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Total Synthesis of Deamido Bleomycin A_2, the Major Catabolite of the Antitumor Agent Bleomycin

Ying Zou, Nour Eddine Fahmi, Corine Vialas, Guy M. Miller, and Sidney M. Hecht*

Contribution from the Departments of Chemistry and Biology, University of Virginia, Charlottesville, Virginia 22901

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Abstract: Metabolic inactivation of the antitumor antibiotic bleomycin is believed to be mediated exclusively via the action of bleomycin hydrolase, a cysteine proteinase that is widely distributed in nature. While the spectrum of antitumor activity exhibited by the bleomycins is believed to reflect the anatomical distribution of bleomycin hydrolase within the host, little has been done to characterize the product of the putative inactivation at a chemical or biochemical level. The present report describes the synthesis of deamido-bleomycin demethyl A_2 (**3**) and deamido bleomycin A_2 (**4**), as well as the respective aglycones. These compounds were all accessible via the key intermediate N^{α}-Boc-N^{β}-[1-amino-3(*S*)-(4-amino-6-carboxy-5-methylpyrimidin-2-yl)propion-3-yl]-(*S*)-β-aminoalanine *tert*-butyl ester (**16**). Synthetic deamido bleomycin A_2 was shown to be identical to the product formed by treatment of bleomycin A_2 with human bleomycin hydrolase, as judged by reversed-phase HPLC analysis and ^{1}H NMR spectroscopy. Deamido bleomycin A_2 was found to retain significant DNA cleavage activity in DNA plasmid relaxation assays and had the same sequence selectivity of DNA cleavage as bleomycin A_2. The most significant alteration of function noted in this study was a reduction in the ability of deamido bleomycin A_2 to mediate double-strand DNA cleavage, relative to that produced by BLM A_2.

The bleomycins are a family of structurally related glycopeptide-derived antibiotics used clinically for the treatment of several malignancies.[1] The bleomycins mediate the oxidative degradation of DNA[2] and RNA,[3] and one or both of these properties likely forms the basis for the antitumor activity associated with this class of agents.

Polynucleotide degradation by bleomycin requires the presence of a metal ion such as Fe^{2+} [4] or Cu^{+},[5] as well as oxygen. For DNA, degradation affords frank strand scission as well as the formation of alkali-labile lesions; both occur in a sequence-selective fashion predominantly involving 5′-GC-3′ and 5′-GT-3′ sequences.[2] RNA degradation occurs with even greater selectivity in a process that appears to involve recognition both of sequence and of shape.[3] As in the case of DNA, RNA degradation involves more than a single mechanistic pathway.[6]

In comparison with the complexity inherent in metallobleomycin activation and polynucleotide degradation, studies of the pharmacokinetics of bleomycin suggest a much simpler picture. The catabolism of bleomycin, in particular, is believed to involve a single metabolic event, the hydrolytic conversion of the β-aminoalanineamide side chain of bleomycin to afford deamido bleomycin, the corresponding carboxylic acid (Figure 1). This transformation is mediated by bleomycin hydrolase, a cysteine proteinase found in numerous eukaryotic tissues.[7] The human enzyme has been cloned and expressed, and characterized in some detail.[8]

In the earliest reports, it was indicated that deamido BLM lacked antitumor activity[9] and had a greatly reduced ability to degrade DNA in a cell free system.[10] However, a later report

* To whom correspondence should be addressed. E-mail: sidhecht@virginia.edu.

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10.1021/ja012741l CCC: $22.00

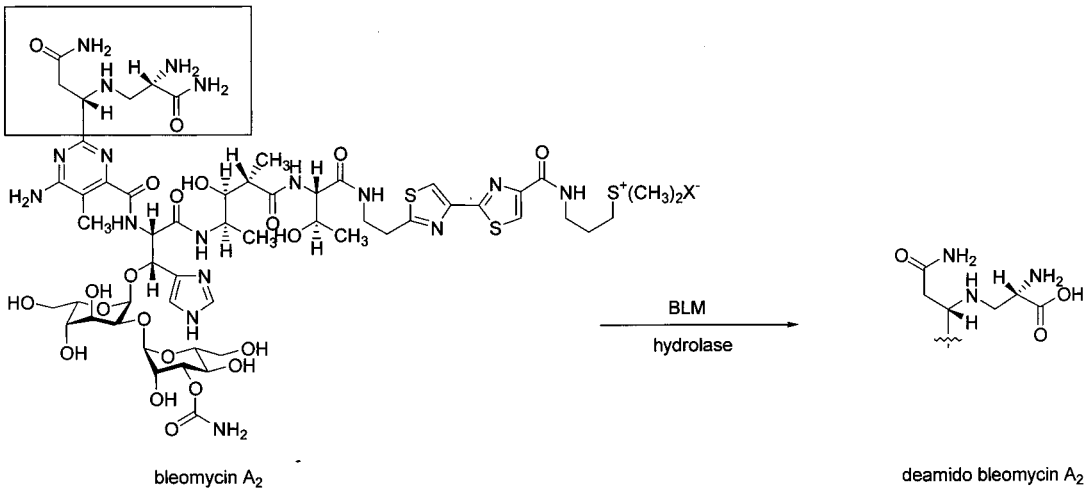

Figure 1. Structure of bleomycin A_2, and conversion to catabolite deamido BLM A_2 via the action of bleomycin hydrolase.

indicated that deamido BLM A_2 retained about one-half the ability of BLM A_2 to produce single-strand DNA breaks, and less than one-eighth the ability to produce double-strand breaks.[11] A potential complication in the study of deamido BLM is the assignment of structure, which was based on the observation of the release of ammonia during treatment with bleomycin hydrolase,[9] but has never been described in detail. While a compound assigned the same structure as deamido BLM was formed as one of several products by partial acid hydrolysis,[12] no synthesis of deamido BLM has been reported.

Presently, we describe the total synthesis of deamido deglyco BLM demethyl A_2 (**1**), deamido deglyco BLM A_2 (**2**), deamido BLM demethyl A_2 (**3**), and deamido BLM A_2 (**4**). Also reported is a careful comparison of synthetic deamido BLM A_2 (**4**) with the product resulting from treatment of BLM A_2 with purified BLM hydrolase. Finally, the ability of BLM analogues **1**−**4** to effect the relaxation of supercoiled plasmid DNA and the sequence selective cleavage of linear duplex DNA in the presence of Fe^{2+} is described.

Results

The target molecules in this study were deamido deglycobleomycin A_2 (**2**) and deamido bleomycin A_2 (**4**) (Figure 2). Also prepared were the corresponding analogues in the BLM demethyl A_2 series (i.e., **1** and **3**, respectively). Syntheses have been reported for deglycobleomycin A_2[13] and bleomycin A_2,[14] and these provided important insights that guided the strategy used for the syntheses of deamido deglycobleomycin A_2 (**2**) and deamido bleomycin A_2 (**4**). The retrosynthetic strategy employed for the synthesis of deamido bleomycin A_2 is outlined in Figure 3. The deamido BLM A_2 molecule can be divided

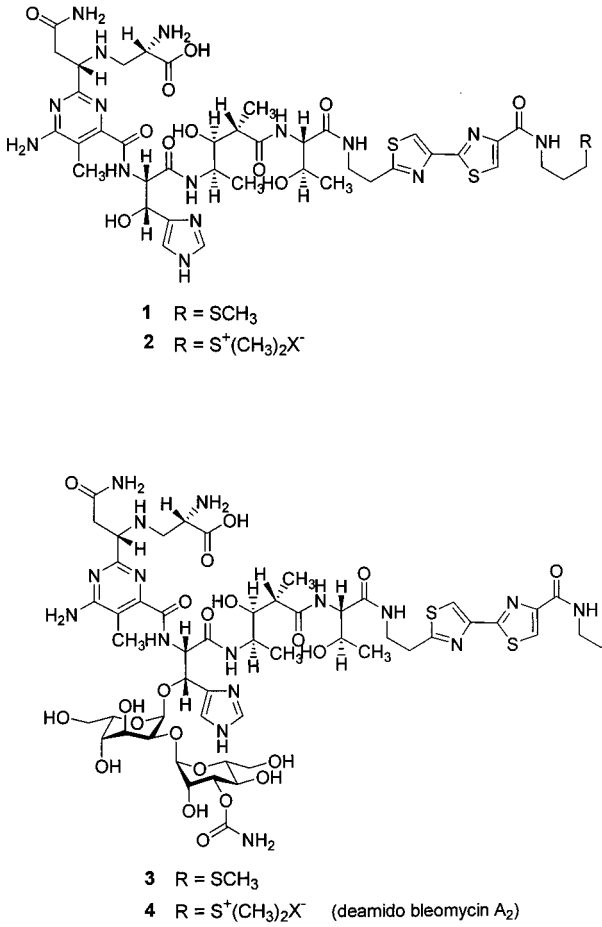

Figure 2. Structures of bleomycin A_2, deamido deglycobleomycin demethyl A_2 (**1**), deamido deglycobleomycin A_2 (**2**), deamido bleomycin demethyl A_2 (**3**), and deamido bleomycin A_2 (**4**).

into two major fragments, a deamido analogue of pyrimidoblamic acid and a pentapeptide disaccharide. The latter was envisioned as being accessible by condensation of the glycosylated β-hydroxyhistidine moiety with the tetrapeptide shown in Figure 3. The syntheses of these intermediates have been described[14] from the constituent carbohydrate and amino acid analogues. Each of these fragments was prepared synthetically using suitable protecting groups. The intermediates so prepared

