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The Disaccharide Moiety of Bleomycin Facilitates Uptake by Cancer Cells

Benjamin R. Schroeder,[†] M. Imran Ghare,[‡] Chandrabali Bhattacharya,[‡] Rakesh Paul,[‡] Zhiqiang Yu,[‡] Paul A. Zaleski,[‡] Trevor C. Bozeman,[‡] Michael J. Rishel,[§] and Sidney M. Hecht*[,‡]

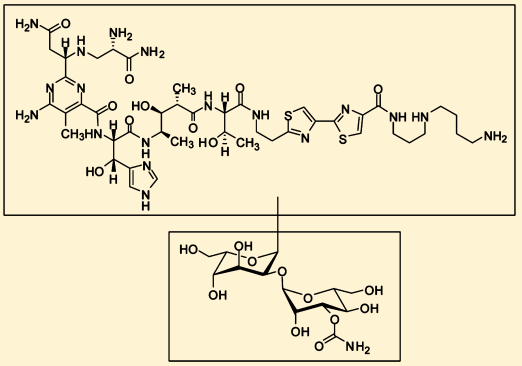
[†]Department of Chemistry, University of Virginia, Charlottesville, Virginia 22904, United States

[‡]Center for BioEnergetics, Biodesign Institute, and Department of Chemistry and Biochemistry, Arizona State University, Tempe, Arizona 85287, United States

[§]GE Global Research, 1 Research Circle, Niskayuna, New York 12309, United States

Supporting Information

ABSTRACT: The disaccharide moiety is responsible for the tumor cell targeting properties of bleomycin (BLM). While the aglycon (deglycobleomycin) mediates DNA cleavage in much the same fashion as bleomycin, it exhibits diminished cytotoxicity in comparison to BLM. These findings suggested that BLM might be modular in nature, composed of tumor-seeking and tumoricidal domains. To explore this possibility, BLM analogues were prepared in which the disaccharide moiety was attached to deglycobleomycin at novel positions, namely, via the threonine moiety or C-terminal substituent. The analogues were compared with BLM and deglycoBLM for DNA cleavage, cancer cell uptake, and cytotoxic activity. BLM is more potent than deglycoBLM in supercoiled plasmid DNA relaxation, while the analogue having the disaccharide on threonine was less active than deglycoBLM and the analogue containing the C-terminal disaccharide was slightly more potent. While having unexceptional DNA cleavage potencies, both glycosylated analogues were more cytotoxic to cultured DU145 prostate cancer cells than deglycoBLM. Dye-labeled conjugates of the cytotoxic BLM aglycons were used in imaging experiments to determine the extent of cell uptake. The rank order of internalization efficiencies was the same as their order of cytotoxicities toward DU145 cells. These findings establish a role for the BLM disaccharide in tumor targeting/uptake and suggest that the disaccharide moiety may be capable of delivering other cytotoxins to cancer cells. While the mechanism responsible for uptake of the BLM disaccharide selectively by tumor cells has not yet been established, data are presented which suggest that the metabolic shift to glycolysis in cancer cells may provide the vehicle for selective internalization.



INTRODUCTION

The bleomycins (BLM) are a family of glycopeptide-derived antitumor antibiotics first isolated from *Streptomyces verticillus*.[1,2] Their antitumor activity derives from their ability to effect DNA cleavage in cancer cells[3−6] and probably reflects the propensity of the compounds to effect double-strand DNA cleavage.[7−10] The bleomycins are also tumor-seeking molecules, as demonstrated initially by the use of bleomycins derivatized with radionuclides[11−16] and more recently in cell culture experiments involving bleomycins and BLM carbohydrates conjugated to microbubbles[17] and dyes.[18]

Bleomycin-mediated DNA strand scission requires oxygen and a metal ion such as Fe or Cu, which activates BLM for DNA degradation.[19,20] The DNA degradation process is initiated by the binding of activated BLM to its DNA substrate;[21] the actual chemistry of DNA degradation has been characterized[2,22−24] and shown to involve the rate-limiting abstraction of a H atom from the C-4′ position of a nucleoside deoxyribose moiety.[25] As shown in Figure 1, the structure of bleomycin (**1**) includes a disaccharide moiety composed of two unusual sugars, L-gulose and 3-*O*-carbamoyl-D-mannose. Interestingly, the disaccharide moiety is not essential for DNA cleavage *in vitro*, as the BLM derivative lacking the disaccharide (deglycoBLM, **2**) cleaves DNA with a sequence selectivity and chemistry quite similar to that of bleomycin itself, albeit with somewhat diminished efficiency.[26−31]

While the disaccharide moiety of BLM is not essential for DNA cleavage, we have recently demonstrated that the disaccharide is sufficient to recapitulate the tumor cell targeting properties of bleomycin.[18] This suggested that bleomycin could potentially be regarded as a modular system composed of a tumor-targeting agent (the disaccharide moiety) and a tumoricidal agent (deglycobleomycin). Consistent with the need for the disaccharide moiety to effect efficient cellular uptake in tumor cells, we find that deglycoBLM is much less

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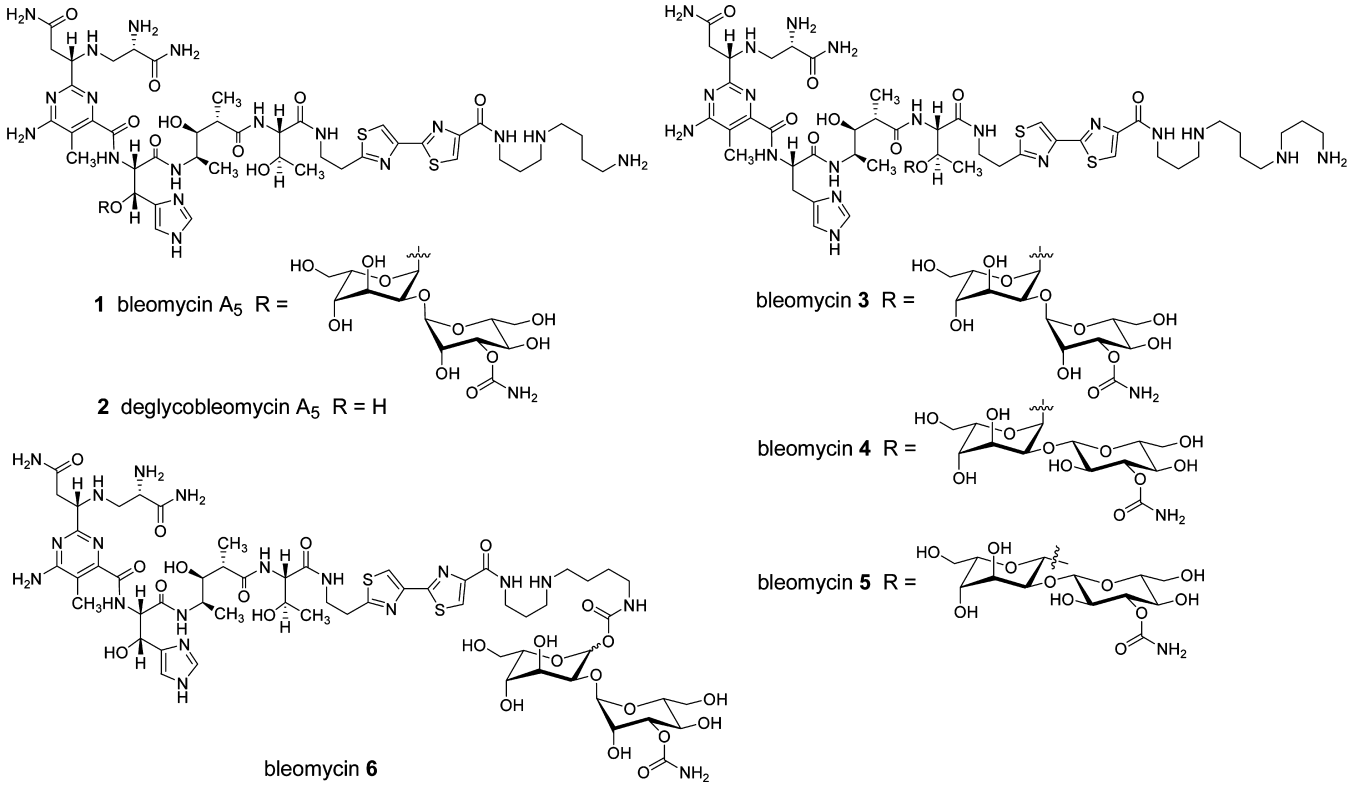

Figure 1. Structures of bleomycin A_5 (**1**), deglycobleomycin A_5 (**2**), and bleomycin analogues **3**–**6**.

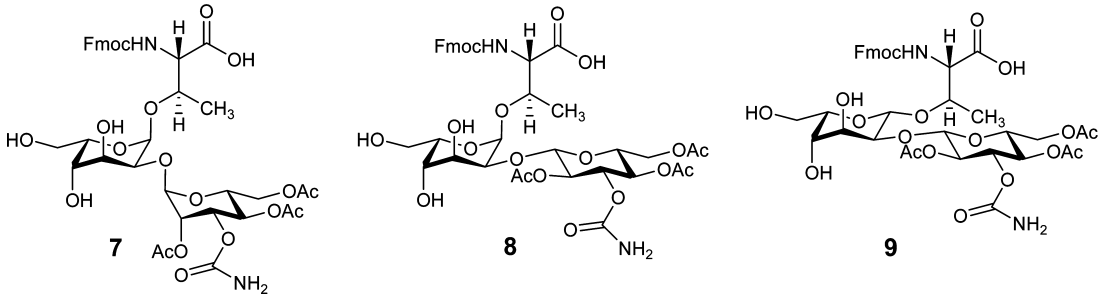

Figure 2. Structures of glycosylated threonine derivatives **7**–**9**, the constituents of bleomycin analogues **3**–**5**, respectively.

cytotoxic than BLM toward cultured cancer cells. To better define the possible modular nature of bleomycin as an antitumor agent, we have prepared derivatives of deglycoBLM in which the BLM disaccharide is attached to the threonine moiety of deglycoBLM (BLM **3**) or to the C-terminal substituent (BLM **6**). Also prepared as negative controls were two BLM derivatives related to **3** in which the disaccharide consisted of two anomers of L-gulose attached to 3-*O*-carbamoyl-D-glucose or linked to deglycoBLM through the threonine moiety (BLMs **4** and **5**).

Evaluation of the analogues for efficiency of DNA cleavage in a plasmid DNA relaxation assay revealed that BLM **6** had potency comparable to deglycoBLM (**2**), and that BLMs **3**–**5** were all significantly less potent. Cytotoxicity testing was carried out in DU145 human prostate cancer cells. In spite of the weak DNA cleavage activity of BLM **3**, it was found to be more cytotoxic than deglycoBLM itself. BLMs **4** and **5**, having disaccharides different than the tumor-targeting disaccharide present in BLM, were minimally cytotoxic. BLM **6**, having the BLM disaccharide attached at the C-terminus, was found to be significantly more cytotoxic than deglycoBLM in spite of its comparable DNA cleavage activity. To verify that the cytotoxicities of the BLM derivatives studied were strongly influenced by cellular uptake, cytotoxic BLMs **1**, **2**, **3**, and **6** were conjugated to the cyanine dye Cy5** (affording **44**, **45**, **47**, and **55**, respectively). The dye-labeled compounds were shown to be internalized by DU145 cells, and the efficiency of uptake was in direct proportion to the observed cytotoxicities. These data underscore the importance of the BLM disaccharide for cellular uptake of this class of antitumor agent and suggest that the cytotoxic activity of deglycoBLM is limited by lack of facile cellular uptake.

Also studied in a preliminary fashion was the possible basis for cancer cell selective targeting and internalization by the carbohydrate moiety of bleomycin. Evidence is presented that upregulation of one or more receptors associated with enhanced glycolysis in tumor cells may provide the means by which the bleomycins target tumor cells selectively.

RESULTS

Synthesis of Bleomycin Analogues. Bleomycin A_5 was an outdated clinical sample; deglycobleomycin A_5 was obtained

