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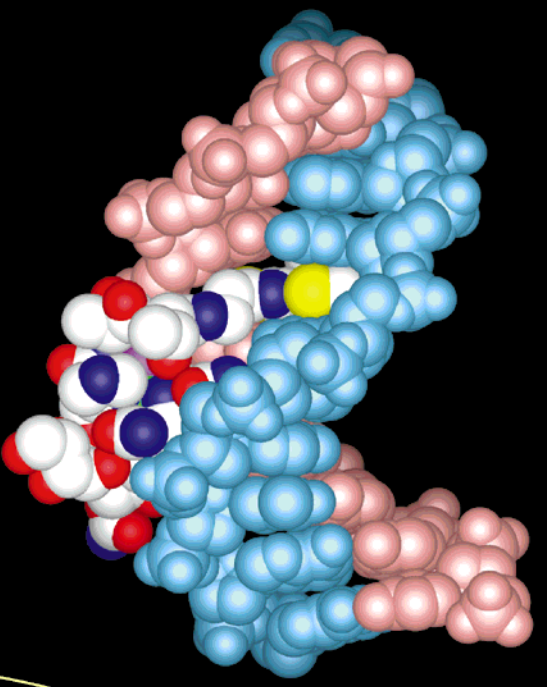

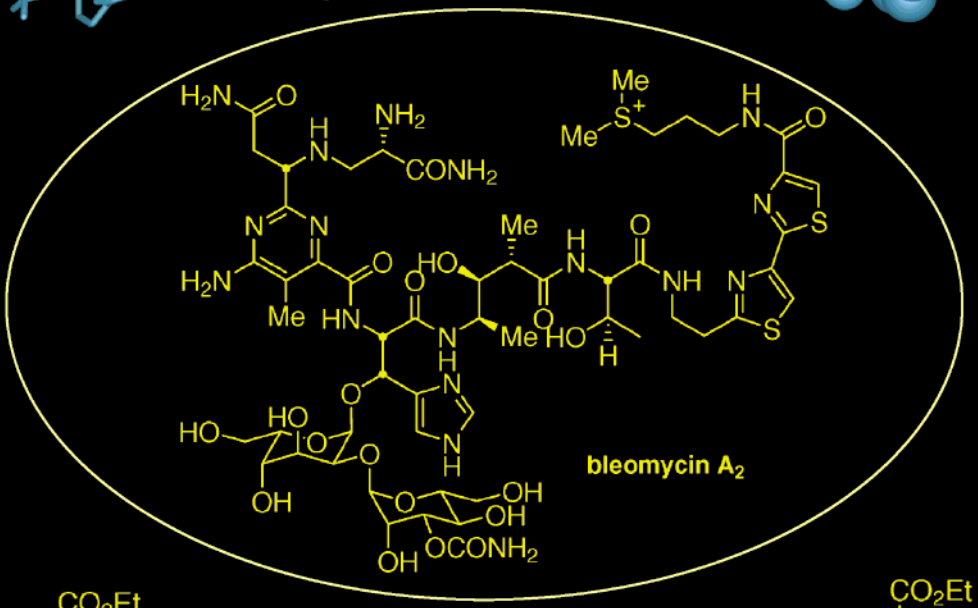

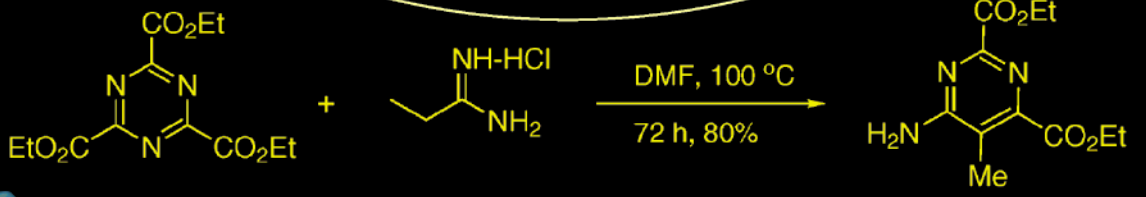

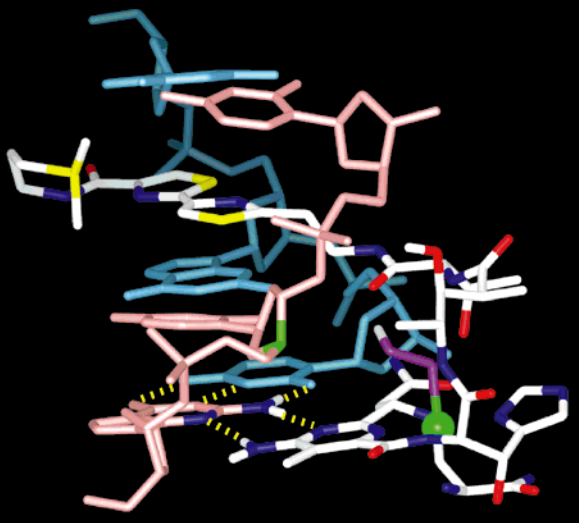

ANGEWANDTE CHEMIE

The binding and cleavage of DNA by bleomycin A_2 was investigated through the use of synthetic analogues.

Bleomycin: Synthetic and Mechanistic Studies

Dale L. Boger* and Hui Cai

Bleomycin A_2 is a clinically employed antitumor agent that derives its properties through the sequence-selective cleavage of DNA in a process that is both metal-ion and O_2 dependent. Highlights of the evolution of a modular total synthesis of bleomycin A_2 amenable to the preparation of an extensive series of analogues are detailed. The implementation of this technology in the preparation of nearly 50 analogues is summarized in efforts that clarify the functional roles of the individual subunits and their substituents. These studies, in conjunction with emerging structural models, define a remarkable combination of functional, structural, and conformational properties embodied in the natural product.

Keywords: antitumor agent · bioorganic chemistry · bleomycin · DNA cleavage · total synthesis

1. Introduction

The bleomycins are a class of glycopeptide antitumor antibiotics that were isolated by Umezawa and co-workers from *Streptomyces verticellus* over 30 years ago (Figure 1).[1] Bleomycin A_2 (**1**), which differs from other naturally occurring bleomycins only in the cationic C-terminus, is the major component (70%) of the clinical anticancer drug Blenoxane, which is used for the treatment of Hodgkin's lymphoma, carcinomas of the skin, head and neck, and testicular cancers.[2–15] Through a series of now classic chemical degradation studies coupled with X-ray crystallographic identification of the products, Umezawa and co-workers established the structure of bleomycin A_2, complete with the assignment of its relative and absolute stereochemistry.[16] The only exception to the X-ray structure determination of the degradation products was the N-terminus. Its early structural assignment, which contained a β-lactam, was revised to its correct structure in 1978[17] and its spectroscopically-derived absolute configuration was confirmed in our synthetic efforts.[18]

Bleomycin A_2 is thought to exert its biological effects through DNA binding and degradation, a process that is metal-ion and oxygen dependent.[19–26] It cleaves double-strand DNA selectively at 5′-GC or 5′-GT sites by minor groove C4′-H atom abstraction and subsequent fragmentation of the deoxyribose backbone (Scheme 1 and Figure 2).[27–32]

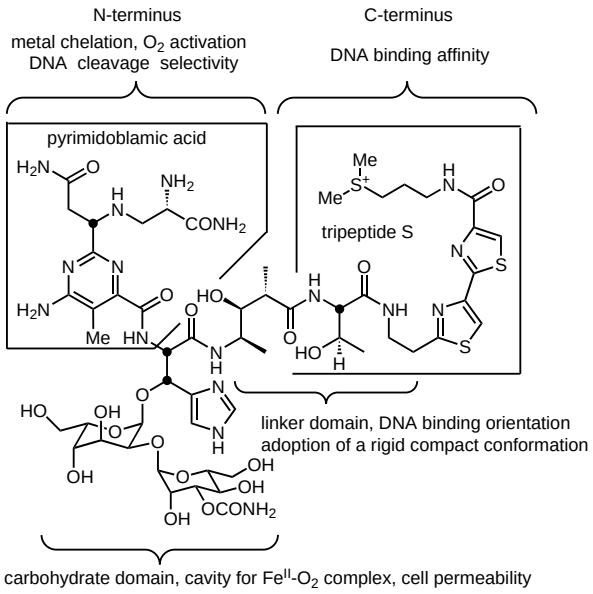

Figure 1. Structure of bleomycin A_2 (**1**) and functional roles of its subunits.

While both single- and double-strand cleavage are observed, the latter has been regarded by many as the biologically more important event.[10b, 33–41] More recently, bleomycin A_2 has been shown to cleave RNA[42, 43] as well as DNA-RNA hybrids,[44] which provide additional nucleic acid targets potentially related to its biological properties.

Each structural unit of bleomycin A_2 contributes importantly to its biological activity (Figure 1). Early and extensive structural,[45–65] biophysical,[66–71] chemical,[6, 72–75] and biological[76, 77] studies on bleomycin A_2 and its derivatives defined the N-terminal pyrimidine, including its β-aminoalanine

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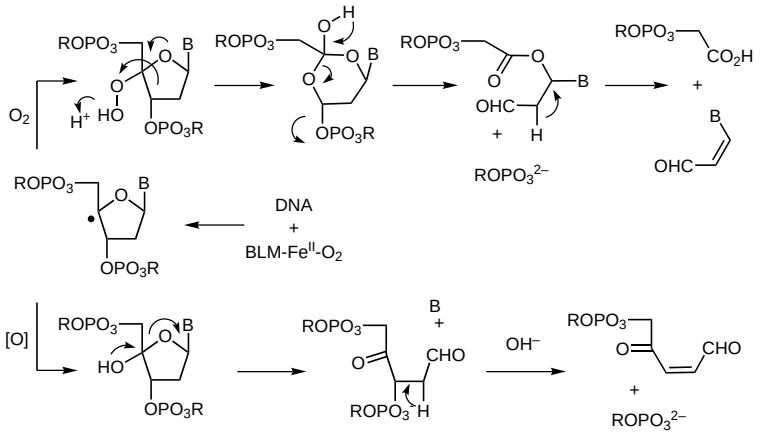

Scheme 1. Primary mechanisms for DNA cleavage induced by bleomycin A_2. B = nucleobase, BLM = bleomycin A_2.

amide side chain and the linked β-hydroxy-L-histidine, as the metal binding domain, and demonstrated that the majority of the DNA binding affinity originates from the C-terminus with the bithiazole and the positively charged sulfonium salt. The role of the carbohydrate domain has been less extensively examined although it is known to enhance biological potency and efficacy;[45, 76, 78, 79] while it does not influence the DNA cleavage selectivity, it does enhance cleavage efficiency. It has also been suggested that the carbohydrate domain facilitates cellular uptake. Although a crystal structure of bleomycin A_2 bound to DNA has not yet become available, several models based on solution structures determined by NMR spectroscopy have been advanced to elucidate the molecular basis of its metal chelation, DNA binding, and cleavage.[52, 53]

Despite efforts over the past three decades, many key questions remained unanswered at the onset of our inves-

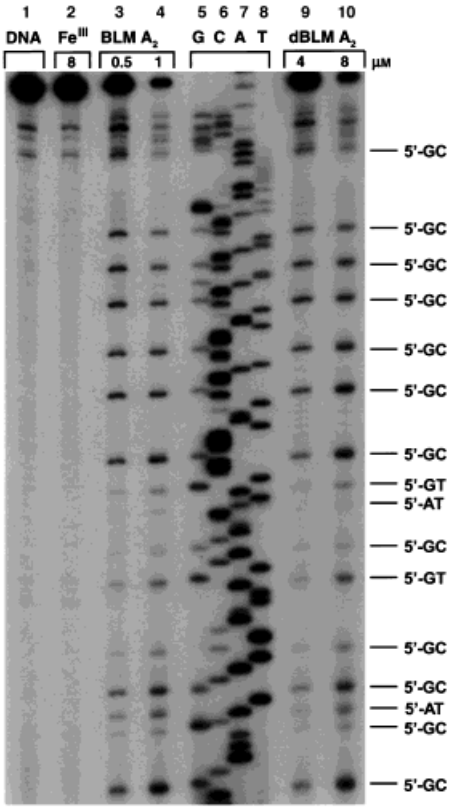

Figure 2. Sequence selective DNA cleavage by bleomycin A_2 (BLM A_2, **1**) and deglycobleomycin A_2 (dBLM A_2, **41**). The products were separated on a polyacrylamide gel by electrophoresis and detected autoradiographically. The untreated DNA was used as a control (lane 1), and its sequence is shown in lanes 5–8. No cleavage was observed on incubation with Fe^{III} ions alone (lane 2). The DNA was cleaved on incubation with BLM A_2 and dBLM A_2 in the presence of Fe^{III} ions (lanes 3 and 4, and 9 and 10, respectively), with the cleavage occurring at the sites labeled and at the concentrations indicated.

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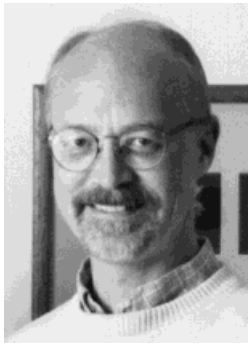

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tigations. Among them was the nature of the metal-binding ligands and their arrangement in the metal complexes. The possible functional roles of the linker domain and its substituents as well as the disaccharide were untested and even the origin of the sequence selectivity of the DNA cleavage and mechanistic aspects of its double-strand (ds) cleavage remained elusive and controversial. Both the C-terminus bithiazole[26, 80a] and the N-terminus metal-binding domain[68, 72, 80b] have been proposed to control the cleavage selectivity, and both bithiazole minor groove binding[26, 80a] or intercalation[66, 67c, 77a, 81, 94] have been advanced. Efforts to address these questions were limited by the accessibility of structural analogues because of the inherent complexity of bleomycins. It was in this context that we initiated a program on bleomycin A_2, which has led to the development of an efficient total synthesis and its application in the subsequent design, synthesis, and evaluation of nearly 50 key analogues. The results of our studies are summarized herein.

2. Total Synthesis of (+)-P-3A and Bleomycin A_2

Preceding our efforts, both Umezawa, Ohno et al.[82] and Hecht et al.[83] detailed pioneering total syntheses of deglycobleomycin A_2 and bleomycin A_2. These efforts have been reviewed elsewhere[6] and served to confirm the structure of the natural product and define challenges associated with its synthesis. Both were unable to control the stereochemistry at the C2 benzylic center of pyrimidoblamic acid and the diastereomer assignment was accomplished by correlation with the natural product, which precluded confirmation of the original assignment. By virtue of its highly substituted and functionalized nature, even the pyrimidoblamic acid preparation proved challenging and overall yields for the approaches were low. Assemblage and introduction of the disaccharide unit proved problematic and glycosidation of a protected β-hydroxy-L-histidine proved modest (ca. 20%) and proceeded with a lack of diastereoselection at the newly introduced anomeric center. Competitive reactions of the highly functionalized intermediates were addressed with the introduction of protecting groups, which added to the length and complexity of the synthesis. These challenges precluded the extrapolation of the studies to the preparation of a large number of key analogues of the natural product. In the intervening period since the disclosures of Umezawa, Ohno et al. and Hecht et al. that culminated in the total syntheses in 1982, advances in synthetic chemistry provided the opportunity to readdress these challenges and develop an approach amenable to analogue synthesis. Key elements of our approach include a concise and diastereoselective preparation of the fully substituted pyrimidine core, the use of recent advances in acyclic stereocontrol for direct introduction of eight of the acyclic stereogenic centers, a convergent assembly of the C-terminus, and a diastereoselective introduction of the disaccharide by enlisting recent advances in glycosidation methodology.

2.1. Construction of the Pyrimidine Core and Total Synthesis of (+)-P-3A[18b, 84]

Central to the efforts was the preparation of the pyrimidine core and its attached side chains that constitute the metal binding domain. In this regard, (+)-P-3A (**2**) served as an appropriate initial synthetic target. It is a microbial product isolated in biosynthetic studies of the bleomycins, whose structure was established in an X-ray structure determination of its Cu^{II} complex **2a** (Figure 3).[85] With the exception of the

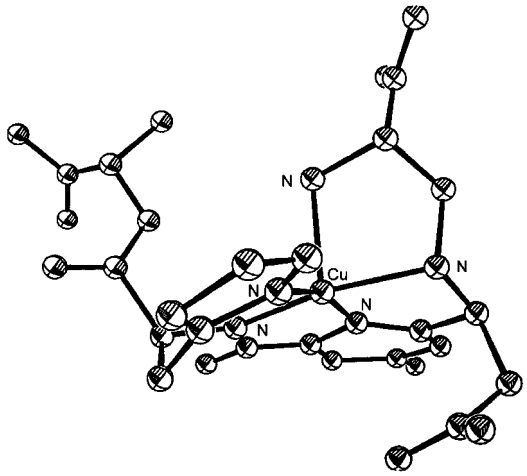
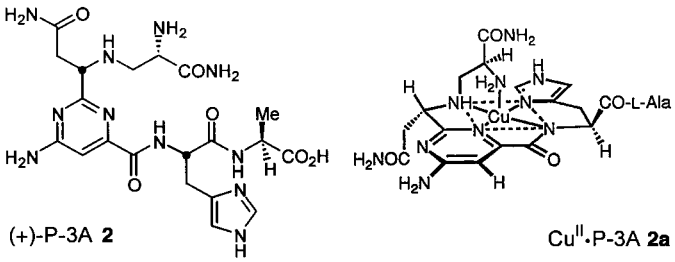


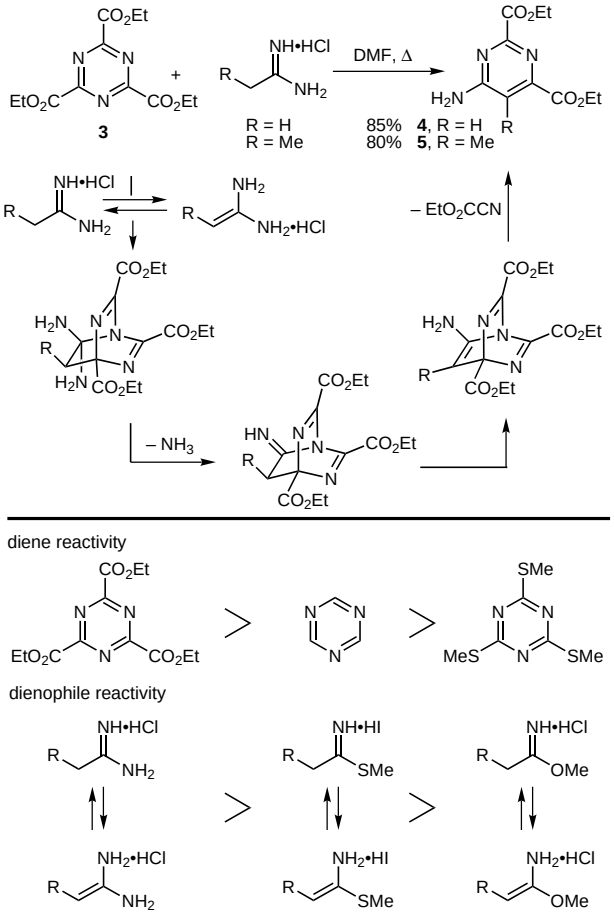
Figure 3. ORTEP representation of the X-ray structure of $Cu^{II} \cdot$ P-3A.