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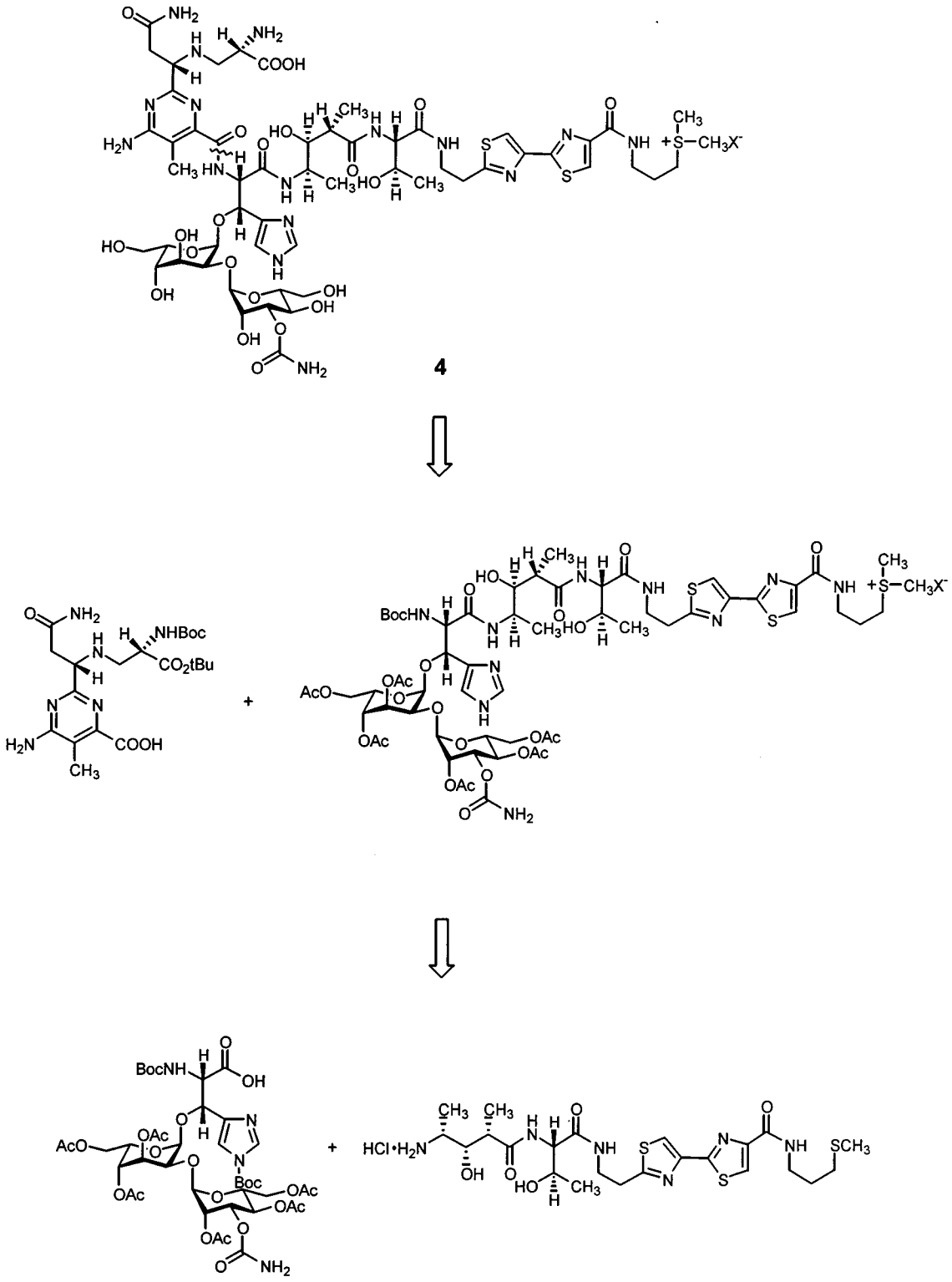
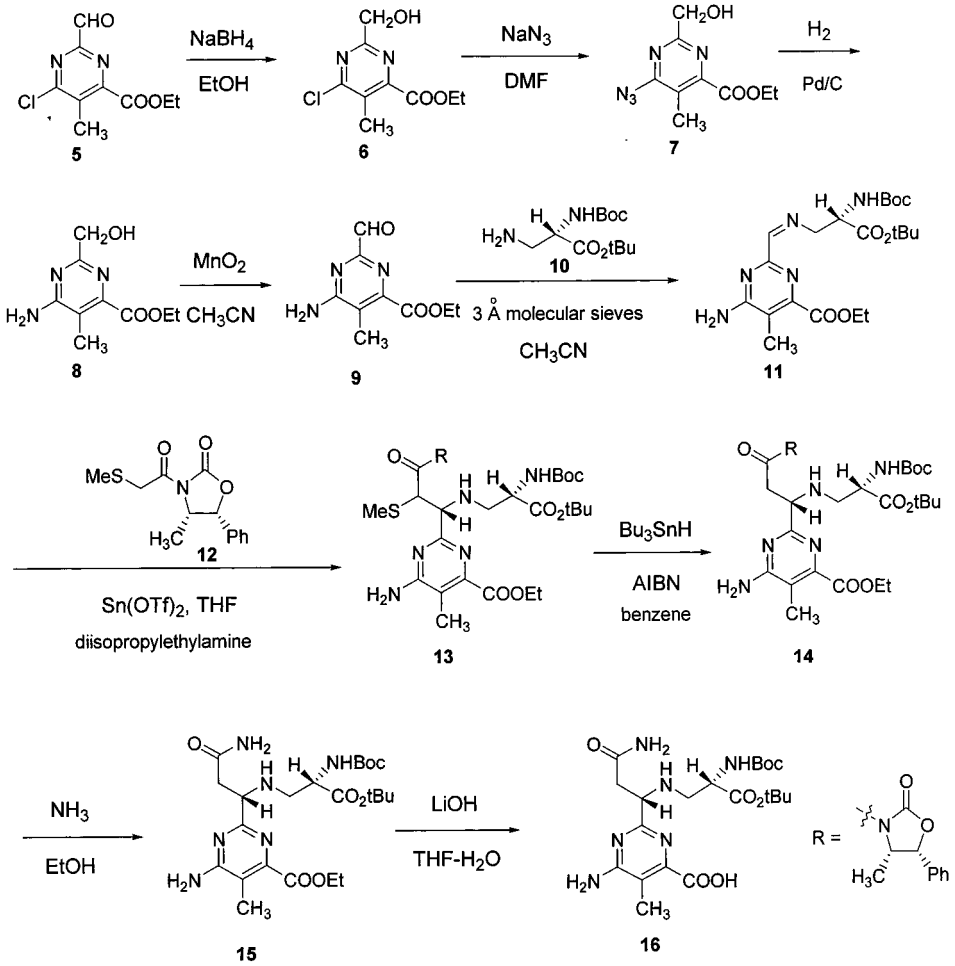


Figure 3. Retrosynthetic pathway for deamido bleomycin A_2 (**4**).

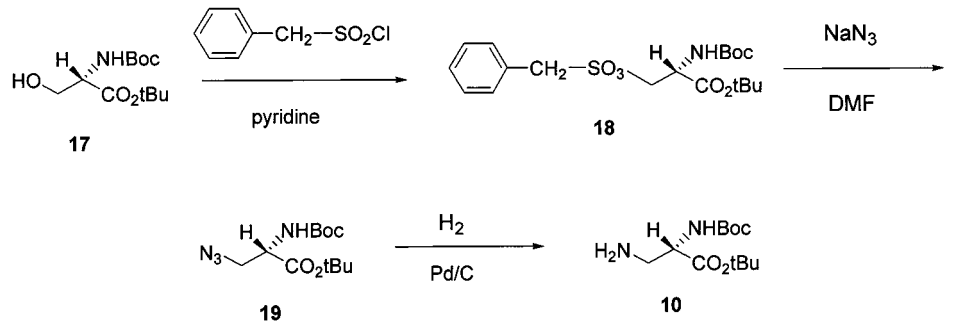
were then coupled sequentially. The deamido BLMs differ from their naturally occurring BLM counterparts only in that the terminal amide of the β-aminoalanineamide moiety has been replaced by a carboxylate. To obtain a pyrimidoblamic acid analogue suitable for the coupling with other fragments, the primary amine of the β-aminoalanineamide moiety was protected by a Boc group, and the carboxylic acid was protected as the *tert*-butyl ester, ultimately allowing for the simultaneous removal of both protecting groups under acidic conditions.[15] In addition, the stability of *tert*-butyl esters under basic conditions and ammonolysis made the use of this group even more convenient.

The route employed for the synthesis of the novel pyrimidoblamic acid analogue **16** is outlined in Schemes 1 and 2. The synthesis of pyrimidoblamic acid has been reported,[16] but the synthesis of analogue **16** proved to be challenging nonetheless.

Scheme 1



Scheme 2



Stereoselective introduction of the β-aminoalanine moiety of pyrimidoblamic acid analogue **16** required the preparation of key intermediate **8** (Scheme 1), according to a procedure reported by the Boger group.[16g] Ethyl 6-chloro-2-formyl-5-methylpyrimidine-4-carboxylate (**5**)[16a,d] was utilized as the starting material for intermediate **8**. The need to introduce an amino group at the 4-position of the pyrimidine moiety made it necessary to first protect the aldehyde functionality. Attempts to protect this group as the 1,3-dioxane derivative were unsuccessful because of poor yields in the protection and deprotection steps (20−40%). Therefore, aldehyde **5** was instead reduced to give compound **6** as a colorless oil in 70% yield by

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treatment with 0.4 equiv of sodium borohydride. Using greater than 0.4 equiv of sodium borohydride promoted the concomitant, undesired reduction of the ethyl carboxylate moiety to a hydroxymethyl group. Treatment of compound **6** with sodium azide in DMF then provided 4-azidopyrimidine **7** as colorless needles after crystallization from ethyl acetate−hexane. Azidopyrimidine **7** exists in equilibrium with the corresponding tetrazole; both forms were observable in the ^{1}H NMR spectrum of **7**.[16f] Hydrogenolysis of azidopyrimidine **7** over 10% palladium-on-carbon afforded key intermediate aminopyrimidine **8** as a colorless powder in quantitative yield. Aminopyrimidine **8** synthesized by this method could be used directly in the next step without further purification. Thus, aminopyrimidine **8** was readily oxidized with freshly prepared manganese dioxide in acetonitrile at 80 °C for 3 h to give aldehyde **9** as a colorless solid in 52% yield.[16g] Aldehyde **9** was not sufficiently stable to be stored and was, therefore, used immediately following its preparation.

For the introduction of the β-aminoalanine substituent, *tert*-butyl (2*S*)-3-amino-2-[(*tert*-butoxycarbonyl)amino]propionate (**10**) was prepared as shown in Scheme 2. Although the synthesis of **10** has been reported,[17a] a simplified procedure[17b] using commercially available *N*-Boc serine *tert*-butyl ester (**17**) was used here. Serine derivative **17** was treated with phenylmethanesulfonyl chloride in pyridine to give compound **18** as a colorless powder in 95% yield ($[\alpha]^{25}_D$ +20.3 (*c* 0.89, $CHCl_3$)). Compound **18** was then treated with sodium azide in DMF, affording azide **19** as a colorless oil in 70% yield ($[\alpha]^{25}_D$ +25.4 (*c* 0.63, $CHCl_3$)). Hydrogenolysis of **19** in the presence of 10% palladium-on-carbon provided the desired amine **10** as a colorless oil in quantitative yield ($[\alpha]^{25}_D$ −22.0 (*c* 0.90, EtOH), lit.[17a] $[\alpha]^{25}_D$ −23.9 (*c* 1.05, EtOH)). Thus, amine **10** was synthesized efficiently from *N*-Boc serine *tert*-butyl ester (**17**) in three steps and 63% overall yield. Amine **10** was stable under N_2 and solidified upon standing. It could also be purified on silica gel if needed.

