNA cleavage including iron,[4] copper,[5] and cobalt[6] among others, but it is iron that is believed to be the major cofactor that promotes the effects of bleomycin in vivo.

Bleomycin is comprised of three functional domains (Figure 1). The metal binding domain, which consists of $\beta$-aminoalanineamide, pyrimidine, and $\beta$-hydroxyhistidine moieties at the N-terminus of this polypeptide-derived antibiotic, is responsible for metal complexation, and oxygen binding and activation, in addition to DNA binding.[2,7] The C-terminus, which encompasses the bithiazole and attached C-terminal substituent, is important for DNA binding,[8] and the disaccharide moiety may provide a metal ligand,[2c,9] and possibly have roles in cell permeability and selective tumor cell recognition.

The mode of DNA binding by metalloBLMs has been the subject of numerous studies. It is generally accepted that the metal binding domain of BLM must reside in the minor groove of DNA, since abstraction of a H atom from C-4′ of deoxyribose in the minor groove is required to initiate DNA degradation.[2] In contrast, the accumulated evidence for the nature of association of the C-terminus of BLM with DNA has provided support for more than one mode of association.[10] In fact, it seems likely that BLM actually does bind to DNA in more than one fashion.[11]

Recently, we described the conjugation of Fe(II)·BLM $A_5$ to a controlled pore glass bead, and found that this conjugated BLM was able to cleave DNA with the same sequence selectivity and an efficiency remarkably similar to that of free Fe(II)·BLM $A_5$.[12] This finding has important implications for the mode of DNA association by BLM, since the conjugated bead is far too large to permit threading intercalation of the C-substituent of BLM.

To further characterize the role of the C-terminus portion of bleomycin in DNA binding, we have extended our study of conjugated BLMs by employing three solid supports for Fe(II)·BLM and also by studying green Co(III)·BLM $A_5$ conjugated to a controlled pore glass bead. BLM $A_5$ was conjugated to controlled pore glass (CPG) beads and to Sepharose 4B, and the abilities of the conjugates to effect DNA cleavage were assessed. Presently we demonstrate that BLM $A_5$ conjugated to CPG-$C_2$ effected plasmid DNA relaxation with an efficiency similar to that of free Fe(II)·BLM $A_5$ itself. In addition, Fe(II)·BLM $A_5$-CPG-$C_2$, Fe(II)·BLM $A_5$-CPG-$C_6$, and Fe(II)·BLM $A_5$-Sepharose 4B were all able to effect sequence selective cleavage of a linear DNA duplex in a fashion identical with free Fe(II)·BLM $A_5$. Also utilized as a substrate for Fe(II)·BLM $A_5$-CPG-$C_2$ was a relaxed covalently closed circular DNA, i.e., a substrate that lacks ends or negative supercoiling, to minimize the chance that cleavage obtained by extensive denaturation of the DNA substrate, which then reformed a duplex around BLM $A_5$-CPG-$C_2$. Also tested was Cu(II)·BLM $A_5$-CPG-$C_2$. In the presence of dithiothreitol, this bleomycin relaxed plasmid DNA and nicked linear duplex DNA in the same fashion as free Cu(II)·BLM $A_5$, and with only modestly diminished potency. Finally, Co(III)·BLM $A_5$-CPG-$C_2$ was also shown to relax a plasmid DNA with an efficiency comparable to that of free Co(III)·BLM $A_5$, and to cleave a linear duplex DNA with a sequence selectivity identical to that of free Co(III)·BLM $A_5$.

## Results and Discussion

Equilibrium constants for the binding of metal-free and metallobleomycins to DNA have been determined by fluorescence spectroscopy [8a,11,13] and equilibrium dialysis[14] to be on

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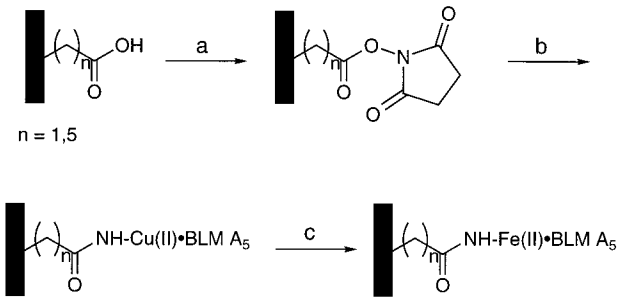
the order of $5 \times 10^5$ $M^{-1}$. There is convincing spectroscopic evidence that bleomycin binds to DNA by more than one mode of association.[11] The hydrophobic component of the interaction is presumably due to the association of the bithiazole moiety with DNA. An electrostatic component of binding likely derives from the interaction of the positively charged C-substituent (e.g., the methylsulfonium moiety in BLM $A_2$) and the negatively charged DNA-phosphate backbone; consistent with this interpretation, BLM demethyl $A_2$ binds to and degrades DNA less efficiently than BLM $A_2$.[15,16]

Numerous studies of the interaction of bleomycin with DNA have focused on the issue of whether the C-terminus binds by intercalation or minor groove binding. Studies to determine whether bleomycin functions as a classical (threading) intercalator include measurements of unwinding and elongation of DNA,[13,14a,16b] and have resulted in mixed conclusions. $^1$H NMR spectroscopy and molecular dynamics calculations have also been used to study the binding of Co(III)·BLM to short DNA duplexes.[17] These studies argue that Co(III)·BLM interacts with DNA by a classically intercalative mode, whereby the bithiazole and C-substituent bind via a partial intercalation and are threaded through the DNA duplex, which is unwound.

Conversely, there is published evidence that bleomycin interacts with DNA by binding in the minor groove, or possibly as a minor groove bound structure in which one thiazole undergoes edgewise (partial) intercalation. These studies are supported by the observations that known minor groove DNA binding molecules, such as distamycin, alter the sequence selectivity[18] of bleomycin, and that DNA substrates containing modifications to the 2-amino group of guanosine in the minor groove of DNA inhibit cleavage at G-pyrimidine sequences by Fe·BLM.[19] In addition, it is well documented that cleavage of DNA involves abstraction of the C-4′ H from the deoxyribose ring, suggesting that the metal binding domain which abstracts the sugar H must be oriented in the minor groove.[2] $^1$H NMR and molecular dynamics calculations using Zn(II)·BLM do not support a classical threading mode of intercalation by BLM, but rather a minor groove binding mode for the bithiazole moiety.[20] Studies with BLM analogues containing chlorinated bithiazoles that effect DNA cleavage via chlorine radicals also strongly support minor groove binding by BLM since the abstracted H atoms lie within the minor groove of DNA.[21] Recently, we have reported that Fe(II)·BLM $A_5$ conjugated to a controlled pore glass bead that is far too large to permit passage through the DNA duplex nonetheless cleaves DNA with a sequence selectivity identical to that of free Fe(II)·BLM $A_5$, and with good efficiency.[12] These results constitute compelling evidence that threading intercalation is not a requirement for DNA cleavage by Fe(II)·BLM.

