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Alteration of the Selectivity of DNA Cleavage by a Deglycobleomycin Analogue Containing a Trithiazole Moiety

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Abstract: The bleomycin (BLM) group of antitumor antibiotics effects DNA cleavage in a sequence-selective manner. Previous studies have indicated that the metal-binding and bithiazole moieties of BLM are both involved in the binding of BLM to DNA. The metal-binding domain is normally the predominant structural element in determining the sequence selectivity of DNA binding, but it has been shown that replacement of the bithiazole moiety with a strong DNA binder can alter the sequence selectivity of DNA binding and cleavage. To further explore the mechanism by which BLM and DNA interact, a trithiazole-containing deglycoBLM analogue was synthesized and tested for its ability to relax supercoiled DNA and cleave linear duplex DNA in a sequence-selective fashion. Also studied was cleavage of a novel RNA substrate. Solid-phase synthesis of the trithiazole deglycoBLM A_5 analogue was achieved using a TentaGel resin containing a Dde linker and elaborated from five key intermediates. The ability of the resulting BLM analogue to relax supercoiled DNA was largely unaffected by introduction of the additional thiazole moiety. Remarkably, while no new sites of DNA cleavage were observed for this analogue, there was a strong preference for cleavage at two 5′-GT-3′ sites when a 5′-^{32}P end-labeled DNA duplex was used as a substrate. The alteration of sequence selectivity of cleavage was accompanied by some decrease in the potency of DNA cleavage, albeit without a dramatic diminution. In common with BLM, the trithiazole analogue of deglycoBLM A_5 effected both hydrolytic cleavage of RNA in the absence of added metal ion and oxidative cleavage in the presence of Fe^{2+} and O_2. In comparison with BLM A_5, the relative efficiencies of hydrolytic cleavage at individual sites were altered.

Introduction

The bleomycin (BLM) family of antitumor antibiotics possesses clinical utility in the treatment of squamous cell carcinomas, lymphomas, and testicular cancer.[1] It is believed that the observed therapeutic activity of BLM results from the metal-dependent oxidative degradation of DNA[2] and possibly RNA.[3] DNA cleavage is known to be sequence-selective, preferentially occurring at 5′-GpC-3′ and 5′-GpT-3′ sequences.[4]

The structure of BLM, exemplified by one member of this family of antitumor agents (BLM A_5 (**1**)), can be subdivided into three domains (Figure 1). The metal-binding domain contains the pyrimidine moiety, which is known to participate in DNA binding as well as chelation to the metal ion cofactor.[5] The carbohydrate moiety may participate in metal ion binding[6] and possibly also in cell surface recognition and uptake.[7] The C-terminus consists of the bithiazole moiety and a positively charged alkyl substituent, both of which take part in DNA binding and possibly sequence recognition.[2]

Numerous structure−activity and molecular modeling studies have contributed to the current view of the molecular mechanism of action of BLM.[8] Key unresolved issues include a complete understanding of the binding interaction of the bithiazole moiety

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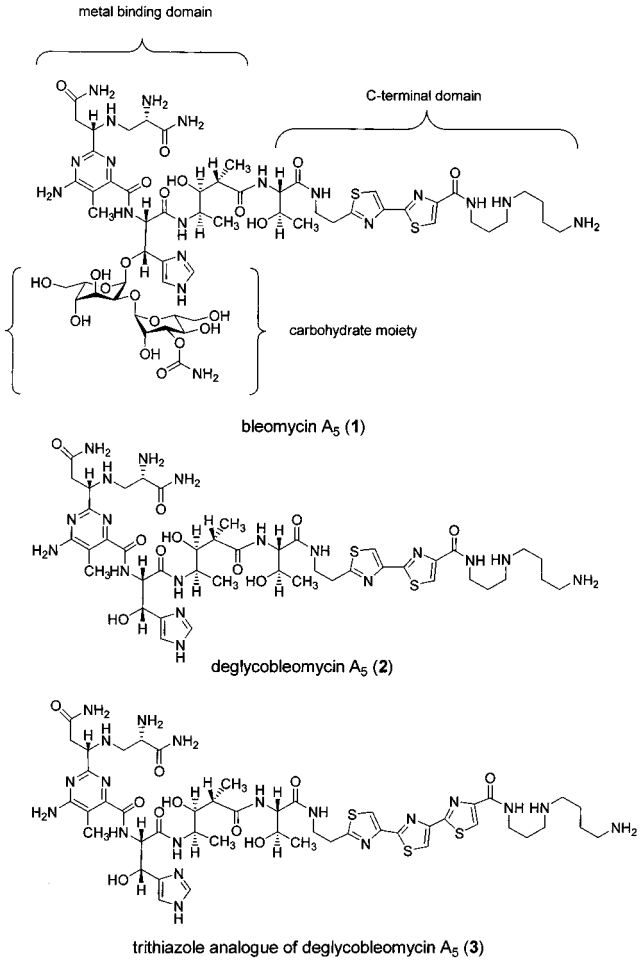

Figure 1. Structures of bleomycin A_5 (**1**), deglycoBLM A_5 (**2**), and a trithiazole analogue of deglycoBLM A_5 (**3**).

with DNA and its possible role in sequence selectivity of DNA cleavage. The interaction between the bithiazole moiety and DNA may entail intercalation, minor-groove binding, or partial intercalation. Evidence has been presented to support each of these possibilities, including numerous molecular models and NMR-based experiments.[9] While there is compelling evidence that the metal-binding domain is primarily responsible for the sequence selectivity of DNA cleavage by BLM, studies with a series of chlorinated bithiazoles and related BLM analogues have clearly established that the bithiazole is also capable of binding to DNA with its own preferred sequence selectivity.[10] We reasoned that replacement of the bithiazole moiety with related

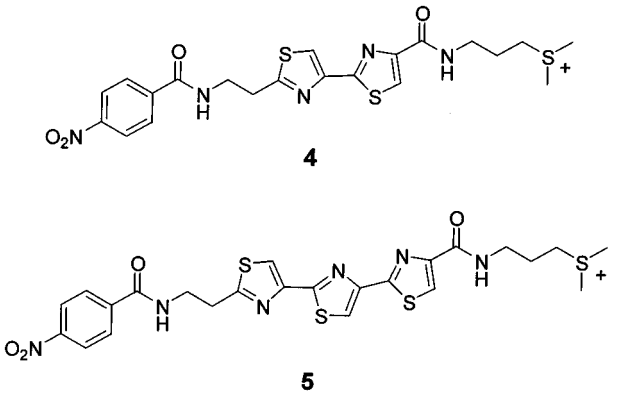

Figure 2. Structures of bithiazole (**4**) and trithiazole (**5**) analogues that mediate sequence-selective DNA cleavage.

unfused aromatic ring systems could alter the sequence selectivity of BLM, as already demonstrated in one case by Ohno and co-workers.[11]

Kuroda and co-workers have prepared a number of model compounds containing one or more thiazoles and incorporating a *p*-nitrobenzoyl group capable of mediating DNA cleavage.[12] Interestingly, a model compound containing a bithiazole moiety (**4**) (Figure 2) cleaved DNA substrates at 5′-AAATN (N not equal to G) sequences, while a structurally related trithiazole (**5**) exhibited selectivity for 5′-GG-3′ and 5′-GA-3′ sequences.

Presently, we describe the synthesis of an analogue of deglycoBLM A_5 containing a trithiazole moiety (**3**). Also described is the ability of **3** to effect the relaxation of supercoiled plasmid DNA in comparison with deglycoBLM A_5 (**2**) and the effect of the additional thiazole moiety on the sequence selectivity of DNA cleavage. The hydrolytic and oxidative cleavage of an RNA substrate is discussed as well.

