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Deglycobleomycin A_6 analogues modified in the methylvalerate moiety

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ABSTRACT

Previous studies have indicated that the methylvalerate subunit of bleomycin (BLM) plays an important role in facilitating DNA cleavage by BLM and deglycoBLM. Eleven methylvalerate analogues have been synthesized and incorporated into deglycoBLM congeners by the use of solid-phase synthesis. The effect of the valerate moiety in the deglycoBLM analogues has been studied by comparison with the parent deglycoBLM A_5 using supercoiled DNA relaxation and sequence-selective DNA cleavage assays. All of the deglycoBLM analogues were found to effect the relaxation of the plasmid DNA. Those analogues having aromatic C4-substituents exhibited cleavage efficiency comparable to that of deglycoBLM A_5. Some, but not all, of the deglycoBLM analogues were also capable of mediating sequence-selective DNA cleavage.

1. Introduction

The bleomycins (BLMs) are glycopeptide antibiotics that have been used clinically to treat several types of cancers, notably squamous cell carcinomas, malignant lymphomas and testicular cancers.[1–3] The antitumor activity of BLM is thought to be related to its degradation of DNA,[4–12] and possibly RNA,[13–19] in the presence of a metal ion and oxygen. BLM, exemplified by bleomycin A_5 and bleomycin A_6 in Figure 1, has four structural domains. The C-terminal domain plays an important role in DNA binding.[20,21] The metal binding domain is responsible for the chelation of a metal ion cofactor and oxygen activation[22,23] and is likely also involved in the sequence selectivity of DNA cleavage.[24,25] The carbohydrate moiety participates in the metal ion binding,[26,27] and also in cell surface recognition[28] and the cellular effects of the drug.[29,30] The linker region, which consists of threonine and methylvalerate organizes the metal binding domain and the DNA binding domain to facilitate productive DNA binding and thereby enable cleavage.[24] This region is of great importance because of this role.[24,31–39] This unique β-hydroxy-γ-amino acid is believed to orient BLM into a rigid and compact conformation essential for DNA interaction.[40] Methyl substitutions at the C2- and C4-positions were suggested to stabilize the compact conformation to allow for efficient DNA interaction[39] while alteration of that conformation can greatly diminish DNA cleavage.[41] Additionally, it has been found that increased steric bulk at the methylvalerate C4-position can alter the efficiency and pattern of DNA cleavage.[42,43]

Deglycobleomycin (Fig. 2), lacking the carbohydrate moiety, exhibits DNA cleavage properties very similar to those of bleomycin, including cleavage potency and selectivity.[40,44,45] Thus, this BLM analogue, which is more readily accessible synthetically than BLM, might facilitate the understanding of the mechanism by which BLM cleaves DNA, potentially leading to the development of improved antitumor agents. Recently, we have constructed a 108-membered deglycoBLM A_6 library on a solid support. Two members of this library, both of which contained phenylmethylvalerate moieties, displayed potencies of supercoiled DNA relaxation greater than that of the parent deglycobleomycin.[42,43] To further investigate the effect of steric hindrance within the valerate moiety on the DNA cleavage efficiency of (deglyco) BLM, we designed a series of valerate analogues to probe the effects of steric bulk at the C2- as well as C4-position, possibly allowing deglycoBLM to have better DNA cleavage efficiency. Herein, we describe the synthesis of these valerate analogues (**2e**–**12e**, Fig. 3). These analogues were incorporated into deglycoBLM using solid-phase synthesis, affording 11 deglycoBLM A_6 analogues (**2**–**12**, Figs. 4–6). These analogues were evaluated for DNA cleavage efficiency and selectivity compared to deglycoBLM itself.

2. Results

2.1. Analogues of methylvalerate to be incorporated into deglycobleomycin A_6

The analogues of interest (Fig. 3) involve modifications that introduce steric bulk at either the C2- or C4-position, or both of these positions. Compared to the native valerate moiety (**1e**) of

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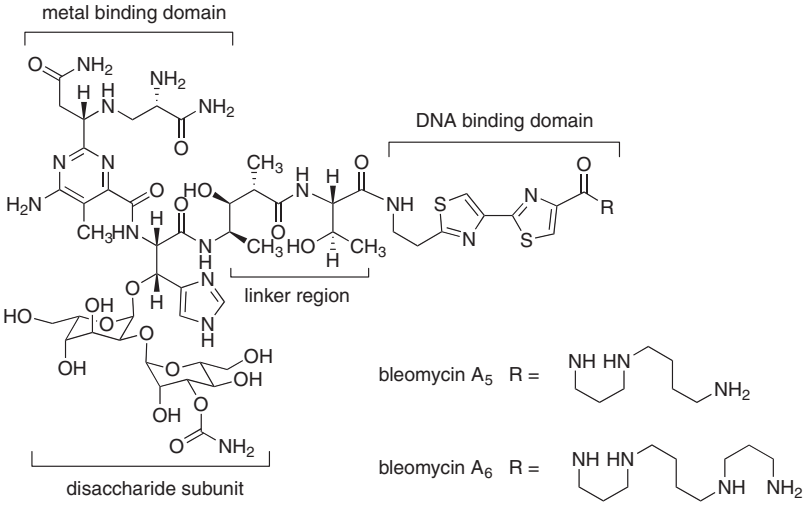


Figure 1. Structures and functional domains of bleomycins A_5 and A_6.

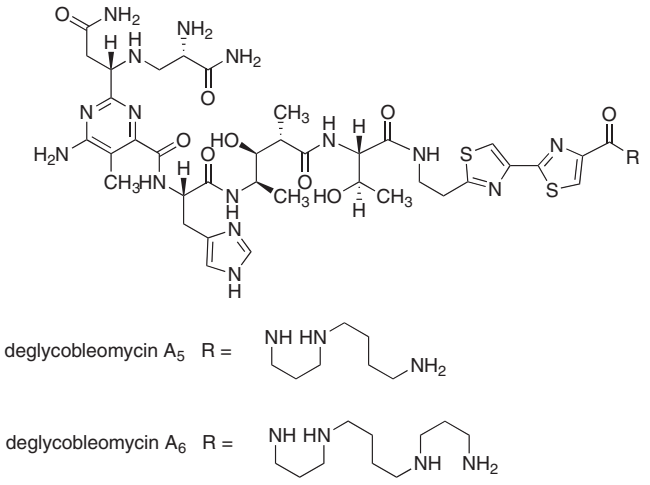


Figure 2. Structures of deglycobleomycins A_5 and A_6.

bleomycin, analogues **2e**–**4e** involve the replacement of the methyl group at the C4-position with isobutyl, *sec*-butyl, and *tert*-butyl groups, respectively. Analogue **2e** has already been prepared and incorporated into deglycoBLM A_6 previously; the resulting deglycoBLM analogue exhibited a potency of relaxation of supercoiled plasmid DNA comparable to that of deglycobleomycin A_6 itself.[42] Analogues **3e** and **4e** were chosen in the hope that steric alterations to the butyl group would affect the cleavage properties of BLM. Analogues **5e**–**8e** involved modification at the C4-position with various cyclic substituents. The incorporation of **6e** has been already reported, and shown to improve the DNA cleavage potency of deglycobleomycin.[42] Based on the structure of **6e**, the phenyl group of **6e** was replaced with a cyclohexyl group to afford **5e**. A potential enhancement of the steric effects of the phenyl group in **6e** was envisioned in analogues **7e** and **8e**, containing additional bromo and methyl groups, respectively, at the *ortho*-position of the phenyl ring of **6e**. The focus was to probe the limit of effects of steric bulk at the C4-position of the methylvalerate moiety. In addition, another three analogues varied at the C2-position (**9e**–**11e**) were prepared to probe the effect of steric bulk at this position, which has been less studied. Finally, a methylvalerate analogue (**12e**) having isobutyl groups at both the C2- and C4-positions was synthesized. This particular analogue provided a deglycoBLM analogue with additional steric bulk for biochemical evaluation.

2.2. Synthesis of Fmoc-methylvalerate moiety

As shown in Scheme 1, the requisite Boc-protected amino acids were converted to their corresponding Weinreb amides.[46,47] Compounds **1a**–**8a** were reduced to their Boc-protected aldehydes **1b**–**8b** by treatment with lithium aluminum hydride, and the crude products were used for the next step without further purification. The aminoaldehydes were condensed with the appropriate Z-boron enolate[31,48] of chiral acyloxazolidinones **13**–**16** to afford aldol condensation products **1c**–**12c** in yields that varied from 48% to 88%. This step demonstrates a particularly effective method for controlling the relative and absolute stereochemistry. The chiral acyloxazolidinones **13**,[42] **14**[49] and **15**[50] were prepared in a single step from the corresponding acyl chlorides and lithiated oxazolidinone, as outlined in Scheme 2. For compound **16**, the N-acylation of oxazolidinone was achieved by using 4-methylvaleric acid activated with pivaloyl chloride.[51] The aldol products were treated with lithium peroxide in 3:1 THF–H_2O at 0 °C.[32,48] Replacement of Boc protecting group with the Fmoc group provided the desired amino acid derivatives **1e**–**12e** in yields ranging from 14% to 95%.

2.3. Solid-phase synthesis of deglycoBLM analogues 2–12

The solid-phase synthesis of deglycoBLM was first reported by our laboratory and represents an efficient method for preparing deglycoBLM[42,52,53] and BLM[17] analogues. In the present case, solid-phase synthesis was used to prepare deglycoBLM analogues **2**–**12**, as outlined in Scheme 3. The synthesis was carried out using a TentaGel amino functionalized resin (preloading of 0.48 mmol/g), followed by the attachment of a hydrazine-labile Dde linker developed by Bycroft et al.[54,55] The linker-bound resin was treated with di-Boc spermine in the presence of Hünig's base in DMF.[56] The amino group was coupled immediately with the Fmoc-protected bithiazole in the presence of HBTU and Hünig's base in DMF. After the resin was washed successively with DMF, CH_2Cl_2 and MeOH, an aliquot of the resin was assayed with the Kaiser test to confirm the absence of free amino groups.[57] The coupling was quantified by Fmoc-cleavage with a solution of 20% piperidine in DMF, which indicated a loading of 0.12 mmol/g, corresponding to a 25% yield for the production of the dipeptide from the initial TentaGel resin.[58] After the Fmoc group was removed from the dipeptide using a solution containing 20% piperidine in DMF, commercially available Fmoc-threonine was coupled to the free amine by the use of HBTU, HOBt and Hünig's base in DMF. Fmoc analysis of the tripeptide indicated that the coupling proceeded in 83% yield. Synthesis