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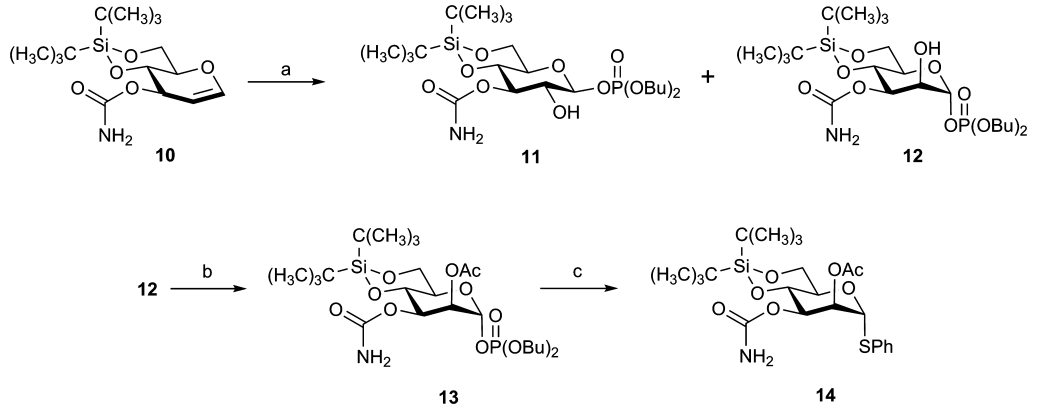
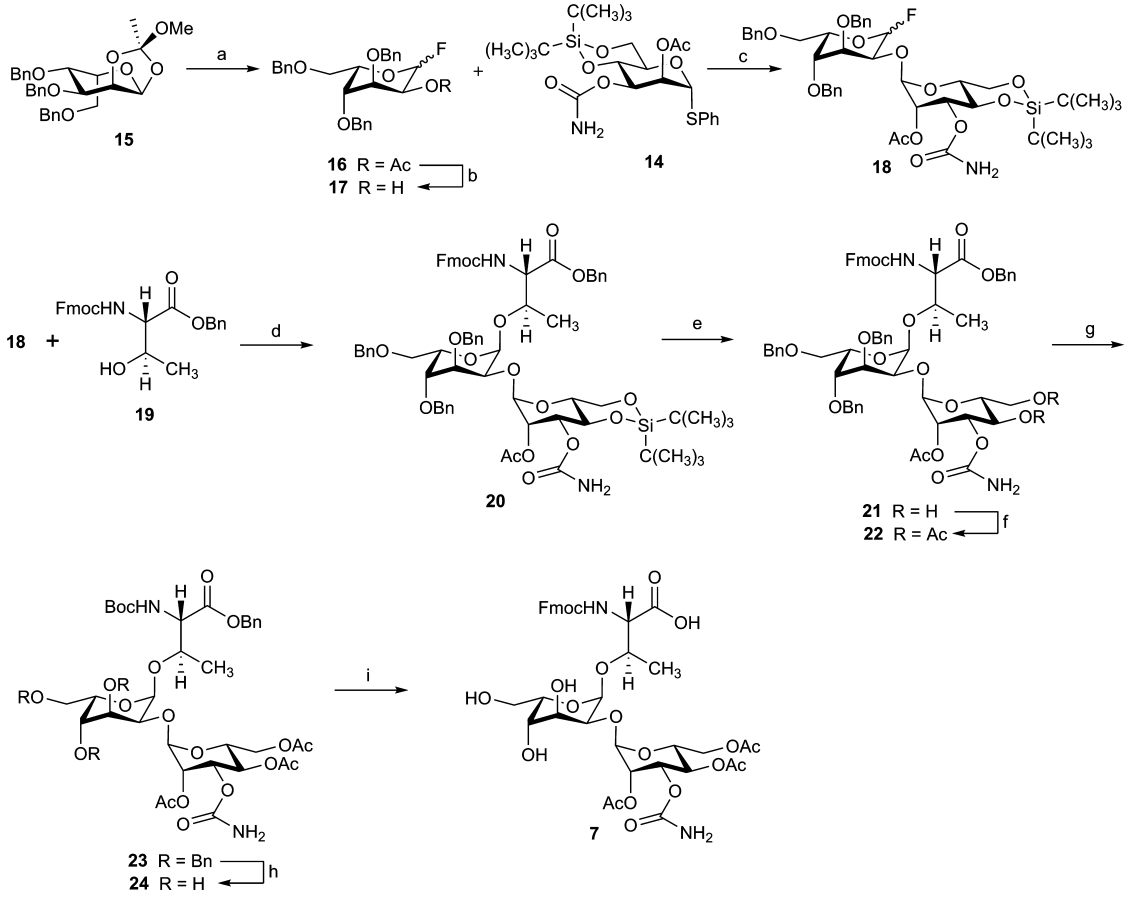
Scheme 1. Synthesis of Key Intermediates 11 and 12 and Conversion of 12 to Mannose Derivative 14[a]



[a]Conditions: (a) methyl trioxorhenium (4 mol %), urea hydrogen peroxide, dibutyl phosphate, pyrazole, 4 Å molecular sieves, CH_2Cl_2 (30% for **11**; 27% for **12**). (b) Ac_2O, pyridine, 0 to 25 °C (93%). (c) PhSH, TMSOTf, 4 Å molecular sieves, CH_2Cl_2, −78 to 25 °C (92%).

Scheme 2. Synthesis of Key Intermediate 7[a]



[a]Conditions: (a) DAST, CH_2Cl_2, 0 to 25 °C (95%). (b) K_2CO_3, MeOH (97%). (c) *N*-Iodosuccinimide, cat. AgOTf, 4 Å molecular sieves, CH_2Cl_2, −78 to −5 °C (91%). (d) Cp_2ZrCl_2, AgOTf, 4 Å molecular sieves, CH_2Cl_2, −15 °C (68%). (e) 48% aq HF, CH_3CN (89%). (f) Ac_2O, pyridine, 0 to 25 °C (99%). (g) KF, triethylamine, Boc_2O, DMF (86%). (h) $Pd(OH)_2/C$, H_2, 1:1 THF−MeOH (95%). (i) (1) 1:1:0.1 TFA−CH_2Cl_2−Me_2S; (2) FmocOSu, 1:1 CH_3CN−sat. aq $NaHCO_3$ (70% over two steps).

by treatment of bleomycin A_5 with HF, followed by purification on reversed-phase HPLC.[32] The syntheses of bleomycin analogues **3**−**5** were accomplished by first preparing the requisite protected glycosylated threonine derivatives **7**−**9**, respectively (Figure 2).

Synthesis of glycosylated threonine derivative **7** began with preparation of the protected 3-*O*-carbamoylated mannose derivative **14** (Scheme 1). O-Carbamoylated D-glucal **10**[33] was treated with methyl trioxorhenium, urea hydrogen peroxide, and dibutyl phosphate to afford the protected dibutyl phosphate derivatives of 3-*O*-carbamoyl-*β*-D-glucopyranose (**11**) and 3-*O*-carbamoyl-*α*-D-mannopyranose (**12**) in 30% and 27% yield, respectively.[34] The latter was O-acetylated to afford **13** as a colorless oil in 93% yield and then treated with

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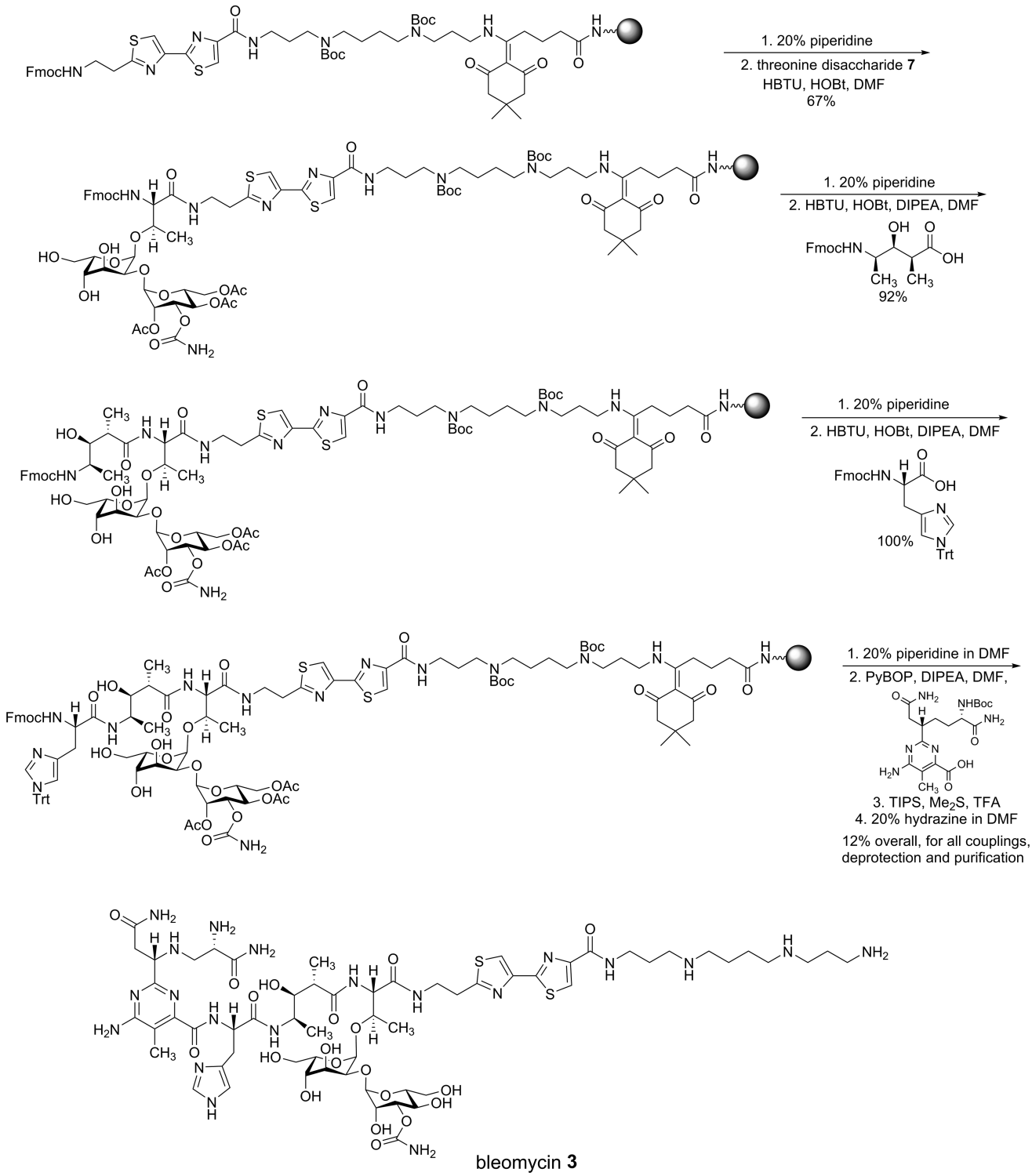
Scheme 3. Solid-Phase Synthesis of Bleomycin 3



thiophenol and trimethylsilyl trifluoromethanesulfonate (TMSOTf), affording **14**, which crystallized as colorless needles in 92% yield.

The perbenzylated orthoacetal of α-L-gulopyranose (**15**)[35] was converted to 3,4,6-tri-*O*-benzyl-α,β-L-gulopyranosyl fluoride (**16**) in 95% yield by treatment with diethylaminosulfur trifluoride (DAST) (Scheme 2). Following O-deacetylation, a solution of gulosyl fluoride **17** and phenylthiomannoside **14** in CH_2Cl_2 was treated with *N*-iodosuccinimide and catalytic silver triflate[36] at low temperature, affording disaccharide **18** in 91% yield as a colorless foam. Treatment of fluorinated disaccharide **18** with Fmoc-L-threonine benzyl ester (**19**)[37] in the presence of Cp_2ZrCl_2 and AgOTf gave glycosylated threonine derivative **20** as a colorless foam in 68% yield. Compound **20** was

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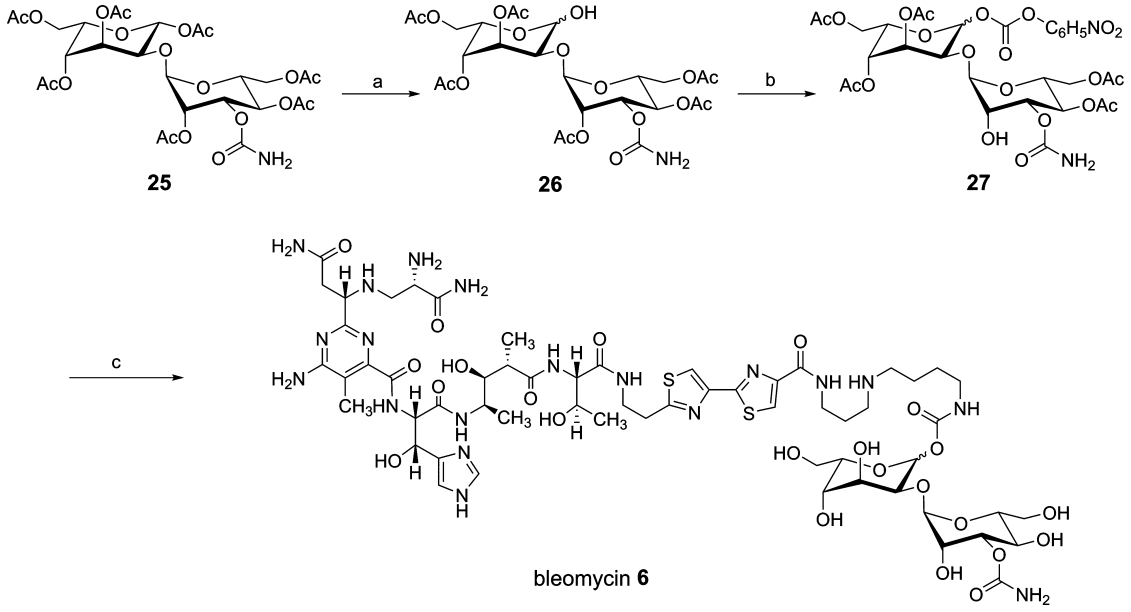
Scheme 4. Synthesis of Bleomycin 6[a]



[a]Conditions: (a) NH_2NH_2 acetate salt (70%). (b) *p*-Nitrophenyl chloroformate, 4-(*N*,*N*-dimethylamino)pyridine, CH_3CN (92%). (c) (1) Cu(II)–deglycoBLM A_5, DMF, NEt_3; (2) NH_2NH_2, DMF; (3) 15% aqueous EDTA (39%).

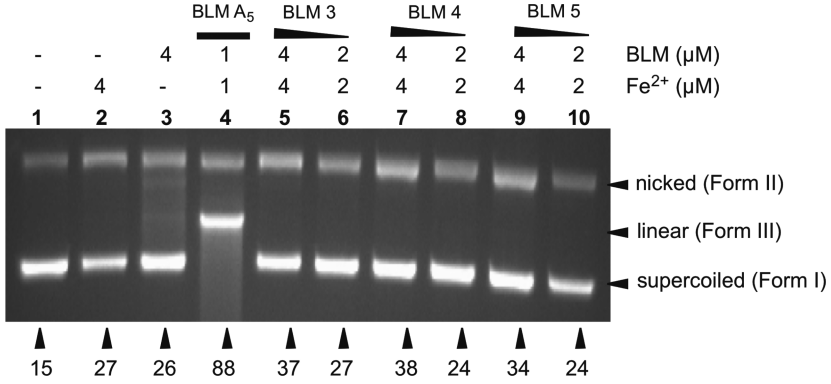


Figure 3. Relaxation of supercoiled pBR322 plasmid DNA by BLM A_5 (**1**) and BLM analogues **3**–**5**. Lane 1, DNA alone; lane 2, 4 μM Fe^{2+}; lane 3, 4 μM BLM A_5 (**1**); lane 4, 1 μM Fe(II)·BLM A_5 (**1**); lane 5, 4 μM Fe(II)·BLM **3**; lane 6, 2 μM Fe(II)·BLM **3**; lane 7, 4 μM Fe(II)·BLM **4**; lane 8, 2 μM Fe(II)·BLM **4**; lane 9, 4 μM Fe(II)·BLM **5**; lane 10, 2 μM Fe(II)·BLM **5**. The numbers below each lane represent the percentage of cleaved DNA relative to all DNA in the lane.

desilylated with 48% HF in water and then bisacetylated with Ac_2O in pyridine. Compound **22** was obtained in 88% overall yield from **20**. Finally, replacement of the fluorenylmethyloxycarbonyl (Fmoc) protecting group with a *tert*-butyloxycarbonyl (Boc) protecting group,[38] debenzylation with H_2 over $Pd(OH)_2/C$, and reinstallation of the Fmoc protecting group afforded **7** as a colorless solid in 57% overall yield. Key intermediate **7** was then converted to BLM analogue **3** by solid-phase synthesis[30,39,40] in an overall yield of 12% for the final four steps of the synthesis and HPLC purification (Scheme 3).