Results

Synthesis of Trithiazole Intermediate 11. The elaboration of key Fmoc-protected trithiazole intermediate **11** (Scheme 1) was accomplished by analogy with the work of Kobayashi et al.[13] *N*-Boc bithiazole methyl ester **7** was synthesized from the previously reported carboxylic acid **6**[14] by acid-catalyzed esterification. Protection of the free amine with di-*tert*-butyl dicarbonate was accomplished in 91% yield. Treatment of *N*-Boc bithiazole methyl ester **7** with ammonium hydroxide in methanol afforded the corresponding amide **8** in 77% yield. The conversion of **8** to the respective thioamide **9** was then effected in 60% yield using Lawesson's reagent.[15] Formation of the third thiazole ring was realized via Hantzsch cyclization[16] with ethyl

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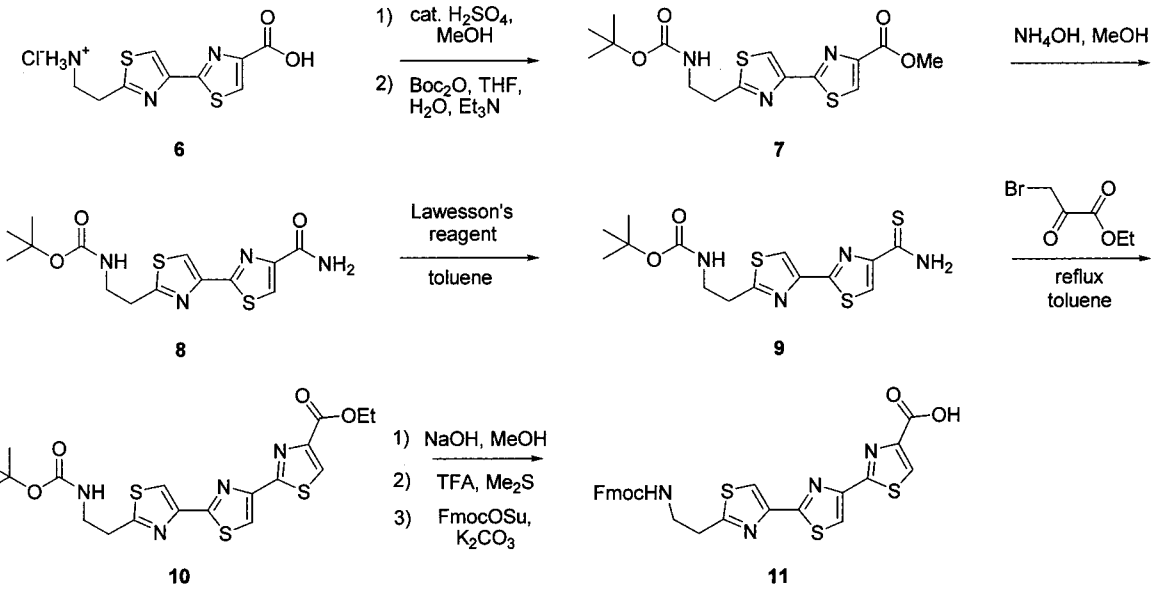
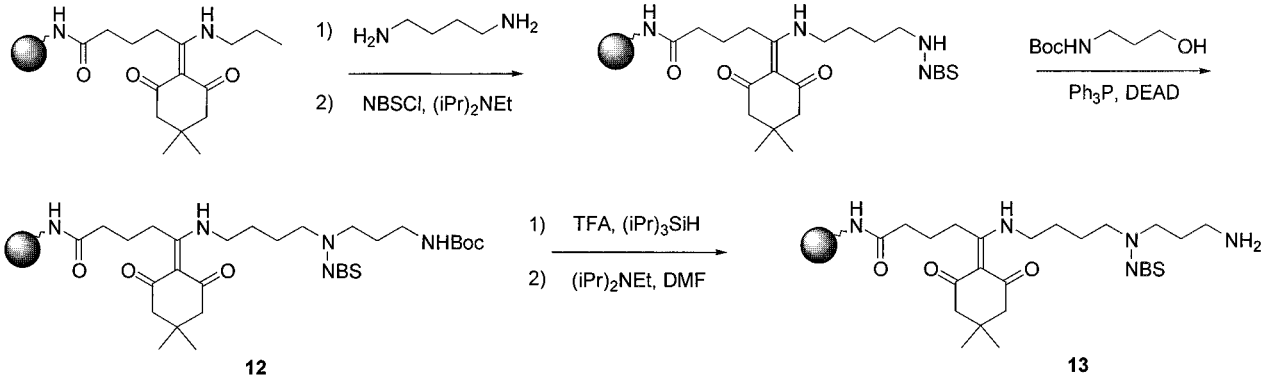
Scheme 1



Scheme 2



bromopyruvate in toluene, providing Boc trithiazole ethyl ester **10** in 43% yield. Saponification of **10** with aqueous sodium hydroxide was followed by CF_3COOH-catalyzed removal of the Boc protecting group. Fmoc protection was then carried out by a standard procedure that employed Fmoc succinimide,[17] affording Fmoc trithiazole **11** as a colorless solid in 79% yield for the final three steps.

Solid-Phase Synthesis of Trithiazole DeglycoBLM Analogue 3. The synthesis of deglycobleomycin **3** was carried out by solid-phase synthesis using a TentaGel resin (preloading of 0.45 mmol/g) and utilizing the versatile Dde linker developed by Bycroft et al.[18] The elaboration of a support containing the spermidine moiety of BLM (**13**) was accomplished as shown in Scheme 2. The TentaGel resin containing the Dde linker was treated with 1,4-diaminobutane to effect transamination, after which that terminal amine was protected with 2-nitrobenzenesulfonyl chloride. Nucleophilic displacement of *N*-Boc-3-aminopropanol under Mitsunobu conditions[19] produced the resin-bound intermediate (**12**) containing the protected spermidine moiety. Removal of the Boc group was carried out immediately prior to the synthesis of the BLM analogue by treatment with CF_3COOH in the presence of triisopropylsilane; subsequent washing with 20% Hunig's base in DMF afforded the resin-bound spermidine (**13**) used for the synthesis of deglycoBLM analogue **3**.

The synthesis of resin-bound tetrapeptide **18** was accomplished using resin **13**, as outlined in Scheme 3. Initially, resin **13** was condensed with Fmoc trithiazole **11** using *O*-benzotriazol-1-yl- *N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) in the presence of Hunig's base in DMF over a period of 30 min. The addition of the Fmoc-protected trithiazole afforded the first opportunity for quantitative measurement of loading. Fmoc cleavage analysis of an aliquot of dry resin indicated that the loading of resin **14** was 0.226 mmol/g. This corresponded to a 75% yield for the production of resin-bound dipeptide **14** over the first six steps. Preparative removal of the Fmoc group from **14** was accomplished using a 20% piperidine solution in DMF. Condensation with commercially available N^{α}-Fmoc-(*S*)-threonine (**15**) was accomplished via the agency HBTU, HOBt, and Hunig's base in DMF, providing resin-bound tripeptide **16** in 90% yield, as judged by subsequent Fmoc analysis. Using conditions as described above, Fmoc removal and coupling with N^{α}-Fmoc-(2*S*,3*S*,4*R*)-4-amino-3-hydroxy-2-methylvalerate (**17**)[20] resulted in the synthesis of resin-bound

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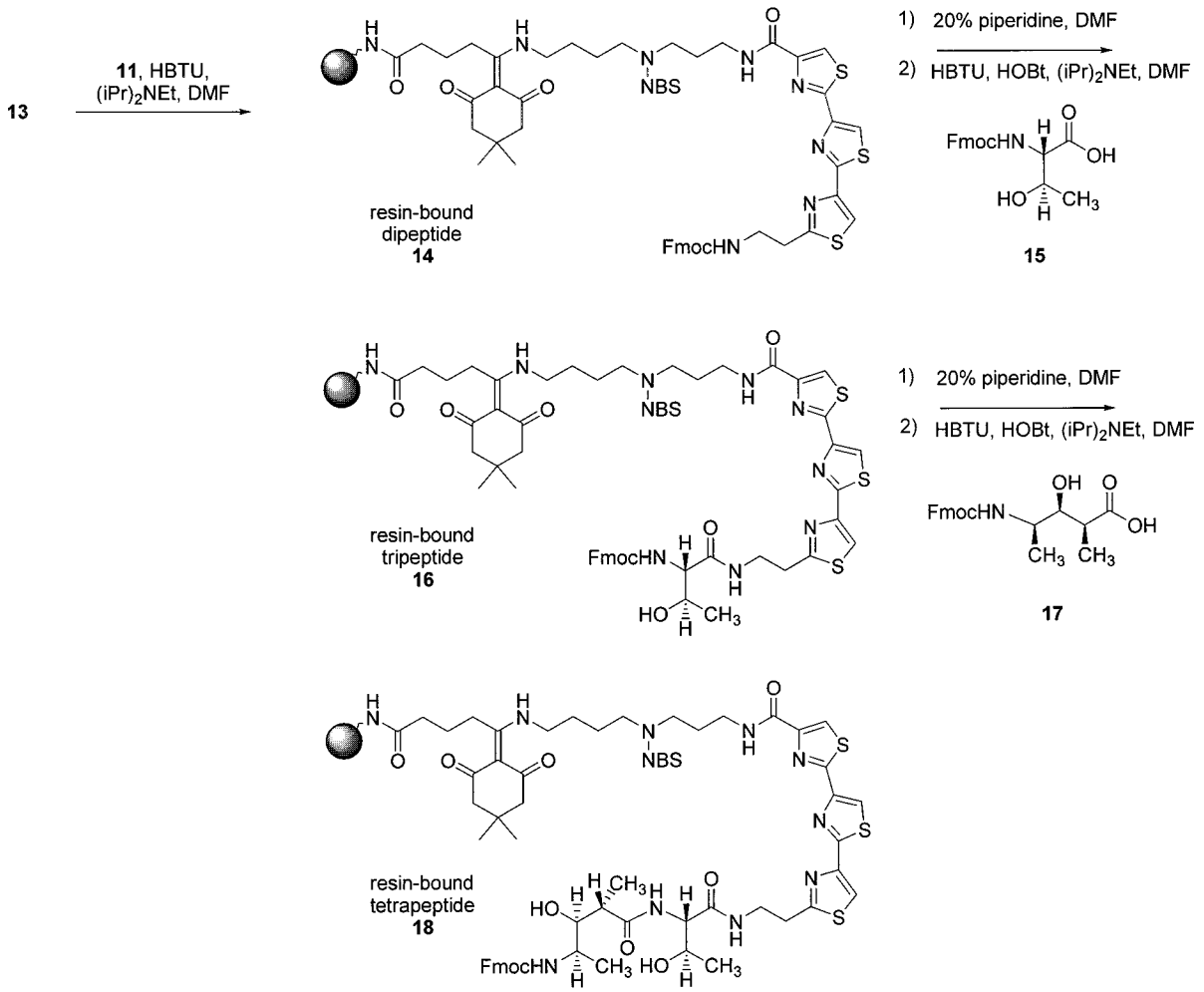
Scheme 3



tetrapeptide **18** in 93% yield, as judged by subsequent Fmoc analysis.

