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Synthesis of Key Analogs of Bleomycin A_2 that Permit a Systematic Evaluation of the Linker Region: Identification of an Exceptionally Prominent Role for the L-Threonine Substituent

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Abstract—The synthesis of a full series of analogs **2b–k** of deglycobleomycin A_2 (**2a**) containing systematic variations in the linker domain of bleomycin A_2 (**1**) is described. The agents **2b–k**, which are not accessible through structural modification of **1** or **2a**, constitute key substructure analogs incorporating deep-seated structural modifications in the linker domain capable of delineating the contribution of the individual backbone substituents to the DNA cleavage efficiency, characteristic DNA cleavage selectivity, and double strand to single strand DNA cleavage ratio. The comparative examination of the DNA cleavage properties of the Fe(II) and Fe(III) complexes of **2a–k** upon activation by O_2–thiol or H_2O_2, respectively, revealed several characteristic features and trends. First, none of the substituents affect the characteristic 5'-GC, 5'-GT > 5'-GA DNA cleavage selectivity of bleomycin A_2. In contrast, an exceptionally prominent role for the L-threonine substituent and an important role for the C4-methyl substituent of the (2*S*,3*S*,4*R*)-4-amino-3-hydroxy-2-methylpentanoic acid subunit were observed on the DNA cleavage efficiency of the agents. Similarly, the L-threonine substituent was found to substantially increase the ratio of double strand to single strand DNA cleavage events (2–3 times). In a w794 DNA cleavage assay, shortening the linker region by two carbons resulted in an exceptionally large reduction in DNA cleavage efficiency (125 times) and provided an agent that was only 1.3 times more effective than Fe(III) indicating that this deep-seated modification essentially destroys the DNA cleavage capabilities of the agent. The L-threonine substituent contributes in an exceptional manner, and its removal resulted in a 25 times reduction in DNA cleavage efficiency. A substantial contribution was observed for the C4-methyl group on the 4-aminobutanoic acid subunit and its removal resulted in a 7 times reduction in DNA cleavage efficiency. Little effect for the C3-hydroxyl and C2-methyl substituents on the 4- aminobutanoic acid subunit was observed (0–2.5 times) and even their inversion of stereochemistry had little impact on DNA cleavage efficiency or selectivity. Notably, the magnitude of the previously unappreciated L-threonine substituent contribution to the DNA cleavage efficiency and on the ratio of double to single strand DNA cleavage events is the largest effect observed to date including the well recognized disaccharide potentiation (6 times) of the DNA cleavage properties. Consequently, the past role and relative importance of the L-threonine subunit and substituent has been underestimated. Moreover, the cumulative effect of the two important linker chain substituents clearly illustrate that the functional role of this domain is much more important than its simply serving as a linker.

Introduction

Bleomycin A_2 (**1**) is the major naturally occurring constituent of the clinical antitumor agent blenoxane thought to derive its therapeutic effects from the catalytic oxidative cleavage of DNA[1–15] or RNA[16] by a process that is metal ion and oxygen dependent (Fig. 1). Since its disclosure, extensive studies have been conducted to identify the functional roles of the structural subunits of **1** that contribute to its DNA binding affinity, selectivity, and oxidative DNA cleavage efficiency and selectivity.[17–37]

Despite these studies, the full role of the linker region and its substituents joining the bithiazole C-terminus responsible for DNA binding affinity and the N-terminus pyrimidoblamic acid segment responsible for metal chelation and oxygen activation is poorly understood. Studies with a select set of bleomycin A_2 analogs disclosed in the studies of Umezawa, Ohno and coworkers have indicated the potential importance of the presence and absolute stereochemistry of the C4 methyl group in the (2*S*,3*S*,4*R*)-4-amino-3-hydroxy-2-methylpentanoic acid subunit[2,29] to DNA cleavage efficiency. In contrast, Hecht and coworkers have shown that substitution of $(gly)_n$, n = 0–4, for the L-threonine subunit causes no significant change in the characteristic 5'-GC, 5'-GT cleavage selectivity of bleomycin A_2 which has suggested that the linker region and especially the L-threonine subunit and its backbone substituents may not be functionally so important.[38]

In recent studies, we prepared a full range of agents constituting the C-terminus segment of bleomycin A_2 including dipeptide S, tripeptide S, tetrapeptide S, pentapeptide S and related analogs.[35] Analogous to prior studies,[39] *N*-BOC-tripeptide S was found to embody the