**Scheme 1.** Method Employed for the Conjugation of BLM $A_5$ to Controlled Pore Glass Beads and Sepharose 4B[a]




[a] Conditions: (a) dioxane, *N*-hydroxysuccimide, *N,N*′-dicyclohexylcarbodiimide; (b) 0.1 M Na acetate, pH 6.5, Cu(II)·BLM $A_5$; (c) 15% aqueous EDTA, then $Fe^{2+}$. Conjugated Co·BLM $A_5$ was prepared by admixture of Co(III)·BLM $A_5$ to the activated resin.

To permit an analysis of the consequences of precluding a potential threading intercalative interaction of the C-terminal substituent of BLM with DNA, a set of conjugated bleomycins was prepared. As shown in Scheme 1, the derivatized resins all contained carboxyalkane tethers. These were activated as the respective *N*-hydroxysuccimide esters and treated with preformed, purified Cu(II)·BLM $A_5$[5] or green Co(III)·BLM $A_5$[6] to effect nucleophilic replacement of the hydroxysuccinimide moiety by an amine within the C-terminal spermidine substituent. The metal ions were coordinated to the primary amine in the $\beta$-aminoalanineamide moiety thus precluding reaction of this functionality with the activated resin. For the preparation of conjugated Fe(II)·BLMs $A_5$, the metal ion was removed from conjugated Cu(II)·BLM $A_5$ via the agency of EDTA and the conjugated metal-free BLM was admixed with $Fe^{2+}$ immediately prior to the DNA cleavage experiments; likewise conjugated Cu(I)·BLM $A_5$ was formed by admixture of DTT to the Cu(II)·BLM conjugate.[5b] In contrast, the conjugated Co(III)·BLM $A_5$ was employed directly for DNA cleavage. As noted in Scheme 1, both Sepharose 4B and controlled pore glass beads were employed for the conjugation of BLM $A_5$ to permit an assessment of the nature of the solid support on the ability of the attached BLM to effect DNA cleavage. Likewise, two tethers of different lengths were also employed to test their possible effects on DNA cleavage by the attached BLMs.

The progress and extent of conjugation of BLM to each of the solid supports was monitored by measuring the supernatant absorption at $A_{292}$ (due to the bithiazole moiety of BLM) as the reaction proceeded. Since the molar absorptivity of this functionality is known,[20b] quantification of the BLM $A_5$ successfully attached to the solid supports could be achieved by comparison of the supernatant concentrations of BLM $A_5$ before and after admixture of the supports containing the *N*-hydroxysuccinide esters. Each of the derivatized supports was washed thoroughly; no BLM $A_5$ release could be detected by $A_{292}$ absorption. All of the conjugated derivatives prepared had C-terminal resin "substituents" far larger than the (cross-section of the) DNA duplex. For example, the CPG beads employed for BLM conjugation were 50−100 $\mu$m ($5-10 \times 10^5$ Å) in size, i.e., between $10^4$ and $10^5$ times larger than the cross-section of a canonical B-form DNA duplex.

That BLM $A_5$ actually was attached to the solid supports via the spermidine C-substituent may be inferred from the known lack of reactivity of N atoms within the metal binding domain of BLM when the antibiotic is bound to metal ions. For example, Cu(II)·BLM demethyl $A_2$ reacts exclusively at the C-terminus

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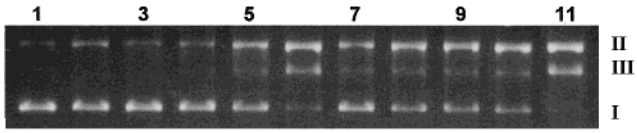


**Figure 2.** Cleavage of supercoiled pSP64 DNA by Fe(II)·BLM $A_5$-CPG-$C_2$. Lane 1, DNA alone; lane 2, 1.5 $\mu$M $Fe^{2+}$; lane 3, 0.01 $\mu$M Fe(II)·BLM $A_5$; lane 4, 0.03 $\mu$M Fe(II)·BLM $A_5$; lane 5, 0.05 $\mu$M Fe(II)·BLM $A_5$; lane 6, 0.08 $\mu$M Fe(II)·BLM $A_5$; lane 7, 0.1 $\mu$M Fe(II)·BLM $A_5$-CPG-$C_2$; lane 8, 0.3 $\mu$M Fe(II)·BLM $A_5$-CPG-$C_2$; lane 9, 0.5 $\mu$M Fe(II)·BLM $A_5$-CPG-$C_2$; lane 10, 1.0 $\mu$M Fe(II)·BLM $A_5$-CPG-$C_2$; lane 11, 1.5 $\mu$M Fe(II)·BLM $A_5$-CPG-$C_2$. The concentrations of BLMs used as conjugates are expressed as the concentration that would have been present in solution had the BLMs not been attached to the solid support.

when treated with a large excess of $CH_3I$ (Supporting Information, Scheme 1).[14b,22] A control experiment was also run using Cu(II)·BLM $A_2$ in the presence of CPG-$C_2$ that had been activated with *N*-hydroxysuccinimide; no conjugated product could be detected.

Initially, Fe(II)·BLM $A_5$-CPG-$C_2$ was tested for its ability to mediate DNA strand scission using supercoiled pSP64 plasmid DNA as a substrate. As shown in Figure 2, Fe(II)·BLM $A_5$-CPG-$C_2$ cleaved DNA perceptibly at 0.1 $\mu$M concentration (lane 7). Increasing concentrations of the Fe(II)·BLM $A_5$-CPG-$C_2$ effected increased conversion of supercoiled (Form I) DNA to nicked circular (Form II) and linear duplex (Form III) DNA (lanes 8−11), with clear conversion to Form III at all tested concentrations.

A number of control experiments were carried out to ensure that the observed DNA cleavage had not been caused by free BLM molecules. These included an experiment in which a sample of conjugated BLM $A_5$-CPG-$C_2$ 100-fold greater than the amount required to effect DNA cleavage (cf. Figure 2) was suspended in deionized, Chelex-treated water and placed in a microcentrifuge tube. The tube was agitated vigorously in a vortex mixer for 15 min, then filtered through a 0.45 $\mu$m nitrocellulose filter and concentrated under vacuum. The residue was employed in an attempted DNA cleavage reaction in the presence of $Fe^{2+}$, as outlined in the Experimental Section. No DNA cleavage was observed, arguing that there was no free BLM present in the conjugated sample even after vigorous agitation. A similar experiment was run on a scale sufficient to permit the supernatant to be concentrated following agitation and used to detect absorption at 292 nm, which is characteristic of the bithiazole moiety of BLM. No free BLM could be detected. Also carried out was an experiment in which a sample of free BLM $A_5$ equal to a few percent of the conjugated BLM $A_5$ required to effect DNA cleavage (Figure 2) was used in a DNA cleavage experiment under the same conditions employed for conjugated BLM $A_5$. No DNA cleavage could be detected, arguing that even a few percent adventitious free BLM in the conjugated BLM sample would be insufficient to effect the observed DNA cleavage. Densitometric analysis of the extent of DNA damage mediated by free Fe(II)·BLM $A_5$ and Fe(II)·BLM $A_5$-CPG-$C_2$ was determined in a similar experiment at each tested concentration (Table 1). Comparison of the extent of cleavage produced by free Fe(II)·BLM $A_5$ and Fe(II)·BLM $A_5$-CPG-$C_2$ indicated that free Fe(II)·BLM $A_5$ cleaves DNA with 3−5-fold greater efficiency. Thus the presence of the large C-terminal "substituent" for the conjugated BLM $A_5$ had only a limited effect on its ability to relax supercoiled plasmid DNA.

