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Deglycobleomycin: Solid-Phase Synthesis and DNA Cleavage by the Resin-Bound Ligand

Kenneth L. Smith, Zhi-Fu Tao, Shigeki Hashimoto, Christopher J. Leitheiser, Xihan Wu, and Sidney M. Hecht*

Departments of Chemistry and Biology, University of Virginia, Charlottesville, Virginia 22901

sidhecht@virginia.edu

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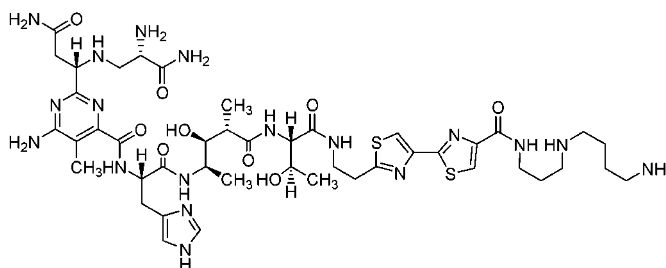
ABSTRACT



A greatly improved solid-phase synthesis of deglycobleomycin using a Dde-based linker is reported. The resin-bound deglycobleomycin could be completely deblocked and assayed for DNA plasmid relaxation, sequence-selective DNA cleavage, and light production from a molecular beacon.

Bleomycin A_5 (**1**, Figure 1) is a member of a class of glycopeptide-derived antitumor antibiotics[1] that are believed to exert their anticancer effects at the level of oxidative damage to DNA[2] and possibly RNA.[3] Biochemical studies have shown that DNA cleavage occurs predominantly at 5′-GC-3′ and 5′-GT-3′ sequences.[4] RNA cleavage is more highly selective and clearly involves the recognition of RNA secondary and tertiary structure as well as sequence.[3] While bleomycin and a number of structural congeners have been prepared,[5] the synthesis of additional agents of this type is an important goal as it should permit the mechanism(s) of polynucleotide recognition and cleavage to be studied in greater detail and lead to the elaboration of agents with improved antitumor activity.

In an effort to facilitate the preparation of additional bleomycin congeners, we have undertaken the solid-phase synthesis of bleomycin. Recently, we reported the synthesis of deglycobleomycin A_5 on a commercially available triphenylmethyl spermidine resin.[6] Due to the acid-labile nature of the trityl linker, this methodology does not allow for the preparation of fully deblocked resin-bound bleomycin analogues, which could be assayed directly for improved DNA cleavage properties while still attached to the resin.[7] The realization of a solid-phase synthesis that allows bleomycin to be fully deblocked and assayed while attached to the resin

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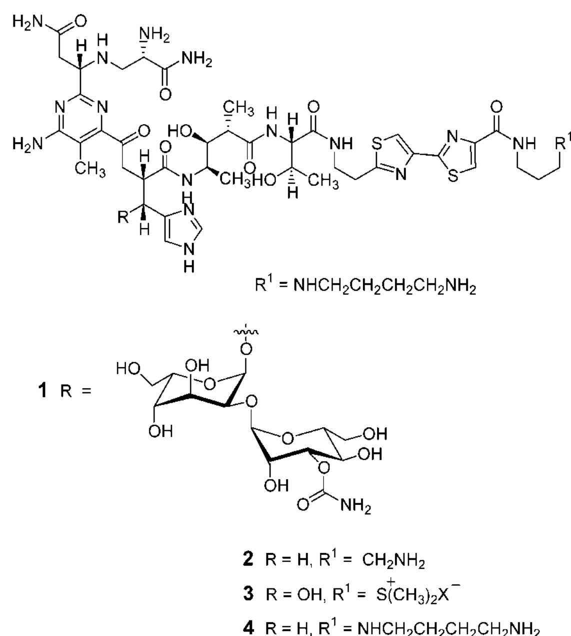

Figure 1. Structures of bleomycin A_5 (**1**), deglycoBLM $A_{2'-a}$ analogue **2**, deglycoBLM A_2 (**3**), and deglycoBLM A_5 analogue **4**.

would be of great advantage for the characterization of bleomycin analogues contained within combinatorial libraries.