A slightly different strategy was used for synthesis of glycosylated threonine **8**. Synthesis began from the protected dibutyl phosphate derivative of 3-*O*-carbamoyl-β-D-glucopyranose (**11**) described above (Scheme 1). Following acetylation, intermediate **28** was converted to the respective phenylthiopyranoside (**29**) in 89% yield by treatment with thiophenol and TMSOTf (Scheme S1, Supporting Information). Fmoc-L-threonine benzyl ester (**19**) and gulopyranosyl fluoride **16** were treated with Cp_2ZrCl_2 and AgOTf, affording α-L-gulopyranosyl threonine derivative **30** as a colorless oil in 38% yield. Also formed in the same reaction was β-L-gulopyranosyl threonine derivative **37** (48% yield), which was employed for the synthesis of glycosylated threonine **9** (Scheme S2, Supporting Information). Following deacetylation of **30**[41] to afford **31**, treatment with **29** in the presence of *N*-iodosuccinimide and AgOTf[36] gave threonyl disaccharide **32** as a colorless foam in 70% yield. Desilylation (48% HF in H_2O, 96% yield) afforded **33**, which was peracetylated and converted to the respective Boc derivative **35** in 89% overall yield. Hydrogenolysis over $Pd(OH)_2/C$ and subsequent restoration of the Fmoc protecting group afforded **8** in 65% overall yield from **35**. Intermediate **8** was then converted to BLM analogue **4**, by analogy with the synthesis of BLM **3** from intermediate **7** (cf. Scheme 3).[30,39,40]

The strategy employed for synthesis of glycosylated threonine derivative **9** (Scheme S2, Supporting Information) was closely analogous to that used for the preparation of **8**. β-L-Gulopyranosylthreonine derivative **37**, obtained in the same

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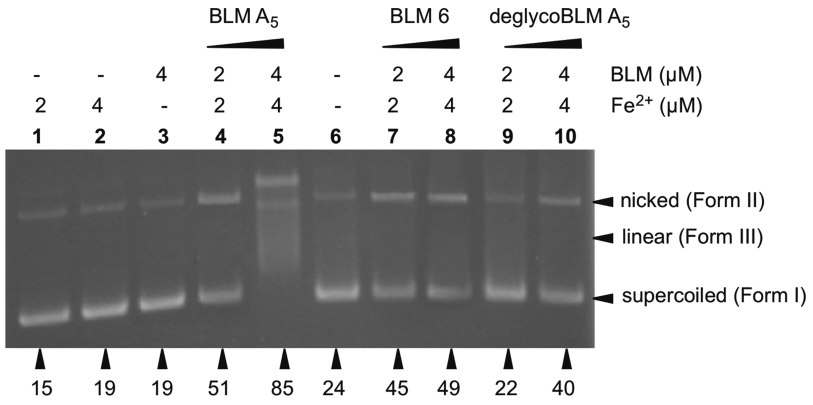

Figure 4. Relaxation of supercoiled pSP64 plasmid DNA by BLM A_5 (**1**) and BLM analogues **2** and **6**. Lane 1, 2 μM Fe^{2+}; lane 2, 4 μM Fe^{2+}; lane 3, 4 μM BLM A_5 (**1**); lane 4, 2 μM Fe(II)·BLM A_5 (**1**); lane 5, 4 μM Fe(II)·BLM A_5 (**1**); lane 6, DNA alone; lane 7, 2 μM Fe(II)·BLM **6**; lane 8, 4 μM Fe(II)·BLM **6**; lane 9, 2 μM Fe(II)·deglycoBLM (**2**); lane 10, 4 μM Fe(II)·deglycoBLM (**2**). The numbers below each lane represent the percentage of cleaved DNA relative to all DNA in the lane.

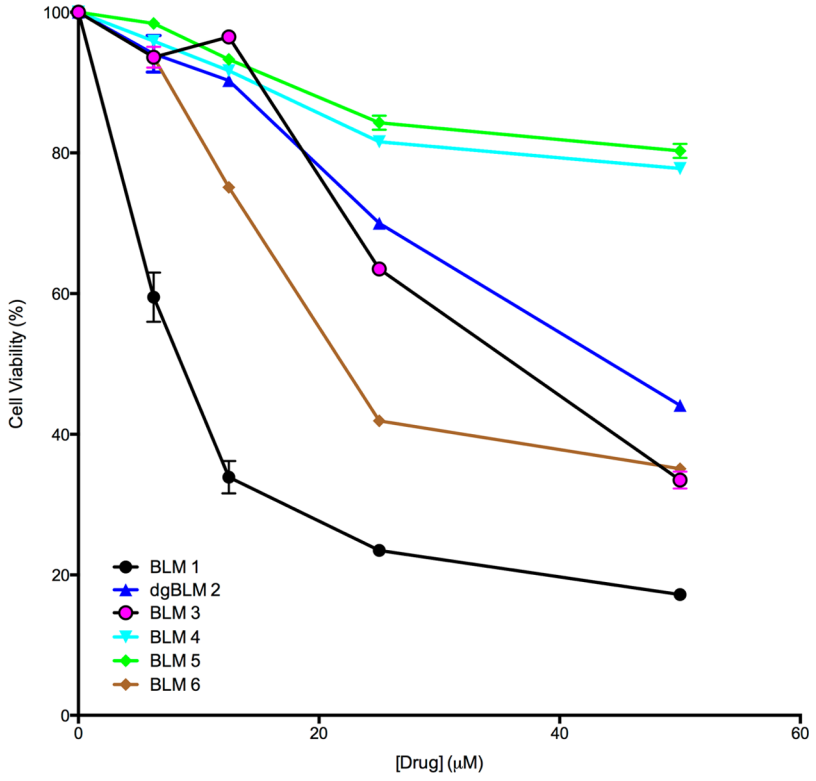

Figure 5. Dose-dependent effects of bleomycins **1**– **6** on the viability of cultured DU145 human prostate cancer cells.

reaction used for synthesis of **30** (vide supra), was deacetylated to afford **38**, the latter of which was combined with phenylthiopyranoside **29** in the presence of *N*-iodosuccinimde and AgOTf, affording glycosylated threonine **39** as a colorless foam in 63% yield. Desilylation and peracetylation gave **41** as a colorless foam in an overall yield of 72%. Replacement of the Fmoc protecting group with a Boc group was effected in 68% yield, and **42** was debenzylated by hydrogenolysis over $Pd(OH)_2/C$, providing **43** as a colorless solid in 97% yield. Reinstallation of the Fmoc protecting group then provided **9** in 70% overall yield from **43**. Intermediate **9** was converted to BLM analogue **5** by analogy with the synthesis of BLM **3** from intermediate **7** (cf Scheme 3).[30,39,40]

Synthesis of BLM analogue **6** was accomplished by use of the peracetylated BLM disaccharide **25**. The anomeric position of the gulose moiety was selectively deacetylated via the agency of the acetate salt of hydrazine to afford **26** in 70% yield, and the disaccharide was then activated as the *p*-nitrophenylcarbonate derivative (**27**) (Scheme 4). Condensation with the Cu(II) salt of deglycoBLM A_5 then afforded a carbamate linkage with the C-terminal substituent, and subsequent deacetylation and demetalation provided BLM **6** in 39% yield.

Biochemical and Biological Evaluation of Bleomycin Analogues. Shown in Figure 3 is the cleavage of supercoiled (form I) plasmid DNA by BLM derivatives **3**, **4**, and **5** in the presence of equimolar Fe^{2+}. While each of the analogues produced more form II (nicked plasmid) DNA than Fe^{2+} alone when used at 4 μM concentration, none mediated DNA cleavage as potently as Fe(II)·BLM A_5. Furthermore, no linear duplex (form III) DNA was apparent, indicating an absence of

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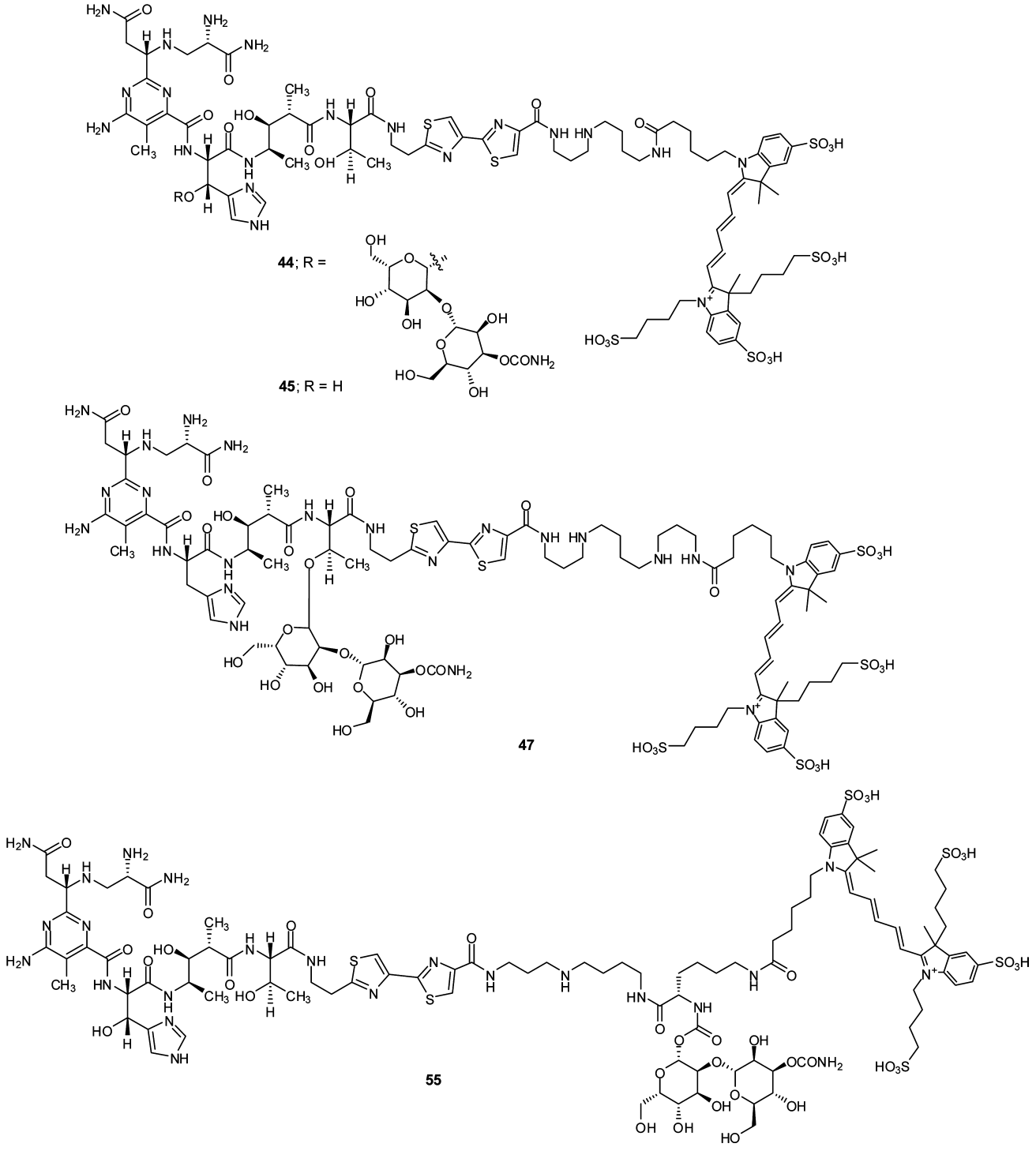

Figure 6. Structures of BLM derivatives conjugated to Cy5**, including analogues of BLM **1** (**44**), deglycoBLM **2** (**45**), BLM **3** (**47**), and BLM **6** (**55**).

double-strand DNA cleavage. DNA cleavage by BLM **6** was also studied and the results are shown in Figure 4. While Fe(II)·BLM **6** was less potent at relaxing plasmid DNA than Fe(II)·BLM A_5, it produced slightly more form II DNA than Fe(II)·deglycoBLM A_5 and more form II DNA than the Fe(II) chelates of BLM derivatives **3**, **4**, or **5**. Thus, BLM analogues **3**–**6** were all capable of DNA strand scission, but **6** was the most potent.

BLMs **1**–**6** were tested for their cytotoxicity toward cultured DU145 human prostate cancer cells (Figure 5). The cells were incubated for 24 h in the presence of four different concentrations of each BLM derivative. Cytotoxicity was determined by use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as a vital dye. As anticipated, BLM A_5 was the most potent of the derivatives tested, presumably reflecting both its efficient cleavage of DNA (Figures 3 and 4) and the presence of the BLM saccharide moiety, which has been shown to enable tumor cell targeting and uptake.[18] DeglycoBLM A_5, lacking the tumor-targeting disaccharide, was cytotoxic to DU145 cells but much less so

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than BLM A_5. BLM **3**, which has the same disaccharide moiety as BLM itself, was slightly more cytotoxic to cultured DU145 cells than deglycoBLM, in spite of the fact that it is slightly less potent in DNA cleavage (cf. Figures 3 and 4). Bleomycin derivatives **4** and **5**, which are isomeric with **3** but do not contain the same disaccharide as BLM, were only very weakly active as cytotoxins. Compound **6**, containing the BLM disaccharide attached to the C-terminal substituent, was less cytotoxic than BLM A_5 but significantly more cytotoxic than deglycoBLM or BLM **3**. Thus, the presence of the BLM disaccharide, attached to either of two positions on deglycoBLM other than the natural linkage via β-hydroxyhistidine, resulted in enhanced cytotoxicity. The rank order of cytotoxicities of BLMs **1**, **3** and **6** was the same as the order of their potencies of DNA cleavage.