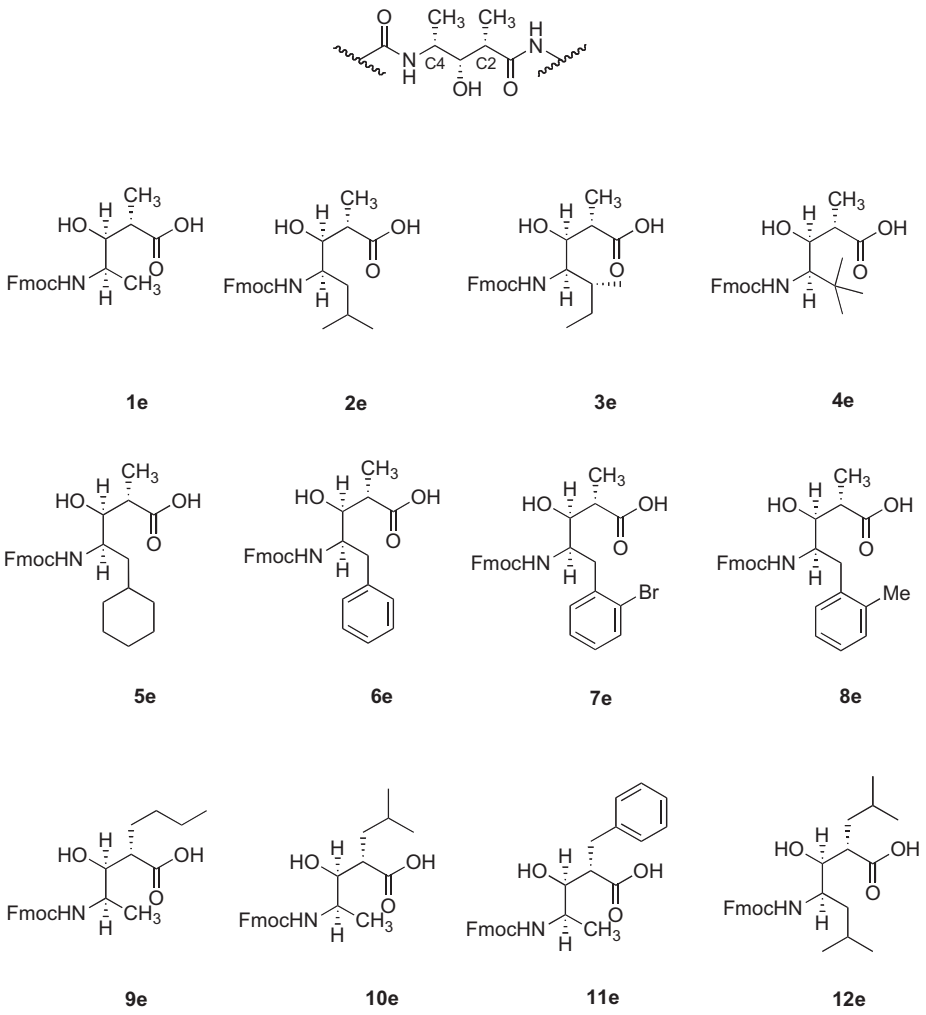
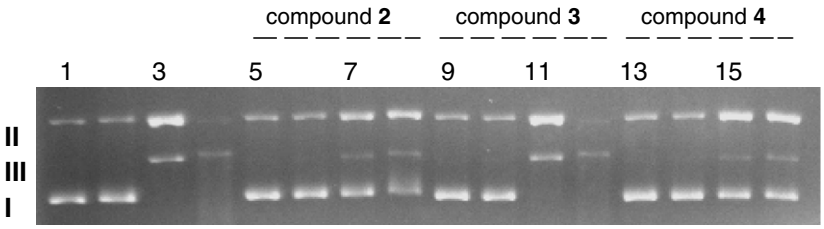


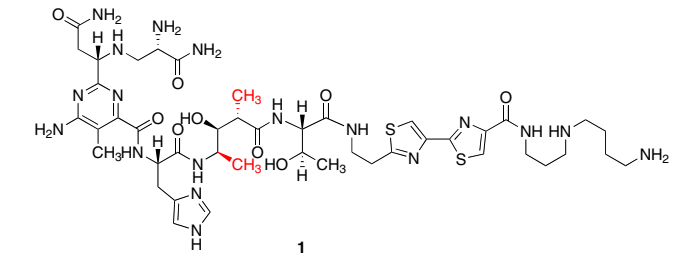
Figure 3. Fmoc-methylvalerate (**1e**) and the Fmoc-methylvalerate analogues (**2e**–**12e**) synthesized for incorporation into deslycobleomycin congeners.

of the tetrapeptide was accomplished by the removal of the Fmoc group from the resin-bound tripeptide and subsequent coupling with valerate analogues **2e**–**12e** (Fig. 3), respectively, in the presence of HBTU, HOBt and Hünig's base in DMF. Fmoc analysis indicated the couplings had proceeded in yields varying from 80% to 98%. The resulting resin was treated with piperidine solution followed by the commercial N^{α}-Fmoc-N^{im}-tritylhistidine using HATU, HOAt and Hünig's base,[59,60] providing the resin-bound pentapeptide in 78% yield. Before the final coupling of the Boc-pyrimidoblamic acid moiety, the structure of the pentapeptide was verified following cleavage of a small sample from the resin with 2% hydrazine in DMF. The resulting solution was concentrated, and analyzed by MALDI-MS. After the molecular weight of the pentapeptide was confirmed, Boc-pyrimidoblamic acid was coupled to the resin using BOP and Hünig's base at 0 °C in the absence of light. The fully protected deglycoBLM underwent the final deprotection of the Boc and trityl groups by the treatment of a 90:5:5 TFA–Me_2S–triisopropylsilane solution,[61] affording the fully functionalized resin-bound deglycobleomycin A_6 analogue. The cleavage of the final products from the resin was accomplished using a solution of 2% hydrazine in DMF. The crude deglycobleomycin analogues were purified by C_{18} reversed phase HPLC. The overall yields of the final coupling, deprotection and cleavage steps varied from 27% to 59%. The purified deglycobleomycin analogues were characterized by ^{1}H NMR spectroscopy and high resolution mass spectrometry.

2.4. Relaxation of supercoiled plasmid DNA by deglycoBLM analogues modified in the valerate moiety

Each deglycoBLM A_6 analogue was evaluated for its potency in relaxing supercoiled plasmid DNA in the presence of equimolar Fe^{2+} (Figs. 4–6). The results showed that all of the deglycoBLM analogues containing modified valerate moieties exhibited the ability to nick supercoiled DNA. Among these, the most potent were deglycoBLMs **3** and **8**, whose potencies of DNA relaxation were comparable to those of deglycoBLM itself (Figs. 4 and 5). Also exhibiting quite strong relaxation of supercoiled plasmid DNA were deglycoBLMs **5**, **6**, **7**, **9**, **10**, **11** and **12**, all of which completely converted the supercoiled DNA substrate to nicked and linear forms when employed at 4 μM concentrations in the presence of equimolar Fe^{2+}. DeglycoBLM analogues **2** and **4** displayed moderate activity in effecting relaxation of supercoiled DNA. It is interesting that some of the analogues (notably **6**, **7**, **8** and **11**) displayed quite efficient plasmid DNA relaxation when employed at 1 μM concentration in the presence of equimolar Fe^{2+}. Comparison of the results obtained for Fe(II)·deglycoBLM A_5 (**1**) in three assays (Figs. 4–6) underscores the intrinsic inter assay variation in the supercoiled plasmid DNA relaxation assay. Thus the failure of analogue **6** to exhibit a greater efficiency of DNA relaxation than **1**, as noted previously,[42] is not entirely surprising. However, the greater efficiency of DNA relaxation upon introduction of ortho substituents into the aromatic ring is notable (cf. **6** vs **7** and **8** in Fig. 5), as is

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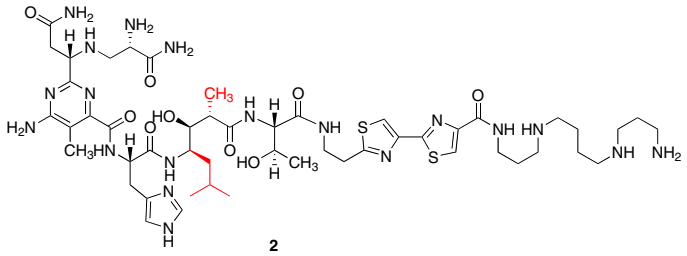
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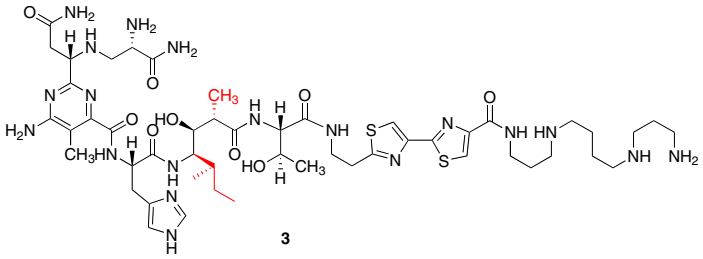
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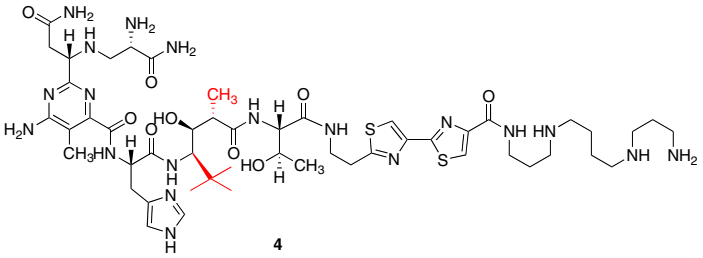
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Figure 4. Relaxation of supercoiled pBR322 plasmid DNA by Fe(II)·deglycoBLM A_6 analogues **2**–**4**. Lane 1, DNA alone; lane 2, 4 μM deglycoBLM A_5 (**1**); lane 3, 2 μM Fe(II)·deglycoBLM A_5 (**1**); lane 4, 4 μM Fe(II)·deglycoBLM A_5 (**1**); lane 5, 4 μM deglycoBLM **2**; lane 6, 1 μM Fe(II)·deglycoBLM **2**; lane 7, 2 μM Fe(II)·deglycoBLM **2**; lane 8, 4 μM Fe(II)·deglycoBLM **2**; lane 9, 4 μM deglycoBLM **3**; lane 10, 1 μM Fe(II)·deglycoBLM **3**; lane 11, 2 μM Fe(II)·deglycoBLM **3**; lane 12, 4 μM Fe(II)·deglycoBLM **3**; lane 13, 4 μM deglycoBLM **4**; lane 14, 1 μM Fe(II)·deglycoBLM **4**; lane 15, 2 μM Fe(II)·deglycoBLM **4**; lane 16, 4 μM Fe(II)·deglycoBLM **4**.

the efficiency of these analogues in DNA relaxation at 1 μM concentration.

