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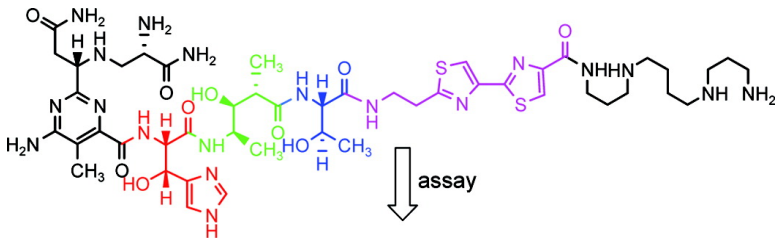

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Biochemical Evaluation of a 108-Member Deglycobleomycin Library: Viability of a Selection Strategy for Identifying Bleomycin Analogues with Altered Properties

Qian Ma, Zhidong Xu, Benjamin R. Schroeder, Wenyue Sun, Fang Wei, Shigeki Hashimoto, Kazuhide Konishi, Christopher J. Leitheiser, and Sidney M. Hecht*

Contribution from the Departments of Chemistry and Biology, University of Virginia, Charlottesville, Virginia 22904

Received April 4, 2007; E-mail: sidhecht@virginia.edu

Abstract: The bleomycins (BLMs) are clinically used glycopeptide antitumor antibiotics that have been shown to mediate the sequence-selective oxidative damage of both DNA and RNA. Previously, we described the solid-phase synthesis of a library of 108 unique analogues of deglycoBLM A_6, a congener that cleaves DNA analogously to BLM itself. Each member of the library was assayed for its ability to effect single- and double-strand nicking of duplex DNA, sequence-selective DNA cleavage, and RNA cleavage in the presence and absence of a metal ion cofactor. All of the analogues tested were found to mediate concentration-dependent plasmid DNA relaxation to some extent, and a number exhibited double-strand cleavage with an efficiency comparable to or greater than deglycoBLM A_6. Further, some analogues having altered linker and metal-binding domains mediated altered sequence-selective cleavage, and a few were found to cleave a $tRNA_3^{Lys}$ transcript both in the presence and in the absence of a metal cofactor. The results provide insights into structural elements within BLM that control DNA and RNA cleavage. The present study also permits inferences to be drawn regarding the practicality of a selection strategy for the solid-phase construction and evaluation of large libraries of BLM analogues having altered properties.

Introduction

The bleomycins (BLMs) are a family of glycopeptide antitumor agents that have been studied extensively due to their ability to effect site-selective nucleic acid degradation in the presence of oxygen and a metal-ion cofactor.[1] Blenoxane, the marketed chemotherapeutic agent composed primarily of BLMs A_2 and B_2 (Figure 1), has been utilized both as a single agent and in combination therapy in the treatment of a number of cancers.[2] The clinical success of BLM has prompted studies of its mechanism of action, as well as identification of those structural features responsible for the biochemical and biological properties of the drug.[3]

It has been thought for some time that the primary therapeutic target of the BLMs is DNA. Early studies involving competitive binding assays identified the minor groove as the preferred binding site of the agent.[4] Further, intermolecular interactions that are responsible for the characteristic 5′-GC-3′ and 5′-GT-3′ sequence selectivity pattern are believed to stem from recognition events involving the purine 2-amino group of the nucleic acid substrate, which is only accessible from the minor groove.[5] BLM-mediated DNA degradation is initiated by H4′ atom abstraction, leading to a radical cascade involving either of two degradation pathways.[6]

More recently, it has been shown that BLMs effect the cleavage of a variety of RNA substrates.[3c,7] Our laboratory has been involved in efforts to define the mechanism(s) by which BLM cleaves RNA,[8] as well as the possible relevance of RNA as a therapeutic target.[9]

To expedite the preparation of BLM analogues, we introduced methods for their construction on a solid support.[10] These efforts resulted in the creation of a combinatorial library consisting of

(1) (a) Umezawa, H.; Maeda, K.; Takeuchi, T.; Okami, Y. *J. Antibiot.* **1966**, *19*, 200. (b) Burger, R. M. *Chem. Rev.* **1998**, *98*, 1153. (c) Claussen, C. A.; Long, E. C. *Chem. Rev.* **1999**, *99*, 2797.

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(4) (a) Sugiura, Y.; Suzuki, T. *J. Biol. Chem.* **1982**, *257*, 10544. (b) Suzuki, T.; Kuwahara, J.; Sugiura, Y. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 917.

(5) (a) Kuwahara, J.; Sugiura, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 2459. (b) Bailly, C.; Waring, M. J. *J. Am. Chem. Soc.* **1995**, *117*, 7311.

(6) (a) Sugiyama, H.; Kilkuskie, R. E.; Hecht, S. M.; van der Marel, G. A.; van Boom, J. H. *J. Am. Chem. Soc.* **1985**, *107*, 7765. (b) Stubbe, J.; Kozarich, J. W. *Chem. Rev.* **1987**, *87*, 1107. (c) Natrajan, A.; Hecht, S. M. In *Molecular Aspects of Anticancer Drug−DNA Interactions*; Neidle, S., Waring, M. J., Eds.; Macmillan Press: London, 1993; pp 197−242.

(7) (a) Carter, B. J.; de Vroom, E.; Long, E. C.; van der Marel, G. A.; van Boom, J. H.; Hecht, S. M. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9373. (b) Carter, B. J.; Holmes, C. E.; Van Atta, R. B.; Dange, V.; Hecht, S. M. *Nucleotides Nucleosides* **1991**, *10*, 215. (c) Hüttenhofer, A.; Hudson, S.; Noller, H. F.; Mascharak, P. K. *J. Biol. Chem.* **1992**, *267*, 24471. (d) Hecht, S. M. *Bioconjugate Chem.* **1994**, *5*, 513.

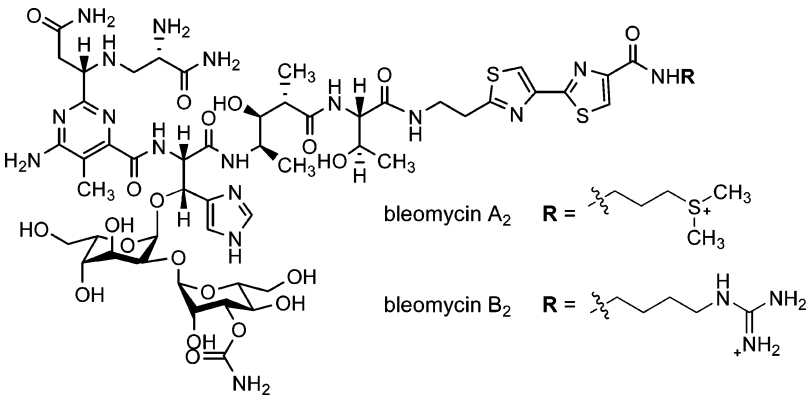

Figure 1. Structures of BLMs A_2 and B_2.

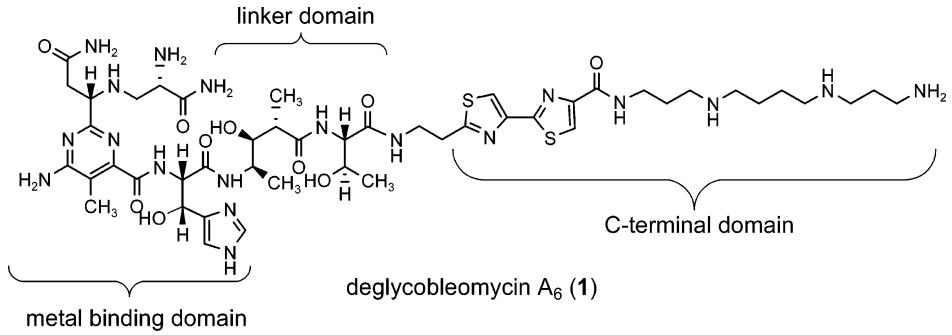

Figure 2. Structure of deglycoBLM A_6 (**1**) illustrating the major domains.

108 analogues of deglycoBLM A_6 (**1**) (Figure 2),[10d] a congener known to exhibit significant sequence-selective DNA cleavage relative to BLM itself.[11] Previous analogue studies have utilized a rational design strategy for the generation of structural variations within the BLM molecule;[11e,f,12] however, the present study employs a selection strategy that permits a systematic survey of the salient features of each individual constituent as well as the biochemical consequence of such substitutions. This is accomplished by systematic variation of each of the four core amino acid constituents of BLM in a combinatorial fashion. All of the analogues are then assayed in each of several assays to characterize their behavior, permitting an analysis of the effect of (multiple) structural changes on the biochemical activities of deglycoBLM.

Studies of BLM analogues by the Ohno et al., Hecht et al., Kozarich et al., Stubbe et al., and Boger et al.[12] have revealed much of what is presently known regarding the functional importance of the various domains of BLM. Herein, we describe a full biochemical evaluation of the DNA and RNA cleavage

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(9) (a) Abraham, A. T.; Lin, J.-J.; Newton, D. L.; Rybak, S.; Hecht, S. M. *Chem. Biol.* **2003**, *10*, 45. (b) Tao, Z.-F.; Konishi, K.; Keith, G.; Hecht, S. M. *J. Am. Chem. Soc*. **2006**, *128*, 14806.

