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Synthesis and DNA Cleavage Activity of a Novel Bleomycin A_5 Glycoconjugate

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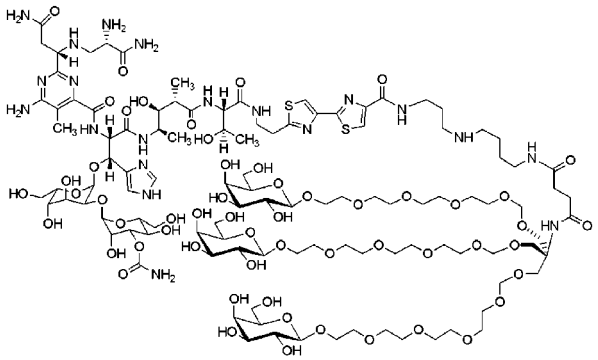
ABSTRACT



To explore the possibility of modifying bleomycin in a fashion that could alter its physiological distribution in a therapeutic setting, a new analogue of bleomycin has been prepared. This analogue is intended to target the asialoglycoprotein receptor on liver cells. Critically, despite the large C-substituent, the bleomycin conjugate was found to degrade DNA in the same fashion as bleomycin A_5 itself, and with only modestly decreased efficiency.

The bleomycins (BLMs) are glycopeptide-derived antitumor agents[1] used clinically for the treatment of several neoplasms;[2] the antitumor activity of these agents is believed to result from their ability to mediate the selective cleavage of DNA[3] and possibly also RNA.[4] While the mechanisms of polynucleotide recognition and cleavage have been studied intensively,[3,4] much less is known about the basis for the selectivity of BLM action as a therapeutic agent. Factors that may influence therapeutic selectivity include selectivity of organ distribution[5] and selective catabolism of the agent by bleomycin hydrolase.[2,6] It is interesting that the cytotoxicity of bleomycin has been reported to be limited by low efficiency of cellular uptake; electropermeabilization dramatically increased the cytotoxicity of the drug.[7]

The asialoglycoprotein receptor,[8] a specific membrane-bound receptor for glycoproteins in mammalian liver cells, recognizes the terminal galactose residues of synthesized

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glycoproteins and effects their internalization. Because of the rapid uptake mediated by the asialoglycoprotein receptor, this constitutes an attractive experimental system in which to study cell targeting.[9] Recently, van Boom and co-workers[10] reported the synthesis of galactosyl glycopeptides and generalized the structures required for the ligands to be recognized by the asialoglycoprotein receptor. It has also been reported[11] that both the hydrophobicity and the type of linker attached to the sugar moiety play important roles in ligand recognition.

Radionuclide conjugates of bleomycin have been shown to localize in tumors,[12] and individual naturally occurring bleomycin congeners have been reported to be distributed in a characteristic fashion to specific organs in mice.[5] Despite the preparation of numerous analogues of bleomycin for defining the structural elements that are essential for DNA and RNA binding and cleavage,[13] no analogous effort has addressed the molecular basis of organ or tumor targeting by bleomycin. Reported herein is the conjugation of BLM A_5 to a cluster galactoside capable of binding and internalization by the asialoglycoprotein receptor on liver cells.

The point of attachment of the cluster galactoside to bleomycin is clearly critical, in that the conjugate must be capable of binding and degrading those DNA and RNA targets responsible for the antitumor activity of the drug. The recent report[14] that conjugation of BLM A_5 to a solid phase support had little effect on its ability to mediate DNA cleavage suggested that the C-terminal spermidine moiety might well constitute an appropriate site for conjugation of the cluster galactoside. Accordingly, we have prepared BLM conjugate **2** for study (Figure 1).

The synthesis of bleomycin conjugate **2** is outlined in Schemes 1 and 2. First, key intermediate **6** was synthesized (Scheme 1). Succinimidyl ester **3**,[15] prepared from monomethyl succinate in 86% yield, was treated with tris(hydroxymethyl)aminomethane (**4**) in anhydrous DMF to afford trihydroxylated amide **5**[16] as a colorless solid in 50% yield. Thiomethylation of **5** with methyl sulfide and benzoyl peroxide[17] in acetonitrile at 0 °C afforded tris methylthiomethyl derivative **6**[16] as a syrup in 38% yield.