Completion of the synthesis of deglycoBLM analogue **3** was accomplished as shown in Scheme 4. Resin-bound tetrapeptide **18** was treated with 20% piperidine in DMF to effect removal of the Fmoc group, followed by coupling with N^{α}-Fmoc-N^{im}-trityl-(*S*)-erythro-β-hydroxyhistidine (**19**)[21] using a combination of *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), 1-hydroxy-7-azabenzotriazole (HOAt), and Hunig's base in DMF. Fmoc analysis indicated a 81% yield for the synthesis of resin-bound pentapeptide **20**. Final Fmoc removal with 20% piperidine in DMF was followed by coupling with N^{α}-Boc-pyrimidoblamic acid (**21**)[23] by employing benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) reagent and Hunig's base in DMF at 0 °C. This coupling was allowed to proceed over a 24 h period to afford protected, resin-bound deglycoBLM **22**.

(20) Synthesized by acid-catalyzed deprotection and Fmoc-succinimide-mediated reprotection of the previously reported Boc-protected derivative. See: Boger, D. L.; Colletti, S. L.; Honda, T.; Menezes, T. F. *J. Am. Chem. Soc.* **1994**, *116*, 5607.

(21) Prepared from (2′*S*,3′*R*,4*R*)-3-[2′-azido-3′-hydroxy-3′-(*N*-(triphenylmethyl)-imidazol-4″-yl) propanoyl]-4-isopropyl-2-oxazolidinone[22] as described in the Experimental Section.

(22) See, e.g. Boger, D. L.; Honda, T.; Menezes, R. F.; Colletti, S. L. *J. Am. Chem. Soc.* **1994**, *116*, 5631.

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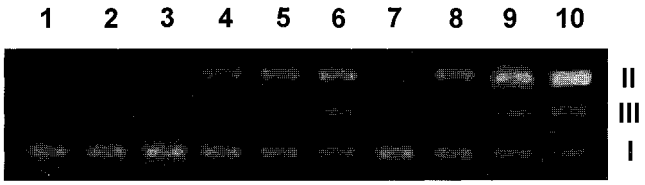
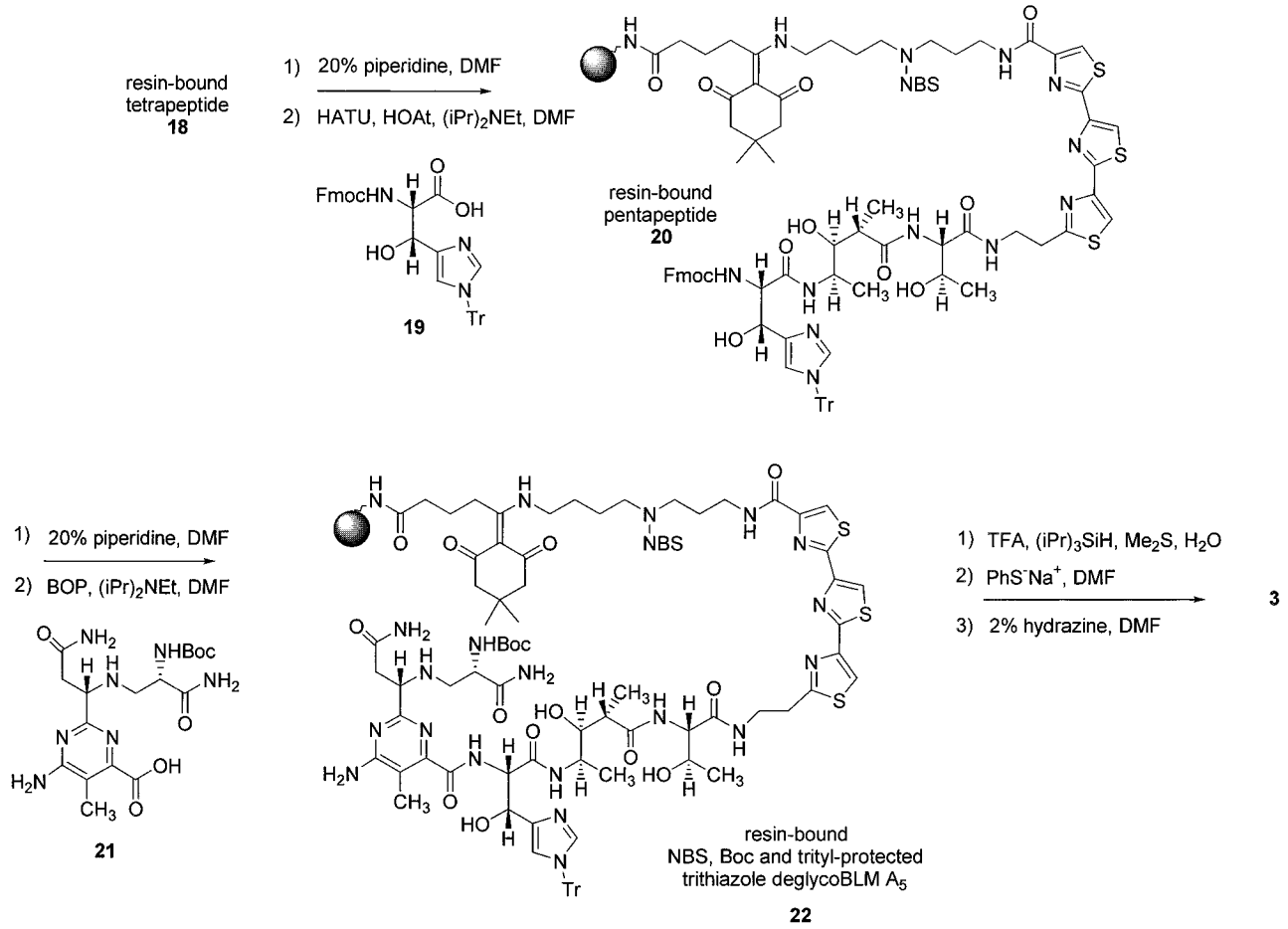


Figure 3. Cleavage of supercoiled pBR322 DNA by a trithiazole-containing bleomycin analogue: lane 1, DNA alone; lane 2, 1.5 μM Fe^{2+}; lane 3, 5 μM deglycoBLM **3**; lane 4, 1 μM deglycoBLM **3** + 1.5 μM Fe^{2+}; lane 5, 3 μM deglycoBLM **3** + 1.5 μM Fe^{2+}; lane 6, 5 μM deglycoBLM **3** + 1.5 μM Fe^{2+}; lane 7, 5 μM deglycoBLM A_5 (**2**); lane 8, 1 μM deglycoBLM A_5 + 1.5 μM Fe^{2+}; lane 9, 3 μM deglycoBLM A_5 + 1.5 μM Fe^{2+}; lane 10, 5 μM deglycoBLM A_5 + 1.5 μM Fe^{2+}.

Deprotection of the trityl and Boc groups was accomplished using CF_3COOH in the presence of dimethyl sulfide, triisopropylsilane, and water. Removal of the NBS group was carried out using repetitive treatments with the sodium salt of thiophenol in DMF. Cleavage of the product from the resin was achieved using a 2% solution of hydrazine in DMF. The ultraviolet spectrum of the crude product indicated that the final coupling had taken place in high yield. Following two successive purifications of the product by C_{18} reversed-phase HPLC, the overall yield of the final coupling, deprotection, and cleavage steps was 42%. Lyophilization provided deglycoBLM analogue **3** as a colorless solid.