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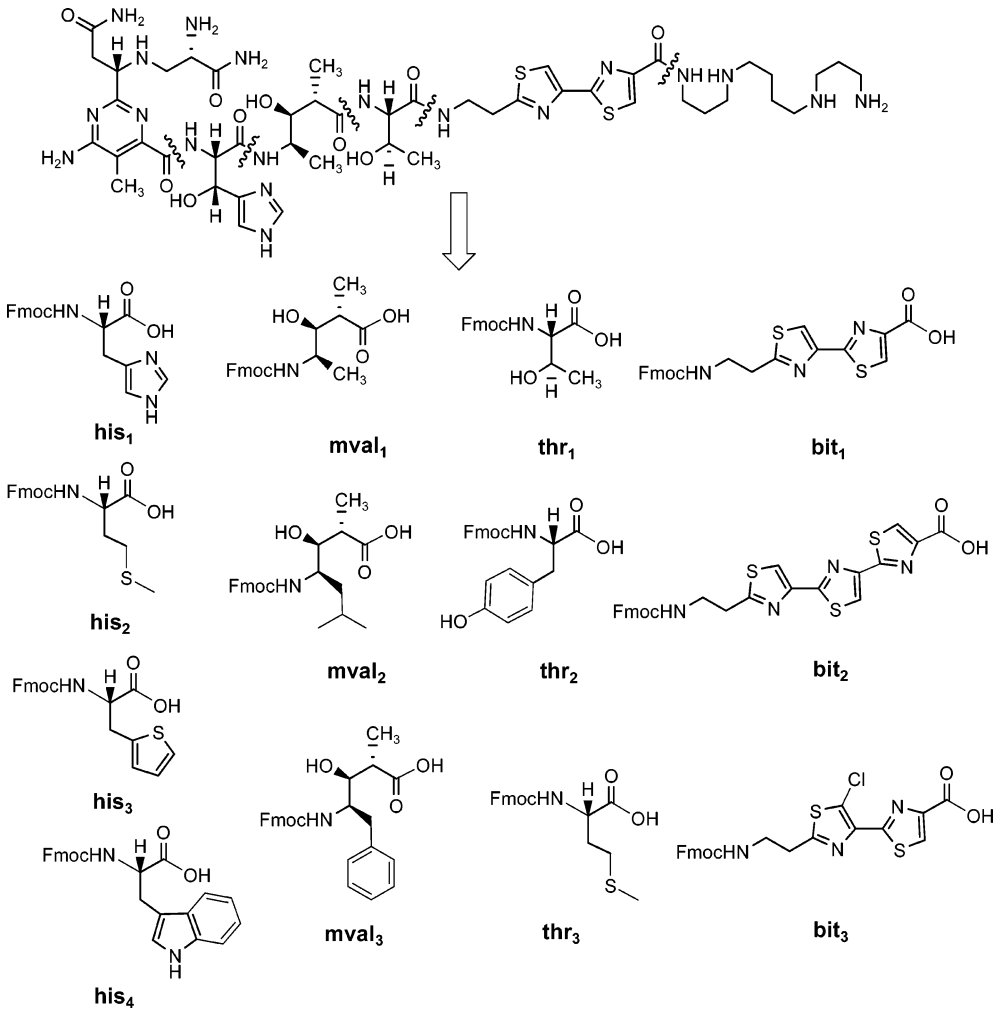


Figure 3. DeglycoBLM fragment analogues used for combinatorial library synthesis.

competency of the aforementioned 108 synthetic analogues of deglycoBLM A_6. This evaluation has permitted a definition of those structural elements that contribute to DNA and RNA cleavage. The data obtained through this study may well facilitate the discovery of analogues of BLM capable of preferentially targeting either DNA or RNA.

Results

Structural analysis of deglycoBLM A_6 reveals a series of amide linkages that can be dissected into five individual amino acid building blocks for use in solid-phase peptide synthesis (Figure 3). For the construction of the present library, substitutions of the bithiazole, threonine, methylvalerate, and β-hydroxyhistidine moieties were made utilizing readily accessible amino acid analogues. Including the individual amino acids native to deglycoBLM, three bithiazole, three threonine, three methylvalerate, and four β-hydroxyhistidine analogues were employed as building blocks in the combinatorial library, so that the contribution of individual constituents could be analyzed systematically (Figure 3).[13] Each deglycoBLM analogue was tested for its ability to effect single- and double-strand cleavage of duplex DNA, sequence-selective DNA cleavage, and RNA cleavage both in the presence and in the absence of a metal ion cofactor. Further, additional observations were made regarding the efficiency of DNA cleavage, as well as the relative binding affinities of the molecules for the DNA substrate.

Relaxation of Supercoiled Plasmid DNA. Each deglycoBLM in the 108-member library was assayed initially to define their ability to cleave pSP64 supercoiled plasmid DNA in the presence of equimolar Fe^{2+}.[14] This assay permitted the assessment of single-strand (Form II) and double-strand (Form III) DNA cleavage activity for each analogue and enabled the identification of those analogues exhibiting the greatest potency. All members of the deglycoBLM combinatorial library were found to mediate concentration-dependent plasmid DNA relaxation to some extent.

In the initial account, two synthetic analogues were described that demonstrated increased DNA cleavage potency as compared to deglycoBLM A_6, affording both single- and double-strand breaks.[10d] Since that time, several additional analogues have been found to mediate relaxation of supercoiled plasmid DNA with a potency at least as great as the parent molecule. For example, deglycoBLMs **4** and **5** effected nearly complete destruction of Form I (supercoiled circular) DNA even at a concentration of 1 μM (Figure 4). In all, 16 analogues were found to relax DNA at least as efficiently as deglycoBLM A_6;

(13) Synthetic methods and physical characterization of individual compounds are described in ref 10d.

(14) (a) Sugiyama, H.; Ehrenfeld, G. M.; Shipley, J. B.; Kilkuskie, R. E.; Chang, L.-H.; Hecht, S. M. *J. Nat. Prod.* **1985**, *48*, 869. (b) Hecht, S. M. *Pure Appl. Chem.* **1989**, *61*, 577.

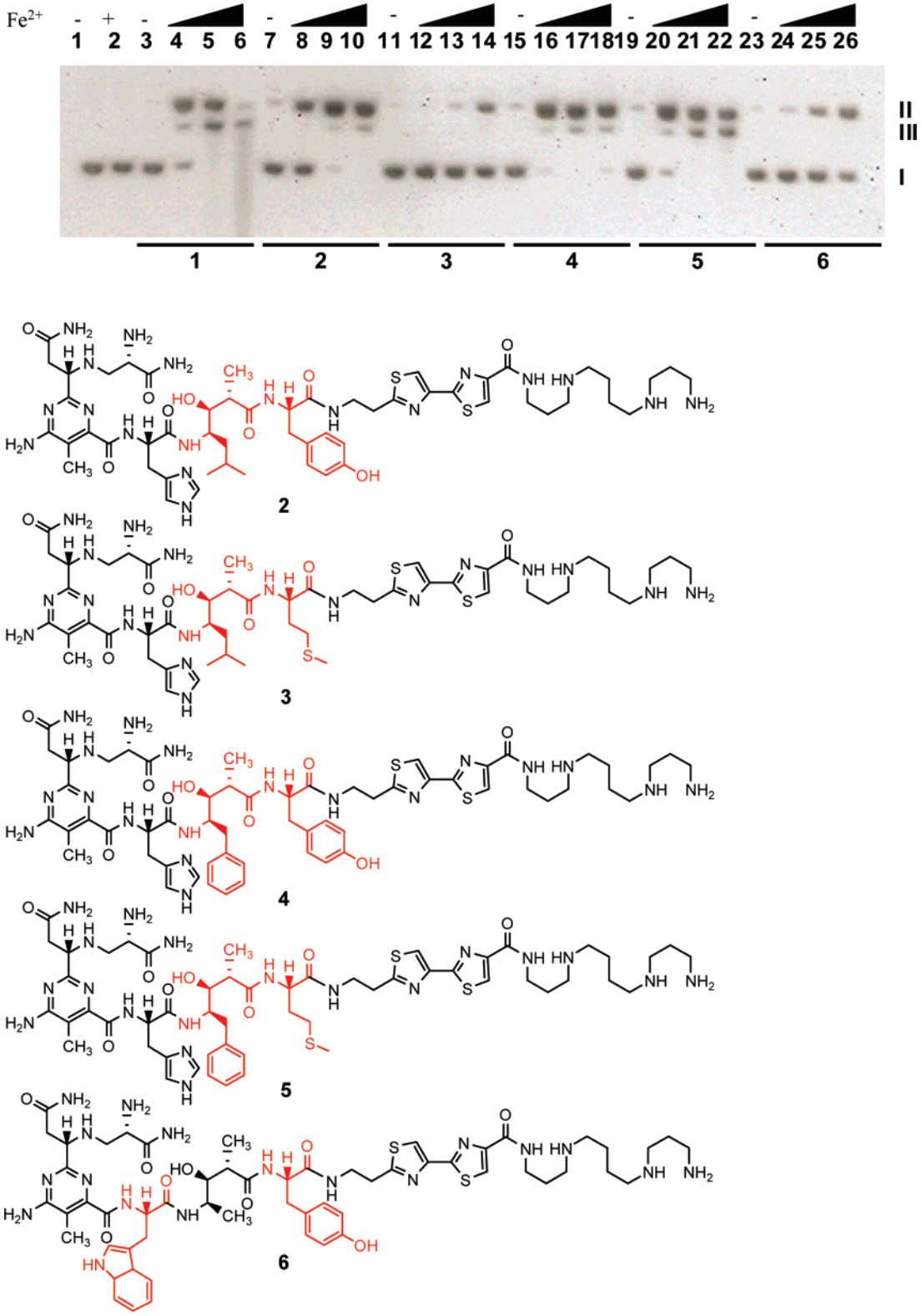


Figure 4. Relaxation of supercoiled pSP64 plasmid DNA by Fe(II)·deglycoBLM A_6 analogues. Lane 1, DNA alone; lane 2, DNA + 4 μM Fe^{2+}; lane 3, DNA + 4 μM deglycoBLM A_6 (**1**); lane 4, 1 μM Fe(II)·deglycoBLM A_6 (**1**); lane 5, 2 μM Fe(II)·deglycoBLM A_6 (**1**); lane 6, 4 μM Fe(II)·deglycoBLM A_6 (**1**); lane 7, 4 μM deglycoBLM **2**; lane 8, 1 μM Fe(II)·deglycoBLM **2**; lane 9, 2 μM Fe(II)·deglycoBLM **2**; lane 10, 4 μM Fe(II)·deglycoBLM **2**; lane 11, 4 μM deglycoBLM **3**; lane 12, 1 μM Fe(II)·deglycoBLM **3**; lane 13, 2 μM Fe(II)·deglycoBLM **3**; lane 14, 4 μM Fe(II)·deglycoBLM **3**; lane 15, 4 μM deglycoBLM **4**; lane 16, 1 μM Fe(II)·deglycoBLM **4**; lane 17, 2 μM Fe(II)·deglycoBLM **4**; lane 18, 4 μM Fe(II)·deglycoBLM **4**; lane 19, 4 μM deglycoBLM **5**; lane 20, 1 μM Fe(II)·deglycoBLM **5**; lane 21, 2 μM Fe(II)·deglycoBLM **5**; lane 22, 4 μM Fe(II)·deglycoBLM **5**; lane 23, 4 μM deglycoBLM **6**; lane 24, 1 μM Fe(II)·deglycoBLM **6**; lane 25, 2 μM Fe(II)·deglycoBLM **6**; and lane 26, 4 μM Fe(II)·deglycoBLM **6**.

the structures of these analogues are shown in Figure S1 (Supporting Information). Interestingly, a few analogues displayed efficient single-strand DNA nicking but showed very little evidence of double-strand cleavage. For example, as shown in Figure 5, deglycoBLM **8** produced strong DNA nicking but failed to produce a linear DNA duplex.