2.5. Sequence-selective DNA cleavage

The deglycoBLM analogues were tested for their ability to effect the sequence-selective cleavage of a linear duplex DNA. This assay was carried out initially using a 5′-^{32}P-end labeled 158-base pair DNA restriction fragment as a substrate (Supplementary data, Fig. S1). As shown in Figure 7, Fe(II)·deglycoBLM A_5 effected sequence-selective cleavage of the DNA substrate at several sites. The strongest cleavage sites at the 5′-end of the labeled strand included two 5′-GC-3′ sites (5′-GC_{11}-3′ and 5′-GC_{35}-3′) and three 5′-GT-3′ sites (5′-GT_{14}-3′, 5′-GT_{17}-3′ and 5′-GT_{27}-3′). These sites were cleaved to some extent by deglycoBLM analogues **2**–**4**. Analogue **3**, which was the most potent of these in the relaxation of supercoiled plasmid DNA, also showed the strongest cleavage of the linear duplex DNA. Additionally, treatment of the DNA substrate with Fe(II)·deglycoBLM analogue **3** effected cleavage at two new sites not apparent with Fe(II)·deglycoBLM A_5 or any of the other analogues in the present study (cf. Fig. 7, lanes 11–13). Specifically, deglycoBLM **3** induced unique cleavage at G_{12} and A_{15} of the

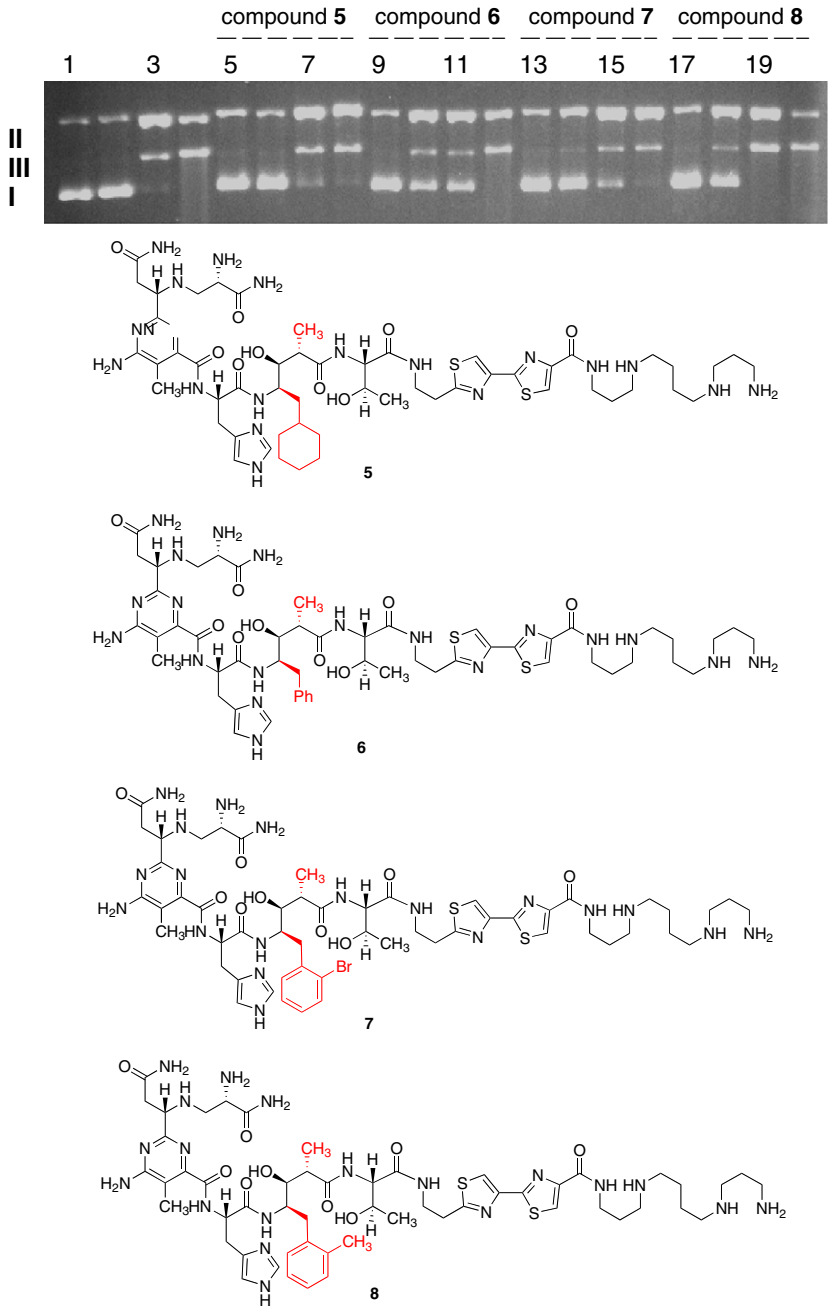


Figure 5. Relaxation of supercoiled pBR322 plasmid DNA by Fe(II)·deglycoBLM A_6 analogues **5**–**8**. Lane 1, DNA alone; lane 2, 4 μM deglycoBLM A_5 (**1**); lane 3, 2 μM Fe(II)·deglycoBLM A_5 (**1**); lane 4, 4 μM Fe(II)·deglycoBLM A_5 (**1**); lane 5, 4 μM deglycoBLM **5**; lane 6, 1 μM Fe(II)·deglycoBLM **5**; lane 7, 2 μM Fe(II)·deglycoBLM **5**; lane 8, 4 μM Fe(II)·deglycoBLM **5**; lane 9, 4 μM deglycoBLM **6**; lane 10, 1 μM Fe(II)·deglycoBLM **6**; lane 11, 2 μM Fe(II)·deglycoBLM **6**; lane 12, 4 μM Fe(II)·deglycoBLM **6**; lane 13, 4 μM deglycoBLM **7**; lane 14, 1 μM Fe(II)·deglycoBLM **7**; lane 15, 2 μM Fe(II)·deglycoBLM **7**; lane 16, 4 μM Fe(II)·deglycoBLM **7**; lane 17, 4 μM deglycoBLM **8**; lane 18, 1 μM Fe(II)·deglycoBLM **8**; lane 19, 2 μM Fe(II)·deglycoBLM **8**; lane 20, 4 μM Fe(II)·deglycoBLM **8**.

duplex DNA. Fe(II)·DeglycoBLM analogues **5**–**8** all produced some cleavage of the linear duplex DNA, albeit to a lesser extent than deglycoBLM **3** (Supplementary data, Fig. S2). The same was true of Fe(II)·deglycoBLM analogues **9**–**12**, as illustrated in Figure S3 (Supplementary data). Significantly, none of these analogues effected cleavage at G_{12} or A_{15}.

Also studied was the cleavage of a 5′-^{32}P-end labeled hairpin DNA that had been identified on the basis of its strong binding to BLM A_5.[62–64] As shown in Figure 8, deglycoBLM analogue **3** cleaved this hairpin DNA with a sequence selectivity quite similar to that of deglycoBLM A_5, albeit not as strongly, while cleavage by analogues **2** and **4** was quite weak. The remaining analogues also cleaved this hairpin DNA rather weakly (data not shown).

3. Discussion

The solid-phase synthesis of BLM analogues[42,52,53] has been employed for the preparation of BLMs containing modifications in the carbohydrate moiety,[17] and for the elaboration of a combinatorial library of deglycoBLMs having 108 analogues which was prepared by substituting four of the five 'amino acid' constituents with structural variants.[42] In both cases, the synthesis of analogues was carried out in parallel series of reactions. Evaluation of the BLM analogues having modified carbohydrates revealed significant differences in their patterns of DNA cleavage and, especially, in their abilities to effect the oxidative degradation of an RNA substrate.[17,42,43] Analysis of the properties of the 108-membered

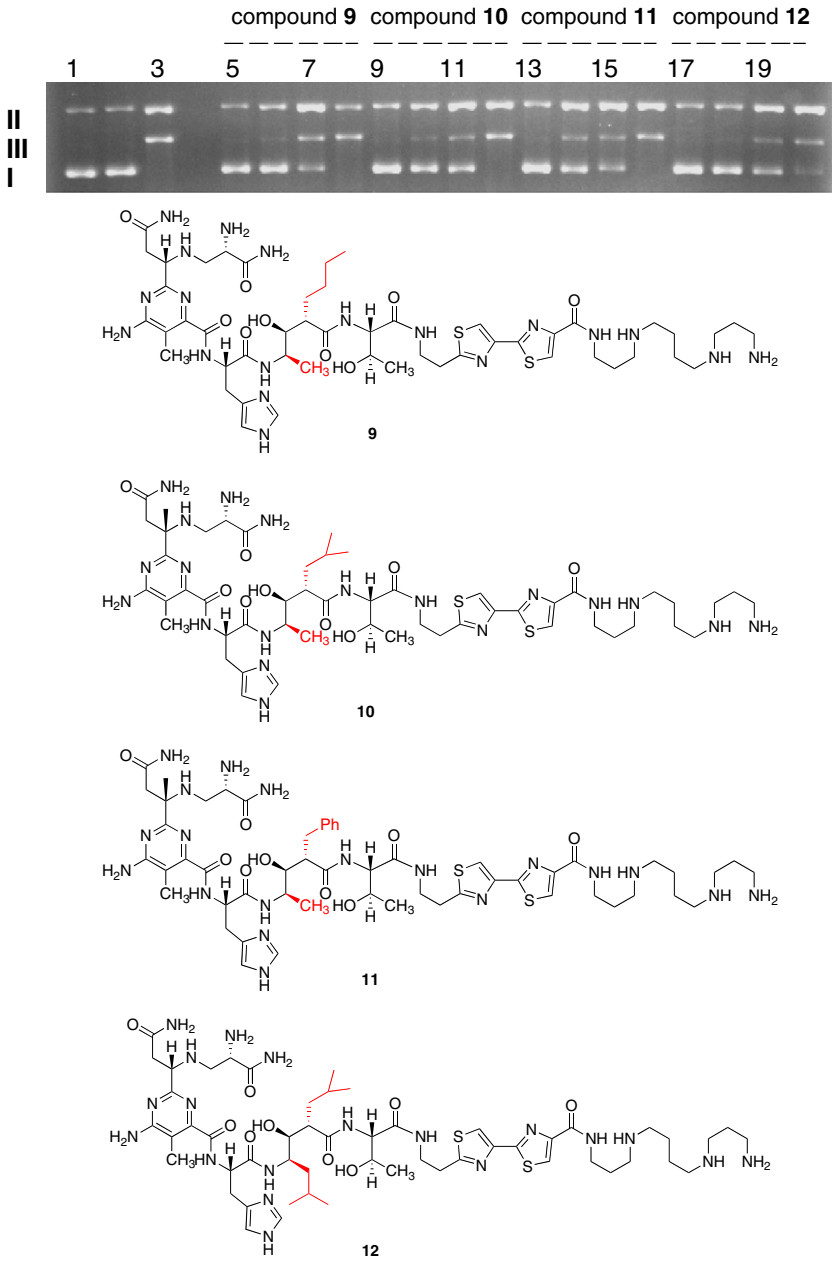
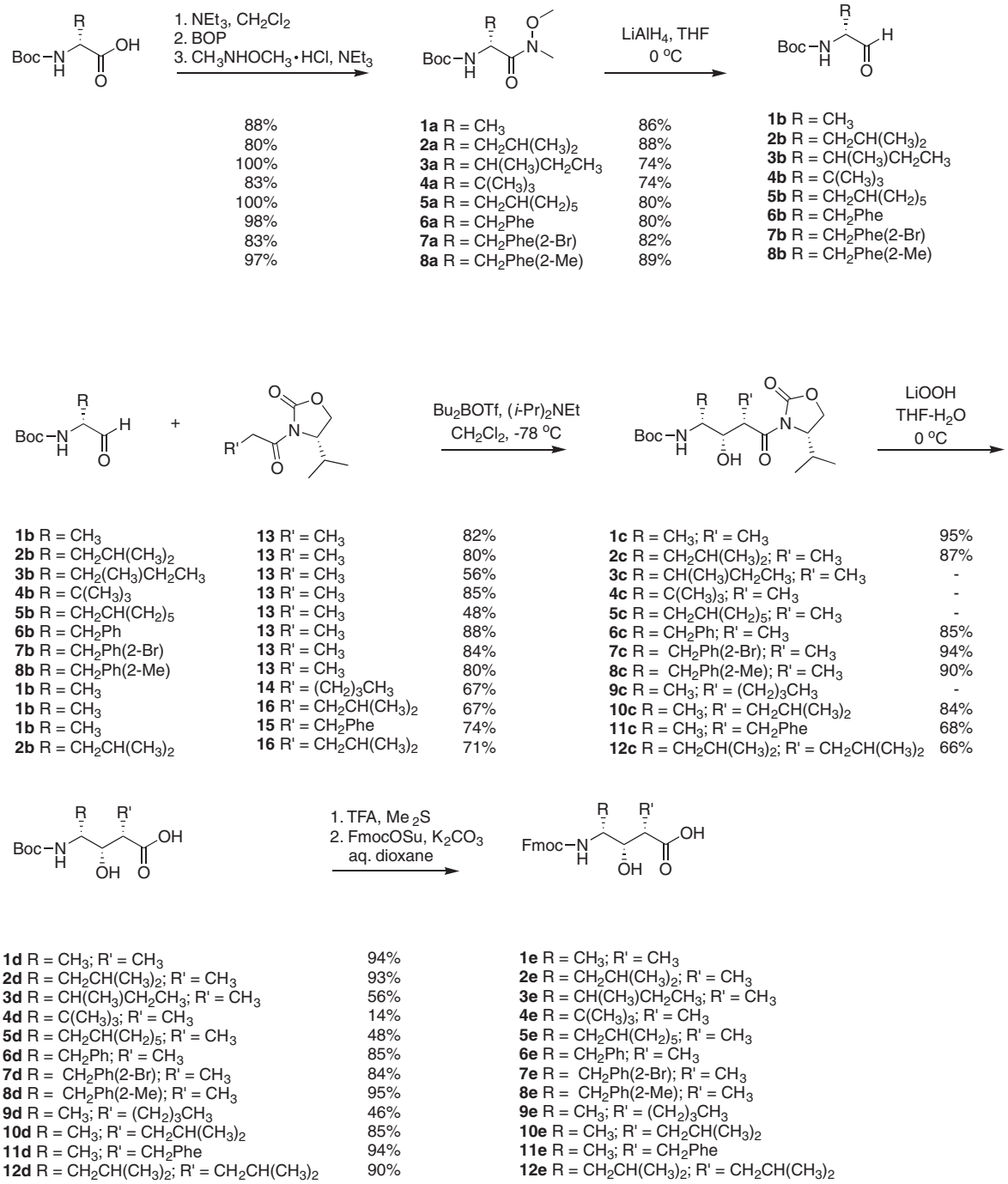