Characterization of DNA Degradation by DeglycoBLM 3. As shown in Figure 3, Fe(II)·deglycoBLM analogue **3** was

Scheme 4



quite effective in mediating the relaxation of supercoiled pBR322 plasmid DNA. In the presence of 3 μM deglycoBLM **3** + 1.5 μM Fe^{2+}, about 50% of the 300 ng of supercoiled DNA utilized was converted to relaxed (form II) DNA. Moreover, 5 μM deglycoBLM **3** + 1.5 μM Fe^{2+} produced a significant amount of form III (linear duplex DNA). Densitiometric analysis of the form III bands for deglycoBLM **3** (lane 6) and deglycoBLM A_5 (lane 10) revealed that **3** retained 80% of the ability of deglycoBLM A_5 to produce double-stranded DNA cleavage.

Also investigated was the ability of deglycoBLM **3** to effect the sequence-selective cleavage of DNA. This was studied using a 5′-^{32}P end-labeled 158-base pair DNA restriction fragment as a substrate. As shown in Figure 4, Fe(II)·deglycoBLM A_5 (**2**) effected sequence-selective cleavage of the DNA substrate at several sites. Five strong cleavage sites were identified by sequence; these included two 5′-GC-3′ sites and three 5′-GT-3′ sites. The strongest cleavage was at 5′-$G_{33}C_{34}$-3′. The same five sites were also cleaved to some extent by Fe(II)·BLM A_5 analogue **3**. However, three of these sites, including 5′-$G_{33}C_{34}$-3′, were cleaved very weakly by this BLM analogue. The ratio of cleavage at 5′-$G_{33}C_{34}$-3′ relative to that at 5′-$G_{54}T_{55}$-3′ was 5:1 for deglycoBLM A_5, as determined by phosphorimager analysis. In comparison, the cleavage ratio at the same two sites was 1:6 for deglycoBLM analogue **3**. Both of the strong cleavage sites for **3** involved 5′-GT-3′ sequences. Additionally, the overall extent of DNA cleavage mediated by analogue **3** was significantly less than that obtained in the presence of **2** (cf. lanes 4−6 and 8−10). The slight decrease in band intensities

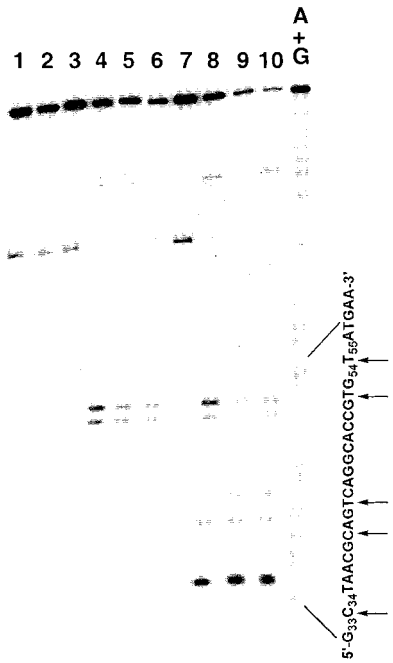


Figure 4. Cleavage of 5′-^{32}P end-labeled 158-base pair DNA duplex by deglycoBLM **3**: lane 1, DNA alone; lane 2, 10 μM Fe^{2+}; lane 3, 10 μM deglycoBLM **3**; lane 4, 1 μM deglycoBLM **3** + 1 μM Fe^{2+}; lane 5, 5 μM deglycoBLM **3** + 5 μM Fe^{2+}; lane 6, 10 μM deglycoBLM **3** + 10 μM Fe^{2+}; lane 7, 10 μM deglycoBLM A_5 (**2**); lane 8, 1 μM deglycoBLM A_5 + 1 μM Fe^{2+}; lane 9, 5 μM deglycoBLM A_5 + 5 μM Fe^{2+}; lane 10, 10 μM deglycoBLM A_5 + 10 μM Fe^{2+}. The bands of intermediate mobility in lanes 1, 2, 3, and 7 were due to nondenatured DNA duplex.

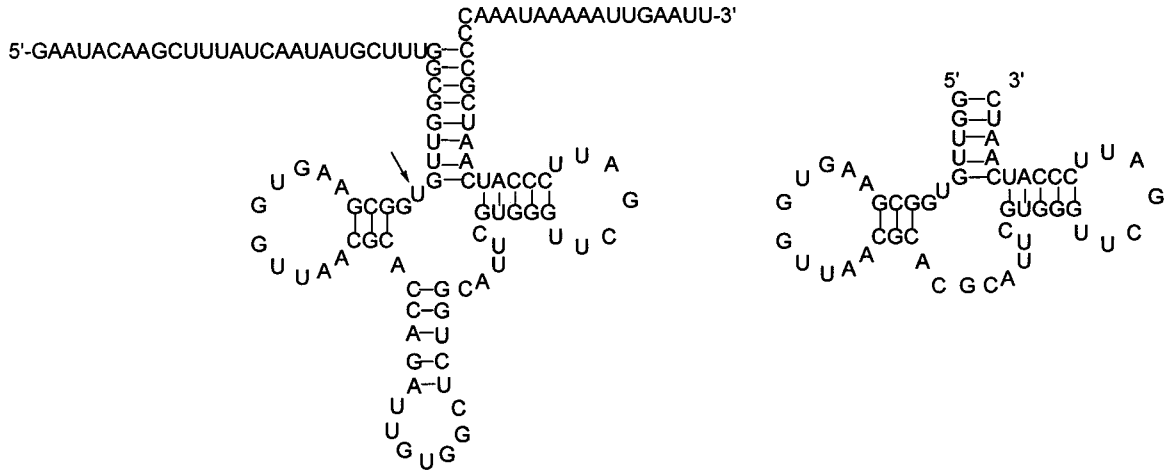


Figure 5. Structures of *B. subtilis* RNA^{His} precursor (left) and a 53-nucleotide RNA (right) that serve as substrates for cleavage by Fe(II)·BLM.

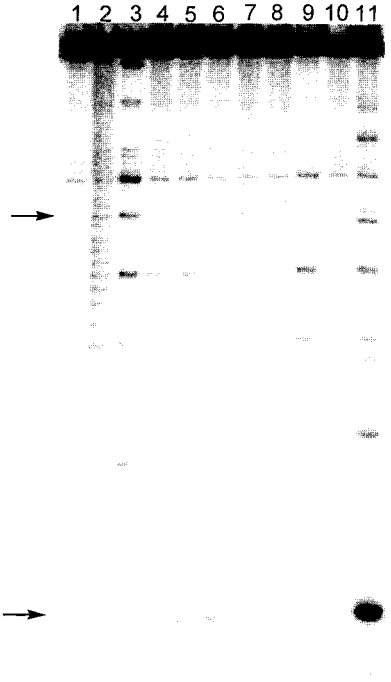


Figure 6. Cleavage of a 53-nucleotide RNA by Fe(II)·BLM: lane 1, RNA alone; lane 2, 100 μM Fe^{2+}; lane 3, 100 μM deglycoBLM **3**; lane 4, 1 μM Fe(II)·deglycoBLM **3**; lane 5, 5 μM Fe(II)·deglycoBLM **3**; lane 6, 10 μM Fe(II)·deglycoBLM **3**; lane 7, 50 μM Fe(II)·deglycoBLM **3**; lane 8, 100 μM Fe(II)·deglycoBLM **3**; lane 9, 10 μM BLM A_5; lane 10, 10 μM Fe^{2+}; lane 11, 10 μM Fe(II)·BLM A_5. The upper arrow illustrates a site of hydrolytic cleavage mediated exclusively by deglycoBLM **3**. The lower arrow illustrates the site of oxidative cleavage mediated both by Fe(II)·deglycoBLM **3** (lanes 4−7) and Fe(II)·BLM A_5 (lane 11) (**1**).

at higher deglycoBLM concentrations was due to multiple cleavage events.

Characterization of RNA Degradation by DeglycoBLM 3. To assess the ability of deglycoBLM **3** to effect RNA degradation, we utilized a synthetic 53-nucleotide RNA that has the same primary sequence as the "core" of *Bacillus subtilis* $tRNA^{His}$ precusor (Figure 5), the latter of which has been shown to be an excellent substrate for oxidative cleavage by Fe(II)·BLM.[3]

As shown in Figure 6 (lane 3), 100 μM deglycoBLM **3** effected cleavage of the RNA substrate at several sites in the absence of added metal ion. Further, these sites of cleavage were greatly diminished by the addition of Fe^{2+}, as already established for BLM itself.[24] Direct comparison with hydrolytic cleavage mediated by 10 μM Fe(II)·BLM A_5 is shown in lane 9. As can be seen, most of the sites of cleavage were the same, although **3** produced cleavage at one site (upper arrow) not readily apparent when BLM A_5 was employed.