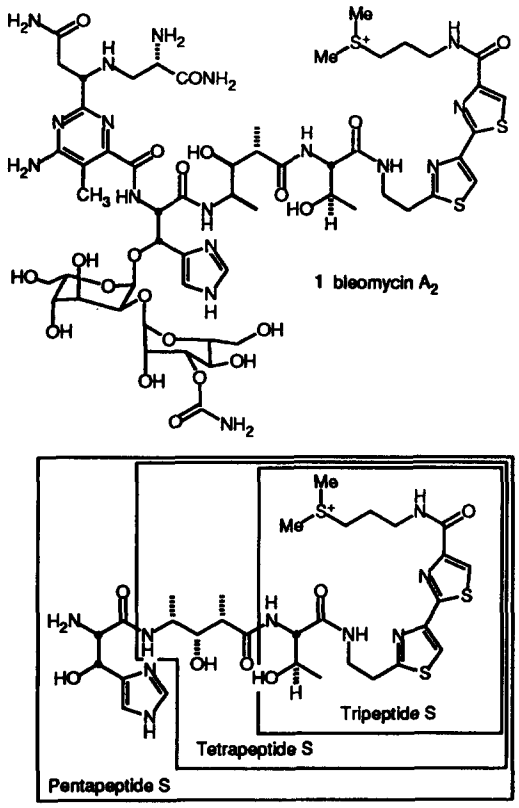


Figure 1.

majority of the DNA binding affinity of the natural agent.[35] In addition, the studies reflected a productive stabilizing binding interaction for the L-threonine subunit and substituent and indicated that the disaccharide, the entire β-hydroxy-L-histidine subunit of pentapeptide S, and the entire pentanoic acid subunit of tetrapeptide S and its substituents do not contribute in a significant manner to DNA binding affinity. The further comparisons of DNA binding constants and apparent binding site sizes suggested that it is the tripeptide S subunit that is fully bound to DNA, that the L-threonine hydroxyethyl substituent detectably affects and stabilizes the interaction with duplex DNA, but that the presence or absence of the tetrapeptide S or pentapeptide S substituents do not substantially alter the apparent binding constant or binding site size.[35] Moreover, the further comparisons of bleomycin A_2 (**1**) and deglycobleomycin A_2 (**2a**) with GABA,Gly-deglycobleomycin A_2 (**2h**) in which all the linker substituents had been removed revealed that they do not affect the binding affinity or characteristic DNA cleavage selectivity (5'-GC, 5'-GT), but that the DNA cleavage efficiency and the ratio of double to single strand DNA cleavage ratios were substantially reduced.[36] Such observations are consistent with the proposal that the linking chain substituents facilitate adoption of a productive bound conformation that leads to DNA cleavage. Moreover, the comparable binding constants of **1**, **2a** and **2h** and their comparable binding site sizes were consistent with models[40] of a bent bound conformation possessing a reverse turn at the tripeptide S–tetrapeptide S juncture with placement of tripeptide S and its substituent fully bound to DNA from the minor groove side. In such a model, the N-terminus extends out of DNA with only the terminal metal chelation subunit making additional stabilizing binding contacts with DNA. The intervening linker region and its substituents which join the C- and N-terminus do not make stabilizing contacts with DNA but may facilitate adoption of a bent bound conformation productive for DNA cleavage. Recent models based on ^{1}H NMR studies of **1** complexed with oligodeoxynucleotides implicate such bent bound conformations.[41,42]

Herein, we detail in the incorporation of the full range of tri- and tetrapeptide S analogs **3**–**4** (Fig. 2) into deglycobleomycin A_2 analogs and the direct, systematic comparative examination of their properties. Notably, the resulting agents **2b–k** are complementary to the series of agents disclosed by Umezawa and Ohno[2] and the $(gly)_n$, n = 0–4, linked agents detailed in the efforts of Hecht and coworkers.[38]

