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Conformationally Constrained Analogues of Bleomycin A_5

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Abstract: The bleomycin (BLM) group antitumor antibiotics are glycopeptide-derived natural products shown to cause sequence selective lesions in DNA. Prior studies have indicated that the linker region, composed of the methylvalerate and threonine residues, may be responsible for a conformational bend in the agent required for efficient DNA cleavage. We have synthesized a number of conformationally constrained methylvalerate analogues and incorporated them into deglycobleomycin A_5 congeners using our recently reported procedure for the solid phase construction of (deglyco)bleomycin and its analogues. These analogues were designed to probe the effects of conformational constraint of the native valerate moiety. Initial experiments indicated that the constrained molecules, none of which mimic the conformation proposed for the natural valerate linker, possessed DNA cleavage activity, albeit with potencies less than that of (deglyco)BLM and lacking sequence selectivity. Further experiments demonstrated that these analogues failed to produce alkali-labile lesions in DNA or sequence selective oxidative damage in RNA. However, two of the conformationally constrained deglycoBLM analogues were shown to mediate RNA cleavage in the absence of added Fe^{2+}. The ability of the analogues to mediate the oxygenation of small molecules was also assayed, and it was shown that they were as competent in the transfer of oxygen to low molecular weight substrates as the parent compound.

Introduction

The bleomycin (BLM) group antibiotics, represented by bleomycin A_5 (Figure 1), are a class of glycopeptide-derived antitumor agents that have been applied clinically for the treatment of a variety of malignancies, including those of the testes and lymph nodes.[1] The agents are composed of four primary structural domains; a metal binding domain responsible for the binding and activation of a metal ion cofactor, a nucleic acid binding domain which contributes the necessary nucleic acid binding affinity to the agent, a linker domain, and a carbohydrate moiety (Figure 1).[2] The bleomycins mediate sequence selective DNA cleavage, which has been shown to require a metal ion cofactor and O_2.[2] The observed sequence selectivity of DNA cleavage by BLM has been suggested by some to arise from selective interactions of the metal binding domain with DNA,[2−4] while others have asserted that sequence selectivity is governed at the interface of the bithiazole portion of the molecule and DNA.[5] BLM can also mediate oxidative RNA cleavage in the presence of Fe^{2+} and oxygen, and this process is also highly selective with regard to the sites cleaved.[6]

Much information has been gathered regarding the structure[7] and unique mechanism of action[2] of the BLMs. This has

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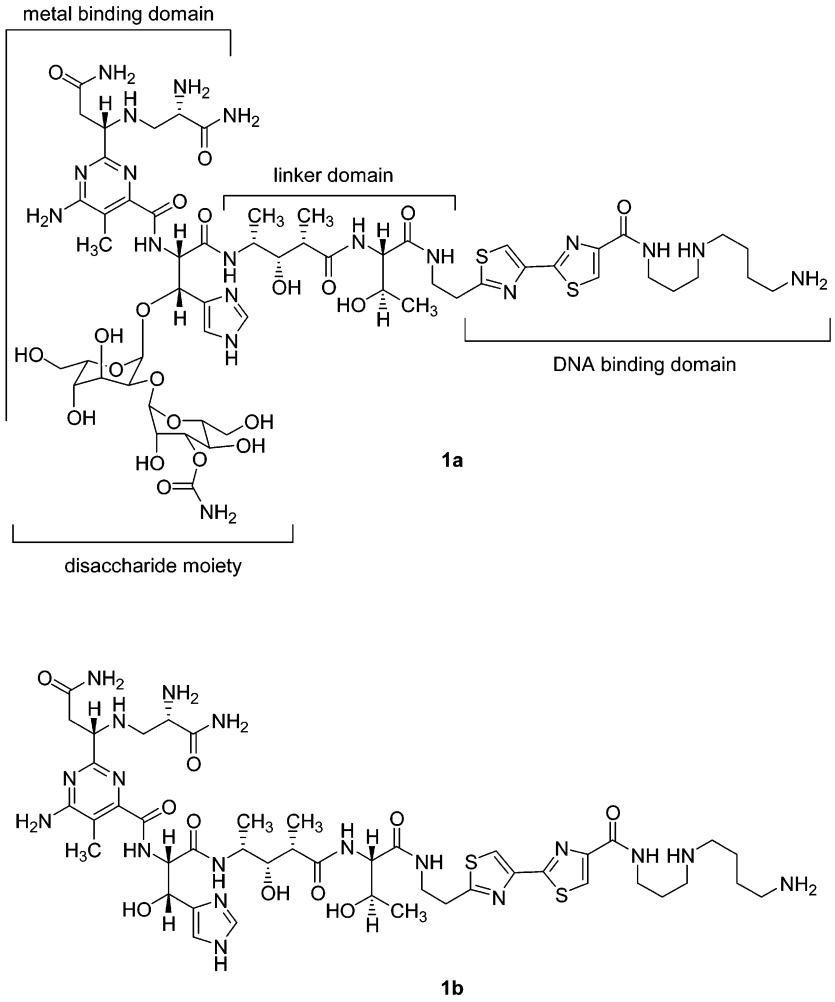

Figure 1. Structures of bleomycin A_5 (**1a**) and deglycobleomycin A_5 (**1b**).

included studies of the linker domain.[2d,4c,7c,8−12] A key finding of the studies focusing on this domain is that the substantial effect of the methylvalerate subunit on BLM cleavage efficiency is of a conformational nature. An analysis of the linker region of BLM has been presented, proposing that this portion of the molecule is responsible for preorganizing BLM into a rigid, compact conformation essential for efficient DNA binding and cleavage (Figure 2).[2d,11] Further, it has been suggested that the insertion of appropriately constrained valerate analogues into the linker region of bleomycin may accelerate rates of DNA cleavage by lowering the entropic cost for DNA binding.[12c] We have synthesized a series of novel conformationally constrained methylvalerate analogues **A**−**F**[13] (Figure 3) intended to probe for alternative DNA bound conformations of the linker region of deglycobleomycin A_5. Although none of these constrained analogues reproduce all aspects of the proposed bound conformation, they serve as an initial query of such conformational features. These analogues were incorporated into deglycoBLM A_5 using our methodology for the synthesis of (deglyco)BLM analogues on a solid support.[14,15] The analogues were assayed for DNA cleavage efficiency and selectivity relative to authentic deglycobleomycin A_5. In addition, the ability of selected analogues to mediate the oxygenation of styrene and the degradation of an RNA were also studied.

Results

Synthesis of Deglycobleomycin Analogues 2a−f. Incorporation of the constrained valerate analogues **A**−**F** (Supporting Information, Schemes S1 and S2) into deglycobleomycin A_5

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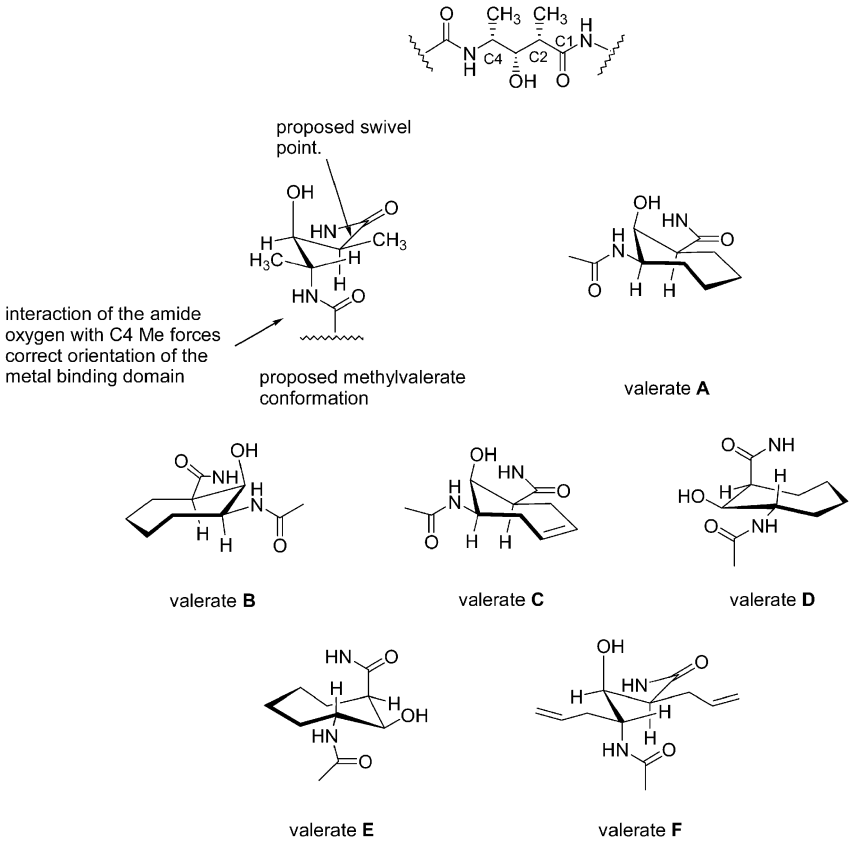

Figure 2. Conformational analyses of the native methylvalerate moiety of deglycobleomycin A_5[11] and valerate analogues **A**−**F**.