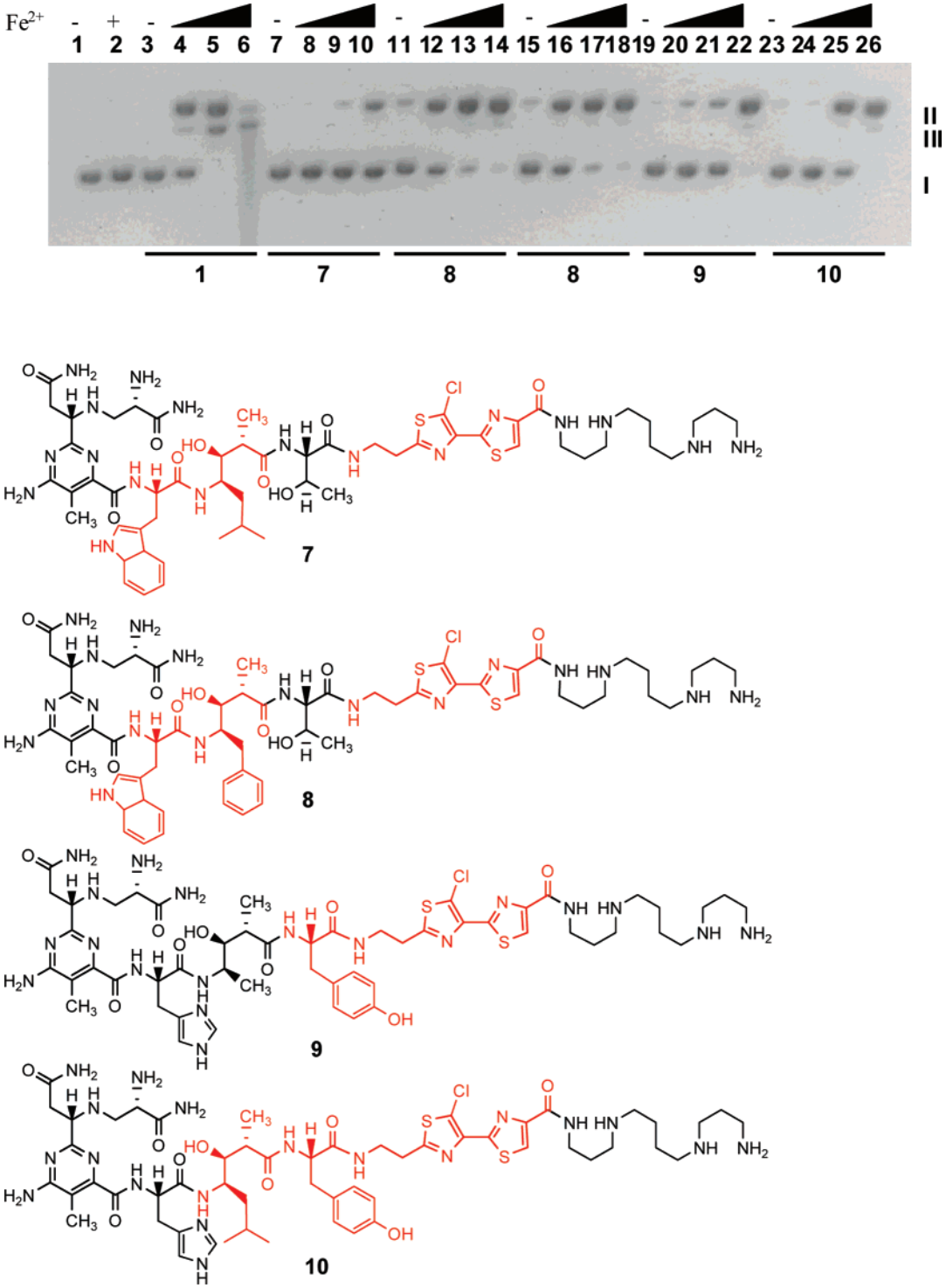
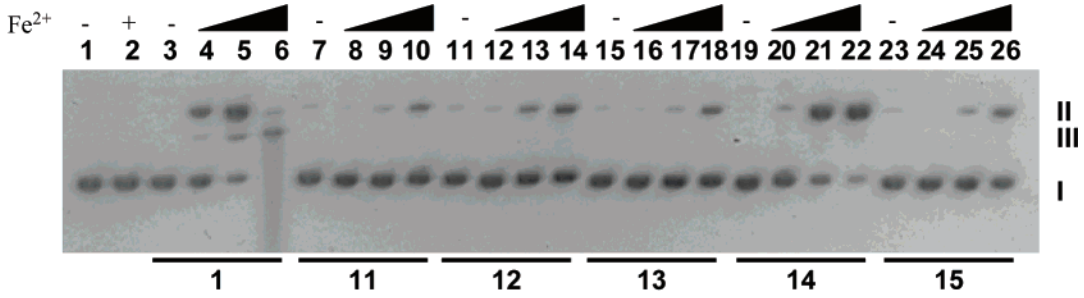


Figure 5. Single-strand DNA cleavage exhibited by Fe(II)·deglycoBLM A_6 analogues. Lane 1, DNA alone; lane 2, DNA + 4 μM Fe^{2+}; lane 3, DNA + 4 μM deglycoBLM A_6 (**1**); lane 4, 1 μM Fe(II)·deglycoBLM A_6 (**1**); lane 5, 2 μM Fe(II)·deglycoBLM A_6 (**1**); lane 6, DNA 4 μM Fe(II)·deglycoBLM A_6 (**1**); lane 7, 4 μM deglycoBLM **7**; lane 8, 1 μM Fe(II)·deglycoBLM **7**; lane 9, 2 μM Fe(II)·deglycoBLM **7**; lane 10, 4 μM Fe(II)·deglycoBLM **7**; lane 11, 4 μM deglycoBLM **8**; lane 12, 1 μM Fe(II)·deglycoBLM **8**; lane 13, 2 μM Fe(II)·deglycoBLM **8**; lane 14, 4 μM Fe(II)·deglycoBLM **8**; lane 15, 4 μM deglycoBLM **8**; lane 16, 1 μM Fe(II)·deglycoBLM **8**; lane 17, 2 μM Fe(II)·deglycoBLM **8**; lane 18, 4 μM Fe(II)·deglycoBLM **8**; lane 19, 4 μM deglycoBLM **9**; lane 20, 1 μM Fe(II)·deglycoBLM **9**; lane 21, 2 μM Fe(II)·deglycoBLM **9**; lane 22, 4 μM Fe(II)·deglycoBLM **9**; lane 23, 4 μM deglycoBLM **10**; lane 24, 1 μM Fe(II)·deglycoBLM **10**; lane 25, 2 μM Fe(II)·deglycoBLM **10**; and lane 26, 4 μM Fe(II)·deglycoBLM **10**.

Further examination of the DNA relaxation data also revealed analogues that produced noticeable shifts of individual DNA bands in the relaxation assay; this suggests that the analogues are capable of enhanced binding to the DNA substrate (Figure 6).[15] For example, deglycoBLM **12**, which contains a tyrosine constituent in place of threonine, a chlorinated bithiazole moiety instead of the natural bithiazole constituent, as well as a phenylmethylvalerate moiety, was found to shift the Form I DNA band when present with equimolar Fe^{2+} at a 4 μM concentration (Figure 6, lanes 11−14). In separate studies, we

(15) Wang, L. K.; Johnson, R. K.; Hecht, S. M. *Chem. Res. Toxicol.* **1993**, *6*, 813.



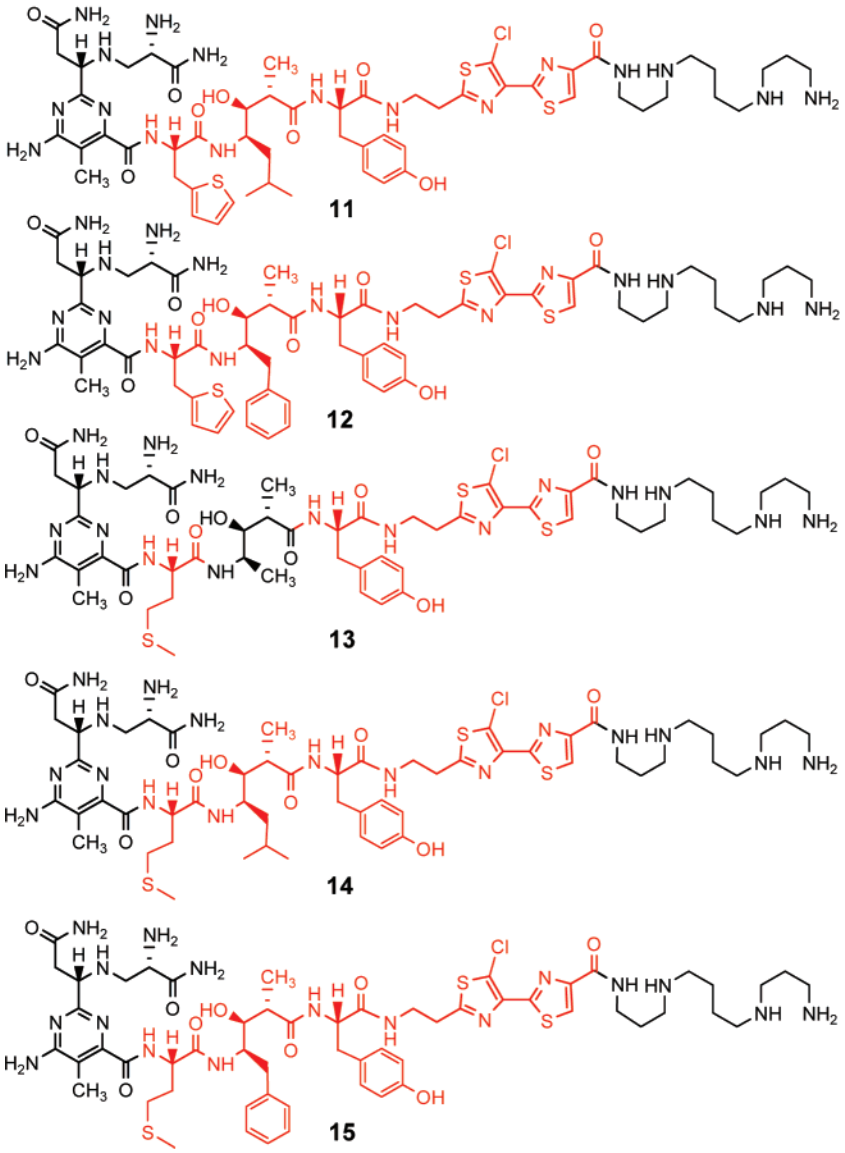
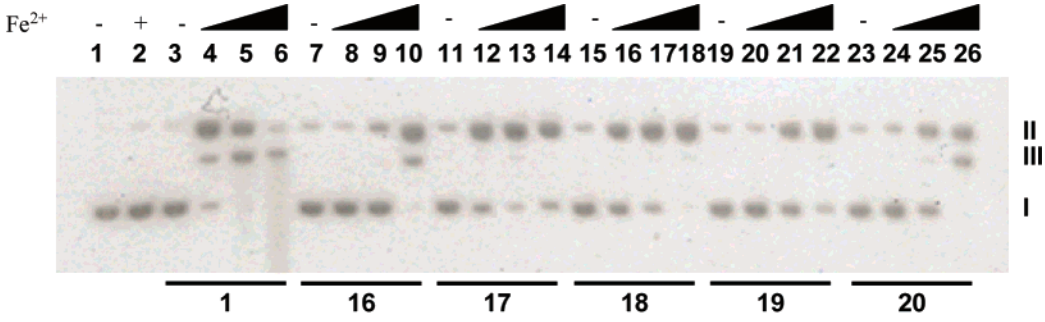