Presently, we report a greatly improved solid-phase synthesis of deglycobleomycins (deglycoBLMs) utilizing a Dde-based linker[8] which has been shown to be stable to conditions used to remove both base- and acid-labile protecting groups. This strategy can potentially provide easy access to the libraries of fully deprotected resin-bound bleomycin derivatives that may be assayed for DNA cleavage prior to their detachment from the solid support. We have demonstrated the utility of this methodology by preparing two different resin-bound deglycobleomycins. Both of these effected DNA cleavage as indicated by agarose and polyacrylamide gel electrophoresis and by molecular beacon assay utilizing fluorescence microscopy.

Resin-bound 1,4-diaminobutane (**5**), which was prepared according to Bycroft et al.[8c,d] (Scheme 1) was utilized as the starting point in the improved synthesis. This was coupled to Fmoc-protected bithiazole **6**[6] which was activated with HBTU[6,9] and diisopropylethylamine in DMF. The resin was shaken for 45 min and then filtered and washed with DMF and CH_2Cl_2. A small sample was utilized in qualitative Kaiser[10] and bromophenol blue tests[11] to ensure that the coupling had taken place. Coupling quantification was carried out by Fmoc cleavage assay[12] and indicated a loading of 0.24 mmol/g, which represented a coupling efficiency of 95%. The resin was treated with 20% piperidine in DMF for 5 min and then filtered and washed with DMF followed by CH_2Cl_2. It was then coupled successively to commercially available Fmoc-threonine (**7**), and then in the same fashion to Fmoc methylvaleric acid **8**;[6] the coupling efficiencies were 94% (0.22 mmol/g) and 95% (0.20 mmol/g), respectively. The resin-bound pentapeptide was prepared in 99% yield (0.18 mmol/g) by treating the resin with piperidine in DMF followed by coupling of Fmoc-histidine (**9**) with HATU. Boc pyrimidoblamic acid (**10**)[13] was coupled to the resin peptide at 0 °C using BOP reagent and $(i\text{-Pr})_2NEt$ in the absence of light for 16 h. The resin was then washed with DMF and CH_2Cl_2 to afford fully deprotected resin-bound oligopeptide **11**. The acid-labile protecting groups were removed by treating the resin with 91:3:3:3 TFA−triisopropylsilane−Me_2S−H_2O for 4 h to afford resin-bound, fully deprotected deglycoBLM analogue **12**. Cleavage of this deglycoBLM analogue from the resin was accomplished by treating the resin three times with 2% hydrazine in DMF for 5 min. The supernatant was reduced in volume, and the product was precipitated from a TFA solution by addition to cold ether. The residue was then dissolved in water; the solution was frozen and lyophilized to afford deglycoBLM analogue **2**. The crude product was purified by C_{18} reversed-phase HPLC; elution was effected with 5−25% CH_3CN in 0.1 M NH_4OAc over a period of 30 min at a flow rate of 1 mL/min. The fraction containing the desired product was collected, frozen, and lyophilized to afford **2** as a colorless solid; yield 1.2 mg (68%).[14] Mass spectral analysis (FAB) revealed a molecular ion ($M^+ + H$) at m/z 1000.4416 ($C_{41}H_{61}N_{17}O_9S_2$ requires 1000.4358).

Since the deglycoBLMs have been reported to degrade DNA with the same sequence selectivity and chemistry as BLM itself, albeit with lesser efficiency,[1,2] deglycoBLM **2** analogue was also characterized by its ability to relax plasmid DNA upon coordination with Fe^{2+} (Figure 2). Relaxation of supercoiled pSP64 plasmid DNA was observed for synthetic Fe(II)·deglycoBLM analogue **2** at 1 and 3 μM