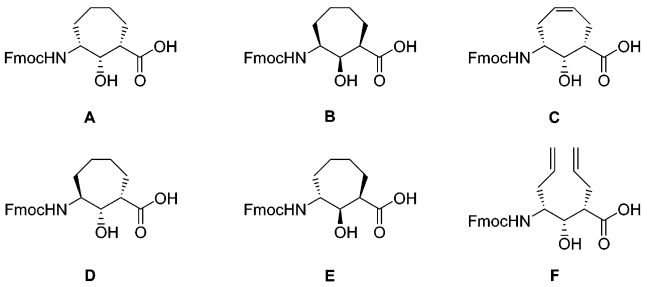

Figure 3. Conformationally constrained valerate analogues **A**−**F**.[13]

congeners was accomplished using our recently described methodology for the solid-phase synthesis of (deglyco)bleomycin A_5.[14,15] This technique has already proven quite efficient in the synthesis of deglycobleomycin A_5 and several analogues[14,15] and is further highlighted here by our construction of deglycoBLMs **2a**−**f** (Figure 4). The solid-phase synthesis of the deglycobleomycin A_5 analogues was accomplished using TentaGel resin (preloading of 0.45 mmol/g). Attachment of the requisite deglycobleomycin A_5 subunits to the solid support was performed using a hydrazine-labile Dde linker of the type described by Bycroft.[16] The elaboration of intermediate **3** (Scheme 1) has been described previously.[15]

Synthesis of resin-bound dipeptide **5** (Scheme 1) was accomplished through the coupling of the resin-bound spermidine moiety **3** and Fmoc bithiazole **4** in the presence of HBTU and Hunig's base in DMF. The addition of **4** permitted the initial quantitative measurement of resin loading. Fmoc cleavage analysis of an aliquot of dry resin treated with a solution of 20% piperidine in DMF indicated a loading of 0.24 mmol/g, corresponding to a 78% yield for the production of the dipeptide **5** from the Dde-linked TentaGel starting material.

Extension to the tripeptide **7** was accomplished by removal of the Fmoc group from **5** and subsequent coupling with commercially available N^{α}-Fmoc-(*S*)-threonine (**6**) in the presence of HBTU, HOBt, and Hunig's base in DMF. Fmoc cleavage analysis of the resin bound product **7** then afforded a loading of 0.21 mmol/g, corresponding to a 90% yield for the synthesis of tripeptide **7** from the dipeptide **5**.

Synthesis of the tetrapeptides **8a**−**f** was accomplished using valerate analogues **A**−**F** (Figure 3), respectively and a synthetic protocol identical to that used for the coupling of N^{α}-Fmoc-(*S*)-threonine (**6**). The use of **A**−**F** in the construction of the tetrapeptides **8a**−**f** gave the desired resin bound precursors in varying yields. Fmoc analysis of the tetrapeptides **8b**, **8c**, and **8d** indicated coupling yields of 91%, 88%, and 90%, respectively. However, the tetrapeptide analogues **8a**, **8e**, and **8f** were formed in much lower yields of 45%, 48%, and 45%, respectively.

The syntheses of the pentapeptide analogues **10a**−**f** (Scheme 2) were effected by Fmoc removal from the corresponding tetrapeptides **8a**−**f** followed by the coupling of the tetrapeptides with N^{α}-Fmoc-N^{im}-trityl-β-hydroxyhistidine (**9**) via the agency of HATU, HOAt, and Hunig's base in DMF. Fmoc analysis of an aliquot of each of the resin bound pentapeptides **10a**−**f** revealed resin loadings corresponding to yields of 93%, 82%, 56%, 50%, 86%, and 89% for the pentapeptides **10a**−**f**, respectively.

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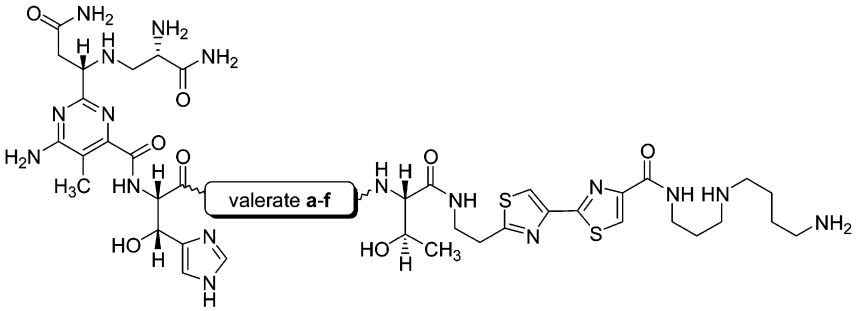


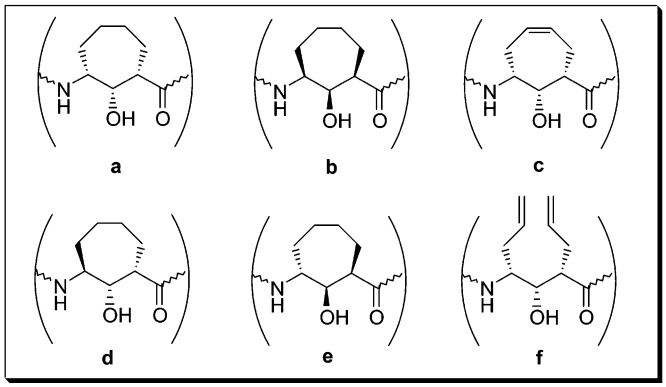
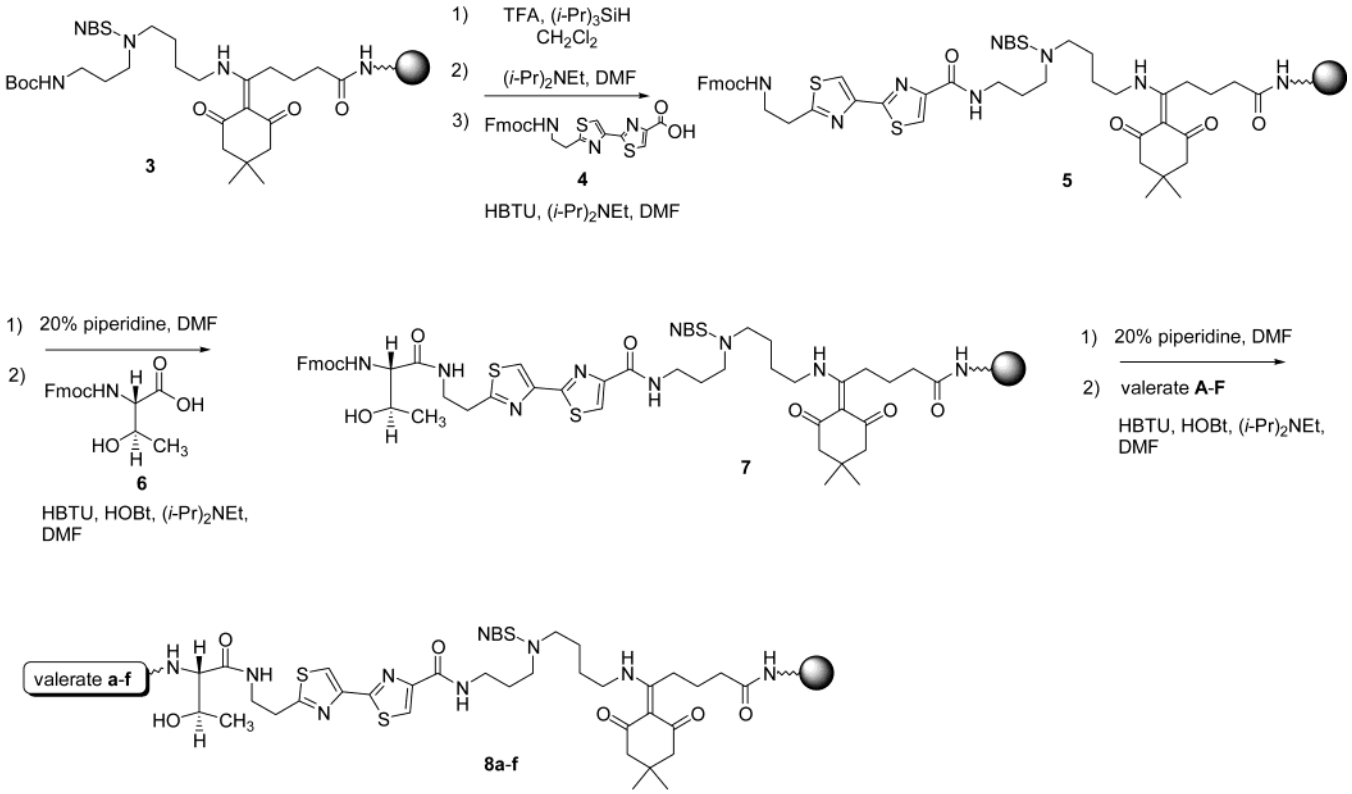


Figure 4. Structures of the constrained deglycobleomycin A_5 analogues **2a**−**2f**.