To provide additional evidence in support of the basis for expression of cytotoxicity by glycosylated deglycoBLMs, the four cytotoxic derivatives were conjugated to the cyanine dye Cy5** (Figure 6). As shown in Figure 7, the dye-labeled conjugate of BLM **1** (**44**) was internalized the most efficiently, while uptake of dye-labeled deglycoBLM **2** (**45**) was the least efficient. The dye-labeled conjugate of BLM **6** (**55**) was internalized more efficiently than the dye-labeled conjugate of BLM **3** (**47**) but less efficiently than that of BLM **1** (**44**). Thus, the rank order of uptake of these conjugates was precisely the same as the rank order of observed cytotoxicities toward DU145 cells.

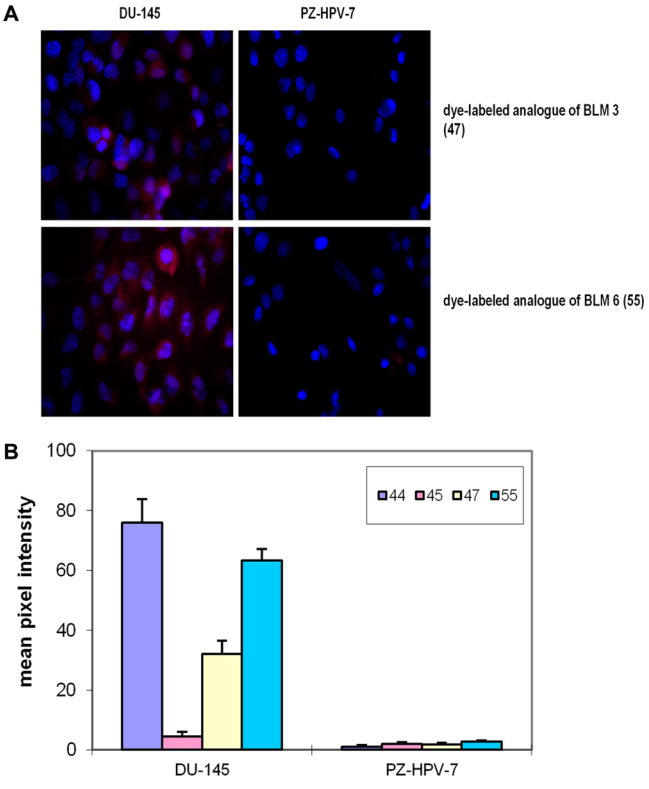

Figure 7. (A) Comparison of the binding/internalization of dye-labeled analogues of BLM **1** (**44**), deglycoBLM **2** (**45**), BLM **3** (**47**), and BLM **6** (**55**) in DU-145 prostate cancer cells and PZ-HPV-7 normal prostate cells. Cells were treated with 25 μM dye-labeled conjugates at 37 °C for 1 h, washed with phosphate-buffered saline (PBS), and fixed with 4% paraformaldehyde. The cell nuclei were stained with diamidino-2-phenylindole (DAPI). Fluorescence imaging was carried out after a 3 s exposure. (B) Quantification of the binding/internalization of dye-labeled conjugates in cultured prostate cancer and normal cells.

In an effort to identify the cellular receptor(s) for BLM and its sugars, we considered the possible involvement of metabolic events characteristic of cancer cells, notably the shift to glycolysis for ATP production and away from mitochondrial oxidative phosphorylation.[42] In an effort to mimic this process in normal cells, normal lung WI-38 cells and normal kidney CCD-1105 KIDTr cells were treated with a sublethal dose of the mitochondrial complex I inhibitor rotenone[43] for 24 h to force the cells to use glycolysis to a greater extent. As shown in Figure 8, following rotenone treatment, both cell lines exhibited the same enhanced update of BLM-Cy5** (**44**) as did the numerous cancer cell lines we have studied.[18]

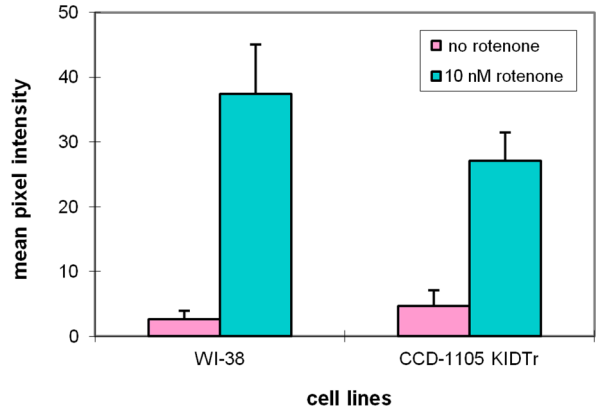

Figure 8. Effect of mitochondrial electron-transport chain (ETC) inhibitor on internalization of the dye-labeled analogue of BLM **1** (**44**) by cultured normal lung WI-38 cells and normal kidney CCD-1105 KIDTr cells. Cells were seeded to 16-well slides for 24 h, and then 25 μM dye conjugate was added to the cells at 37 °C for 1 h after the cells had been treated with 10 nM rotenone for 24 h. Cells were washed with PBS and fixed with 4% paraformaldehyde. Fluorescence imaging was carried out with a 3 s exposure time.

The facilitative glucose transporter GLUT1 is known to be inducible[44] and is subject to inhibition both by cytochalasin B[45] and phloretin,[46] although neither of these compounds is specific for this receptor. As shown in Figure 9, both of these known GLUT1 inhibitors effected substantial and dose-dependent inhibition of BLM-Cy5** binding/uptake in SW-480 colon cancer cells.

DISCUSSION

The present study was designed to define the contributions of the carbohydrate moiety of bleomycin to the DNA-cleaving and cytotoxic actions of this clinically used antitumor antibiotic. DeglycoBLM, the bleomycin analogue lacking the carbohydrate moiety, has been studied fairly extensively and shown to cleave duplex DNA by the same chemistry as BLM and with similar sequence selectivity.[29,47] However, the potency of supercoiled DNA relaxation by deglycoBLM is 2−3-fold less than for BLM, and much less double-strand cleavage is apparent for deglycoBLM.[26−31] Two studies in which the disaccharide moiety was replaced with a single sugar afforded BLM analogues with diminished potency of DNA cleavage[48,49] as well as a lesser ability to cleave RNA.[49] Thus, the disaccharide moiety attached to BLM via β-hydroxyhistidine significantly potentiates DNA cleavage and enables double-strand DNA cleavage and RNA cleavage.

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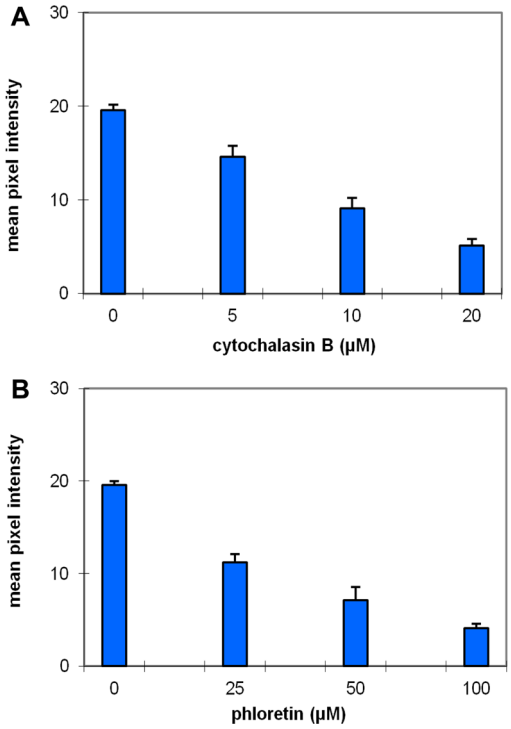

Figure 9. Effect of GLUT1 inhibitors on internalization of the dye-labeled analogue of BLM **1** (**44**) by SW-480 colon cancer cells. Cells were pretreated with different concentrations of (A) cytochalasin B or (B) phloretin at 37 °C for 2 h before incubation with 25 μM dye-labeled conjugate and then washed with PBS and fixed with 4% paraformaldehyde. Fluorescence imaging was carried out with a 2 s exposure time.

The present study in DU145 prostate cancer cells revealed that deglycoBLM was significantly less cytotoxic than BLM. It was initially unclear whether this diminished cytotoxic potential of deglycoBLM was due to diminished potency of DNA cleavage, lack of significant double-strand cleavage, or lack of tumor cell targeting and uptake. The recent study from our laboratory documenting tumor targeting and uptake by the disaccharide moiety itself[18] underscores the ambiguity intrinsic to defining the role(s) of the BLM disaccharide.

In order to gain insights into possible roles of the disaccharide, the present study considers the possibility that BLM may be modular in nature, composed of a carbohydrate moiety responsible for tumor targeting and uptake and an aglycon that mediates DNA strand scission. This hypothesis was tested by preparing analogues of BLM in which the disaccharide moiety is attached to BLM through structural elements other than the β-hydroxyhistidine moiety. If BLM actually is modular in nature, it might be anticipated that the disaccharide would still target tumor cells and the aglycon would still exert a cytotoxic effect via DNA strand scission in spite of the altered relative orientations of these two structural elements.

Analogue **3** differs from BLM in that it has the normal BLM disaccharide attached through the threonine moiety. This position has been shown to be reasonably tolerant of substitution,[50−52] although it has also been demonstrated that threonine is spatially close to a structural element in BLM that requires a specific conformation for optimal expression of DNA cleavage activity.[52−54] Analogues **4** and **5**, containing disaccharides isomeric to those in **3**, were anticipated to have DNA cleavage activities comparable to **3** but to lack any tumor targeting/uptake properties. Finally, BLM analogue **6** has a disaccharide moiety identical with that of BLM but attached to the end of the C-terminal substituent by means of a carbamate linkage. It was anticipated that the disaccharide itself would have little effect on the efficiency of DNA cleavage, such that the potency of DNA cleavage should be similar to that of deglycoBLM A_5 (**2**).

Synthesis of BLM Analogues. BLM analogues **3**−**5** were prepared by solid-phase synthesis essentially as described[30,39,40] using glycosylated threonine building blocks **7**−**9**, respectively. The synthesis of key intermediate **7** was accomplished by condensation of the activated disaccharide (**18**) with Fmoc-threonine benzyl ester (**19**). This was converted to **7** in several steps by the introduction of appropriate protecting groups for solid-phase synthesis (Scheme 2). A slightly different strategy was employed for the synthesis of **8** and **9**. Condensation of gulopyranosyl fluoride **16** with Fmoc-threonine benzyl ester (**19**) afforded a mixture of anomeric gulopyranosylthreonine derivatives **30** and **37** (Schemes S1 and S2, Supporting Information), which were separated by flash chromatography on a silica gel column. Intermediate **30** eluted subsequent to **37** from the silica gel column when washed with 2:1 hexanes−ethyl acetate. Following deacetylation to afford **31**, treatment with phenylthioglucoside **29** afforded key intermediate **32** (Scheme S1, Supporting Information). Protecting group optimization then gave intermediate **8**. The same strategy was used to convert **37** to disaccharide **39**, the latter of which was converted to intermediate **9** (Scheme S2, Supporting Information). Compounds **7**−**9** were then employed for the syntheses of BLM analogues **3**−**5** by solid-phase synthesis (Scheme 3).[30,39,40]

BLM analogue **6** was accessible by modification of deglycoBLM A_5. DeglycoBLM A_5 was treated with 1 equiv of Cu^{2+} to form a chelate in which the amino group of the β-aminoalanineamide substituent is rendered unreactive,[55,56] permitting the primary amine at the C-terminus of deglycoBLM A_5 to react selectively with electrophilic species. Thus, condensation of Cu(II)·deglycoBLM A_5 with the activated *p*-nitrophenylcarbonate derivative of BLM disaccharide (**27**) afforded Cu(II)·BLM **6**, which was demetalated and purified by reversed-phase HPLC (Scheme 4).

Evaluation of BLM Analogues. As shown in Figure 3, Fe(II)·BLMs **3**, **4**, and **5** all effected the relaxation of supercoiled pBR322 plasmid DNA when used at 4 μM concentration, but they did so rather weakly and without producing detectable double-strand cleavage. Thus, unlike BLM itself, which cleaves DNA more potently than deglycoBLM and with more associated double-strand breaks, introduction of the BLM disaccharide by attachment at the threonine moiety actually diminished DNA strand scission. This is consistent with reports that the linker region of BLM must assume a specific conformation to exhibit optimal DNA cleavage properties;[52−54] the introduction of a large substituent to threonine in the form of the disaccharide presumably precludes folding into the conformation optimal for DNA cleavage. For Fe(II)·BLM **6**, more significant DNA nicking was observed, as shown in Figure 4. This experiment employed supercoiled pSP64 plasmid DNA, also demonstrating that the source of the plasmid DNA was unimportant. The potency of cleavage was slightly greater than that of Fe(II)·deglycoBLM A_5. Furthermore, as might have been anticipated, introduction of the BLM saccharide within the C-terminal substituent had no effect on

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the extent of double-strand cleavage by **6** relative to deglycoBLM.

The cytotoxicity of the modified BLMs was studied in comparison with that of BLM A_5 and deglycoBLM A_5, by use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as a vital dye to assess cell viability. As shown in Figure 5, deglycoBLM A_5 was significantly less cytotoxic than BLM A_5, potentially due to multiple factors including its lower potency as a DNA cleaving agent, its propensity to produce fewer double-strand DNA breaks, and the absence of the tumor cell targeting disaccharide moiety. BLM analogue **3** was slightly more cytotoxic than deglycoBLM A_5 at the highest two concentrations tested. Since this analogue cleaves DNA no more potently than deglycoBLM A_5, its greater cytotoxic potential must be due to the tumor selectivity and uptake associated with the presence of the BLM disaccharide in **3**, even though the disaccharide is present in a "non-native" position. Consistent with this interpretation was the very weak cytotoxic activity of BLM analogues **4** and **5**, which have disaccharides in the same position as **3** and cleave DNA as well as **3** but whose disaccharides lack tumor-targeting properties.