Oxidative cleavage by **1** and **3** were also compared (lanes 4−7 and 11, respectively). The predominant cleavage site observed for Fe(II)·BLM A_5 was the same as that produced by deglycoBLM **3**, although the latter cleaved the RNA with lesser efficiency.

Discussion

Experiments from a few laboratories have demonstrated convincingly that in addition to its role in binding metal ions and dioxygen and in mediating the chemical transformations catalyzed by BLM, the metal-binding domain of BLM (Figure 1) is the primary determinant of the sequence selectivity of DNA cleavage by BLM.[5] There is general agreement that the metal-binding domain must associate with DNA in the minor groove, since C4′-H of deoxyribose must be abstracted by an activated metallobleomycin in order to initiate the chemical transformations that are actually observed.[2]

In contrast, the specific molecular role(s) played by the bithiazole moiety in supporting BLM-mediated DNA degradation is uncertain. While not the primary mediator of sequence-selective DNA cleavage by BLM, studies involving photoinduced DNA cleavage by chlorobithiazoles and structurally related deglycoBLMs incorporating chlorinated bithiazole moieties have made it clear that the bithiazole moiety of BLM may also exhibit sequence-selective DNA binding;[10] it seems likely that strongly preferred sites of DNA binding and cleavage by BLM may include those that accommodate the DNA binding preferences of both the metal binding and C-terminal domains of BLM.

Although the metal-binding domain of BLM is the primary determinant of sequence-selective DNA cleavage, it is clear that replacement of the bithiazole moiety with an unfused aromatic ring system that binds strongly to DNA can alter the importance of the structural elements responsible for determining sequence selectivity. Ohno and co-workers demonstrated that the introduction of an oligopyrrole moiety in lieu of the bithiazole altered

(24) Keck, M. V.; Hecht, S. M. *Biochemistry* **1995**, *34*, 12029.

the sequence selectivity of DNA cleavage by BLM to AT-rich regions, although the efficiency of DNA cleavage was diminished significantly.[11]

To permit better definition of the way in which the C-terminal domain of BLM contributes to the sequence selectivity of DNA binding and cleavage, we have employed solid-phase synthesis[25] to prepare a deglycoBLM A_5 analogue (**3**) in which the bithiazole moiety has been replaced by a trithiazole. In a previous study of oligothiazoles incorporating a nitrobenzoyl group as a DNA cleaving agent, the number of thiazoles had a significant effect on the sequence selectivity of DNA cleavage.[12] Specifically, the presence of a third thiazole ring (cf. compounds **4** and **5**, Figure 2) altered the sequence selectivity of DNA cleavage for these compounds from 5′-AAATN-3′ (N not equal to G) regions to 5′-GG-3′ and 5′-GA-3′ regions. It was of interest to determine whether the introduction of a third thiazole ring into a BLM analogue would have an analogous effect on the sequence selectivity of DNA cleavage.

As shown in Figure 3, the introduction of a third thiazole moiety into deglycoBLM A_5 afforded a species capable of relaxing supercoiled plasmid DNA and producing linear duplex (form III) DNA. The proportions of forms II and III DNA produced were not much different than that resulting from cleavage by deglycoBLM A_5 (**2**). The latter is quite unusual; most modifications of BLM structure, including removal of the disaccharide moiety, have resulted in significantly less efficient DNA cleavage and a smaller proportion of form III DNA.[22]

No less striking were the results obtained when a linear DNA duplex was employed as a substrate for cleavage by BLM analogues **2** and **3**. As shown in Figure 4, trithiazole analogue **3** cleaved DNA efficiently at only two of the five sites cleaved efficiently by deglycoBLM analogue **2**. Although analogous effects had previously been noted for several bithiazole-containing BLM congeners,[26] and also for Fe(II)·BLM vs Cu(I)·BLM,[27] the differences in the present case are much more pronounced. This finding argues strongly that while not the primary mediator of sequence-selective cleavage by BLM, the bithiazole + C-substituent does contribute to the observed efficiency of DNA cleavage at certain sites.

In comparison with other BLM analogues that exhibited significant alterations in the patterns of DNA cleavage, deglycoBLM **3** retained much of the potency associated with the parent deglycoBLM (**2**) to which it was related structurally. The proportion of single- vs double-strand DNA cleavage was also largely unaffected.

The pattern of RNA cleavage produced by deglycoBLM **3** is also worthy of note. Analogue **3** mediated both hydrolytic[24] and oxidative cleavage of a 53-nucleotide RNA identical with the core region of *B. subtilis* $tRNA^{His}$. The latter undergoes oxidative cleavage at a single site by Fe(II)·BLM (U_{35}; see arrow in Figure 5) and the 53-nucleotide RNA substrate is apparently cleaved at the analogous (i.e., U_6) site by Fe(II)·BLM A_5. Fe(II)·deglycoBLM **3** also cleaved the 5′-^{32}P end-labeled substrate at this site, although only weakly. More interesting was the hydrolytic cleavage mediated by **3** in the absence of added Fe^{2+}. Several sites were cleaved efficiently, including one site not cleaved to a significant extent by 10 μM Fe(II)·BLM A_5 (cf. lanes 3 and 9, upper arrow). Thus, the presence of the trithiazole moiety also effected some changes in the sequence selectivity of RNA cleavage by (deglyco)BLM.

As we have noted previously, it seems likely that the 5′-G·pyr-3′ specificity noted for cleavage of B-form DNA by bleomycin reflects the fact that these sequences correspond to the widest, shallowest part of the DNA minor groove.[28] Physicochemical studies of the binding of metalloBLMs to the minor groove suggest that the metal-binding domain of BLM is actually somewhat larger than the minor groove;[9] thus, binding to 5′-G·pyr-3′ sequences would best accommodate this ligand. The differences noted for BLMs **2** and **3** in the specific 5′-G·pyr-3′ sequences targeted the most efficiently obviously must reflect the behavior of the trithiazole moiety in **3** vs the bithiazole moiety in **2**. That the differences are not dramatic argues that the fundamental binding mode for the bithiazole moiety in **2** and trithiazole moiety in **3** are the same. In this context, it may be noted that a minor groove binding mode could readily be envisioned for these two moieties, while one might anticipate that threading intercalation seems unlikely to be optimal for both the bi- and trithiazole moieties.

Experimental Section

General Methods. NMR chemical shifts are referenced to $(CD_3)_2$-SO at 2.50 ppm or $CDCl_3$ at 7.26 ppm or D_2O at 4.79 ppm. Mass determination was accomplished by electrospray ionization on a Finnigan 3200 quadrupole mass spectrometer. Elemental analyses were performed by Atlantic Microlab, Inc. HPLC purifications were performed on a Varian Associates HPLC using an Alltech Alltima C_{18} reversed-phase column (250 × 10 mm, 5 μm).

TentaGel resin, HBTU, HOBt, BOP, Fmoc-threonine, and Fmoc-succinimide were purchased from Novabiochem. DMF was purchased from Acros Organics. All other chemicals were purchased from Aldrich Chemical Co. Deuterated NMR solvents were purchased from Cambridge Isotope. Agarose was obtained from Bethesda Research Laboratories. Acrylamide, *N*,*N*′-methylenebisacrylamide and ethidium bromide were purchased from Sigma Chemicals. All solvents were of analytical grade. Tetrahydrofuran was distilled from potassium metal and benzophenone ketyl prior to use. Methanol was dried over 3 Å 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 the appropriate reaction solvent prior to usage.

Yield determinations for reactions performed on the solid support were accomplished following Fmoc cleavage by UV measurement of the dibenzylfulvene−piperidine adduct formed upon treatment of the resin with piperidine.[29] The molar 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.

2′-[2-(*tert*-Butoxycarbonyl)amino]ethyl-2,4′-bithiazole-4-carboxylate Methyl Ester (7). To a solution of 10.0 g (34.3 mmol) of 2′-(2-amino)ethyl-2,4′-bithiazole-4-carboxylate (**6**) in 100 mL of methanol was added 2 mL of concentrated sulfuric acid. The mixture was heated to reflux for 3 h, and the cooled reaction mixture was neutralized with 18 mL of triethylamine and concentrated under diminished pressure. To the resulting mixture was added 75 mL of water, 75 mL of THF, and 11.2 g (51.3 mmol) of di-*tert*-butyl dicarbonate. The reaction mixture was then stirred for 72 h and concentrated under diminished pressure. The resulting aqueous phase was extracted with ethyl acetate.