Scheme 1



Production of resin-bound deglycoBLM A_5 analogues **12a**−**f** was accomplished by a final Fmoc deprotection followed by coupling of the pentapeptides to Boc pyrimidoblamic acid (**11**)[17] utilizing the BOP reagent and Hunig's base at 0 °C. The coupling was performed overnight and in the absence of light. Acid-mediated deprotection of the Boc and trityl groups was accomplished through two successive 20-min exposures of the functionalized resin to trifluoroacetic acid in the presence of triisopropylsilane and dimethyl sulfide. Removal of the 2-nitrobenzenesulfonyl (NBS) group was accomplished by treatment

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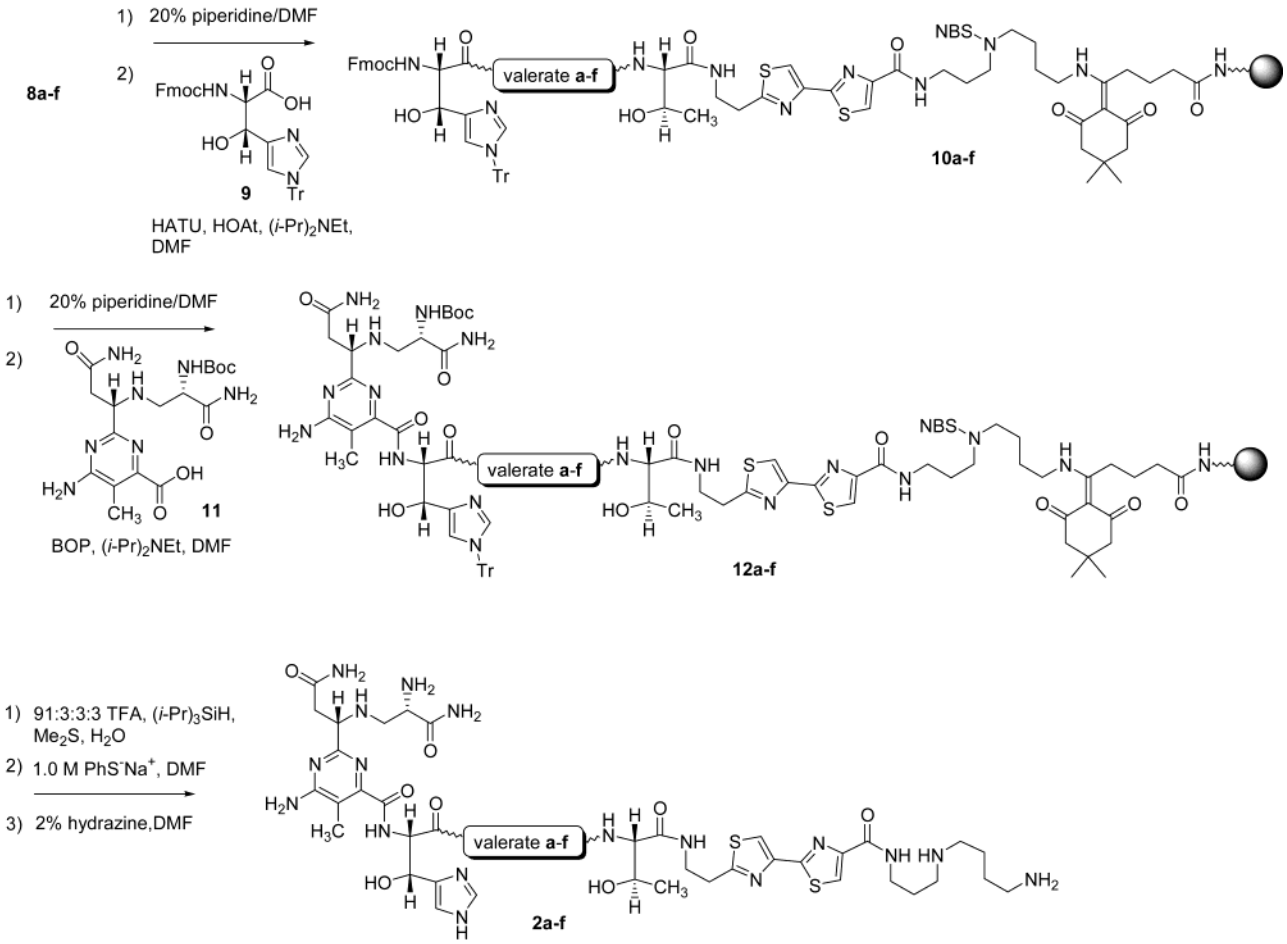
Scheme 2



with a 1 M solution of sodium thiophenoxide in DMF over a period of 2 h. Cleavage of the products **2a**−**f** from the resin was performed via treatment with a 2% solution of hydrazine in DMF. Precipitation of the product from CF_3COOH solution with diethyl ether, followed by HPLC purification, afforded the desired deglycoBLM A_5 analogues **2a**−**f** as colorless amorphous solids. The yields for the final coupling, deprotection, and purification for each analogue were quantified by UV analysis and were found to be 22%, 18%, 9%, 35%, 37%, and 6% for deglycoBLM A_5 analogues **2a**−**f**, respectively.

DNA Degradation by Deglycobleomycin A_5 Analogues Modified in the Valerate Moiety. The synthetic deglycoBLM analogues **2a**−**f** were assayed for their ability to relax supercoiled plasmid DNA (Figure 5). All of the analogues synthesized showed at least some ability to nick the circular DNA at a 10 μM concentration in the presence of Fe^{2+}. A sample of 10 μM deglycobleomycin A_5 in the presence of 1.5 μM Fe^{2+} (lane 15) quantitatively converted Form I (supercoiled) DNA to Form II (relaxed circular) and Form III (linear duplex DNA). As is clear from lane 1, the DNA employed for this study contained some nicked species. The conformationally constrained deglycoBLM analogues **2a**−**e**, in the presence of a metal ion cofactor (lanes 4, 6, 8, 10, and 12), converted supercoiled DNA to relaxed circular DNA to an extent approximately 15% greater than that observed for untreated DNA. The unconstrained analogue **2f** (lane 14) was much more efficient than any of the constrained analogues, relaxing all of the available supercoiled DNA and producing a significant quantity (12%) of Form III DNA. Deglycobleomycin A_5 itself (lane 15) was only slightly more

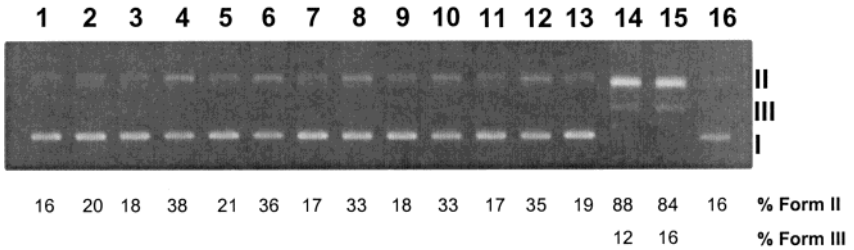


Figure 5. Cleavage of supercoiled pBR322 DNA by valerate-modified deglycobleomycin A_5 analogues. The incubation time was 30 min at 37 °C. Lane 1, DNA alone; lane 2, 1.5 μM Fe^{2+}; lane 3, 10 μM **2a**; lane 4, 10 μM **2a** + 1.5 μM Fe^{2+}; lane 5, 10 μM **2b**; lane 6, 10 μM **2b** + 1.5 μM Fe^{2+}; lane 7, 10 μM **2c**; lane 8, 10 μM **2c** + 1.5 μM Fe^{2+}; lane 9, 10 μM **2d**; lane 10, 10 μM **2d** + 1.5 μM Fe^{2+}; lane 11, 10 μM **2e**; lane 12, 10 μM **2e** + 1.5 μM Fe^{2+}; lane 13, 10 μM **2f**; lane 14, 10 μM **2f** + 1.5 μM Fe^{2+}; lane 15, 10 μM deglycoBLM A_5 + 1.5 μM Fe^{2+}; lane 16, 10 μM deglycoBLM A_5.