Initial attempts to condense the tris(thiomethyl) derivative **6** with the known galactopyranosyl derivative **7**,[10a] prepared from commercially available galactose pentaacetate in four steps and 40% overall yield, in the presence of NIS/TfOH[10a] failed to afford the desired trigalactosyl intermediate **8**. Likewise, the use of MeOTf[18] as a catalyst also failed to afford **8**.

(9) While the internalized ligands are initially contained within an endosomal compartment separate from the cytoplasm, and potentially susceptible to lysosome-mediated degradation, it is known that some of the ligands escape degradation and thus are potentially available for intracellular delivery.[8]

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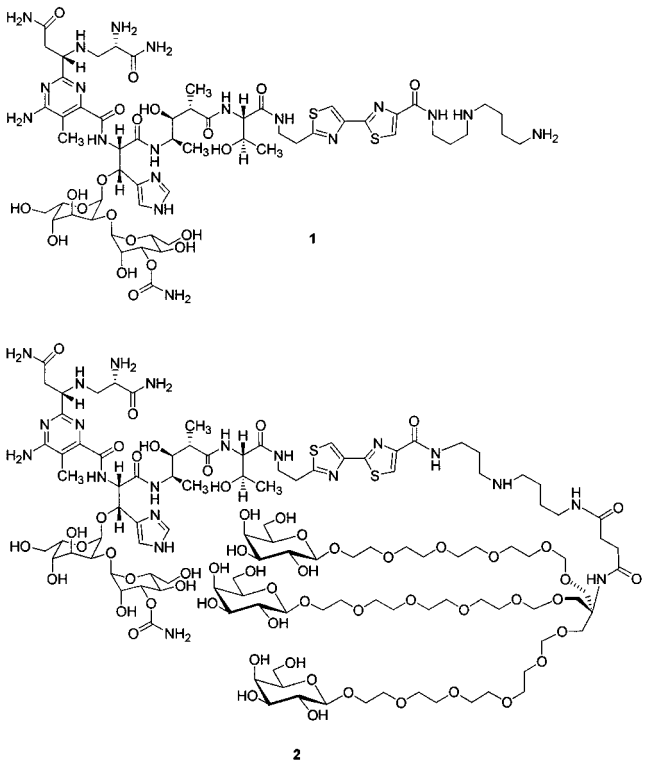
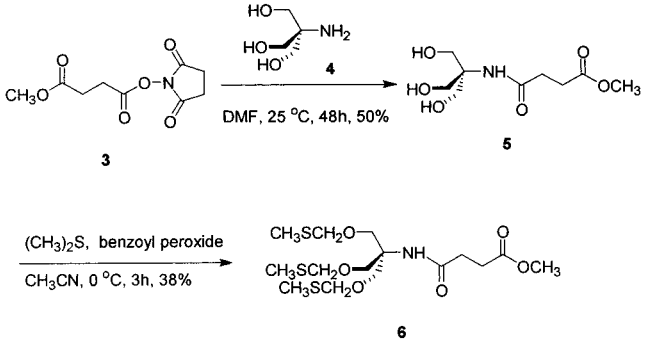


Figure 1. Structures of bleomycin A_5 (**1**) and bleomycin A_5 glycoconjugate **2**.