**Table 1.** Conversion of Supercoiled PSP64 Plasmid DNA to Form II and Form III DNA in the Presence of Fe(II)·BLM $A_5$-CPG-$C_2$

| | DNA products (%) | |
|---|---|---|
| bleomycin ($\mu$M) | Form II | Form III |
| free Fe(II)·BLM $A_5$ | | |
| 0.05 | 4.6 | 0 |
| 0.1 | 6.9 | 3.3 |
| 0.3 | 7.0 | 5.1 |
| 0.5 | 11.0 | 7.5 |
| conjugated Fe(II)·BLM $A_5$-CPG-$C_2$ | | |
| 0.3 | 3.5 | 1.2 |
| 0.5 | 3.8 | 2.3 |
| 1.0 | 4.6 | 5.8 |
| 1.5 | 11.0 | 5.9 |
| 2.0 | 12.3 | 6.2 |

It is interesting that activation of conjugated Fe(II)·BLM $A_5$ can be achieved without the addition of any external reductant, despite clear evidence that Fe(II)·BLM requires an additional electron for activation.[18b,23] In the absence of external reductants, activation normally involves the collision of two (oxygenated) Fe(II)·BLMs, which are believed to disproportionate to give 1 equiv of activated Fe·BLM and 1 equiv of Fe(II)·BLM. In the present case, the beads (50−100 $\mu$m) contained about $4.3 \times 10^3$ fmol of BLM $A_5$ per bead. Assuming a spherical shape for the beads and a random distribution of BLMs on the surface of the beads, the average distance between BLMs was thus ∼10−20 Å, which should permit Fe(II)·BLM activation via bimolecular collision. Alternatively, we have noted that $Fe^{2+}$ can rapidly be displaced from Fe(II)·BLM by $Cu^{2+}$ [24] and it seems not unlikely that Fe(II)·BLM is ordinarily in rapid equilibrium with free $Fe^{2+}$ and BLM. We have shown previously that $Fe^{2+}$ can serve as a source reducing equivalents for activating Fe(II)·BLM,[25] such that conjugated Fe(II)·BLM $A_5$ could also be activated by this mechanism.

Previously, we have shown that Fe·BLM $A_5$-CPG-$C_2$-mediated DNA cleavage occurred in a sequence-selective fashion. A 158-base pair DNA duplex derived from plasmid pBR322 by treatment with restriction endonuclease *Hin*dIII was 5′-$^{32}$P end labeled and then treated with Fe(II)·BLM $A_5$ and Fe(II)·BLM $A_5$-CPG-$C_2$. Identical sequence selectivity was observed for the free and conjugated Fe(II)·BLMs.[12] To assess the DNA cleavage properties of Fe(II)·BLM $A_5$-Sepharose 4B and Fe(II)·BLM $A_5$-CPG-$C_6$, the same 158-base pair, 5′-$^{32}$P end labeled DNA fragment from plasmid pBR322 was employed as a substrate for these tethered BLMs. Degradation of the 5′-end labeled DNA fragment was carried out by incubation with 10 $\mu$M Fe(II)·BLM $A_5$-Sepharose 4B, Fe(II)·BLM $A_5$-CPG-$C_6$, and Fe(II)·BLM $A_5$-CPG-$C_2$. Although BLM was conjugated to each of three resins having different tether lengths and properties, all three conjugated bleomycins cleaved DNA in a sequence-selective fashion identical to that mediated by free Fe(II)·BLM $A_5$ itself; prominent among the cleavage sites were 5-$G_{82}T_{83}$-3′ and 5′-$G_{84}T_{85}$-3′ (Figure 3, lanes 4, 7, 9, and 11). Analysis of Figure 3 indicates that free Fe(II)·BLM $A_5$ cleaved the substrate DNA with severalfold greater efficiency than the conjugated bleomycins. Interestingly, quantification of the gel indicated that for the conjugated BLMs cleavage was most

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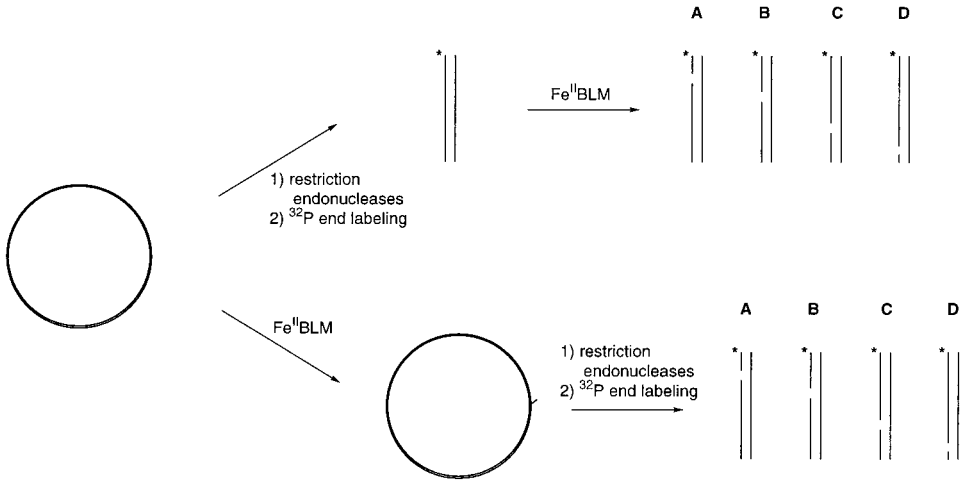
**Scheme 2.** Cleavage of a Relaxed, Covalently Closed Circular DNA and Analysis of the Sites of Cleavage




efficient in the case of Fe(II)·BLM $A_5$-CPG-$C_2$, which exhibited 10−30% more DNA damage than the conjugated bleomycins containing longer tethers.