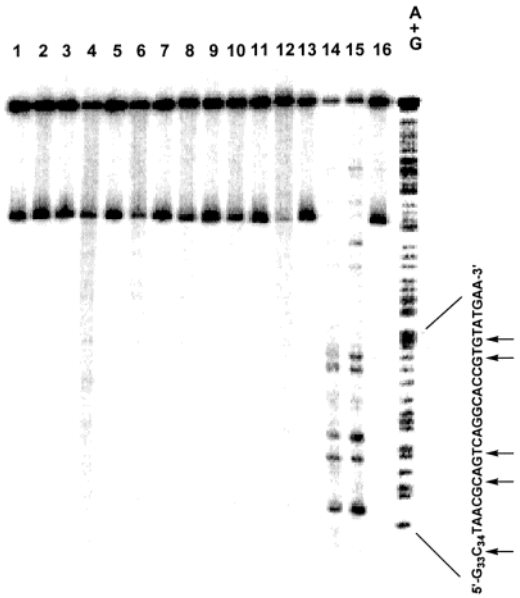


Figure 6. Cleavage of 5′-^{32}P end-labeled 158-base-pair DNA duplex by valerate-modified deglycobleomycin A_5 analogues **2a**−**f**. Lane 1, DNA alone; lane 2, 10 μM Fe^{2+}; lane 3, 10 μM **2a**; lane 4, 10 μM **2a** + 10 μM Fe^{2+}; lane 5, 10 μM **2b**; lane 6, 10 μM **2b** + 10 μM Fe^{2+}; lane 7, 10 μM **2c**; lane 8, 10 μM **2c** + 10 μM Fe^{2+}; lane 9, 10 μM **2d**; lane 10, 10 μM **2d** + 10 μM Fe^{2+}; lane 11, 10 μM **2e**; lane 12, 10 μM **2e** + 10 μM Fe^{2+}; lane 13, 10 μM **2f**; lane 14, 10 μM **2f** + 10 μM Fe^{2+}; lane 15, 10 μM deglycoBLM A_5 + 10 μM Fe^{2+}; lane 16, 10 μM deglycoBLM A_5. The band of intermediate mobility in lanes 1−13 and 16 was due to adventitious nondenatured DNA duplex.

effective at relaxing the plasmid DNA substrate, producing 84% Form II and 16% Form III DNA under the same conditions.

The constrained analogues were assayed for sequence selectivity of DNA cleavage using a 5′-^{32}P end-labeled 158-nucleotide DNA oligonucleotide substrate (Figure 6). As shown, the constrained analogues **2a**−**e** cleaved the 158-base-pair oligonucleotide weakly and at essentially every position, with no clear sequence selectivity. The unconstrained analogue **2f**, on the other hand, cleaved DNA with a sequence selectivity essentially identical to that of deglycobleomycin A_5.

In addition to frank DNA strand scission, BLM is also known to create alkali-labile lesions;[2] these have been shown to be more prominent at low oxygen tension and in the presence of certain modified DNA substrates.[18] To determine whether the modified deglycoBLM analogues **2a**−**2f** produced a greater proportion of such alkali-labile lesions, two arbitrarily chosen analogues (**2a** and **2c**) were also tested for their ability to produce sequence selective alkali-labile lesions in a 5′-^{32}P end-labeled 158-nucleotide DNA oligonucleotide substrate (Figure 7). The analogues tested showed no ability to produce sequence selective lesions in DNA, analogous to those produced by Fe(II)•deglycoBLM A_5 (Figure 7, lane 1). While those tested deglycoBLM analogues **2** mainly produced cleavage bands at every position in the DNA substrate, it is interesting that some of these bands were somewhat enhanced in relative intensity following alkali treatment and were at sites different than those produced by deglycoBLM A_5.

With the apparent lack of DNA cleavage sequence selectivity by **2a**−**2e** established, we turned our attention to an RNA

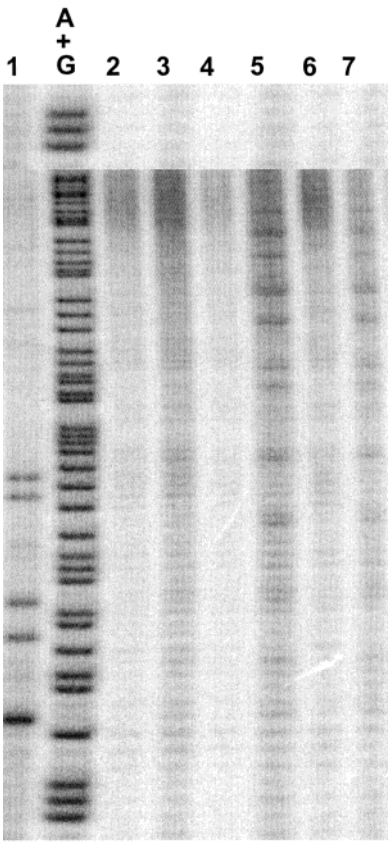


Figure 7. Assay for alkalai-labile lesions produced in a 5′-^{32}P end-labeled 158-base-pair DNA duplex by valerate-modified deglycoBLM analogues **2a** and **2c**. Samples were initially incubated for 30 min at 37 °C and then treated with 0.1 M piperidine for 20 min at 90 °C. Lane 1, 1 μM deglycobleomycin A_5 + 10 μM Fe^{2+}; lane 2, DNA alone; lane 3, 10 μM Fe^{2+}; lane 4, 10 μM **2a**; lane 5, 10 μM **2a** + 10 μM Fe^{2+}; lane 6, 10 μM **2c**; lane 7, 10 μM **2c** + 10 μM Fe^{2+}.

substrate to ascertain if any sequence selectivity could be established for the constrained bleomycin analogues using these types of substrates. As shown in Figure 8, in the presence of Fe^{2+} no sequence selective oxidative RNA cleavage was observed for any of the analogues, including **2f** which had exhibited sequence selective DNA cleavage (Figure 6, lane 14). It may be noted, however, that RNA cleavage by BLM exhibits a concentration optimum[6b] (cf Figure 8, lanes 12 and 13), and it is conceivable that some of the deglycoBLM analogues **2** may produce RNA lesions under narrowly defined conditions of concentration.

Keck and Hecht et al.[19] have shown previously that BLM can effect the cleavage of $tRNA^{Phe}$ in the absence of Fe^{2+} by a process that must involve phosphoryl transfer involving the 2′-OH groups in the RNA substrate at the sites of cleavage. The actual cleavage sites occurred at all pyrimidine−purine sites not involving modified nucleosides; the most efficient cleavage was at the five 5′-CA-3′ sequences.

As shown in Figure 8, deglycoBLM analogues **2b** and **2e**, chosen to be representative of the constrained analogues, effected cleavage of the 53-nucleotide RNA substrate employed here in the absence of added Fe^{2+} but not in its presence, suggesting the occurrence of "hydrolytic" cleavage analogous to that observed previously for BLM itself.[19] Interestingly, the

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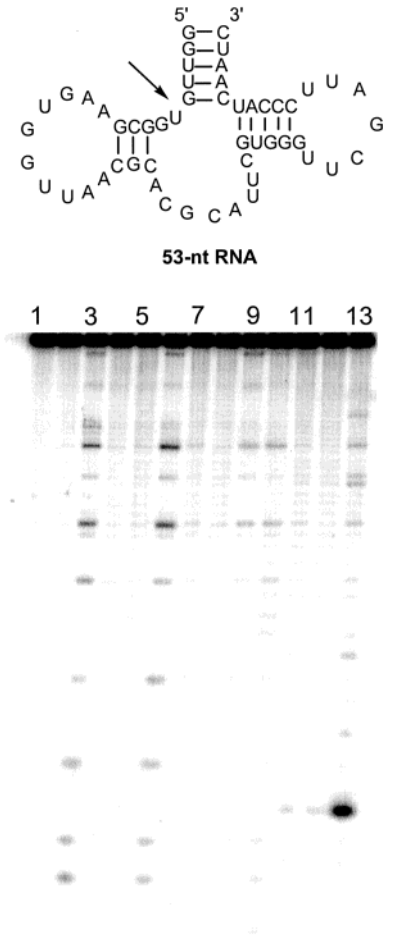

Figure 8. Cleavage of a 53-nt RNA by valerate-modified deglycobleomycin A_5 analogues **2b**, **2e**, and **2f**. Lane 1, RNA alone; lane 2, 100 μM Fe^{2+}; lane 3, 100 μM **2b**; lane 4, 10 μM **2b** + 10 μM Fe^{2+}; lane 5, 100 μM **2b** + 100 μM Fe^{2+}; lane 6, 100 μM **2e**; lane 7, 10 μM **2e** + 10 μM Fe^{2+}; lane 8, 100 μM **2e** + 100 μM Fe^{2+}; lane 9, 100 μM **2f**; lane 10, 10 μM **2f** + 10 μM Fe^{2+}; lane 11, 100 μM **2f** + 100 μM Fe^{2+}; lane 12, 100 μM deglycoBLM A_5 + 100 μM Fe^{2+}; lane 13, 10 μM bleomycin A_5 + 10 mM Fe^{2+}. The position of cleavage (arrow) obtained with deglycoBLM A_5 in lane 13 was inferred by analogy with the cleavage of *Bacillus subtilis* $tRNA^{His}$ precursor transcript.[6b]

efficiency of this process was significantly better for constrained deglycoBLM analogues **2b** and **2e** than for unconstrained analogue **2f**.