Figure 6. Relaxation of supercoiled pBR322 plasmid DNA by Fe(II)·deglycoBLM A_6 analogues **9**–**12**. Lane 1, DNA alone; lane 2, 4 μM deglycoBLM A_5 (**1**); lane 3, 2 μM Fe(II)·deglycoBLM A_5 (**1**); lane 4, 4 μM Fe(II)·deglycoBLM A_5 (**1**); lane 5, 4 μM deglycoBLM **9**; lane 6, 1 μM Fe(II)·deglycoBLM **9**; lane 7, 2 μM Fe(II)·deglycoBLM **9**; lane 8, 4 μM Fe(II)·deglycoBLM **9**; lane 9, 4 μM deglycoBLM **10**; lane 10, 1 μM Fe(II)·deglycoBLM **10**; lane 11, 2 μM Fe(II)·deglycoBLM **10**; lane 12, 4 μM Fe(II)·deglycoBLM **10**; lane 13, 4 μM deglycoBLM **11**; lane 14, 1 μM Fe(II)·deglycoBLM **11**; lane 15, 2 μM Fe(II)·deglycoBLM **11**; lane 16, 4 μM Fe(II)·deglycoBLM **11**; lane 17, 4 μM deglycoBLM **12**; lane 18, 1 μM Fe(II)·deglycoBLM **12**; lane 19, 2 μM Fe(II)·deglycoBLM **12**; lane 20, 4 μM Fe(II)·deglycoBLM **12**.

deglycoBLM library revealed some differences in the specific sites of DNA cleavage. Some of the analogues exhibited double-strand cleavage of supercoiled plasmid DNA with an efficiency at least comparable to that of the parent deglycoBLM and two analogues mediated relaxation of plasmid DNA to a greater extent than the parent deglycoBLM. A few deglycoBLM analogues were found to cleave a $tRNA_3^{Lys}$ transcript in the presence and absence of a metal ion cofactor.

One especially interesting facet of the combinatorial library, which became apparent during the biochemical evaluation, was that when replacement of a single amino acid building block with a structural analogue rendered the resulting (deglyco) BLM dysfunctional in DNA cleavage, no (combination of) variations in other amino acids in the prototype structure could restore function.[42,43] This was shown to be true both for the threonine and β-hydroxyhistidine moieties of (deglyco) BLM.[42,43]

The very interesting properties of the (deglyco) BLM analogues resulting from systematic structure alteration of one or more constituents of (deglyco) BLM suggests that larger combinatorial libraries might well afford BLM analogues with really unique properties. However, the finding that a single amino acid replacement leading to a dysfunctional (deglyco) BLM would likely render every BLM analogue containing that modified amino acid dysfunctional, argues for the need to assure that every modified amino acid building block employed in the combinatorial library can in principle support BLM analogue function. In the present study, 11 structural analogues of the methylvalerate moiety of BLM (**2e**–**12e**) (Fig. 3) were prepared as Fmoc derivatives (Scheme 1) and introduced



88%	**1a** R = CH_3	86%
80%	**2a** R = $CH_2CH(CH_3)_2$	88%
100%	**3a** R = $CH(CH_3)CH_2CH_3$	74%
83%	**4a** R = $C(CH_3)_3$	74%
100%	**5a** R = $CH_2CH(CH_2)_5$	80%
98%	**6a** R = CH_2Phe	80%
83%	**7a** R = $CH_2Phe(2\text{-}Br)$	82%
97%	**8a** R = $CH_2Phe(2\text{-}Me)$	89%

1b R = CH_3
2b R = $CH_2CH(CH_3)_2$
3b R = $CH(CH_3)CH_2CH_3$
4b R = $C(CH_3)_3$
5b R = $CH_2CH(CH_2)_5$
6b R = CH_2Phe
7b R = $CH_2Phe(2\text{-}Br)$
8b R = $CH_2Phe(2\text{-}Me)$

1b R = CH_3	**13** R' = CH_3	82%
2b R = $CH_2CH(CH_3)_2$	**13** R' = CH_3	80%
3b R = $CH_2(CH_3)CH_2CH_3$	**13** R' = CH_3	56%
4b R = $C(CH_3)_3$	**13** R' = CH_3	85%
5b R = $CH_2CH(CH_2)_5$	**13** R' = CH_3	48%
6b R = CH_2Ph	**13** R' = CH_3	88%
7b R = $CH_2Ph(2\text{-}Br)$	**13** R' = CH_3	84%
8b R = $CH_2Ph(2\text{-}Me)$	**13** R' = CH_3	80%
1b R = CH_3	**14** R' = $(CH_2)_3CH_3$	67%
1b R = CH_3	**16** R' = $CH_2CH(CH_3)_2$	67%
1b R = CH_3	**15** R' = CH_2Phe	74%
2b R = $CH_2CH(CH_3)_2$	**16** R' = $CH_2CH(CH_3)_2$	71%

1c R = CH_3; R' = CH_3	95%
2c R = $CH_2CH(CH_3)_2$; R' = CH_3	87%
3c R = $CH(CH_3)CH_2CH_3$; R' = CH_3	-
4c R = $C(CH_3)_3$; R' = CH_3	-
5c R = $CH_2CH(CH_2)_5$; R' = CH_3	-
6c R = CH_2Ph; R' = CH_3	85%
7c R = $CH_2Ph(2\text{-}Br)$; R' = CH_3	94%
8c R = $CH_2Ph(2\text{-}Me)$; R' = CH_3	90%
9c R = CH_3; R' = $(CH_2)_3CH_3$	-
10c R = CH_3; R' = $CH_2CH(CH_3)_2$	84%
11c R = CH_3; R' = CH_2Phe	68%
12c R = $CH_2CH(CH_3)_2$; R' = $CH_2CH(CH_3)_2$	66%

1d R = CH_3; R' = CH_3	94%
2d R = $CH_2CH(CH_3)_2$; R' = CH_3	93%
3d R = $CH(CH_3)CH_2CH_3$; R' = CH_3	56%
4d R = $C(CH_3)_3$; R' = CH_3	14%
5d R = $CH_2CH(CH_2)_5$; R' = CH_3	48%
6d R = CH_2Ph; R' = CH_3	85%
7d R = $CH_2Ph(2\text{-}Br)$; R' = CH_3	84%
8d R = $CH_2Ph(2\text{-}Me)$; R' = CH_3	95%
9d R = CH_3; R' = $(CH_2)_3CH_3$	46%
10d R = CH_3; R' = $CH_2CH(CH_3)_2$	85%
11d R = CH_3; R' = CH_2Phe	94%
12d R = $CH_2CH(CH_3)_2$; R' = $CH_2CH(CH_3)_2$	90%

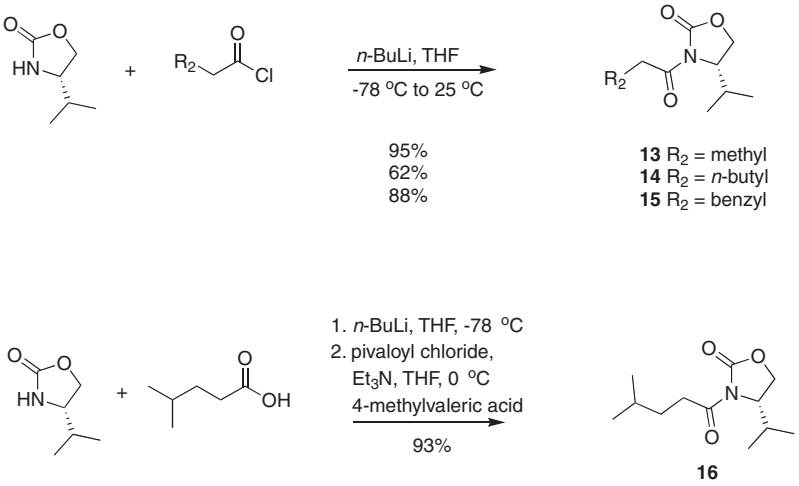
1e R = CH_3; R' = CH_3
2e R = $CH_2CH(CH_3)_2$; R' = CH_3
3e R = $CH(CH_3)CH_2CH_3$; R' = CH_3
4e R = $C(CH_3)_3$; R' = CH_3
5e R = $CH_2CH(CH_2)_5$; R' = CH_3
6e R = CH_2Ph; R' = CH_3
7e R = $CH_2Ph(2\text{-}Br)$; R' = CH_3
8e R = $CH_2Ph(2\text{-}Me)$; R' = CH_3
9e R = CH_3; R' = $(CH_2)_3CH_3$
10e R = CH_3; R' = $CH_2CH(CH_3)_2$
11e R = CH_3; R' = CH_2Phe
12e R = $CH_2CH(CH_3)_2$; R' = $CH_2CH(CH_3)_2$

Scheme 1.

as single modifications in each of 11 deglycoBLM analogues (Scheme 3). Each of the newly prepared deglycoBLM analogues was then compared with deglycoBLM A_5 in each of three types of DNA cleavage assays.

Shown in Figures 4–6 are the results of nicking of supercoiled pBR322 plasmid DNA. The introduction of a single nick at any site on the supercoiled circular (Form I) DNA results in a relaxed circular (Form II) DNA which migrates differently when analyzed by agarose gel electrophoresis. All of the deglycoBLM analogues retained their ability to nick the supercoiled plasmid DNA. The introduction of nicks on both strands of the plasmid DNA within relatively close proximity in DNA sequence produces linear duplex (Form III) DNA. While Form III DNA can result from the random accumulation of numerous single-strand nicks, BLM is known to produce actual double-strand DNA cleavage with reasonable efficiency.[8–10] This produces Form III DNA under conditions where the nicking of Form I DNA is incomplete.