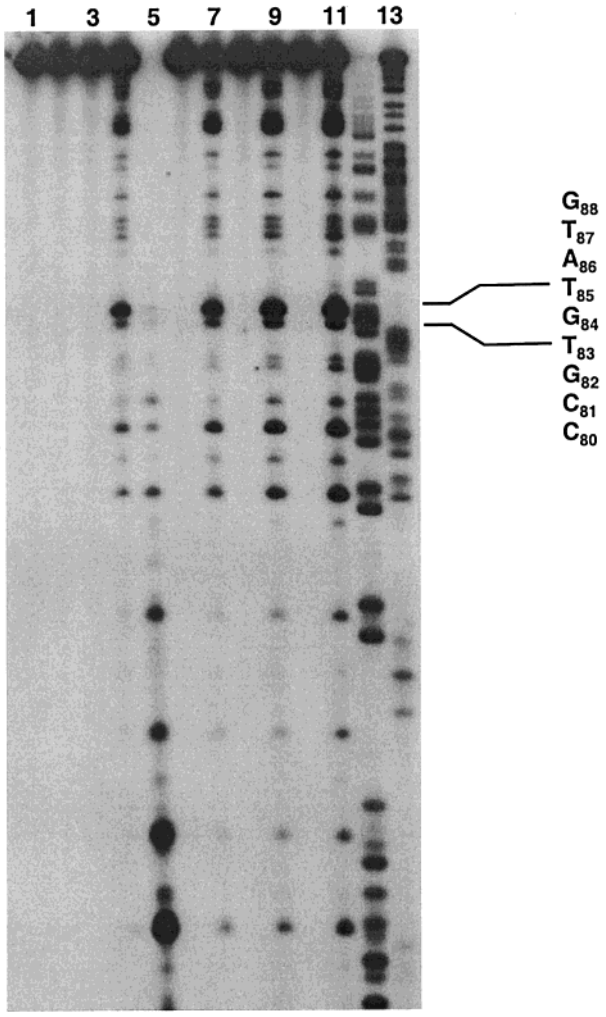



**Figure 3.** Cleavage of 5′-$^{32}$P labeled 158-base pair DNA duplex by conjugated Fe(II)·BLMs. Lane 1, DNA alone; lane 2, 10 $\mu$M $Fe^{2+}$; lane 3, 10 $\mu$M BLM $A_5$; lane 4, 1 $\mu$M Fe(II)·BLM $A_5$; lane 5, 10 $\mu$M Fe(II)·BLM $A_5$; lane 6, 10 $\mu$M BLM $A_5$-Sepharose 4B; lane 7, 10 $\mu$M Fe(II)·BLM $A_5$-Sepharose 4B; lane 8, 10 $\mu$M BLM $A_5$-CPG-$C_6$; lane 9, 10 $\mu$M Fe(II)·BLM $A_5$-CPG-$C_6$; lane 10, 10 $\mu$M BLM $A_5$-CPG-$C_2$; lane 11, 10 $\mu$M Fe(II)·BLM $A_5$-CPG-$C_2$; lane 12, Maxam−Gilbert G lane; lane 13, C lane.

This indicates that the lesser efficiency of cleavage by the conjugated Fe(II)·BLMs does not result from the proximity of the large C-terminal "substituent" to BLM, precluding normal interaction with DNA. Plausibly, the observed diminution of cleavage may be related to the localization of BLM to the surface of the resins. Since each resin particle contains $\sim 10^{12}$−$10^{13}$ BLM molecules, the effective concentrations of the resin particles in the reaction mixture are quite low and the encounter of DNA substrate molecules may well be less efficient.

While the separation of DNA strands in relaxed DNA is not favorable energetically,[26] it is nonetheless possible in principle that the results observed in Figure 3 could have resulted from separation of individual strands of the 158-base pair DNA duplex, which then rehybridized around the conjugated Fe(II)·BLMs. To preclude this possibility, we carried out the cleavage of relaxed, covalently closed pBR322 plasmid DNA, which lacks ends and is unlikely to undergo strand separation sufficient to allow threading intercalation of the conjugated Fe(II)·BLMs. As outlined in Scheme 2, the relaxed plasmid was treated with Fe(II)·BLM $A_5$-CPG-$C_2$ to afford an average of less than one nick per plasmid, followed by digestion with restriction endonuclease *Hin*dIII and 5′-$^{32}$P end labeling. The 5′-$^{32}$P end labeled product was then used for sequence analysis in direct comparison with the same restriction fragment that had been excised from plasmid pBR322 and 5′-$^{32}$P end labeled prior to treatment with Fe(II)·BLM $A_5$-CPG-$C_2$. As shown in Figure 4, Fe(II)·BLM $A_5$-CPG-$C_2$-mediated cleavage of the relaxed plasmid occurred at the same major sites (5′-$G_{82}T_{83}$-3′ and 5′-$G_{84}T_{85}$-3′) cleaved by free Fe(II)·BLM $A_5$. As is also apparent in Figure 4, when Fe(II)·BLM $A_5$-CPG-$C_2$ was utilized at a concentration 10-fold greater than free Fe(II)·BLM $A_5$, it gave DNA cleavage to a somewhat greater extent than the free Fe(II)·BLM $A_5$ (Figure 4, lanes 3 and 4). Thus Fe(II)·BLM $A_5$-CPG-$C_2$ can clearly cleave a DNA substrate with which it is quite unlikely to form a threading intercalated complex.

Coupling of the green Co(III)·BLM $A_5$ to a controlled pore glass bead was also accomplished as described in Scheme 1. Displacement of succinimide group from the activated resin in the presence of 0.1 M sodium acetate, pH 6.5, resulted in the conjugation of Co(III)·BLM $A_5$ to the controlled pore glass bead with a coupling efficiency of 32 $\mu$g/mg of resin.

The ability of Co(III)·BLM $A_5$-CPG-$C_2$ to relax plasmid DNA was measured in direct comparison with free Co(III)·BLM $A_5$.

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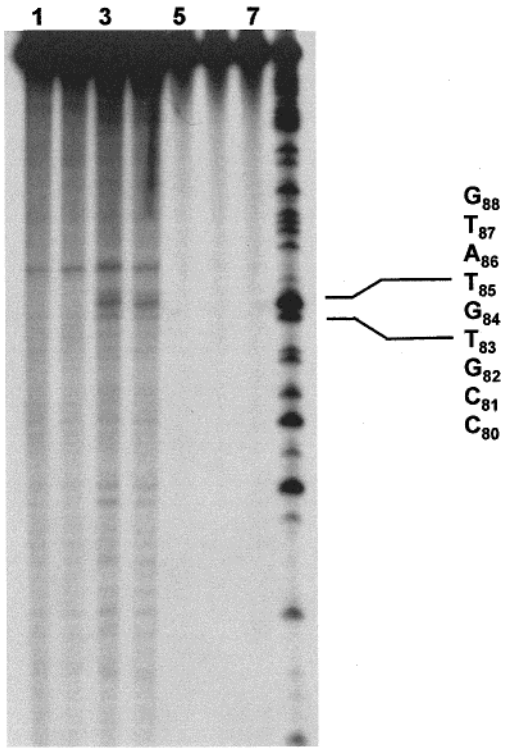


**Figure 4.** Cleavage of relaxed, covalently closed circular pBR322 DNA by Fe(II)·BLM $A_5$-CPG-$C_2$. Lane 1, DNA alone; lane 2, 20 $\mu$M $Fe^{2+}$; lane 3, 20 $\mu$M Fe(II)·BLM $A_5$-CPG-$C_2$; lane 4, 2 $\mu$M Fe(II)·BLM $A_5$; lane 5, $^{32}$P-labeled 158-bp pBR322 DNA duplex alone; lane 6, 1 $\mu$M $Fe^{2+}$; lane 7, 1 $\mu$M BLM $A_5$; lane 8, 1 $\mu$M Fe(II)·BLM $A_5$.