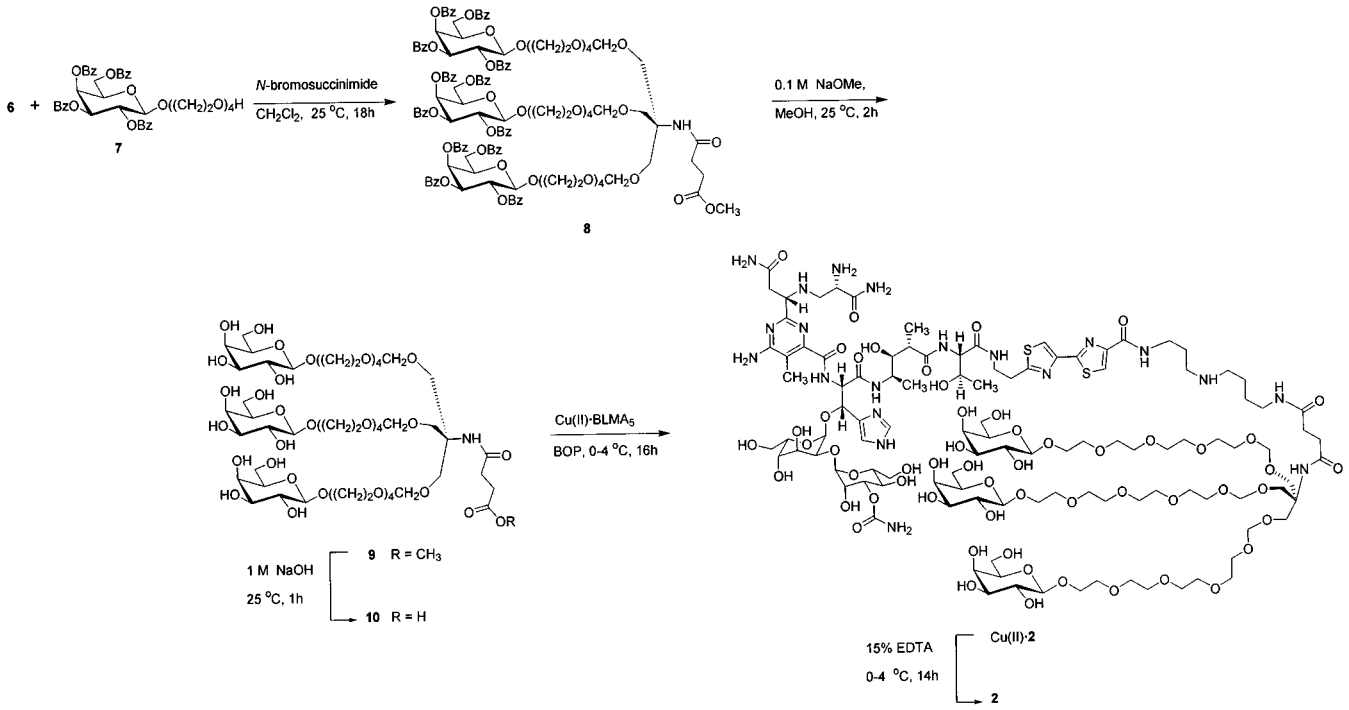
Compound **6** was then condensed with galactopyranosyl derivative **7** in the presence of *N*-bromosuccinimide[19] to obtain the desired cluster galactoside **8**[20,21] (Scheme 2). Intermediate **8** was debenzoylated by treatment with sodium methoxide in methanol. Trigalactosyl derivative **9**[20] was obtained in 74% yield and a good state of purity following column chromatography on silica gel. Finally, methyl ester **9** was saponified using 1 M sodium hydroxide in 3:1 water−dioxane[10b] to afford carboxylate **10**[20] in 48% yield.

The condensation of carboxylate **10** and the Cu(II) complex of bleomycin A_5[22] was carried out via the agency of benzotriazol-1-yloxy tris(dimethylamino)phosphonium hexafluorophosphate (BOP reagent)[23] (Scheme 2). The crude

Scheme 1. Synthesis of Methylthiomethyl Derivative **6**



Scheme 2. Synthesis of Bleomycin A_5 Glycoconjugate **2**



product was purified by C_{18} reversed-phase HPLC. The desired Cu(II) chelate of **2**[20] was recovered from the appropriate fractions by lyophilization as a bluish solid in 25% yield. Demetalation was effected by treatment of Cu(II)·**2** with 15% Na_2EDTA. Purification by C_{18} reversed-phase HPLC afforded the desired BLM A_5 glycoconjugate **2** in 63% yield.