C5 methyl group, **2** contains the functionalized pyrimidine core of bleomycin A_2 and represents the simplest member of this class of agents. Although still an unresolved matter, the



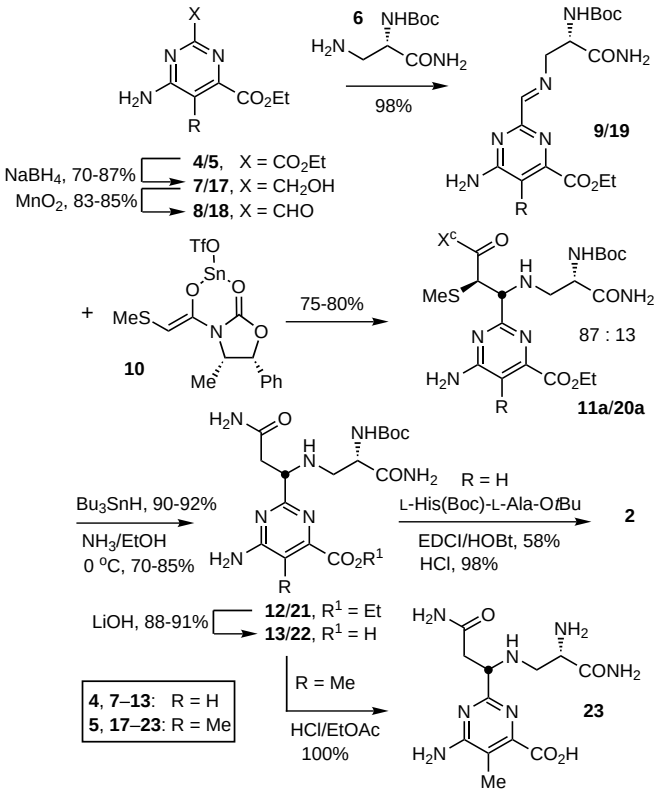
Cu^{II} complex of P-3A is taken to represent the metal-ligated atoms of the iron and copper complexes of bleomycin A_2 and their activated intermediates. Presumably, oxygen complexation and activation occurs *trans* to the axial amine of the distorted square-planar metal complex. Since the disclosure of the X-ray structure in 1978 by Umezawa, spectroscopic studies of various metal complexes as well as those of simple models have been described.[31a, 68, 73, 74] While most, including model Co^{III}-OOH metal complexes,[52] support the ligated atoms observed with Cu^{II}-P-3A, alternatives have been advanced for bleomycin A_2. The potential for the disaccharide carbamoyl group to replace the axial amine as a ligand has been implicated in studies of Zn^{II}[53, 60a, 61a] and Fe^{II}-(CO)[60b] metal complexes, and the complexation of the deprotonated amide had been questioned.[61a, b]

Key to the synthesis of **2** was the preparation of a fully substituted pyrimidine at the core of the structure. An effective one-step approach was devised based on an inverse electron demand Diels–Alder reaction of 1,3,5-triazines[86] (Scheme 2). The in-situ thermal reaction of amidines with symmetrical 1,3,5-triazines[87] provides substituted 4-aminopyrimidines in excellent yields; the reacton proceeds with an amidine to 1,1-diaminoethene tautomerization, [4+2] cycloaddition with the 1,3,5-triazine, loss of ammonia from the Diels–Alder adduct, imine to enamine tautomerization, and loss of ethyl cyanoformate through a retro Diels–Alder reaction. A comparative examination of amidines, thioimidates, and imidates revealed that amidines are uniquely suited for use in this reaction cascade. The reaction proceeds best with the amidine hydrochloride salts at intermediate reaction temperatures in polar, aprotic solvents. The reaction is invariant to the ratio of dienophile–diene used, and it is subject to triazine substituent effects, which are characteristic of an inverse electron demand Diels–Alder reaction.[87]



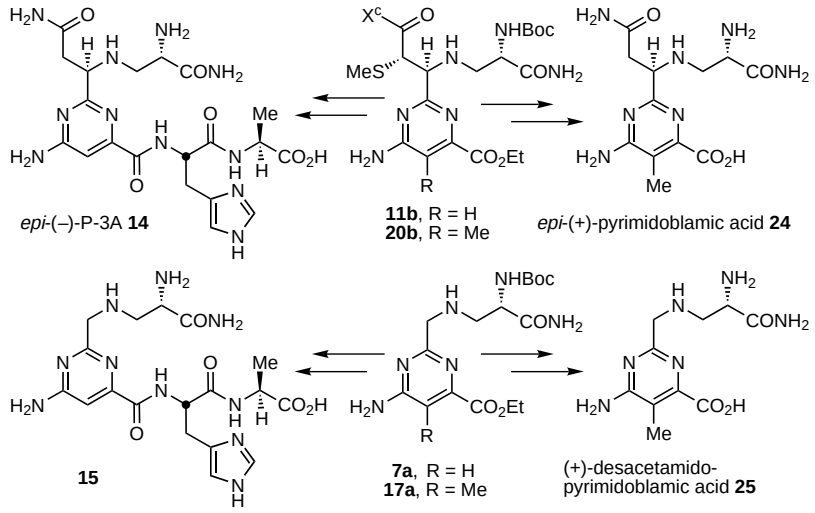
Scheme 2. Diels-Alder reaction of 1,3,5-triazine with amidine. DMF = dimethylformamide.

Based on this cyclization, 2,4,6-triethoxycarbonyl-1,3,5-triazine (**3**) was treated with acetamidine hydrochloride to provide **4** (Scheme 2). A selective differentiation of the C2 and C4 ethyl esters was accomplished through low temperature $NaBH_4$ reduction of the more electrophilic C2 ester to provide **7** (Scheme 3). The remaining strategic element was the stereocontrolled introduction of the C2-acetamido side chain. Previous studies employed nonselective approaches that required a separation of the resulting 1:1 mixture of diastereomers. In our studies, optically active *N*-acyloxazolidinones were found to provide a diastereoselective imine addition reaction suitable for the introduction of a C2-acetamido side chain.[88] Thus, oxidation of **7** with MnO_2 followed by condensation of aldehyde **8** with N^{α}-Boc-β-amino-L-alanine amide (**6**) provided **9**. Treatment of imine **9** with the stannous (*Z*)-enolate **10** provided the desired *anti* imine adduct **11a** accompanied by a small amount of the alternative and separable *anti* addition product **11b** (87:13). Reductive desulfurization of the major diastereomer **11a** and aminolysis afforded **12**. Ethyl ester hydrolysis and coupling of **13** with L-His(Boc)-L-Ala-O*t*Bu provided the penultimate precursor in a reaction that was conveniently conducted without protection of the unreactive aryl amine or hindered secondary amine. A final acid-catalyzed deprotection provided (+)-P-3A (**2**).



Scheme 3. Total synthesis of (+)-P-3A (**2**) and (−)-pyrimidoblamic acid (**23**). Boc = *tert*-butoxycarbonyl, EDCI = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, HOBt = 1-hydroxy-1*H*-benzotriazole, Tf = trifluoromethanesulfonyl, X^C = chiral oxazolidinonyl.

The minor *anti* addition product that possesses the unnatural stereochemistry at the C2 benzylic center was converted to *epi*-(−)-P-3A (**14**)[18b] and the product (**7a**) of the displacement of the primary tosylate derived from **7** with **6** was converted into (−)-desacetamido P-3A (**15**, Scheme 4).[84]

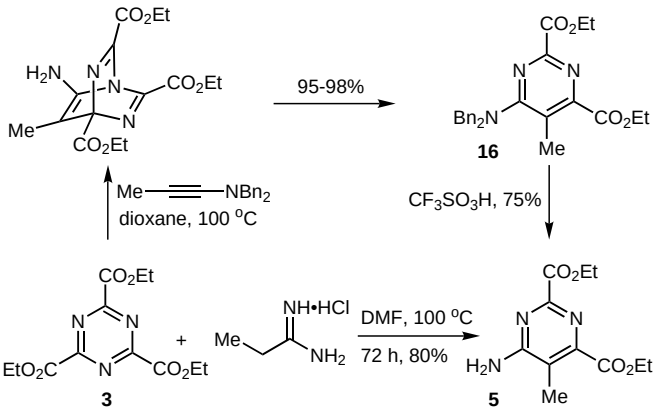


Scheme 4. Synthesis of *epi*-(−)-P-3A (**14**), (−)-desacetamido-P-3A (**15**), *epi*-(+)-pyrimidoblamic acid (**24**), and (+)-desacetamidopyrimidoblamic acid (**25**).

2.2. Synthesis of (−)-Pyrimidoblamic Acid[18d]

The core structure of (−)-pyrimidoblamic acid was assembled through two complementary [4+2] cycloadditions of 2,4,6-triethoxycarbonyl-1,3,5-triazine (**3**) (Scheme 5). Thermal treatment of **3** with 1-(*N*,*N*-dibenzylamino)propyne and acid-catalyzed debenzylation of **16** provided **5.** Alternatively, **5** was derived more directly through the one-pot reaction cascade of an inverse electron demand Diels–Alder reaction between **3** and propionamidine hydrochloride.

Differentiation of the C2 and C4 esters was accomplished by selective reduction of the sterically more accessible and electronically more reactive C2 ester of **5** to provide **17**



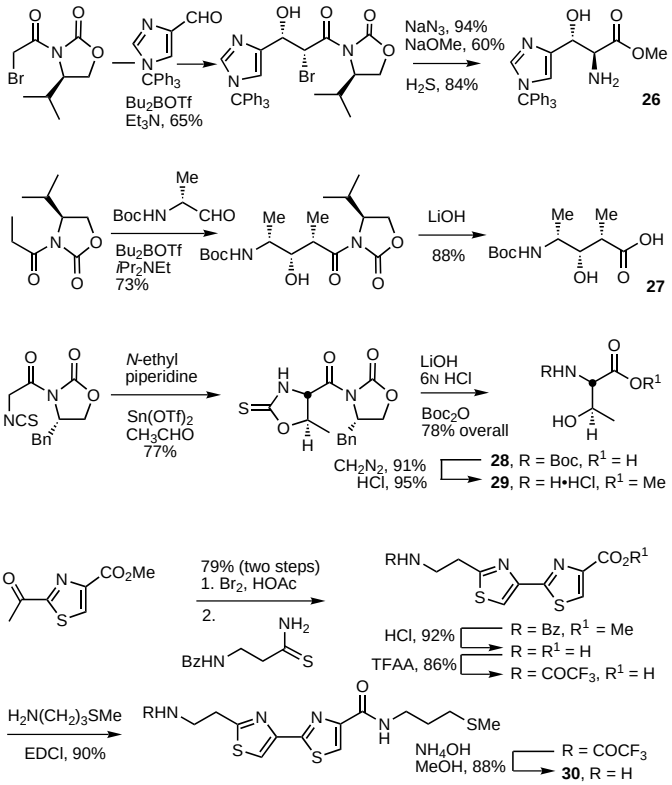
Scheme 5. Synthesis of the pyrimidine core. Bn = benzyl.

(Scheme 3). Diastereoselective addition of the stannous (*Z*)-enolate **10** with imine **19**, generated by MnO_2 oxidation and subsequent condensation of **18** with **6**, provided **20a** as the major *anti* addition product (87:13 diastereoselection). Reductive desulfurization, aminolysis, ester hydrolysis, and deprotection of the *N*-Boc group afforded (−)-pyrimidoblamic acid (**23**). An important ramification of these studies was the confirmation of Umezawa's spectroscopic assignment of the absolute configuration at the C2 benzylic center.

The minor *anti* diastereomer **20b** derived from the imine addition reaction was converted into *epi*-(+)-pyrimidoblamic acid (**24**). Displacement of the primary tosylate derived from **17** with **6** gave the substitution product **17a**, which was then used to prepare (+)-desacetamidopyrimidoblamic acid (**25**, Scheme 4).

2.3. Assembly of the C Terminus: Synthesis of Tri-, Tetra-, and Pentapeptide S[18c, 89]

Concise diastereocontrolled syntheses of the C-terminus subunits **26**–**30**, which incorporated seven of the acyclic stereogenic centers were accomplished as described in Scheme 6. The *erythro*-β-hydroxy-L-histidine subunit **26** was prepared in four steps through adaptation of the approach of Ohno et al.[90] with mod-



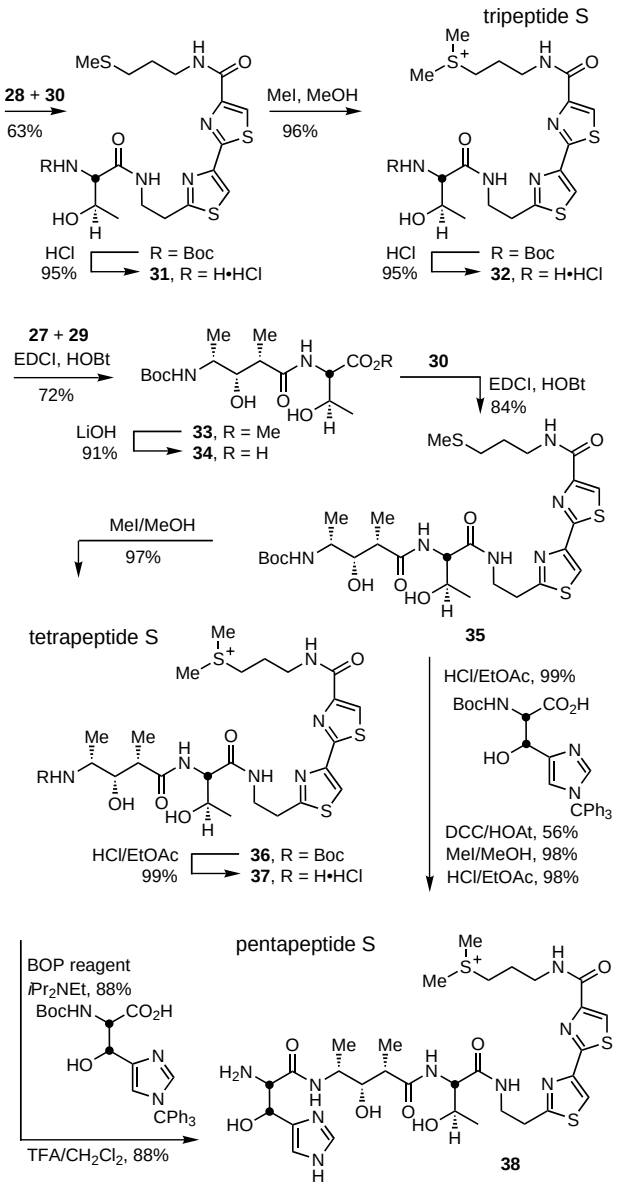
Scheme 6. Synthesis of the C-terminus subunits. Bz = benzoyl, TFAA = trifluoroacetic anhydride.

ifications in which the competitive retro aldol reaction was suppressed during the azide displacement reaction. The (2*S*,3*S*,4*R*)-4-amino-3-hydroxy-2-methylpentanoic acid subunit **27** was synthesized in two steps through a diastereoselective *syn* aldol addition of a boron-(*Z*)-enolate with *N*-Boc-D-amino-1-propanol followed by hydrolysis of the chiral auxiliary. Similarly, the L-threonine subunits **28** and **29** were prepared through diastereoselective *syn* aldol addition of a *N*-acyloxazolidinone stannous (*Z*)-enolate with acetaldehyde following a procedure detailed by Evans et al.[91] The bithia-

zole **30** was prepared following the approach of Sakai et al.,[92] but with minor modifications, and a direct carbodiimide-promoted condensation of 3-(methylthio)propylamine proved more convenient than the reported two-step procedure with the acid chloride. The final deprotection was conducted as detailed by Hecht et al.[83b] to provide **30**.

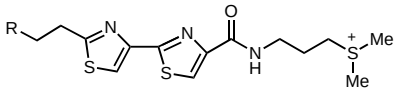
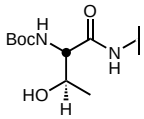
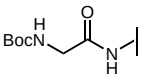
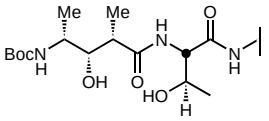
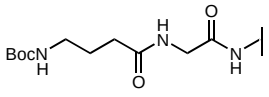
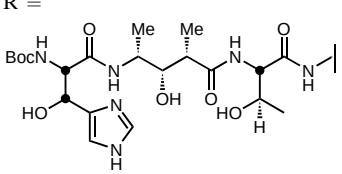
Tripeptide S (**32**) was assembled by coupling **28** with **30** followed by S-methylation and N-Boc deprotection (Scheme 7). Complementary to a linear synthesis of tetrapeptide S based on the coupling of a tripeptide S precursor and **27** as detailed in the efforts of Umezawa et al. and Hecht et al.,[82, 83] a convergent preparation was employed in our studies. Coupling of **27** with **29** (→**33**) followed by methyl ester hydrolysis (→**34**), further coupling with **30**, S-methylation, and N-Boc deprotection provided tetrapeptide S (**37**). While the introduction of the sulfonium salt was postponed to a latter stage in preceding efforts, its earlier introduction provided a more convergent synthesis, simplified the purification of coupling products, and did not introduce competitive side reactions. Similarly, N-Boc deprotection of **35**, coupling with N^{α}-Boc-β-hydroxy-L-His(CPh_3), S-methylation, and acid-catalyzed deprotection provided pentapeptide S (**38**). The direct coupling of **37** with N^{α}-Boc-β-hydroxy-L-His(CPh_3) and acid-catalyzed deprotection also provided pentapeptide S in a sequence that was unaffected by the incorporation of the sulfonium salt.[105] Notably, this provided the fully functionalized C-terminus with no protecting groups.

One issue addressed with the C-terminus and its analogues was the role the linking chain and its substituents may play in the expression of the bleomycin A_2 properties. Central to any interpretation is their impact on the DNA binding affinity and selectivity. Consequently, the calf thymus DNA binding constants and the binding site size for bleomycin A_2, deglycobleomycin A_2, the Boc derivatives of di-, tri-, tetra-, pentapeptide S, and their analogues were determined through measurement of the quenching of the bithiazole fluorescence upon binding.[18c] The Boc derivatives were selected for study rather than the free amines since they closely reflect the subunits as they are found in the natural product. Representative comparisons are summarized in Table 1 and over 15



Scheme 7. Synthesis of tri-, tetra-, and pentapeptide S. BOP = 1-benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate, DCC = *N*,*N*′dicyclohexylcarbodiimide.

Table 1. Apparent DNA binding constants of bleomycin A_2 (**1**) and key partial structures to calf thymus DNA.