DNA cleavage was observed at a concentration of Co(III)·BLM $A_5$-CPG-$C_2$ as low as 0.2 $\mu$M (Supporting Information, Figure 1). At 1.0 $\mu$M concentration, Co(III)·BLM $A_5$-CPG-$C_2$ afforded detectable amounts of Form III DNA. Comparison of the amounts of cleavage produced by free Co(III)·BLM $A_5$ and Co(III)·BLM $A_5$-CPG-$C_2$ indicated that the conjugated analogue was approximately 2- to 3-fold less potent as a DNA cleaving agent.

Shown in Figure 5 are the results of cleavage of a 5′-$^{32}$P end labeled 158-base pair DNA duplex by Co(III)·BLM $A_5$-CPG-$C_2$. The samples were irradiated with a 500 W mercury−xenon lamp at 2 °C for 5 min. As shown in lane 4, Co(III)·BLM $A_5$-CPG-$C_2$ mediated DNA strand scission in a sequence-selective fashion identical to free Co(III)·BLM $A_5$. In the presence of 1 $\mu$M Co(III)·BLM $A_5$-CPG-$C_2$, the 5′-$^{32}$P end labeled duplex was cleaved efficiently at a number of sites, including the 5′-GT-3′ sequences cleaved by Fe(II)·BLM $A_5$ (Figure 5). As in the case of Fe(II)·BLM $A_5$, conjugation of Co(III)·BLM $A_5$ reduced its potency of DNA cleavage severalfold.

In addition to conjugated Fe(II)·BLM $A_5$ and Co(III)·BLM $A_5$, we also tested the ability of conjugated Cu(I)·BLM $A_5$ to effect the sequence-selective cleavage of DNA. Following activation of Cu·BLM as previously established,[5b] we found that the conjugated and free Cu(I)·BLMs $A_5$ gave essentially identical patterns of DNA cleavage using a 158-bp duplex DNA derived from pBR322 as a substrate (Figure 6). Again, cleavage by free Cu(I)·BLMs $A_5$ was severalfold more efficient than that by its conjugated analogue, but the results were otherwise the same. Thus three different metallobleomycins all retain their ability to cleave DNA in a sequence-selective fashion when conjugated to a C-substituent that is far too large to permit threading intercalation of the C-substituent of BLM.

Two additional facets of DNA cleavage by the conjugated metalloBLMs were explored. The first was the actual chemistry

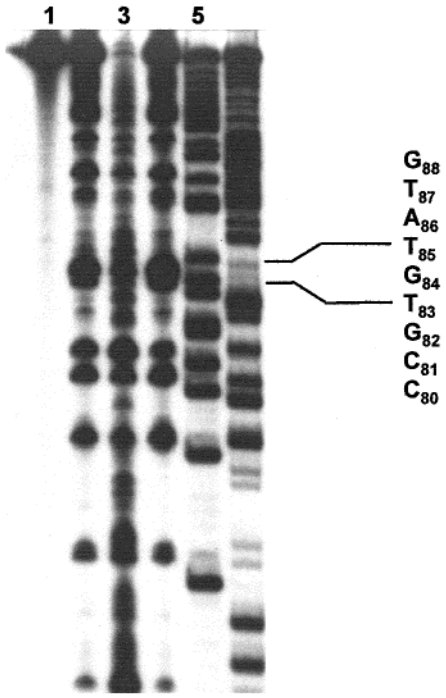


**Figure 5.** Cleavage of 5′-$^{32}$P labeled 158-bp pBR322 DNA duplex by Co(III)·BLM $A_5$-CPG-$C_2$. The reaction mixtures were irradiated with a 500 W mercury−xenon lamp at 2 °C for 5 min. Lane 1, DNA alone; lane 2, 0.1 $\mu$M Co(III)·BLM $A_5$; lane 3, 1.0 $\mu$M Co(III)·BLM $A_5$; lane 4, 1.0 $\mu$M Co(III)·BLM $A_5$-CPG-$C_2$; lane 5, Maxam−Gilbert G lane; lane 6, C lane.

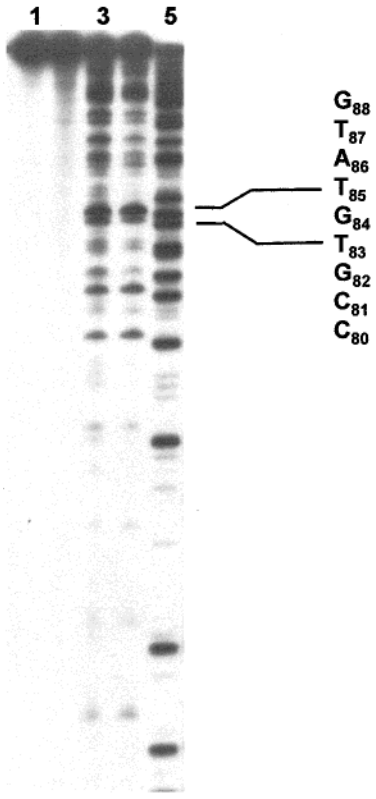


**Figure 6.** Cleavage of 5′-$^{32}$P labeled 158-bp pBR322 DNA duplex by Cu(II)·BLM $A_5$-CPG-$C_2$. All lanes contained 1 mM DTT and 0.5 $\mu$M sonicated calf thymus DNA. Lane 1, DNA alone; lane 2, 10 $\mu$M $Cu^{2+}$; lane 3, 1 $\mu$M Cu(II)·BLM $A_5$; lane 4, 10 $\mu$M Cu(II)·BLM $A_5$-CPG-$C_2$; lane 5, G lane.

of DNA cleavage. It is known that under ambient conditions, DNA cleavage affords frank strand breaks as the predominant

product, which contain phosphoroglycolate termini at the 3′-ends.[2] Also formed are alkali-labile lesions, which are converted to 3′-phosphate termini under the conditions employed for sequencing gels.[2,27] As shown on a well-resolved sequencing gel (Supporting Information, Figure 2), both free Fe(II)·BLM $A_5$ and Fe(II)·BLM $A_5$-CPG-$C_2$ gave bands corresponding to both products. Thus conjugation of Fe(II)·BLM $A_5$ to CPG does not alter its chemistry of DNA degradation.