The ability of glycoconjugate **2** to degrade DNA was evaluated initially using supercoiled pSP64 plasmid DNA. As shown in Figure 2, significant cleavage was obtained using 0.5 μM Fe(II)·BLM conjugate **2**, and greater than 50% conversion to form II (nicked circular) and III (linear duplex) DNA resulted from the use of 1 μM Fe(II)·BLM conjugate **2**. Comparison with DNA relaxation mediated by Fe(II)·BLM A_5 (**1**) indicated that conjugate **2** was approximately one-half as potent as BLM A_5 in effecting DNA relaxation (cf. lanes 5 and 8, and lanes 6 and 9).

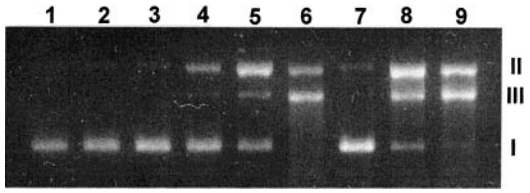


Figure 2. Relaxation of supercoiled pSP64 plasmid DNA by BLM A_5 glycoconjugate **2** in the presence of Fe^{2+}. Twenty-five microliter reaction mixtures contained 300 ng of plasmid DNA in 10 mM Na cacodylate, pH 7.0, at 0 °C. After incubation for 15 min, the reaction mixtures were analyzed on a 1% agarose gel: lane 1, DNA alone; lane 2, 1 μM Fe^{2+}; lane 3, 1 μM BLM **2**; lane 4, 0.5 μM BLM **2** + 1 μM Fe^{2+}; lane 5, 1 μM BLM **2** + 1 μM Fe^{2+}; lane 6, 2 μM BLM **2** + 1 μM Fe^{2+}; lane 7, 1 μM BLM **1**; lane 8, 0.5 μM BLM **1** + 1 μM Fe^{2+}; lane 9, 1 μM BLM **1** + 1 μM Fe^{2+}.

The sequence selectivity of DNA cleavage by Fe(II)·BLM glycoconjugate **2** is shown in Figure 3 in direct comparison

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(16) Compound **5**: ^{1}H NMR (D_2O) δ 2.58−2.60 (s, 4H), 3.67 (s, 3H) and 3.73 (s, 6H); ^{13}C NMR (D_2O) δ 28.7, 30.1, 51.8, 59.9, 61.5, 174.7 and 175.4; mass spectrum (chemical ionization, methane) 236 $(M + H)^+$. Anal. Calcd for $C_9H_{17}NO_6$: C, 45.95; H, 7.28; N, 5.95. Found: C, 45.80; H, 7.12; N, 6.13. Compound **6**: ^{1}H NMR ($CDCl_3$) δ 2.14 (s, 9H), 2.47 (t, 2H), 2.62 (t, 2H), 3.68 (s, 3H), 3.84 (s, 6H), 4.64 (s, 6H) and 5.92 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.1, 29.2, 31.5, 51.8, 58.9, 67.1, 75.9, 171.2 and 173.2; mass spectrum (chemical ionization, methane) 416 $(M + H)^+$. Anal. Calcd for $C_{15}H_{29}NO_6S_3$: C, 43.36; H, 7.04; N, 3.37. Found: C, 43.62; H, 7.04; N, 3.33.

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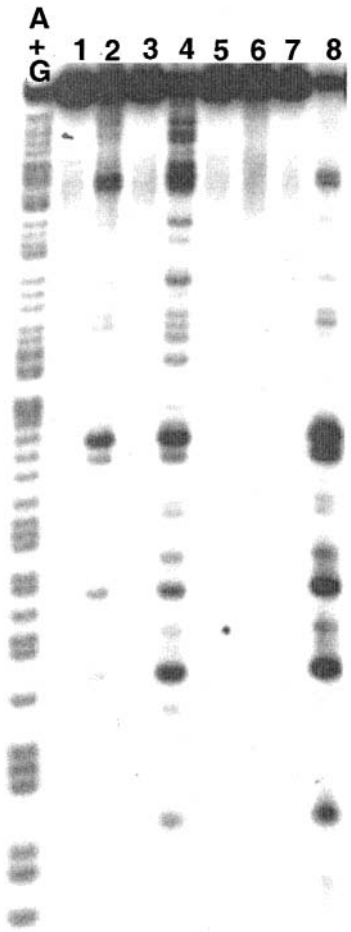