Figure 6. DNA band shifts caused by deglycoBLM A_6 analogues. Lane 1, DNA alone; lane 2, DNA + 4 μM Fe^{2+}; lane 3, DNA + 4 μM deglycoBLM A_6 (**1**); lane 4, 1 μM Fe(II)·deglycoBLM A_6 (**1**); lane 5, 2 μM Fe(II)·deglycoBLM A_6 (**1**); lane 6, 4 μM Fe(II)·deglycoBLM A_6 (**1**); lane 7, 4 μM deglycoBLM **11**; lane 8, 1 μM Fe(II)·deglycoBLM **11**; lane 9, 2 μM Fe(II)·deglycoBLM **11**; lane 10, 4 μM Fe(II)·deglycoBLM **11**; lane 11, 4 μM deglycoBLM **12**; lane 12, 1 μM Fe(II)·deglycoBLM **12**; lane 13, 2 μM Fe(II)·deglycoBLM **12**; lane 14, 4 μM Fe(II)·deglycoBLM **12**; lane 15, 4 μM deglycoBLM **13**; lane 16, 1 μM Fe(II)·deglycoBLM **13**; lane 17, 2 μM Fe(II)·deglycoBLM **13**; lane 18, 4 μM Fe(II)·deglycoBLM **13**; lane 19, 4 μM deglycoBLM **14**; lane 20, 1 μM Fe(II)·deglycoBLM **14**; lane 21, 2 μM Fe(II)·deglycoBLM **14**; lane 22, 4 μM Fe(II)·deglycoBLM **14**; lane 23, 4 μM deglycoBLM **15**; lane 24, 1 μM Fe(II)·deglycoBLM **15**; lane 25, 2 μM Fe(II)·deglycoBLM **15**; and lane 26, 4 μM Fe(II)·deglycoBLM **15**.

have found that the presence of positively charged groups in the linker region can produce an analogous effect.[16]

The plasmid DNA relaxation data were evaluated further to identify those analogues capable of producing Form III (linear duplex) DNA. Eleven deglycoBLMs produced at least as much linear duplex DNA as deglycoBLM A_6 itself (Figure S2). As illustrated in Figure 7, deglycoBLM **20** produced amounts of Form III DNA at least equal to that produced by deglycoBLM A_6 itself. Those analogues that produced the greatest amount

(16) Stevens, W.; Akkari, R.; Hecht, S. M., unpublished results.



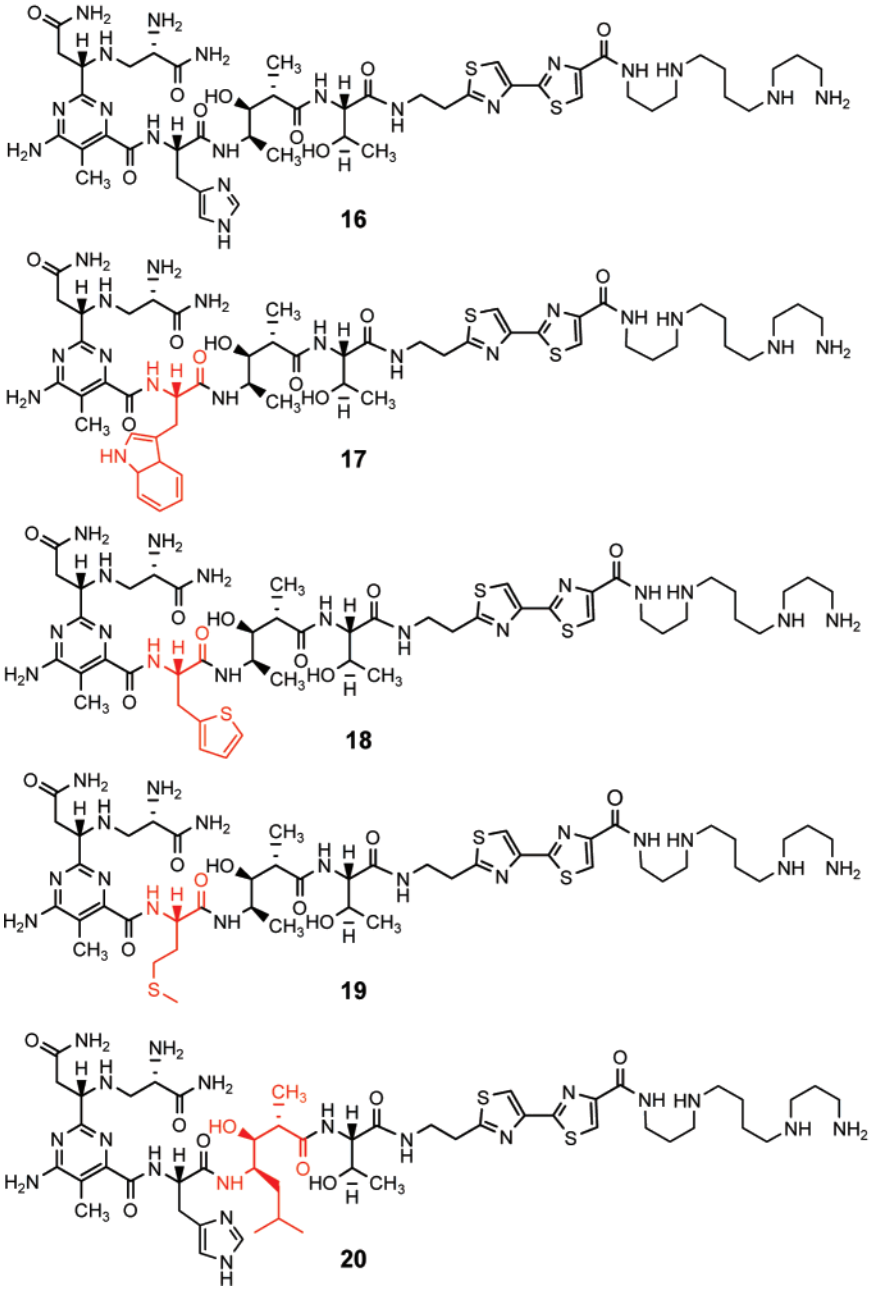


Figure 7. Double-strand DNA cleavage exhibited by Fe(II)·deglycoBLM A_6 analogues. Lane 1, DNA alone; lane 2, DNA + 4 μM Fe^{2+}; lane 3, DNA + 4 μM deglycoBLM A_6 (**1**); lane 4, 1 μM Fe(II)·deglycoBLM A_6 (**1**); lane 5, 2 μM Fe(II)·deglycoBLM A_6 (**1**); lane 6, 4 μM Fe(II)·deglycoBLM A_6 (**1**); lane 7, 4 μM deglycoBLM **16**; lane 8, 1 μM Fe(II)·deglycoBLM **16**; lane 9, 2 μM Fe(II)·deglycoBLM **16**; lane 10, 4 μM Fe(II)·deglycoBLM **16**; lane 11, 4 μM deglycoBLM **17**; lane 12, 1 μM Fe(II)·deglycoBLM **17**; lane 13, 2 μM Fe(II)·deglycoBLM **17**; lane 14, 4 μM Fe(II)·deglycoBLM **17**; lane 15, 4 μM deglycoBLM **18**; lane 16, 1 μM Fe(II)·deglycoBLM **18**; lane 17, 2 μM Fe(II)·deglycoBLM **18**; lane 18, 4 μM Fe(II)·deglycoBLM **18**; lane 19, 4 μM deglycoBLM **19**; lane 20, 1 μM Fe(II)·deglycoBLM **19**; lane 21, 2 μM Fe(II)·deglycoBLM **19**; lane 22, 4 μM deglycoBLM **19**; lane 23, 4 μM deglycoBLM **20**; lane 24, 1 μM Fe(II)·deglycoBLM **20**; lane 25, 2 μM Fe(II)·deglycoBLM **20**; and lane 26, 4 μM Fe(II)·deglycoBLM **20**.

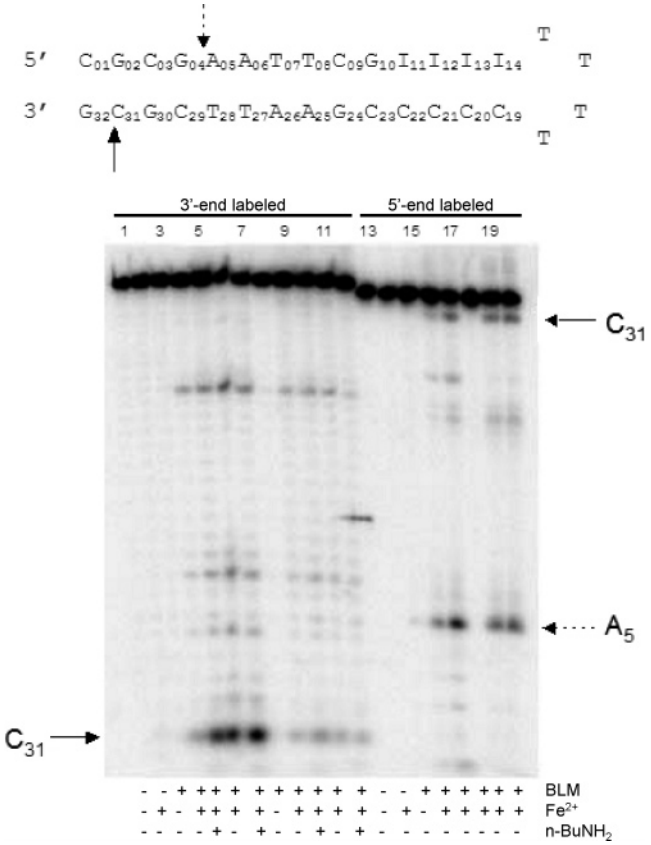


Figure 8. Products of the reaction of Fe(II)·BLM with a 5′- or 3′-^{32}P-end labeled hairpin substrate. Reactions were allowed to proceed for 30 min at 0 °C. Lanes 1−12, [3′-^{32}P]-end labeled DNA; lanes 13−20, [5′-^{32}P]-end labeled DNA. Lane 1, hairpin DNA alone; lane 2, 40 μM Fe^{2+}; lane 3, 20 μM BLM A_5; lane 4, 10 μM Fe(II)·BLM A_5; lane 5, 10 μM Fe(II)·BLM A_5, followed by treatment with *n*-butylamine; lane 6, 20 μM Fe(II)·BLM A_5; lane 7, 20 μM Fe(II)·BLM A_5, followed by treatment with *n*-butylamine; lane 8, 40 μM deglycoBLM A_6 (**1**); lane 9, 20 μM Fe(II)·deglycoBLM A_6; lane 10, 20 μM Fe(II)·deglycoBLM A_6, followed by treatment with *n*-butylamine; lane 11, 40 μM Fe(II)·deglycoBLM A_6; lane 12, 40 μM Fe(II)·deglycoBLM A_6, followed by treatment with *n*-butylamine; lane 13, hairpin DNA alone; lane 14, 40 μM Fe^{2+}; lane 15, 20 μM BLM A_5; lane 16, 10 μM Fe(II)·BLM A_5; lane 17, 20 μM Fe(II)·BLM A_5; lane 18, 40 μM deglycoBLM A_6 (**1**); lane 19, 20 μM Fe(II)·deglycoBLM A_6; and lane 20, 40 μM Fe(II)·deglycoBLM A_6. The left solid arrow denotes the products of the reaction of Fe(II)·BLM with the [3′-^{32}P]-end labeled substrate at cytosine$_{31}$. The right solid arrow denotes the products of reaction of Fe(II)·BLM with the [5′-^{32}P]-end labeled substrate at cytosine$_{31}$. The dashed arrow denotes the products formed by cleavage at A_5.

of double-strand cleavage of the supercoiled substrate were further assayed for their ability to cleave a 32-nucleotide DNA hairpin substrate (vide infra).