Pertinent to the studies detailed herein, the disaccharide of bleomycin A_2 does not contribute to the DNA binding affinity or the characteristic 5'-GC, 5'-GT DNA cleavage selectivity although it enhances the DNA cleavage efficiency 2–5 times. Moreover, recent studies have shown that demannosylbleomycin A_2 and bleomycin A_2 are virtually indistinguishable[34] indicating that the removal of the terminal 2-*O*-(3-*O*-carbamoyl)-α-D-mannose inclusive of the putative carbamoyl sixth metal ligand has no effect on the DNA binding and cleavage properties of the natural agent. This has suggested that the major effect of the disaccharide on the DNA cleavage properties is derived from the pocket it forms in conjunction with the C2 acetamido side chain for O_2 binding and activation and the protection it affords the reactive iron–oxo or perferryl intermediate of activated bleomycin. Consequently, the effects of the linker substituent modifications have been examined with a full range of deglycobleomycin A_2 analogs in lieu of the less synthetically accessible bleomycin A_2 analogs with expectations that the comparisons will be relevant and accurate. The comparison of a subset of the results of **2a–k** with a small set of bleomycin A_2 analogs supports this expectation.

Synthesis of the Modified Deglycobleomycin A_2 Agents 2b–k

Following protocols introduced in the total synthesis of bleomycin A_2 (**1**)[37] and deglycobleomycin A_2 (**2a**),[36] acid-catalyzed deprotection (3M HCl–EtOAc, 25 °C, 1 h, 95–100%) of the *N*-BOC derivatives **3**[35] cleanly provided the hydrochloride salts of **4** (Scheme 1). Direct coupling of **4** (1.5–2.0 equiv.) with **6**[34,36] (1.0–1.1 equiv. DCC, 1.0–1.1 equiv. HOAt, 1.5–2.0 equiv $NaHCO_3$, DMF, 25 °C, 70 h, 50–73%) provided **7**. Notably, this coupling was conducted without deliberate protection of the free hydroxyls or the pyrimidoblamic acid C6 or N^{β}-amines and with the terminal sulfonium salt installed in the substrate. Final acid-

catalyzed deprotection of **7** (20% CF_3CO_2H–CH_2Cl_2, 0 °C, 4 h, 64–95%) provided the 10 agents **2a-j**.

Following a similar protocol, **3k** was converted to **2k** as outlined in Scheme 2.

DNA Cleavage Properties of 2a–2k

The initial study of the relative efficiency of DNA cleavage was conducted with the Fe(II) complexes of