Oxidation of Styrene by Deglycobleomycin A_5 Analogues. In addition to their ability to mediate oxidative damage of DNA and RNA substrates, bleomycin and some of its analogues have also been shown to be capable of supporting the oxygenation of a number of low molecular weight substrates including *cis*-stilbene and styrene and also the hydroxylation of aromatic substrates such as naphthalene and anisole.[4a,4e,20] To determine whether the constrained analogues **2** were bleomycin-like in their capacity to deliver oxygen to small molecules, we tested one of the analogues for its ability to convert styrene to phenylacetaldehyde and styrene oxide in the presence of Fe^{3+} and hydrogen peroxide. The results from this experiment are shown in Table 1. The data clearly demonstrate that analogue **2d**, chosen to be respresentative of the constrained analogues, was as efficient as bleomycin and more efficient than deglycoBLM A_5 in mediating the conversion of styrene to phenylacetaldehyde (**13**) and styrene oxide (**14**).

Discussion

Oxidative damage of DNA by bleomycin can be viewed as a two-step process.[21] Bleomycin first coordinates a metal cofactor and oxygen and undergoes activation to afford a species capable of oxidative DNA degradation. The drug then binds to DNA at a preferred cleavage site, leading to abstraction of the C-4′ H from the deoxyribose moiety, which is apparently the rate-limiting event in the oxidative strand cleavage of a nucleic acid substrate.[22] It is generally accepted that interactions between the activated metal binding domain and the minor groove of the bound DNA duplex are of primary importance in maintaining the sequence selectivity observed for Fe•BLM.[3a,4c,23] Although analogues of the BLM metal binding domain have been shown to mediate sequence selective cleavage of DNA,[3] it has been demonstrated that the actual metal binding subunit of deglycobleomycin alone was unable to produce significant sequence selective DNA cleavage in the presence of Fe^{2+}.[4a,h]

The C-terminus of the agent, although not the primary determinant of DNA sequence selectivity, has been shown to be responsible for the majority of the observed DNA binding affinity of bleomycin.[8] Further, studies of the photoinduced cleavage of DNA by chlorobithiazoles and structurally related deglycobleomycin analogues incorporating chlorinated bithiazole moieties have suggested that the bithiazole moiety itself may in fact exhibit some sequence selective DNA binding.[4f,24] This selectivity, however, was found not to be the same as that associated with oxidatively induced Fe•BLM lesions. This may explain why certain GpY sequences are cleaved more efficiently by bleomycin than others, as those sites best suited to accommodate the preferences of both the metal binding domain and the bithiazole moiety may be cleaved with the greatest efficiency.

The linker domain, while not implicated directly in sequence selective DNA binding or cleavage, has been demonstrated to be important for maintaining the efficiency with which bleomycin cleaves DNA.[11] Although alterations to this region have been shown to have a significant impact on DNA cleavage efficiency, they have not been reported to result in a change in DNA sequence selectivity. In short, the function of the linker domain has been suggested to be one of a conformational nature, directing the agent into a conformation amenable to the efficient, sequence selective cleavage of DNA. Consistent with this analysis, it has been suggested that the appropriate conforma-

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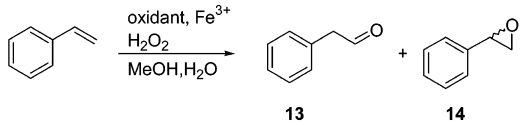
Table 1. Styrene Oxidation by BLM, DeglycoBLM A_5, and DeglycoBLM Analogue **2d**[a,b]



	compound **13**	compound **14**	compound **13**	compound **14**	compound **13**	compound **14**
oxidant	40 min		80 min		120 min	
bleomycin	4.8 ± 1.3	0.6 ± 0.2	2.9 ± 0.8	0.2 ± 0.06	2.9 ± 0.8	0.09 ± 0.03
deglycoBLM A_5	2.9 ± 0.8	0.5 ± 0.2	2.7 ± 0.8	0.2 ± 0.05	2.1 ± 0.6	0.05 ± 0.02
deglycoBLM **2d**	5.0 ± 1.5	1.1 ± 0.3	4.8 ± 1.3	0.7 ± 0.2	2.9 ± 0.7	0.3 ± 0.08

[a] Reaction conditions, 0.73 mM Fe^{3+}, 73 mM styrene, 1.6 mM ethyl benzoate (internal standard), 0.72 mM BLM, deglycobleomycin A_5 or **2d**, and 15 mM H_2O_2. The reactions were maintained at 4 °C for the times indicated. [b] Results are in mmol/L; reported values are the average of three experiments.

tional constraint within the linker domain could lower the entropic cost for DNA binding and thus facilitate cleavage.[12c]

Boger et al.[11c] reported an insightful, quantitative conformational analysis of the linker region in which they stressed the importance of the valerate C4 methyl substituent in permitting the proper orientation of the metal binding domain with respect to the bound DNA substrate. Further, these authors identified a swivel point within the valerate allowing for ca. 120° of rotation between C1 and C2. This swivel point was suggested to allow the bleomycins "access to several related DNA bound conformations" depending on the microstructure of the DNA complex.

Based on this analysis we prepared the valerate analogues shown in Figures 2 and 3 and the respective deglycoBLMs (Figure 4). These analogues were chosen for their ability to provide an initial query of requisite conformations within the methylvalerate moiety and also because they were reasonably accessible synthetically. While none of these analogues closely resembles the conformation proposed to facilitate the formation of a compact linker region associated with efficient DNA cleavage, the existence of swivel points that may provide BLM access to related DNA bound conformations suggested that alternate BLM conformations might conceivably also produce DNA damage.

We utilized our solid phase synthesis strategy[14,15] to elaborate the deglycobleomycin A_5 analogues (**2a**−**f**) in which the valerate moiety was replaced with the valerate analogues **A**−**F**, respectively. It has been proposed that the construction of appropriately constrained analogues should facilitate accelerated rates of DNA cleavage, as the energy for DNA binding should be lowered significantly.[12c] It was of interest to us to see whether any of the constrained analogues **2a**−**e** was capable of eliciting the desired effect even though none of them embody all of the conformational constraints of the natural valerate linker.

As demonstrated in Figure 5, the constrained analogues **2a**−**e** exhibited greatly reduced abilities to relax supercoiled plasmid DNA to produce circular (Form II) DNA. Further, in no case was any linear duplex (Form III) DNA observed. On the other hand, the unconstrained analogue **2f**, prepared as a control, relaxed supercoiled plasmid DNA to produce both circular and linear duplex DNA with an efficiency quite comparable to that of deglycobleomycin A_5 itself. To ensure that the modifications to deglycobleomycin A_5 had not affected its ability to complex with a metal ion cofactor and activate bound oxygen, the analogue **2d** was tested for its ability to oxygenate styrene to afford phenylacetaldehyde (**13**) and styrene oxide (**14**).[20] The analogue performed similarly to authentic BLM and deglycobleomycin A_5 (Table 1), indicating that the ability of this analogue to form an activated metal complex had not been impaired.

The sequence selectivity of DNA cleavage by analogues **2a**−**f** was assayed using a 5′-^{32}P end-labeled linear DNA duplex (Figure 6). The results showed that the constrained analogues **2a**−**e** cleaved the linear DNA substrate at essentially every position, while the unconstrained analogue **2f** maintained the sequence selectivity of the parent deglycoBLM. Analogues **2a** and **2c** were studied further to see whether they formed alkali-labile lesions in the DNA substrate. As shown in Figure 7, none of these analogues produced alkali-labile lesions at the same sites as deglycoBLM A_5, but there were sites at which some enhancement of cleavage may have occurred.

There is accumulating evidence that RNA may also be a therapeutically important target for BLM.[6] Accordingly, it seemed important to characterize RNA cleavage by the conformationally constrained deglycobleomycin analogues. In fact, an interesting result was obtained when analogues **2b** and **2e** were tested for their ability to cleave RNA. The substrate employed was a synthetic RNA identical in sequence with the core region of the *B. subtilis* $tRNA^{His}$ precursor, the latter of which undergoes efficient cleavage by Fe•BLM predominantly at a single site.[6b] The 53-nt RNA has also been shown to undergo efficient cleavage by Fe•BLM at what is presumably the analogous position.[15] While neither **2b** nor **2e** effected cleavage of this RNA under oxidative conditions, the metal free analogues both effected RNA cleavage at a number of sites via a "hydrolytic" process, as has been noted previously for BLM itself.[19] Further, nonoxidative RNA cleavage by **2b** and **2e** was considerably more efficient than that mediated by unconstrained analogue **2f**. The observation that analogues **2b** and **2e** were able to produce sequence selective RNA cleavage in the absence of a metal cofactor indicates that the binding mode for this process is different than that required for oxidative RNA cleavage.