Condensation of aldehyde **9** with amine **10** in dry acetonitrile over powdered 3 Å molecular sieves provided imine **11** as a light yellow foam in 95% yield. This product was used directly in the next step without purification because of its instability on silica gel ($[\alpha]^{25}_D$ −20.6 (*c* 0.85, CH_2Cl_2)) (Scheme 1). Nondiastereoselective introduction of the C2-acetamido side chain which required the separation of a 1:1 mixture of diastereomers has been reported.[16e,f] Stereoselective introduction of the side chain could be achieved by diastereoselective addition of optically active enolates with imines as reported by Boger et al.[16g] It was found that under optimum reaction conditions the imine addition reaction employing (4*S*,5*R*)-3-((methylthio)-acetyl)-4-methyl-5-phenyl-2-oxazolidinone (**12**) as the chiral auxiliary gave a diastereomer leading to pyrimidoblamic acid as the major product.[16g,18] Thus, oxazolidinone **12** was synthesized following a known procedure.[19] The diastereoselective addition of the enolate of oxazolidinone **12** to imine **11** was carried out in the presence of 2.0 equiv of $Sn(OTf)_2$ and 4.0 equiv of *N*,*N*-diisopropylethylamine. The reaction was found to be complete in 2 h, as monitored by silica gel TLC. Purification of the crude product by chromatography on silica gel provided compound **13** as a colorless foam in 45% yield ($[\alpha]^{25}_D$ −19.3 (*c* 0.41, CH_2Cl_2)). Removal of the methylthio group was effected by treatment with 10 equiv of tributyltin hydride and AIBN in benzene at 80 °C for 2 h, affording compound **14** as a colorless foam in 72% yield ($[\alpha]^{25}_D$ −24.3 (*c* 0.30, CH_2Cl_2)). The use of less than 10 equiv of tributyltin hydride was insufficient to effect completion of the reaction; prolonged reaction times led to the decomposition of compound **13** and formed **14**. Treatment of compound **14** with an ethanolic solution of NH_3 at 0 °C led to selective ammonolysis of the carbonyl moiety in the propionate substituent, affording propionamide **15** as a colorless foam in 58% yield ($[\alpha]^{25}_D$ −25.9 (*c* 0.37, EtOH)). Saponification of **15** with 1 N LiOH in THF−H_2O at 0 °C provided pyrimidoblamic acid analogue **16** as a colorless powder in 75% yield ($[\alpha]^{25}_D$ −33.1 (*c* 0.22, H_2O)).

Scheme 3 shows the route employed for the synthesis of deamido deglycobleomycin demethyl A_2 (**1**). Pentapeptide TFA salt **20** ($[\alpha]^{25}_D$ +10.8 (*c* 0.38, CH_3OH), lit.[13c] $[\alpha]^{25}_D$ +7.3 (*c* 1, CH_3OH)) was synthesized according to a reported procedure.[13c] Pentapeptide **20** was coupled with pyrimidoblamic acid analogue **16** using BOP reagent in the presence of *N*,*N*-diisopropylethylamine in anhydrous DMF to give fully protected deamido deglycobleomycin demethyl A_2 (**21**) as a colorless powder in 69% yield.

To ensure that both Boc and *tert*-butyl groups could be removed smoothly from compound **21**, pyrimidoblamic acid analogue **16** was used as a model system to test the reaction conditions to be used for the deprotection of **21**. It was found that treatment of pyrimidoblamic acid analogue **16** with 2:1 TFA−dimethyl sulfide or 25% TFA in CH_2Cl_2 at 0 °C for 3 h resulted only in partial deprotection of the substrate as indicated by ^{1}H NMR spectroscopy. It was surmised that the Boc group was removed completely, while the *tert*-butyl group underwent only partial cleavage. It has been reported that *tert*-butyl groups are more stable than Boc groups in acidic media.[20] Therefore, **16** was treated with 2:1 TFA−dimethyl sulfide or 25% TFA in CH_2Cl_2 at 0 °C for 0.5 h and then at 25 °C for 2 h. Compound **16** underwent complete deprotection under these conditions as suggested by its ^{1}H NMR spectrum. The product was characterized further by high-resolution FAB mass spectrometry after purification by HPLC on an Alltima analytical C_{18} reversed-phase column using CH_3OH−0.1 M NH_4OAc, pH 5.5, as the mobile phase. Treatment of **21** with 2:1 TFA−dimethyl sulfide at 0 °C for 1 h and then at 25 °C for 3 h provided deamido deglycobleomycin demethyl A_2 (**1**) as a colorless solid in 60% yield after purification on a C_{18} reversed-phase column using CH_3OH-H_2O as the mobile phase. Alternatively, crude product **1** was desalted on an Amberlite XAD-2 column. The copper chelate of **1** was then purified on a CM Sephadex C-25 column.[1b,14e] The resulting copper complex of **1** was treated with 10% EDTA solution to remove the copper and then desalted on a C_{18} reversed-phase column to afford **1** as a colorless powder ($[\alpha]^{25}_D$ +22 (*c* 0.10, CH_3OH), $[\alpha]^{25}_D$ +16.2 (*c* 0.13, H_2O)).

The conversion of synthetic BLM demethyl A_2 to BLM A_2 by methylation of its Cu(II) chelate has been reported; the lack

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(18) Boger, D. L.; Honda, T. *Tetrahedron Lett.* **1993**, *34*, 1567.
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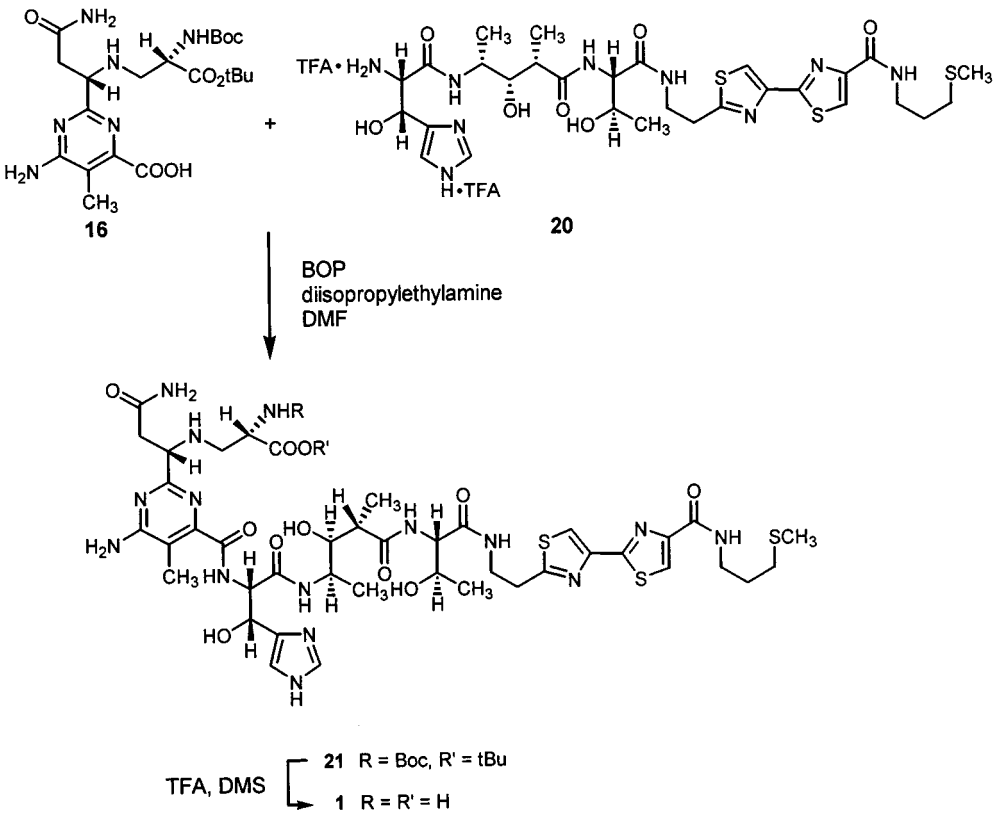
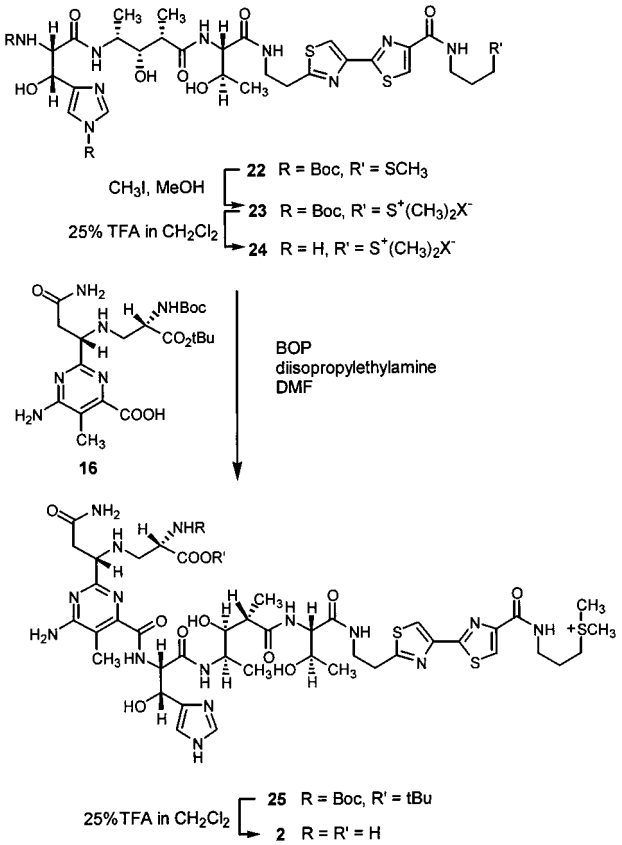
Scheme 3



of methylation of the pyrimidoblamic acid moiety has been attributed to coordination of the primary amine by Cu(II).[14e,21] However, whether the primary amine or the carboxylic acid in the pyrimidoblamic acid moiety of deamido BLM A_2 was coordinated with Cu(II) in its Cu(II)-complex seemed uncertain.[22] Therefore, it was unclear whether formation of the Cu(II)·deamido BLM A_2 complex would preclude methylation of the primary amine during the conversion of **1** to **2**. Alternatively, the condensation of Boc pyrimidoblamic acid with the dimethylsulfonium derivative of the pentapeptide could potentially provide Boc deglycobleomycin A_2 directly.[14c,d] Accordingly, di-Boc pentapeptide **22** ($[\alpha]^{25}_D$ +10.8 (*c* 0.38, CH_3OH), lit.[13c] $[\alpha]^{25}_D$ +7.3 (*c* 1, CH_3OH)) was synthesized according to our reported procedure.[13c] Compound **22** was then treated with iodomethane in methanol for 19 h to afford di-Boc pentapeptide **23** as a light yellow solid in quantitative yield (Scheme 4). Control of the reaction time was critical because prolonged reaction times resulted in the formation of significant amounts of an undefined side product. Treatment of **23** with 25% TFA in CH_2Cl_2 at 0 °C for 3 h provided pentapeptide **24**[13a,23] as a colorless powder in 75% yield ($[\alpha]^{25}_D$ +5.8 (*c* 0.30, CH_3OH), lit.[23] $[\alpha]^{25}_D$ +6.3 (*c* 0.20, CH_3OH)).[23] The condensation of compound **24** with pyrimidoblamic acid analogue **16** using BOP reagent in the presence of *N*,*N*-diisopropylethylamine in DMF gave the protected deamido deglycobleomycin A_2 **25**. Treatment of **25** with 25% TFA in CH_2Cl_2 at 0 °C for 15 min, and then at 25 °C for 3 h, afforded deamido deglycobleomycin A_2 (**2**) as a