DeglycoBLM-Mediated Cleavage of a DNA Hairpin Substrate. There has been considerable interest in the mechanism by which BLM mediates efficient double-strand DNA cleavage[17] and associated efforts to characterize the ability of BLM analogues to mediate efficient double-strand DNA cleavage.[18] Recently, we have described a 32-nucleotide DNA hairpin substrate (Figure 8), which, when alternatively ^{32}P-end labeled at the 5′- or 3′-ends and allowed to react with Fe(II)·BLM, provides unequivocal evidence for double-strand cleavage.[3f] As shown in Figure 8 for BLM A_5 and deglycoBLM A_6 (**1**), treatment of the 3′-^{32}P-end labeled substrate resulted in a major cleavage band corresponding to C_{31} of the hairpin oligonucleotide, as well as other minor sites (lanes 4−7 and 9−12). Cleavage of the 5′-^{32}P-end labeled substrate yielded a band corresponding to C_{31} (solid arrow in Figure 8) and a second cleavage band corresponding to position A_5 (dashed arrow in Figure 8). Because the cleavage sites are positioned in proximity on opposing strands of the formed DNA duplex, the observation of cleavage at these two sites is consistent with a coordinated double-strand cleavage event. This possibility was analyzed by quantification of the cleavage bands.

Keck et al.[3f] utilized phosphorimager analysis of the cleavage of the same DNA hairpin substrate, as well as subsequent alkali treatment to determine the percentage of cleavage events that led directly to double-strand DNA cleavage. Cleavage events (frank strand scission) involving the 3′-^{32}P end labeled hairpin oligonucleotide at C_{31} following treatment by 40 μM Fe(II)·deglycoBLM A_6 (Figure 8, lane 11) were determined to account for 10.7% of the total DNA in the lane (Table 1). Following *n*-butylamine treatment to reveal alkali-labile lesions (Figure 8, lane 12),[19] the total product increased to 14.2%, indicating that 3.5% of the initial DNA modifications led to the formation of abasic site lesions. Thus, the ratio of glycolate-abasic lesions was approximately 3:1. Quantification of the frank DNA cleavage band at C_{31} of the 5′-^{32}P-end labeled oligonucleotide (Figure 8, lane 20) was found to account for 8.7% of the total DNA in the lane. From these data, it can be calculated that approximately 14% of the total damage initiated at C_{31} led to double-strand cleavage of the hairpin oligonucleotide. The analogous figure for Fe(II)·BLM A_5 was 40%, in good agreement with earlier findings for BLM A_2.[3f]

Those members of the deglycoBLM library that exhibited a proclivity for double-strand cleavage of the supercoiled DNA greater than or equivalent to deglycoBLM A_6 were also tested for their ability to cleave the hairpin oligonucleotide. Unfortunately, none of the compounds gave cleavage bands strong enough to be quantified accurately. No trend toward enhanced double-strand cleavage was noted.

Sequence-Selective DNA Cleavage. It was of great interest to determine whether differences in the structure of BLM could bring about alterations in the sequence selectivity of DNA cleavage. Toward this end, each of the 108 deglycoBLM analogues was tested for the ability to effect sequence-selective DNA cleavage. The analysis was carried out using a 222-base pair 3′-^{32}P-end labeled *Hin*dIII-*Pvu*II restriction fragment of pSP64 plasmid DNA prepared by modification of an earlier method.[15] As shown in Figure 9, Fe(II)·deglycoBLM A_6 (**1**) (lanes 4−6) itself showed several sites of cleavage, mainly at the 5′-GC-3′ and 5′-GT-3′ sites as expected. Treatment of the substrate with Fe(II)·deglycoBLM analogue **21** effected cleavage at several new sites (see lanes 8−10 in Figure 9). Specifically, analogue **21** showed strong cleavage at the 5′-GA-3′ sites (A_{136} and A_{171}) in addition to weaker cleavage at the 5′-AC-3′ and 5′-AA-3′ sites. Analogues **22**−**25** also displayed an altered

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(18) See, e.g., refs 11e and 12l,n,s,t,u.

(19) Sugiyama, H.; Xu, C.; Murugesan, N.; Hecht, S. M.; van der Marel, G. A.; van Boom, J. H. *Biochemistry* **1988**, *27*, 58.

Table 1. Analysis of DNA Damage Initiated at Cytidine$_{31}$ of the Hairpin Oligonucleotide Substrate

nature of DNA damage at C_{31}	DNA cleavage (% total DNA)	
	BLM A_5	*deglycoBLM* A_6
3′-end labeled DNA		
total DNA damage	27.5	14.2
frank strand scission	14.5	10.7
alkali labile lesion	13.0	3.5
5′-end labeled DNA		
frank strand scission	6.9	8.7
percentage of lesions leading to double-strand cleavage	40	14

sequence selectivity of DNA cleavage, although with reduced efficiency as compared to deglycoBLMs **1** and **21**. Not only were new sites of cleavage observed, but several of the sites cleaved by Fe(II)·deglycoBLM A_6 were not cleaved by analogue **21** or any of the synthetic analogues tested in Figure 9. The specific sites of DNA oligonucleotide cleavage are shown explicitly in Figure 10.

It is interesting that many of the analogues assayed for sequence selectivity of DNA cleavage showed little or no cleavage of the ^{32}P-end labeled DNA duplex. This reflects the much greater sensitivity of the plasmid DNA cleavage assay. With the exception of the BLMs shown in Figure 9, the remaining analogues found to be capable of cleaving the 222-mer oligonucleotide all contained the natural histidine constituent in the metal-binding domain and showed little variation in sequence selectivity.

Transfer RNA as a Substrate for Cleavage by DeglycoBLM. The cleavage of tRNA substrates by BLM has been found to produce two types of cleavage sites: hydrolytic cleavage involving phosphoryl transfer[20] and oxidative cleavage.[3c,7,8] The chemistry involved in both degradation pathways has been studied extensively and has been shown to be dependent upon both the sequence and the secondary structure of the substrate in addition to the structural features of the drug itself.[8b,21]

Each of the 108 deglycoBLM analogues was tested individually for cleavage using a $tRNA_3^{Lys}$ transcript[9b] as a substrate to determine the facility with which single and multiple substitutions within deglycoBLM A_6 could produce RNA cleavage sites. Figure 11 illustrates five analogues of deglycoBLM A_6, as well as BLM A_5, that were tested for their ability to produce cleavage sites within the tRNA substrate both in the presence and in the absence of a metal cofactor. As seen in lane 5 in Figure 11, in the presence of Fe(II)·BLM A_5, strong oxidative cleavage of the substrate was obtained at sites G_{44}, T_{54}, U_{66}, and C_{74}. The appearance of these products was dependent on the presence of Fe^{2+}. Products of the oxidative degradation of RNA substrates, specifically by the BLMs, have been shown to contain mostly 5′-phosphate and either 3′-phosphate or 3′-phosphoroglycolate fragments resulting from oxidative destruction of the ribose ring system.[7a,8a,22]

In contrast to the oxidative cleavage of $tRNA_3^{Lys}$ by BLM A_5, some analogues of deglycoBLM A_6 were found to produce efficient hydrolytic cleavage at C_{56} and C_{75} of the substrate. Previous reports have indicated that analogous sites in yeast $tRNA^{Phe}$ are particularly susceptible to hydrolytic cleavage both by BLM A_2[20] as well as constrained analogues of deglycoBLM A_6.[8h] However, the present study is the first to employ $tRNA_3^{Lys}$ as a substrate for deglycoBLM. Generally occurring at pyrimidine-purine sites, the phosphoryl transfer event has been found to closely mirror the cleavage of RNAs by various chemical nucleases.[23] It is significant to note that in all cases, the hydrolytic cleavage appears at the lone 5′-CA-3′ junction within a single-stranded region of the tRNA substrate. It is possible that in the context of this single-stranded element, the 5′-CA-3′ sequence is predisposed to be especially labile to hydrolysis effects[24] and that deglycoBLM acts as a ribonuclease mimetic to produce cleavage of the phosphodiester bond (vide infra). There was virtually no evidence of oxidative cleavage by any of the 108 analogues of deglycoBLM A_6 that were tested in this assay (data not shown).

The deglycoBLM analogues showing strong hydrolytic cleavage at C_{56} and C_{75}, namely, **17**, **25**, and **26** (Figure 11), were found to do so both in the presence and in the absence of Fe^{2+} (cf. lanes 7 vs 8 and 13 vs 14). This result is not without precedent, as previous studies have shown evidence of deglycoBLM-mediated RNA hydrolysis in the presence of Fe^{2+}, albeit with significantly diminished efficiency.[8g]

Discussion

The development of solid-phase methodology for the synthesis of the BLM group of antitumor antibiotics has greatly facilitated the synthesis of large numbers of analogues for biochemical evaluation.[10] Indeed, it is now possible to produce hundreds of analogues differing at single or multiple sites in the peptide backbone of BLM by simple, iterative solid-phase peptide coupling reactions. Previous efforts in the preparation of BLM analogues have involved single substitutions within BLM to enable the characterization of the role of individual structural elements.[11,12] This study marks the first detailed biochemical evaluation of analogues having multiple substitutions within the deglycoBLM molecule. The careful analysis of the biochemical properties of a combinatorial library thus enables the evaluation of a selection paradigm for identifying BLMs with altered properties.