A more provocative finding concerns the ability of the conjugated Fe·BLMs to effect double-strand DNA cleavage. As is apparent in Figure 2, conjugated Fe(II)·BLM $A_5$ produced Form III DNA, the formation of which could well involve double-strand DNA cleavage. An experiment carried out under conditions rigorously amenable to analysis of double strand cleavage (Table 1) indicated that both free and conjugated Fe(II)·BLM $A_5$ gave double strand cleavage of DNA to quite similar extents. Recent publications based on a study of Co(III)·BLMs have suggested that double strand cleavage, if it involves a single metalloBLM molecule, may involve a reorganization or relocation of BLM structure on the DNA duplex.[28] The present results with conjugated BLM $A_5$ clearly limit the orientational changes accessible to BLM for effecting double strand DNA cleavage. While it is possible that the postulated change in orientation is still accessible to the conjugated Fe·BLM $A_5$, that more than one conjugated Fe·BLM $A_5$ molecule is involved in double-strand cleavage, or that the DNA substrate moves relative to conjugated Fe·BLM $A_5$, it may also be the case that double-strand cleavage can result from a BLM molecule bound to DNA that involves minimal relative motion of the reactive partners.[29]

As is clear from the foregoing results, the conjugation of Fe(II)·BLM $A_5$ and Co(III)·BLM $A_5$ to solid supports has no effect on the sequence selectivity or chemistry of DNA cleavage, and surprisingly little effect on the efficiency of DNA cleavage. While it seems clear from $^1$H NMR studies of free Co(III)·BLM bound to DNA that this species binds in an energetically favored orientation that involves threading intercalation,[17] the present results make it clear that threading intercalation cannot be required for DNA cleavage by Fe(II)·BLM, Cu(I)·BLM, or Co(III)·BLM. This is underscored by the finding that the efficiency of DNA cleavage following conjugation of Cu(I)·BLM or Co(III)·BLM to controlled pore glass beads resulted in no greater diminution of cleavage efficiency than the analogous conjugation of Fe(II)·BLM.

In contrast to a threading intercalation mechanism, which seems clearly excluded for the conjugated BLMs, all of the data obtained to date for the conjugated BLMs are consistent with a model in which the bithiazole associates with the DNA substrate in the DNA minor groove or else by edgewise (partial) intercalation of one or both thiazole rings from an otherwise minor groove bound structure. This model is also consistent with our earlier finding that bleomycin analogues bearing one or two Cl atoms attached to the bithiazole moiety mediated normal sequence-selective DNA cleavage in the presence of $Fe^{2+}$, but produced DNA damage in the minor groove following photoinduced homolysis of the C−Cl bonds in the chlorobithiazole moiety.[21] The present findings thus parallel studies of Zn(II)·BLM[20] and phleomycin,[30] neither of which seem likely to bind to DNA by intercalation.

## Experimental Section

**General Experimental Procedures.** Calf intestinal phosphatase was obtained from Boehringer Mannheim Biochemicals. Restriction endonuclease *Eco*RV was purchased from GIBCO BRL and *Hin*dIII was from Promega. Plasmid pBR322 was purchased from New England Biolabs; relaxed plasmid pBR322 was from Lucent Ltd. (Leicester University Centre for Enterprise). T4 Polynucleotide kinase was obtained from Amersham; [$\gamma$-$^{32}$P]ATP (7000 Ci/mmol) was from ICN Pharmaceuticals. Sepharose 4B derivatized with 6-amino hexanoic acid and activated as the *N*-hydroxysuccinimide ester (loading 10−14 $\mu$mol/mL; 1 g swells to ∼3 mL) was purchased from Sigma; carboxymethyl CPG (pore volume 1.8 mL/g; pore size 630 Å; loading 162 $\mu$mol/g; surface area ∼60 $M^2$/g; bulk density ∼0.35 g/mL) and carboxypentyl CPG (pore volume 0.9 mL/g; pore size ∼600 Å; loading 127 $\mu$mol/g; surface area ∼70 $M^2$/g; bulk density ∼0.5 g/mL) were purchased from Prime Synthesis. Bleomycin $A_5$ was obtained with the assistance of Dr. Li-He Zhang, Beijing University.

Agarose gel electrophoresis was carried out in 40 mM Tris-acetate buffer, pH 7.8, containing 5 mM EDTA. The agarose gel loading solution contained 30% glycerol and 0.05% (w/v) bromophenol blue. Quantification of these gels was carried out by densitometry, taking care to utilize gels that had not been overexposed. The interexperiment variation was estimated to be no greater than 5%. Polyacrylamide gel electrophoresis was carried out in 90 mM Tris-borate buffer, pH 8.3, containing 5 mM EDTA.[31] The polyacrylamide gel loading solution contained 80% formamide, 2 mM EDTA, 0.05% (w/v) xylene cyanol, and 0.05% (w/v) bromophenol blue. Chemical sequencing was carried out according to the method of Maxam and Gilbert.[32] Quantification of the polyacrylamide gels was carried out by phosphorimager analysis using a Molecular Dynamics 400E Phosphorimager equipped with ImageQuant version 3.2 software. Distilled and deionized water from a Milli-Q system was treated with Chelex resin (Sigma Chemicals) and used for all aqueous solutions and manipulations. Autoradiography was carried out with Kodak X-Omat film at −80 °C.

**Synthesis of Cu(II)·BLM $A_5$.** An aqueous solution containing 5.0 mg (3.5 $\mu$mol) of BLM $A_5$ was treated with 5.6 mg (3.9 $\mu$mol) of $CuSO_4$ and the combined solution was maintained at 0−4 °C for 30 min. The resulting solution was purified on a C-18 column (12 × 3 cm) by washing with 500 mL of water, then with 300 mL of 4:1 MeOH−2 mM HCl. After combining the fractions that were blue in color, the sample was concentrated under diminished pressure to remove excess MeOH. The product was then lyophilized to obtain Cu(II)·BLM $A_5$[5] as a blue powder: yield 4.9 mg (92%).

**Synthesis of Green Co(III)·BLM $A_5$.** Co(III)·BLM $A_5$ was synthesized and purified by slight modification of previously described procedures.[6,17,33] An aqueous solution containing 5.0 mg (3.5 $\mu$mol) of BLM $A_5$ was treated with 1 equiv of $CoCl_2 \cdot 6H_2O$ and the solution was adjusted to pH 7.0 with dilute NaOH and allowed to stand at room temperature overnight. The mixture of products was purified by semipreparative reversed-phase C-18 HPLC; elution employed 0.1 M ammonium acetate, pH 5.5, with a linear gradient from 10 to 15% acetonitrile over a 45-min period at a flow rate of 3.0 mL/min. Detection was at 290 nm. The retention times for the brown and green complexes were 18.4 and 22.2 min, respectively.

**Coupling of Cu(II)·BLM $A_5$ to Sepharose 4B.** The Sepharose 4B beads (25 mg) were added to 1.0 mL of 0.1 M sodium acetate, pH 6.5, containing 1.99 mg (1.3 $\mu$mol) of Cu(II)·BLM $A_5$ and the resulting suspension was stirred rapidly at 0−4 °C. The solution was monitored at 292 nm by ultraviolet spectroscopy. After 38 h, the coupled Cu(II)·

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BLM $A_5$-Sepharose 4B was washed with 4.0 mL of 0.1 M sodium acetate, pH 6.5, and subsequently washed extensively with water (5 mL) to remove any traces of free Cu(II)·BLM $A_5$. Demetalation was accomplished by stirring with 15% aqueous EDTA (5 mL) at 4 °C overnight; further washing with 20 mL of water facilitated the removal of salts. The extent of bead derivatization was determined from the supernatant buffer after the coupling reaction was complete; the absorption maximum at 292 nm is characteristic for bleomycin and the known molar absorptivity ($\epsilon$ 14500 $M^{-1}cm^{-1}$)[20] permitted calculation of the amount of bleomycin that had failed to undergo coupling. The remainder of the material was assumed to have undergone coupling.