Scheme 4



colorless powder, after purification on a C_{18} reversed-phase column using CH_3OH-H_2O as the mobile phase ($[\alpha]^{25}_D$ +22.5 (*c* 0.12, H_2O)).

(21) Roy, S. N.; Orr, G. A.; Brewer, C. F.; Horwitz, S. B. *Cancer Res.* **1981**, *41*, 4471.
(22) Sugiura, Y.; Muraoka, Y.; Fujii, A.; Takita, T.; Umezawa, H. *J. Antibiot.* **1979**, *32*, 756.
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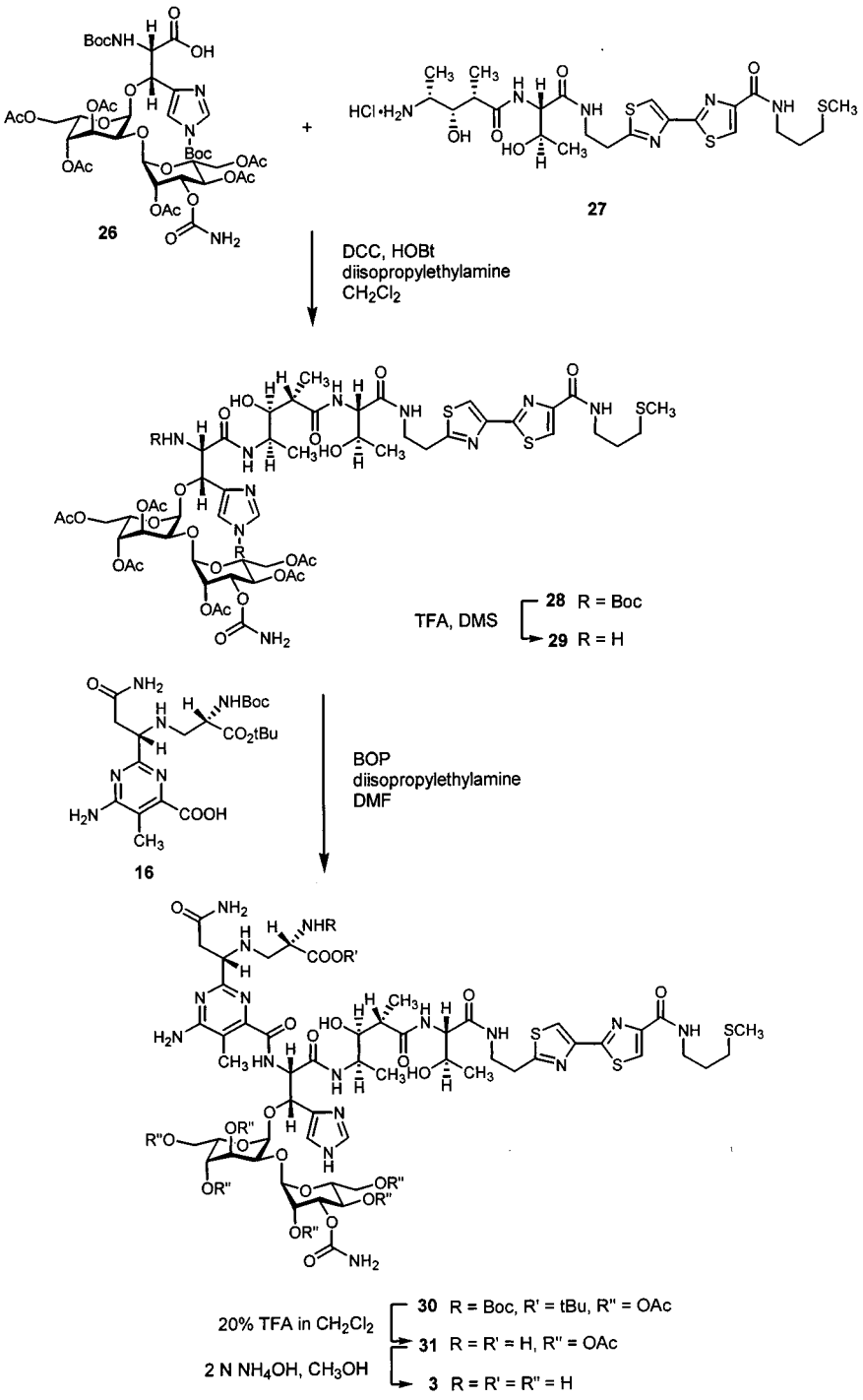
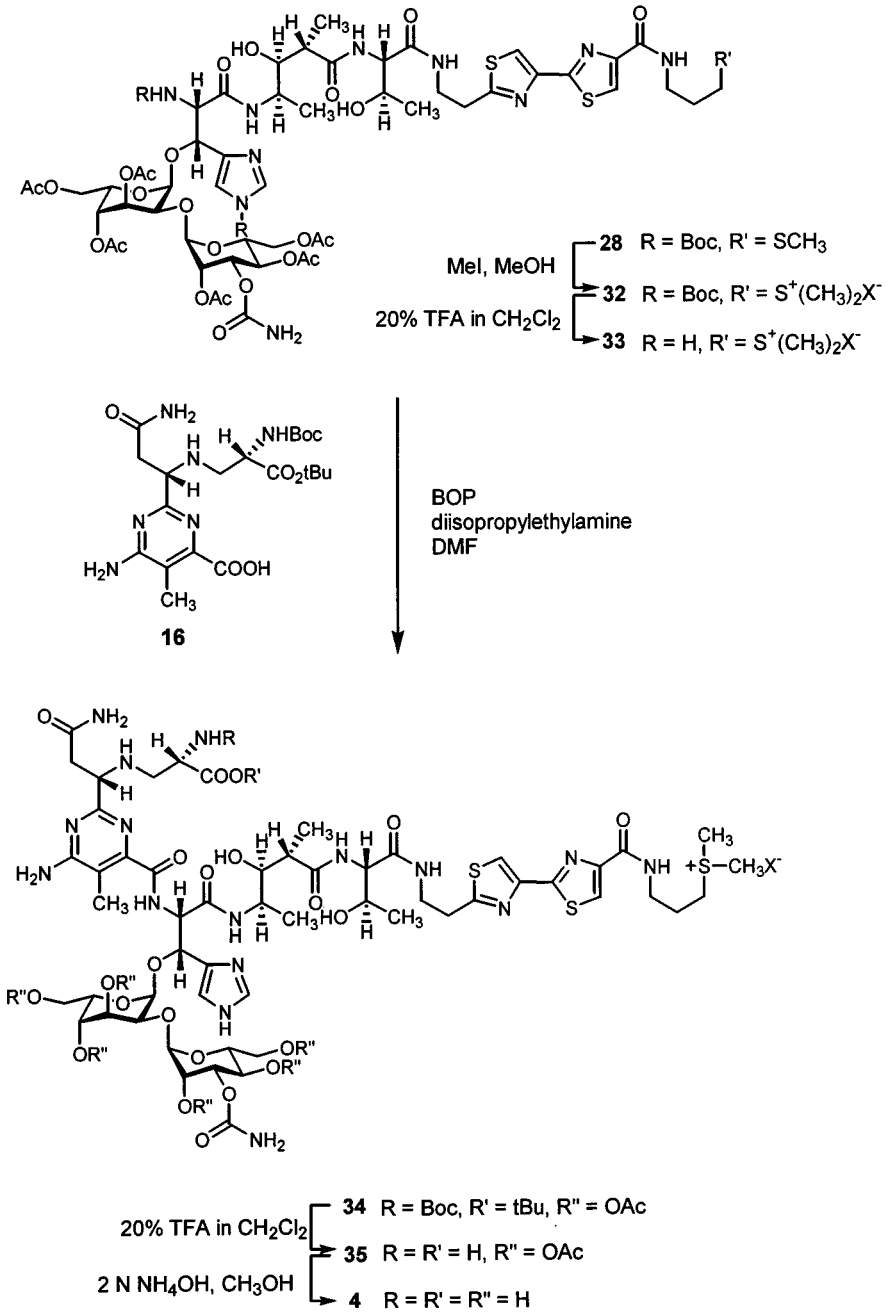
Scheme 5



The route used for the synthesis of deamido BLM demethyl A_2 is outlined in Scheme 5.[14a,b] Histidinyl disaccharide **26**[14c] and tetrapeptide hydrochloride **27** ($[\alpha]^{25}_D$ −3.3 (*c* 0.40, CH_3OH), lit.[24] $[\alpha]^{25}_D$ −4 (*c* 0.2, CH_3OH))[13a,c,24−26] were synthesized following known procedures. Tetrapeptide **27** was then condensed with histidinyl disaccharide **26** via the agency of DCC-HOBt in the presence of *N*,*N*-diisopropylethylamine in dry CH_2Cl_2 at 25 °C for 5 h, affording di-Boc pentapeptide **28** in 55% yield ($[\alpha]^{25}_D$ +2.8 (*c* 0.18, CH_3OH)). Di-Boc pentapeptide **28** was treated with a 2:1 mixture of trifluoroacetic acid and dimethyl sulfide to give pentapeptide disaccharide **29** as a colorless solid in 57% yield after purification on an Amberlite XAD-2 column ($[\alpha]^{25}_D$ +15.6 (*c* 0.09, CH_3OH)). Condensation of pyrimidoblamic analogue **16** with pentapeptide disaccharide **29** was carried out using BOP reagent in the presence of *N*,*N*-diisopropylethylamine in anhydrous DMF at 25 °C for 2 h. This provided fully protected deamido BLM demethyl A_2 (**30**) in 78% yield after purification on a C_{18} reversed-phase column ($[\alpha]^{25}_D$ +2.6 (*c* 0.23, CH_3OH)). Treatment of **30** with 20% TFA in CH_2Cl_2 at 25 °C for 2 h effected removal of the Boc and

(24) Boger, D. L.; Colletti, S. L.; Takeshi, H.; Menezes, R. F. *J. Am. Chem. Soc.* **1994**, *116*, 5607.
(25) Levin, M. D.; Subrahamanian, K.; Katz, H.; Smith, M. B.; Burlett, D. J.; Hecht, S. M. *J. Am. Chem. Soc.* **1980**, *102*, 1452.
(26) McGowan, D. A.; Jordis, U.; Minster, D. K.; Hecht, S. M. *J. Am. Chem. Soc.* **1977**, *99*, 8078.