At least two possibilities are consistent with the behavior observed for deglycoBLM analogues **2a**−**2e**. The first is that deviations from an optimal folded conformation of the linker domain may greatly affect the orientation of the metal binding domain with respect to its DNA substrate. The specific structural constraints employed in the present study thus precluded the adoption of a conformation necessary for efficient DNA binding and cleavage. The second possibility is that some conformational flexibility per se within the linker domain may be necessary for efficient sequence selective binding and cleavage of DNA

and RNA substrates. Accordingly, the conformational constraints imposed in analogues **2a**−**2e** may have precluded efficient cleavage. In this context, it may be mentioned that a parallel study employed analogues of deglycoBLM having conformationally constrained replacements for the methylvalerate moiety based on the model of Zn(II)•deglycoBLM bound to DNA;[25,26] no sequence selective DNA cleavage was obtained.[27] The failure of several conformationally constrained deglycoBLM analogues to mediate sequence selective DNA cleavage raises the possibility that the compact valerate conformation adopted by (deglyco)BLM itself[11] may be uniquely suited to orient the molecule in a conformation conducive to selective DNA cleavage. In principle, the lack of sequence selective DNA and RNA cleavage by analogues **2a**−**2e** might also reflect unfavorable steric interactions with substrate not present for deglycobleomycin itself. However, this seems unlikely, based both on the sequence selective cleavage mediated by analogue **2f** and also by the recent finding that deglycobleomycin analogues having C4 substituents of increased size within the methylvalerate moiety actually exhibited enhanced cleavage of supercoiled plasmid DNA.[28] Clearly, it may be possible to decide among the foregoing possibilities by preparing constrained analogues that more closely mimic the proposed preferred conformation.

Finally, it may be noted that the assays employed in the present study measured the cleavage of DNA and RNA, an event that is believed to be preceded by selective binding of these substrates. While **2a**−**2e** failed to mediate sequence selective oxidative cleavage of the single DNA and RNA substrates tested in this initial study, it seems possible that these analogues may still exhibit DNA and RNA binding. This possibility is underscored by the fact that H atom abstraction from DNA is the rate-limiting step in DNA cleavage even for BLM itself.[22] Accordingly, these analogues could exhibit productive binding and cleavage of non-B-form DNA structures such as DNA triplexes[29] and bulges,[30] not to mention RNA structures not cleaved efficiently by BLM itself.[6c] In this context, it is interesting to note that McLean et al.[31] found that Co•BLM and Fe•BLM, which have the same valerate moiety, nonetheless exhibited different patterns of DNA cleavage; each cleaved DNA at some positions not cleaved by the other metallobleomycin. This argues that not all activated BLMs exhibit the same unique DNA binding pattern and conversely that one may anticipate that individual BLM analogues could exhibit a particular facility for the cleavage of a structurally unique DNA or RNA substrate.

Also worthy of comment are the implications of the present study for models of BLM binding and cleavage published previously. Assuming that the failure of analogues **2a**−**2e** to mediate sequence selective DNA cleavage is not due to their lack of flexibility, the present results support the thesis that stabilization of a rather narrowly defined conformation of the methylvalerate moiety is essential for sequence selective DNA cleavage. However, it is perhaps somewhat surprising that conformational alterations around the proposed swivel points[11b,c] did not result in DNA cleavage, if only at altered positions.

A related issue is the proposal[12b,c,32] that both Fe(II)•BLMs and Co(III)•BLMs, which were both used to develop the binding model being tested in this study, interact with and cleave their nucleic acid substrates in the same fashion. There is compelling evidence that this is not the case. DNA cleavage by Fe•BLM is a dark reaction, while cleavage by Co(III)•BLM requires light.[33] The affinity of Co(III)•BLM for its DNA substrate is 10-fold greater than that of Fe(II)•BLM.[33b] The specificities of these two metallobleomycins were compared by examining the relative extents of cleavage of each DNA strand in a DNA duplex; neither the selectivity nor the extent of cleavage by the metallobleomycins at specific sites along the DNA backbone was comparable.[31] Specifically, Fe(II)•BLM exhibited characteristic GT and GC selectivity, while Co(III)•BLM showed a preponderance of cleavage at GA and at every GT sequence. The cleavage pattern for Co(III)•BLM was sequence selective but different than that of Fe(II)•BLM. Co(III)•BLM has also been reported to produce less double-strand cleavage than Fe(II)•BLM.[33b] Additionally, it was suggested that the actual mechanism of cleavage by the two metallobleomycins is different, an assertion that is consistent with differences in the nature of the degradation products formed,[34] as well as differences in the isotope effects associated with DNA degradation.[22,35] Further, each metalloBLM cleaved DNA at sites not cleaved by the other metalloBLM.[31] The data argues strongly that the two metallobleomycins utilize different chemistry for DNA cleavage and exhibit different sequence selectivity for DNA cleavage and that caution should be exercised when making generalizations regarding commonalities in the behavior of different metallobleomycin species.

Experimental Section

General Methods. Mass determination was accomplished by electrospray ionization on a Finnigan 3200 Quadrupole mass spectrometer. HPLC purifications were performed on a Varian Associates HPLC using an Altech Alltima C_{18} reversed phase column (250 × 10 mm, 5 μm). TentaGel resin, HBTU, HOBt, BOP, and N^{α}-Fmoc-(*S*)-threonine were purchased from Novabiochem. Microspin G-25 columns were obtained from Qiagen. DMF was purchased from Acros Organics. All other reagents were purchased from Aldrich Chemical Co. The 53-nucleotide RNA substrate was purchased from Dharmacon Research. All solvents were of analytical grade. Tetrahydrofuran was distilled from potassium metal and benzophenone ketyl prior to use. Methanol was dried over 3 Å molecular sieves for 48 h prior to use. All synthetic transformations were carried out under dry argon or nitrogen. Dry resin was swollen in CH_2Cl_2 and then in the appropriate reaction solvent prior to use.

Yield determination for reactions performed on solid support was accomplished by UV quantification of the dibenzylfulvene−piperidine adduct formed upon treatment of the resin with piperidine.[36] The molar

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absorptivities of 5540 M^{-1} at 290 nm and 7300 M^{-1} at 300 nm were used to calculate the loading from a known weight of dry resin.

Resin-Bound Dipeptide 5. To 500 mg of swollen Nbs and Boc protected spermidine resin (**3**) was added a 10% solution of triisopropylsilane in 2 mL of CH_2Cl_2 and 1 mL of CF_3COOH. The reaction mixture was stirred for 1 h, and then the solvent was removed by filtration and the entire process was repeated. The resin was washed with a 10% solution of triisopropylsilane in CH_2Cl_2. The solvent was removed by filtration and the resin was washed successively with three 20-mL portions of CH_2Cl_2, three 20-mL portions of DMF, and three 20-mL portions of a 10% solution of Hunig's base in DMF. An aliquot of the resin was removed, and a qualitative Kaiser free amine test[37] was performed, indicating the presence of a free amine. The NBS-protected spermidine resin **3** was suspended in a solution of 421 mg (0.9 mmol) of Fmoc protected bithiazole **4**, 341 mg (0.9 mmol) of HBTU, and 240 μL (170 mg, 1.4 mmol) of Hunig's base in 2 mL of DMF. The reaction mixture was stirred for 30 min, and then the solvent was removed by filtration and the resin was washed with three 20-mL portions of DMF, three 20-mL portions of CH_2Cl_2, and three 20-mL portions of methanol. An aliquot of the resin was removed, and a qualitative Kaiser free amine test was performed, indicating the absence of a free amine. An aliquot of the putative dipeptide **5** was cleaved from the resin with a solution of 2% hydrazine in DMF. After removal of the solvent under diminished pressure, the residue was dissolved in CF_3COOH and the solution was treated with diethyl ether to effect precipitation of the cleaved product: mass spectrum (electrospray), *m*/*z* 568.3 $(M + H)^+$, theoretical *m*/*z* 568.7 $(M + H)^+$. An aliquot of the resin was subjected to Fmoc cleavage analysis, which indicated a loading of 0.24 mmol/g (corresponding to a 78% overall yield over the first six steps). The resin was dried under diminished pressure.