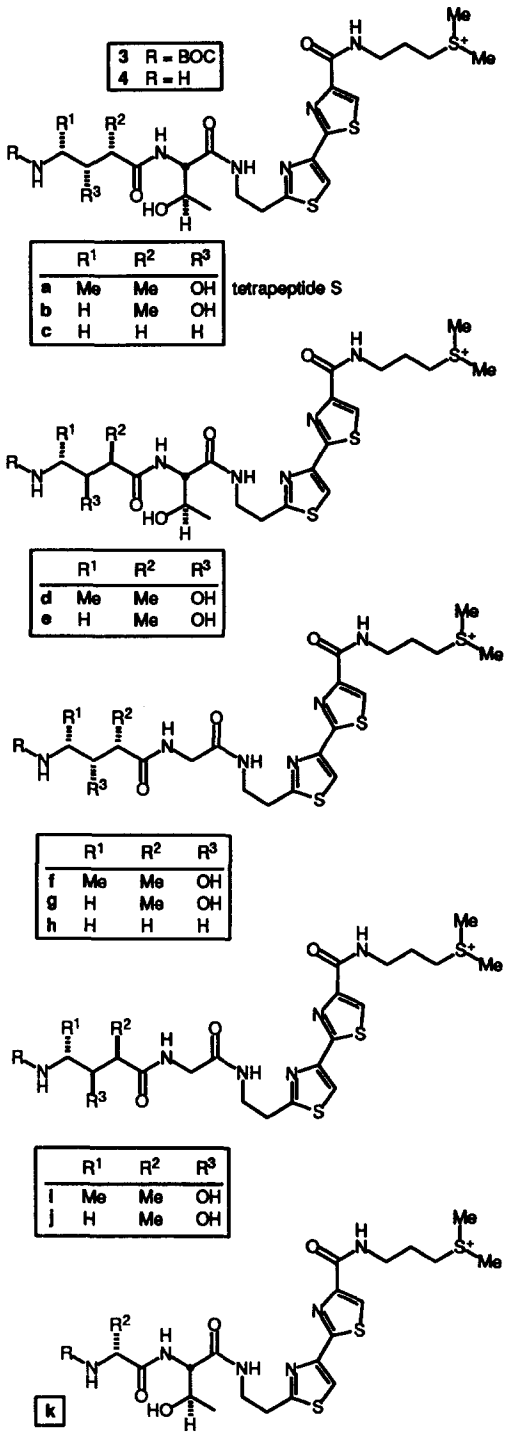
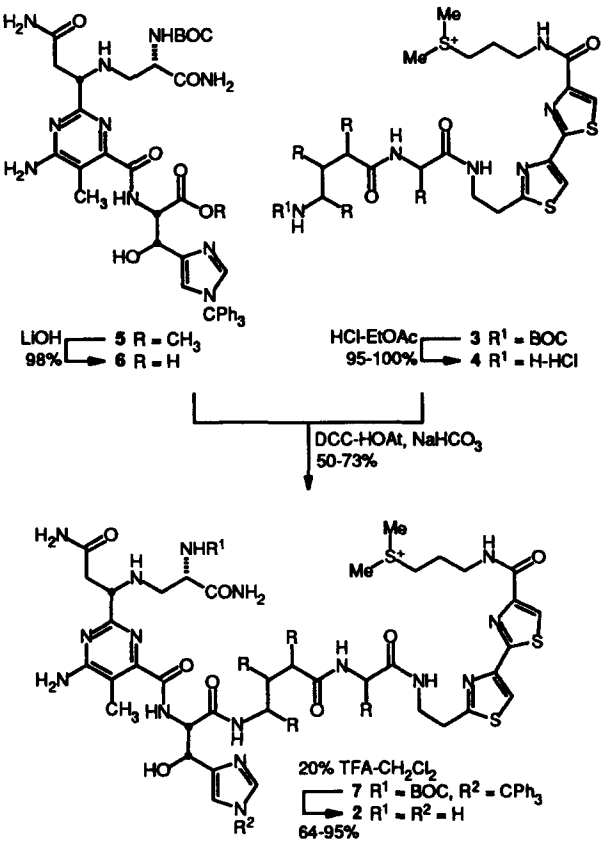
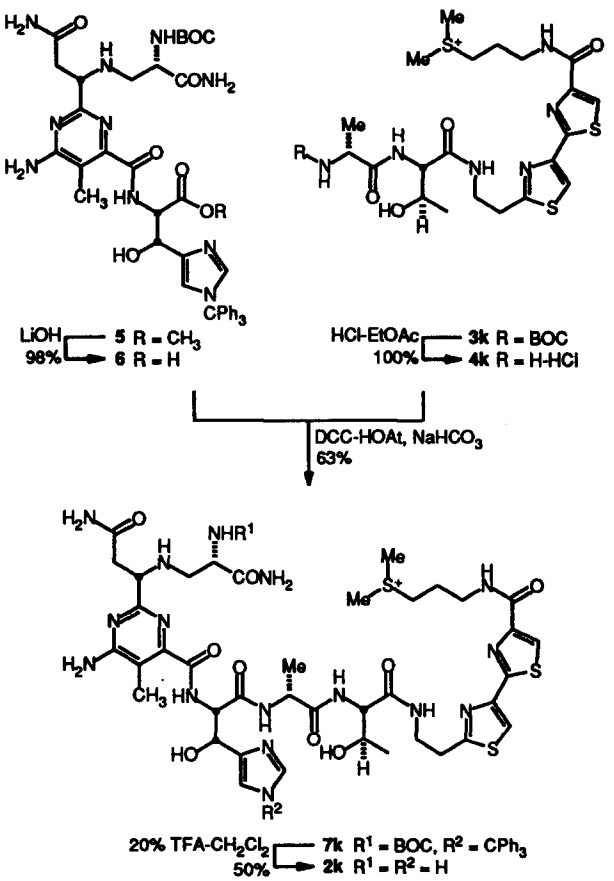


Figure 2.



Scheme 1.



Scheme 2.

2a–k in the presence of O_2 and 2-mercaptoethanol as an appropriate reducing agent. The assessment was made through the determination of the relative concentrations of the Fe(II) complex required to produce comparable single and double strand cleavage of supercoiled ΦX174 DNA (Form I) to produce relaxed (Form II) and linear (From III) DNA, respectively (Fig. 3). Like Fe(II)–bleomycin A_2 (**1**), the Fe(II) complexes of **2a–k** produced both single and double strand cleavage of supercoiled DNA and the results are summarized in Table 1. The lack of DNA cleavage by the agents alone in the absence of Fe(II) in control studies is consistent with expectations that the agents are cleaving DNA by a metal-dependent oxidative process in a manner analogous to **1**.