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(26) Shipley, J. B.; Hecht, S. M. *Chem. Res. Toxicol.* **1988**, *1*, 25.
(27) Ehrenfeld, G. M.; Shipley, J. B.; Heimbrook, D. C.; Sugiyama, H.; Long, E. C.; van Boom, J. H.; van der Marel, G. A.; Oppenheimer, N. J.; Hecht, S. M. *Biochemistry* **1987**, *26*, 931.
(28) Dickerson, R. E. In *Structure and Methods*, vol. 3; *DNA and RNA. Proceedings of the Sixth Conversation in Biomolecular Stereodynamics;* Sarma, R. H.; Sarma, M. H., Eds.; Academic Press: Schenectady, NY, 1990; pp 1−38.
(29) (a) Fields, G. B.; Noble, R. L. *Int. J. Pept. Protein Res.* **1990**, *35*, 161. (b) Gordeev, M. F.; Luehr, G. W.; Hui, H. C.; Gordon, E. M.; Patel, D. V. *Tetrahedron* **1998**, *54*, 15879.

The combined organic extract was dried ($MgSO_4$), filtered, and concentrated under diminished pressure to give bithiazole **7** as a colorless solid: yield 11.5 g (91%); silica gel TLC R_f 0.35 (1:1 hexanes−EtOAc); ^{1}H NMR ($CDCl_3$) δ 1.43 (s, 9H), 3.22 (t, 2H), 3.61 (q, 2H), 3.97 (s, 3H), 5.04 (s, 1H), 8.05 (s, 1H), and 8.20 (s, 1H).

Anal. Calcd for $C_{15}H_{19}N_3O_4S_2$: C, 48.76; H, 5.18; N, 11.37. Found: C, 48.81; H, 5.17; N, 11.37.

***N*-Boc Bithiazole Carboxamide 8.** To a solution of 8.04 g (21.8 mmol) of bithiazole methyl ester **7** in 200 mL of methanol was added 100 mL (14.8 N, 1.48 mol) of concentrated ammonium hydroxide. The reaction was stirred at room temperature for 12 h and then concentrated under diminished pressure. The resulting solid was purified by crystallization from ethyl acetate and hexanes, affording bithiazole **8** as a colorless solid: yield 5.96 g (77%); ^{1}H NMR ($(CD_3)_2SO$) δ 1.30 (s, 9H), 3.10 (t, 2H, J = 6 Hz), 3.26 (t, 2H, J = 6 Hz), 6.98 (m, 1H), 7.61 (s, 1H), 7.74 (s, 1H), 8.12 (s, 1H), and 8.21 (s, 1H); ^{13}C NMR ($(CD_3)_2SO$) δ 28.27, 33.05, 77.86, 117.65, 124.36, 147.40, 151.12, 155.55, 161.95, 162.26, and 169.42; MS (FAB), *m*/*z* 355.0890 ($C_{14}H_{19}N_4O_3S_2$ requires 355.0899).

***N*-Boc Bithiazole Thiocarboxamide 9.** To a suspension of 5.00 g (14.1 mmol) of bithiazole carboxamide **8** in 50 mL of toluene was added 4.42 g (10.9 mmol) of Lawesson's reagent. The resulting mixture was heated to reflux for 3 h and concentrated under diminished pressure. The residue was partitioned between 200 mL of water and 200 mL of CH_2Cl_2. The aqueous phase was extracted twice with 200-mL portions of CH_2Cl_2. The combined organic fraction was dried ($MgSO_4$), filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column. Elution with 1:1 hexanes−ethyl acetate afforded bithiazole **9** as a colorless solid: yield 3.16 g (60%); silica gel TLC R_f 0.41 (1:1 hexanes−EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 1.31 (s, 9H), 3.10 (t, 2H, J = 6 Hz), 3.27 (t, 2H, J = 6 Hz), 6.98 (m, 1H), 8.16 (s, 1H), 8.40 (s, 1H), 9.57 (s, 1H), and 10.05 (s, 1H); ^{13}C NMR ($(CD_3)_2SO$) δ 28.25, 33.08, 77.86, 117.98, 127.39, 147.35, 154.31, 155.59, 161.43, 169.51, and 189.03; MS (FAB), *m*/*z* 371.0672 ($C_{14}H_{19}N_4O_2S_3$ requires 371.0670).

Boc Trithiazole Ethyl Ester 10. To a suspension of 5.16 g (13.9 mmol) of bithiazole thiocarboxamide **9** in 100 mL of toluene was added 2.50 mL (3.89 g, 17.9 mmol) of ethyl bromopyruvate. The reaction mixture was stirred at ambient temperature for 8 h. A second portion of 2.50 mL (17.9 mmol) of ethyl bromopyruvate was added, the reaction mixture was heated to reflux for 2 h, and the cooled reaction mixture was then concentrated under diminished pressure. The resulting residue was dissolved in 150 mL of THF and 150 mL of water. To this solution was added 3.05 g (13.9 mmol) of Boc anhydride and 4 mL (2.90 g, 28.7 mmol) of technical grade triethylamine to ensure that the primary amine was protected. The reaction mixture was stirred at ambient temperature for 48 h and then concentrated to a small volume under diminished pressure. The remaining aqueous phase was extracted with three portions of CH_2Cl_2. The combined organic fraction was dried ($MgSO_4$), filtered, concentrated under diminished pressure and then washed with methanol to afford trithiazole **10** as an off-white solid: yield 2.78 g (43%); silica gel TLC R_f 0.38 (1:1 hexanes−EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 1.26 (t, 3H, J = 7 Hz), 1.28 (m, 9H), 3.10 (t, 2H, J = 6 Hz), 3.26 (t, 2H, J = 6 Hz), 4.26 (q, 2H, J = 7 Hz), 7.01 (m, 1H), 8.22 (s, 1H), 8.35 (s, 1H), and 8.54 (s, 1H); ^{13}C NMR ($(CD_3)_2SO$) δ 14.28, 28.27, 33.03, 60.98, 77.87, 118.31, 118.93, 129.76, 146.87, 147.07, 148.40, 148.42, 160.73, 163.19, 166.99, and 169.51.

Anal. Calcd for $C_{19}H_{22}N_4O_4S_3$: C, 48.91; H, 4.75. Found: C48.97; H, 4.74.

***N*-Fmoc Trithiazole Carboxylate 11.** To a suspension of 2.68 g (5.74 mmol) of trithiazole ethyl ester **10** in 50 mL of methanol was added 4.60 mL (23.0 mmol) of aqueous 5 M NaOH. The reaction mixture was stirred for 3 h and then concentrated under diminished pressure. To this residue was added 30 mL of dimethyl sulfide and 10 mL of trifluoroacetic acid. The reaction mixture was stirred at room temperature for 12 h and then concentrated under diminished pressure and coevaporated with portions of chloroform. To the residue was added 50 mL of 10% aqueous K_2CO_3 and 2 mL of dioxane. To this mixture was added a solution of 1.84 g (5.45 mmol) of Fmoc succinimide in 50 mL of dioxane. The reaction mixture was stirred for 3 h and then concentrated under diminished pressure. The resulting precipitate was washed with acetone and water and then dried, providing Fmoc trithiazole **11** as an off-white solid: yield 2.43 g (76%); silica gel TLC R_f 0.72 (1:1 EtOAc−MeOH); ^{1}H NMR ($(CD_3)_2SO$) δ 3.14 (m, 2H), 3.38 (m, 2H), 4.18 (m, 1H), 4.26 (m, 2H), 7.22−7.32 (m, 4H), 7.38 (m, 1H), 7.61 (d, 2H, J = 8 Hz), 7.82 (d, 2H, J = 8 Hz), 8.22 (s, 1H), 8.30 (s, 1H), and 8.46 (s, 1H); ^{13}C ($(CD_3)_2SO$) δ 32.89, 46.77, 65.39, 118.27, 118.71, 120.18, 125.17, 127.05, 127.58, 127.65, 129.29, 140.79, 143.90, 146.94, 148.18, 148.58, 162.06, 163.12, 166.99, and 169.45; mass spectrum (FAB), *m*/*z* 561.0727 ($C_{27}H_{21}N_4O_4S_3$ requires 561.0725).

Spermidine Resin 12. To a suspension of 1.01 g (0.40 mmol/g) of swollen Dde-modified TentaGel resin in 2 mL of DMF was added 2.00 g (22.7 mmol) of 1,4-diaminobutane. The mixture was shaken for 12 h, then the solvent was removed by filtration, and the resin was washed with three 30-mL portions of DMF, three 30-mL portions of CH_2Cl_2, and three 30-mL portions of DMF. The treatment with 1,4-diaminobutane and subsequent washing was repeated a second time and an aliquot was removed; a qualitative Kaiser free-amine test[30] showed the presence of free amine. The resin was washed with three 30-mL portions of THF and three 30-mL portions of CH_2Cl_2. To a suspension of resin in 3 mL of CH_2Cl_2 was added 516 μL (383 mg, 3.0 mmol) of Hunig's base. The resin was shaken for 30 s and 440 mg (2.0 mmol) of 2-nitrobenzenesulfonyl chloride was added. The reaction mixture was shaken for 12 h and the solvent was removed by filtration. The resin was washed with three 30-mL portions of CH_2Cl_2, three 30-mL portions of THF, and three 30-mL portions of CH_2Cl_2. The treatment with 2-nitrobenzenesulfonyl chloride and subsequent washing procedure was repeated a second time and an aliquot (<1 mg) was removed; a qualitative Kaiser free-amine test showed the absence of free amine. The resin was suspended in a solution containing 315 mg (1.80 mmol) of Boc-protected 3-aminopropanol and 474 mg (1.80 mmol) of triphenylphosphine in 3 mL of THF. The mixture was shaken for 30 s, and 283 μL (1.80 mmol) of diethyl azodicarboxylate was added. The reaction mixture was shaken for 12 h in the absence of light, and then solvent was removed by filtration. The treatment with Boc-protected 3-aminopropanol, triphenylphosphine, and diethyl azodicarboxylate was repeated a second time. Resin **12** was washed with three 30-mL portions of THF, three 30-mL portions of CH_2Cl_2, and another three 30-mL portions of THF. This washing procedure was repeated a second time and an aliquot was removed; a qualitative Kaiser free-amine test indicated the absence of free amine. The resin was dried under diminished pressure.