**Formation of Active *N*-Hydroxysuccinimide Ester of Carboxypentyl CPG.** Carboxypentyl CPG (5.0 mg) was added to a mixture of 0.66 mg (3.2 $\mu$mol; 5 equiv) of *N*,*N*′-dicyclohexylcarbodimide and 0.36 mg (3.2 $\mu$mol; 5 equiv) of *N*-hydroxysuccinimide in 0.5 mL of dioxane. The reaction mixture was stirred at 25 °C for 70 min. The CPG was stirred and washed on a coarse Buchner funnel with eight volumes of dioxane over a 10-min period, followed by four volumes of methanol over a 5-min period to remove the precipitated dicyclohexylurea. The activated CPG was washed further with three volumes of dioxane. After drying the derivatized CPG briefly for 10 min under suction, the slightly moist cake of activated CPG was kept in a sealed bottle at −20 °C for further use.

**Formation of Active *N*-Hydroxysuccinmide Ester of Carboxymethyl CPG.** Carboxymethyl CPG (5.0 mg) was added to a mixture of 0.85 mg (4.1 $\mu$mol; 5 equiv) of *N*,*N*′-dicyclohexylcarbodimide and 0.47 mg (4.1 $\mu$mol; 5 equiv) of *N*-hydroxysuccinimide in 0.5 mL of dioxane. The reaction mixture was stirred at 25 °C for 70 min. The CPG was stirred and washed on a coarse Buchner funnel with eight volumes of dioxane over a 10-min period, followed by four volumes of methanol over a 5-min period to remove the precipitated dicyclohexylurea. The activated CPG was washed further with three volumes of dioxane. After drying the derivatized CPG briefly for 10 min under suction, the slightly moist cake of activated CPG was kept in a sealed bottle at −20 °C for further use.

**Coupling of Cu(II)·BLM $A_5$ to the Activated Carboxypentyl CPG.** The moist activated CPG (5.0 mg) was added to 0.5 mL of 0.1 M sodium acetate, pH 6.5, containing 0.9 mg (0.60 $\mu$mol) of Cu(II)·BLM $A_5$ and the reaction mixture was stirred rapidly at 0−4 °C for 48 h. The course of the coupling was monitored by UV spectroscopy at 292 nm. The coupled Cu(II)·BLM $A_5$-CPG-$C_6$ was washed with 4 mL of 0.1 M sodium acetate, pH 6.5, and subsequently with 5 mL of water to remove traces of free Cu(II)·BLM $A_5$. Demetalation was accomplished by stirring with 1.0 mL of 15% aqueous EDTA at 4 °C overnight; further washing with water facilitated the removal of salts. The extent of derivatization (determined as for the Sepharose 4B-linked BLM) was approximately 48 $\mu$g/mg of bead.

**Coupling of Cu(II)·BLM $A_5$ to the Activated Carboxymethyl CPG.** The moist activated carboxymethyl CPG (5.0 mg) was added to 0.5 mL of 0.1 M sodium acetate, pH 6.5, containing 1.0 mg (0.67 $\mu$mol) of Cu(II)·BLM $A_5$ and the reaction mixture was stirred rapidly at 0−4 °C for 48 h. The course of the coupling was monitored by UV spectroscopy at 292 nm. The coupled Cu(II)·BLM $A_5$-CPG-$C_2$ was washed with 4 mL of 0.1 M sodium acetate, pH 6.5, and subsequently washed extensively with water (5 mL) to remove any traces of free Cu(II)·BLM $A_5$. Demetalation was accomplished by stirring with 1 mL of 15% aqueous EDTA at 4 °C overnight; further washing with water (10 mL) facilitated the removal of salts. The extent of derivatization was approximately 52 $\mu$g/mg of bead.

**Coupling of Co(III)·BLM $A_5$ to the Activated CPG.** The moist activated CPG (5.0 mg) was added to 0.1 M sodium acetate, pH 6.5, containing 0.49 mg (0.32 $\mu$mol) of Co(III)·BLM $A_5$ and the reaction mixture was stirred rapidly at 0−4 °C for 48 h. The course of the coupling was monitored by ultraviolet spectroscopy at 292 nm. The coupled Co(III)·BLM $A_5$-CPG was washed with 0.1 M sodium acetate, pH 6.5, and subsequently with water to remove traces of free Co(III)·BLM $A_5$. The extent of derivatization (determined as for the Sephorose 4B-linked BLM) was approximately 32 $\mu$g/mg of bead.

**Relaxation of Plasmid DNA by Fe(II)·BLM $A_5$ and Fe(II)·BLM $A_5$-CPG-$C_2$.** Reactions were carried out in 10 $\mu$L (total volume) of 10 mM sodium cacodylate, pH 7.0, containing 250 ng (0.13 pmol) of pSP64 plasmid DNA and the appropriate concentrations of Fe(II)·BLM $A_5$ or Fe(II)·BLM $A_5$-CPG-$C_2$. Reaction mixtures were initiated by the addition of 1 mM DTT, incubated at 37 °C for 30 min and then treated with 5 $\mu$L of loading solution (30% glycerol containing 0.05% (w/v) bromophenol blue) and applied to a 1% agarose gel containing 1 $\mu$g/mL of ethidium bromide. Horizontal gel electrophoresis was carried out in 90 mM Tris borate buffer, pH 8.3, containing 1 mM EDTA at 105 V for 3 h (UV−visualization).

**Preparation of a 5′-$^{32}$P End Labeled DNA Restriction Fragment.** A 158-base pair 5′-$^{32}$P end labeled DNA restriction fragment was prepared as described[22] starting from 25 $\mu$g of pBR322 plasmid DNA.

**Degradation of 5′-$^{32}$P End Labeled DNA Duplexes by Conjugated Fe·Bleomycins.** Reactions were carried out in 10 $\mu$L (total volume) of 10 mM Na cacodylate, pH 7.0, containing 5′-$^{32}$P end labeled DNA duplex ($4 \times 10^4$ cpm) and the appropriate concentrations of conjugated Fe(II)·BLM $A_5$. Reaction mixtures were agitated and incubated at 4 °C for 15 min, and then quenched by the addition of 4 $\mu$L of 80% formamide loading solution containing 2 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue. The resulting solution was heated at 90 °C for 10 min, chilled on ice, and then applied to a 14% denaturing polyacrylamide gel. The gel was run at 50 W for 1.75 h, then visualized by autoradiography.