The results obtained from the supercoiled DNA relaxation assay were examined carefully to define those analogues that exhibited interesting or novel properties. While each of the deglycoBLM analogues exhibited concentration-dependent relaxation of supercoiled DNA, some analogues were clearly better

(20) Keck, M. V.; Hecht, S. M. *Biochemistry* **1995**, *34*, 12029.
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(23) (a) Kierzek, R. *Nucleic Acids Res.* **1992**, *20*, 5073. (b) Kierzek, R. *Nucleic Acids Res.* **1992**, *20*, 5079. (c) Podyminogin, M. A.; Vlassov, V. V.; Giegé, R. *Nucleic Acids Res.* **1993**, *21*, 5950.
(24) Dock-Bregeon, A. C.; Moras, D. *Cold Spring Harbor Symp. Quant. Biol.* **1987**, *52*, 113.

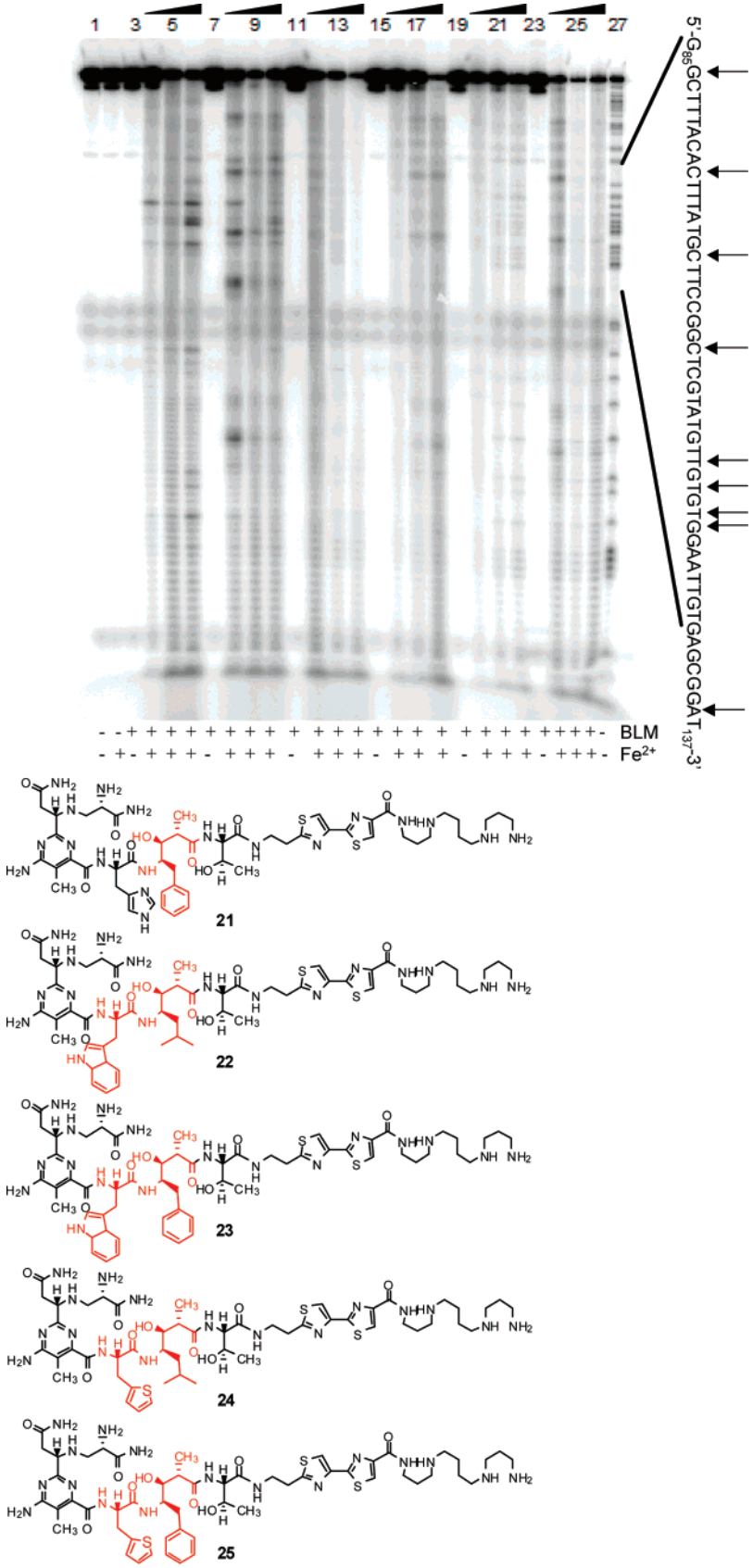

Figure 9. Sequence-selective cleavage of [3′-^{32}P]-end labeled 222-base pair DNA duplex by deglycoBLM analogues **21**−**25**: lane 1, DNA alone; lane 2, DNA + 10 μM Fe^{2+}; lane 3, 10 μM deglycoBLM **1**; lane 4, 1 μM Fe(II)·deglycoBLM **1**; lane 5, 5 μM Fe(II)·deglycoBLM **1**; lane 6, 10 μM Fe(II)·deglycoBLM **1**; lane 7, 10 μM deglycoBLM **21**; lane 8, 1 μM Fe(II)·deglycoBLM **21**; lane 9, 5 μM Fe(II)·deglycoBLM **21**; lane 10, 10 μM Fe(II)·deglycoBLM **21**; lane 11, 10 μM deglycoBLM **22**; lane 12, 1 μM Fe(II)·deglycoBLM **22**; lane 13, 5 μM Fe(II)·deglycoBLM **22**; lane 14, 10 μM Fe(II)·deglycoBLM **22**; lane 15, 10 μM deglycoBLM **23**; lane 16, 1 μM Fe(II)·deglycoBLM **23**; lane 17, 5 μM Fe(II)·deglycoBLM **23**; lane 18, 10 μM Fe(II)·deglycoBLM **23**; lane 19, 10 μM deglycoBLM **24**; lane 20, 1 μM Fe(II)·deglycoBLM **24**; lane 21, 5 μM Fe(II)·deglycoBLM **24**; lane 22, 10 μM Fe(II)·deglycoBLM **24**; lane 23, 10 μM deglycoBLM **25**; lane 24, 1 μM Fe(II)·deglycoBLM **25**; lane 25, 5 μM Fe(II)·deglycoBLM **25**; lane 26, 10 μM Fe(II)·deglycoBLM **25**; and lane 27, DNA sequencing lane.

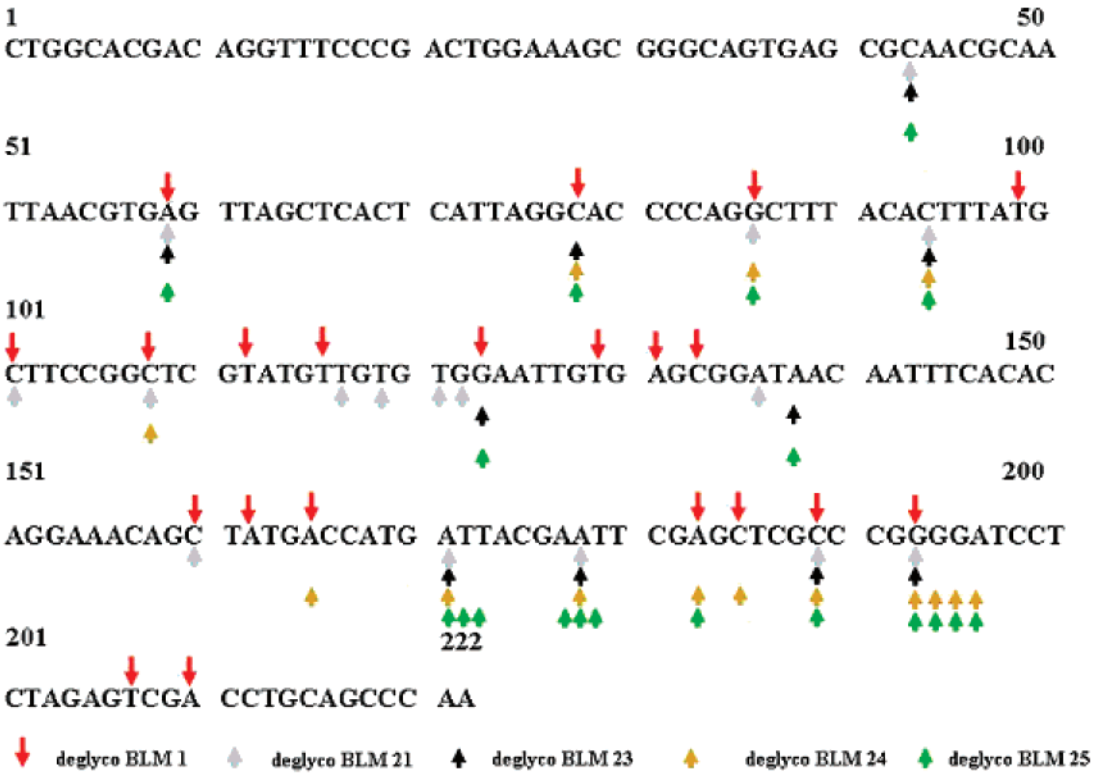

Figure 10. Histogram indicating the sequence selectivity of DNA oligonucleotide cleavage by Fe(II)·deglycoBLM **1** and analogues **21** and **23**−**25**.

than others. Generally, BLMs that contained multiple substitutions were found to effect DNA relaxation to a lesser extent than those that retained much of their natural constitution. However, there were clear exceptions to this pattern. There were multiply substituted analogues of deglycoBLM that relaxed supercoiled plasmid DNA with enhanced efficiency (Figure 4) and that afforded either reduced (Figure 5) or enhanced (Figure 7) amounts of Form III (linear duplex) DNA.

Analysis of the plasmid DNA relaxation assay also allowed qualitative conclusions to be reached concerning the effects of the individual amino acid building blocks that were inserted into the BLM backbone. For the analogues containing substitutions of the histidine moiety, DNA cleavage efficiency was found to follow the trend $his_1 > his_2 > his_3 > his_4$ (cf. Figure 3) with deglycoBLM analogues containing histidine itself being the most efficient. The histidine building blocks were logically chosen to contain heteroatoms that would be capable of forming metal coordination sites. It was observed that substitutions of the histidine moiety led to the greatest decrease in the efficiency of supercoiled plasmid DNA relaxation of all of the building blocks utilized in the library. This result is likely due to the diminished ability of the non-imidazole containing histidine building blocks to stabilize binding of the metal ion cofactor in a fashion conducive to DNA strand scission. Interestingly, several deglycoBLM analogues containing the nonaromatic methionine substitution (his_2) effected plasmid DNA relaxation to a greater extent than those containing aromatic constituents. While it has been well-established that methionine residues in proteins such as cytochrome *c* can interact favorably with coordinated iron,[25] there is little precedent for histidine substitutions in BLMs involving nonaromatic residues. Further, deglycoBLM analogues containing tryptophan (His_4) were found to exhibit only a slight relaxation of supercoiled DNA, supporting earlier observations by Boger et al.[12p] and by Kozarich, Stubbe and coworkers[26] that the imidazole N^1-H tautomer of natural histidine, functioning as an N^π metal chelator, is involved in metal complexation.