Resin-Bound Dipeptide 14. To 300 mg of swollen spermidine resin **12** was added a 10% solution of triisopropylsilane in 2 mL of CH_2Cl_2 and 1 mL of CF_3COOH. The reaction mixture was allowed to stir for 1 h, then the solvent was removed by filtration, and the $(^iPr)_3SiH$/TFA treatment was repeated. The resin was washed with 20 mL of a 10% solution of isopropylsilane in CH_2Cl_2, and then the solvent was removed by filtration and the resin was washed successively with three 30-mL portions of CH_2Cl_2, three 30-mL portions of DMF, and three 30-mL portions of a 10% solution of Hunig's base in DMF. An aliquot was removed; a qualitative Kaiser free-amine test showed the presence of free amine. The resin **13** was suspended in a solution of 175 mg (0.31 mmol) of Fmoc-protected trithiazole **11**, 119 mg (0.31 mmol) of HBTU, and 110 μL (0.63 mmol) of Hunig's base in 2 mL of DMF. The resulting mixture was stirred for 30 min, then the solvent was removed by filtration, and the resin was washed successively with three 30-mL portions of DMF, three 30-mL portions of CH_2Cl_2, and again with three 30-mL portions of DMF. An aliquot was removed; a qualitative Kaiser free-amine test showed the absence of free amine. An aliquot of the putative "dipeptide" was cleaved from the resin with a 1-mL solution containing 2% hydrazine in DMF. After concentration, the residue was

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dissolved in 1 mL of CF_3COOH and the solution was treated with 10 mL of ether to effect precipitation of the cleaved product. ESI mass spectroscopy showed a molecular ion at *m*/*z* 651.3, consistent with the presence of the desired product. An aliquot was used for Fmoc cleavage analysis and revealed a loading of 0.226 mmol/g (corresponding to a 75% overall yield for the first six steps). The resin was dried under diminished pressure.

Resin-Bound Tripeptide 16. To 290 mg (0.226 mmol/g) of swollen dipeptide resin **14** was added 3 mL of a 20% solution of piperidine in DMF. The reaction mixture was shaken for 10 min, then the solvent was 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 three times and resin washed successively with three 30-mL portions of DMF, three 30-mL portions of CH_2Cl_2, and another three 30-mL portions of DMF. An aliquot was removed; a qualitative Kaiser free-amine test showed the presence of free amine. The resin was suspended in a solution containing 68 mg (0.20 mmol) of Fmoc-protected threonine (**15**), 103 mg (0.20 mmol) of HBTU, 26 mg (0.19 mmol) of HOBt, and 70 μL (51.9 mg, 0.40 mmol) of Hunig's base in 2 mL of DMF. The reaction mixture was allowed to stir for 30 min, then the solvent was removed by filtration, and the resin was washed with three 30-mL portions of DMF, three 30-mL portions of CH_2Cl_2, and three 30-mL portions of methanol. An aliquot was removed; a qualitative Kaiser free-amine test showed the absence of free amine. An aliquot was cleaved from the resin with a solution of 2% hydrazine in DMF. After concentration, the residue was dissolved in CF_3COOH and the solution was treated with ether to effect precipitation of the cleaved product. ESI mass spectrometry gave a molecular ion at *m*/*z* 752.3, consistent with the presence of desired "tripeptide" **16**. An aliquot was removed for Fmoc cleavage analysis and revealed a loading of 0.197 mmol/g (corresponding to a 90% yield from resin-bound dipeptide **14**, calculated on the basis of a theoretical loading of 0.219 mmol/g). The resin was dried under vacuum.

Resin-Bound Tetrapeptide 18. To 280 mg (0.197 mmol/g) of swollen tripeptide resin **16** was added 2 mL of a 20% solution of piperidine in DMF. The reaction mixture was shaken for 10 min, then the solvent was removed by filtration, and the resin was washed with 10 mL of a 20% piperidine solution in DMF. The piperidine treatment and subsequent washing procedure was repeated three times, and resin was washed successively with three 30-mL portions of DMF, three 30-mL portions of CH_2Cl_2, and again with three 30-mL portions of DMF. An aliquot was removed; a qualitative Kaiser free-amine test showed the presence of free amine. The resin was suspended in a solution containing 61 mg (0.17 mmol) of Fmoc-protected valerate **17**,[20] 64 mg (0.17 mmol) of HBTU, 22 mg (0.16 mmol) of HOBt, and 58 μL (43 mg, 0.33 mmol) of Hunig's base in 2 mL of DMF. The reaction mixture was stirred for 30 min, then the solvent was removed, and the resin was washed successively with three 30-mL portions of DMF, three 30-mL portions of CH_2Cl_2, and three 30-mL portions of methanol. An aliquot was removed; a qualitative Kaiser free-amine test showed the absence of free amine. An aliquot was cleaved from the resin with a solution containing 2% hydrazine in DMF. After concentration, the residue was dissolved in CF_3COOH and the solution was treated with ether to effect precipitation of the cleaved product. ESI mass spectrometry indicated a molecular ion at *m*/*z* 881.21, consistent with the presence of "tetrapeptide" **18**. An aliquot was removed for Fmoc cleavage analysis and revealed a loading of 0.176 mmol/g (corresponding to a 93% yield from the resin-bound tripeptide **16**, calculated on the basis of a theoretical loading of 0.189 mmol/g). The resin was dried under vacuum.

N_α-Fmoc-N^{im}-trityl-(*S*)-erythro-β-hydroxyhistidine (19). To a solution of 0.75 g (1.4 mmol) of (2′*S*,3′*R*,4*R*)-3-[2′-azido-3′-hydroxy-3′-(*N*-(triphenylmethyl)imidazol-4″-yl)propanoyl]-4-isopropyl-2-oxazolidinone[25] in 60 mL of a 4:1 THF−H_2O mixture at 25 °C was added 0.28 g (6.8 mmol) of LiOH·H_2O. The reaction mixture was stirred for 40 min and then quenched by the addition of 15 mL of 1 N HCl to pH ≈2.5. The acidic mixture was extracted with three 25-mL portions of EtOAc and two 30-mL portions of $CHCl_3$. The combined organic extract was washed with two 25-mL portions of brine, and the excess solvent was removed under diminished pressure to give a colorless foam. The residue was dissolved in 50 mL of a 9:1 THF−H_2O mixture, to which was carefully added 45 mg of 10% Pd on carbon, 0.56 g (4.1 mmol) of K_2CO_3, and 1.36 g (4.05 mmol) of FmocOSu. The reaction mixture was carefully vacuum purged five times with H_2 and then stirred under an H_2 atmosphere for 16 h. The suspension was filtered through a pad of Celite to remove the catalyst, and then the Celite pad was rinsed thoroughly with 100 mL of a 90:8:2 $CHCl_3$−MeOH−AcOH solution, followed by 25 mL of toluene. The excess solvent was carefully removed under diminished pressure and the remaining residue was coevaporated with two additional 25-mL portions of toluene. The crude product was an off-white foam. The crude product was purified by flash chromatography on a silica gel column (35 × 5 cm). Elution with 90:8:2 $CHCl_3$−MeOH−AcOH gave the product in a semipure form; this was purified further by flash chromatography on a silica gel column (15 × 2 cm). Elution with 80:20:0.1 EtOAc−hexanes−acetic acid provided the product as a colorless oil. The product was precipitated from EtOAc and petroleum ether to give a colorless powder: yield 0.56 g (63%); mp 133 °C (dec); silica gel TLC R_f 0.24 (90:8:2 $CHCl_3$−MeOH−AcOH); $[\alpha]^{22}_D$ +35.7° ± 1.2° (*c* 1.03, $CHCl_3$); ^{1}H NMR ($(CD_3)_2SO$) δ 4.19 (m, 2H), 4.33 (m, 1H), 4.57 (d, 1H, J = 5.2 Hz), 5.30 (s, 1H), 6.30 (s, 1H), 6.96 (s, 1H), 7.08 (m, 6H), 7.31 (m, 14H), 7.58 (d, 2H, J = 7.0 Hz), and 7.75 (d, 2H, J = 7.8 Hz); ^{13}C NMR ($CDCl_3$) δ 46.67, 60.38, 67.04, 67.17, 119.56, 127.71, 124.91, 125.01, 126.78, 127.35, 128.08, 128.29, 129.28, 136.75, 137.59, 140.56, 140.84, 143.32, 143.51, 156.68, and 171.31; MS (FAB), *m*/*z* 636.2474 $(M + H)^+$ ($C_{40}H_{34}N_3O_5$ requires 636.2498).