Figure 3. Strand scission of a linear DNA duplex by BLM A_5 conjugate **2** in the presence of Fe^{2+}. A 5′-^{32}P end labeled 158-bp DNA duplex was incubated with Fe(II)·BLM at 4 °C for 15 min and then analyzed on a 10% denaturing polyacrylamide gel: lane 1, 0.5 μM BLM **2**; lane 2, 0.5 μM BLM **2** + 20 μM Fe^{2+}; lane 3, 0.25 μM BLM **1**; lane 4, 0.25 μM BLM **1** + 20 μM Fe^{2+}; lane 5, DNA alone; lane 6, 20 μM Fe^{2+}; lane 7, 1 μM BLM **2**, lane 8, 1 μM BLM **2** + 20 μM Fe^{2+}.

with that mediated by Fe(II)·BLM A_5 (**1**). The substrate was a 158-base pair 5′-^{32}P end labeled DNA duplex that has been employed previously as a substrate for Fe(II)·BLM.[14] As is clear from the figure, Fe(II)·BLM glycoconjugate **2** exhibited the same sequence selectivity of DNA cleavage as BLM A_5 (**1**); the potency of cleavage by **2** was only 2−3-fold less than that obtained with **1**. Thus, the introduction of a glycosylated substituent at the C-terminus of BLM A_5 had no effect on the sequence selectivity of DNA cleavage and only a modest effect on cleavage potency, consistent with observations made for BLM derivatives conjugated to solid supports via the C-terminus.[14]

Bleomycin derivative **2** was tested for its cytotoxic potential in direct comparison with BLM A_5 (**1**). In preliminary experiments, **2** exhibited cytotoxicity comparable to BLM A_5 toward cultured human epidermal carcinoma A253 cells. Experiments designed to evaluate the ability of BLM glycoconjugate **2** to interact selectively with liver cells are underway and will be reported in due course.

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(20) Compound **8**: ESI-MS, *m/z* 2610.3 $(M + Na)^+$ ($C_{138}H_{149}O_{48}NNa$ requires 2610.9). Compound **9**:^{13}C NMR (D_2O) δ 28.6, 30.2, 51.8, 60.5, 65.8, 66.3, 68.2, 68.9−69.3 [C from tris(tetraethylene glycol)], 70.3, 72.2, 74.7, 95.0, 102.4, 173.9 and 175.2; ESI-MS, *m/z* 1362.8 $(M + Na)^+$ ($C_{54}H_{101}O_{36}NNa$ requires 1362.6). Compound **10**: ESI-MS, *m/z* 1348.7 $(M + Na)^+$ ($C_{53}H_{99}O_{36}NNa$ requires 1348.6). Compound Cu(II)·**2**: ESI-MS, *m/z* 2809.4 $(M)^+$ ($C_{110}H_{186}O_{56}N_{20}S_2Cu$ requires 2810.1). Compound **2**: ESI-MS, *m/z* 2770.3 $(M + Na)^+$ ($C_{110}H_{186}O_{56}N_{20}S_2Na$ requires 2770.2).

(21) Compound **8** was obtained as a mixture, contaminated predominantly with compounds resulting from the condensation of only one or two molecules of galactoside **7** with intermediate **6**. A sample greatly enriched in **8** was obtained in 50% yield following chromatography on silica gel and then on Sephadex LH-20.

(22) Prepared by admixture of $CuSO_4·5H_2O$ to an aqueous solution of bleomycin A_5, thus forming a derivative in which only the C-terminal substituent could undergo acylation.[14]

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