Figure 2. Cleavage of supercoiled pSP64 DNA by deglycoBLM analogue **2** and resin-bound deglycoBLM analogue **12**. A 25-μL reaction mixture containing 200 ng (0.11 pmol) of pSP64 plasmid DNA in 6.7 mM sodium cacodylate, pH 7.0, was incubated at 37 °C for 30 min and then analyzed on a 1.0% agarose gel. Lane 1, DNA alone; lane 2, 1.5 μM Fe^{2+}; lane 3, 3 μM deglycoBLM A_2 (**3**); lane 4, 1 μM deglycoBLM A_2 (**3**) + 1.5 μM Fe^{2+}; lane 5, 3 μM deglycoBLM A_2 (**3**) + 1.5 μM Fe^{2+}; lane 6, 3 μM deglycoBLM analogue **2**; lane 7, 1 μM deglycoBLM **2** + 1.5 μM Fe^{2+}; lane 8, 3 μM deglycoBLM analogue **2** + 1.5 μM Fe^{2+}; lane 9, 3 μM resin-bound deglycoBLM analogue **12**; lane 10, 3 μM resin-bound deglycoBLM analogue **12** + 1.5 μM Fe^{2+}.

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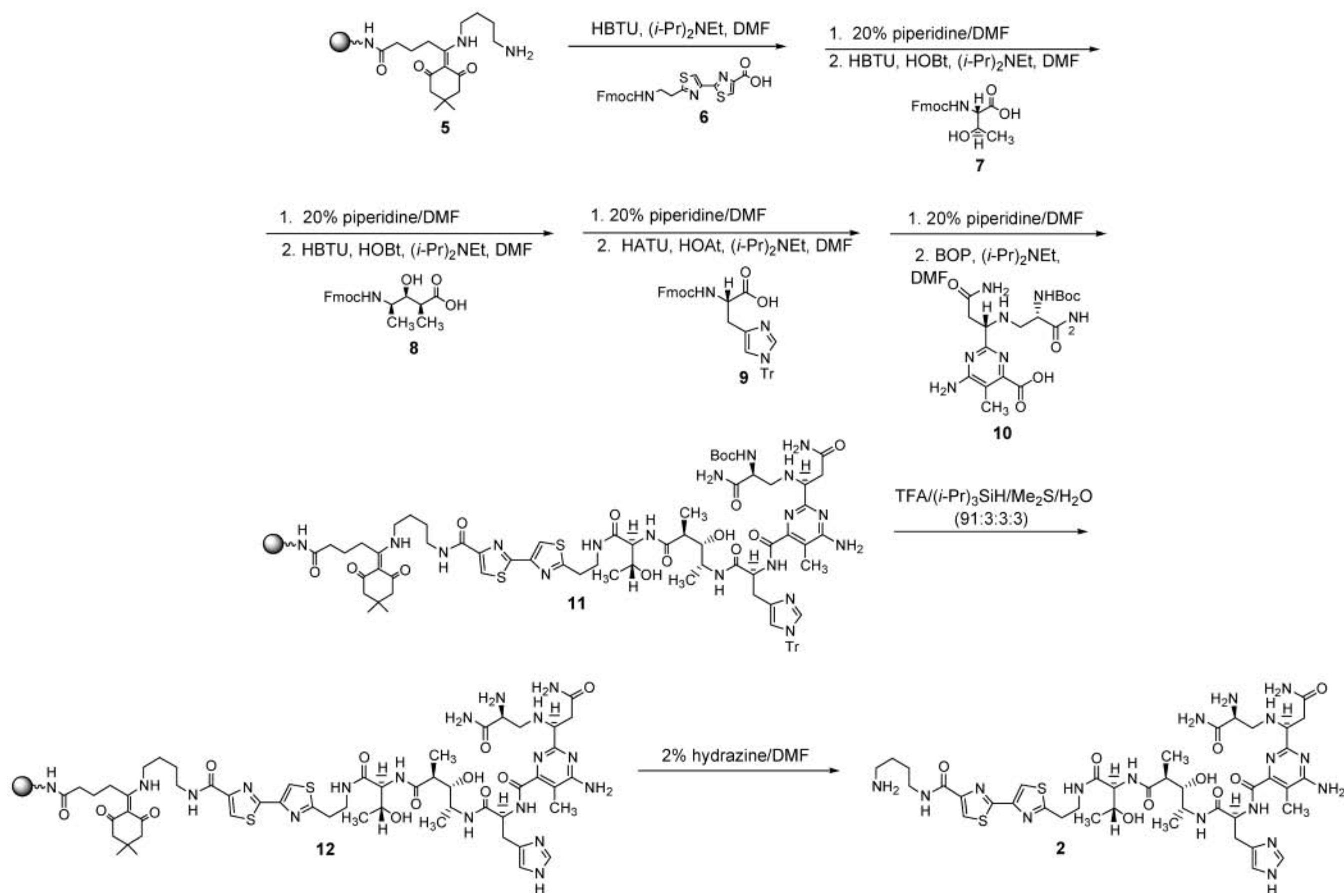
Scheme 1. Solid-Phase Synthesis of DeglycoBLM Analogue **2**[a]