Substitutions of the methylvalerate moiety were discovered to follow a much different trend, namely, $mval_2 > mval_3 > mval_1$, with analogues containing the natural methylvalerate constituent being the least active in the relaxation of supercoiled DNA. It was originally proposed that increased steric bulk at the C-4 position might lead to preferential interactions with the DNA substrate as compared to the natural methylvalerate moiety.[10d] Indeed, the hydrophobic isopropylmethylvalerate constituent and the aromatic phenylmethylvalerate substituents conferred significant increases in the cleavage efficiency of derived deglycoBLM analogues.[10d] NMR[3e] and molecular modeling studies[12t] have shown that the methylvalerate moiety must adopt a rigid conformation for optimal interaction with DNA in which the C-4 methyl group extends toward the substrate. The presence of such hydrophobic constituents seems to facilitate the adoption of such a conformation, thus increasing the potential to support favorable noncovalent interactions with the DNA backbone.

The threonine building blocks were selected with the hope of further defining the structural parameters of the linker domain that can affect BLM function. Substitutions of the threonine

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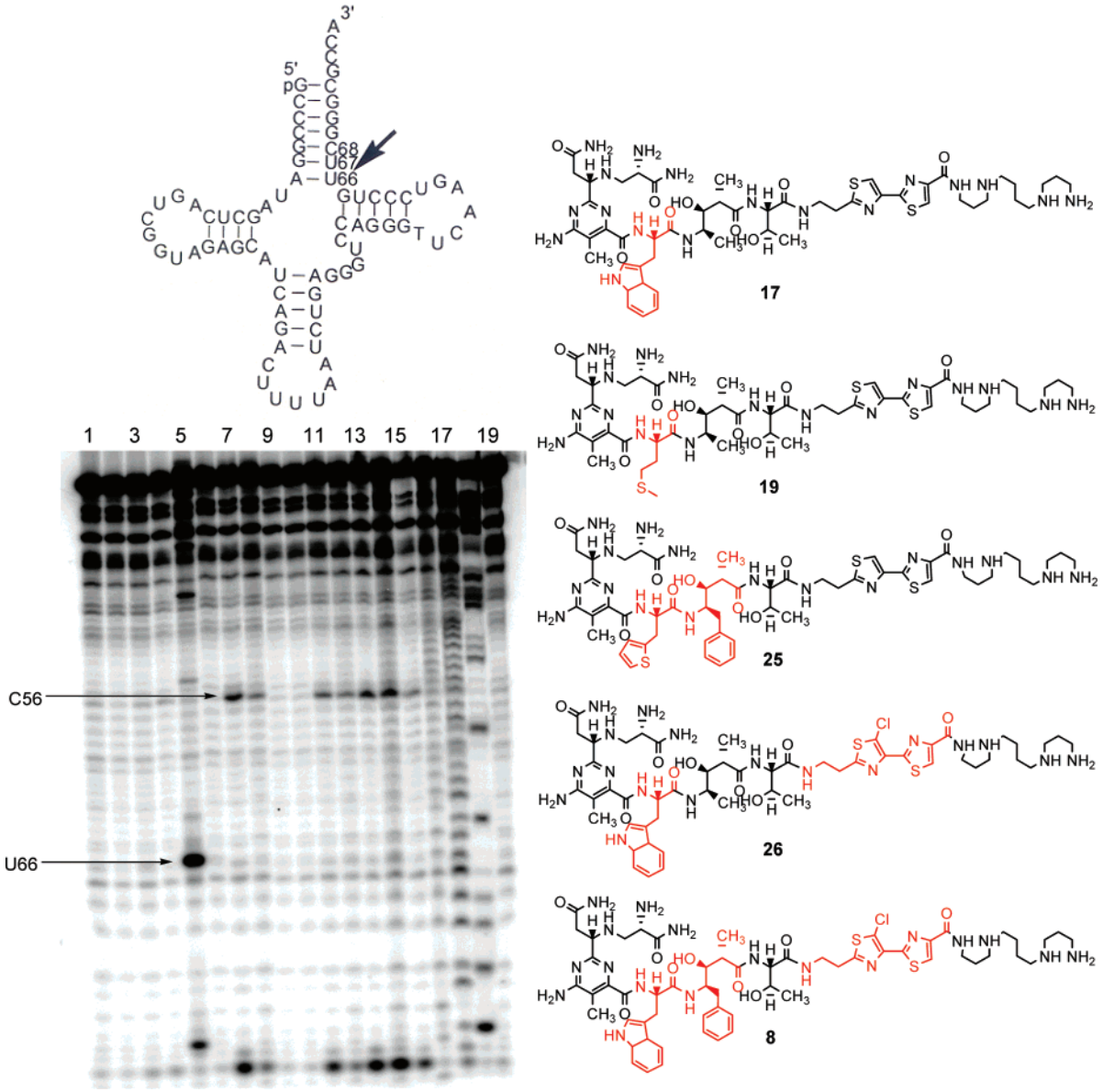

Figure 11. Cleavage of a $tRNA_3^{Lys}$ transcript by BLM A_5 and deglycoBLM A_6 analogues. Lane 1, $tRNA_3^{Lys}$ transcript alone; lane 2, 25 μM Fe^{2+}; lane 3, 25 μM BLM A_5; lane 4, 5 μM BLM A_5; lane 5, 25 μM Fe(II)·BLM A_5; lane 6, 5 μM Fe(II)·BLM A_5; lane 7, 25 μM deglycoBLM **17**; lane 8, 25 μM Fe(II)·deglycoBLM **17**; lane 9, 25 μM deglycoBLM **19**; lane 10, 25 μM Fe(II)·deglycoBLM **19**; lane 11, 25 μM deglycoBLM **25**; lane 12, 25 μM Fe(II)·deglycoBLM **25**; lane 13, 25 μM deglycoBLM **26**; lane 14, 25 μM Fe(II)·deglycoBLM **26**; lane 15, 25 μM deglycoBLM **8**; lane 16, 25 μM Fe(II)·deglycoBLM **8**; lane 17, alkaline hydrolysis; lane 18, RNase T_1; and lane 19, RNase T_2.

moiety showed the following trend: $thr_2 > thr_1 > thr_3$. Once again, the presence of an aromatic constituent in the linker domain led to a greater efficiency of DNA cleavage. Previous studies have left little doubt that the linker domain of BLM plays a major role in the formation of favorable intramolecular interactions amenable to optimal DNA cleavage;[11e,12d,26,27] however, aromatic substitutions in this domain had never before been explored. Examination of the results obtained for deglycoBLM A_6 analogue **12**, containing aromatic residues in each of the modified building blocks varied in this study, showed a noticeable DNA band shift in the relaxation assay, which argues that π-stacking interactions within the linker domain with the substrate could lead to increased affinity.

Finally, the bithiazole moiety contributed to the efficiency of supercoiled plasmid DNA relaxation in the following order: $bit_1 > bit_2 > bit_3$. Previous studies by our laboratory described the alteration of sequence selectivity[8f] and DNA cleavage in the presence of light[12w,28] for substitutions involving trithiazole and chlorobithiazole analogues, respectively. In the present study, most of the deglycoBLM A_6 analogues containing the trithiazole and chlorobithiazole building blocks retained much of their capacity for supercoiled DNA relaxation but showed little, if any, enhancement in reactivity. The fact that substitutions involving these building blocks had little effect on the overall DNA relaxation supports the belief that this portion of the molecule is involved in the DNA-binding event rather than the actual cleavage event per se.

Keck et al. have previously described the use of a DNA hairpin to provide unequivocal evidence for double-strand cleavage of DNA by BLM.[3f] The 32-nucleotide DNA hairpin

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shown in Figure 8 had previously been found to undergo extensive cleavage at C_{31}, with a linked secondary cleavage site at A_5. Alternative labeling of the hairpin substrate at the 5′- and 3′-ends permitted quantification of the extent to which DNA damage inflicted initially at C_{31} also resulted in coupled cleavage at A_5; for BLM A_2, 43% of the damage at C_{31} led to double-strand DNA cleavage.[3f] Repetition of this experiment with BLM A_5 revealed that 40% of the lesions initiated at C_{31} resulted in double-strand cleavage at A_5. The prototype BLM analogue in the present study, deglycoBLM A_6 (**1**), was also studied and found to effect only about 14% double-strand cleavage (Table 1). The lesser efficiency of deglycoBLM in effecting double-strand cleavage was fully consistent with earlier reports by Boger et al.[11e,12l,n,s,t,u] In spite of the fact that a number of the deglycoBLM analogues prepared for evaluation here were found to relax supercoiled plasmid DNA at least as efficiently as deglycoBLM A_6, efforts to use several of these species to cleave the hairpin DNA failed to produce sufficiently strong bands to permit reliable analysis of the extent of double-strand cleavage.

Each of the deglycoBLM A_6 analogues examined in this study was tested for sequence selectivity of DNA cleavage utilizing a double-strand 222-bp DNA duplex. While all of the deglycoBLMs exhibited concentration-dependent relaxation of supercoiled DNA, due to the lesser sensitivity of the assay, only a smaller number produced robust cleavage of the linear DNA duplex. With the exception of those analogues in Figure 9, none of the synthetic analogues containing methionine, thienylalanine, or tryptophan substitutions of the natural histidine moiety were able to cleave the oligonucleotide substrate above the background level of cleavage produced by Fe^{2+}.

Those synthetic analogues containing a histidine moiety generally showed sequence-selective cleavage at 5′-GC-3′ and 5′-GT-3′ sequences within the DNA oligonucleotide. Interestingly, deglycoBLM analogue **21**, which had previously been reported to relax supercoiled plasmid DNA more efficiently than deglycoBLM A_6 itself,[10d] was also found to afford an altered pattern of cleavage of the linear duplex DNA substrate (Figure 9). As shown in Figure 10, this included sites cleaved by deglycoBLM but not any of the analogues (e.g., T_{99}, T_{112}, T_{129}, A_{162}, T_{207}, and A_{210}) and other sites cleaved by one or more analogues but not by the parent deglycoBLM (e.g., C_{94}, A_{138}, A_{171}, A_{178}, G_{194}, G_{195}, and A_{196}). This finding would not have been anticipated based on what was previously known about the structural features in BLM responsible for sequence-selective DNA cleavage and thereby strongly supports the use of combinatorial libraries as a source of BLM analogues having novel properties in nucleic acid cleavage.