BLM analogue **6**, having the BLM disaccharide appended to the C-terminal substituent, was intermediate in cytotoxicity between BLM A_5 and deglycoBLM A_5. Since BLM **6** cleaved DNA with a potency only slightly greater than that of deglycoBLM A_5 and produced no more double-strand cleavage than deglycoBLM A_5, its greater cytotoxicity can most logically be ascribed to the presence of the tumor-selective BLM disaccharide. This was true in spite of the fact that the disaccharide was attached to the molecule in a position quite different than that in BLM itself. This thesis was tested by preparing cyanine dye-labeled conjugates of the cytotoxic BLMs (**44**, **45**, **47**, and **55**, corresponding to BLMs **1**, **2**, **3**, and **6**, respectively) (Figure 6). As shown in Figure 7, the efficiencies of cellular uptake of these four BLM analogues were in precisely the same order as their cytotoxic potencies.

In the aggregate, these results strongly support the conclusion that BLM is modular, consisting of an aglycon responsible for DNA cleavage, connected to a tumor-selective disaccharide. It may be noted that the foregoing analysis presumes that all of the BLM derivatives studied kill DU145 cells by the same mechanism involving DNA cleavage. While there is no direct evidence in support of this assumption, it may be noted that a study of the relative potencies of DNA cleavage and cellular cytotoxicity of a number of BLM analogues failed to indicate a strict correlation between potency of DNA cleavage in vitro and cellular cytotoxicity but provides some general support for this assumption.[6]

The cytotoxicity of BLM, which is believed to form the basis for its antitumor activity, is often attributed to its ability to mediate double-strand breaks in DNA. While less potent than BLM A_5 as cytotoxic agents, deglycoBLM A_5 (**2**) and BLMs **3** and **6** all mediated killing of cultured DU145 cells. The potency of the three compounds as cytotoxins was strongly dependent on the presence of the BLM disaccharide moiety somewhere in their structure, and on their potency of supercoiled plasmid DNA relaxation. However, all three compounds exhibited minimal double-strand cleavage of plasmid DNA. An interesting issue not addressed in the present study is whether BLMs such as **2**, **3**, and **6** possess a mechanism for double-strand cleavage of chromatin, which is not apparent when simpler DNAs are used as substrates. A recent report from our laboratory suggests a mechanism by which this might occur.[57] Specifically, we have shown that DNAs strongly bound by Fe·BLMs undergo double-strand cleavage predominantly by a novel mechanism involving closely spaced independent single-strand cleavages. It may well be the case that such double-strand cleavage reactions constitute the molecular basis for antitumor activity of BLM. Such double-strand cleavage reactions would not be apparent in the analysis provided in Figures 3 and 4.

The broad selectivity for tumor cells noted for BLM and its sugars suggests that the targeting must involve some process common to all or most cancers. One well-known characteristic of tumor cells is the Warburg effect, that is, the propensity of tumor cells to produce increased amounts of ATP via glycolysis rather than by mitochondrial oxidative phosphorylation.[42] The shift to glycolysis is accompanied by upregulation of glucose transporters[44] to provide the greater amounts of glucose required to support increased glycolysis. In fact, treatment of two normal cell lines with the mitochondrial complex I inhibitor rotenone forced these cells to utilize increased glycolysis in the same fashion as tumor cells and resulted in their greatly enhanced ability to incorporate BLM-Cy5** (**44**) (Figure 8). In support of the possible involvement of glucose transporters in the binding and uptake of BLM (sugars), it was found (Figure 9) that GLUT1 inhibitors cytochalasin B and phloretin mediated dose-dependent inhibition of the binding/uptake of BLM-Cy5** (**44**) by SW-480 colon cancer cells. At least one sugar transporter (GLUT5) has been reported to facilitate the cellular uptake of a simple sugar (fructose) conjugated to a cyanine dye (Cy5.5).[58]

■ CONCLUSIONS

The effect of adding the BLM disaccharide to different positions of deglycoBLM was studied. Attachment to the β-hydroxyhistidine moiety, as in the natural product, significantly potentiated both DNA cleavage and cytotoxicity toward cultured DU145 human prostate cancer cells. When the BLM disaccharide was attached to the threonine moiety, DNA cleavage activity was significantly diminished but the cytotoxicity toward DU145 cells was enhanced nonetheless, arguing that the cytotoxicity of deglycoBLM is severely limited by lack of cellular uptake. Two analogues in which close structural analogues of the BLM disaccharide were attached to threonine exhibited similar DNA cleavage but essentially no cytotoxicity, consistent with the thesis that both facilitated uptake and DNA cleavage are essential to the expression of cytotoxicity by bleomycin. An analogue having the BLM saccharide attached to the C-terminal substituent exhibited relatively good DNA cleavage and cytotoxicity, entirely consistent with the need for efficient uptake and DNA cleavage as prerequisites for cytotoxicity. The relationship between (BLM sugar-mediated) cell uptake and cytotoxicity was confirmed by measuring the actual extent of incorporation of cytotoxic BLM analogues following conjugation to a cyanine dye. These findings confirm that BLM is a modular molecule, composed of a tumor cell targeting moiety (the saccharide) attached to a cytotoxic DNA-cleaving domain (the BLM aglycon). This finding implies that the BLM saccharide moiety may be able to deliver other cytotoxins selectively to tumor cells. The uptake mechanism is suggested to involve one or more cell surface receptors involved in glucose transport in tumor cells, which are upregulated in tumor cells to support their enhanced use of glycolysis.

EXPERIMENTAL PROCEDURES

Synthesis of Bleomycin Analogues. *4,6-Di-tert-butyl-O-silylene-3-O-carbamoyl-β-D-glucopyranosyl Dibutyl Phosphate (**11**).* To a stirred solution of 424 mg (1.70 mmol) of methyl trioxorhenium (MTO) and 4 Å molecular sieves in 150 mL of CH_2Cl_2 were added sequentially 11.9 g (126 mmol) of urea hydrogen peroxide (UHP), 10.0 mL (10.6 g, 50.0 mmol) of dibutyl phosphate, and 1.43 g (21.0 mmol) of pyrazole. The resulting yellow solution was stirred for 10 min, followed by addition of 14.0 g (42.0 mmol) of 3-*O*-carbamoyl-4,6-di-*O*-*tert*-butylsilylene-D-glucal (**10**).[33] The reaction mixture was stirred for 4 h and then filtered through a pad of Celite. The filtrate was concentrated under diminished pressure, and the residue was purified by flash chromatography on a silica gel column (44 × 4 cm). Elution with ethyl acetate gave **11** as a colorless foam: yield 7.22 g (30%); silica gel thin-layer chromatography (TLC) R_f 0.58 (ethyl acetate); $[\alpha]_D^{21}$ −18.8 (*c* 0.78, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.85−0.96 (m, 6H), 0.93 (s, 9H), 0.99 (s, 9H), 1.31−1.38 (m, 4H, *J* = 7.8 Hz), 1.56−1.64 (m, 4H, *J* = 7.2 Hz), 3.43−3.57 (m, 2H), 3.77−3.84 (t, 1H, *J* = 9.6 Hz), 3.84−3.91 (t, 1H, *J* = 9.9 Hz), 3.99−4.12 (m, 4H), 4.14−4.20 (m, 1H), 4.24−4.29 (m, 1H), 4.86−4.92 (t, 1H, *J* = 9.3 Hz), 5.0 (br s, 2H), and 5.30 (t, 1H, *J* = 7.8 Hz); ^{13}C NMR ($CDCl_3$) δ 13.5, 18.5, 19.9, 22.6, 26.9, 27.3, 31.95, 32.04, 66.0, 67.9, 68.06, 68.14, 71.0, 73.2, 73.3, 74.1, 77.8, 98.8 (d), and 157.8; ^{31}P NMR ($CDCl_3$) δ −1.53; mass spectrum (fast atom bombardment, FAB) *m*/*z* 556.1703 (M + H)$^+$ ($C_{23}H_{47}O_{10}NSiP$ requires 556.1706).

*4,6-Di-tert-butyl-O-silylene-3-O-carbamoyl-α-D-mannopyranosyl Dibutyl Phosphate (**12**).* The reaction was carried out in the same flask as for **11**. Elution of the silica gel column with ethyl acetate gave **12** as a colorless foam: yield 6.38 g (27%); silica gel TLC R_f 0.39 (ethyl acetate); $[\alpha]_D^{22}$ +34.4 (*c* 0.84, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.88−0.93 (m, 6H), 0.95 (s, 9H), 1.01 (s, 9H), 1.31−1.45 (m, 4H), 1.58−1.69 (m, 4H), 3.67−3.82 (dt, 1H, *J* = 9.3 and 8.7 Hz), 3.91−4.18 (m, 8H), 4.25 (t, 1H, *J* = 9.3 Hz), 4.89−4.96 (dd, 1H, *J* = 6.6 and 3.3 Hz), 5.28 (br s, 2H), and 5.58−5.60 (dd, 1H, *J* = 5.1 and 1.5 Hz); ^{13}C NMR ($CDCl_3$) δ 13.5, 18.6, 19.9, 22.5, 26.8, 27.3, 32.03, 32.10, 66.2, 68.0, 68.2, 69.2, 69.5, 71.3, 73.0, 98.0, and 156.6; ^{31}P NMR ($CDCl_3$) δ −2.03; mass spectrum (FAB) *m*/*z* 556.1704 (M + H)$^+$ ($C_{23}H_{47}O_{10}NSiP$ requires 556.1706).

*4,6-Di-tert-butyl-O-silylene-2-O-acetyl-3-O-carbamoyl-α-D-mannopyranosyl Dibutyl Phosphate (**13**).* To a cooled (0 °C) solution containing 0.412 g (0.741 mmol) of **12** in 1.5 mL of pyridine was added 0.140 mL (0.151 g, 0.148 mmol) of acetic anhydride. The reaction mixture was allowed to warm to room temperature and was stirred overnight. The solution was diluted with 20 mL of EtOAc and poured into 20 mL of ice water. The mixture was extracted with three 20 mL portions of EtOAc, and the combined organic extract was washed sequentially with three 25 mL portions of 0.5 M HCl and 25 mL of saturated aqueous $NaHCO_3$. The organic layer was then dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (36 × 2 cm). Elution with 1:1 hexanes−ethyl acetate gave **13** as a colorless oil: yield 0.411 g (93%); silica gel TLC R_f 0.23 (1:1 hexanes−ethyl acetate); $[\alpha]_D^{21}$ +11.0 (*c* 1.12, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.85−0.89 (m, 6H), 0.92 (s, 9H), 0.99 (s, 9H), 1.28−1.41 (m, 4H), 1.56−1.66 (m, 4H), 2.08 (s, 3H), 3.81−4.11 (m, 8H), 5.00 (br s, 2H), 5.06−5.10 (dd, 1H, *J* = 6.3 and 3.3 Hz), 5.25 (dd, 1H, *J* = 1.8 and 1.8 Hz), and 5.46−5.49 (dd, 1H, *J* = 4.8 and 1.8 Hz); ^{13}C NMR ($CDCl_3$) δ 13.4, 18.5, 19.8, 20.6, 22.5, 26.7, 27.2, 32.0, 32.1, 65.9, 67.8, 67.9, 69.0, 69.8, 70.6, 71.6, 95.2, 155.7, and 169.4; ^{32}P NMR ($CDCl_3$) δ −1.91; mass spectrum (FAB) *m*/*z* 598.2810 (M + H)$^+$ ($C_{25}H_{49}O_{11}NSiP$ requires 598.2813).

*4,6-Di-tert-butyl-O-silylene-2-O-acetyl-3-O-carbamoyl-1-phenylthio-α-D-mannopyranose (**14**).* To a cooled (−78 °C) solution containing 2.62 g (4.38 mmol) of **13** and 4 Å molecular sieves in 15 mL of CH_2Cl_2 were added sequentially 711 μL (723 mg, 6.57 mmol) of thiophenol and 1.98 mL (2.43 g, 10.9 mmol) of TMSOTf. The reaction mixture was allowed to warm to ambient temperature and was quenched by the addition of 10 mL of saturated aqueous $NaHCO_3$. The reaction mixture was diluted with 100 mL of CH_2Cl_2, washed sequentially with 50 mL of H_2O and 50 mL of brine, and then dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (41 × 3 cm). Elution with 2:1 hexanes−ethyl acetate gave **14** as colorless needles following crystallization from hot petroleum ether: yield 2.00 g (92%); silica gel TLC R_f 0.50 (2:1 hexanes−ethyl acetate); mp 89−91 °C; $[\alpha]_D^{22}$ +87.8 (*c* 1.08, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.02 (s, 9H), 1.05 (s, 9H), 2.12 (s, 3H), 3.96 (t, 1H, *J* = 10.2 Hz), 4.06 (m, 1H), 4.19 (t, 1H, *J* = 9.6 Hz), 4.37 (m, 1H), 5.10 (dd, 1H, *J* = 6.3 and 3.3 Hz), 5.11 (br s, 2H), 5.40 (s, 1H), 5.50 (d, 1H, *J* = 3.3 Hz), 7.26−7.33 (m, 3H), and 7.44 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 20.0, 20.9, 22.6, 26.9, 27.3, 66.1, 68.7, 71.8, 72.2, 72.4, 86.4, 127.8, 129.1, 131.7, 133.2, 155.9, and 169.8; mass spectrum (FAB) *m*/*z* 498.1984 (M + H)$^+$ ($C_{23}H_{36}O_7NSiS$ requires 498.1982).