Agent	Formula	K_B [10^5 M^{-1}][a]	L [bp][b]
bleomycin A_2	**1**	1.0	3.8
deglycobleomycin A_2	**41**	1.1	3.9
			
dipeptide S	R = 	0.10	2.2
tripeptide S	R = 	0.26	3.6
	R = 	0.18	2.7
tetrapeptide S	R = 	0.21	3.7
	R = 	0.20	3.5
pentapeptide S	R = 	0.23	4.2

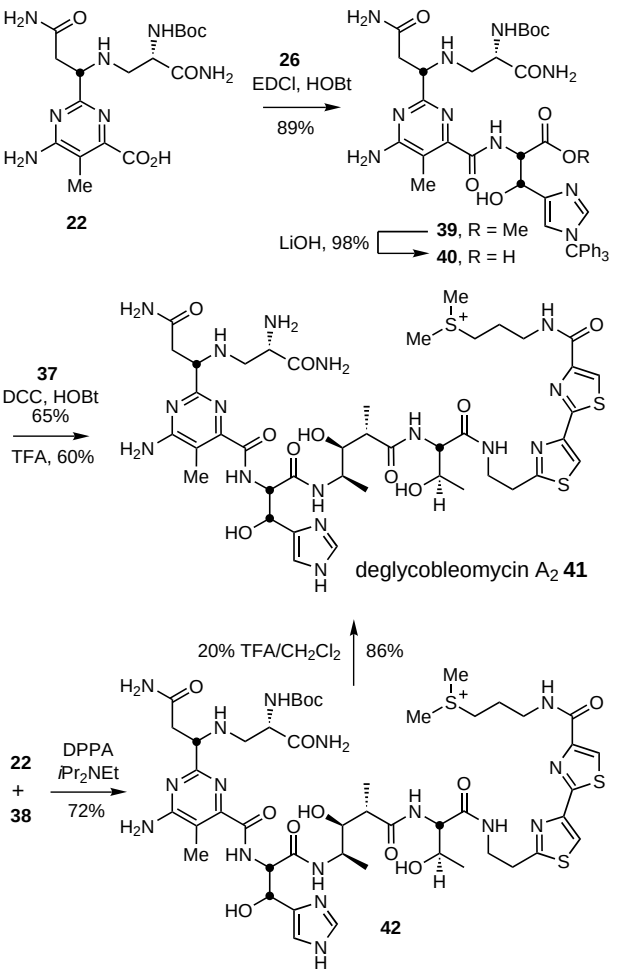
[a] K_B = apparent binding constant. [b] L = length of the binding site (base pairs) in DNA.

analogues of the C-terminus were examined, which complement and extend past studies.[66, 77, 93, 94] *N*-Boc-tripeptide S, *N*-Boc-tetrapeptide S, and *N*-Boc-pentapeptide S exhibited near identical binding constants, which were greater than that of *N*-Boc-dipeptide S and only slightly lower than that of bleomycin A_2 or deglycobleomycin A_2. The comparisons indicate that the disaccharide is not contributing to the binding affinity, that the majority of the affinity is derived from tripeptide S, that the substituted pentanoic acid and β-hydroxy-L-histidine subunits are not contributing to DNA binding affinity, and infer that the N-terminus pyrimidoblamic acid subunit contributes the remaining affinity that is observed with deglycobleomycin A_2. The observation that removal of the hydroxyethyl substituent of tripeptide S lowers the binding affinity, while the removal of all the tetrapeptide S substituents does not, suggests that the L-threonine subunit productively interacts with DNA, that the L-threonine hydroxyethyl substituent may attenuate the binding, and that the remaining linking chain substituents do not contribute to binding. With the possible exception of the L-threonine substituent, this suggests that their effects on cleavage efficiency are not a consequence of stabilizing binding interactions with DNA. In addition, the binding site sizes corresponded nicely to the estimated size of bleomycin A_2 independently established by other techniques.[66, 77, 93, 94] Consistent with conclusions drawn from the binding constants, the binding site sizes also suggested that the tripeptide S subunit is fully bound to DNA, that the L-threonine substituent may detectably affect the interaction with DNA, but that the remaining tetrapeptide S or pentapeptide S substituents do not substantially alter the binding site size or tripeptide S binding mode. Alternative substitutions of the pentanoic acid subunit had little effect on the binding affinity ($K_B = 0.18 - 0.23$ versus $0.21 \times 10^5\,M^{-1}$) or binding site size (3.5 – 3.9 versus 3.7 base pairs (bp)) and greater variations were observed with agents lacking the tripeptide S hydroxyethyl substituent.

Although these studies preceded recent NMR structural models,[52, 53] the observations were interpreted to indicate that the significant reductions in the DNA-cleavage efficiencies observed with agents that incorporated modifications in the linking chain were not the result of an altered binding affinity or altered tripeptide S binding mode, but rather were related to conformational effects of the linking chain substituents and implicated a compact, DNA-bound conformation, which enlisted a turn at the threonine – valerate site.[18c] With the availability of recent structural models, this may now be more definitively attributed to their role in the preorganization of bleomycin A_2 into such a compact conformation productive for DNA cleavage (see Section 3.3).

2.4. Total Syntheses of Deglycobleomycin A_2[18a, e]

With the key intermediates available, the synthesis of deglycobleomycin A_2 was in hand. Coupling of N^{α}-Boc-pyrimidoblamic acid (**22**) with **26** provided **39** (Scheme 8). Hydrolysis of the ester and coupling of **40** with tetrapeptide S (**37**) that contained the intact sulfonium salt, followed by deprotection afforded deglycobleomycin A_2 (**41**). Alternatively, the coupling of pentapeptide S (**38**) with **22** proceeded smoothly to provide N^{α}-Boc-deglycobleomycin A_2 (**42**) when treated with DPPA.[105] This was accomplished with the sulfonium salt installed, with only one protecting group in the reacting substrates, without deliberate protection of the imidazole, and without observation of competitive imidazole acylation. Acid-catalyzed deprotection of **42** provided deglycobleomycin A_2 (**41**). Typically, the more convergent synthesis with **40** has been employed in our preparation of deglycobleomycin A_2 analogues, while the approach that proceeds through **42** is especially convenient for those that incorporate changes in the pyrimidoblamic acid subunit.

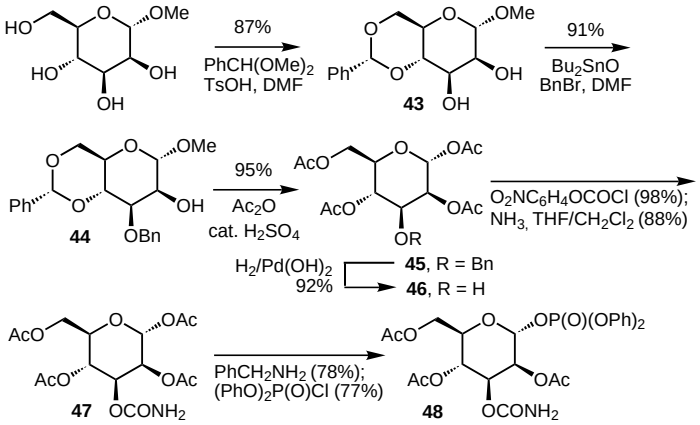


Scheme 8. Total syntheses of deglycobleomycin A_2. DPPA = diphenylphosphorylazide.

2.5. Disaccharide Preparation and Total Synthesis of Bleomycin A_2[18f]

The D-mannose subunit **48** was prepared from D-mannose itself through selective C3-functionalization. In addition, the intermediate **48**, which serves as the mannose-derived glycosyl donor, was designed to use a C2 acetate to control the stereochemistry of the disaccharide glycosidation reaction. Alkylation of the 2,3-*O*-dibutylstannylene derived from the reaction of **43** with Bu_2SnO and benzyl bromide cleanly

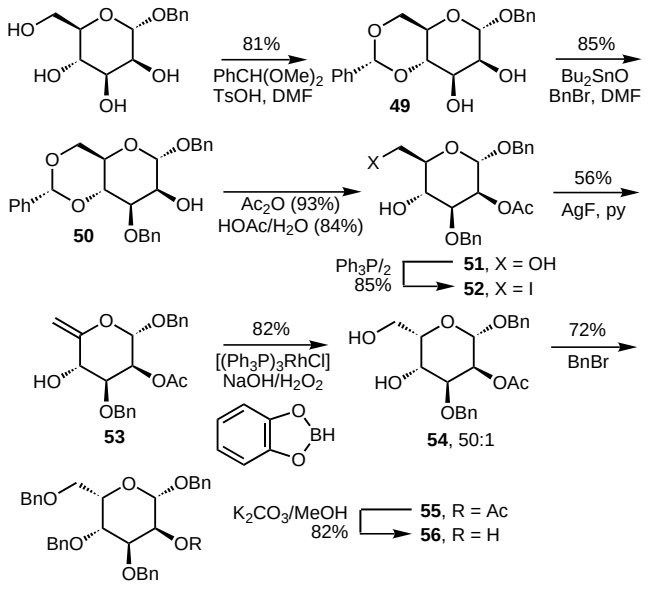
provided **44**, which is derived by exclusive alkylation of the equatorial C3 alcohol (Scheme 9). Single step peracetylation cleanly afforded the pentaacetate **45**. Following protocols introduced by Hecht et al.,[83] debenzylation of **45** with Pearlman's catalyst and conversion of **46** into the *p*-nitrophenylcarbonate followed by aminolysis provided **47** in 62% overall yield from D-mannose. Activation of **47** as a glycosyl donor was accomplished through treatment with HBr/HOAc or sequential treatment with benzylamine and $(PhO)_2P(O)Cl$ to provide the glycosyl bromide or glycosyl diphenylphosphate **48**, respectively.



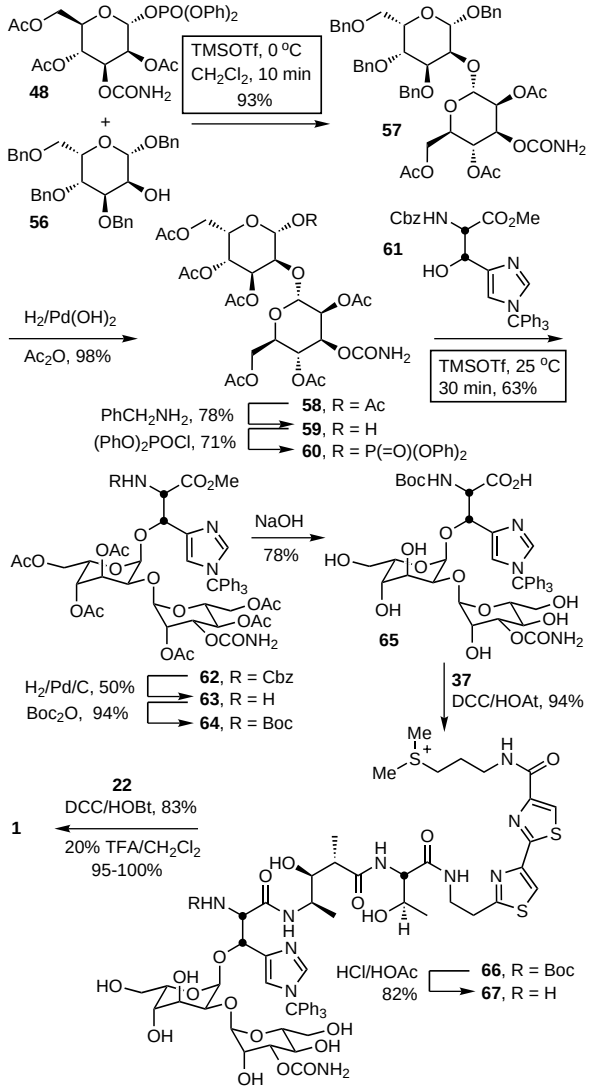
Scheme 9. Synthesis of the D-mannose subunit. Ts = toluene-4-sulfonyl.

Past efforts to prepare a suitably protected L-gulose derivative employed a six-step synthesis from the rare L-gulose (3–4% overall yield) or the successive head-to-tail inversion of α-D-glucofuranose with interconversion of the C1 and C6 oxidation states.[82, 83] Our approach used a D-mannose precursor with the correct C1–C4 stereochemistry and a simple inversion of the C5 stereochemistry for interconversion of D-mannose to L-gulose. Thus, protection of the C4 and C6 hydroxyl groups of benzyl α-D-mannopyranoside to give acetal **49**, followed by selective equatorial C3 hydroxyl benzylation through alkylation of the intermediate 2,3-*O*-dibutylstannylene provided **50** (Scheme 10). Acetylation of the C2 alcohol followed by acetal hydrolysis and conversion of **51** into the corresponding primary iodide followed by HI elimination provided **53**. Rh^I-catalyzed hydroboration and oxidation of **53** cleanly provided **54**, with inversion of the C5 stereochemistry in a reaction that proceeded with a diastereoselection of $\geq$50:1. Benzylation of **54** followed by acetate methanolysis provided **56** and a suitable L-gulose glycosyl acceptor for disaccharide coupling.

Disaccharide formation, with **56** serving as the glycosyl acceptor and **48** serving as the glycosyl donor, cleanly provided **57** (Scheme 11). The use of the diphenylphosphate **48** provided **57** under milder reaction conditions (0 versus 25 °C), in much shorter reaction times (10 min versus 10–36 h), and in higher conversions (94 versus 74–78%) relative to the glycosyl bromide. Notably, the carbamoyl group could be taken through this glycosidation reaction without protection or competitive reactions. The exclusive retention of the α linkage may be attributed to neighboring group participation of the mannose C2′ acetate group, which provides clean



Scheme 10. Synthesis of the L-gulose subunit. Py = pyridine.



Scheme 11. Final stages of total synthesis of bleomycin A_2 (**1**). Cbz = benzyloxycarbonyl, HOAt = 7-aza-1-hydroxy-1*H*-benzotriazole, TMS = trimethylsilyl.

retention of the C1′ stereochemistry. Debenzylation of **57** followed by acetylation provided **58** in superb conversions, and activation as the α-glycosyl diphenylphosphate **60** was accomplished by sequential treatment with benzylamine and $(PhO)_2P(O)Cl$. The O-glycosidation reaction was conducted with **60** and *N*-Cbz-β-hydroxy-L-His(CPh_3) methyl ester (**61**) to provide adduct **62** under exceptionally mild conditions (25 °C, 30 min) in excellent conversion (63 %) as a greater than 13:1 mixture of the desired α and undesired β linked anomers. The initial approaches that enlisted glycosyl halide activation required prolonged reaction times, proceeded in much lower conversions, and produced a mixture of diastereomers. The high diastereoselectivity of this glycosidation reaction may be attributed to the low reactivity of the glycosyl acceptor, which favors formation of the most stable α anomer, and mechanistic characteristics of a glycosyl phosphate acceptor, which favor inversion of the stereochemistry at the reacting anomeric center. Selective Cbz deprotection without competitive hydrogenolysis of the trityl protecting group, N-Boc protection of the amine, and exhaustive ester hydrolysis provided **65** suitably protected for sequential couplings with tetrapeptide S and N^{α}-Boc-pyrimidoblamic acid.

The coupling of **65** with tetrapeptide S (**37**) provided **66** in superb yield without deliberate protection of the disaccharide or the hydroxyl groups of tetrapeptide S and was conducted with the sulfonium salt installed. Mild acid treatment of **66** under conditions defined by Sieber and Riniker[95] cleanly provided **67** by removal of the Boc protecting group without deglycosidation or removal of the trityl protecting group. Subsequent coupling of **67** with N^{α}-Boc-pyrimidoblamic acid (**22**) and final acid-catalyzed deprotection provided bleomycin A_2 (**1**), identical in all respects with the natural material.

3. Probing the Functional Roles of the Bleomycin A_2 Subunits

The completion of a modular and convergent total synthesis of bleomycin A_2 provided a unique foundation upon which many analogues that contained single-point changes were prepared in efforts to answer key questions concerning metal chelation, oxygen activation, DNA binding, and the origin of sequence-selective DNA cleavage.

3.1. The Carbohydrate Domain

Of all the bleomycin A_2 subunits the role of the disaccharide is the most poorly understood. Although it is known not to impact on the cleavage selectivity (5′-GC, 5′-GT), it does account for subtle differences in the relative selectivity among the available sites.[76, 96] It is also known to enhance biological potency and efficacy and make significant contributions to the DNA cleavage efficiency and ratio of ds to ss cleavage. Although there are many plausible explanations, the mannose C3-carbamoyl group had been implicated in the metal complexation that affected the structure and reactivity of the metal complexes.[6, 45a, 61a,b] It had also been suggested that the bulky disaccharide serves the role, in conjunction with the C2-acetamido side chain, of forming one side of a pocket to protect the reactive intermediates.[45b, 73f, 78] To start addressing these questions, the impact of the individual disaccharide subunits was assessed through the synthesis and evaluation of three key analogues.

3.1.1. Demannosylbleomycin A_2[97]

The analogue **77** lacks the terminal α-D-mannopyranoside unit and permits the establishment of its role, including that of the carbamoyl group, as a putative sixth ligand for metal complexation. To insure that the liberated L-gulose C2 alcohol would not affect the comparisons, it was capped as a methyl ether. The agent was prepared through diastereoselective O-glycosidation of **61** with the glycosyl diphenylphosphate of 3,4,6-tri-*O*-acetyl-2-*O*-methyl-β-L-gulopyranose (**70**), followed by adjustment or removal of the protecting groups and sequential couplings with tetrapeptide S and N^{α}-Boc-pyrimidoblamic acid (Scheme 12). The glycosidation reaction proceeded under exceptionally mild conditions (−15 °C, 30 min) in superb yield (65–72 %) with clean inversion of the stereochemistry at the glycosyl C1 position ($\geq$20:1 α:β) to provide **71**. Both the low reactivity of the glycosyl acceptor **61** and mechanistic features of a glycosyl phosphate donor, which favor inversion of the stereochemistry at the reacting center, account for the clean generation of **71**.

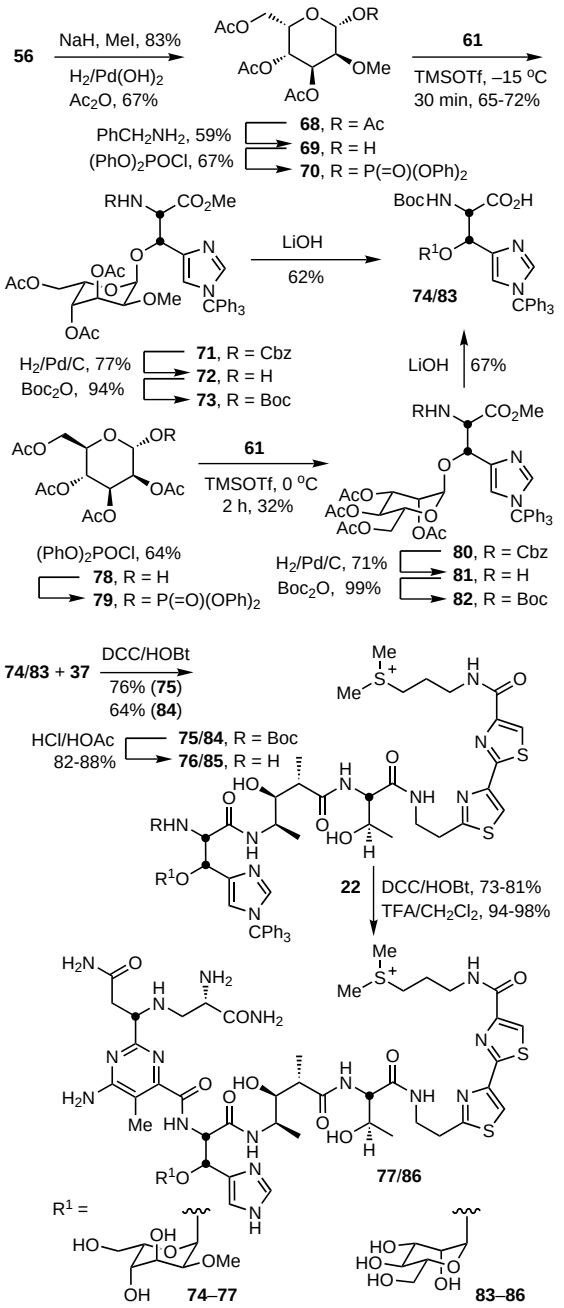
3.1.2. α-D-Mannopyranosyldeglycobleomycin A_2[97]

The analogue **86** bears a monosaccharide in which the single C5 stereocenter of α-L-gulopyranoside has been inverted to provide a linked α-D-mannopyranoside. Glycosidation of **61** with the glycosyl diphenylphosphate of 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranose, which proceeded with net retention of the glycosyl C1 stereochemistry by virtue of participation of the neighboring C2 acetate group, provided **80** without detection of isomeric products (Scheme 12). Adjustment or removal of the protecting groups and sequential couplings with **37** and **22** provided **86**.