As noted in Figures 4–6, deglycoBLM analogues **3** and **8** were the most potent in effecting the nicking of supercoiled plasmid DNA; both were at least as potent as deglycoBLM A_5 itself. In comparison, deglycoBLMs **2** and **4** were the least potent analogues in effecting DNA relaxation, with the remaining seven analogues (**5**, **6**, **7**, **9**, **10**, **11** and **12**) displaying intermediate potencies. Four of the deglycoBLM analogues (**6**, **7**, **8** and **11**) were more efficient at



Scheme 2.

nicking the plasmid DNA to afford Form II DNA when used at the lowest (1 μM) concentration tested, and analogues **6** and **11** also produced a significant amount of linear duplex (Form III) DNA when tested at 1 μM concentration in the presence of equimolar Fe^{2+} (Figs. 5 and 6), suggesting a greater propensity to produce double-strand DNA nicks.

At a structural level, certain observations can also be made about the effects of substitution patterns in the methylvalerate moiety on the DNA nicking efficiency of the resulting deglycoBLM analogues. For example, deglycoBLM analogues **2**–**4** are isomers, differing only in the nature of the butyl substituent at position 4 of the methylvalerate moiety. Nonetheless, deglycoBLM **3** was one of the two most potent analogues studied while **2** and **4** were the least potent. The deglycoBLMs having an aromatic substituent at position 2 or 4 of the methylvalerate moiety (**6**, **7**, **8** and **11**) all produced efficient conversion of Form I DNA to Form II DNA. DeglycoBLMs **6** and **11**, both of which had benzyl substituents within the methylvalerate moiety, produced the greatest amount of Form III DNA. These structural observations can help to guide the choice of additional methylvalerate analogues for preparation and evaluation. The present results do not establish the molecular basis for the change in DNA relaxation properties noted above. However, the strong correlation between the presence of aromatic substituents at position 2 or 4 of the methylvalerate moiety, and the observation of altered DNA cleavage properties suggests that the aromatic substituents may alter the nature of the interaction of these analogues with DNA. There is presently little published data on the dynamics of bleomycin–DNA interaction, and the lack of dramatic shifts of the DNA bands in Figures 4–6 argues against strongly enhanced binding of the analogues to DNA. Therefore, the most likely explanation for the altered binding is a qualitative difference in the orientation of the individual analogues on the bound DNA, leading to an alteration in the facility of DNA cleavage chemistry. Ongoing X-ray crystallographic studies[65] of bleomycin interaction with DNA oligonucleotides will no doubt provide data pertinent to this type of issue in due course.

The second DNA cleavage assay employed a 158-base pair 5′-^{32}P end-labeled DNA duplex that has been employed extensively for studies of DNA cleavage by BLM.[53,66] As shown in Figure 7, Fe(II)·deglycoBLM A_5 produced cleavage at a number of sites, including the expected 5′-GT-3′ and 5′-GC-3′ sites, as well as 5′-AT_9-3′ and 5′-AT_{21}-3′. All 11 deglycoBLMs effected DNA strand scission, producing cleavage bands at the same positions as deglycoBLM A_5. One congener, deglycoBLM **3**, produced additional new cleavage bands at 5′-CG_{12}-3′ and 5′-TA_{15}-3′ sequences. In common with the potency of these analogues at plasmid DNA relaxation, deglycoBLM **3** also produced strong cleavage of the 158-bp DNA duplex.

Recently, we have described a strategy for selecting 64-nucleotide hairpin DNAs that are bound strongly by BLM. The binding and cleavage of such strongly bound DNAs is of potential therapeutic relevance because the low clinical dose of BLM makes it likely that DNA is in large excess relative to BLM when the drug is used for antitumor therapy. Under such circumstances, only strongly bound sites on DNA should be occupied by BLM, and only a subset of these bound sites can be cleaved.

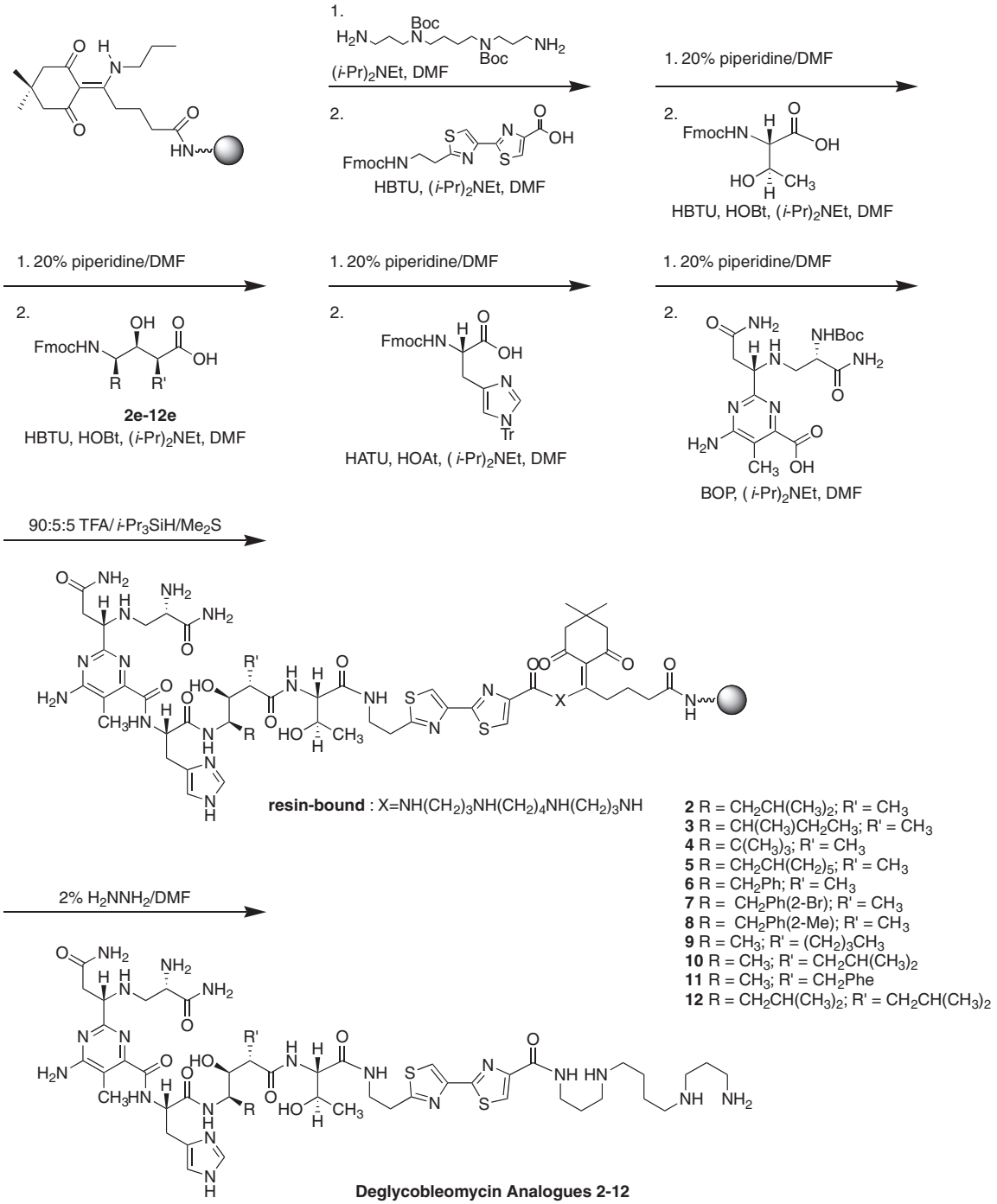
While the characterization of the cleavage of such DNAs by Fe·BLM is ongoing, the first two reports[63,64] make it clear that such strongly bound DNAs are cleaved at sites quite different than those of randomly chosen DNAs,[53,66] that alkali labile lesions[67] are more prominent, and that BLM analogues (such as deglycoBLM) exhibit cleavage patterns that are significantly different from those displayed by BLM itself.[63,64]

Accordingly, one of the strongly bound hairpin DNAs has been employed as a substrate for cleavage by deglycoBLMs **2**–**12**. As shown in Figure 8, deglycoBLM analogue **3**, which gave fairly strong cleavage of the 158-base pair DNA duplex, was also the most efficient at mediating cleavage of the 64-nucleotide hairpin DNA substrate shown to be bound and cleaved strongly by BLM A_5. Unlike the linear DNA duplex, which was cleaved by **3** at two new sites not apparent using deglycoBLM A_5, the pattern of cleavage of the hairpin DNA was the same for **3** and for deglycoBLM A_5.

4. Conclusions

Eleven deglycoBLM analogues, differing only within the methylvalerate moiety, have been prepared and characterized. Detailed assessments of their ability to cleave DNA have also been conducted using three different assays to determine the effect of methylvalerate substituents on the DNA cleavage properties. An evaluation of the deglycoBLM analogues revealed that modification either at the C2- or C4-position did not diminish the ability of deglycoBLM to relax supercoiled DNA significantly. Replacement of C4-methyl group of the methylvalerate with a *sec*-butyl group provide an analogue that exhibited new sequence-selective DNA cleavage properties.

Two of the analogues were also found to produce greater amounts of Form II DNA when employed at low concentration as Fe(II) chelates, suggesting more efficient double-strand cleavage.



2 R = $CH_2CH(CH_3)_2$; R' = CH_3
3 R = $CH(CH_3)CH_2CH_3$; R' = CH_3
4 R = $C(CH_3)_3$; R' = CH_3
5 R = $CH_2CH(CH_2)_5$; R' = CH_3
6 R = CH_2Ph; R' = CH_3
7 R = $CH_2Ph(2\text{-}Br)$; R' = CH_3
8 R = $CH_2Ph(2\text{-}Me)$; R' = CH_3
9 R = CH_3; R' = $(CH_2)_3CH_3$
10 R = CH_3; R' = $CH_2CH(CH_3)_2$
11 R = CH_3; R' = CH_2Phe
12 R = $CH_2CH(CH_3)_2$; R' = $CH_2CH(CH_3)_2$

Scheme 3.

5. Experimental section

5.1. Chemistry

5.1.1. General methods

Chemicals and solvents were of reagent grade and were used without further purification. All reactions involving air or mois ture-sensitive reagents or intermediates were performed under an argon atmosphere. Flash chromatography was carried out using Silicycle 200–400 mesh silica gel. Analytical TLC was carried out using 0.25 mm EM Silica Gel 60 F_{250} plates that were visualized by irradiation (254 nm) or by staining with *p*-anisaldehyde stain. HPLC separations were performed on a Waters 600 series HPLC multi-solvent delivery system using a Kratos 747 UV detector. The crude products were purified on an Alltech Alltima C_{18} reversed phase semi-preparative (250 × 10 mm, 5 μm) HPLC column using aq 0.1% TFA and CH_3CN mobile phases. A linear gradient was employed (100:0 0.1% aq TFA/CH_3CN→40:60 0.1% aq TFA/CH_3CN) over a period of 30 min at a flow rate of 4 mL/min. ^1H and ^{13}C NMR spectra were obtained using Inova 400, Inova 500 or VNMRS 800 MHz Varian instruments. Chemical shifts were reported in parts per million (ppm, δ) referenced to the residual ^1H resonance of the solvent ($CDCl_3$, 7.26 ppm). ^{13}C spectra were referenced to the residual ^{13}C resonance of the solvent ($CDCl_3$, 77.0 ppm). Splitting patterns were designated as follows: s, singlet; br, broad; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. High resolution mass