*2-O-Acetyl-3,4,6-tri-O-benzyl-α,β-L-gulopyranosyl Fluoride (**16**).* To a cooled (0 °C) solution containing 1.00 g (1.97 mmol) of 3,4,6-tri-*O*-benzyl-1,2-*O*-(1-methoxyethylidene)-α-L-gulopyranose (**15**)[35] in 20 mL of dry CH_2Cl_2 was added dropwise 287 μL (350 mg, 2.17 mmol) of DAST. The solution was allowed to warm slowly to ambient temperature and stirred for 3 h. The solution was then diluted with 50 mL of CH_2Cl_2 and poured into 50 mL of ice water. The aqueous layer was extracted with three 25 mL portions of CH_2Cl_2, and the combined organic extract was washed sequentially with 50 mL of H_2O, 50 mL of saturated aqueous $NaHCO_3$, and 50 mL of brine and then dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (36 × 2 cm). Elution with 7:3 hexanes−ethyl acetate gave **16** as a pale yellow oil: yield 927 mg (95%); silica gel TLC R_f 0.84 (1:1 hexanes−ethyl acetate); $[\alpha]_D^{21}$ +41.4 (*c* 0.97, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 2.09 (s, 3H), 3.63−3.74 (m, 1H), 4.07 (q, 1H, *J* = 3.9 Hz), 4.34 (dt, 1H, *J* = 4.5 and 1.8 Hz), 4.82−4.63 (m, 6H), 5.16 (m, 1H), 5.54 (d, 0.5H, *J* = 7.2 Hz), 5.72 (d, 1H, *J* = 7.2 Hz), and 7.26−7.39 (m, 15H); ^{13}C NMR ($CDCl_3$) δ 20.8, 68.2, 70.3 (0.5C), 70.6 (0.5C), 72.6, 73.2, 73.4, 73.7 (0.5C), 73.8 (0.5C), 104.5 (0.5C), 107.3 (0.5C), 127.7, 127.8, 127.9, 128.1, 128.3, 128.4, 137.28, 137.32, 137.7, and 169.7; mass spectrum (FAB) *m*/*z* 493.2029 (M − H)$^+$ ($C_{29}H_{30}O_6F$ requires 493.2026).

*3,4,6-Tri-O-benzyl-α,β-L-gulopyranosyl Fluoride (**17**).* To a solution of 394 mg (797 μmol) of acetylated glycosyl fluoride **16** in 10 mL of dry MeOH was added 16.0 mg (119 μmol) of K_2CO_3. The solution was stirred for 4 h and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (34 × 1 cm). Elution with 2:1 hexanes−ethyl acetate gave **17** as a pale yellow oil: yield 348 mg (97%); silica gel TLC R_f 0.71 (1:1 hexanes−ethyl acetate); $[\alpha]_D^{21}$ −3.26 (*c* 0.38, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 2.57 (d, 1H, *J* = 8.1 Hz), 3.69 (m, 3H), 3.87 (m, 2H), 4.22 (dt, 1H, *J* = 4.8 and 1.8 Hz), 4.48−4.62 (m, 6H), 5.29 (d, 0.5H, *J* = 6.6 Hz), 5.47 (d, 0.5H, *J* = 6.6 Hz), and 7.25−7.41 (m, 15H); ^{13}C NMR ($CDCl_3$) δ 68.1, 68.6 (0.5C), 68.9 (0.5C), 72.67, 72.76 (0.5C), 72.80 (0.5C), 73.0, 73.2, 73.4, 76.3 (0.5C), 76.4 (0.5C), 107.2 (0.5C), 110.0 (0.5C), 127.66, 127.76, 127.81, 127.98, 128.05, 128.11, 128.3, 128.4, 128.5, 137.2, 137.3, and 137.7; mass spectrum (FAB), *m*/*z* 451.1920 (M − H)$^+$ ($C_{27}H_{28}O_5F$ requires 451.1921).

*3,4,6-Tri-O-benzyl-2-O-[4,6-di-tert-butyl-O-silylene-3-O-carbamoyl-2-O-acetyl-α-D-mannopyranosyl]-α,β-L-gulopyranosyl Fluoride (**18**).* To a cooled (−78 °C) solution containing 4 Å molecular sieves, 625 mg (1.38 mmol) of gulosyl fluoride **17**, and 687 mg (1.38 mmol) of phenylthiomannoside **14** in 8 mL of CH_2Cl_2 were added 466 mg (2.07 mmol) of *N*-iodosuccinimide and 71.0 mg (276 μmol) of AgOTf in the dark. The reaction mixture was allowed to warm to −5 °C and was quenched with 10 mL of saturated aqueous $NaHCO_3$ 90 s after the color changed from colorless to deep purple. The reaction mixture was diluted with 25 mL of CH_2Cl_2 and poured into 25 mL of saturated aqueous $Na_2S_2O_3$. The aqueous layer was extracted with three 50 mL portions of CH_2Cl_2. The combined organic extract was washed with 50 mL of H_2O and then dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (36 × 2 cm). Elution with 2:1 hexanes−ethyl acetate gave **18** as a colorless foam: yield 1.05 g (91%); silica gel TLC R_f 0.51 (2:1 hexanes−ethyl acetate); $[\alpha]_D^{22}$ +8.9 (*c* 0.39, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.96 (s, 9H), 1.05 (s, 9H), 2.17 (s, 3H), 3.53 (br s, 1H), 3.62 (m, 2H), 3.79 (m, 1H), 3.88 (m, 2H),

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4.11 (m, 4H), 4.25 (t, 1H, J = 6.6 Hz), 4.39−4.70 (m, 6H), 4.80 (s, 2H), 5.21 (m, 2H), 5.45 (d, 0.5H, J = 6.9 Hz), 5.63 (d, 0.5H, J = 6.9 Hz), 7.14 (m, 2H), and 7.26−7.36 (m, 13H); ^{13}C NMR ($CDCl_3$) δ 19.9, 20.9, 22.5, 26.8, 27.2, 66.1, 67.4, 68.1, 70.7, 71.4, 72.1, 72.8, 73.0, 73.1, 73.4, 73.6, 74.2, 95.2, 105.1 (0.5C), 108.0 (0.5C), 127.6, 127.7, 127.8, 127.9, 128.0, 128.27, 128.36, 128.41, 137.5, 137.7, 156.0, and 170.0; mass spectrum (FAB) m/z 862.3627 $(M + Na)^+$ ($C_{44}H_{58}O_{12}NSiFNa$ requires 862.3610).

*3,4,6-Tri-O-benzyl-2-O-[4,6-di-tert-butyl-O-silylene-3-O-carbamoyl-2-O-acetyl-α-D-mannopyranosyl]-α-L-gulopyranosyl-N^α-(9-fluorenylmethoxycarbonylamino)-L-threonine Benzyl Ester (**20**).* To a cooled (−15 °C) solution containing 4 Å molecular sieves, 187 mg (433 μmol) of Fmoc-L-threonine benzyl ester (**19**), and 400 mg (476 μmol) of fluorinated disaccharide **15** in 3 mL of CH_2Cl_2 were added 380 mg (1.30 mmol) of Cp_2ZrCl_2 and 334 mg (1.30 mmol) of AgOTf. The reaction mixture was stirred at −15 °C for 3 h until complete consumption of the starting glycosyl fluoride was observed. The solution was filtered through a pad of Celite, and the filtrate was concentrated under a stream of N_2. The residue was purified by flash chromatography on a silica gel column (48 × 3 cm). Elution with 2:1 hexanes−ethyl acetate gave **20** as a colorless foam: yield 376 mg (68%); silica gel TLC R_f 0.74 (1:1 hexanes−ethyl acetate); $[\alpha]_D^{21}$ −15.7 (c 0.17, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.98 (s, 9H), 1.07 (s, 9H), 1.35 (d, 3H, J = 6.0 Hz), 2.16 (s, 3H), 3.52−3.55 (m, 2H), 3.65 (d, 1H, J = 2.7 Hz), 3.82 (s, 1H), 3.88 (dd, 1H, J = 5.4 and 4.2 Hz), 3.97 (m, 1H), 4.05 (t, 1H, J = 3.6 Hz), 4.11−4.25 (m, 6H), 4.33−4.56 (m, 10H), 4.66 (s, 1H), 4.78 (s, 1H), 4.96 (d, 1H, J = 3.6 Hz), 5.14 (d, 1H, J = 2.1 Hz), 5.19−5.25 (m, 2H), 6.53 (d, 1H, J = 8.1 Hz), 7.20−7.34 (m, 24H), 7.61 (d, 2H, J = 7.5 Hz), and 7.72 (d, 2H, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 16.6, 19.8, 21.0, 22.6, 26.8, 27.3, 47.0, 59.2, 65.7, 66.3, 67.0, 67.2, 68.0, 68.5, 70.3, 70.7, 70.8, 71.4, 72.1, 72.5, 72.8, 73.0, 73.2, 74.4, 77.2, 95.1, 95.6, 119.7, 125.3, 125.4, 127.0, 127.4, 127.5, 127.7, 127.9, 128.0, 128.1, 128.2, 128.4, 128.65, 128.71, 137.7, 137.95, 138.02, 141.1, 144.0, 144.1, 155.6, 157.0, 169.8, and 170.5; mass spectrum (FAB) m/z 1251.5475 $(M + H)^+$ ($C_{70}H_{83}O_{17}N_2Si$ requires 1251.5461).

*3,4,6-Tri-O-benzyl-2-O-[3-O-carbamoyl-2-O-acetyl-α-D-mannopyranosyl]-α-L-gulopyranosyl-N^α-(9-fluorenylmethoxycarbonylamino)-L-threonine Benzyl Ester (**21**).* To a solution of 180 mg (143 μmol) of threonine **20** in 3 mL of acetonitrile in a plastic tube was carefully added 100 μL (5.00 mmol) of 48% HF in H_2O. The solution was stirred overnight and then poured slowly into a mixture of 20 g of ice and 20 mL of saturated aqueous $NaHCO_3$. The aqueous layer was extracted with three 50 mL portions of ethyl acetate, and the combined organic extract was washed with 50 mL of H_2O. The organic layer was then dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (38 × 1 cm). Elution with 88:10:2 chloroform−methanol−acetic acid gave **21** as a colorless foam: yield 0.142 g (89%); silica gel TLC R_f 0.50 (88:10:2 chloroform−methanol−acetic acid); $[\alpha]_D^{22}$ −5.2 (c 0.14, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.18 (d, 2H, J = 6.0 Hz), 1.28 (br s, 1H), 2.08 (s, 3H), 3.51 (dd, 1H, J = 5.4 and 4.5 Hz), 3.64 (m, 1H), 3.76−3.94 (m, 7H), 4.09 (br s, 2H), 4.17 (m, 1H), 4.23 (t, 1H, J = 6.3 Hz), 4.40−4.59 (m, 9H), 4.64 (br s, 2H), 4.99 (br s, 3H), 5.10 (d, 1H, J = 8.4 Hz), 5.12−5.24 (q, 2H, J = 12.3 and 11.7 Hz), 5.35 (s, 1H), 6.72 (d, 1H, J = 8.1 Hz), 7.19−7.40 (m, 24H), 7.65 (d, 2H, J = 6.9 Hz), and 7.75 (d, 2H, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 15.3, 20.9, 29.6, 47.1, 59.0, 62.6, 66.7, 67.5, 68.7, 70.1, 71.1, 71.8, 72.4, 72.7, 73.2, 73.4, 93.1, 97.2, 119.8, 125.0, 125.2, 127.1, 127.5, 127.6, 127.7, 127.8, 128.0, 128.1, 128.3, 135.3, 137.8, 138.0, 141.1, 143.8, 143.9, 156.4, 157.1, 169.8, and 171.0; mass spectrum (FAB) m/z 1111.4447 $(M + H)^+$ ($C_{62}H_{67}O_{17}N_2$ requires 1111.4439).

*3,4,6-Tri-O-benzyl-2-O-(3-O-carbamoyl-2,4,6-tri-O-acetyl-α-D-mannopyranosyl)-α-L-gulopyranosyl-N^α-(9-fluorenylmethoxycarbonylamino)-L-threonine Benzyl Ester (**22**).* To a cooled (0 °C) solution containing 0.102 g (0.092 mmol) of **21** in 2 mL of pyridine was added 0.043 mL (0.047 g, 0.485 mmol) of acetic anhydride. The reaction mixture was allowed to warm to ambient temperature and then stirred overnight. The solution was diluted with 25 mL of EtOAc and poured into 10 mL of ice water. The aqueous layer was extracted with three 25 mL portions of EtOAc, and the combined organic extract was washed sequentially with three 50 mL portions of 0.5 M HCl, 50 mL of H_2O, and 50 mL of brine and then dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (48 × 2 cm). Elution with 1:1 hexanes−ethyl acetate gave **22** as a colorless oil: yield 0.109 g (99%); silica gel TLC R_f 0.55 (1:1 hexanes−ethyl acetate); $[\alpha]_D^{21}$ −17.4 (c 0.15, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.33 (d, 3H, J = 6.0 Hz), 2.01 (s, 3H), 2.06 (s, 3H), 2.15 (s, 3H), 3.48−3.59 (m, 2H), 3.69 (d, 1H, J = 2.4 Hz), 3.85 (t, 1H, J = 3.3 Hz), 4.05 (m, 3H), 4.21−4.52 (m, 10H), 4.56−4.68 (m, 3H), 4.83 (d, 3H, J = 3.6 Hz), 4.99 (d, 1H, J = 3.6 Hz), 5.08−5.19 (m, 2H), 5.30 (m, 3H), 6.43 (d, 1H, J = 8.1 Hz), 7.19−7.37 (m, 24H), 7.60 (d, 2H, J = 7.5 Hz), and 7.72 (d, 2H, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 16.6, 20.59, 20.64, 20.9, 47.0, 59.2, 62.3, 65.7, 67.0, 67.2, 68.1, 69.0, 69.8, 69.9, 72.0, 72.2, 72.7, 73.0, 73.2, 74.4, 94.8, 96.1, 119.7, 125.2, 125.3, 126.95, 126.99, 127.48, 127.55, 127.64, 127.8, 127.9, 128.00, 128.1, 128.2, 128.4, 135.4, 137.6, 137.9, 141.1, 143.8, 144.0, 155.1, 157.0, 169.7, 169.9, 170.4, and 170.5; mass spectrum (FAB) m/z 1195.4641 $(M + H)^+$ ($C_{66}H_{71}O_{19}N_2$ requires 1195.4651).