3.1.3. Deshydroxydeglycobleomycin A_2[98]

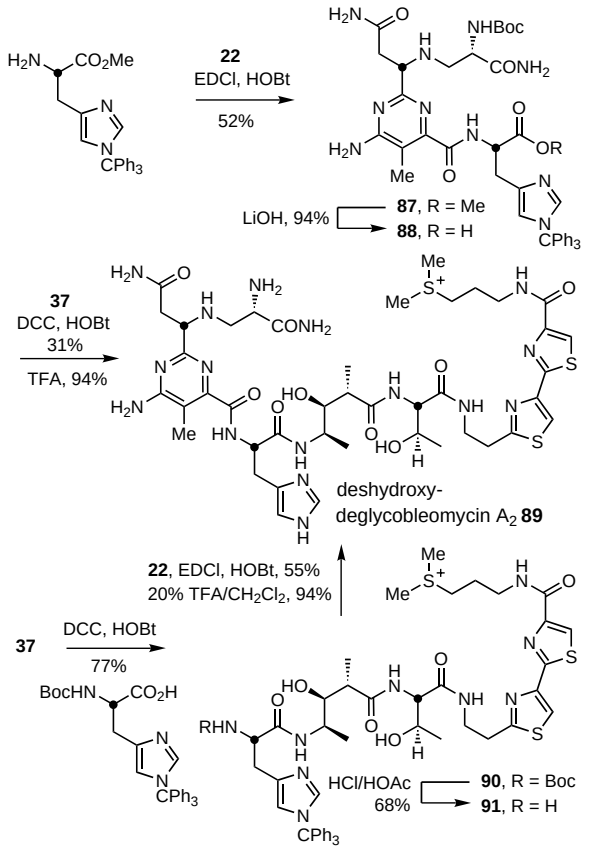
It was conceivable that the distinction between bleomycin A_2 and deglycobleomycin A_2 rested not with the removal of the disaccharide but rather with the liberation of the free alcohol of the β-hydroxy-L-histidine subunit. Therefore, deshydroxydeglycobleomycin A_2 (**89**), in which this alcohol was removed, was prepared for comparison (Scheme 13). Two approaches were used. The first was conducted by the coupling of **88** with tetrapeptide S and suffered partial racemization. Simply reversing the order of couplings such that the L-His carboxylate group was activated with a *N*-carbamoyl instead of the *N*-acyl group provided a more effective route to **89**.

The DNA cleavage efficiency, selectivity, and ds:ss cleavage ratio of demannosylbleomycin A_2 (**77**) proved similar or indistinguishable from bleomycin A_2 (**1**), which indicated that the terminal 2-*O*-(3-*O*-carbamoyl)-α-D-mannopyranoside, inclusive of the carbamoyl group, has little impact on the DNA

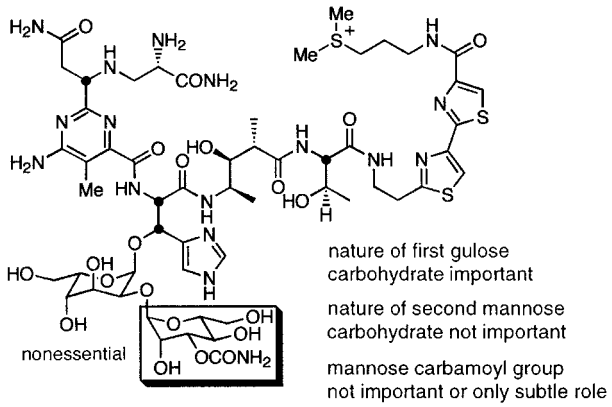


Scheme 12. Synthesis of demannosylbleomycin A_2 (**77**) and α-D-mannosyldeglycobleomycin A_2 (**86**).

cleavage properties (Figure 4). In contrast, the decreased efficiency of DNA cleavage and ds:ss cleavage ratio of **86**, which proved less effective than even deglycobleomycin A_2, indicate that the first carbohydrate of the disaccharide may greatly influence the properties. Similarly, deshydroxydeglycobleomycin A_2 (**89**) proved indistinguishable or slightly less effective than deglycobleomycin A_2, which indicates that it is the removal of the disaccharide and not the release of the L-histidine β-hydroxyl group that is responsible for its diminished DNA-cleavage properties. Importantly, these studies implicate the α-L-gulopyranoside and not the α-D-mannopyranoside inclusive of the carbamoyl group as the important disaccharide component required for full potentiation of the DNA-cleavage efficiency of bleomycin A_2.



Scheme 13. Synthesis of deshydroxydeglycobleomycin A_2 (**89**).



Agent	Efficiency	ds:ss	Selectivity
bleomycin A_2 (**1**)	2 – 5	1 : 6	5'-GC, 5'-GT > 5'-GA
demannosyl bleomycin A_2 (**77**)	2 – 4	1 : 7	5'-GC, 5'-GT > 5'-GA
α-D-mannosyl deglycobleomycin A_2 (**86**)	0.6 – 1	1 : 20	5'-GC, 5'-GT > 5'-GA
deglycobleomycin A_2 (**41**)	1	1 : 12	5'-GC, 5'-GT > 5'-GA
deshydroxy deglycobleomycin A_2 (**89**)	0.75	1 : 12	5'-GC, 5'-GT > 5'-GA

Figure 4. DNA cleavage properties of bleomycin A_2 derivatives with modified disaccharide subunits. The results for bleomycin A_2 (**1**) and deglycobleomycin A_2 (**41**) show that the presence of the complete disaccharide subunit increases the cleavage efficiency two- to fivefold while the ratio of double strand to single strand cleavage doubles.

In support of the studies, recent structural studies from the Stubbe group completed subsequent to our studies demonstrated that the Co^{III}-OOH complexes of bleomycin A_2 and deglycobleomycin A_2 bind to an oligonucleotide cleavage site in identical manners, but **41** binds with a lower affinity (30 ×).[52] The molecules adopt a compact conformation in the minor groove with partial intercalation of the bithiazole at the base pair 3′ to the site of cleavage and with the metal-bound peroxide positioned in close proximity to the C4′ hydrogen atom of the cleavage site. The disaccharide is located on the outer face of the minor groove and shields the complex from solvent (Figure 5). The primary amino group of the β-amino-

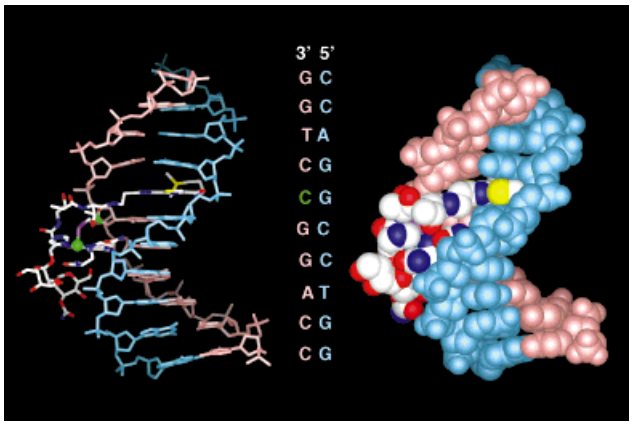


Figure 5. Model of Co^{III}-OOH bleomycin A_2 bound to DNA as determined by NMR spectroscopy.[52]

alanineamido side chain of pyrimidoblamic acid but not the mannose C3-carbamoyl group is the axial ligand. These and related studies suggest that the mannose C3-carbamoyl group probably does not contribute to metal complexation, that the disaccharide may contribute protective stabilization and possibly enhanced facility for adoption of a productive DNA-bound conformation, and that deglycobleomycin A_2 may serve as a simplified model that provides relevant information on the interaction of bleomycin A_2 with DNA.

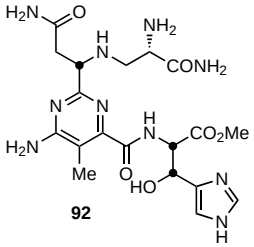
The observation that the Co^{III}-OOH complex of bleomycin A_2 bound 30 times more effectively than deglycobleomycin A_2 at a single oligonucleotide cleavage site was attributed to weak, nonselective contacts of the terminal mannose with the hydrophobic chain of the DNA backbone. The generality of such observations, which appear to contradict the effective properties of **77**, which lacks this terminal sugar, is under study.

3.2. The Metal Binding Domain

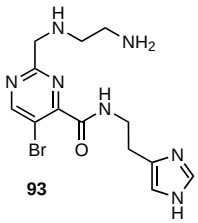
Central to the properties of bleomycin A_2 is the metal chelation and subsequent O_2 activation. A commonly accepted depiction of the metal chelation is derived from the X-ray structure of $Cu^{II} \cdot$P-3A, which shows the primary and secondary amine groups of the β-aminoalanineamide side chain, the N1 atom of the pyrimidine, the N3 atom of the imidazole from L-histidine, and its deprotonated amide are coordinated to the metal in a square-planar complex with the primary amine occupying an axial coordination site[85] (see Figure 3). NMR[52–65] and related spectroscopic studies[66–72] on a range of bleomycin A_2 metal complexes have contributed to the consensus that the pyrimidine, imidazole, and secondary amine are bound to the metal. While our studies together with those of others suggest that the mannose C3-carbamoyl group does not contribute to metal chelation and that the axial ligand is the primary amine of the β-aminoalanine, the additional metal ligand(s) remained ambiguous. In addition, the origin of the DNA cleavage selectivity remained unresolved at the start of our studies although the contribution that the metal binding domain makes to DNA binding affinity and polynucleotide recognition have been an active topic of investigation.[68, 72, 80] Both the C-terminus[26, 80a] and the N-terminus metal binding domain[68, 72, 80b] has been suggested to be independently responsible for the 5′-GC/5′-GT cleavage selectivity. Consequently, a number of agents that directly address the issues of metal chelation and origin of DNA recognition have been examined.

3.2.1. *Full Metal Binding Domain*[100]

One important issue addressed in early studies was the DNA-cleavage properties of the metal binding domain itself. Although both Hecht et al.[72] and later Mascharak et al.[68] have advanced the proposal that the metal binding domain controls the DNA-cleavage selectivity, a structural origin of the polynucleotide recognition was not defined. Moreover, reports from Hecht et al.[5b] that Fe complexes of **92**, the metal



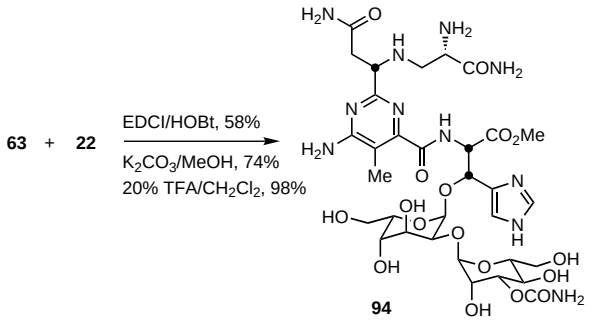
92



93

binding domain alone, and related agents that constitute the N-terminus fail to cleave DNA above the background level of iron cleavage, while the simplified models including **93** described by Mascharak et al. produce a cleavage pattern similar, but not identical, to bleomycin A_2 remained unresolved.[99] In efforts to address these issues and to assess the polynucleotide recognition features of the metal binding domain, the synthesis of the fully functionalized N-terminus metal binding domain complete with the linked 2-*O*-(3-*O*-carbamoyl-α-D-mannopyranosyl)-α-L-gulopyranosyl disaccharide (**94**), was conducted.[100]

The synthesis is outlined in Scheme 14 from **22** and **63**, which were prepared in nine steps (20–25% overall) and seven steps (16–19% overall), respectively. Coupling of N^{α}-Boc-pyrimidoblamic acid (**22**) with **63**, followed by methanolysis of the six O acetate groups by treatment with powdered K_2CO_3 in CH_3OH, and acid-catalyzed deprotection provided **94**. For comparison, **92** was prepared by the acid-catalyzed deprotection of **39** (20% TFA/CH_2Cl_2, 95%).



Scheme 14. Synthesis of the full metal binding domain of bleomycin A_2 (**94**).

Unlike **92** the Fe^{II} complex of the full metal binding domain **94** was found to cleave DNA well above the background level of cleavage and only 10 times less efficiently than deglycobleomycin A_2, but to do so in a nonsequence-specific manner with a significantly reduced ds:ss cleavage ratio (Figure 6).[100] Thus, although it may play a dominant role in determining the DNA cleavage selectivity when incorporated into the full natural product structure (see Section 3.2.8), the metal binding domain alone failed to exhibit the sequence selective DNA cleavage characteristic of bleomycin A_2.

Non-selective DNA cleavage by the metal binding domain

Agent	Efficiency	ds:ss	Selectivity
bleomycin A_2 (**1**)	2 – 5	1 : 6	5'-GC, 5'-GT > 5'-GA
deglycobleomycin A_2 (**41**)	1	1 : 12	5'-GC, 5'-GT > 5'-GA
94	0.1	1 : 48	none
92	<0.04	nd	none
Fe^{III}	0.04	1 : 98	none

Figure 6. DNA cleavage properties of the metal binding domain. The complete metal binding domain alone exhibits no DNA cleavage selectivity. nd = not determined.

3.2.2. (+)-P-3A, epi-(−)-P-3A, and (−)-Desacetamido P-3A

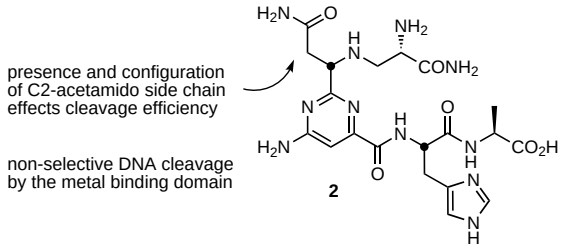
Similarly, (+)-P-3A (**2**) along with *epi*-(−)-P-3A (**14**) and (−)-desacetamido-P-3A (**15**) were examined as being representative of the metal binding domain.[18b, 84] (+)-P-3A (**2**) proved to be surprisingly effective at cleaving DNA, being even more effective than **92** or **94**. The Fe^{II} complexes of all three agents produced both ss and ds cleavage although with a decreased propensity for ds cleavage and no sequence selectivity (Figure 7). $Fe^{II} \cdot$ **2** proved to be only three to five times less efficient than deglycobleomycin A_2 and was three to five times more efficient than $Fe^{II} \cdot$ **14** and $Fe^{II} \cdot$ **15**. The results indicated not only that the metal binding domain is insufficient for sequence selective DNA cleavage, but also provided the first observation that the C2-acetamido side chain significantly effects cleavage efficiency although it is not involved in metal chelation.

3.2.3. L-Histidine Amide Replacements[98]

Potentiometric titration of the bleomycin copper complex in Umezawa's studies indicated that one deprotonated functional group occupies a metal coordination site at pH values between 4 and 9.[85] Since the amide group of the histidine unit is in a favorable position to coordinate to a metal center, it was inferred that the deprotonated amide nitrogen atom constituted one coordination site despite the relative pK_a of imidazole (14.4) and an amide (17) in the absence of a metal.[85] This was supported by the X-ray crystal structure of $Cu^{II} \cdot$ P-3A (**2a**) and more recently by those of simple model complexes, which illustrate L-histidine imidazole N^{π} complexation and deprotonated amide N^{σ} complexation.[68] While studies have addressed, and seem to confirm, this[69b, c] related spectroscopic studies conducted on a range of metal complexes have also suggested that the histidine amide group may not always be involved and cast doubt on the site of the deprotonated metal ligand.[61a, b] The evaluation of two amide replacement analogues was conducted in order to address the inferred importance of the secondary amide of L-histidine. The first analogue **96** incorporates a *N*-methylamide group. The *N*-methylamide is only capable of amide N^{π} complexation and is incapable of deprotonation and metal complexation through N^{σ} coordination. Similarly, the ester replacement in **97** is incapable of providing the deprotonated amide for metal coordination. The appropriately protected *N*-methyl-L-histidine methyl ester **95** for use in the synthesis of **96** was prepared from L-His(CPh_3)-OMe by sequential reductive benzylation and methylation followed by hydrogenolysis of the benzylamine group (Scheme 15). Coupling of **95** with N^{α}-Boc-pyrimidoblamic acid (**22**) led to introduction of the tertiary amide. Methyl ester hydrolysis, coupling with tetrapeptide S (**37**) and acid-catalyzed deprotection provided **96**.

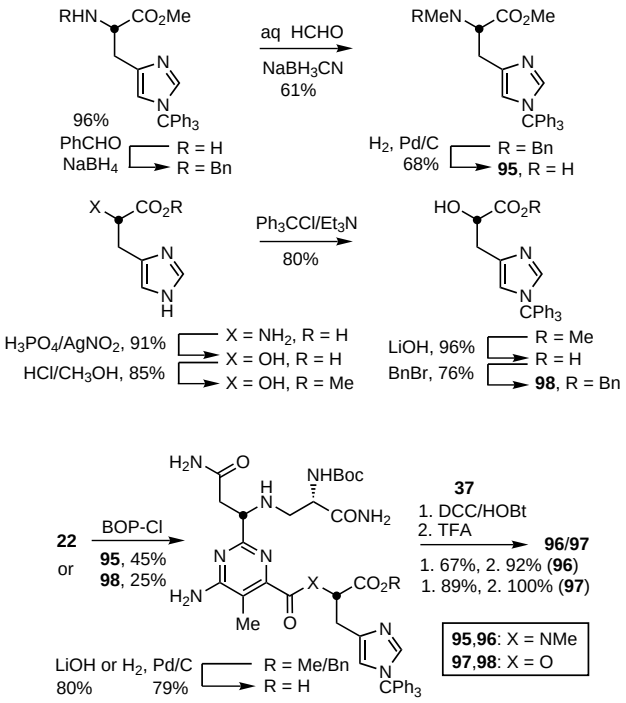
The synthesis of **97** required the selectively protected alcohol **98**, which could be deprotected after coupling with **22** without epimerization or competitive hydrolysis of the newly formed ester group. For this reason the benzyl ester **98** was selected and prepared from L-histidine (Scheme 15). Coupling of **98** with N^{α}-Boc-pyrimidoblamic acid (**22**) provided the linked ester, which was unusually sensitive to hydrolysis. Benzyl ester deprotection, coupling of the carboxylic acid with tetrapeptide S (**37**), and acid-catalyzed deprotection provided **97**.

The bleomycin analogues **96** and **97** that contain a *N*-methylamide and an ester group, respectively, in place of the secondary amide group of L-His were found to cleave DNA,



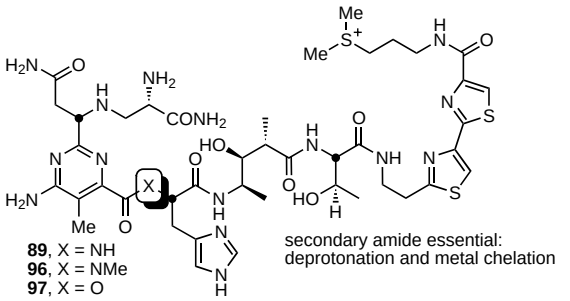
Agent	Efficiency	ds:ss	Selectivity
bleomycin A_2 (**1**)	2 – 5	1 : 6	5'-GC, 5'-GT > 5'-GA
deglycobleomycin A_2 (**41**)	1	1 : 12	5'-GC, 5'-GT > 5'-GA
(+)-P-3A (**2**)	0.3 - 0.2	1 : 30	none
epi-(–)-P-3A (**14**)	0.07	1 : 38	none
(–)-desacetamido P-3A (**15**)	0.07	1 : 40	none

Figure 7. DNA cleavage properties of (+)-P-3A and related agents.



Scheme 15. Synthesis of bleomycin A_2 derivatives by replacement of the NH of the L-histidine amide group with *N*-Me or O. BOP-Cl = benzotriazolyloxytris(dimethylamino)phosphonium chloride.

but to do so in a nonsequence-selective fashion with a substantially reduced efficiency and a diminished ds:ss cleavage ratio that were only slightly greater than that of free Fe^{III} ions (Figure 8). These observations are consistent with the proposal that the deprotonated secondary amide of L-histidine is required for functional metal chelation and activity.