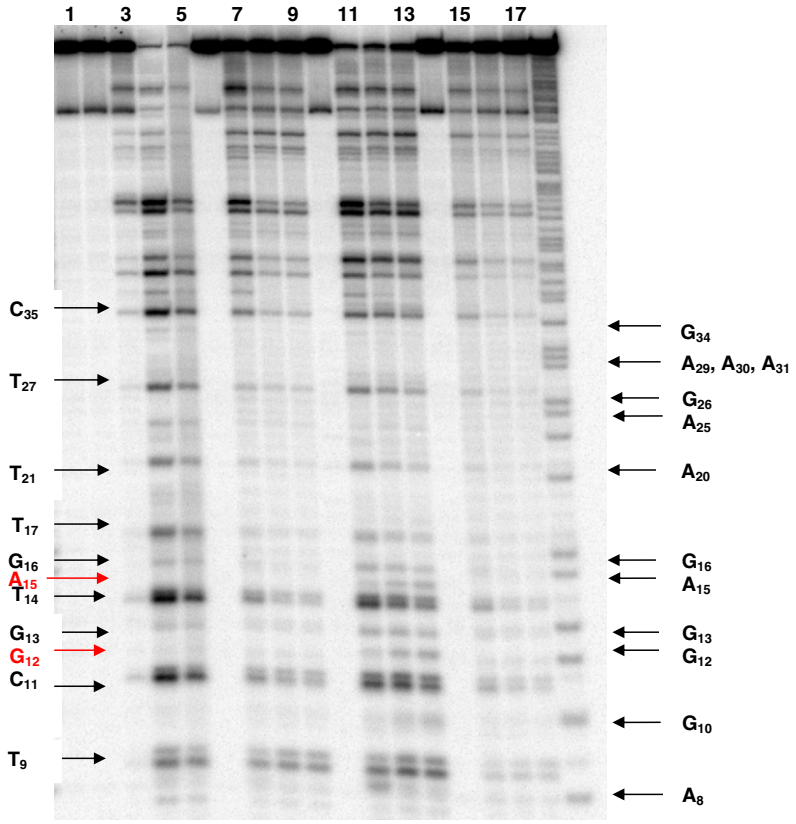


Figure 7. Cleavage of a [5′-^{32}P]-end labeled 158-base pair DNA duplex by deglycoBLM A_5 and deglycoBLM analogues **2**–**4**. Lane 1, DNA alone; lane 2, 10 μM deglycoBLM A_5 (**1**); lane 3, 1 μM Fe(II)·deglycoBLM A_5 (**1**); lane 4, 5 μM Fe(II)·deglycoBLM A_5 (**1**); lane 5, 10 μM Fe(II)·deglycoBLM A_5 (**1**); lane 6, 10 μM deglycoBLM **2**; lane 7, 1 μM Fe(II)·deglycoBLM **2**; lane 8, 5 μM Fe(II)·deglycoBLM **2**; lane 9, 10 μM Fe(II)·deglycoBLM **2**; lane 10, 10 μM deglycoBLM **3**; lane 11, 1 μM Fe(II)·deglycoBLM **3**; lane 12, 5 μM Fe(II)·deglycoBLM **3**; lane 13, 10 μM Fe(II)·deglycoBLM **3**; lane 14, 10 μM deglycoBLM **4**; lane 15, 1 μM Fe(II)·deglycoBLM **4**; lane 16, 5 μM Fe(II)·deglycoBLM **4**; lane 17, 10 μM Fe(II)·deglycoBLM **4**; lane 18, G + A lane.

spectra were obtained in the Arizona State University CLAS High Resolution Mass Spectrometry Laboratory.

5.1.2. Solid-phase synthesis of deglycobleomycins

5.1.2.1. General procedure for the attachment of bithiazole to the solid support. To a suspension containing 1.0 g (0.48 mmol/g) of NovaSyn TentaGel amino functionalized resin was added a solution containing 0.89 g (2.20 mmol) of Boc-protected spermine and 460 μL (2.64 mmol) of Hünig's base in 4 mL of DMF. After 24 h, the resin was filtered, and washed for 30 sec each with three 5-mL portions of DMF, three 5-mL portions of CH_2Cl_2, and then three 5-mL portions of DMF. A solution containing 0.63 g (1.32 mmol) of Fmoc-bithizaole,[42] 0.51 g (1.32 mmol) of HBTU, and 460 μL (2.64 mmol) of Hünig's base in 2 mL of DMF was added. After 30 min, the resin was filtered, and washed for 30 sec each with three 5-mL portions of DMF, three 5-mL portions of CH_2Cl_2, and three 5-mL portions of methanol. The resulting resin was dried under diminished pressure over KOH pellets. Quantitative Fmoc cleavage analysis[58] indicated a loading of 0.12 mmol/g (25% over three steps).

5.1.2.2. General procedure for the attachment of threonine to the resin-bound dipeptide. To a suspension containing 200 mg of bithiazole-functionalized resin was added, sequentially for 5 min each, three 4-mL solutions containing 20% piperidine in DMF. The resulting resin was washed for 30 sec each with three 5-mL portions of DMF, three 5-mL portions of CH_2Cl_2, and then three 5-mL portions of DMF. A solution containing 27.0 mg (0.079 mmol) of commercially available Fmoc-threonine, 29.7 mg (0.079 mmol) of HBTU, 12.1 mg (0.079 mmol) of HOBt, and 28 μL (0.16 mmol) of Hünig's base in 2 mL of DMF was added. After 30 min, the resin was filtered and washed for 30 s each with three 5-mL portions of DMF, three 5-mL portions of CH_2Cl_2, and three 5-mL portions of methanol. The resulting resin was dried under diminished pressure over KOH pellets. Quantitative Fmoc cleavage analysis[58] indicated a loading of 0.10 mmol/g (83%).

5.1.2.3. General procedure for the attachment of methylvalerate (analogues) to the resin-bound tripeptide. To a suspension containing 200 mg of the derivatized resin was added sequentially for 5 min each, three 4-mL solutions containing 20% piperidine in DMF. The resulting resin was washed for 30 s each with three 5-mL portions of DMF, three 5-mL portions of CH_2Cl_2, and then three 5-mL portions of DMF. A solution containing 35 mg (77 μmol) of an Fmoc-methylvalerate (analogue), 29 mg (77 μmol) of HBTU, 12 mg (77 μmol) of HOBt, and 27 μL (0.16 mmol) of Hünig's base in 2 mL of DMF was added. After 30 min, the resin was filtered and washed for 30 s each with three 5-mL portions of DMF, three 5-mL portions of CH_2Cl_2, and three 5-mL portions of methanol. The resulting resin was dried under diminished pressure over KOH pellets. Quantitative Fmoc cleavage analysis[58] indicated a loading of 0.09 mmol/g (90%).

5.1.2.4. General procedure for the attachment of histidine to the resin-bound tetrapeptide. To a suspension containing 100 mg of the derivatized resin was added sequentially for 5 min each, three 4-mL solutions containing 20% piperidine in DMF.

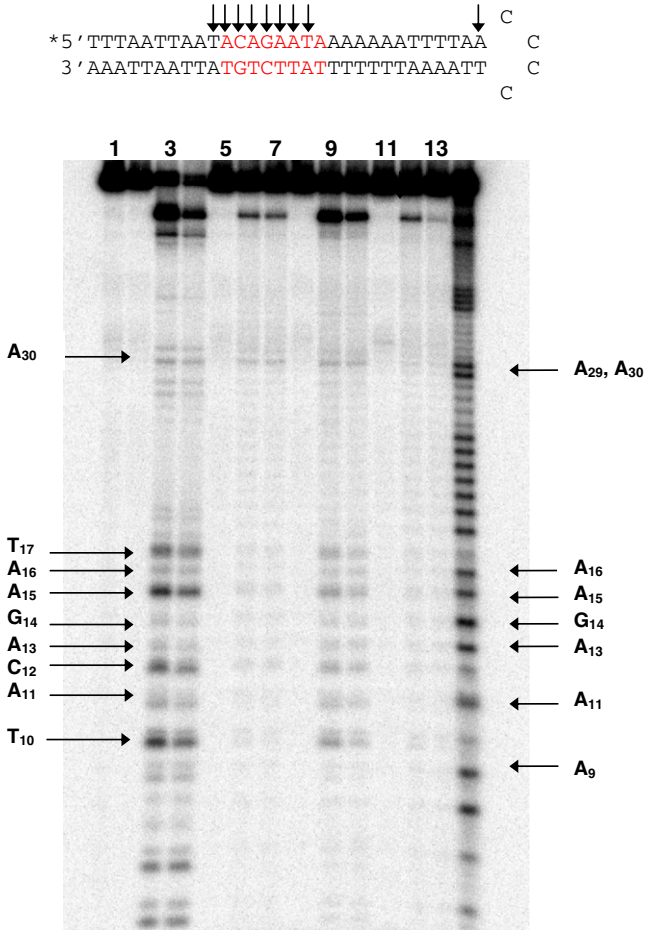


Figure 8. Cleavage of a [5′-^{32}P]-end labeled 64-nucleotide hairpin DNA by deglycoBLM A_5 and deglycoBLM analogues **2**–**4**. Lane 1, DNA alone; lane 2, 20 μM deglycoBLM A_5 (**1**); lane 3, 5 μM Fe(II)·deglycoBLM A_5 (**1**); lane 4, 20 μM Fe(II)·deglycoBLM A_5 (**1**); lane 5, 20 μM deglycoBLM **2**; lane 6, 5 μM Fe(II)·deglycoBLM **2**; lane 7, 20 μM Fe(II)·deglycoBLM **2**; lane 8, 20 μM deglycoBLM **3**; lane 9, 5 μM Fe(II)·deglycoBLM **3**; lane 10, 20 μM Fe(II)·deglycoBLM **3**; lane 11, 20 μM deglycoBLM **4**; lane 12, 5 μM Fe(II)·deglycoBLM **4**; lane 13, 20 μM Fe(II)·deglycoBLM **4**; lane 14, G + A lane.

The resulting resin was washed for 30 s each with three 5-mL portions of DMF, three 5-mL portions of CH_2Cl_2, and then three 5-mL portions of DMF. A solution containing 30 mg (49 μmol) of commercially available Fmoc-histidine, 19 mg (49 μmol) of HATU, 7.0 mg (49 μmol) of HOAt, and 17 μL (97 μmol) of Hünig's base in 2 mL of DMF was added. After 30 min, the resin was filtered and washed for 30 s each with three 5-mL portions of DMF, three 5-mL portions of CH_2Cl_2, and three 5-mL portions of methanol. The resulting resin was dried under diminished pressure over KOH pellets. Quantitative Fmoc cleavage analysis[58] indicated a loading of 0.07 mmol/g (78%).

5.1.2.5. General procedure for the synthesis of deglycobleomycin analogues. To a suspension containing 40 mg of the derivatized resin was added sequentially for 10 min each, three 1-mL solutions containing 20% piperidine in DMF. The resulting resin was washed for 30 s each with three 5-mL portions of DMF, three 5-mL portions of CH_2Cl_2, and three 5-mL portions of DMF. The resin was then added to a 10-mL round bottom flask containing 1 mL of DMF and cooled to 0 °C for 10 min. A mixture containing 5.0 mg (12 μmol) of Boc-pyrimidoblamic acid[32,68] and 16 mg (36 μmol) of BOP reagent was added to the resin with an additional 1 mL of DMF. The reaction mixture was cooled for an additional 10 min, followed by the addition of 13 μL (72 μmol) of Hünig's base. After 16 h, the resin was filtered and washed for 30 s each with three 5-mL portions of DMF, three 5-mL portions of CH_2Cl_2, and three 5-mL portions of methanol. The resulting resin was dried under diminished pressure over KOH pellets.