*3,4,6-Tri-O-benzyl-2-O-(3-O-carbamoyl-2,4,6-tri-O-acetyl-α-D-mannopyranosyl)-α-L-gulopyranosyl-N^α-(t-butoxycarbonylamino)-L-threonine Benzyl Ester (**23**).* To a solution containing 0.101 g (0.085 mmol) of threonine disaccharide **22** in 2 mL of *N*,*N*-dimethylformamide (DMF) were added sequentially 0.034 g (0.592 mmol) of KF, 0.025 mL (0.018 g, 0.179 mmol) of Et_3N, and 0.024 g (0.111 mmol) of $(Boc)_2O$. The reaction mixture was stirred at ambient temperature until complete consumption of the starting material was observed by silica gel TLC analysis. The solution was diluted with 25 mL of EtOAc and washed with 10 mL of H_2O. The aqueous layer was back-extracted with three 10 mL portions of EtOAc, and the combined organic extract was washed sequentially with 25 mL of saturated aqueous $NaHCO_3$ and 25 mL of brine and then dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (46 × 2 cm). Elution with 2:1 hexanes−ethyl acetate gave **23** as a colorless oil: yield 0.078 g (86%); silica gel TLC R_f 0.68 (3:1 EtOAc−hexanes); $[\alpha]_D^{22}$ −18.1 (c 0.16, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.25 (d, 3H, J = 5.7 Hz), 1.40 (s, 9H), 2.05 (s, 3H), 2.07 (s, 3H), 2.15 (s, 3H), 3.44−3.55 (m, 2H), 3.66 (s, 1H), 3.82 (t, 1H, J = 3.3 Hz), 3.98 (t, 1H, J = 3.3 Hz), 4.01−4.13 (m, 2H), 4.21 (t, 1H, J = 6.0 Hz), 4.33−4.49 (m, 7H), 4.56−4.68 (m, 2H), 4.83 (s, 2H), 4.95 (d, 1H, J = 3.6 Hz), 5.08 (s, 2H), 5.29 (m, 3H), 5.76 (d, 2H, J = 8.4 Hz), 7.17 (m, 2H), and 7.26−7.30 (m, 18H); ^{13}C NMR ($CDCl_3$) δ 16.3, 20.6, 20.8, 20.9, 28.2, 58.7, 62.3, 65.8, 66.9, 68.2, 68.9, 69.9, 72.2, 72.7, 72.8, 73.0, 73.2, 74.8, 79.7, 94.6, 96.6, 127.5, 127.6, 127.9, 128.1, 128.2, 128.4, 135.5, 137.7, 138.0, 138.2, 155.1, 156.3, 169.8, 169.9, 170.5, and 170.6; mass spectrum (FAB) m/z 1073.4507 $(M + H)^+$ ($C_{56}H_{69}O_{19}N_2$ requires 1073.4495).

*2-O-(3-O-Carbamoyl-2,4,6-tri-O-acetyl-α-D-mannopyranosyl)-α-L-gulopyranosyl-N^α-(t-butoxycarbonylamino)-L-threonine (**24**).* A suspension containing 150 mg (140 μmol) of threonine disaccharide **23** and 20 mg of $Pd(OH)_2$/C in 10 mL of 1:1 tetrahydrofuran (THF)−MeOH was purged with H_2 and maintained under a H_2 atmosphere overnight. The reaction mixture was then diluted with MeOH and filtered through a pad of Celite, and the filtrate was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (39 × 1 cm). Elution with 83:15:2 chloroform−methanol−acetic acid gave **24** as a colorless solid: yield 94 mg (95%); silica gel TLC R_f 0.45 (3:2 ethyl acetate−methanol); $[\alpha]_D^{22}$ +1.7 (c 0.24, MeOH); ^{1}H NMR (methanol-d_4) δ 1.12 (d, 3H, J = 4.8 Hz), 1.35 (s, 9H), 1.94 (s, 3H), 1.95 (s, 3H), 2.03 (s, 3H), 3.65 (m, 3H), 3.90−4.01 (m, 6H), 4.08−4.19 (m, 2H), 4.34 (br s, 1H), 4.90 (d, 1H, J = 3.3 Hz), 4.95 (s, 1H), 5.15 (m, 1H), and 5.27 (s, 1H); ^{13}C NMR (methanol-d_4) δ 16.1, 20.7, 20.8, 22.1, 28.8, 61.2, 62.9, 63.7, 67.6, 68.0, 69.7, 70.5, 70.8, 71.2, 71.5, 74.6, 80.4, 96.1, 97.0, 158.2, 158.4, 171.4, 171.6, 172.4, and 177.7; mass spectrum (FAB) m/z 735.2430 $(M + Na)^+$ ($C_{28}H_{44}O_{19}N_2Na$ requires 735.2436).

2-O-(3-O-Carbamoyl-2,4,6-tri-O-acetyl-α-D-mannopyranosyl)-α-L-gulopyranosyl-N^α-(9-fluorenylmethoxycarbonylamino)-L-threo-

nine (7). To a solution containing 140 mg (196 μmol) of **24** in 2 mL of CH_2Cl_2 were added 0.2 mL of Me_2S and 2 mL of CF_3COOH. The reaction mixture was maintained at ambient temperature until complete consumption of the starting material was observed by silica gel TLC analysis. The reaction mixture was diluted with 5 mL of toluene and then concentrated under diminished pressure. The residue was then dissolved in 2 mL of saturated aqueous $NaHCO_3$. To the solution was added a solution containing 67.0 mg (200 μmol) of FmocOSu in 2 mL of CH_3CN. The reaction mixture was stirred under N_2 for 8 h and then diluted with 25 mL of EtOAc. The solution was washed with 25 mL of 0.5 M HCl, and the aqueous layer was back-extracted with three 25 mL portions of EtOAc. The combined organic extract was then dried ($MgSO_4$) and concentrated under diminished pressure. Purification by flash chromatography on a silica gel column gave **7** as a colorless solid: yield 114 mg (70% over two steps); silica gel TLC R_f 0.17 (85:13:2 chloroform−methanol−acetic acid); $[\alpha]_D^{21}$ +23.4 (*c* 0.10, MeOH); ^{1}H NMR (methanol-d_4) δ 1.31 (d, 3H, J = 6.3 Hz), 2.05 (s, 3H), 2.13 (s, 3H), 2.30 (s, 3H), 3.75 (d, 2H, J = 5.7 Hz), 3.87 (d, 1H, J = 3.0 Hz), 4.07−4.16 (m, 5H), 4.22−4.39 (m, 5H), 4.50 (dd, 1H, J = 3.6 and 2.4 Hz), 5.02 (d, 1H, J = 3.3 Hz), 5.09 (d, 1H, J = 1.2 Hz), 5.29 (d, 2H), 5.42 (m, 1H), 7.10−7.20 (m, 2H), 7.30 (t, 2H, J = 7.2 Hz), 7.37 (t, 2H, J = 6.9 Hz), 7.70 (d, 2H, J = 7.2 Hz), and 7.76 (d, 2H, J = 6.9 Hz); ^{13}C NMR (methanol-d_4) δ 16.6, 20.7, 60.2, 62.5, 63.7, 67.5, 67.6, 68.3, 69.0, 70.4, 70.8, 71.20, 71.23, 71.4, 74.7, 96.7, 97.1, 120.9, 126.3, 126.4, 128.2, 128.8, 129.2, 129.9, 142.5, 145.2, 145.3, 158.1, 159.3, 171.4, 171.6, 172.5, and 174.1; mass spectrum (electrospray) m/z 835.2766 $(M + H)^+$ ($C_{38}H_{47}O_{19}N_2$ requires 835.2773).

Solid-Phase Synthesis of Deglycobleomycin Analogue 3. *Resin-Bound Spermine Tripeptide.* To 0.366 g (loading of 0.082 mmol/g) of swollen NovaSyn TentaGel amino resin functionalized with the Fmoc-protected bithiazole moiety and BLM A_6 C-substituent (Scheme 3)[39,40] was added a 20% solution of piperidine in 2 mL of DMF. The reaction mixture was shaken for 5 min, and the solvent was removed by filtration. The resin was washed successively with three 10 mL portions of CH_2Cl_2, three 10 mL portions of MeOH, and three 10 mL portions of DMF. The piperidine deblocking step was repeated twice and the resin was washed successively with three 10 mL portions of CH_2Cl_2, three 10 mL portions of MeOH, and three 10 mL portions of DMF. An aliquot of the resin was removed, and a qualitative Kaiser free amine test indicated the presence of a free amine. The resin was suspended in a solution of 0.068 g (0.180 mmol) of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 0.150 g (0.180 mmol) of threonine disaccharide **7**, 0.027 g (0.180 mmol) of hydroxybenzotriazole (HOBt), and 0.063 mL (0.360 mmol) of *N*,*N*-diisopropylethylamine (DIPEA) in 2 mL of DMF. The reaction mixture was shaken for 45 min, and the solvent was removed by filtration. The resin was washed successively with three 10 mL portions of CH_2Cl_2, three 10 mL portions of MeOH, and three 10 mL portions of DMF. An aliquot of the resin was removed, and a qualitative Kaiser free amine test indicated the absence of a free amine. An aliquot of the resin was subjected to Fmoc cleavage assay analysis, which indicated a loading of 0.032 mmol/g (corresponding to 42% coupling efficiency). The resin was dried under diminished pressure and carried on to the methylvalerate coupling step: mass spectrum (matrix-assisted laser desorption ionization time-of-flight, MALDI-TOF) m/z 1108.3 $(M + H)^+$ (theoretical m/z 1108.3).

Resin-Bound Spermine Tetrapeptide. To 0.355 g (loading of 0.032 mmol/g) of swollen tripeptide resin was added a 20% solution of piperidine in 2 mL of DMF. The reaction mixture was shaken for 5 min, and the solvent was removed by filtration. The resin was washed successively with three 10 mL portions of CH_2Cl_2, three 10 mL portions of MeOH, and three 10 mL portions of DMF. The piperidine deblocking step was repeated twice and the resin was washed successively with three 10 mL portions of CH_2Cl_2, three 10 mL portions of MeOH, and three 10 mL portions of DMF. An aliquot of the resin was removed, and a qualitative Kaiser free amine test indicated the presence of a free amine. The resin was suspended in a solution of 0.021 g (0.057 mmol) of Fmoc-methylvalerate,[39,40] 0.022 g (0.057 mmol) of HBTU, 0.009 g (0.057 mmol) of HOBt, and 0.020 mL (0.114 mmol) of Hunig's base (DIPEA) in 2 mL of DMF. The reaction mixture was stirred for 45 min, and the solvent was removed by filtration. The resin was washed successively with three 10 mL portions of CH_2Cl_2, three 10 mL portions of MeOH, and three 10 mL portions of DMF. An aliquot of the resin was removed, and a qualitative Kaiser free amine test indicated the absence of a free amine. An aliquot of the resin was subjected to Fmoc cleavage assay analysis, which indicated a loading of 0.029 mmol/g (corresponding to 94% coupling efficiency). The resin was dried under diminished pressure and carried on to the histidine coupling step: mass spectrum (MALDI-TOF) m/z 1237.4 $(M + H)^+$ (theoretical m/z 1237.4).

Resin-Bound Spermine Pentapeptide. To 0.350 g (loading of 0.029 mmol/g) of swollen tetrapeptide resin was added a 20% solution of piperidine in 2 mL of DMF. The reaction mixture was shaken for 10 min, and then the solvent was removed by filtration. The resin was washed successively with three 10 mL portions of CH_2Cl_2, three 10 mL portions of MeOH, and three 10 mL portions of DMF. The piperidine deblocking step was repeated twice and the resin was washed successively with three 10 mL portions of CH_2Cl_2, three 10 mL portions of MeOH, and three 10 mL portions of DMF. An aliquot of the resin was removed, and a qualitative Kaiser free amine test indicated the presence of a free amine. The resin was suspended in a solution of 0.031 g (0.050 mmol) of Fmoc-tritylhistidine, 0.019 g (0.050 mmol) of 2-(7-aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), 0.007 g (0.050 mmol) of 1-hydroxy-7-azabenzotriazole (HOAt), and 0.017 mL (0.100 mmol) of Hunig's base in 2 mL of DMF. The reaction mixture was stirred for 45 min, and the solvent was removed by filtration. The resin was washed successively with three 10 mL portions of CH_2Cl_2, three 10 mL portions of MeOH, and three 10 mL portions of DMF. An aliquot of the resin was removed, and a qualitative Kaiser free amine test indicated the absence of a free amine. An aliquot of the resin was subjected to Fmoc cleavage assay analysis, which indicated a loading of 0.051 mmol/g. The resin was dried under diminished pressure: mass spectrum (MALDI-TOF) m/z 1616.5 $(M + H)^+$ (theoretical m/z 1616.5).

Deglycobleomycin Analogue 3. To a suspension containing 0.118 g of pentapeptide resin were added three 0.5 mL solutions containing 20% piperidine in DMF for 10 min each. The resulting resin was washed for 30 s each with seven 5 mL portions of DMF, five 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 5.00 mg (11.6 μmol) of Boc-pyrimidoblamic acid[59−61] and 19.0 mg (34.8 μmol) of (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) 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 12 μL (9.0 mg, 70 μmol) of Hunig'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 MeOH. The resin was dried under vacuum for 2 h. To a suspension containing 0.076 g of resin-bound fully protected deglycoBLM A_6 precursor was added a solution containing 200 μL of triisopropylsilane and 200 μL of methyl sulfide. After 5 min, 4 mL of trifluoroacetic acid (TFA) was added to the suspension. After 4 h, the resin was filtered and washed with five 3 mL portions of CH_2Cl_2 and three 3 mL portions of DMF. The resulting resin was treated with 0.5 mL of a solution containing 20% hydrazine in DMF. The resin was filtered and then treated with three 0.5 mL portions of 20% hydrazine in DMF for an additional 10 min each. The eluate was collected and concentrated under diminished pressure. The resulting oil was dissolved in 0.1% aqueous CF_3COOH, frozen, and lyophilized. The crude product was purified on an Alltech Alltima C_{18} reversed-phase semipreparative (250 × 10 mm, 5 μm) HPLC column by use of 0.1% aqueous CF_3COOH and CH_3CN mobile phases. A linear gradient was employed (90:10 0.1% aqueous CF_3COOH−CH_3CN → 70:30 0.1% aqueous CF_3COOH−CH_3CN) over a period of 30 min at a flow rate of 4 mL/min. Fractions containing the desired product eluted at 13.2 min and were collected, frozen, and lyophilized to give **3** as a colorless foam: yield 1.1 mg (12%); ^{1}H NMR ($CDCl_3$) δ 0.93 (d, 3H, J = 7.0

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Hz), 1.05 (d, 3H, J = 6.5 Hz), 1.06 (d, 3H, J = 7.5 Hz), 1.66 (m, 4H), 1.90−1.97 (m, 7H), 2.54−2.60 (m, 3H), 2.93−3.04 (m, 10H), 3.15 (dd, 1H, J = 7.5 and 7.0 Hz), 3.20 (t, 1H, J = 4.5 Hz), 3.41 (m, 1H), 3.54−3.59 (m, 4H), 3.58−3.66 (m, 2H), 3.73−3.79 (m, 4H), 3.88 (t, 1H, J = 6.0 Hz), 3.94 (t, 1H, J = 4.0 Hz), 4.02 (m, 2H), 4.15 (m, 1H), 4.39 (d, 1H, J = 3.5 Hz), 4.49 (d, 1H, J = 2.5 Hz), 4.57 (d, 1H, J = 1.5 Hz), 4.96 (d, 1H, J = 4.0 Hz), 7.24 (s, 1H), 7.94 (s, 1H), 8.10 (s, 1H), and 8.55 (s, 1H); mass spectrum (electrospray) m/z 1481.5 $(M + H)^+$, theoretical m/z 1481.7; mass spectrum (TOF ES+) m/z 1481.6633 $(M+H)^+$ ($C_{60}H_{97}N_{20}O_{20}S_2$ requires 1481.6631).