Resin-Bound Pentapeptide 20. To 260 mg (0.176 mmol/g) of swollen tetrapeptide resin **18** was added 3 mL of a 20% solution of piperidine in DMF. The reaction mixture was shaken for 10 min, then the solvent was removed by filtration, and the resin was washed with 10 mL of a 20% piperidine solution. The piperidine treatment and subsequent washing procedure was repeated three times, and the resin was washed with three 30-mL portions of DMF, three 30-mL portions of CH_2Cl_2, and three additional 30-mL portions of DMF. An aliquot was removed; a qualitative Kaiser free-amine test showed the presence of free amine. The resin was suspended in a solution containing 87 mg (0.14 mmol) of Fmoc-protected β-hydroxyhistidine (**19**), 52 mg (0.14 mmol) of HATU, 20 mg (0.15 mmol) of HOAt, and 48 μL (36 mg, 0.28 mmol) of Hunig's base in 2 mL of DMF. The reaction mixture was stirred for 30 min, then the solvent was removed, and the resin was washed successively with three 30-mL portions of DMF, three 30-mL portions of CH_2Cl_2, and three 30-mL portions of methanol. An aliquot was removed; a qualitative Kaiser free-amine test showed the absence of free amine. An aliquot was cleaved from the resin with a solution containing 2% hydrazine in DMF. After concentration, the residue was dissolved in CF_3COOH and the solution was treated with ether to effect precipitation of the cleaved, detritylated product. ESI mass spectrometry showed the presence of a molecular ion at *m*/*z* 1034.5, consistent with the presence of "pentapeptide" **20**. An aliquot was removed for Fmoc cleavage analysis and revealed a loading of 0.127 mmol/g (corresponding to a 81% yield from the tetrapeptide **18**, calculated on the basis of a theoretical loading of 0.156 mmol/g). The resin was dried under vacuum.

Trithiazole DeglycoBLM 3. To 100 mg (0.127 mmol/g) of swollen pentapeptide resin **20** was added 3 mL of a 20% solution of piperidine in DMF. The reaction mixture was shaken for 10 min, then the solvent was removed, and the resin was washed with 10 mL of 20% aqueous piperidine in DMF. The piperidine treatment and subsequent washing procedure was repeated three times and the resin was washed with three 30-mL portions of DMF, three 30-mL portions of CH_2Cl_2, and again with three 30-mL portions of DMF. An aliquot was removed; a qualitative Kaiser free-amine test showed the presence of free amine. The resin was suspended in solution of 8 mg (0.019 mmol) of Boc pyrimidoblamic acid (**21**),[23] 22 mg (0.05 mmol) of BOP reagent, and

13 μL (10 mg, 0.07 mmol) of Hunig's base in 1 mL of DMF at 0 °C. The reaction mixture was stirred for 12 h, then the solvent was removed by filtration, and the resin was washed successively with three 30-mL portions of DMF, three 30-mL portions of CH_2Cl_2, and three 30-mL portions of methanol. An aliquot was removed; a qualitative Kaiser free-amine test showed the absence of free amine. An aliquot was cleaved from the resin with a solution containing 2% hydrazine in DMF. After concentration, the residue was dissolved in CF_3COOH and the solution was treated with ether to effect precipitation of the cleaved product lacking trityl and Boc protecting groups. ESI mass spectrometry indicated a molecular ion having *m*/*z* 1341.5, consistent with the presence of trithiazole deglycoBLM **22**. To 80.0 mg of swollen resin was added 0.1 mL of triisopropylsilane, 0.1 mL of dimethyl sulfide, and 0.1 mL of water. The reaction mixture was shaken for 30 s and 1 mL of CF_3COOH was added. The reaction mixture was shaken for 2 h, then the solvent was removed by filtration, and the resin was washed successively with three 30-mL portions of DMF, three 30-mL portions of CH_2Cl_2, and three additional 30-mL portions of DMF. The resin was suspended in 1.5 mL of a 1 M solution of sodium benzenethiolate in DMF. The solution was shaken for 30 min and the solvent was then removed by filtration The treatment with sodium benzenethiolate was repeated, then the solvent was filtered, and the resin was washed successively with three 30-mL portions of DMF, three 30-mL portions of CH_2Cl_2, and another three 30-mL portions of DMF. The product was cleaved from resin by treatment with a 2% hydrazine solution in DMF; the crude ultraviolet spectrum had an absorption at 300 nm; the ratio of A_{240}/A_{300} indicated that the final coupling had proceeded in good yield. ^{1}H NMR of the crude sample containing a known concentration of *tert*-butyl alcohol as an internal standard indicated that the coupling of Boc pyrimidoblamic and proceeded in 70% yield (as judged by integration of the methyl resonance in pyrimidoblamic acid vs those in *tert*-butyl alcohol). The sample was lyophilized, yielding a colorless, solid product. Purification was achieved by C_{18} reversed-phase HPLC using a linear gradient of 0.1% CF_3COOH containing increasing amounts of CH_3CN (0 → 25 min, linear gradient from 13% → 22% CH_3CN at a flow rate of 4 mL/min: t_R 14.8 min); this gave trithiazole deglycoBLM analogue **3** as a colorless solid following lyophilization (yield 4.9 mg, 41.7% overall for the pyrimidoblamic acid coupling, deprotection and cleavage from the resin): ^{1}H NMR (D_2O) δ 0.99−1.04 (m, 6H), 1.07 (d, 3H, J = 6.8 Hz), 1.61−1.71 (m, 4H), 1.86 (s, 3H), 1.95 (m, 2H), 2.52 (m, 1H), 2.64−2.66 (m, 2H), 2.93−3.09 (m, 8H), 3.17−3.25 (m, 2H), 3.41−3.47 (m, 2H), 3.52−3.59 (m, 2H), 3.65−3.68 (m, 1H), 3.78−3.81 (m, 1H), 3.96−4.15 (m, 4H), 4.76 (d, 1H, J = 7.9 Hz), 5.13 (d, 1H, J = 7.5 Hz), 7.37 (s, 1H), 8.01 (s, 1H), 8.10 (s, 1H), 8.14 (s, 1H) and 8.63 (s, 1H); MS (electrospray), *m*/*z* 1156.5 (M^+) ($C_{47}H_{69}N_{19}O_{10}S_3$ requires 1156.4).

Relaxation of Supercoiled DNA by a Trithiazole-Containing Deglycobleomycin Analogue. Reactions were carried out in 25 μL (total volume) of 10 mM sodium cacodylate buffer, pH 7.0, containing 300 ng (4.32 nM plasmid concentration, 18.8 μM base pair concentration) of pBR322 plasmid DNA and the appropriate concentrations of BLM and Fe^{2+}. 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 (15 × 9 × 0.5 cm) 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 V 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 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 autoradiography 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 a Trithiazole-Containing Bleomycin Analogue. 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, 0.1 nM duplex concentration) and the appropriate concentrations of BLM and Fe^{2+}. Each reaction was initiated by the simultaneous addition of solutions of BLM and Fe^{2+} to the buffered solution containing DNA. The reaction mixture was 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 (31 cm × 38.5 cm × 0.4 mm, containing 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.[31]

Cleavage of a 53-Nucleotide 5′-^{32}P End-Labeled RNA by a Trithiazole-Containing Bleomycin Analogue. The 53-nucleotide RNA was 5′-^{32}P end-labeled essentially as described.[32] 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, 0.32 nM concentration) and the appropriate concentrations of reagents as indicated in the legend to Figure 6. Each reaction was initiated by the simultaneous addition of solutions of BLM and Fe^{2+} to the buffered solution containing RNA. Each reaction mixture was incubated at 23 °C for 30 min, and then the reaction mixture was frozen in dry ice, lyophilized, and dissolved in a gel loading solution [80% formamide, 2 mM EDTA, 0.05% (w/v) xylene cyanol, and 0.05% (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 (31 cm × 38.5 cm × 0.4 mm). Electrophoresis was carried out at 45 W for 2 h. The gel was analyzed by the use of a Molecular Dynamics Phosphorimager.

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