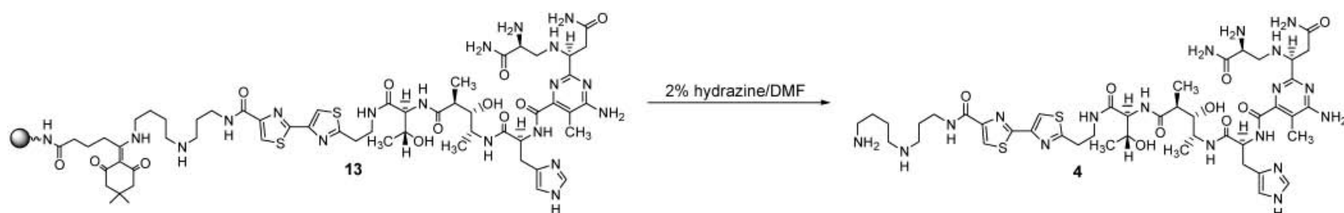
[a] Reagents, Fmoc = 9-fluorenylmethoxycarbonyl, HBTU = 2(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, DMF = *N*,*N*-dimethylformamide, HOBt = *N* -hydroxybenzotriazole, TFA = trifluoroacetic acid, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOAt = 1-hydroxy-7-azabenzotriazole.

concentrations and was greater at 3 μM concentration. The cleavage at 1 μM concentration was not much greater than in the presence of Fe^{2+} alone, but the latter is not entirely suitable as a control in that admixture of (deglyco)BLM results in the chelation of free Fe^{2+}. In the absence of added Fe^{2+}, little nicking of Form I DNA was noted (lane 6). As observed in lanes 9 and 10, resin-bound Fe(II)·deglycoBLM analogue **12** also effected DNA relaxation, but to a lesser extent than the respective free Fe(II)·deglycoBLM analogue (**2**), as noted previously for resin-bound and free BLM A_5.[7]

Further characterization of synthetic deglycoBLM analogue **2** (Figure 3) involved the cleavage of a 5′-^{32}P end-labeled 158-base pair DNA duplex; this revealed sequence selective DNA cleavage (lane 4) which was the same as that mediated by Fe(II)·deglycoBLM A_2 (**3**) (lane 6). In the absence of Fe^{2+} no cleavage was observed (lanes 3 and 5).

In the belief that the DNA cleavage activity of resin-bound deglycoBLM analogue **12** might be limited by the absence of any positively charged group within the C-terminus of the synthetic oligopeptide to facilitate DNA binding, we also prepared deglycoBLM A_5 analogue **13** in analogy with Scheme 1, albeit using CPG as a solid support (Scheme 2). Cleavage of the product from the CPG beads with 2% hydrazine afforded deglycoBLM analogue **4**.