**Cleavage of Relaxed Covalently Closed Circular DNA by Fe(II)·BLM $A_5$-CPG-$C_2$.** Reaction mixtures (10 $\mu$L total volume) contained 5 $\mu$g of relaxed covalently closed circular pBR322 DNA, 10 mM Na cacodylate, pH 7.0, and 20 $\mu$M Fe(II)$(NH_4)_2(SO_4)_2$, or 20 $\mu$M Fe(II)·BLM $A_5$-CPG-$C_2$, or 2 $\mu$M Fe(II)·BLM $A_5$. Reaction mixtures were incubated at room temperature for 15 min with agitation, then quenched with 5 $\mu$L of EDTA to a final concentration of 50 $\mu$M. A 0.5-$\mu$L aliquot of the samples was mixed with 30% glycerol loading solution (containing 0.05% (w/v) bromophenol blue) and loaded onto a 1% agarose gel containing ethidium bromide (1 $\mu$g/mL). The gel was run at 100 V for 45 min and visualized by UV.

**Preparation of a 5′-$^{32}$P End Labeled DNA Restriction Fragment after Treatment with Conjugated Bleomycin.** The remaining material from the above reactions was incubated with 20 units of restriction endonuclease *Hin*dIII in a reaction mixture (40 $\mu$L total volume) containing 6 mM Tris-Cl, pH 7.5, 6 mM $MgCl_2$, 100 mM NaCl, and 1 mM dithiothreitol. The reaction mixtures were incubated at 37 °C for 2 h and the DNA was recovered by ethanol precipitation.

The linearized DNA (14 pmol of 5′ termini) was dephosphorylated with 1 unit of calf intestinal alkaline phosphatase in a reaction mixture (140 $\mu$L total volume) containing 50 mM Tris-Cl, pH 7.5, and 0.1 mM EDTA. The reaction mixture was incubated at 37 °C for 1 h then terminated by heating at 65 °C for 15 min to inactivate the enzyme. The reaction mixture was extracted once with 50:48:2 phenol equilibrated with Tris, pH 7.8-chloroform-isoamyl alcohol and then once with ether. The DNA was recovered by ethanol precipitation.

The dephosphorylated DNA was 5′-$^{32}$P end labeled in a reaction mixture (30 $\mu$L total volume) containing 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 10 mM $\beta$-mercaptoethanol, and 2.3 $\mu$M [$\gamma$-$^{32}$P]ATP (0.32 mCi). T4 Polynucleotide kinase (9 units) was added to initiate the reaction. The reaction mixture was incubated at 37 °C for 1 h, then terminated by heating at 65 °C for 10 min to inactivate the enzyme.

The 5′-$^{32}$P end labeled DNA was digested with 100 units of restriction endonuclease *Eco*RV in a reaction mixture (150 $\mu$L total volume) containing 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, and 50 mM NaCl. The reaction mixture was incubated at 37 °C for 3 h. Fifty microliters of loading solution (30% glycerol, 0.05% xylene cyanol, and 0.05% bromophenol blue) was added and the sample was applied to an 8% native polyacrylamide gel. Electrophoresis was carried out at 10 W for 2−3 h. The gel was visualized by autoradiography, the band of interest was excised from the gel, and the DNA was eluted into 2 M $LiClO_4$ at 37 °C for 12 h. The solution was filtered to remove gel pieces and the DNA was recovered by ethanol precipitation.

A portion of the DNA (400 000 cpm) from each reaction was mixed with a equal volume of loading solution (containing 0.05% xylene cyanol and 0.05% bromophenol blue) and analyzed on a 16% denaturing PAG run at 50W for 2 h.

**Relaxation of Plasmid DNA by Co(III)·BLM $A_5$ and Co(III)·BLM $A_5$-CPG-$C_2$.** Reactions were carried out in 10 $\mu$L (total volume)

of 10 mM sodium cacodylate, pH 7.0, containing 250 ng (0.126 pmol) of pSP64 plasmid DNA and the appropriate concentrations of Co(III)·BLM $A_5$ and Co(III)·BLM $A_5$-CPG-$C_2$. Reaction mixtures were incubated at 2 °C for 5 min and agitated while irradiating with a 500 W mercury−xenon lamp, and then treated with 5 $\mu$L of loading solution (30% glycerol with 0.05% (w/v) bromophenol blue) and applied to a 1% agarose gel containing 1 $\mu$g/mL of ethidium bromide. Horizontal gel electrophoresis was carried out in 90 mM Tris borate buffer, pH 8.3, containing 1 mM EDTA at 150 V for 3 h (UV−visualization).

**Degradation of $^{32}$P-End Labeled DNA Duplexes by Co(III)·BLM $A_5$-CPG-$C_2$.** Reactions were carried out in 10 $\mu$L (total volume) of 10 mM sodium cacodylate, pH 7.0, containing 5′-$^{32}$P labeled DNA duplex ($4 \times 10^4$ cpm) and the appropriate concentrations of Co(III)·BLM $A_5$-CPG-$C_2$. Reactions mixtures were incubated at 2 °C for 5 min by mixing and irradiation with a 500 W mercury−xenon lamp in the presence of Pyrex and $H_2O$ filters, and quenched by the addition of 4 $\mu$L of 80% formamide loading solution containing 2 mM EDTA, 0.5% (w/v) of bromophenol blue, and 0.05% (w/v) of xylene cyanol. The resulting solution was heated at 90 °C for 10 min, chilled on ice, and then applied to a 16% denaturing polyacrylamide gel. The gel was run at 50 W for 2.25 h, then visualized by autoradiography.

**Degradation of a 5′-$^{32}$P End Labeled DNA Duplex by Cu(I)·Bleomycin.** Reactions were carried out in 10 $\mu$L (total volume) of 10 mM sodium cacodylate, pH 7.0, containing $^{32}$P-labeled DNA duplex ($4 \times 10^4$ cpm), sonicated calf thymus DNA (0.5 $\mu$M DNA nucleotide concentration) containing the indicated concentration of conjugated or free Cu(II)·BLM $A_5$ in the presence of 1 mM dithiothreitol. The Cu(II)·BLM $A_5$ + DTT were preincubated as described.[5b] The solution containing activated Cu·BLM was then added to the DNA−buffer mixture to initiate the reaction. Reaction mixtures were incubated at 4 °C for 15 min with agitation and quenched by the addition of 4 $\mu$L of 80% formamide loading solution containing 2 mM EDTA. The resulting solution was heated at 90 °C for 10 min, chilled on ice, and then applied to a 20% denaturing polyacrylamide gel. The gel was run at 50 W for 2.5 h, then visualized by autoradiography.

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**Supporting Information Available:** Figures showing the relaxation of supercoiled plasmid DNA by Co(III)·BLM $A_5$-CPG-$C_2$, the resolution of DNA cleavage products formed by Fe(II)·BLM-CPG-$C_2$, and a scheme showing the preferred position of methylation of Cu(II)·BLM demethyl $A_2$ (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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