Scheme 6



tert-butyl groups, affording partially deprotected deamido BLM demethyl A_2 **31** as a colorless solid in 45% yield after purification on a C_{18} reversed-phase column ($[\alpha]^{25}_D$ −8.0 (*c* 0.49, CH_3OH)). Deacetylation of **31** with 2 N ammonium hydroxide in methanol[14e] afforded deamido BLM demethyl A_2 (**3**) in 45% yield ($[\alpha]^{25}_D$ +21.5 (*c* 0.13, CH_3OH), $[\alpha]^{25}_D$ +17.3 (*c* 0.15, H_2O)).

The route employed for the synthesis of deamido BLM A_2 is shown in Scheme 6.[14a,b] Di-Boc pentapeptide disaccharide **28** was methylated by the use of iodomethane in methanol for 36 h. Sulfonium salt **32** was obtained as a light yellow solid in 92% yield. Deprotection of **32** was accomplished with 20% TFA in CH_2Cl_2 at 0 °C for 2 h, affording pentapeptide disaccharide **33** as a colorless solid in 74% yield ($[\alpha]^{25}_D$ +2.65 (*c* 1.36, CH_3OH)). Pyrimidoblamic acid analogue **16** was then condensed with **33** using BOP reagent in the presence of *N*,*N*-diisopropylethylamine in anhydrous DMF at 25 °C for 2 h. Fully protected deamido BLM A_2 **34** was obtained as a colorless solid in 83% yield ($[\alpha]^{25}_D$ +8.3 (*c* 0.12, CH_3OH)). Deprotection of **34** with 20% TFA in CH_2Cl_2 gave partially deprotected deamido BLM A_2 **35** as a colorless solid in 64% yield ($[\alpha]^{25}_D$ −7.1 (*c* 0.28, CH_3OH)). Deacetylation of **35** was accomplished with 2 N NH_4OH at 25 °C for 1.5 h to afford deamido BLM A_2 (**4**) as a colorless solid in 68% yield ($[\alpha]^{25}_D$ +25.4 (*c* 0.13, CH_3OH)).

To obtain an authentic sample of the catabolite of bleomycin, a sample of BLM A_2 was treated with human bleomycin hydrolase, essentially as described.[27] Aliquots of the incubation mixture were removed after 2.5, 5, and 24 h and analyzed by reversed-phase HPLC. As shown in Figure 4, there was a time-

(27) Sebti, S. M.; DeLeon, J. C.; Ma, L.-T.; Hecht, S. M.; Lazo, J. S. *Biochem. Pharmacol.* **1989**, *38*, 141.

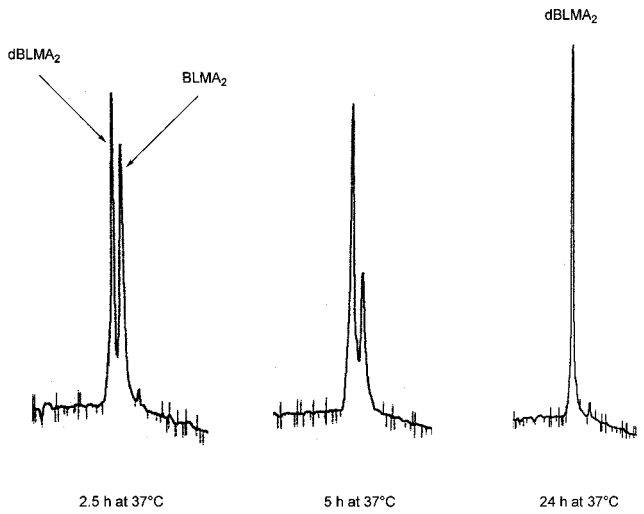


Figure 4. Conversion of BLM A_2 to deamido bleomycin A_2 by bleomycin hydrolase.

dependent conversion of BLM A_2 to a species that eluted more quickly from a C_{18} reversed-phase HPLC column. Co-injection of the BLM A_2 catabolite with authentic synthetic deamido bleomycin A_2 (**4**) afforded a single HPLC peak (Supporting Information Figure 1). Although the BLM A_2 catabolite prepared in this fashion was not absolutely pure as judged by ^{1}H NMR spectroscopy, the major product had a ^{1}H NMR spectrum that was superimposable on that of synthetic deamido BLM A_2 (**4**).

The synthetic and enzymatically prepared samples of deamido BLM A_2 were tested for their ability to effect the nicking of supercoiled pBR322 plasmid DNA. As shown in Figure 5, the synthetic and enzymatically derived samples mediated Fe(II)-dependent relaxation of a supercoiled plasmid DNA, and did so to essentially the same extent. While deamido BLM A_2 was less potent than Fe(II)·BLM A_2 itself (cf. lanes 4−7 vs lanes 9−13 and 15−19), it was clearly quite active in the DNA relaxation assay.

Also studied was the ability of deamido BLM A_2 to effect the degradation of linear duplex DNA. As shown in Figure 6, synthetic and enzymatically derived deamido BLM A_2 both effected Fe(II)-dependent DNA cleavage, and did so to essentially the same extent. While the extent of DNA degradation mediated by Fe(II)·deamido BLM A_2 was somewhat less than that obtained with Fe(II)·BLM A_2, the extent of cleavage was still quite significant. Moreover, the sequence selectivity of DNA cleavage obtained using Fe(II)·BLM A_2 and Fe(II)·deamido BLM A_2 was the same. In preliminary assays, the relative potencies of DNA cleavage by BLM A_2 and deamido BLM A_2 were not affected to any significant extent as a function of pH (data not shown).

Discussion

Central to the synthesis of deamido BLM A_2 was the preparation of pyrimidoblamic acid analogue **16** (Scheme 1). The Boc and *tert*-butyl groups were selected to protect the primary amine and carboxylic acid of β-aminoalanine moiety, respectively, so that both protecting groups could be removed simultaneously under acidic conditions. Boger et al. reported that stereoselective introduction of β-aminoalanine moiety could lead to such an analogue efficiently.[16g] Compound **8** was a key precursor for the diastereoselective imine addition reaction. It was synthesized from compound **5**, which we utilized previously for the synthesis of pyrimidoblamic acid.[16f] In this fashion, compound **8** could be prepared in large quantity and a state of purity suitable for the next reaction without further purification.

N-Acyloxazolidinone chiral auxiliaries have been used successfully in diastereoselective imine addition reactions for the synthesis of pyrimidoblamic acid.[16g] The addition of the enolate of (4*S*,5*R*)-3-((methylthio)acetyl)-4-methyl-5-phenyl-2-oxazolidinone (**12**) to imine **11** gave a mixture of diastereoisomers, the composition of which depended on the structure of the imine and enolate substituent as well as the specific reaction conditions.[16g,18,28] It has been reported that this reaction could provide a major diastereomer having the same absolute configuration at the α-methine carbon as BLM.[16g] Because imine **11** was different from that used for the synthesis of pyrimidoblamic acid itself, the stereochemistry of the α-methine carbon of the major product **13** could not be assigned definitively. Therefore, the major product was converted to the respective pyrimidoblamic analogue **16**, and the stereochemistry of the α-methine carbon of this compound was determined unambiguously by comparison of its CD spectrum with those of pyrimidoblamic acid and *epi*-pyrimidoblamic acid (Supporting Information Figure 2).[28] It was found that compound **16** had the same stereochemistry at the α-methine carbon as pyrimidoblamic acid, indicating that compound **16** was the desired pyrimidoblamic acid analogue required for the preparation of deamido BLMs **1**−**4**.

Deamido BLM A_2 was elaborated by the sequential coupling of suitably protected disaccharide, β-hydroxyhistidine, methylvalerate, and threonylbithiazole derivatives with the new pyrimidoblamic acid analogue. The sequence of couplings greatly affects the efficiency of the synthesis of BLMs. The coupling strategy for the fragments followed that reported previously.[13,14] Accordingly, key intermediate pentapeptide disaccharide **28** could be obtained by the coupling of histidinyl disaccharide **26**[14e] with tetrapeptide hydrochloride **27**[24] in good yield. This method helped to minimize requirements for the less accessible compound **26** and made the whole synthesis more convergent.