Two immediate and important trends are evident from the comparisons of **2a–k**. First, **2k** which lacks two carbons of the full carbon backbone of the linker region is substantially less efficient in the DNA cleavage reaction than the remainder of the agents. It was 14 times less effective than deglycobleomycin A_2 (**2a**) and substantially less effective than **2b–e** which contain the L-threonine substituent. Moreover, it was only 1.75 times more efficient than Fe(II) itself indicating that this deep-seated modification in the linker region severely compromises the DNA cleavage efficiency. Second, the L-threonine substituent contributes substantially to the DNA cleavage efficiency of the natural product. The comparisons of analogous pairs **2a/2f**, **2b/2g**, **2c/2h**, **2d/2i**, and **2e/2j** which contain or lack the L-threonine side chain indicate that its presence increases the DNA cleavage efficiency 3–5 times. No other single substituent in the linker region exhibits this same magnitude of effect. Removal of all three of the 4-aminobutanoic acid substituents (**2a** versus **2c** and **2f** versus **2h**) resulted only in a 2–3 times reduction in DNA cleavage efficiency. Of these, the majority of the effect resides in the presence of the C4 methyl group. Its removal accounts for a 1.4–2 times reduction in DNA cleavage efficiency in the direct assessment of its importance made through the comparison of **2a** versus **2b** and **2f** versus **2g**. In an interesting additional test of the relative contribution of the C4 methyl substituent, the presence of the (4*R*)-methyl substituent within the series **2d** versus **2e** and **2i** versus **2j** where the stereo-

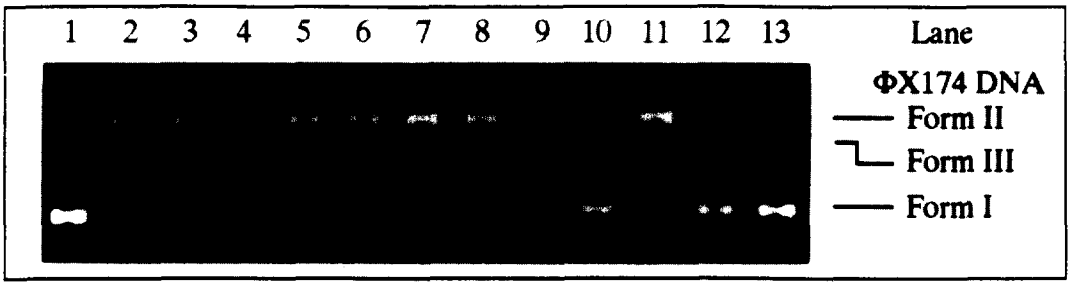


Figure 3. Cleavage of supercoiled ΦX174 DNA by Fe(II)–deglycobleomycin A_2 (**2a**), Fe(II)–**2b**, Fe(II)–**2c** and Fe(II)–**2f**. Solutions contained 0.25 μg supercoiled ΦX174 DNA (1.4×10^{-8} M) in 50 mM Tris–HCl (pH 8.0) containing 10 mM 2-mercaptoethanol. The DNA cleavage reactions were run for 60 min at 25 °C and electrophoresis was conducted at 50 V (3 h) on a 1.0% agarose gel containing 0.1 $\mu g \cdot mL^{-1}$ ethidium bromide. Lane 1, control ΦX174 DNA, 95% Form I (supercoiled), 5% Form II (circular); lanes 2–4, 4.0, 2.0 and 1.0 μM Fe(II)–deglycobleomycin A_2 (**2a**); lanes 5–7, 16.0, 8.0 and 4.0 μM Fe(II)–**2b**; lanes 8–10, 16.0, 8.0 and 4.0 μM Fe(II)–**2c**; lanes 11–13, 16.0, 8.0 and 4.0 μM Fe(II)–**2f**. Form I = supercoiled DNA, Form II = relaxed DNA (single-strand cleavage), Form III = linear DNA (double-strand cleavage). Direct fluorescence quantitation of the DNA in the presence of ethidium bromide was conducted using a Millpore BioImage 60S RFLP system visualized on a UV (312 nm) transilluminator taking into account the relative fluorescence intensities of Forms I–III ΦX174 DNA (Form II and III fluorescence intensities are 0.7 times that of Form I).