Resin-Bound Tripeptide 7. To 480 mg (0.24 mmol/g) of swollen dipeptide resin **5** was added 3 mL of a 20% solution of piperidine in DMF. The reaction mixture was shaken for 10 min, and the solvent was then removed by filtration and the resin was washed with 10 mL of a 20% solution of piperidine in DMF. The piperidine deblocking and subsequent wash procedure was repeated 3 times, and the resin was washed successively with three 20-mL portions of DMF, three 20-mL portions of CH_2Cl_2, and three 20-mL portions of DMF. An aliquot of the resin was removed, and a qualitative Kaiser free amine test was performed, indicating the presence of a free amine. The resin was suspended in a solution of 230 mg (0.7 mmol) of N^{α}-Fmoc-(*S*)-threonine (**6**), 255 mg (0.7 mmol) of HBTU, 91 mg (0.7 mmol) of HOBt, and 205 μL (152 mg, 1.2 mmol) of Hunig's base in 2 mL of DMF. The reaction mixture was stirred for 30 min, and the solvent was then removed by filtration. The resin was washed with three 20-mL portions of DMF, three 20-mL portions of CH_2Cl_2, and three 20-mL portions of methanol. An aliquot of the resin was removed, and a qualitative Kaiser free amine test was performed on this aliquot, indicating the absence of a free amine. An aliquot of the putative tripeptide **7** was cleaved from the resin with a solution of 2% hydrazine in DMF. After removal of the solvent under diminished pressure, the residue was dissolved in CF_3COOH and the solution was treated with diethyl ether to effect precipitation of the cleaved product: mass spectrum (electrospray), *m*/*z* 669.4 $(M+H)^+$, theoretical *m*/*z* 669.8 $(M + H)^+$. An aliquot of the resin was removed for Fmoc cleavage analysis and revealed a loading of 0.21 mmol/g (corresponding to an overall 90% yield from the dipeptide). The resin was dried under diminished pressure.

Resin-Bound Tetrapeptide 8a. To 220 mg (0.18 mmol/g) of swollen tripeptide resin **7** was added 3 mL of a 20% solution of piperidine in DMF. The reaction mixture was shaken for 10 min, and the solvent was then removed by filtration and the resin was washed with 10 mL of a 20% solution of piperidine in DMF. The piperidine deblocking and subsequent wash procedure was repeated 3 times, and the resin was then washed successively with three 20-mL portions of DMF, three 20-mL portions of CH_2Cl_2, and three 20-mL portions of DMF. An aliquot of the resin was removed, and a qualitative Kaiser free amine test was performed, indicating the presence of a free amine. The resin was suspended in a solution of 110 mg (0.3 mmol) of the Fmoc protected, constrained methylvalerate analogue **A**,[13] 106 mg (0.3 mmol) of HBTU, 37 mg (0.3 mmol) of HOBt, and 95 μL (70 mg, 0.5 mmol) of Hunig's base in 2 mL of DMF. The reaction mixture was stirred for 30 min, and the solvent was then removed by filtration. The resin was washed with three 20-mL portions of DMF, three 20-mL portions of CH_2Cl_2, and three 20-mL portions of methanol. An aliquot of the resin was removed, and a qualitative Kaiser free amine test was performed, indicating the absence of a free amine. An aliquot of the putative tetrapeptide **8a** was cleaved from the resin using a solution of 2% hydrazine in DMF. After removal of the solvent under diminished pressure, the residue was dissolved in CF_3COOH and the solution was treated with diethyl ether to effect precipitation of the cleaved product: mass spectrum (electrospray), *m*/*z* 824.4 $(M + H)^+$, theoretical *m*/*z* 825.0 $(M + H)^+$. An aliquot of the resin was removed for Fmoc cleavage analysis and revealed a loading of 0.08 mmol/g (corresponding to a 45% yield from the tripeptide). The resin was dried under diminished pressure.

Resin-Bound Pentapeptide 10a. To 200 mg (0.08 mmol/g) of swollen tetrapeptide resin **8a** was added 3 mL of a 20% solution of piperidine in DMF. The reaction mixture was shaken for 10 min, and the solvent was then removed by filtration and the resin was washed with 10 mL of a 20% solution of piperidine in DMF. The piperidine deblocking and subsequent wash procedure was repeated 3 times, and the resin was then washed successively with three 20-mL portions of DMF, three 20-mL portions of CH_2Cl_2, and three 20-mL portions of DMF. An aliquot of the resin was removed, and a qualitative Kaiser free amine test was performed, indicating the presence of a free amine. The resin was suspended in a solution of 51 mg (0.08 mmol) of N^{α}-Fmoc-N^{im}-trityl-β-hydroxyhistidine (**9**),[15] 30 mg (0.08 mmol) of HATU, 11 mg (0.08 mmol) of HOAt, and 28 μL (21 mg, 0.16 mmol) of Hunig's base in 2 mL of DMF. The reaction mixture was stirred for 30 min, and the solvent was then removed by filtration. The resin was washed successively with three 20-mL portions of DMF, three 20-mL portions of CH_2Cl_2, and three 20-mL portions of methanol. An aliquot of the resin was removed, and a qualitative Kaiser free amine test was performed, indicating the absence of a free amine. An aliquot of the putative pentapeptide **10a** was cleaved from the resin with a solution of 2% hydrazine in DMF. After removal of the solvent under diminished pressure, the residue was dissolved in CF_3COOH and the solution was treated with diethyl ether to effect precipitation of the cleaved product: mass spectrum (electrospray), *m*/*z* 977.4 $(M + H)^+$, theoretical *m*/*z* 978.1 $(M + H)^+$. Fmoc cleavage analysis on an aliquot of the resin revealed a loading of 0.067 mmol/g (corresponding to an overall 93% yield from the tetrapeptide). The resin was dried under diminished pressure.

Deglycobleomycin Analogue 2a. To 150 mg (0.067 mmol/g) of swollen pentapeptide resin **10a** was added 3 mL of a 20% solution of piperidine in DMF. The reaction mixture was shaken for 10 min, and the solvent was then removed by filtration and the resin was washed with 10 mL of a 20% solution of piperidine in DMF. The piperidine deblocking and subsequent wash procedure was repeated 3 times, and the resin was then washed successively with three 20-mL portions of DMF, three 20-mL portions of CH_2Cl_2, and three 20-mL portions of DMF. An aliquot of the resin was removed, and a qualitative Kaiser free amine test was performed, indicating the presence of a free amine. The resin was suspended in a solution of 11 mg (0.03 mmol) of Boc pyrimidoblamic acid (**11**),[17] 31 mg (0.07 mmol) of BOP reagent, and 20 μL (15 mg, 0.1 mmol) of Hunig's base in 1 mL of DMF at 0 °C. The reaction mixture was stirred for 12 h, and the solvent was then removed by filtration. The resin was washed successively with three

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20-mL portions of DMF, three 20-mL portions of CH_2Cl_2, and three 20-mL portions of methanol. The resin was dried under diminished pressure giving putative resin-bound hexapeptide **12a**. To 80 mg of swollen resin **12a** was added 0.1 mL of triisopropylsilane and 0.1 mL of dimethyl sulfide. The reaction mixture was agitated for 30 s, and then 1 mL of CF_3COOH was added. The reaction mixture was shaken for 3 h, and the solvent was then removed by filtration. The resin was washed successively with three 20-mL portions of DMF, three 20-mL portions of CH_2Cl_2, and three 20-mL portions of DMF. The resin was suspended in 1.5 mL of a 1.0 M solution of sodium thiophenoxide in DMF. The solution was shaken for 30 min, and the solvent was then removed by filtration. The sodium thiophenoxide treatment was repeated, and the solvent was once again removed by filtration. The resin was washed successively with three 20-mL portions of DMF, three 20-mL portions of CH_2Cl_2, and three 20-mL portions of DMF. The product was cleaved from the resin by treatment with 10 mL of a 2% hydrazine solution in DMF. After removal of the solvent under diminished pressure, the residue was dissolved in CF_3COOH and the product was precipitated by the addition of diethyl ether. The precipitate was then dissolved in 0.1% aqueous CF_3COOH and lyophilized, yielding a colorless, solid product. Purification of the crude product was effected by C_{18} reversed phase HPLC (250 × 10 mm, 5μ). Elution with a linear gradient of 0.1% CF_3COOH containing increasing amounts of acetonitrile (13%→22% acetonitrile over a period of 25 min, at a flow rate of 4 mL/min; t_r 13.2 min) provided deglycobleomycin A_5 analogue **2a** as a colorless solid following lyophilization: yield 1.51 mg (26% for pyrimidoblamic acid coupling, deprotection, cleavage from the resin and HPLC purification); ^{1}H NMR (D_2O) δ 0.94 (d, 3H, J = 9.5 Hz), 1.46 (m, 4H), 1.59 (m, 4H), 1.75 (s, 3H), 1.87 (m, 3H), 2.42 (d, 1H, J = 5.5 Hz), 2.45 (m, 1H), 2.56 (m, 1H), 2.83 (m, 4H), 2.94 (m, 4H), 3.05 (m, 2H), 3.37 (m, 3H), 3.46 (m, 1H), 3.78 (m, 2H), 3.92 (m, 1H), 3.98 (m, 2H), 4.05 (m, 1H), 4.72 (m, 3H), 5.08 (d, 2H, J = 12.5 Hz), 7.32 (s, 1H), 7.83 (s, 1H), 8.04 (s, 1H), and 8.55 (s, 1H); mass spectrum (electrospray), m/z 1099.5 $(M + H)^+$, theoretical m/z 1100.3 $(M + H)^+$, HRMS (FAB), m/z 1099.5040 $(M + H)^+$ ($C_{46}H_{71}N_{18}O_{10}S_2$ requires 1099.5042).