One unique feature of BLM in treating cancer is its lack of significant hepatic, renal, and bone marrow toxicities that have been associated with other anticancer drugs.[29] However, there are two major limitations to the use of BLM as an antitumor agent in a clinical setting: side effects such as BLM-induced pulmonary fibrosis[9,30,31] and tumor resistance.[31,32] Bleomycin hydrolase, a cysteine protease of the papain superfamily, is believed to be pertinent to these limitations. This enzyme can metabolize BLM, reportedly by hydrolyzing the carboxamide of the β-aminoalanineamide moiety of BLM to a carboxylic acid to provide deamido BLM as the product.[9,29−33] Bleomycin hydrolase has been found to be expressed in bacteria, yeast, birds, reptiles, and mammals. The DNA cleavage activity of

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(31) Bennett, J. M.; Reich, S. D. *Ann. Intern. Med.* **1979**, *90*, 945.
(32) (a) Sebti, S. M.; Lazo, J. S. *Pharmacol. Ther.* **1988**, *38*, 321. (b) Lazo, J. S.; Sebti, S. M. In *Anticancer Drug Resistance*; Kessel, D., Ed.; CRC Press: Boca Raton, FL, 1989; p 267 ff.
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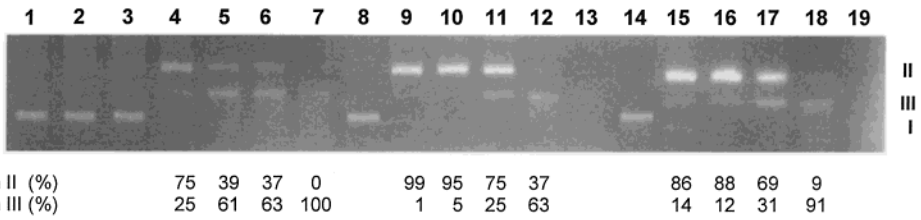

Figure 5. Relaxation of supercoiled pBR322 plasmid DNA by deamido bleomycin A_2. Lane 1, DNA alone; lane 2, 2 μM Fe^{2+}; lane 3, 10 μM BLM A_2 alone; lanes 4−7, 1, 2.5, 5, and 10 μM BLM A_2, respectively, in the presence of 2 μM Fe^{2+}; lane 8, 50 μM synthetic deamido BLM A_2 (**4**) alone; lanes 9−13, 2.5, 5, 10, 20, and 50 μM synthetic deamido BLM A_2, respectively, in the presence of 2 μM Fe^{2+}; lane 14, 50 μM BLM A_2 catabolite alone; lanes 15−19, 2.5, 5, 10, 20, and 50 μM BLM A_2 catabolite, respectively, in the presence of 2 μM Fe^{2+}. The percentage of forms II and III DNA present in each lane is indicated.

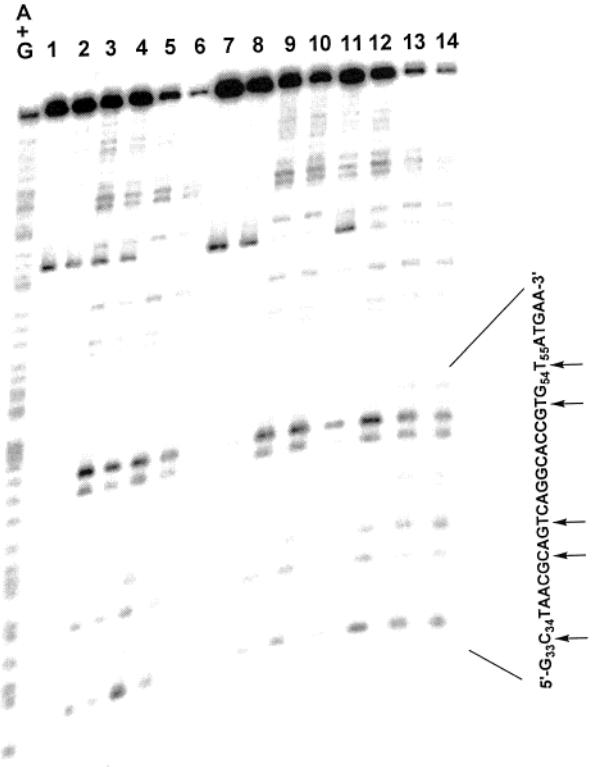

Figure 6. Cleavage of a 5′-^{32}P end-labeled 158-base pair DNA duplex by deamido bleomycin A_2. Lane 1, DNA alone; lane 2, 10 μM Fe^{2+}; lane 3, 10 μM BLM A_2; lanes 4−6, 1, 5, and 10 μM Fe(II)·BLM A_2, respectively; lane 7, 10 μM synthetic deamido BLM A_2 (**4**); lanes 8−10, 1, 5, and 10 μM synthetic Fe(II)·deamido BLM A_2, respectively; lane 11, 10 μM BLM A_2 catabolite; lanes 12−14, 1, 5, and 10 μM Fe(II)·BLM A_2 catabolite, respectively. The bands migrating $\sim^1/_4$ of the length of the gel in lanes 1−4, 6, 7, and 11 were due to undenatured duplex DNA.

deamido BLM was initially reported to be much less than that of BLM.[10] It was found, for example, that the DNA cleavage activity of deamido PEP, a BLM congener that is more potent in inhibiting tumor cells but less pulmonary toxic than BLM, was 1% of that of the respective BLM at pH 7.4.[10] On the other hand, Huang et al.[11] later reported that deamido BLM A_2 retained a significant fraction of the ability of BLM A_2 to degrade DNA when the two were compared, especially in regards to single-strand cleavage. However, the latter workers noted a substantial decrease in the extent of double-strand cleavage which could plausibly account for the loss of antitumor activity by deamido BLM.

A pH-dependent ligand change in Fe·deamido BLM, relative to that in Fe·BLM, has been suggested on the basis of the observation that the retention time of the deamido BLM A_2·Cu(II) complex on the CM-Sephadex column was intermediate between those of the Cu(II) complexes of BLM A_2 and BLM demethyl A_2.[22] At the outset of our studies, it seemed plausible that a ligand change of this type could be responsible for the diminished activity reported for deamido BLM.

BLM has been demonstrated to be metabolized extensively by a variety of organs in vivo[9,34] and in vitro.[9,32b,35] The level of bleomycin hydrolase activity appears to play an important role in protecting organs from BLM-induced toxicity. It has been shown that pulmonary fibrosis results from the lack of metabolism of BLM by bleomycin hydrolase in the lung.[34−36] In contrast, whether the ability of bleomycin hydrolase in tumor cells to metabolize BLM to deamido BLM contributes to tumor resistance to BLM treatment has been less well defined. Studies employing different tumor cell lines or methods to measure tumor response to BLM treatment have sometimes led to conflicting conclusions.[9,10,37] In one study that employed clonigenic tumor cells, no correlation was observed between bleomycin hydrolase activity and the sensitivity of human tumors to BLM.[37c] In other reports, however, BLM-resistant tumors were shown to have an enhanced ability to metabolize BLM and underwent less DNA cleavage by BLM than BLM-sensitive tumors in vivo and in vitro.[37b,e]

Deamido BLM was the only metabolite of BLM found in tumor homogenates from any species.[9,30] However, the metabolism of BLM by human tumors grown in vivo was found to give other metabolites in addition to deamido BLM A_2; this mixture was unable to degrade DNA.[34,37e] This indicated either that bleomycin hydrolase could produce other BLM metabolites in addition to deamido BLM in vivo or that other enzymes were also capable of metabolizing BLM in vivo. Despite the conflicting results, the aggregate studies tend to show that bleomycin hydrolase converts BLM predominantly to deamido BLM, that the latter has at least somewhat diminished DNA cleavage activity relative to BLM, and that BLM-resistant tumors have high levels of bleomycin hydrolase. However, there is a lack of direct evidence to correlate tumor resistance to BLM treatment with diminished DNA cleavage activity of deamido BLM. This reflects both the paucity of data that defines the

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(35) Ohnuma, T.; Holland, J. F.; Masuda, H.; Waligunda, J. A.; Goldberg, G. A. *Cancer* **1974**, *33*, 1230.
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cellular and nuclear uptake of deamido BLM and its detailed characterization as a DNA cleaving agent, as well as the reality that cellular loci other than DNA may also be important for the expression of antitumor activity by BLM.[38] For example, BLM can cleave a variety of RNA substrates.[3] The interaction of BLM with RNA rather than DNA has been postulated to be a possible source of the cytotoxic effects of BLM.[3,39]

In an effort to support the thesis that tumor resistance to BLM treatment results from its catabolic conversion to deamido BLM, it was first necessary to evaluate the DNA cleavage activity of deamido BLM in a cell free system, relative to that of BLM itself. Definitive interpretation of the results also demanded that the structure assigned to deamido BLM be verified. Therefore, we have carried out the synthesis of deamido BLM to facilitate the study of its properties.

In the present study, we have prepared four deamido BLM derivatives in an unambiguous fashion, including deamido BLM A_2 (**4**). Each of the BLM analogues was purified by C_{18} reversed-phase HPLC following synthesis. Comparison of synthetic **4** with the product resulting from degradation of BLM A_2 with bleomycin hydrolase was carried out by HPLC for the synthetic and enzymatically derived compounds in the presence and absence of Cu^{2+}, and at different pH values. This analysis indicated no difference in behavior between the two samples. Enzymatically derived BLM A_2 had the same ^{1}H NMR spectrum as the authentic synthetic sample of **4**; these were found to be identical. As shown in Figure 5, synthetic and enzymatically derived deamido BLM A_2 had the same potency in an assay designed to measure relaxation (i.e., nicking) of a supercoiled plasmid DNA. Also noted in the figure is a comparison of DNA cleavage potential with that of BLM A_2. As is clear from the figure, deamido BLM A_2 has significant DNA cleavage activity at pH 7.0, although less than that of BLM A_2. The percentage of form II (nicked circular) and form III (linear duplex) DNA present is given in each lane. As noted previously by Huang et al.,[11] deamido BLM A_2 produced less double-strand DNA breakage than did BLM A_2. Interestingly, unlike the results noted by Huang et al., we found little pH dependence of DNA cleavage by deamido BLM. For example, at pH 6.0, treatment of supercoiled pBR322 DNA with 10 μM **4** afforded 76% form II DNA and 24% form III DNA (vs values of 75 and 25%, respectively, shown in Figure 5 at pH 7.0). At pH 8.0, the respective values were 74 and 26%.

Also studied was the cleavage of a 5′-^{32}P end-labeled DNA duplex by deamido BLM A_2 (Figure 6). As in the experiments involving DNA plasmid relaxation, the synthetic and enzymatically derived samples of deamido BLM A_2 exhibited the same potency and sequence selectivity of DNA cleavage. For example, synthetic Fe(II)·deamido BLM A_2 (**4**) effected the conversion of 1, 42, and 64% of the linear DNA duplex substrate to sequence specific cleavage products of concentrations of 1, 5, and 10 μM, respectively. While this derivative was somewhat less potent than BLM A_2 in mediating DNA cleavage, the sequence selectivity of cleavage was unchanged.

While the diminution of DNA cleavage potential that accompanies the catabolic conversion of BLM to deamido BLM may conceivably be sufficient to account for the observed "inactivation" of BLM as a cytotoxic agent by BLM hydrolase, it is important to note that the foregoing experiments were carried out in a cell free system. Altered facility of cell uptake could also contribute to the diminished cytotoxicity of deamido BLM. Additionally, the pH dependence of DNA cleavage by deamido BLM A_2 may help to define the issue of coordination geometry of the ligand about the metal ions required for DNA cleavage[22] and is under investigation. The chemistry of DNA cleavage and the ability of deamido BLM to effect RNA degradation are also under active investigation and will be reported in due course.