Earlier studies have shown definitive oxidative cleavage of some RNAs by Fe(II)·BLMs; however, deglycoBLMs have been found to produce mostly hydrolytic cleavage, especially in the absence of any metal cofactor. These results are reflected in the present study. As shown in Figure 11, the few compounds that were able to cleave the $tRNA_3^{Lys}$ substrate did so by a hydrolytic process. Hydrolysis occurred in a nonrandom fashion that was specific for substrate sequence and possibly conformation; all cleavage was observed in single-stranded regions having the sequence 5′-CA-3′. This sequence specificity has been observed previously for RNA substrates following exposure to congeners of BLM.[8f−h,20] Dock-Bregeon and Moras,[24] and later Kierzek,[23a,b] observed that certain sequences within an RNA substrate may be predisposed to hydrolytic cleavage due to surrounding sequence contexts and positioning within the secondary structure of the oligonucleotide, especially within bulge-loop regions. Recently, several groups have exploited this putative lability and generated artificial ribonucleases capable of the sequence-selective hydrolytic cleavage of RNA substrates.[23,29]

It is also possible that structural motifs present in the synthetic analogues of deglycoBLM A_6 provide a basic functionality that could effect deprotonation of the ribose 2′-OH and thus lead to RNA hydrolysis. A previous study supported a role for the imidazole residue of the histidine moiety in RNA hydrolysis;[20] however, analysis of the synthetic deglycoBLMs that mediated $tRNA_3^{Lys}$ hydrolysis in this study indicated that none contained this structural element. It is likely that a combination of inherent RNA substrate lability and nucleobase specific alteration of RNA structure due to some interaction of BLM with the RNA leads to productive hydrolysis of the phosphodiester backbone of RNA. From a structural standpoint, the most important domain required for oxidative cleavage of RNA substrates appears to be the carbohydrate domain, which is clearly absent in each of the analogues used in this study.

The primary goal of the present work was to test the thesis that systematic, simultaneous alteration of individual amino acid constituents in (deglyco) BLM could be used to identify BLM analogues having altered properties in specific assays of BLM function. The results of this study make it clear that analogues altered in any of a number of different properties can be identified in this fashion. It is also of interest to analyze the activity data in terms of those specific structural changes that lead to the alteration of biochemical properties. The effects of changes of single constituents on the several biochemical activities reported here are described previously, and parallel findings have been reported in many earlier studies. A unique feature of the present study is the ability to discern the patterns of changes of one or more amino acid constituents that lead to alterations of specific activities. For example, while substitution of $mval_2$ or $mval_3$ for naturally occurring $mval_1$ tended to enhance the DNA relaxation potential, this effect was affected substantially by the nature of the amino acid in the adjacent portion (normally threonine in BLM). Thus, analogues **4** and **5**, containing tyrosine and methionine, respectively, in lieu of threonine, afforded clearly enhanced cleavage (Figure 4). Plausibly, such data can be used to further the conformational analysis of the methylvalerate moiety as it relates to facilitation of DNA binding and cleavage.[3e,12t]

Likewise, it seems likely that the enhanced DNA binding of deglycoBLM **12** to DNA (Figure 5) is a consequence of the presence of numerous aromatic substituents, a principal that has not been recognized previously in analogues containing only a single altered constitutent. Comparison of some of the analogues that produced an altered sequence selectivity of DNA cleavage (Figures 9 and 10) revealed that most of the analogues exhibiting this property had alterations both to the β-hydroxyhistidine and to the methylvalerate constituents, arguing for a change in the nature of (preferred) DNA binding by certain analogues altered in these constituents. Perhaps the most interesting observation

(29) (a) Putnam, W. C.; Daniher, A. T.; Trawick, B. N.; Bashkin, J. K. *Nucleic Acids Res.* **2001**, *29*, 2199. (b) Kuznetsova, I. L.; Zenkova, M. A.; Gross, H. J.; Vlassov, V. V. *Nucleic Acids Res.* **2005**, *33*, 1201.

at this level was the alterations that led to enhanced hydrolytic cleavage of $tRNA_3^{Lys}$ at positions C_{56} and C_{75}. Of the three analogues that mediated strong hydrolytic cleavage, **17** was altered only at the site containing β-hydroxyhistidine in BLM. Analogue **25** was altered at this position (but not with the same amino acid substitution as in **17**). Analogue **26** contained the same alteration of the β-hydroxyhistidine as **17** but had a second alteration within the bithiazole moiety. The triply altered analogue **8**, containing substitutions present in **17**, **25**, and **26**, did not exhibit exceptionally strong hydrolytic cleavage of $tRNA_3^{Lys}$. Thus, alteration of single constituents in (deglyco) BLM cannot be relied upon to identify combinations of changes that can alter the properties of (deglyco) BLM. In this sense, the BLM molecule, with its many potential degrees of conformational freedom, presents a unique challenge from the perspective of the medicinal chemist and one that is amenable to study by the combinatorial approach described here.

Conclusion

Evaluation of a synthetic combinatorial library of deglycoBLMs revealed a number of unexpected outcomes, including the finding that several analogues exhibited a greater potency than deglycoBLM itself in relaxing supercoiled plasmid DNA. The two methylvalerate analogues employed in lieu of the natural methylvalerate found in BLM both increased the efficiency of DNA relaxation, and the replacement of the threonine moiety by tyrosine also tended to improve the efficiency of DNA cleavage. While none of the deglycoBLM analogues exhibited improved oxidative cleavage of a $tRNA_3^{Lys}$ substrate, several mediated efficient hydrolytic cleavage of the substrate at two CA sequences. Four deglycoBLM analogues also exhibited altered patterns of DNA cleavage. Thus, the selection paradigm was quite successful in identifying deglycoBLM analogues having significantly altered properties.

Experimental Procedures

General Methods. $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was purchased from EMD Chemicals. Sodium cacodylate was purchased from Sigma Aldrich. Restriction endonucleases *Hin*dIII, *Pvu*II, and Klenow fragment (3′ → 5′ exo$^-$) were purchased from New England Biolabs. Deoxyadenosine 5′-[α-^{32}P]triphosphate, triethylammonium salt were obtained from Amersham Biosciences.

Relaxation of Supercoiled Plasmid DNA by DeglycoBLM Analogues. The DNA relaxation assays were carried out in 15 μL (total volume) of 50 mM Tris-HCl buffer, pH 8.0, containing 200 ng of pSP64 plasmid DNA and the appropriate concentrations of BLM and freshly prepared Fe^{2+} solutions. Reaction mixtures were incubated at 25 °C for 50 min. The reactions were quenched by the addition of 3 μL of loading dye [40% sucrose containing 0.25% (w/v) Bromophenol Blue and 1 mM EDTA] and applied to a 1% agarose gel. Horizontal gel electrophoresis was carried out in 90 mM Tris-borate buffer, pH 8.3, containing 1 mM disodium EDTA at 150 V for 3 h. The gel was stained in 0.5 μg/mL ethidium bromide solution for 40 min. The DNA bands were visualized under UV light.

Preparation of a 3′-^{32}P-End Labeled DNA Restriction Fragment. Plasmid pSP64 DNA (50 μg) was incubated with restriction endonucleases *Pvu*II and *Hin*dIII (20 units separately) in a 100 μL reaction mixture according to the manufacturer's protocol. The digestion reaction was carried out at 37 °C for 3 h. The linearized DNA was elongated using [α-^{32}P]-dATP at the 3′-terminus of the *Hin*dIII cleavage site by 5 units of the Klenow polymerase (3′ → 5′ exo$^-$) at room temperature for 30 min and then applied to an 8% native polyacrylamide gel after adding 20 μL of loading dye [30% glycerol, 0.025% (w/v) Bromophenol Blue, and 0.025% (w/v) xylene cyanol]. Electrophoresis was carried out at 10 W for 2 h. The DNA was visualized by autoradiography, and the band of interest was excised from the gel and then recovered by the crush and soak method. The 222-bp 3′-^{32}P-end labeled DNA was finally dissolved in distilled water.

Fe(II)·BLM-Mediated Cleavage of the DNA Oligonucleotide. Reactions were carried out in 5 μL (total volume) of 10 mM sodium cacodylate, pH 7.0, containing 3′-^{32}P-end labeled DNA ($\sim 1.5 \times 10^4$ cpm) and the appropriate concentrations of deglycoBLM analogue and Fe^{2+}. Reaction mixtures were incubated at 4 °C for 30 min and then quenched by the addition of 2 μL of loading dye (98% formamide, 10 mM EDTA, 0.025% (w/v) xylene cyanol, and 0.025% (w/v) Bromophenol Blue), heated at 90 °C for 10 min, and then chilled on ice. The solutions were applied to an 8% denaturing polyacrylamide gel. Electrophoresis was carried out at 50 W for 2 h. The gel was analyzed using a phosphorimager (Molecular Dynamics).

Radiolabeling of $tRNA_3^{Lys}$. The $tRNA_3^{Lys}$, prepared by in vitro transcription,[7a] was [3′-^{32}P]-end labeled with [3′-^{32}P]pCp and T_4 RNA ligase. It was prepared in a reaction mixture (25 μL total volume) in 50 mM Tris-HCl, pH 7.8, containing 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 40 units of T_4 RNA ligase, 0.125 mCi [^{32}P]pCp, 6 μg of $tRNA_3^{Lys}$, and 11 μL of diethylpyrocarbonate-treated water. The solution was incubated at 4 °C for 24 h, and radiolabeled $tRNA_3^{Lys}$ was purified by 8% polyacrylamide gel electrophoresis.

Cleavage of [3′-^{32}P]-End Labeled $tRNA_3^{Lys}$ by BLM A_5 and the DeglycoBLM Analogues. $tRNA_3^{Lys}$ cleavage reactions were carried out in 5 μL (total volume) of 3′-^{32}P labeled $tRNA_3^{Lys}$ (2×10^5 cpm, 0.5−5 μM), containing 10 mM sodium phosphate, pH 7.0, and the appropriate concentrations of reagents as indicated (Figure 11). Each reaction was initiated by the simultaneous addition of BLM and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (freshly prepared solution) to the buffered solution containing $tRNA_3^{Lys}$. Each reaction mixture was incubated at room temperature for 45 min, and then the reaction mixture was dissolved in a gel loading buffer (98% formamide, 2 mM EDTA, 0.025% xylene FF, and 0.025% Bromophenol Blue). The resulting solution was heated at 70 °C for 5 min and then applied to a 20% polyacrylamide gel (31 cm × 38.5 cm × 0.4 cm) and subjected to electrophoresis at 50 W for 2 h. The gel was analyzed using a phosphorimager.

Cleavage of a ^{32}P-End Labeled DNA Hairpin Substrate. The 5′- and [3′-^{32}P]-end labeling, treatment with Fe(II)·deglycoBLMs, and product analyses were carried out as described previously.[3f]

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Supporting Information Available: Structures of selected deglycoBLM derivatives and complete ref 11a. This material is available free of charge via the Internet at http://pubs.acs.org.

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