Deglycobleomycin Analogue ***4***. The crude product from the solid-phase synthesis (Supporting Information) was purified on an Alltech Alltima C_{18} reversed-phase semipreparative (250 × 10 mm, 5 μm) HPLC column by the use of 0.1% CF_3COOH and CH_3CN mobile phases. A linear gradient was employed (90:10 0.1% aqueous CF_3COOH−CH_3CN → 70:30 0.1% aqueous CF_3COOH−CH_3CN) over a period of 30 min at a flow rate of 4 mL/min. Fractions containing the desired product eluted at 13.3 min and were collected, frozen, and lyophilized to give **4** as a colorless foam: yield 2.0 mg (22%); ^{1}H NMR ($CDCl_3$) δ 1.00 (d, 3H, J = 6.3 Hz), 1.13 (d, 3H, J = 4.8 Hz), 1.15 (d, 3H, J = 5.7 Hz), 1.75 (m, 5H), 1.98−2.07 (m, 8H), 2.62 (m, 2H), 2.98 (m, 2H), 3.03−3.14 (m, 12H), 3.20−3.30 (m, 6H), 3.40 (t, 2H, J = 8.7 Hz), 3.50 (m, 6H), 3.64−3.68 (m, 5H), 3.69−3.73 (m, 3H), 3.81−3.96 (m, 8H), 5.06 (m, 2H), 4.13 (t, 1H, J = 3.0 Hz), 4.27 (m, 2H), 4.46 (d, 1H, J = 2.4 Hz), 4.65 (m, 4H), 5.17 (d, 1H, J = 3.3 Hz), 7.32 (s, 1H), 8.02 (d, 1H, J = 0.9 Hz), 8.18 (d, 1H, J = 1.2 Hz), and 8.63 (s, 1H); mass spectrum (electrospray) m/z 1481.6 $(M + H)^+$, theoretical m/z 1481.7; mass spectrum (TOF ES+), m/z 1481.6631 (M + H) ($C_{60}H_{97}N_{20}O_{20}S_2$ requires 1481.6631).

Deglycobleomycin Analogue ***5***. The crude product from the solid-phase synthesis (Supporting Information) was purified on an Alltech Alltima C_{18} reversed-phase semipreparative (250 × 10 mm, 5 μm) HPLC column by use of 0.1% CF_3COOH and CH_3CN mobile phases. A linear gradient was employed (90:10 0.1% aqueous CF_3COOH−CH_3CN → 70:30 0.1% aqueous CF_3COOH−CH_3CN) over a period of 30 min at a flow rate of 4 mL/min. Fractions containing the desired product eluted at 14.0 min and were collected, frozen, and lyophilized to give **5** as a colorless foam: yield 0.2 mg (2%); mass spectrum (electrospray) m/z 1481.6 $(M + H)^+$, theoretical m/z 1481.7.

*3,4,6-Tri-O-acetyl-2-O-(2,4,6-tri-O-acetyl-3-O-carbamoyl-α-*D*-mannopyranosyl)-α,β-*L*-gulopyranoside (**26**).*[62,63] To a solution of 83.0 mg (0.12 mmol) of 1,3,4,6-tetra-*O*-acetyl-2-*O*-(2,4,6-tri-*O*-acetyl-3-*O*-carbamoyl-α-D-mannopyranosyl)-α-L-gulopyranoside (**25**) in 2 mL of dry DMF was added 15.0 mg (0.16 mmol) of the acetate salt of hydrazine at 0 °C. The reaction mixture was warmed to room temperature and stirred for 1 h, at which time analysis by silica gel TLC indicated that the starting material had been completely consumed. The reaction mixture was diluted with 50 mL of EtOAc, washed with three 15 mL portions of brine, dried over Na_2SO_4, filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 2 cm). Elution with 1:3 hexanes−ethyl acetate afforded **26** as a colorless oil: yield 55 mg (70%); silica gel TLC R_f 0.30 (1:2 hexanes−ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 2.02 (s, 3H), 2.04 (s, 3H), 2.09 (s, 3H), 2.10 (s, 3H), 2.12 (s, 3H), 2.15 (s, 3H), 3.73 (dd, 1H, J = 8.0 and 3.4 Hz), 4.12 (m, 3H), 4.34 (m, 1H), 4.95 (m, 5H), 5.10 (m, 1H), 5.16 (d, 1H, J = 3.3 Hz), 5.25 (m, 1H), and 5.37 (m, 1H).

*1-O-[(p-Nitrophenyl)oxycarbonyl]-3,4,6-tri-O-acetyl-2-O-(2,4,6-tri-O-acetyl-3-O-carbamoyl-α-*D*-mannopyranosyl)-α,β-*L*-gulopyranoside (**27**).* To a solution of 25 mg (39 μmol) of **26** in 1 mL of dry acetonitrile were added 7.0 mg (58 μmol) of 4-(*N*,*N*-dimethylamino)-pyridine and a solution containing 12 mg (58 μmol) of *p*-nitrophenyl chloroformate in 125 μL of acetonitrile. The reaction mixture was stirred at room temperature for 1 h, at which time silica gel TLC analysis showed complete consumption of the starting material. The reaction mixture was concentrated under diminished pressure, dissolved in 30 mL of ethyl acetate, and then washed successively with 10 mL of H_2O, 10 mL of 1 N aqueous HCl, and 10 mL of brine. The ethyl acetate layer was dried over Na_2SO_4, filtered, and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 2 cm). Elution with 1:1 → 1:2 hexanes−ethyl acetate afforded the carbonate **27** as a colorless oil: yield 28 mg (92%); silica gel TLC R_f 0.38 (2:3 hexanes−ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.94 (s, 3H), 2.06 (s, 3H), 2.10 (s, 3H), 2.15 (s, 3H), 2.16 (s, 3H), 2.21 (s, 3H), 4.11 (m, 6H), 4.41 (m, 1H), 4.69 (br s, 2H), 5.04 (m, 2H), 5.16 (m, 2H), 5.29 (m, 1H), 5.48 (m, 1H) 5.83 (d, 1H, J = 8.4 Hz), 7.47 (dd, 1H, J = 6.8 and 2 Hz), and 8.31 (dd, 1H, J = 6.8 and 2 Hz); ^{13}C NMR ($CDCl_3$) δ 20.78, 20.84, 20.95, 61.4, 62.0, 65.6, 65.9, 67.5, 69.2, 69.7, 69.8, 70.0, 72.0, 95.3, 95.5, 122.1, 125.6, 146.0, 151.1, 155.1, 155.2, 169.3, 169.3, 169.6, 169.8, 170.5, and 170.7; mass spectrum (ESI) m/z 825.1821 $(M + Na)^+$ ($C_{32}H_{38}N_2O_{22}Na$ requires m/z 825.1808).

DeglycoBLM Analogue ***6***. To a solution containing 1.0 mg (1.2 μmol) of **27** and 1.0 mg (0.9 μmol) of Cu(II)·deglycobleomycin A_5 in 0.5 mL of anhydrous DMF was added 5.0 μL (3.7 mg; 36 μmol) of dry triethylamine, and the reaction mixture was shaken at room temperature for 3 h. Fifty microliters (51 mg; 1.6 mmol) of hydrazine was added and the reaction mixture was shaken at room temperature for 1 h and then concentrated under diminished pressure. The reaction mixture was diluted with 0.2 mL of 1:1 0.1% aqueous CF_3COOH−CH_3CN and purified on an Alltech Alltima C_{18} reversed-phase semipreparative (250 × 10 mm, 5 μm) HPLC column by the use of 0.1% aqueous CF_3COOH and CH_3CN mobile phases. A linear gradient was employed (99:1 0.1% aqueous CF_3COOH−CH_3CN → 50:50 0.1% aqueous CF_3COOH−CH_3CN) over a period of 35 min at a flow rate of 3 mL/min. Fractions containing the desired product eluted at 17.0 min and were collected, frozen, and lyophilized to give the Cu(II) chelate of **6** as a light blue solid: yield 0.53 mg (39%). Following demetalation with 15% aqueous EDTA, 0.20 mg (40%) of **6** was isolated as a colorless solid; mass spectrum (MALDI-TOF) m/z 1484.9 $(M + H)^+$ (theoretical m/z 1484.6); mass spectrum (ESI) m/z 742.7995 $(M + 2H)^{2+}$ ($C_{58}H_{91}N_{19}O_{23}S_2$ requires m/z 742.7983).

Biochemical Experiments. *Cleavage of Supercoiled Plasmid DNA by Modified DeglycoBLM A_6 Analogues.* Two hundred nanograms of supercoiled pBR322 or pSP64 plasmid DNA was treated with the appropriate concentrations of freshly prepared Fe^{2+} [from $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$] and (deglyco)BLM solutions in a 15 μL reaction mixture (total volume) containing 50 mM Tris-HCl, pH 8.0. Reactions were incubated at 25 °C for 30 min and were then quenched by the addition of 3 μL of native gel loading buffer containing 30% glycerol and 0.125% (w/v) bromophenol blue. Five microliters of the reaction mixture was loaded onto a 1% agarose gel containing 9 mM Tris−borate buffer, pH 8.3, and 320 μM disodium ethylenediaminetetraacetate (Na_2EDTA). The gel was run at 96 V for 2 h in a buffer solution containing 9 mM Tris−borate buffer, pH 8.3, and 320 μM Na_2EDTA. The gel was then stained in the running buffer solution supplemented with 0.5 μg/mL ethidium bromide for 1 h, followed by destaining in water for 15 min. Gels were then analyzed with a UV transilluminator.

MTT Assay.[64] DU145 (American Type Culture Collection) human prostate cancer cells were grown in log-phase mode in Eagle's minimal essential medium (MEM; Gibco) supplemented with 10% fetal bovine serum and 1% penicillin−streptomycin. Cells were trypsinized and seeded into a 96-well plate in 100 μL of culture medium at 5000 cells/well. Cells were incubated for 24 h, followed by removal of medium and replacement with medium containing each bleomycin derivative at the appropriate concentration to the wells. The samples contained dimethyl sulfoxide (DMSO) at a final concentration of 5%. Following incubation with each compound for 24 h, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added at a concentration of 5 mg/mL in phosphate-buffered saline, pH 7.4. After incubation for 3 h at 37 °C, the culture medium was removed, and the crystals were dissolved in 200 μL of DMSO. The 96-well plate was read in a multiwell plate reader with an absorbance filter of 560 nm and a reference filter of 670 nm. Data were analyzed by the use of QtiPlot for determination of linear fit. Cell viability was determined by the following equation: $OD_{560}/[OD_{560}(\text{positive control}) - OD_{560}(\text{negative control})]$.

Cell Growth Conditions. SW480 colon cancer cells (ATCC CCL-228) were cultured in RPMI 1640 medium (Gibco, Grand Island, NY)

supplemented with 10% fetal bovine serum (HyClone, South Logan, UT) and 1% penicillin−streptomycin antibiotic mixture (Cellgro, Manassas, VA). DU145 (ATCC HTB-81) prostate cancer cells, PZ-HPV-7 (ATCC CRL-2221) normal prostate cells, WI-38 (ATCC CCL-75) normal lung cells, and CCD-1105 KIDTr (ATCC CRL-2305) normal kidney cells were grown in MEM medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin−streptomycin mix antibiotic supplement. The cells were incubated at 37 °C under a humidified atmosphere of 5% CO_2 and 95% air.

Fluorescence Microscopy. Fluorescence images were acquired on a Zeiss Axiovert 200 M inverted microscope fitted with an AxioCam MRm camera equipped with a 300 W xenon lamp (Sutter, Novato, CA) and Cy5 and Cy7 cyanine filter (Chroma, Bellows Falls, VT). The cells were grown on 16-well Lab-Tek glass chamber slides at a cell density of 5000 cells/well (Thermo Scientific, Waltham, MA) at 37 °C for 48 h. Cells were rinsed twice with phosphate-buffered saline (PBS) and the medium was replaced with RPMI 1640 (no phenol red) when the cell density reached 70% confluence. Cells were treated with dye-labeled conjugates to the final desired concentration. Cells were incubated at 37 °C for 1 h, washed with PBS, and then fixed with 4% paraformaldehyde at 37 °C for 5 min. Finally, the slide was mounted with Prolong Antifade Gold reagent with DAPI (Invitrogen). All images were recorded and the target cells were counted by use of a 40× oil objective. To ensure accurate intensity measurements, the exposure time and laser time were kept identical. Pixel intensity was quantified by the use of AxioVision Release 4.7 version software, and the mean pixel intensity was generated as gray level.

■ ASSOCIATED CONTENT

Supporting Information

Four schemes and additional experimental procedures for synthesis and characterization of BLM analogues **28**−**36**, **8**, **37**−**43**, **9**, **4**, **5**, **46**, **47**, **50**, **52**, **54**, and **55**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

sid.hecht@asu.edu

Notes

The authors declare the following competing financial interest(s): Sidney Hecht is a consultant to Isis Pharmaceuticals.

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