5.1.2.6. General procedure for the cleavage of deglycoBLM analogues from the resin. To a suspension containing 35–50 mg of resin-bound fully protected deglycobleomycin A_6 analogue was added a solution containing 200 μL of triisopropylsilane and 200 μL of dimethyl sulfide. After 5 min, 3.6 mL of trifluoroacetic acid (TFA) was added to the suspension. After 4 h, the resin was filtered and washed for 30 s each with three 5-mL portions of DMF, three 5-mL portions of CH_2Cl_2, and three 5-mL portions of DMF. The resulting resin was treated with 0.5 mL of 2% hydrazine in DMF. The resin was filtered and then treated with three 0.5-mL portions of 2% hydrazine in DMF solution for an additional 10 min. The eluate was collected and concentrated under diminished pressure. The resulting oil was dissolved in 0.1% aq TFA, frozen and lyophilized.

5.1.3. Characterization of deglycobleomycin analogues

5.1.3.1. Deglycobleomycin analogue 2. HPLC retention time (using the conditions described under Section 5.1.1): 15.1 min; colorless solid; yield 1.8 mg (55%); ^{1}H NMR (D_2O) δ 0.53 (s, 1H), 0.65 (s, 1H), 0.90 (d, 3H, J = 6.4 Hz), 1.02 (d, 3H, J = 6.4 Hz), 1.16 (m, 2H), 1.63–1.64 (m, 4H), 1.88–1.95 (m, 7H), 2.45–2.48 (m, 1H), 2.57–2.64 (m, 2H), 2.94–3.01 (m, 12H), 3.09–3.11 (m, 2H), 3.14–3.19 (m, 4H), 3.38–3.41 (m, 2H), 3.44–3.47 (m, 2H), 3.53 (t, 1H, J = 5.6 Hz), 3.56–3.60 (m, 2H), 3.68–3.70 (m, 2H), 3.88–3.90 (m, 2H), 3.97–3.98 (m, 1H), 4.02 (t, 1H, J = 5.6 Hz), 4.12 (d, 1H, J = 4.2), 7.24 (s, 1H), 7.96 (s, 1H), 8.09 (s, 1H) and 8.58 (s, 1H); mass spectrum (MALDI-TOF), m/z 1178.4 $(M+Na)^+$ (theoretical 1178.6); mass spectrum (ESI), m/z 1156. 5977 $(M+H)^+$ ($C_{50}H_{82}N_{19}O_9S_2$ requires 1156.5984).

5.1.3.2. Deglycobleomycin analogue 3. HPLC retention time: 14.5 min; colorless solid; yield 1.5 mg (47%); ^{1}H NMR (D_2O) δ 0.15 (d, 3H, J = 7.2 Hz), 0.30 (d, 3H, J = 7.2 Hz), 0.57 (d, 3H, J = 7.2 Hz), 0.59 (d, 3H, J = 6.4 Hz), 0.85–0.87 (m, 2H), 1.08–1.19 (m, 3H), 1.28–1.29 (m, 4H), 1.53–1.61 (m, 7H), 2.22–2.29 (m, 3H), 2.59–2.66 (m, 9H), 2.71–2.73 (m, 1H), 2.78–2.84 (m, 3H), 3.05 (t, 2H, J = 5.6 Hz), 3.14–3.33 (m, 3H), 3.31–3.33 (m, 1H), 3.50 (dd, 1H, J = 5.6 and 3.2 Hz), 3.62–3.68 (m, 3H), 3.75 (d, 1H, J = 4.0 Hz), 4.34 (t, 1H, J = 7.2 Hz), 6.95 (s, 1H), 7.60 (s, 1H), 7.67 (s, 1H) and 8.25 (s, 1H); mass spectrum (MALDI-TOF), m/z 1156.6 $(M+H)^+$ (theoretical 1156.6); mass spectrum (ESI), m/z 1156.5991 $(M+H)^+$ ($C_{50}H_{82}N_{19}O_9S_2$ requires 1156.5984).

5.1.3.3. Deglycobleomycin analogue 4. HPLC retention time (using the conditions described under Section 5.1.1): 14.3 min; colorless solid; yield 0.98 mg (40%); ^{1}H NMR (D_2O) δ 0.78–1.00 (m, 15H), 1.60 (m, 4H), 1.85–1.91 (m, 7H), 2.08 (m, 1H), 2.53–2.54 (m, 1H), 2.90–2.98 (m, 14H), 3.03–3.05 (m, 2H), 3.11–3.18 (m, 3H), 3.34–3.38 (m, 2H), 3.46–3.53 (m, 3H), 3.83–3.85 (m, 2H), 4.18 (d, 1H, J = 7.2 Hz), 4.29 (t, 1H, J = 7.2 Hz), 7.23 (s, 1H), 7.96 (s, 1H), 8.08 (s, 1H) and 8.50 (s, 1H); mass spectrum (MALDI-TOF), m/z 1156.4 $(M+H)^+$ (theoretical 1156.6); mass spectrum (ESI), m/z 1156.5991 $(M+H)^+$ ($C_{50}H_{82}N_{19}O_9S_2$ requires 1156.5984).

5.1.3.4. Deglycobleomycin analogue 5. HPLC retention time: 15.7 min; colorless solid; yield 1.9 mg (59%); ^{1}H NMR (D_2O) δ 1.00 (d, 3H, J = 6.4 Hz), 1.11 (d, 3H, J = 7.2 Hz), 1.19–1.26 (m, 2H), 1.35 (d, 3H, J = 11.2 Hz), 1.71–1.55 (m, 5H), 1.71–1.73 (m, 4H), 1.96–2.03 (m, 9H), 2.53–2.55 (m, 1H), 2.62–2.65 (m, 2H), 2.92–2.97 (m, 2H), 3.02–3.09 (m, 12H), 3.05 (dd, 1H, J = 8.0 and 7.2 Hz), 3.24–3.29 (m, 4H), 3.46–3.48 (m, 1H), 3.52–3.55 (m, 1H), 3.56–3.61 (m, 1H), 3.61–3.68 (m, 1H), 3.69–3.83 (m, 2H),

3.84–3.98 (m, 1H), 4.23 (d, 1H, *J* = 5.6 Hz), 4.29 (t, 1H, *J* = 7.2), 7.33 (s, 1H), 8.05 (s, 1H), 8.17 (s, 1H) and 8.66 (s, 1H); mass spectrum (MALDI-TOF), *m*/*z* 1218.5 $(M+Na)^+$ (theoretical 1218.6); mass spectrum (ESI), *m*/*z* 1196.6289 $(M+H)^+$ ($C_{53}H_{86}N_{19}O_9S_2$ requires 1196.6297).

5.1.3.5. Deglycobleomycin analogue 6. HPLC retention time: 14.7 min; colorless solid; yield 1.4 mg (47%); ^1H NMR (D_2O) δ 0.97 (d, 3H, *J* = 6.4 Hz), 1.07 (d, 3H, *J* = 7.2 Hz), 1.63–1.64 (m, 4H), 1.84–1.95 (m, 7H), 2.35 (t, 1H, *J* = 12.0 Hz), 2.56–2.60 (m, 3H), 2.73–2.76 (m, 2H), 2.94–3.01 (m, 14H), 3.15 (t, 2H, *J* = 5.6 Hz), 3.32–3.38 (m, 4H), 3.53 (t, 1H, *J* = 6.4 Hz), 3.66 (t, 1H, *J* = 5.6 Hz), 3.96–4.03 (m, 4H), 4.19 (d, 1H, *J* = 5.6 Hz), 4.55 (t, 1H, *J* = 6.4 Hz), 6.73 (s, 1H), 6.98 (d, 1H, *J* = 8.0 Hz), 7.02 (t, 1H, *J* = 7.2 Hz), 7.08 (d, 1H, *J* = 8.0 Hz), 7.91 (s, 1H), 8.06 (s, 1H) and 8.38 (s, 1H); mass spectrum (MALDI-TOF), *m*/*z* 1212.4 $(M+Na)^+$ (theoretical 1212.6); mass spectrum (ESI), *m*/*z* 1190.5833 $(M+H)^+$ ($C_{53}H_{80}N_{19}O_9S_2$ requires 1190.5828).

5.1.3.6. Deglycobleomycin analogue 7. HPLC retention time: 19.0 min; colorless solid; yield 1.2 mg (27%); ^1H NMR (D_2O) δ 0.99 (d, 3H, *J* = 7.2 Hz), 1.09 (d, 3H, *J* = 7.2 Hz), 1.63–1.64 (m, 4H), 1.83–1.95 (m, 7H), 2.40 (t, 1H, *J* = 12.0 Hz), 2.53–2.61 (m, 3H), 2.66–2.69 (m, 1H), 2.94–3.01 (m, 14H), 3.15–3.20 (m, 2H), 3.31–3.36 (m, 2H), 3.50–3.53 (m, 1H), 3.63–3.67 (m, 2H), 3.84–3.85 (m, 1H), 3.93–3.94 (m, 1H), 4.01–4.04 (m, 1H), 4.22 (d, 1H, *J* = 4.8 Hz), 4.23–4.25 (m, 1H), 4.55 (t, 1H, *J* = 6.4 Hz), 6.82 (s, 1H), 6.87 (t, 1H, *J* = 8.0 Hz), 6.96 (d, 1H, *J* = 7.2 Hz), 7.01 (t, 1H, *J* = 7.2 Hz), 7.05 (d, 1H, *J* = 8.0 Hz), 7.87 (s, 1H), 8.01 (s, 1H) and 8.43 (s, 1H); mass spectrum (MALDI-TOF), *m*/*z* 1290.7 and 1292.0 $(M+Na)^+$ (theoretical 1290.5 and 1292.5); mass spectrum (ESI), *m*/*z* 1268.4986 and 1270.4956 $(M+H)^+$ ($C_{53}H_{79}N_{19}O_9S_2Br$ requires 1268.4933 and 1270.4913).

5.1.3.7. Deglycobleomycin analogue 8. HPLC retention time: 15.5 min; colorless solid; yield 1.5 mg (47%); ^1H NMR (D_2O) δ 0.98 (d, 3H, *J* = 6.4 Hz), 1.08 (d, 3H, *J* = 7.2 Hz), 1.63–1.64 (m, 4H), 1.85–1.95 (m, 7H), 2.00 (s, 3H), 2.33 (t, 1H, *J* = 12.0 Hz), 2.58–2.66 (m, 4H), 2.74 (dd, 1H, *J* = 9.6 and 5.6 Hz), 2.80 (dd, 1H, *J* = 11.2 and 3.2 Hz), 2.94–2.97 (m, 12H), 3.00 (t, 2H, *J* = 8.0 Hz), 3.31–3.37 (m, 2H), 3.51–3.59 (m, 2H), 3.67 (t, 1H, *J* = 7.2 Hz), 3.99 (t, 1H, *J* = 5.6 Hz), 4.04–4.07 (m, 2H), 4.08–4.11 (m, 1H), 4.20 (d, 1H, *J* = 4.8 Hz), 4.55 (t, 1H, *J* = 7.2 Hz), 6.72 (s, 1H), 6.84–6.87 (m, 2H), 6.90 (d, 2H, *J* = 7.2 Hz), 7.90 (s, 1H), 8.03 (s, 1H) and 8.39 (s, 1H); mass spectrum (MALDI-TOF), *m*/*z* 1226.6 $(M+Na)^+$ (theoretical 1226.6); mass spectrum (ESI), *m*/*z* 1204.5968 $(M+H)^+$ ($C_{54}H_{82}N_{19}O_9S_2$ requires 1204.5984).