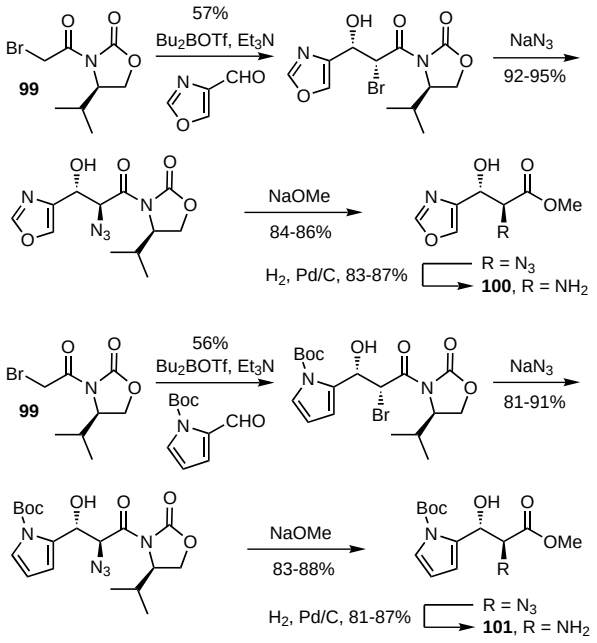


Agent	Efficiency	ds:ss	Selectivity
bleomycin A_2 (**1**)	2 – 5	1 : 6	5'-GC, 5'-GT > 5'-GA
deglycobleomycin A_2 (**41**)	1	1 : 12	5'-GC, 5'-GT > 5'-GA
89, X = NH	0.75	1 : 12	5'-GC, 5'-GT > 5'-GA
96, X = NMe	0.08	1 : 61	none
97, X = O	0.13	1 : 49	none
Fe^{III}	0.04	1 : 98	none

Figure 8. DNA cleavage properties of bleomycin analogues with modified amide groups on L-histidine. The results show that an unsubstituted amide group is essential.

3.2.4. Oxazole-, Pyrrole-, and Desimidazole-Deglycobleomycin A_2[101]

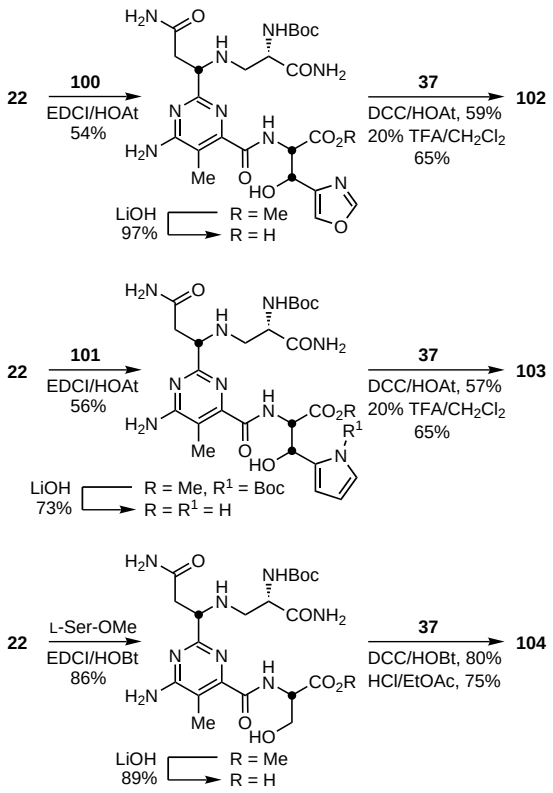
The N3 atom of imidazole of the *erythro*-β-hydroxy-L-histidine subunit functions as a key ligand in the metal complexes and has been assumed to play a pivotal role in the oxygen-activation properties. In conjunction with our studies on the amide group of β-hydroxy-L-histidine, the issue of complexation of the N3 atom of the imidazole ring with either N^{σ} or N^{π} metal coordination was addressed with the evaluation of **102** – **104**. The oxazole analogue is only capable of N^{π} metal complexation through a form related to the N^1-H imidazole tautomer of bleomycin A_2, while the pyrrole analogue may mimic the N^{σ} metal complexation capabilities of the imidazole N^3-H tautomer. The analogue **104**, which incorporates L-serine in place of the L-histidine subunit, lacks the imidazole group altogether. The synthesis of **102** and **103** required the protected *erythro*-β-hydroxy-L-histidine analogues **100** and **101**. Both were prepared according to the approach implemented for the authentic subunit by using the diastereoselective *syn* aldol addition of the optically active α-bromoacetyloxazolidinone **99** (Scheme 16).



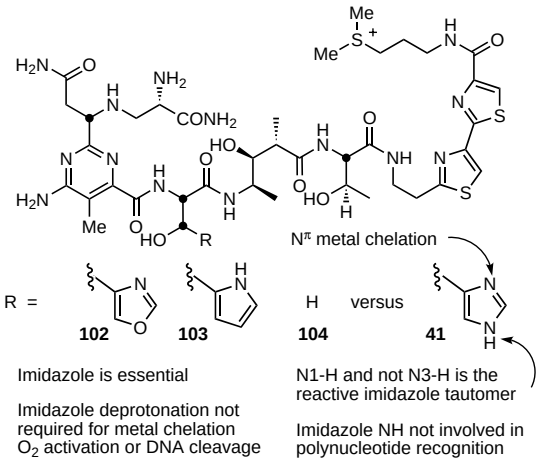
Scheme 16. Preparation of the L-His subunit analogues.

Coupling of **100** with N^{α}-Boc-pyrimidoblamic acid (**22**) followed by methyl ester hydrolysis, coupling of the resulting carboxylic acid with tetrapeptide S (**37**) and acid-catalyzed deprotection provided **102**. The incorporation of **101** and L-Ser-OCH_3 into analogues **103** and **104** was accomplished similarly (Scheme 17).

The oxazole analogue **102**, which is incapable of N^{σ} metal chelation, was found to behave analogous to, and only slightly less effectively, than deglycobleomycin A_2. The observation of sequence-selective cleavage confirmed that imidazole N^{π} metal chelation is sufficient for functional reactivity (Figure 9). The effective substitution of the O-1 oxazole atom for a N-1 histidine atom also illustrates that this group does not require deprotonation upon metal complexation, oxygen activation, or the ensuing oxidation reactions, that the functional bleomycin A_2 tautomer is the N^1-H tautomer, and that the N^1-H atom does not contribute to the polynucleotide



Scheme 17. Synthesis of bleomycin derivatives by replacement of imidazole group of the L-histidine subunit.



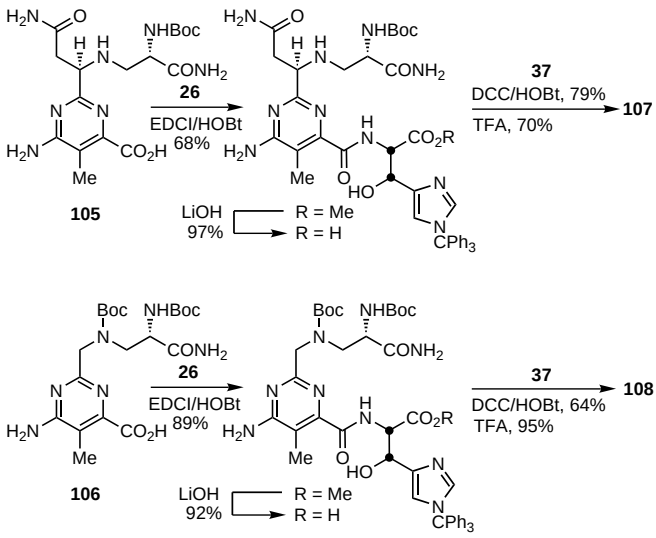
Agent	Efficiency	ds:ss	Selectivity
bleomycin A_2 (**1**)	2 – 5	1 : 6	5'-GC, 5'-GT > 5'-GA
deglycobleomycin A_2 (**41**)	1	1 : 12	5'-GC, 5'-GT > 5'-GA
102, R = oxazol	0.5	1 : 26	5'-GC, 5'-GT > 5'-GA
103, R = pyrrol	0.15	1 : 44	none
104, R = H	0.08	1 : 57	none
Fe^{III}	0.06	1 : 98	none

Figure 9. DNA cleavage properties of bleomycin derivatives with imidazole analogues of the histidine subunit. For further information see the text.

recognition through H-bonding to the phosphate backbone or nucleotide bases. In contrast, the pyrrole analogue **103**, which is incapable of N^{π} chelation and possesses the capabilities of functioning as a N^{σ} donor, was found to cleave DNA but did so in a nonsequence-selective fashion with a significantly reduced efficiency and ds:ss cleavage ratio, both only slightly above background Fe itself. Similarly, **104** proved to be an ineffective agent, which confirmed the requirement for the imidazole group. The cleavage of DNA by **103**, like that of **104**, may be mediated through Fenton chemistry (H_2O_2 and a Fe(II) salt) with generation of diffusible oxidants including hydroxyl radicals. These observations support N^{π}, not N^{σ}, coordination through the N^1-H imidazole tautomer for the functional activity of bleomycin A_2. The more recent structural studies of Stubbe et al. conducted on the Co^{III}-OOH complex of bleomycin A_2 also illustrate, and would seem to confirm, the N^{π} complexation of the histidine imidazole group and the complexation from the deprotonated amide group.[52]

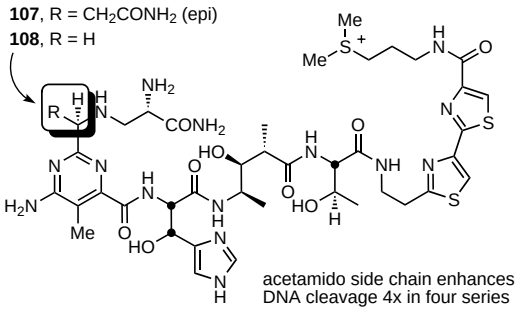
3.2.5. epi-*Deglycobleomycin A_2 and Desacetamidodeglycobleomycin A_2*[18d,e, 102]

The C2-acetamido side chain is not intimately involved in the key metal chelation and subsequent oxygen activation and yet appears to be important to the natural product since *epi*-bleomycin A_2[47] exhibits diminished biological activity and a reportedly altered DNA cleavage selectivity.[96] Studies on (+)-P-3A and related agents also revealed a significant role for the C2-acetamido side chain in the DNA cleavage efficiency.[84b, 102] Moreover, β-elimination of the β-aminoalanineamide activated by the C2-acetamido side chain may contribute to the inherent instability of bleomycin A_2. Thus, the removal of the C2-acetamido side chain could be anticipated to eliminate this degradation pathway and enhance the stability of the agent.[48, 49] In efforts to address the role of the C2-acetamido side chain, *epi*-deglycobleomycin A_2 (**107**) and desacetamidodeglycobleomycin A_2 (**108**) were prepared. Coupling of **105**, epimeric with natural pyrimidoblamic acid at the C2-acetamido side chain center, and **106**, lacking the side chain altogether, with **26** followed by methyl ester hydrolysis, coupling with tetrapeptide S (**37**) and deprotection provided *epi*-deglycobleomycin A_2 (**107**) and desacetamidodeglycobleomycin A_2 (**108**), respectively (Scheme 18).



Scheme 18. Synthesis of bleomycin analogues with modifications in the C2-acetamido side chain.

$Fe^{II} \cdot$ **107** and $Fe^{II} \cdot$ **108** proved indistinguishable and both displayed a substantially diminished DNA cleavage efficiency and ds:ss cleavage ratio relative to deglycobleomycin A_2 (Figure 10). The selectivity of the DNA cleavage was unaffected by the removal or epimerization of the C2-acetamido side chain. Since both **107** and **108** behave identically, it is not the unnatural configuration of **107** that diminishes cleavage efficiency. Rather, the results suggest a productive role for the natural C2-acetamido side chain that increases DNA cleavage



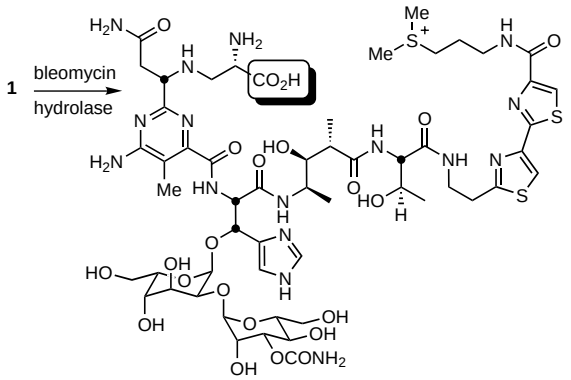
Agent	Efficiency	ds:ss	Selectivity
bleomycin A_2 (**1**)	2 – 5	1 : 6	5'-GC, 5'-GT > 5'-GA
deglycobleomycin A_2 (**41**)	1	1 : 12	5'-GC, 5'-GT > 5'-GA
107, R = CH_2CONH_2 (*epi*)	0.25	1 : 29	5'-GC, 5'-GT > 5'-GA
108, R = H	0.25	1 : 29	5'-GC, 5'-GT > 5'-GA

Figure 10. DNA cleavage by bleomycin derivatives containing C2-acetamido side chain modifications. The presence of the acetamido side chain enhances the DNA cleavage fourfold, as seen by a comparison of the data for **41** with those of **107** and **108**, those for **2** with those for **14** and **15** (Figure 7), those for **116**/**117** with those for **118**/**119** (Figure 14), as well as those for **131** with those for **132** (Figure 16).

efficiency and significantly increases the ratio of ds:ss DNA cleavage without affecting the cleavage selectivity. Although there are a number of attractive explanations for these observations, the trends are identical with those of (+)-P-3A (**2**), *epi*-(−)-P-3A (**14**), and (−)-desacetamido-P-3A (**15**) where the agents were found to cleave DNA with no sequence selectivity. This observation suggests that the role of the C2-acetamido side chain may not be the result of a specific DNA interaction at a particular cleavage site. Rather, the observations would be consistent with a productive role in increasing DNA cleavage by stabilizing the activated metal complex, increasing its catalytic turnover, or diminishing decomposition perhaps through protecting the activated metal complex from solvent. However, the structural studies of Stubbe et al. on the Co^{III}-OOH complex of deglycobleomycin A_2 suggest an additional provocative explanation.[52] In this model, a highly organized H-bond network that involves the threonine NH, carbonyl, and hydroxyl group with the metal-bound hydroperoxide and the metal binding domain is observed. Both the C2-acetamido side chain carbonyl and the metal coordinated β-amino group of the pyrimidoblamic acid side chain are H-bonded to the threonine hydroxyl group and potentially contribute to the stability of a compact DNA-bound conformation. This could enhance cleavage efficiency by either stabilization of the activated metal complex or by enhancing the adoption of a conformation productive for DNA cleavage (see Figures 13 and 19). In addition, the carboxamide NH_2 group of the C2-acetamide side chain may be H bonded to the C2 carbonyl group of the cytosine that is base paired with the guanine at the cleavage site. In principle, this provides a third H bond between the metal binding domain and the cleavage site GC base pair. The loss of this H bond could also account for the diminished cleavage efficiency but the results of our studies would indicate that it is not intimately required for recognition of the GC pair (see Section 3.2.8).

3.2.6. Descarboxamidodeglycobleomycin A_2[18e]

The terminal α-carboxamide found in the pyrimidoblamic acid side chain has long been suggested not to be involved in metal chelation and has no obvious role in the oxygen activation and DNA cleavage reactions. It does, however, undergo a rapid in-vivo hydrolysis that is catalyzed by bleomycin hydrolase [Eq. (1)].[48, 103, 104] The resulting

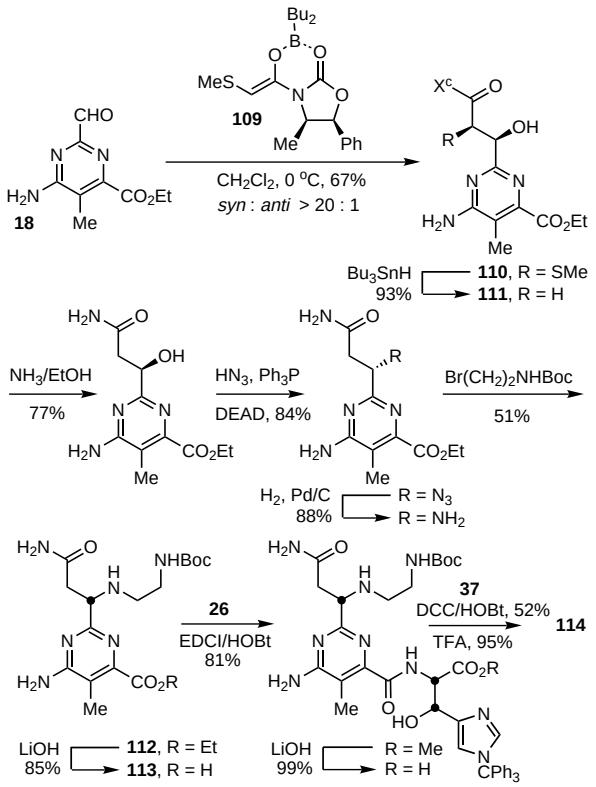


(1)

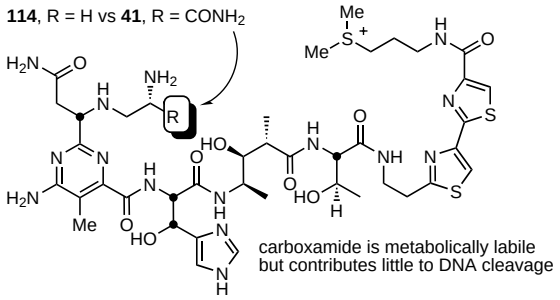
carboxylic acid displaces the metal-bound primary amine to serve as one tightly bound ligand of the inactivated metal complex. To determine if there was an unappreciated role for the metabolically labile carboxamide group, **114** was prepared for evaluation.

A modified approach was devised to prepare the pyrimidoblamic acid analogue **113** that lacked the carboxamide group. Treatment of **18** with the di-*n*-butylboronyl-(*Z*)-enolate **109** provided (2*R*,3*S*)-*syn*-**110** as the only detectable product (67 %, > 20:1; Scheme 19). Reductive desulfurization effected by Bu_3SnH (93 %), aminolysis (77 %), and Mitsunobu activation of the alcohol and azide displacement with inversion of the stereochemistry proceeded in excellent yield (84 %) with no loss of stereochemical integrity at the reaction center. The reverse order of Mitsunobu activation and azide displacement of the alcohol followed by aminolysis of the *N*-acyloxazolidinone also proceeded well but in lower conversions (51 and 40 %, respectively). Reduction of the azide, alkylation with *N*-Boc-2-bromoethylamine, and hydrolysis provided **113**. Coupling of **113** with **26**, methyl ester hydrolysis, coupling with tetrapeptide S (**37**), and deprotection provided **114**.

The comparison of **114** with deglycobleomycin A_2 proved interesting. The analogue was only 1.6 times less effective at cleaving DNA, only slightly less effective at producing ds



Scheme 19. Synthesis of descarboxamidodeglycobleomycin A_2 (**114**). DEAD = diethylazodicarboxylate, X^C = oxazolidinonyl.