Oxidation of Styrene by Fe(III)•BLM and Analogues. A reaction mixture containing 0.4 mg (0.28 μmol) of bleomycin and 0.14 mg (0.28 μmol) of ferric perchlorate in 220 μL of water was treated with 100 μL of a methanol solution that was 277 mM in styrene and 6.21 mM in ethyl benzoate. The resulting solution was cooled to 4 °C, and 62.7 μL of an aqueous 91.5 mM H_2O_2 stock solution was added to bring the final concentration of the reaction mixture to 15 mM in H_2O_2; this resulted in a total reaction volume of 383 μL. Aliquots (127 μL each) were taken from the reaction mixture at 40-min intervals. Each aliquot was diluted with 300 μL of H_2O and then extracted with three 100-μL portions of $CHCl_3$. The combined organic extract was dried over anhydrous $MgSO_4$, filtered, and analyzed by gas chromatography. Injections (10 μL, splitless mode) were applied to a 5% phenylsiloxane capillary column (0.25 mm ID × 30 m). The products were detected using a flame ionization detector. The following temperature program was employed at a carrier gas (helium) flow rate of 1.0 mL/min: 60 °C for 22 min; 60 → 85 °C at 10 °C/ min; then 85 °C for 15 min. Under these conditions, the observed retention times (calculated from the elution of an injected air peak) were as follows: styrene, 4.6 min; phenylacetaldehyde, 15.4 min; styrene oxide, 18.8 min; ethyl benzoate, 28.9 min.

Relaxation of Supercoiled DNA by Modified Deglycobleomycin A_5 Analogues. Reactions were carried out in 25 μL (total volume) of 10 mM sodium cacodylate buffer, pH 7.0, containing 300 ng of pBR322 plasmid DNA and the appropriate concentrations of BLM and Fe^{2+}. The Fe^{2+} solutions were freshly prepared from $Fe^{II}(NH_4)_2(SO_4)_2$•$6H_2O$ for all experiments. Reaction mixtures were incubated at 37 °C for 30 min. The reactions were quenched by the addition of 5 μL of loading dye (30% glycerol containing 0.125% (w/v) bromophenol blue) and applied to a 1% agarose gel containing 0.5 μg/mL ethidium bromide. Horizontal gel electrophoresis was carried out in 9 mM Tris-borate buffer, pH 8.3, containing 320 μM disodium EDTA, at 156 W for 2 h. The DNA bands were visualized under UV light.

Preparation of a 5′-^{32}P End-Labeled DNA Restriction Fragment. Plasmid pBR322 (25 μg) was incubated with 100 units of restriction endonuclease *Hin*dIII in a 100 μL (total volume) reaction mixture containing 5 mM NaCl, 1 mM Tris-HCl, pH 7.9, 1 mM $MgCl_2$, and 0.1 mM dithiothreitol (DTT). The digestion reaction was carried out at 37 °C for 3 h. The DNA was recovered by ethanol precipitation. The linearized DNA was dephosphorylated with 1 unit of calf intestinal alkaline phosphatase in a reaction mixture (200 μL total volume) containing 10 mM NaCl, 5 mM Tris-HCl, pH 7.9, 1 mM $MgCl_2$, and 0.1 mM DTT. The reaction mixture was incubated at 37 °C for 1 h. The enzyme was inactivated by heating at 75 °C for 10 min in 5 mM EDTA. The DNA was then recovered by ethanol precipitation following phenol extraction. The dephosphorylated DNA was 5′-^{32}P-end-labeled by incubation with 20 units of T4 polynucleotide kinase in a reaction mixture (50 μL total volume) containing 7 mM Tris-HCl, pH 7.6, 1 mM $MgCl_2$, 0.5 mM DTT, and 0.5 mCi of [γ-^{32}P]ATP. The reaction mixture was incubated at 37 °C for 1 h. The enzyme was inactivated by heating at 65 °C for 20 min. Unreacted [γ-^{32}P]ATP was removed by the use of a microspin G-25 column and the DNA was recovered by ethanol precipitation. The 5′-^{32}P end-labeled DNA was digested with 100 units of restriction endonuclease *Eco*RV in a reaction mixture (150 μL total volume) containing 10 mM NaCl, 5 mM Tris-HCl, pH 7.9, 1 mM $MgCl_2$ and 0.1 mM DTT. The reaction medium was incubated at 37 °C for 3 h, and the enzyme was then inactivated by heating at 80 °C for 20 min. The sample was desalted using a microspin G-25 column. The sample was applied to an 8% native polyacrylamide gel after addition of 50 μL of loading dye (50% glycerol (w/v), 25 mM EDTA, 0.25% bromophenol blue). Electrophoresis was carried out at 10 W for 3.5 h. The DNA was visualized by audioradiography, and the band of interest was excised from the gel. The 158-base-pair 5′-^{32}P end-labeled DNA duplex was eluted into 2 M $LiClO_4$ at 37 °C for 12 h and finally recovered by ethanol precipitation.

Cleavage of 5′-^{32}P End-Labeled DNA Duplex by Deglycobleomycin Analogues. Reactions were carried out in 20 μL (total volume) of 10 mM sodium cacodylate buffer, pH 7.0, containing ^{32}P end-labeled DNA (~3 × 10^4 cpm) and the appropriate concentrations of BLM and Fe^{2+}. Reaction mixtures were incubated at 4 °C for 30 min and lyophilized. The samples were dissolved in 5 μL of loading dye (80% formamide, 2 mM EDTA, 1% (w/v) xylene cyanol and 1% (w/v) bromophenol blue), heated at 90 °C for 10 min, and then chilled on ice. The solutions were finally applied to a 10% denaturing polyacrylamide gel (7 M urea). Electrophoresis was carried out at 50 W for 2 h. The gel was analyzed using a phosphoimager (Molecular Dynamics). The bands were correlated with those produced according to a Maxam−Gilbert A+G sequencing protocol.[38]

Alkali-Induced Cleavage of 5′-^{32}P End-Labeled DNA Duplex by Deglycobleomycin Analogues. Reactions were carried out in 20 μL (total volume) of 10 mM sodium cacodylate, pH 7.0, containing ^{32}P end-labeled DNA (~3 × 10^4 cpm) and the appropriate concentrations of BLM and Fe^{2+}. Reaction mixtures were incubated at 37 °C for 30 min, treated with 0.1 M piperidine for 20 min at 90 °C, and then lyophilized. The samples were dissolved in 5 μL of loading dye (80% formamide, 2 mM EDTA, 1% (w/v) xylene cyanol and 1% (w/v) bromophenol blue), heated at 90 °C for 10 min, and then chilled on ice. The solutions were applied to a 10% denaturing polyacrylamide gel (7 M urea). Electrophoresis was carried out at 50 W for 2 h. The gel was analyzed using a phosphorimager (Molecular Dynamics). The bands were correlated with those produced according to a Maxam−Gilbert A+G sequencing protocol.[38]

(38) Maxam, A. M.; Gilbert, W. *Methods Enzymol.* **1980**, *65*, 499.

Cleavage of a 53-Nucleotide 5′-^{32}P End-Labeled RNA by Deglycobleomycin A_5 Analogues. The 53-nt RNA was radiolabeled in essentially the same manner as described.[39] RNA cleavage reactions were carried out in 10 μL (total volume) of 10 mM sodium phosphate, pH 7.0, containing 5′-^{32}P end-labeled RNA (5×10^4 cpm) and the appropriate concentrations of reagents as indicated in the legend to Figure 8. Each reaction mixture was incubated at 23 °C for 30 min and then frozen in dry ice, lyophilized, and dissolved in a gel loading solution (80% formamide, 2 mM EDTA, 0.5% (w/v) xylene cyanol, and 0.5% (w/v) bromophenol blue). The resulting solution was heated at 70 °C for 3 min, chilled on ice, and then applied to a 20% denaturing polyacrylamide gel. The gel was analyzed by the use of a phosphorimager.

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Supporting Information Available: Synthetic procedures for valerate analogues **A**−**F**, **8b**−**8f**, **10b**−**10f**, and **2b**−**2f**. This material is available free of charge via the Internet at http://pubs.acs.org.

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