5.1.3.8. Deglycobleomycin analogue 9. HPLC retention time: 16.7 min; colorless solid; yield 1.8 mg (56%); ^1H NMR (D_2O) δ 0.61 (t, 3H, *J* = 7.2 Hz), 0.89 (d, 3H, *J* = 7.2 Hz), 0.96 (d, 3H, *J* = 5.6 Hz), 1.03–1.06 (m, 2H), 1.38–1.40 (m, 2H), 1.53–1.54 (m, 2H), 1.64 (m, 2H), 1.86–1.95 (m, 8H), 2.35–2.36 (m, 1H), 2.60–2.64 (m, 3H), 2.94–3.01 (m, 14H), 3.09–3.18 (m, 4H), 3.37–3.41 (m, 3H), 3.52–3.56 (m, 4H), 4.02 (t, 1H, *J* = 5.6 Hz), 4.11 (d, 1H, *J* = 4.0 Hz), 7.21 (s, 1H), 7.92 (s, 1H), 8.08 (s, 1H) and 8.53 (s, 1H); mass spectrum (MALDI-TOF), *m*/*z* 1178.0 $(M+Na)^+$ (theoretical 1178.6); mass spectrum (ESI), *m*/*z* 1156.5979 $(M+H)^+$ ($C_{50}H_{82}N_{19}O_9S_2$ requires 1156.5984).

5.1.3.9. Deglycobleomycin analogue 10. HPLC retention time: 17.6 min; colorless solid; yield 1.4 mg (43%); ^1H NMR (D_2O) δ 0.65 (d, 3H, *J* = 6.4 Hz), 0.69 (d, 3H, *J* = 6.4 Hz), 0.90 (d, 3H, *J* = 7.2 Hz), 0.95 (d, 3H, *J* = 6.4 Hz), 1.21–1.29 (m, 3H), 1.42–1.48 (m, 2H), 1.64 (m, 4H), 1.87–1.95 (m, 7H), 2.41–2.44 (m, 1H), 2.57–2.67 (m, 2H), 2.91–3.03 (m, 12H), 3.06–3.18 (m, 4H), 3.38–3.49 (m, 2H), 3.41–3.56 (m, 3H), 3.67–3.69 (m, 2H), 3.93 (t, 1H, *J* = 5.6 Hz), 4.11 (d, 1H, *J* = 4.8 Hz), 7.21 (s, 1H), 7.93 (s, 1H), 8.08 (s, 1H) and 8.53 (s, 1H); mass spectrum (MALDI-TOF), *m*/*z* 1178.4 $(M+Na)^+$ (theoretical 1178.6); mass spectrum (ESI), *m*/*z* 1156.5995 $(M+H)^+$ ($C_{50}H_{82}N_{19}O_9S_2$ requires 1156.5984).

5.1.3.10. Deglycobleomycin analogue 11. HPLC retention time: 17.2 min; colorless solid; yield 1.0 mg (30%); ^1H NMR (D_2O) δ 0.53 (d, 3H, *J* = 6.4 Hz), 0.93 (d, 3H, *J* = 6.4 Hz), 1.63–1.64 (m, 4H), 1.86–1.94 (m, 7H), 2.52–2.59 (m, 2H), 2.61 (t, 1H, *J* = 4.8 Hz), 2.69–2.72 (m, 1H), 2.82–2.86 (m, 1H), 2.93–3.01 (m, 14H), 3.09–3.14 (m, 3H), 3.17–3.20 (m, 2H), 3.27–3.49 (m, 4H), 3.66–3.68 (m, 2H), 3.78 (t, 1H, *J* = 6.4 Hz), 3.89 (d, 1H, *J* = 4.8 Hz), 6.95 (d, 2H, *J* = 7.2 Hz), 6.99–7.00 (m, 3H), 7.23 (s, 1H), 7.92 (s, 1H), 8.07 (s, 1H) and 8.54 (s, 1H); mass spectrum (MALDI-TOF), *m*/*z* 1212.4 $(M+Na)^+$ (theoretical 1212.6); mass spectrum (ESI), *m*/*z* 1190.5823 $(M+H)^+$ ($C_{53}H_{80}N_{19}O_9S_2$ requires 1190.5828).

5.1.3.11. Deglycobleomycin analogue 12. HPLC retention time: 16.4 min; colorless solid; yield 1.2 mg (36%); ^1H NMR (D_2O) δ 0.53 (d, 3H, *J* = 7.2 Hz), 0.59 (d, 3H, *J* = 6.4 Hz), 0.65–0.66 (m, 9H), 0.91–0.92 (m, 5H), 1.15–1.25 (m, 4H), 1.40–1.42 (m, 1H), 1.63–1.64 (m, 2H), 1.88–1.95 (m, 7H), 2.57–2.61 (m, 3H), 2.93–3.01 (m, 12H), 3.07–3.11 (m, 1H), 3.17–3.20 (m, 2H), 3.38–3.40 (m, 2H), 3.44–3.50 (m, 2H), 3.57–3.60 (m, 1H), 3.65–3.67 (m, 1H), 3.89 (t, 1H, *J* = 6.4 Hz), 3.92–3.93 (m, 1H), 3.99–4.02 (m, 1H), 4.15 (d, 1H, *J* = 4.8 Hz), 7.23 (s, 1H), 7.97 (s, 1H), 8.09 (s, 1H) and 8.57 (s, 1H); mass spectrum (MALDI-TOF), *m*/*z* 1220.5 $(M+Na)^+$ (theoretical 1220.6); mass spectrum (ESI), *m*/*z* 1198.6442 $(M+H)^+$ ($C_{53}H_{88}N_{19}O_9S_2$ requires 1198.6454).

5.2. Biochemical evaluation

5.2.1. Materials and methods

pBR322 plasmid DNA, and restriction endonucleases *Hin*dIII and *Eco*RV, and T4 polynucleotide kinase were purchased from New England Biolabs. Thermosensitive alkaline phosphatase and GeneJet PCR purification kit were purchased from Fermentas. [γ-^{32}P]ATP was purchased from Perkin Elmer. $Fe(NH_4)_2(SO_4)_2{\cdot}H_2O$ was purchased from Sigma Aldrich and used to prepare Fe^{2+} solutions immediately prior to use. Chelex 100 was purchased from Sigma Aldrich and used to remove adventitious Fe^{2+} from solutions prior to experiments. The hairpin DNA was purchased from Integrated DNA Technologies, Inc.

5.2.2. Cleavage of supercoiled pBR322 plasmid DNA by valerate-modified deglycoBLM A_6 analogues

Two hundred nanograms of supercoiled pBR322 plasmid DNA was treated with the appropriate concentration of freshly prepared Fe^{2+} and deglycoBLM solutions in 15 μL (total volume) of 50 mM Tris–HCl buffer, pH 8.0. The reaction mixtures were incubated at room temperature for 30 min and then quenched by the addition of 3 μL of native gel loading buffer (30% glycerol, 0.125% (w/v) bromophenol blue). Five microliters of each reaction mixture was loaded onto a 1% agarose gel made in 90 mM Tris–borate, pH 8.3, containing 1 mM sodium EDTA and run at 96 V for 2 h. The gel was then stained in the same buffer solution containing 0.5 μg/mL ethidium bromide for 1 h followed by destaining in water for 15 min. The gels were analyzed using a UV transilluminator.

5.2.3. [5′-^{32}P]-end labeling and purification of a 158-base pair DNA restriction fragment[69]

Twenty-five micrograms of pBR322 plasmid DNA was digested at 37 °C for 3 h with 50 U of restriction endonuclease *Hin*dIII in a final volume of 80 μL of 10 mM Tris–HCl, pH 7.9, containing 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM dithiothretol. The linearized DNA was

dephosphorylated by treatment with 25 U of thermosensitive alkaline phosphatase in 120 μL (total volume) of 10 mM Tris–HCl, pH 8.0, containing 0.1 M KCl, 5 mM $MgCl_2$, 0.02% Trition X-100 and 0.1 mg/mL bovine serum albumin. The reaction mixture was incubated at 37 °C for 30 min followed by heat inactivation at 75 °C for 5 min. The reaction mixture was then purified with a GeneJET PCR purification kit following the manufacturer's protocol (Fermentas). The dephosphorylated DNA was then [5′-^{32}P]-end labeled by incubation with 20 U of T4 polynucleotide kinase in addition to 0.10 mCi [γ-^{32}P]ATP (specific activity 6000 Ci (222 TBq)/mmol) in 80 μL (total volume) of 70 mM Tris–HCl buffer, pH 7.6, containing 10 mM $MgCl_2$ and 5 mM dithiothreitol. The reaction mixture was incubated at 37 °C for 1 h followed by heat inactivation of the enzyme at 65 °C for 20 min. Unincorporated [γ-^{32}P]ATP was removed using a GeneJET PCR purification kit. The [5′-^{32}P]-end labeled DNA was digested for 3 h at 37 °C with 50 U of restriction endonuclease *Eco*RV in a final volume of 70 μL. This digestion resulted in a [5′-^{32}P]-end labeled fragment having 158-base pairs that was purified essentially as described.[42,69]

5.2.4. Sequence selective cleavage of [5′-^{32}P]-end labeled 158-base pair DNA duplex by valerate-modified deglycoBLM A_6 analogues

A sample of [5′-^{32}P]-end labeled 158-base pair DNA (30,000 cpm) was treated with the appropriate concentrations of Fe^{2+} and deglycoBLM solutions in 10 μL (total volume) of 50 mM Tris–HCl buffer, pH 8.0. Reactions were incubated at room temperature for 30 min followed by removal of the supernatant under diminished pressure. Ten microliters of denaturing gel loading buffer (80% formamide, 2 mM EDTA, 1% (w/v) bromophenol blue and 1% (w/v) xylene cyanol FF) was added to the DNA pellet and the solution was heated at 90 °C for 10 min followed by chilling on ice. Five microliters of each sample was loaded onto a denaturing gel (16% polyacrylamide, 7 M urea) and run at 1800 V for 3.5 h. Cleavage sites were confirmed by comparison with the reaction products obtained through the Maxam-Gilbert G + A sequencing protocol.[70] Gels were visualized using a phosphorimager (Molecular Dynamics).

5.2.5. [5′-^{32}P]-end labeling and purification of hairpin DNA

Ten pmol of 64-nt hairpin DNA was [5′-^{32}P]-end labeled by incubation with 20 U of T4 polynucleotide kinase and 0.06 mCi [γ-^{32}P]ATP (specific activity 6000 Ci (222 TBq)/mmol) in 50 uL (total volume) of 70 mM Tris–HCl buffer, pH 7.6, containing 10 $MgCl_2$ and 5 mM dithiothreitol. The reaction mixture was incubated at 37 °C for 1 h followed by heat inactivation of the enzyme at 65 °C for 20 min. [5′-^{32}P]-end labeled 64-nt hairpin DNA was purified by 16% polyacrylamide gel electrophoresis carried out at 1800 V for 2.5 h.

5.2.6. Sequence selective cleavage of [5′-^{32}P]-end labeled hairpin DNA by the modified deglycoBLM A_6 analogues

A sample of [5′-^{32}P]-end labeled hairpin DNA (30,000 cpm) was treated with the appropriate concentrations of Fe^{2+} and deglycoBLM solutions in 10 μL (total volume) of 50 mM Tris–HCl buffer, pH 8.0. Reactions were incubated at room temperature for 30 min followed by removal of the supernatant under diminished pressure. Ten microliters of denaturing gel loading buffer (80% formamide, 2 mM EDTA, 1% (w/v) bromophenol blue and 1% (w/v) xylene cyanol FF) was added to the DNA pellet and the solution was heated at 90 °C for 10 min, followed by chilling on ice. Five microliters of each sample was loaded onto a denaturing gel (16% polyacrylamide, 7 M urea) and run at 1800 V for 2.5 h. Cleavage sites were confirmed by comparison with the reaction products obtained through the Maxam-Gilbert G + A sequencing protocol.[70] Gels were visualized using a phosphorimager (Molecular Dynamics).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.04.047.

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