Experimental Section

Ethyl 4-Chloro-2-(hydroxymethyl)-5-methylpyrimidine-6-carboxylate (6). To a solution of 8.59 g (37.6 mmol) of **5**[16a] in 150 mL of absolute ethanol was added 500 mg (13.2 mmol) of $NaBH_4$ at 0−5 °C. The reaction mixture was stirred at 0−5 °C for 30 min and then treated with 10 mL of 10% hydrochloric acid. The reaction mixture was stirred for 10 min and diluted with 100 mL of dichloromethane. The reaction mixture was washed successively with 30 mL of saturated sodium bicarbonate solution and 80 mL of brine, then dried ($MgSO_4$), and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (30 × 4 cm). Elution with 1:3 ethyl acetate−hexane gave **6** as a colorless oil: yield 6.0 g (70%). Silica gel TLC R_f 0.50 (1:1 ethyl acetate−hexane). ^{1}H NMR ($CDCl_3$): δ 1.43 (t, 3H, J = 7.0 Hz), 2.50 (s, 3H), 3.26 (br s, 1H), 4.74 (q, 2H, J = 7.0 Hz), and 4.83 (s, 2H). ^{13}C NMR ($CDCl_3$): δ 13.69, 14.61, 62.36, 63.74, 126.50, 156.61, 163.34, 164.03, and 166.16. Mass spectrum (FAB), m/z 231.1 $(M + H)^+$. Mass spectrum (FAB), m/z 231.0544 $(M + H)^+$ ($C_9H_{12}ClN_2O_3$ requires 231.0536).

Ethyl 4-Azido-2-(hydroxymethyl)-5-methylpyrimidine-6-carboxylate (7). To a solution containing 6.0 g (26.1 mmol) of **6** in 250 mL of DMF at 0−5 °C was added 5.14 g (79.1 mmol) of sodium azide. The reaction mixture was stirred at 0−5 °C for 6 h and then poured into 1.2 L of water. The reaction mixture was extracted with three 300 mL portions of ethyl acetate. The combined organic layer was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 5 cm). Elution with 1:4 ethyl acetate−hexane gave fractions which afforded a solid upon concentration of the solvent; this had λ_{max} 268 nm; crystallized from ethyl acetate−hexane to give **7** as colorless needles: yield 5.3 g (86%). mp 115−116 °C. Silica gel TLC R_f 0.50 (95:5 dichloromethane−methanol). UV (MeOH) λ_{max} 268 nm. ^{1}H NMR ($CDCl_3$): δ 1.47 (m, 3H), 2.28, 3.01 (2 s, 3H), 3.34−3.42 (m, 1H), 4.42−4.55 (m, 2H), and 4.78, 5.36 (2 d, 2H). ^{13}C NMR ($CDCl_3$): δ 13.80, 59.87, 62.41, 123.71, 146.75, 151.39, 163.46, and 165.40. IR ($CHCl_3$): 3501, 3016, 2142, and 1729 cm^{-1}. Mass spectrum (FAB), m/z 238.2 $(M + H)^+$. Mass spectrum (FAB), m/z 238.0943 $(M + H)^+$ ($C_9H_{12}N_5O_3$ requires 238.0938).

Ethyl 4-Amino-2-(hydroxymethyl)-5-methylpyrimidine-6-carboxylate (8).[16g] A mixture of 2.6 g (11 mmol) of **7** and 240 mg of 10% palladium-on-carbon in 135 mL of absolute ethanol was stirred at 25 °C under 1 atm of hydrogen for 2 h. The catalyst was filtered through Celite and washed with ethanol. The filtrate was concentrated under diminished pressure to give amine **8** as a colorless powder: yield 2.2 g (95%). mp 149−151 °C (lit.[16g] mp 169 °C). Silica gel TLC R_f 0.10 (95:5 dichloromethane−methanol). UV (MeOH) λ_{max} 236 and 284 nm. ^{1}H NMR ($CDCl_3$): δ 1.41 (t, 3H, J = 7.0 Hz), 2.22 (s, 3H), 3.63 (br s, 1H), 4.43 (q, 2H, J = 7.0 Hz), 4.62 (s, 2H), and 5.19 (br s, 2H). Mass spectrum (chemical ionization), m/z 212 $(M + H)^+$.

4-Amino-6-carboethoxy-5-methylpyrimidine-2-carboxaldehyde (9).[16g] A solution of 200 mg (0.95 mmol) of **8** in 25 mL of acetonitrile was treated with 1.2 g of freshly made MnO_2. The reaction mixture was heated to 75 °C and stirred for 2 h. The cooled mixture was filtered through Celite and washed with acetonitrile. The filtrate was concen-

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trated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (8 × 1 cm). Elution with 4:1 ethyl acetate−hexane gave **9** as an amorphous solid: yield 80 mg (40%). Silica gel TLC R_f 0.40 (93:7 dichloromethane−methanol). ^{1}H NMR ($CDCl_3$): δ 1.44 (t, 3H, J = 7.0 Hz), 2.33 (s, 3H), 4.48 (q, 2H, J = 7.0 Hz), 6.49 (br s, 2H), and 9.89 (s, 1H). Mass spectrum (chemical ionization), m/z 210 $(M + H)^+$.

***N*$^\alpha$-(*tert*-Butoxycarbonyl)-*N*$^\beta$-[[(4-amino-6-(carboethoxy)-5-methylpyrimidin-2-yl)methylene]amino]-(*S*)-*β*-aminoalanine *tert*-Butyl Ester (11).** To a solution containing 135 mg (0.645 mmol) of **9** and 168 mg (0.645 mmol) of **10** in 15 mL of dry acetonitrile was added 3.7 g of 3 Å molecular sieves. The reaction mixture was stirred under N_2 for 24 h and then filtered through Celite. The filtrate was concentrated under diminished pressure to give imine **11** as a colorless foam: yield 282 mg (97%). $[\alpha]^{25}_D$ −20.6 (*c* 0.85, CH_2Cl_2). ^{1}H NMR (C_6D_6): δ 1.32 (s, 9H), 1.40 (s, 9H), 1.43 (t, 3H, J = 7.0 Hz), 2.34 (s, 3H), 3.84 (dd, 1H, J = 14.0, 3.0 Hz), 4.00 (q, 2H, J = 7.0 Hz), 4.02−4.06 (m, 1H), 4.56−4.59 (m, 1H), 5.88 (d, 1H, J = 9.0 Hz), and 8.27 (s, 1H). ^{13}C NMR (C_6D_6): δ 12.48, 14.17, 54.06, 79.78, 82.66, 114.63, 152.93, 155.30, 158.00, 163.77, 164.64, 166.04, and 171.61. Mass spectrum (FAB), m/z 452.3 $(M + H)^+$. Mass spectrum (FAB), m/z 452.2493 $(M + H)^+$ ($C_{21}H_{34}N_5O_6$ requires 452.2509).

***N*$^\alpha$-(*tert*-Butoxycarbomyl)-*N*$^\beta$-[3(*S*)-(4-amino-6-carboethoxy-5-methylpyrimidin-2-yl)-1-[(4*S*, 5*R*)-4-methyl-5-phenyl-2-oxazolidinyl]-2-methylthiopropion-3-yl]-(*S*)-*β*-aminoalanine *tert*-Butyl Ester (13).** To a solution containing 407 mg (976 μmol) of stannous trifluoromethanesulfonate in 1.5 mL of THF at −78 °C was added slowly a solution containing 129 mg (488 μmol) of oxazolidinone **12** in 1.5 mL of THF followed by 187 μL (138.8 mg, 1.07 mmol) of diisopropylethylamine. The reaction mixture was allowed to warm to −20 °C and was then stirred for 1 h. The reaction mixture was again cooled to −78 °C. To this mixture was added slowly a solution containing 110 mg (244 μmol) of imine **11** in 1.5 mL of THF. The reaction mixture was warmed to −5 °C and stirred for 2 h. The reaction mixture was poured into 20 mL of 2:1 CH_2Cl_2−saturated sodium bicarbonate solution and then filtered through Celite. The filtrate was washed with 20 mL of brine, dried ($MgSO_4$), and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 4 cm). Elution with 1:2 ethyl acetate−hexane gave **13** as a colorless solid: yield 79 mg (45%). Silica gel TLC R_f 0.47 (1:1 ethyl acetate−hexane). $[\alpha]^{25}_D$ −19.3 (*c* 0.41, CH_2Cl_2). UV (MeOH) λ_{max} 236 and 286 nm. ^{1}H NMR (C_6D_6): δ 0.93 (t, 3H, J = 7.5 Hz), 0.97 (d, 3H, J = 6.0 Hz), 1.17 (s, 9H), 1.26 (s, 9H), 1.86 (s, 3H), 2.30 (s, 3H), 2.69 (dd, 1H, J = 13.0, 4.5 Hz), 2.93 (dd, 1H, J = 13.0, 4.0 Hz), 4.00 (q, 2H, J = 7.0 Hz), 4.24 (m, 1H), 4.31 (dq, 1H, J = 8.5, 7.5 Hz), 4.48 (d, 1H, J = 11.0 Hz), 4.55 (d, 1H, J = 6.5 Hz), 5.43 (br s, 1H), 5.52 (d, 1H, J = 11.0 Hz), 5.71 (d, 1H, J = 8.5 Hz), and 6.80−6.93 (m, 5H). ^{13}C NMR (C_6D_6): δ 11.7, 11.9, 14.1, 14.8, 27.9, 28.4, 46.6, 49.3, 55.3, 55.5, 61.4, 65.8, 78.6, 78.8, 81.0, 111.2, 126.0, 134.0, 153.4, 154.5, 156.0, 164.3, 166.4, 166.6, 170.8, and 171.0. Mass spectrum (FAB), m/z 717.4 $(M + H)^+$. Mass spectrum (FAB), m/z 717.3296 $(M + H)^+$ ($C_{34}H_{49}N_6O_9S$ requires 717.3281).