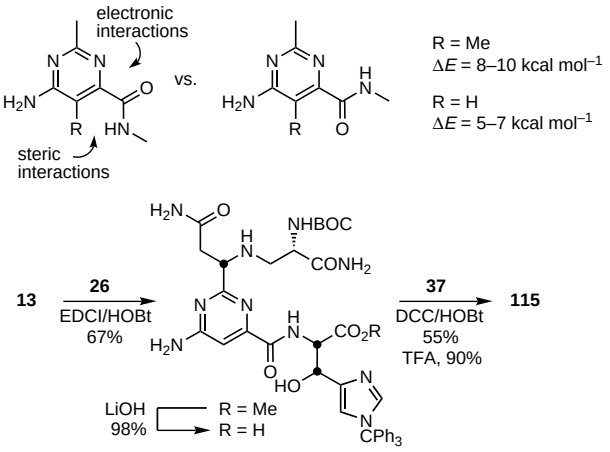
Agent	Efficiency	ds:ss	Selectivity
bleomycin A_2 (**1**)	2 – 5	1 : 6	5'-GC, 5'-GT > 5'-GA
deglycobleomycin A_2 (**41**)	1	1 : 12	5'-GC, 5'-GT > 5'-GA
114, R = H	0.6	1 : 18	5'-GC, 5'-GT > 5'-GA

Figure 11. DNA cleavage by descarboxamidodeglycobleomycin A_2 (**114**).

versus ss cleavage, and no differences in the cleavage selectivity were observed (Figure 11). These small distinctions suggest analogues that lack the carboxamide group might represent more potent, more efficacious, or longer acting agents as a result of their increased stability toward bleomycin hydrolase inactivation.

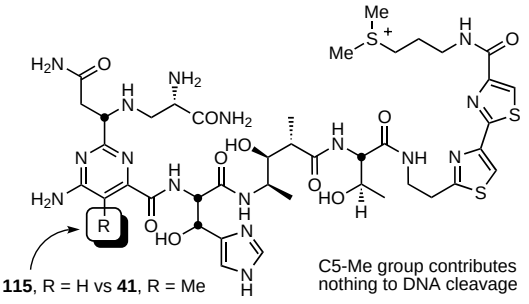
3.2.7. C5-desmethyldeglycobleomycin A_2[18e]

The C5-desmethyldeglycobleomycin A_2 (**115**) was prepared to assess the role of the methyl substituent on the pyrimidine ring. A steric interaction of the methyl group with the *ortho* carboxamide group may favor a conformation that enhances metal coordination, oxygen activation, and DNA cleavage. However, the inherent electronic destabilization of the *syn* conformation derived from lone pair – lone pair repulsion coupled with an inherent destabilizing steric interaction between a NHR group and a H atom of the *syn* conformation of **115** should ensure that it adopts the *anti* conformation ($\Delta E = 5.4 - 7.2$ kcal mol^{-1}; Scheme 20). Consequently, the C5-methyl substituent was anticipated to be unnecessary. This was easily addressed with the use of **13**, which was available from our synthesis of P-3A. Thus, the coupling of **13** with **26**, methyl ester hydrolysis, coupling with tetrapeptide S (**37**), and acid-catalyzed deprotection provided **115**.



Scheme 20. Synthesis of C5-desmethyldeglycobleomycin A_2 (**115**).

The comparison of **115** with **41** proved exceptionally good (Figure 12). The two agents were indistinguishable and exhibited the same DNA cleavage efficiency, nearly the same ratio of ds:ss cleavage, and the same cleavage selectivity, which indicated that the C5-methyl substituent does not contribute productively to the properties of bleomycin A_2.



Agent	Efficiency	ds:ss	Selectivity
bleomycin A_2 (**1**)	2 – 5	1 : 6	5'-GC, 5'-GT > 5'-GA
deglycobleomycin A_2 (**41**)	1	1 : 12	5'-GC, 5'-GT > 5'-GA
115, R = H	1	1 : 18	5'-GC, 5'-GT > 5'-GA

Figure 12. DNA cleavage by C5-desmethyldeglycobleomycin A_2 (**115**).

3.2.8. Dimethylamino- and Desaminodeglycobleomycin A_2: Confirmation of the Origin of Sequence Selective DNA Cleavage[105]

The role of the C4 amino group of pyrimidine was probed through the synthesis and evaluation of **116** and **117** as well as their epimers **118** and **119** in which the amine was replaced

with a tertiary *N*,*N*-dimethylamine or removed altogether. In addition to the impact this may have on the metal chelation and oxygen-activation properties, their examination allowed the assessment of a potential key H bond from this primary C4 amine to the guanine N3 atom defined in the beautiful structural studies of Stubbe et al.[52] These studies highlighted two previously unrecognized H bonds between the pyrimidine ring of the metal binding domain of bleomycin A_2 and guanine of the 5′-GC/5′-GT cleavage sites. The N3 atom of pyrimidoblamic acid was found to be H bonded to the nonbase pairing hydrogen atom of the C2-amine group of guanine and one of its C4-amine hydrogen atoms was H bonded to the N3 atom of guanine to provide a triplex-like recognition interaction in the minor groove (Figure 13). This interaction potentially provides the basis for the sequence selective cleavage of DNA and explains the requirement for the guanine C2-amine group for 5′-GC/5′-GT cleavage.[26b, 44c, 76c, 76d, 106]

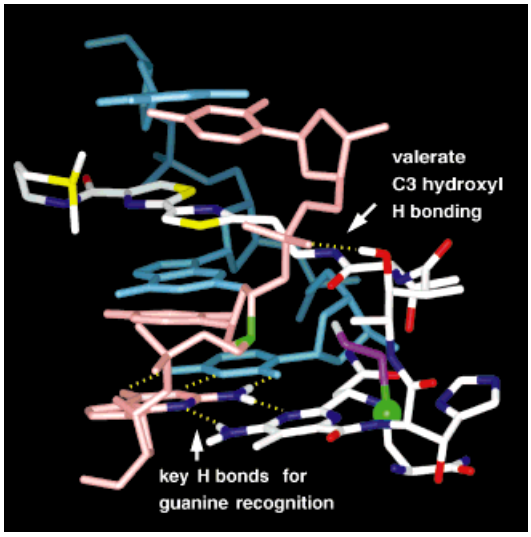
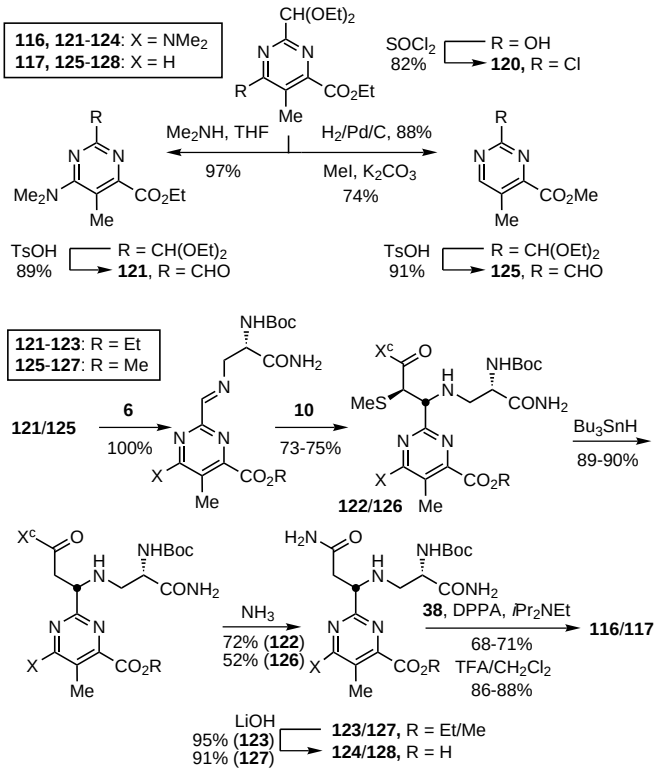


Figure 13. Expanded view of the complex structure determined by NMR spectroscopy (see Figure 5) illustrating the two H bonds between pyrimidoblamic acid (C4 amino group and N3 atom) and the guanine at the cleavage site. Also highlighted are the H bond between the valerate C3-OH subunit to DNA, the C4′-H abstraction site (green), the Co^{III} · OOH subunit (green ball and violet stick), and the rigid, compact conformation of the linker domain. For clarity, the disaccharide subunit is not shown.

The synthesis of **116** from an appropriately substituted pyrimidine was addressed after unsuccessful efforts to prepare **123** by reductive methylation or alkylation of the ethyl ester of N^{α}-Boc-pyrimidoblamic acid. Thus, dimethylamine displacement of the 4-chloro substituent of **120** provided cleanly the corresponding 4-dimethylaminopyrimidine, and subsequent acetal hydrolysis afforded the aldehyde **121** as a key intermediate for diastereoselective introduction of the side chain (Scheme 21). Condensation with **6** followed by addition of the stannous (*Z*)-enolate **10** provided **122** (87:13 diastereoselection). Reductive removal of the thiomethyl group, aminolysis of the *N*-acyloxazolidinone, followed by ester hydrolysis provided **124**. Coupling of **124** with pentapeptide S (**38**) and acid-catalyzed deprotection of the single *N*-Boc protecting group provided **116**.

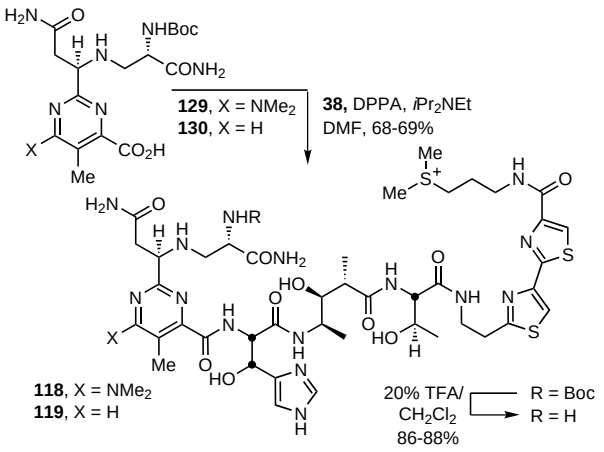


Scheme 21. Synthesis of dimethylaminodeglycobleomycin A_2 (**116**) and desaminodeglycobleomycin A_2 (**117**). X^C = oxazolidonyl.

The synthesis of **128** and its incorporation into **117** was accomplished after unsuccessful efforts at reductive deamination of a pyrimidoblamic acid precursor (Scheme 21). Reductive dechlorination of **120** under conditions that resulted in ethyl ester hydrolysis followed by reesterification and acetal hydrolysis provided the key aldehyde intermediate **125**. Condensation with **6** followed by addition of the stannous (*Z*)-enolate **10** provided **126** (89:11 diastereoselection). Reductive desulfurization, aminolysis, and ester hydrolysis afforded **128**. Coupling of **128** with pentapeptide S (**38**) and acid-catalyzed deprotection provided **117**.

Complementary to these efforts the minor diastereomers derived from the imine addition reactions, which possess the unnatural stereochemistry at the C2-acetamido side chain, were converted into **129** and **130** and incorporated into the epimeric analogues **118** and **119** (Scheme 22).

In initial efforts to characterize the properties of the agents, the ability of their Fe^{III} complexes to mediate the oxidation of styrene was investigated.[72b, 107] Although the pyrimidine C4-amine group of bleomycin A_2 is not directly engaged in the metal chelation, the electronic character of C4 substituents has been shown to affect the O_2-activation properties.[6, 68h, 73] The examination of **116** and **117** and their epimers revealed the same product distribution with all analogues and deglycobleomycin A_2, which indicates that all give rise to competent iron-oxo intermediates (Table 2). The relative efficiencies of the two dimethylamino analogues (**116** and **118**) were nearly indistinguishable from **41** itself, while those of the desamino analogues (**117** and **118**) were slightly lower. This is consistent with expectations that an electron-donating C4



Scheme 22. Synthesis of the epimeric analogues **118** and **119**.

Table 2. Styrene oxidation by Fe^{III} complexes of bleomycin A_2 analogues.[a]

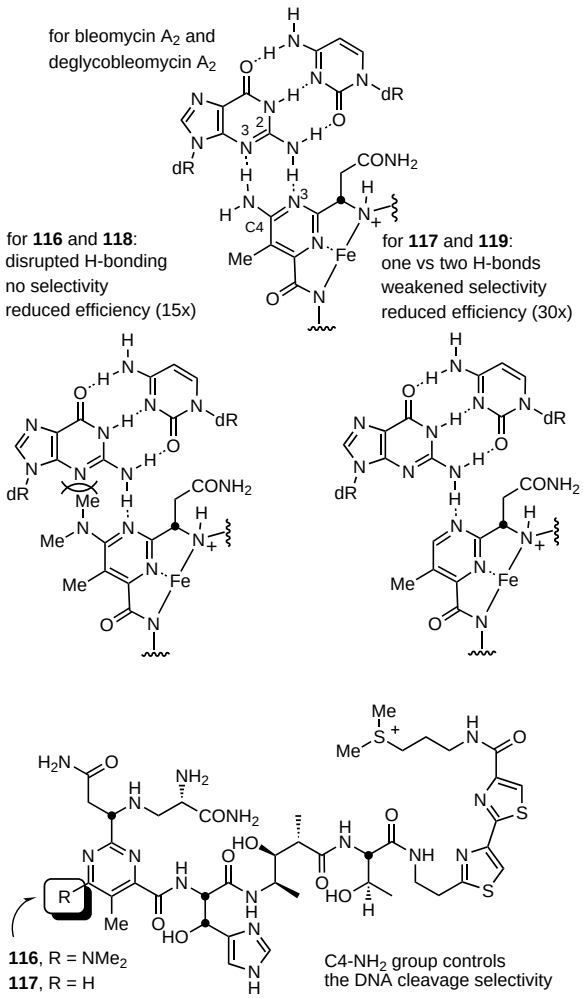
Agent	*c*(styrene oxide) [mM]	*c*(phenylacetaldehyde) [mM]	Ratio	*c*(total product) [mM]	Relative efficiency
Fe^{III} · **41**	1.80	1.32	1.36	3.12	1.0
Fe^{III} · **116**	1.67	1.21	1.38	2.88	0.92
Fe^{III} · **117**	1.11	0.88	1.26	1.99	0.64
Fe^{III} · **118**	1.55	1.00	1.55	2.55	0.82
Fe^{III} · **119**	1.07	0.79	1.35	1.86	0.59
Fe^{III}[b]	0	0	–	0	0
Fe^{III} · **41**[c]	0	0	–	0	0

[a] 500 μM Fe^{III}-agent, 50 mM styrene, 30 mM H_2O_2, 0 °C, 1.5 h, 80 % CH_3OH in H_2O. [b] 500 μM Fe^{III} under identical conditions as [a] and with H_2O_2 present. [c] Same as in [a], but without H_2O_2.

substituent on pyrimidine would improve the O_2-activation properties of the metal complexes. However, all were effective and provided roughly 4–6 oxidations per Fe^{III} complex.

The DNA cleavage properties of the agents were especially revealing. The analogue **116** and its epimer **118** with a dimethylamino substituent exhibited a substantially diminished DNA cleavage efficiency (10–15 ×), reduced ratio of ds versus ss cleavage**,** and a complete loss of cleavage selectivity (Figure 14). These observations indicate that substitution with the C4 dimethylamino group not only precludes the formation of a H bond between the C4-amino group and the guanine N3 atom but also sterically prevents formation of the remaining H bond between the guanine C2 amino group and the pyrimidine N3 atom, which destroys both the cleavage efficiency and selectivity of the agent.

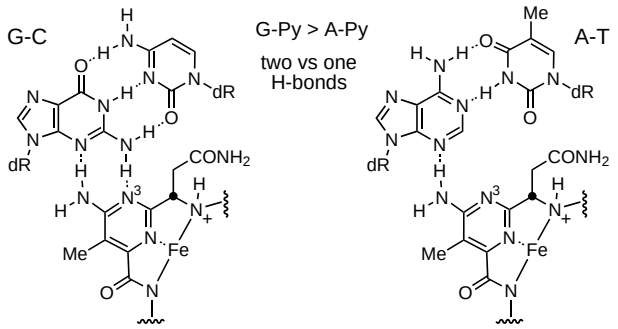
The analogues **117** and **119** exhibited an even greater diminished DNA cleavage efficiency (30 ×) with altered and sometimes lost sequence selectivity. Even in the instances where the selectivity was not substantially altered, its detection required assay conditions of 4 °C instead of the usual 25–37 °C. In addition, the cleavage at minor 5′-AT sites essentially disappeared. This suggests a reduced binding interaction and is consistent with the participation of the C4 amino group of pyrimidine in the formation of one of the two critical H bonds of the minor groove triplex-like recognition.



Agent	Efficiency	ds:ss	Selectivity
bleomycin A_2 (**1**)	2 – 5	1 : 6	5′-GC, 5′-GT > 5′-GA
deglycobleomycin A_2 (**41**)	1	1 : 12	5′-GC, 5′-GT > 5′-GA
	0.08	1 : 53	none
	0.06	1 : 45	weak 5′-GC, 5′-GT > 5′-GA
118, R = NMe_2 (*epi*)	0.04	1 : 61	none
119, R = H (*epi*)	0.03	1 : 48	weak 5′-GC, 5′-GT > 5′-GA

Figure 14. DNA cleavage by bleomycin A_2 analogues with modified pyrimidine C4-amino groups. The results show that this group is essential to control the selectivity of the DNA cleavage.

Both these observations are consistent with the involvement of the C4 amino group in a pair of H bonds in a triplex-like recognition of the guanine at the cleavage site and provide direct evidence for its critical role in the polynucleotide recognition. In addition, these observations have further implications on the inherent DNA cleavage selectivity of bleomycin A_2 itself. Bleomycin A_2 not only cleaves essentially all 5′-GT, 5′-GC sites in DNA, but also cleaves 5′-AT, 5′-AC sites albeit less effectively (25–40 % versus 100 %) and does so with a weaker efficiency (Figure 15). Although the statistically minor cleavage sites compiled in Figure 15 could result from ds cleavage that originates from primary sites on the complementary strand, the more general cleavage preference of 5′-GPy > 5′-APy (Py = pyrimidine base) may be attributed



Dinucleotide cleavage frequency

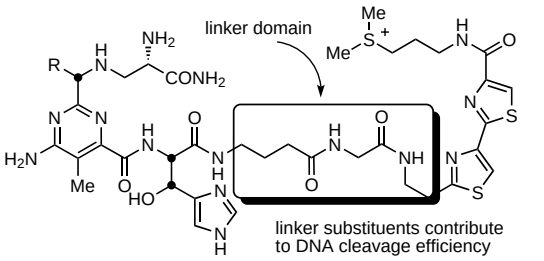
Site	% cleaved	Site	% cleaved	Site	% cleaved	Site	% cleaved
5'-GC	100	5'-AC	28	5'-TC	0	5'-CC	0
5'-GT	100	5'-AT	39	5'-TT*	8	5'-CT*	5
5'-GA	79	5'-AA	13	5'-TA*	7	5'-CA	0
5'-GG	0	5'-AG	0	5'-TG	0	5'-CG	0

Figure 15. Further implications for bleomycin A_2 cleavage selectivity. The frequency of the cleavage of the dinucleotides is shown. The sites marked with a star are secondary ds-DNA cleavage sites.

to an analogous reduced binding affinity at the 5′-APy sites that result from one versus two triplex-like H bonds to adenine (Figure 15). Thus, although the properties of **117** might have been unanticipated based on the triplex-like H bond model, its behavior shed further light on the origin of the inherent 5′-GPy > 5′-APy cleavage selectivity of bleomycin A_2 itself. These results complement the studies where Waring and co-workers showed that the cleavage is diminished but not lost when guanine is replaced with inosine and that adenine cleavage is greatly increased when it is replaced with 2-aminoadenine.[76] Both of these single site changes in the DNA also support the basis of the H-bonding specificity.