Scheme 2. Conversion of Resin-Bound DeglycoBLM Analogue **13** to **4**



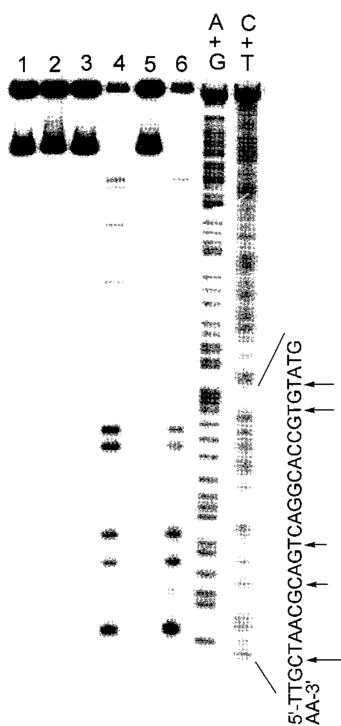


Figure 3. Cleavage of a 5′-^{32}P end-labeled, 158-base pair DNA duplex by synthetic deglycoBLM analogue **2** in the presence of Fe^{2+}. A 25-μL reaction mixture containing 10^4 cpm of ^{32}P end-labeled duplex and the indicated reagents was incubated in 10 mM sodium cacodylate, pH 7.0, at 37 °C for 30 min and then analyzed on a 10% denaturing polyacrylamide gel. Lane 1, DNA alone; lane 2, 15 μM Fe^{2+}; lane 3, 15 μM deglycoBLM analogue **2**; lane 4, 15 μM deglycoBLM analogue **2** + 15 μM Fe^{2+}; lane 5, 15 μM deglycoBLM A_2 (**3**); lane 6, 15 μM deglycoBLM A_2 (**3**) + 15 μM Fe^{2+}.

DNA cleavage by resin-bound deglycoBLM analogue **13** could be monitored by the use of a 5′-^{32}P end-labeled DNA (not shown), which demonstrated a cleavage pattern identical to that shown for **2** in Figure 3, and also by molecular beacon[15] assay utilizing fluorescence microscopy (Figure 4). The molecular beacon sequence was 5′d($CGCT_3A_7GCG$); the beacon contained the fluorophore fluorescein at the 5′-end and the quencher dabcyl at the 3′-end. This DNA hairpin has been shown to fluoresce following BLM-mediated DNA cleavage.[16] As can be seen in the figure, the green fluorescence emission from the conjugated CPG was observed by irradiating the cleavage mixture with visible light.

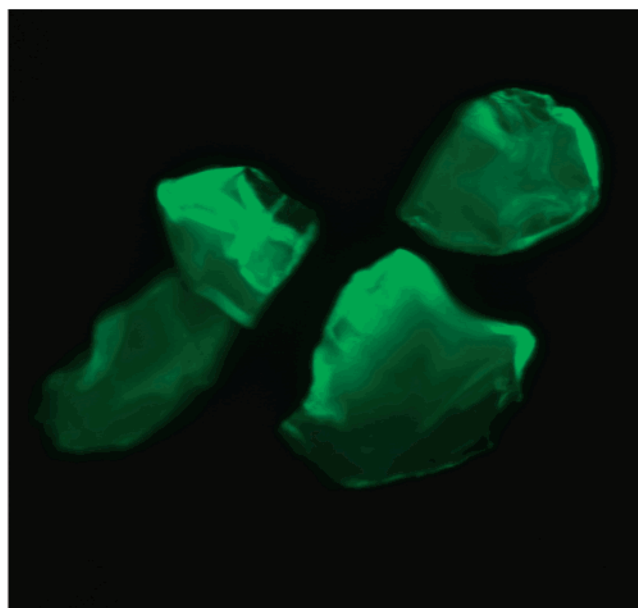


Figure 4. Fluorescence microscopy (1 h incubation) of the beacon DNA treated with CPG-bound Fe(II)·deglycoBLM analogue **13**. Beacon DNA cleavage for visualization was carried out with the beads embedded in a 1.5% agarose gel medium containing the beacon DNA to retard diffusion of the formed fluorescent products from the surface of the beads. The CPG beads were ~100 μm in size.

The foregoing experiments demonstrate the feasibility of preparing bleomycin analogues attached to the solid support in good yield and in assaying the derived species before or after removal from the resin. The realization of these goals should permit the construction and assay of bead-bound BLM combinatorial libraries, thereby greatly facilitating the identification of bleomycin analogues having improved properties.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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