***N*$^\alpha$-(*tert*-Butoxycarbonyl)-*N*$^\beta$-[3(*S*)-4-amino-6-carboethoxy-5-methylpyrimidin-2-yl)-1-[(4*S*, 5*R*)-4-methyl-5-phenyl-2-oxazolidinyl]-propion-3-yl]-*S*-*β*-aminoalanine *tert*-Butyl Ester (14).** To a solution containing 36.5 mg (51 μmol) of **13** in 0.7 mL of dry benzene were added 137 μL (148 mg, 510 μmol) of Bu_3SnH and 4.5 mg (27.4 μmol) of AIBN. The reaction mixture was heated to 80 °C and stirred for 2 h. The cooled reaction mixture was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 1 cm). Gradient elution with 0 → 3% methanol in dichloromethane gave **14** as a colorless foam: yield 17.3 mg (72%). Silica gel TLC R_f 0.30 (95:5 dichloromethane−methanol). $[\alpha]^{25}_D$ −24.3 (*c* 0.30, CH_2Cl_2). UV (MeOH) λ_{max} 236 and 286 nm. ^{1}H NMR (C_6D_6): δ 0.82 (d, 3H, J = 6.5 Hz), 1.05 (t, 3H, J = 7.0 Hz), 1.37 (s, 9H), 1.40 (s, 9H), 1.95 (s, 3H), 2.81 (dd, 1H, J = 11.0, 2.0 Hz), 3.20 (dd, 1H, J = 12.0, 4.5 Hz), 3.42 (dd, 1H, J = 15.0, 5.5 Hz), 3.69 (br d, 1H, J = 5.5 Hz), 3.88 (dd, 1H, J = 15.0, 9.5 Hz), 4.14 (q, 2H, J = 7.0 Hz), 4.35 (dq, 1H, J = 8.0, 6.5 Hz), 4.41−4.53 (m, 1H), 4.87 (d, 1H, J = 7.5 Hz), 5.56 (br s, 1H), 6.07 (d, 1H, J = 8.0 Hz), and 6.96−7.05 (m, 5H). ^{13}C NMR (C_6D_6): δ 11.6, 14.1, 14.6, 28.0, 28.4, 41.5, 49.1, 54.9, 55.4, 61.5, 62.0, 78.6, 78.9, 81.1, 110.3, 126.0, 134.2, 153.2, 155.0, 156.0, 164.1, 166.4, 168.0, 171.0, and 171.3. Mass spectrum (FAB), m/z 671.4 $(M + H)^+$. Mass spectrum (FAB), m/z 671.3399 $(M + H)^+$ ($C_{33}H_{47}N_6O_9$ requires 671.3404).

***N*$^\alpha$-(*tert*-Butoxycarbonyl)-*N*$^\beta$-[1-amino-3(*S*)-(4-amino-6-(carboethoxy)-5-methypyrimidin-2-yl)propion-3-yl]-(*S*)-*β*-aminoalanine *tert*-Butyl Ester (15).** To a solution containing 25.1 mg of **14** (37 μmol) in 2 mL of absolute ethanol at 0 °C was added 5 mL of saturated ethanolic NH_3 solution. The reaction mixture was stirred at 0 °C for 1.5 h and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 1 cm). Gradient elution with 3 → 10% methanol in dichloromethane gave **15** as a colorless foam: yield 11 mg (58%). Silica gel TLC R_f 0.34 (9:1 dichloromethane−methanol). $[\alpha]^{25}_D$ −25.9 (*c* 0.37, EtOH). ^{1}H NMR (CD_3OD): δ 1.31 (t, 3H, J = 7.5 Hz), 1.36 (s, 9H), 1.37 (s, 9H), 2.06 (s, 3H), 2.47 (dd, 1H, J = 15.0, 8.0 Hz), 2.54 (dd, 1H, J = 14.5, 5.5 Hz), 2.74 (d, 2H, J = 5.5 Hz), 3.89 (dd, 1H, J = 8.0, 5.5 Hz), 4.03 (t, 1H, J = 5.5 Hz), and 4.33 (q, 2H, J = 7.5 Hz). ^{13}C NMR (CD_3OD): δ 11.8, 14.2, 28.0, 28.5, 42.0, 49.0, 55.5, 61.2, 61.6, 79.3, 81.3, 110.9, 154.0, 156.2, 164.6, 166.4, 167.3, 171.3, and 174.6. Mass spectrum (FAB), m/z 511.3 $(M + H)^+$. Mass spectrum (FAB), m/z 511.2885 $(M + H)^+$ ($C_{23}H_{39}N_6O_7$ requires 511.2880).

***N*$^\alpha$-(*tert*-Butoxycarbonyl)-*N*$^\beta$-[1-amino-3(*S*)-(4-amino-6-carboxy-5-methylpyrimidin-2-yl)propion-3-yl]-(*S*)-*β*-aminoalanine *tert*-Butyl Ester (16).** To a solution containing 33.7 mg (66 μmol) of **15** in 1 mL of 3:1:1 THF−CH_3OH−H_2O was added 132 μL of 1 N LiOH. The mixture was stirred at 0 °C for 2 h. After evaporation of most of the THF and CH_3OH, the aqueous phase was acidified to pH 4−5 with 10% citric acid. The residue was applied to an Amberlite XAD-2 column (8 × 1 cm). The column was washed successively with water and methanol. The combined methanol fraction was concentrated to dryness. The residue was dissolved in 1 mL of water, frozen, and lyophilized to give **16** as a colorless powder: yield 23.8 mg (75%). Silica gel TLC R_f 0.45 (4:1:1 BuOH−AcOH−H_2O). $[\alpha]^{25}_D$ −33.1 (*c* 0.22, H_2O). ^{1}H NMR (CD_3OD): δ 1.44 (s, 9H), 1.46 (s, 9H), 2.21 (s, 3H), 2.82 (dd, 1H, J = 16.0, 8.0 Hz), 2.90 (dd, 1H, J = 16.0, 4.0 Hz), 3.09 (dd, 1H, J = 12.5, 8.0 Hz), 3.31 (m, 1H), and 4.27−4.34 (m, 2H). ^{13}C NMR (D_2O): δ 10.8, 26.6, 27.1, 36.2, 46.1, 51.7, 58.7, 81.7, 84.4, 107.3, 157.0, 158.6, 159.4, 163.7, 169.4, 172.3, and 173.6. Mass spectrum (FAB), m/z 483.3 $(M + H)^+$. Mass spectrum (FAB), m/z 483.2561 $(M + H)^+$ ($C_{21}H_{35}N_6O_7$ requires 483.2567).

Deamido Bleomycin A_2 (4). A solution of 8 mg (3.9 μmol) of partially deprotected bleomycin A_2 **35** in 1 mL of methanol was treated with 1 mL of 2 N NH_4OH at 0 °C. The reaction mixture was stirred at 25 °C for 2 h and then concentrated under diminished pressure. The solution was then acidified to pH 2−3 with 0.1 N HCl and purified by flash chromatography on a C_{18} reversed-phase column (6 × 1 cm). Gradient elution with 0 → 30% methanol−water gave **4** as a colorless powder: yield 4.0 mg (68%). Silica gel TLC R_f 0.22 (10:9:1 methanol−10% ammonium acetate solution−10% ammonium hydroxide). $[\alpha]^{25}_D$ +25.4 (*c* 0.13, methanol). UV λ_{max} 238 (sh) and 291 nm. ^{1}H NMR (D_2O): δ 1.10−1.12 (m, 9H), 2.02 (s, 3H), 2.22 (qn, 2H, J = 7.0 Hz), 2.46−2.48 (m, 1H), 2.60−2.71 (m, 2H), 2.72−2.79 (m, 1H), 2.83−2.88 (m, 1H), 2.93 (s, 6H), 3.27 (t, 2H, J = 6.5 Hz), 3.40 (t, 2H, J = 6.5 Hz), 3.53−4.12 (m, 22H), 4.23 (d, 1H, J = 4.5 Hz), 4.80 (m, 1H), 5.02 (br s, 1H), 5.04 (d, 1H, J = 6.5 Hz), 5.26−5.29 (m, 2H), 7.28 (s, 1H), 7.80 (s, 1H), 8.04 (s, 1H), and 8.24 (s, 1H). Mass spectrum (FAB), m/z 1415.5 (M^+). Mass spectrum (FAB), m/z 1415.5030 (M^+) ($C_{55}H_{83}N_{16}O_{22}S_3$ requires 1415.5046).

Treatment of Bleomycin A_2 with Bleomycin Hydrolase. To 500 μg of bleomycin A_2 in 250 μL of 20 mM Tris-HCl, pH 7.5, was added 10 μg of human bleomycin hydrolase. The reaction mixture was incubated at 37 °C, and aliquots (2 μL) were removed at predetermined times, diluted with 40 μL of MeOH and 10 μL of 7.5 mM $CuSO_4$, and then analyzed by reversed-phase HPLC. HPLC analysis was carried out on an analytical (250 mm × 4.6 mm) C_{18} reversed-phase column. The column was washed with a linear gradient of 1 → 60% CH_3CN in 0.1% trifluoroacetic acid at a flow rate of 1 mL/min over a period of 45 min. The eluate was monitored at 290 nm.

Relaxation of Supercoiled Plasmid DNA by Deamido BLM A_2. The DNA relaxation assays were carried out in 25 μL (total volume) of 10 mM sodium cacodylate buffer, pH 7.0, containing 300 ng of pBR322 plasmid DNA and the concentrations of BLM and Fe^{2+} shown in the legend to Figure 5. The reaction mixtures were incubated at 37 °C for 30 min, and then quenched by the addition of 5 μL of loading dye (30% glycerol containing 0.125% (w/v) bromophenol blue) and applied to a 1% agarose gel containing 0.5 μg/mL ethidium bromide. Horizontal gel electrophoretic analysis was carried out in 9 mM Tris-borate buffer, pH 8.3, containg 320 μM disodium EDTA (156 W, 2 h). The DNA bands were visualized under UV light.

Cleavage of a 5′-^{32}P End-Labeled DNA Duplex by Deamido Bleomycin A_2. DNA cleavage reactions were carried out in 20 μL (total volume) of 10 mM sodium cacodylate, pH 7.0, containing a 5′-^{32}P end-labeled 158-bp DNA (~3×10^4 cpm) and the concentrations of BLM and Fe^{2+} shown in the legend to Figure 6. The reaction mixture was incubated at 4 °C for 30 min and lyophilized. The samples were dissolved in 5 μL of loading dye (80% formamide, 2 mM EDTA, 1% (w/v) xylene cyanol, and 1% (w/v) bromophenol blue), heated at 90 °C for 10 min, and then chilled on ice. The solutions were then applied to a 10% denaturing polyacrylamide gel (7 M urea). Electrophoretic analysis was carried out at 50 W for 2 h. The gel was analyzed using a (Molecular Dynamics) phosphorimager. The bands were correlated with those produced according to a Maxam−Gilbert A + G sequencing protocol.[40]

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Supporting Information Available: Synthesis of key intermediate **10** and BLM analogues **1**, **2**, and **3**. CD spectrum of compound **16** and HPLC analysis of synthetic deamido BLM A_2 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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