3.3. The Linker Domain

At the onset of our efforts the full role of the linker region joining the bithiazole C terminus and the N-terminus pyrimidoblamic acid was poorly understood. The importance of the presence and absolute stereochemistry of the C4 methyl group was first disclosed in the studies of Umezawa, Ohno, and co-workers on the DNA cleavage efficiency of a select set of bleomycin A_2 analogues.[6, 73] Similarly, Hecht and co-workers have shown that the L-threonine subunit and its backbone substituents are not important to the cleavage selectivity although they appeared to have an impact on the cleavage efficiency.[24d, 42b] In studies conducted concurrent with our synthesis of deglycobleomycin A_2, **131** and **132** were prepared in which all the linker substituents had been removed.[18e, 108] Their examination revealed that the substituents do not adversely affect the DNA binding affinity (calf thymus DNA $K_B = 2.4 \times 10^5\,M^{-1}$ versus $1-1.1 \times 10^5\,M^{-1}$ for **1** and **41**) or cleavage selectivity, but that the cleavage efficiency (ca. 10–100 times) and the ds:ss cleavage ratio were substantially reduced (Figure 16).[18e] Such observations are consistent with our early proposal that the chain substituents of the linker facilitate the adoption of a compact bound conformation with at least one turn at the tripeptide S/



Agent	Efficiency	ds:ss	Selectivity
bleomycin A_2 (**1**)	2 – 5	1 : 6	5'-GC, 5'-GT > 5'-GA
deglycobleomycin A_2 (**41**)	1	1 : 12	5'-GC, 5'-GT > 5'-GA
131, R = CH_2CONH_2	0.1	1 : 33	5'-GC, 5'-GT > 5'-GA
132, R = H	0.03	1 : 35	5'-GC, 5'-GT > 5'-GA

Figure 16. Cumulative effect of the substituents in the linker domain. These contribute to the efficiency of DNA cleavage.

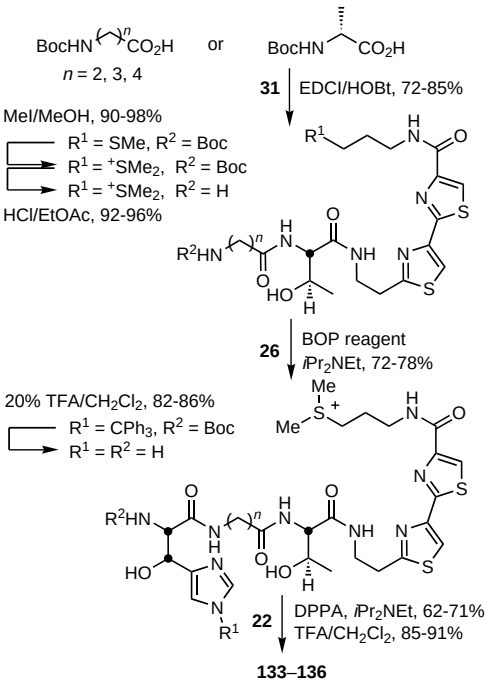
tetrapeptide S juncture. In efforts to address the contribution of the individual substituents of the linker domain and accurately define the effects, an extensive series of linker analogues was examined.

3.3.1. Valerate Subunit

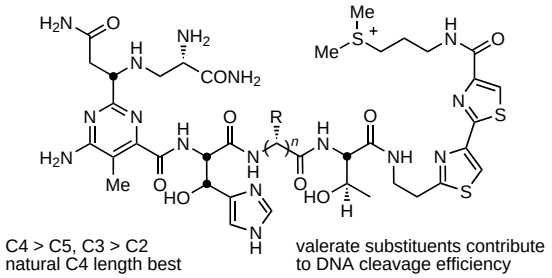
3.3.1.1. Effect of Linker Length[109]

The analogues **133**–**136** that incorporate 2–5 carbon linkers in place of the valerate subunit were prepared as outlined in Scheme 23. Their examination permitted an assessment of the impact of the length of the valerate subunit.

DNA cleavage studies revealed a well-defined relationship of C4 > C5, C3 > C2 where the length of the natural valerate linker was established to be best (Figure 17). This is consistent



Scheme 23. Synthesis of bleomycin A_2 analogues with variable linker lengths.



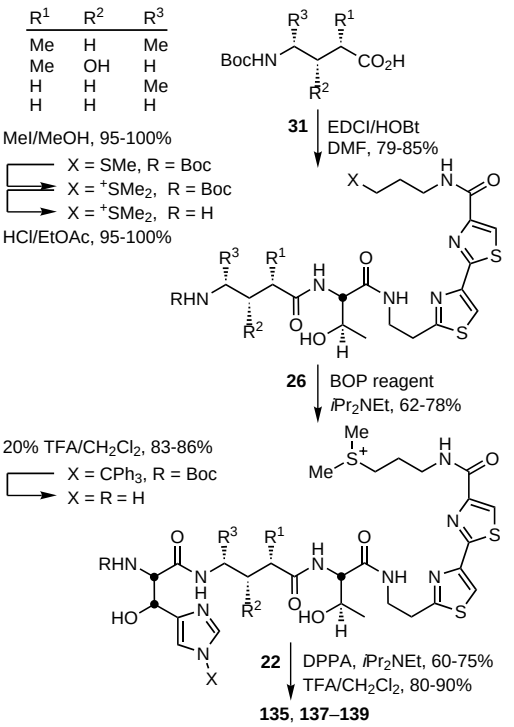
Agent	Efficiency	ds:ss	Selectivity
bleomycin A_2 (**1**)	2 – 5	1 : 6	5'-GC, 5'-GT > 5'-GA
deglycobleomycin A_2 (**41**)	1	1 : 12	5'-GC, 5'-GT > 5'-GA
133, *n* = 1, R = Me	0.07	-	not done
134, *n* = 2, R = H	0.08	1 : 43	5'-GC, 5'-GT > 5'-GA
135, *n* = 3, R = H	0.13	1 : 31	5'-GC, 5'-GT > 5'-GA
136, *n* = 4, R = H	0.08	1 : 38	5'-GC, 5'-GT > 5'-GA

Figure 17. DNA cleavage by bleomycin A_2 analogues with various lengths of the valerate subunit.

with the adoption of a well-defined DNA-bound conformation where those agents that possess a valerate substitute shorter than four carbon atoms may have progressively more difficulty in adopting conformations that span the linker region. In turn, those longer than four carbon atoms possess greater flexibility and less opportunity to adopt the productive conformation required for selective cleavage.

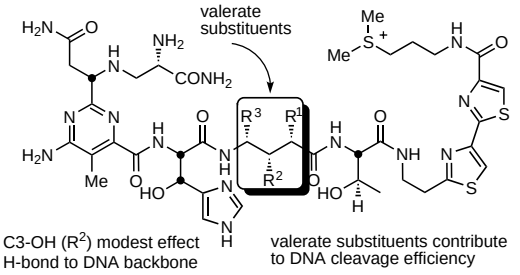
3.3.1.2. Substituent Effects[109]

Examination of **137** – **139** along with **135** allowed for a clear definition of the substituent effects. Analogue **137** permitted the assessment of the valerate C3-hydroxyl group. The examination of **138** – **139** was anticipated to clarify the role of the C2 and C4 methyl substituents, which had been identified as important in our previous studies.[109] The analogues were prepared through the coupling of the pentapeptide S analogues with the modified valerate subunits with N^{α}-Boc-pyrimidoblamic acid (Scheme 24).

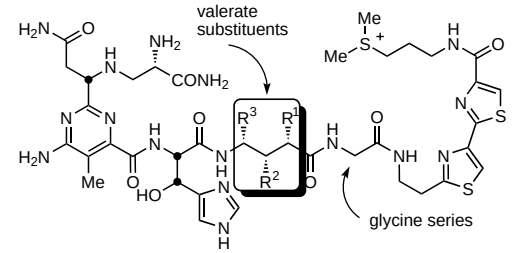


Scheme 24. Synthesis of bleomycin A_2 analogues containing the key valerate substituents.

The DNA cleavage properties of the valerate substituent analogues are summarized in Figure 18. Analogue **137** was only slightly less effective (0.8 ×) than deglycobleomycin A_2 at cleaving DNA, while **138** was substantially less efficient (0.4 ×). Thus, removal of the C4 methyl group had a more significant impact than the C3 hydroxyl group. Removal of the C2 methyl and C3 hydroxyl group in **139** led to an even greater (fivefold) reduction, which indicated that the C4 methyl group alone cannot account for the cleavage efficiency induced by the substituents. Further removal of the remaining C4 methyl substituent with **135** resulted in a still greater reduction in the cleavage efficiency (0.13 ×). Analogous comparisons were made in a glycine versus threonine series where in addition to highlighting the significant effect of the threonine side chain, which is described in the following section, the same trends for the valerate substituents were observed (Figure 18).



Agent	Efficiency	ds:ss	Selectivity
bleomycin A_2 (**1**)	2 – 5	1 : 6	5'-GC, 5'-GT > 5'-GA
deglycobleomycin A_2 (**41**)	1	1 : 12	5'-GC, 5'-GT > 5'-GA
R^1 R^2 R^3			
137, Me H Me	0.8	1 : 14	5'-GC, 5'-GT > 5'-GA
138, Me OH H	0.4	1 : 15	5'-GC, 5'-GT > 5'-GA
139, H H Me	0.2	1 : 25	5'-GC, 5'-GT > 5'-GA
135, H H H	0.13	1 : 31	5'-GC, 5'-GT > 5'-GA



Agent	Efficiency	ds:ss	Selectivity
R^1 R^2 R^3			
144, Me OH Me	0.2	1 : 29	5'-GC, 5'-GT > 5'-GA
140, Me OH H	0.15	1 : 30	5'-GC, 5'-GT > 5'-GA
131, H H H	0.1	1 : 33	5'-GC, 5'-GT > 5'-GA

Figure 18. DNA cleavage by bleomycin A_2 analogues with modified valeric acid subunits. The valerate substituents contribute to the efficiency of the DNA cleavage.

The cumulative substituent effects are substantial and highlight an unappreciated role they play in the preorganization of bleomycin A_2 into a rigid, compact conformation suitable for DNA cleavage. The valerate subunit of both free and DNA-bound Co^{III}-OOH bleomycin A_2 and deglycobleomycin A_2 adopts an identical, well-defined, and rigid conformation (Figure 19). Important characteristics of this conformation are two turns, one at the C2 center and one at the C4 center. Diagnostic of this conformation is the small coupling constant for C2-H/C3-H ($J = 1.8 \pm 1.2$ Hz) and the large coupling constant for C3-H/C4-H ($J = 9.5 \pm 1.2$ Hz).[52]

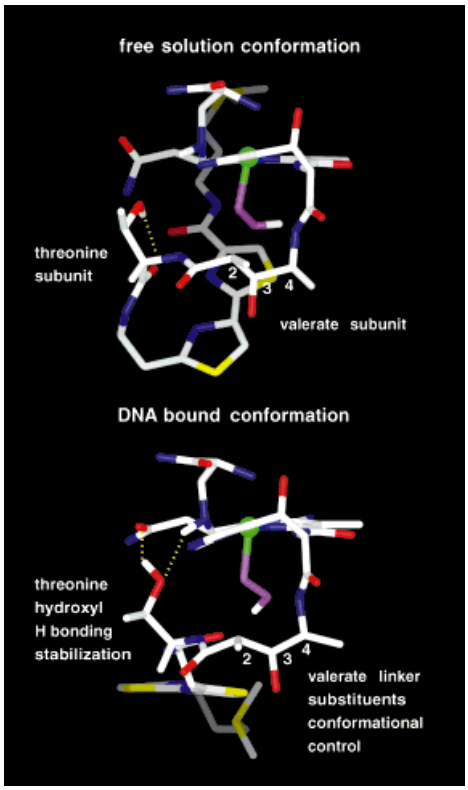


Figure 19. Models of the linker domain taken from the structures determined by NMR spectroscopy of free and DNA-bound Co^{III}-OOH bleomycin A_2.[52]

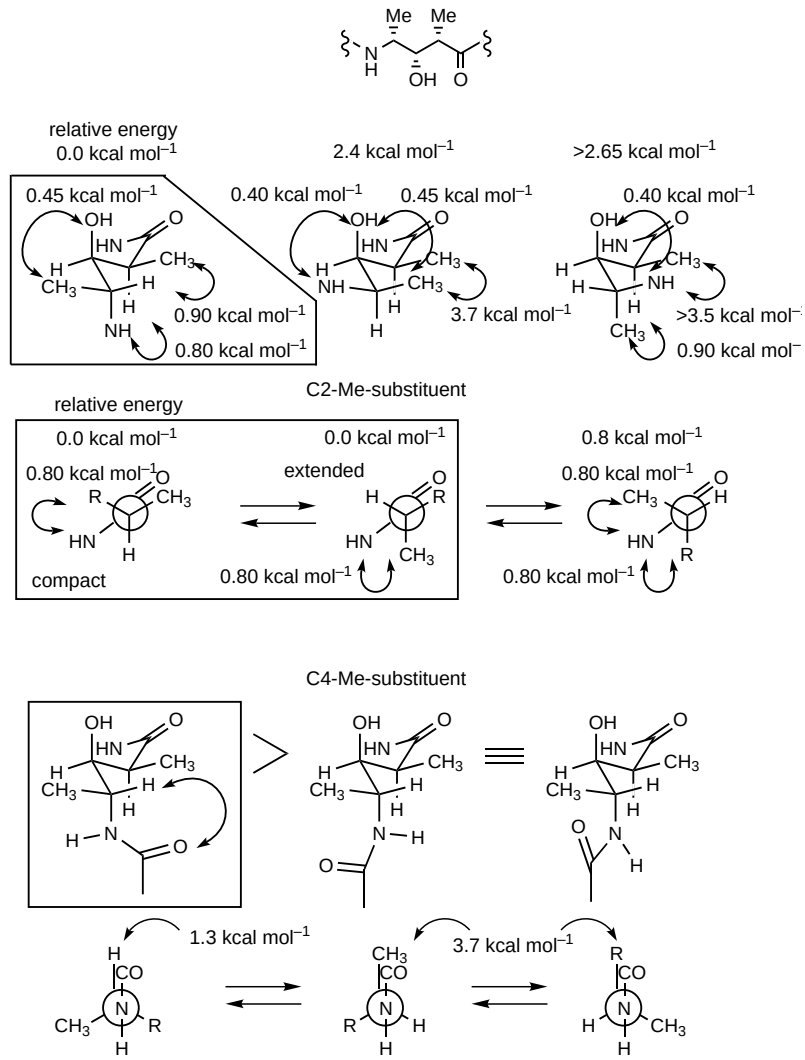


Figure 20. Conformational effects of the valerate substituents.

The C2 site within the valerate could adopt two accessible conformations (Figure 20). The eclipsed conformation between the carbonyl and the C2 methyl group was found to be favored, presumably through adoption of H bonds from the valerate NH and the threonine NH to the metal-bound hydroperoxide (1.8 Å, 155° and 1.9 Å, 152°, respectively). This is further reinforced for the DNA-bound agents by a H bond from the C3 hydroxyl group to the DNA phosphate backbone (see Figure 13).[52] Alternative conformations about the C3 ($\geq 2.65 - 3.35$ kcal mol^{-1}) or C4 centers ($> 2.4 - 2.65$ kcal mol^{-1}) are much less stable and constitute noncontributing conformations. The C4 methyl substituent when combined with the presence of the C2 methyl group, must adopt the extended orientation to induce a second turn in the valerate subunit at the amide bond that links the histidine subunit ($\geq 2.4 - 2.65$ kcal mol^{-1}). Just as important as this turn is the placement of the amide group, the conformation in which its carbonyl group eclipses the H atom is also preferred (ΔE ca. 2.4 kcal mol^{-1}) and sets a defined orientation for the histidine subunit and the entire metal binding domain. In total, this provides the rigid core structure about which the DNA binding domain and the metal chelation domain are linked.

The C3 hydroxyl group is engaged in a H bond with a DNA backbone phosphate group that spans the intercalation site, which may contribute to the affinity and alignment of the DNA-bound agent. Its removal has little effect on the conformation about the C3 center ($\Delta E_{\text{alternatives}} = 2.25 - 2.45$ kcal mol^{-1}) or the C4 center ($\Delta E_{\text{alternatives}} = 2.0 - 2.7$ kcal mol^{-1}), and it is the lost intermolecular H bond with the nonbridging oxygen atom of the phosphate backbone that may account for the modest decrease in the cleavage efficiency with **137** ($0.8\times$). With the exception of this effect, the remainder of the results correlate remarkably well with the ability of the agents to adopt the compact conformation implicated in the structural studies of Stubbe et al. (Table 3). The cumulative effect is substantial and suggests that an important functional role of the valerate substituents is to

Table 3. Effects of the valerate substituents.

Agent	R^1	R^2	R^3	$\Delta E_{rel.}$[a] [kcal mol^{-1}]	Z[b]
41	Me	OH	Me	0.0	1.0
137	Me	H	Me	0.0[c]	0.8[c]
138	Me	OH	H	0.4[d]	0.4[d]
139	H	H	Me	0.8	0.2
135	H	H	H	1.6	0.13

[a] $\Delta E_{rel.} = E$(low energy conformation) $- E$(DNA-bound conformation adopted by Co^{III}-OOH BLM A_2). [b] Z is the relative DNA cleavage efficiency. [c] Lacks the capability for a intermolecular H bond from C3-OH to the DNA phosphate group (see text). [d] Possesses two equivalent amide orientations of the L-histidine subunit in which the H atoms are in eclipsed positions versus one which contributes further to a reduction in the cleavage efficiency (see text).

preorganize bleomycin A_2 into a rigid, compact conformation productive for DNA cleavage.

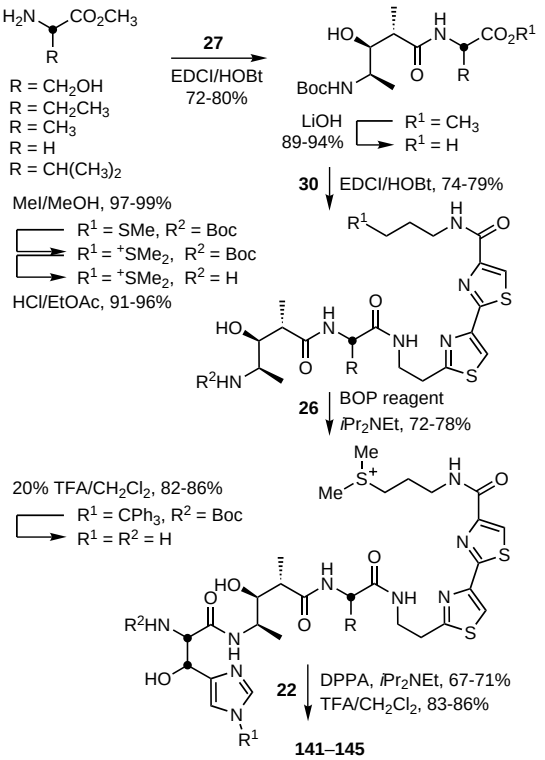
3.3.2. The Threonine Subunit

3.3.2.1. Substituent Effects[109, 110]

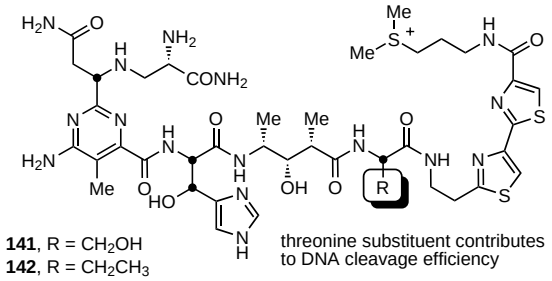
In our efforts to understand the role of the linker domain and following initial studies that indicated that the L-threonine side chain contributed substantially to the cleavage efficiency, a complete series of side-chain analogues (**141**–**145**) was examined to systematically dissect the subtle contributions of this subunit. In part, this was assumed to be a consequence of conformational effects imposed by the side chain that induced a turn at the threonine–valerate junction and facilitated the adoption of a compact conformation. In addition, the threonine carbonyl group of the DNA bound conformation, but not the free solution conformation, of the bleomycin Co^{III}-OOH complex was found to be positioned to accept a H bond from the terminal oxygen atom of the hydroperoxide ligand in the structural model of Stubbe et al., which implicated additional roles for the L-threonine subunit. Similarly, the threonine hydroxyl group was engaged in a network of H bonding with the metal binding domain, which further contributes to the stabilization of the compact conformation. The approach to **141**–**145** entailed the synthesis of the pentapeptide S analogues with five altered side chains and a final coupling with N^{α}-Boc-pyrimidoblamic acid (Scheme 25).