Table 1. DNA cleavage properties of **2a–2k**: supercoiled ΦX174 DNA, Fe(II) complexes, O_2 activation, mercaptoethanol initiation

agent	relative efficiency of DNA cleavage[a]	ratio of double to single strand DNA cleavage[b]
1, bleomycin A_2	2.0–5.0	1:6
2a, deglycobleomycin A_2	1.0	1:12
2b	0.50	1:15
2c	0.30	1:13
2d	0.60	1:13
2e	0.20	1:15
2f	0.20	1:29
2g	0.15	1:30
2h	0.10	1:33
2i	0.13	1:28
2j	0.07	1:27
2k	0.07	1:25
Fe(II)	0.04	1:98

[a]Relative efficiency of supercoiled ΦX174 DNA cleavage.
[b]Ratio of double to single strand cleavage of supercoiled ΦX174 DNA cleavage calculated as $F_{II} = n_2 \exp(-n_2)$, $F_I = \exp[-(n_1 + n_2)]$.

chemistry of the natural (3*S*)-hydroxy and (2*S*)-methyl substituents has been inverted to the unnatural (3*R*,2*R*) configurations led to the identical 2–3 times potentiation of the DNA cleavage efficiency. The further removal of the 3-hydroxy and 2-methyl substituents on the 4-aminobutanoic acid segment led to only a 1.5–1.6 times reduction in cleavage efficiency (**2b** versus **2c** and **2g** versus **2h**) indicating their small contribution. Moreover, the inversion of the stereochemistry of the 3-hydroxy and 2-methyl substituents similarly resulted in only a surprisingly small 1.5–1.7 times reduction in DNA cleavage efficiency (**2a** versus **2d**, **2f** versus **2i**) and provided agents that were essentially equivalent to those lacking the substituents altogether (**2c** versus **2e**, **2f** versus **2i**).

Although both single and double strand DNA lesions result from the oxidative cleavage of DNA by bleomycin A_2, the latter has been considered the more significant biological event.[3] Consequently, the ratio and relative extent of double to single strand DNA cleavage was established for **2a–k** through a kinetic study of the production of circular and linear DNA employing the Fe(II) complexes and supercoiled ΦX174 DNA.[33,34,36] Typical results are illustrated in Figure 4 and the results of the full study are summarized in Table 1. The reactions show initial fast kinetics in the first 1–10 min and a subsequent decreasing rate of DNA cleavage which may reflect conversion to a less active or inactive agent or metal complex reactivation kinetics. We assumed a Poisson distribution for the formation of single and double strand breaks to calculate the average number of double and single strand cuts per DNA molecule using the Freifelder–Trumbo equation.[43] The data for the first few minutes (1–2 min) could be fitted to a linear equation, and the ratios of double to single strand cuts observed with the Fe(II) complexes of **2a–k** are summarized in Table 1. A theoretical ratio of approximately 1:100 is required in order for the linear DNA to be the result of the random accumulation of single strand breaks within the 5386 base pair size of ΦX174 DNA assuming that sequential cleavage on the complementary strands within 15 base pairs is required for formation of linear DNA. Experimentally, Fe(II) alone produced a ratio of 1:98 double strand to single strand breaks under the conditions of the assay. A single clear trend or effect was observed in the comparisons of **2a–k** and is due to the presence of the L-threonine substituent. The agents **2a–e** all exhibited comparable double to single strand DNA cleavage ratios (1:12 to 1:15) as did the agents **2f–j** (1:28 to 1:33) and those bearing the L-threonine substituent (**2a–e**) exhibited a clear 2–3 times higher ratio for the double strand DNA cleavage.