DNA cleavage studies revealed that variations in the L-threonine subunit substituents had no impact on the cleavage selectivity, but did have a large effect on the cleavage efficiency (Figure 21). This was consistent with our initial studies that indicated that the effect was both substantial and general when threonine was replaced with glycine (see Figures 16 and 18). In addition, the glycine analogue **144** was shown to oxidize styrene nearly as effectively as deglycobleomycin A_2 ($0.5\times$) at an initial rate that was not distinguishable, albeit with twofold fewer turnovers in the catalytic reaction.

The studies defined a clear potentiating role for the L-threonine hydroxyl group, highlighted the importance of the presence of a substituent on the L-threonine subunit ($R \neq H$), and suggested the effect is unusually sensitive to the size of the



Scheme 25. Preparation of bleomycin A_2 analogues with threonine subunit modifications.



Agent	Efficiency	ds:ss	Selectivity
bleomycin A_2 (**1**)	2 – 5	1 : 6	5'-GC, 5'-GT > 5'-GA
deglycobleomycin A_2 (**41**)	1	1 : 12	5'-GC, 5'-GT > 5'-GA
141, R = CH_2OH	1	1 : 17	5'-GC, 5'-GT > 5'-GA
142, R = CH_2CH_3	0.5	1 : 21	5'-GC, 5'-GT > 5'-GA
143, R = CH_3	0.4	1 : 24	5'-GC, 5'-GT > 5'-GA
144, R = H	0.2	1 : 31	5'-GC, 5'-GT > 5'-GA
145, R = $CH(CH_3)_2$	0.17	1 : 25	5'-GC, 5'-GT > 5'-GA

Figure 21. DNA cleavage of bleomycin A_2 analogues with threonine subunits modifications.

substituent ($R = CH_3 \cong Et > iPr$). A remarkably good correlation was observed between the cleavage efficiencies and the calculated Boltzmann probabilities[111] of adopting the L-threonine subunit local conformation observed with free Co^{III}-OOH bleomycin A_2 and, with the exception of valine, with the total distribution within the free and DNA-bound conformations of Co^{III}-OOH bleomycin A_2 (Table 4). This suggests that the L-threonine substituent facilitates preorga-

Table 4. Effects of substituents on the threonine subunit.

Agent	Z[a]	P_B[b] $\Psi = 20$ to 130°	P_B[b] $\Psi = 130$ to -140°	P_B[b] (total)
Thr, R = $CH(OH)CH_3$	1.0	0.798	0.153	0.951
Ser, R = CH_2OH	1 – 0.9	0.776	0.087	0.863
Abu, R = Et	0.50	0.605	0.191	0.796
Ala, R = CH_3	0.45	0.589	0.138	0.727
Gly, R = H	0.20	0.425	0.049	0.474
Val, R = *i*Pr	0.17	0.390	0.335	0.725

[a] Z is the relative DNA cleavage efficiency. [b] P_B = Boltzmann probability of having the peptide binding angle Ψ (taken from ref. [111], for AcN-X-NHMe). Free Co^{III}-OOH BLM A_2 ($\Phi = -121.5^\circ$, $\Psi = 120.4^\circ$). DNA-bound Co^{III}-OOH BLM A_2 ($\Phi = -145.5^\circ$, $\Psi = 173.9^\circ$).

nization of bleomycin A_2 into a compact conformation productive for DNA cleavage (see Figure 19). The additional important role of the hydroxyl group may be attributed to either intramolecular H bonding to the threonine carbonyl group or, more provocatively, to the distal carbonyl group of the C2-acetamido side chain of pyrimidoblamic acid to stabilize a preorganized conformation of activated bleomycin productive for DNA cleavage (see Figure 19). This could, in part, also explain the potentiating role of the acetamide side chain (ca. 4 ×, see Figure 10). However, we cannot rule out an intermolecular H bond with DNA itself, although structural models to date have not provided evidence for such an interaction.

The simpler effect of the presence of a substituent is consistent with an important role in restricting the available conformations accessible to the agent (Figure 22). In the presence of a substituent (R ≠ H), a dominant effect of restricting the valerate – threonine amide group to a single orientation ($\Phi = -120^\circ$) induces a turn in the linker domain. Removal of this substituent (R = H) results in five accessible conformations, at least three of which may be nonproductive for DNA cleavage. Moreover, as the size of the substituent increases, the conformations nonproductive for DNA cleavage become increasingly favored and result in diminished cleavage (R = Me ≅ Et > *i*Pr).

3.3.2.2. *Replacement of the Threonine Amide Group*[112]

The NMR studies of the Co^{III}-OOH complex of both free and DNA-bound bleomycin revealed that they may benefit from a H bond from the threonine NH group to the proximal oxygen atom of the metal-bound hydroperoxide (Figure 23).[52] This H bond could stabilize the productive bound conformation of the activated agent, fix the position of the reacting Fe-oxo intermediate for abstraction of the C4′ hydrogen atom, stabilize the metal-bound hydroperoxide, or contribute to catalysis of the reaction by assisting homolytic oxygen – oxygen bond cleavage. In light of this interaction of the threonine NH group, we examined **146** and its epimer **147** in which the threonine amide group was replaced with a *N*-methyl amide group. The synthesis of **146** was accomplished through preparation of the tetrapeptide **152** with the *N*-methyl-L-threonine subunit (Scheme 26). NMe-Thr-OMe was prepared from L-Thr-OMe by sequential reductive benzylation and methylation followed by reductive debenzylation. Its

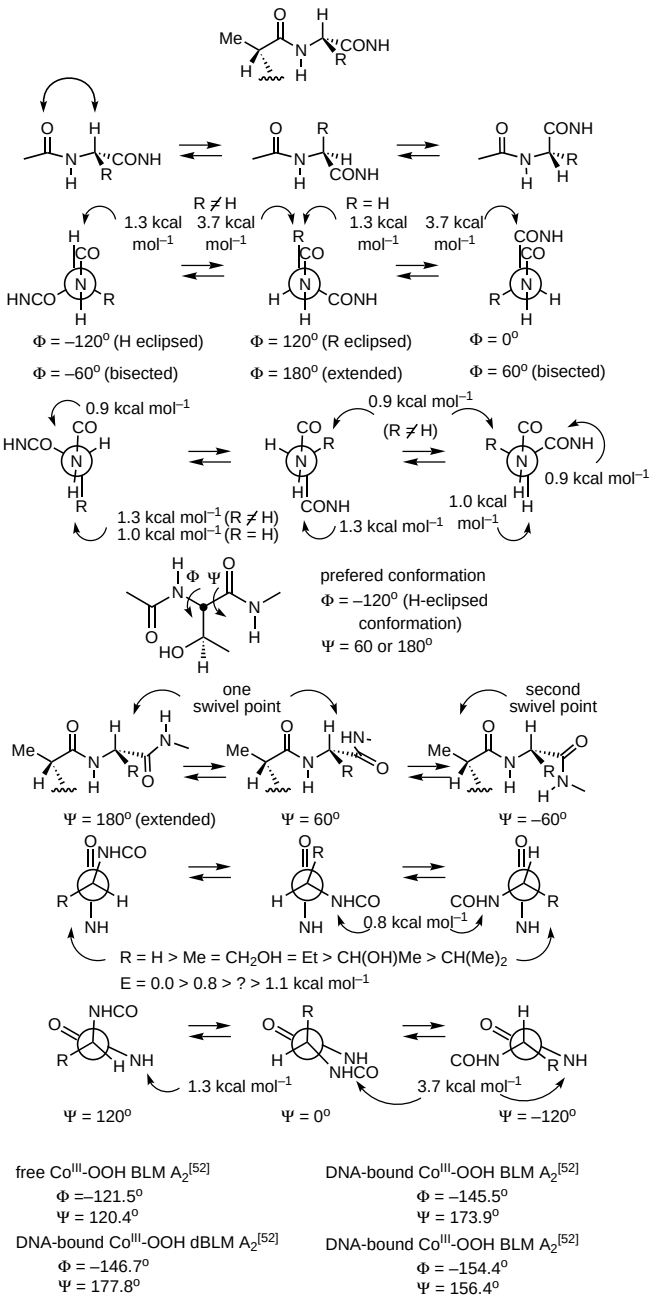


Figure 22. Conformational effects of the threonine subunit.

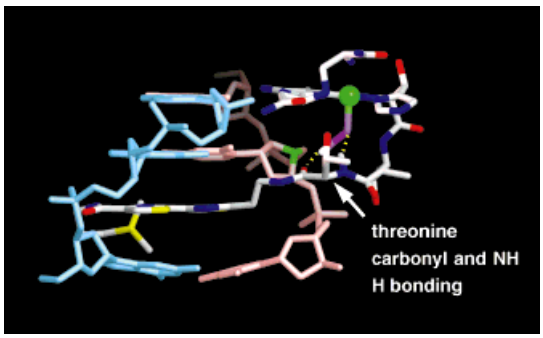
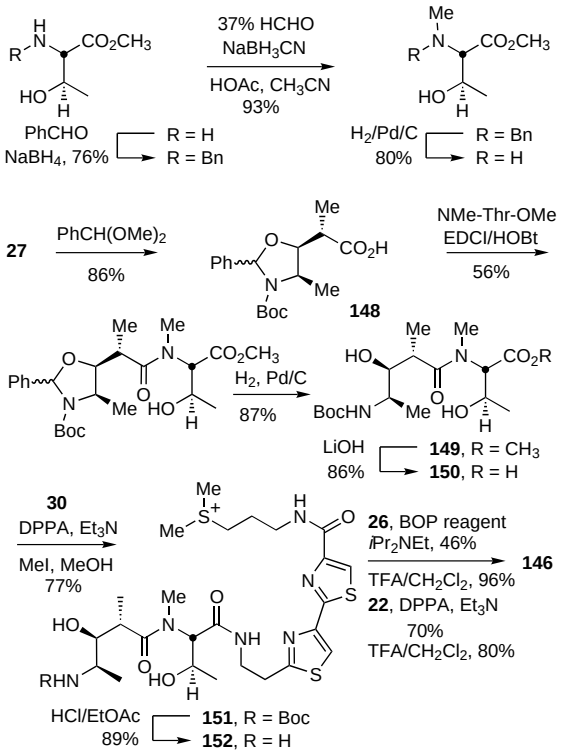


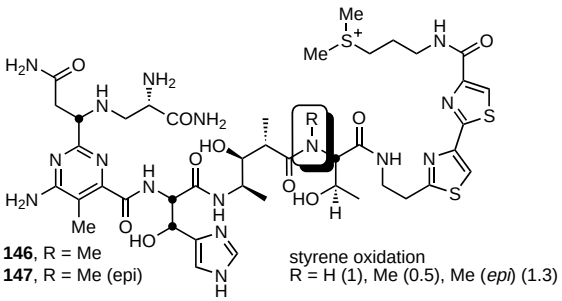
Figure 23. Model derived from ^1H NMR spectroscopy highlighting the threonine NH and carbonyl H bonds to the metal-bound hydroperoxide of Co^{III}-OOH bleomycin A_2.[52]



Scheme 26. Synthesis of bleomycin A_2 analogues with *N*-methyl substituted threonine amide groups.

coupling with **27** failed to provide a useful approach to **149**, and **27** preferentially closed to the corresponding five-membered *N*-Boc lactam. Consequently, the coupling was accomplished with the cyclic N,O-acetal **148** prepared by treatment of **27** with benzaldehyde dimethyl acetal. Coupling of **148** with NMe-Thr-OMe after cleavage of the N,O-acetal provided the key dipeptide **149**. Methyl ester hydrolysis followed by coupling with **30** provided **151** and small amounts of the separable diastereomer. The analogue **146** was assembled by sequential couplings to introduce the *erythro*-β-hydroxy-L-histidine and pyrimidoblamic acid subunits after formation of the sulfonium salt. Conducting the same sequence on the minor diastereomer of **152** provided **147** in comparable conversions.

DNA cleavage studies revealed that N-methylation of the threonine amide group dramatically reduced the DNA cleavage efficiency ($10-15\times$), weakened and nearly abolished the inherent DNA cleavage selectivity, but had little effect on the inherent oxidation capabilities of the activated Fe^{III} complexes (Figure 24). These results along with those in the preceding section not only suggest that the unsubstituted amide group is essential, but that the conformational or stabilization effects of the H bond of the threonine NH group account for the observations rather than a role related to oxidation catalysis. This is consistent with a previously unrecognized prominent role for the threonine NH group and suggests a potential importance for the H bond to the Fe^{III}-OOH complex of bleomycin or a subsequent Fe-oxo intermediate implicated the structural models of Stubbe et al.[52]



Agent	Efficiency	ds:ss	Selectivity
bleomycin A_2 (**1**)	2 – 5	1 : 6	5'-GC, 5'-GT > 5'-GA
deglycobleomycin A_2 (**41**)	1	1 : 12	5'-GC, 5'-GT > 5'-GA
146, R = Me	0.08	1 : 58	weak 5'-GC, 5'-GT > 5'-GA
147, R = Me (*epi*)	0.08	1 : 53	none
Fe^{III}	0.04	1 : 98	none

Figure 24. DNA cleavage properties of bleomycin A_2 with modified L-Thr amide groups.

3.4. The C-Terminus DNA Binding Domain

The importance of the C-terminus cation and its electrostatic contribution to DNA binding is well recognized from studies of *S*-desmethyldeglycobleomycin A_2[24d, 72a] (**153**) and bleomycinic acid,[96] both of which lack the terminal sulfonium salt. To assess the relative impact of the substitution of a sulfoxide for the sulfonium salt deglycobleomycin A_1 (**154**), the aglycone of a naturally occurring bleomycin, was prepared and evaluated. Despite the importance of this comparison, especially in light of the reported altered DNA cleavage selectivity of bleomycinic acid, neither synthetic nor naturally derived deglycobleomycin A_1 had been described previously.

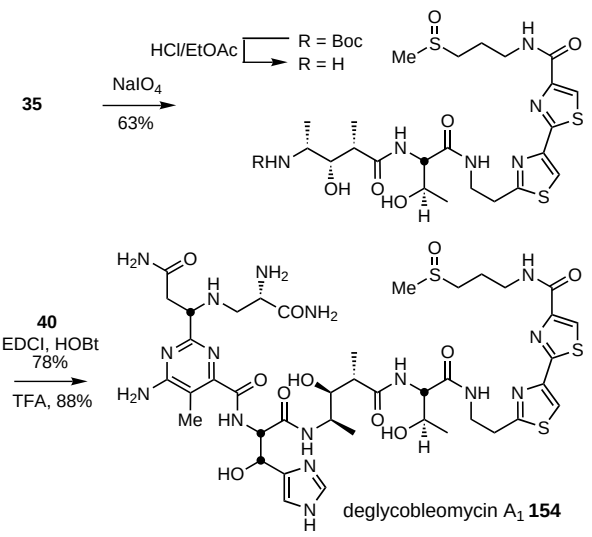
3.4.1. Deglycobleomycin A_1[18e]

Deliberate oxidation of the tetrapeptide S precursor **35** proceeded cleanly to provide the sulfoxide (Scheme 27). Acid-catalyzed deprotection, coupling with **40**, and acid-catalyzed deprotection provided deglycobleomycin A_1 (**154**).

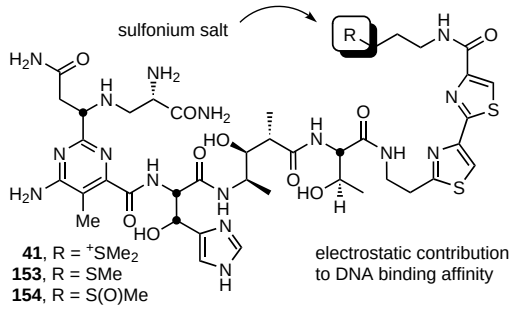
The DNA cleavage studies demonstrated an important, productive role for the terminal sulfoxide or sulfonium salt (Figure 25). Removal of the positive charge resulted in a tenfold decrease in DNA cleavage efficiency although the sequence selectivity was not altered. Introduction of the sulfoxide group reestablished some but not all of the cleavage efficiency. In the structural model of Stubbe et al. the cation threads through the base pairs into the major groove with the perpendicular intercalation of the bithiazole unit, and is in electrostatic contact with the negatively charged phosphate backbone.

4. Summary and Outlook

Our studies have focused on the elucidation of the functional roles of bleomycin subunits through the synthesis and



Scheme 27. Total synthesis of deglycobleomycin A_1 (**154**).



Agent	Efficiency	ds:ss	Selectivity
bleomycin A_2 (**1**)	2 – 5	1 : 6	5'-GC, 5'-GT > 5'-GA
deglycobleomycin A_2 (**41**)	1	1 : 12	5'-GC, 5'-GT > 5'-GA
153, R = SMe	0.1	nd	5'-GC, 5'-GT > 5'-GA
154, R = S(O)Me	0.3	1 : 28	5'-GC, 5'-GT > 5'-GA
Fe^{III}	0.04	1 : 98	none

Figure 25. DNA cleavage properties of bleomycin A_2 analogues with modified sulfonium groups.

evaluation of key structural analogues. These studies demonstrated the essential roles of the amide nitrogen atom of β-hydroxy-L-histidine and the imidazole N^{π} atom in metal chelation, the important role of tripeptide S and the C-terminus sulfonium cation in providing the majority of the binding affinity, and the unrecognized subtle substituent and conformational effects of the linker domain in preorganization and stabilization of a compact conformation implicated in DNA cleavage. Detailed assessments of the pyrimidoblamic acid substituents and side chains established little or no role for the C5 methyl and terminal α-carboxamide group, and an important subtle role for the C2-acetamide side chain. This latter role potentially involves H bonding of the carboxamide NH_2 group to the C2 carbonyl group of cytosine that is base paired with the guanine at the cleavage site, and H bonding of the carbonyl group to the threonine hydroxyl group. The former would contribute to the stability of the binding at a cleavage site while the latter reinforces adoption of a compact conformation productive for DNA cleavage. Most important was the demonstration of the C4 amine group on pyrimidine in H bonding, DNA recognition, and as the source of the DNA cleavage selectivity. Based on these studies, new or rigid analogues of bleomycin A_2 that can potentially effect the preorganization of the productive bound conformation, affect or alter the sequence selectivity of the DNA cleavage, or affect the double strand versus single strand DNA cleavage events are being pursued in our laboratories. The integral details of the role of the disaccharide subunit remain to be established and a systematic examination of the intercalating bithiazole group have not yet been conducted. Specifically labeled materials (for example, N^{15}) are being pursued in efforts to unambiguously define the metal chelation. Finally, details of the second cleavage reaction of double-strand DNA, as well as knowledge of the subtle details of the catalytic activation and degradation of bleomycin, would assist in the design of more effective agents.

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