The selectivity of DNA cleavage and a second assessment of the relative DNA cleavage efficiency were examined within duplex w794 DNA and its complement w836 DNA[44] by monitoring strand cleavage of singly ^{32}P 5'-end-labeled double-stranded DNA[45] after exposure to the Fe(III) complexes of the agents following

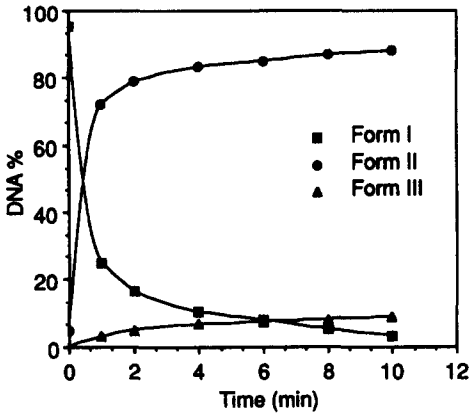


Figure 4. The kinetics of supercoiled ϕX174 DNA cleavage by Fe(II)–**2f** (8.0 μM). Each of the freshly prepared Fe(II)–**2f** complex solutions was treated with a DNA solution containing 0.25 μg of supercoiled ΦX174 DNA (1.4×10^{-8} M) in 50 mM Tris–HCl buffer solution (pH 8.0) containing 10 mM 2-mercaptoethanol. The thoroughly mixed solutions were incubated at 25 °C for 60, 40, 30, 20, 15, 10, 8, 6, 4, 2 and 1 min, respectively, quenched, and the subsequent electrophoresis was conducted at 50 V (3 h) on a 1.0% agarose gel containing 0.1 $\mu g \cdot mL^{-1}$ ethidium bromide. The percentage of Form I, II and III DNA present at each time point was based on the direct fluorescence quantitation of the DNA in the presence of ethidium bromide using a Millipore BioImage 60S RFLP system visualized on a UV (312 nm) transilluminator taking into account the relative fluorescence intensities of Form I–III ΦX174 DNA. Form I = supercoiled DNA, Form II = relaxed DNA (single-strand cleavage), Form III = linear DNA (double-strand cleavage).

activation with H_2O_2[46] in 10 mM phosphate buffer (pH 7.0). Incubation of the labeled DNA with the Fe(III) complexes of **2a–k** in the presence of H_2O_2 led to DNA cleavage. Removal of the agent by EtOH precipitation of the DNA, resuspension of the treated DNA in aqueous buffer, and high resolution polyacrylamide gel electrophoresis (PAGE) of the resultant DNA under denaturing conditions adjacent to Sanger sequencing standards permitted the identification of the sites of DNA cleavage. An extensive range of conditions for the DNA cleavage reactions were examined including variations in reaction temperature, time, buffer, and pH. Among the conditions examined, **2a–k** were found to cleave DNA most effectively when the reactions were conducted at 37 °C for 30 min in 10 mM phosphate–10 mM KCl buffer at pH 7.0.[34,36]

Each agent exhibited DNA cleavage at the same characteristic sites as bleomycin A_2 (**1**). A statistical treatment of the observed and available dinucleotide DNA cleavage sites detected for **2a–k** is summarized in Table 2 and the observed sites of DNA cleavage within w794 and w836 DNA are illustrated in Figure 5. The typical w794 PAGE that is shown in Figure 6 for **2a–g** illustrates clearly that no distinguishing differences in the DNA cleavage sequence selectivity were observed between the agents.

Quantitation of the consumption of the labeled DNA representing an accurate measure of the extent of DNA cleavage provided an additional assessment of the relative efficiency of DNA cleavage under a second set of conditions. The results of the quantitative assessment

for w794 DNA are summarized in Table 3 and take into account the different concentrations of complex employed in the DNA cleavage reaction. The same order and the same relative quantitative trends in the DNA cleavage efficiency were observed with the w794 DNA protocol that were observed with supercoiled ΦX174 DNA although this assay is more sensitive and the absolute magnitudes of the differences were magnified. This reflects the two different procedures employed for agent activation and initiation of DNA cleavage and the conditions of the assay.

Table 2. Summary of DNA cleavage sites for the Fe(III) complexes of **2a–k** within w794 and w836 DNA

cleavage sites	no. of cleavage sites	total no. of dinucleotide sites	%
5'-GC	29	29	100
5'-GT	5	5	100
5'-GA	11	14	79
5'-GG	0	28	0
5'-AT	7	18	39
5'-AC	2	7	28
5'-AA	3	24	13
5'-AG	0	22	0
5'-TT	1	13	8
5'-TA	1	15	7
5'-TC	0	19	0
5'-TG	0	10	0
5'-CT	1	20	5
5'-CC	0	38	0
5'-CA	0	18	0
5'-CG	0	17	0

First, **2k** which lacks two carbons of the full carbon backbone of the linker region proved to be 125 times less effective than deglycobleomycin A_2 (**2a**) and only 1.3 times more effective than Fe(III) itself clearly indicating that this deep-seated modification shortening the linking chain essentially destroys the DNA cleavage capabilities of the agent. Similarly, the L-threonine substituent contributes in an exceptional manner to the DNA cleavage efficiency of the natural agents. The comparison of **2a** versus **2f** revealed a 25 times reduction in DNA cleavage efficiency with the removal of this single substituent. This constitutes the largest effect observed with a single substituent in the linker region and the largest effect observed to date with any bleomycin substituent including the disaccharide. The analogous pairs **2b/2g**, **2c/2h**, **2d/2i** and **2e/2j** show a similar trend (4–24 times reduction) although the magnitude of the effect is diminished as the agents become increasingly less effective at cleaving DNA. A third, but more modest effect observed was the potentiation provided by the C4 methyl substituent on the 4-aminopentanoic and subunit. The comparison of **2a**, **2b** revealed a 7 times reduction in DNA cleavage efficiency. Removal of all three of the 4-aminopentanoic acid substituents (**2a** versus **2c**) resulted in the same 7 times reduction in DNA cleavage efficiency indicating that the C3-hydroxyl and C2-methyl substituent contributions in the absence of the C4-methyl group are negligible. Consistent with this observation, the inversion of the stereochemistry of both the C3-hydroxyl and C2-methyl (**2a** versus **2d** and **2c** versus **2e**) only resulted in a surprisingly small 2.5 times reduction in DNA cleavage efficiency and the further removal of the C4-methyl group from **2d** (**2d** versus **2e**) resulted in the analogous 6–7 times reduction in DNA cleavage efficiency observed in the **2a/2b** comparison. Comparable effects were observed in the **2f–j** series lacking the L-threonine substituent but the magnitude of the effects was diminished due to the intrinsically less efficient DNA cleavage of the series.

Discussion

Three important features within the linker domain of bleomycin A_2 were detected in the comparisons of **2a–k**. First, shortening the linker backbone by two carbons substantially reduced and virtually destroyed the DNA cleavage capabilities of the agents indicating that such a deep-seated modification is detrimental to their properties. Although limited to the comparison of **2k** with **2a**, the studies suggest that the length of the linker chain can be much more important to the productive DNA cleavage properties than previously recognized.[38] Moreover, the results clearly illustrate that it is not sufficient to just link the C- and N-terminus of bleomycin thereby delivering the reactive oxidizing agent to DNA but rather that the nature of that linkage may have a profound effect on the DNA cleavage capabilities of the resulting agent.

The second extraordinary and previously unappreciated feature within the linker domain is the contribution that the L-threonine substituent makes to the productive DNA cleavage properties. It was found to have a major impact on both the DNA cleavage efficiency and the ratio of double strand to single strand DNA cleavage

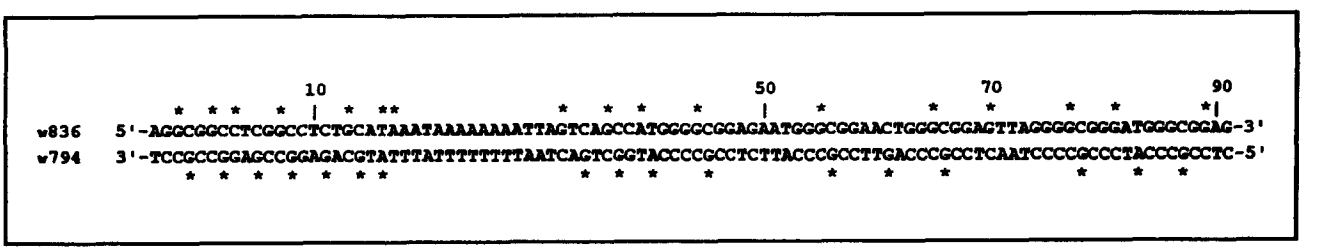


Figure 5. Summary of cleavage sites for **2a–i** within w794 and w836 DNA. The missing terminal regions of the DNA not shown from which some of the data for Table 2 was taken represent the non-overlapping regions not present on the complementary clones.

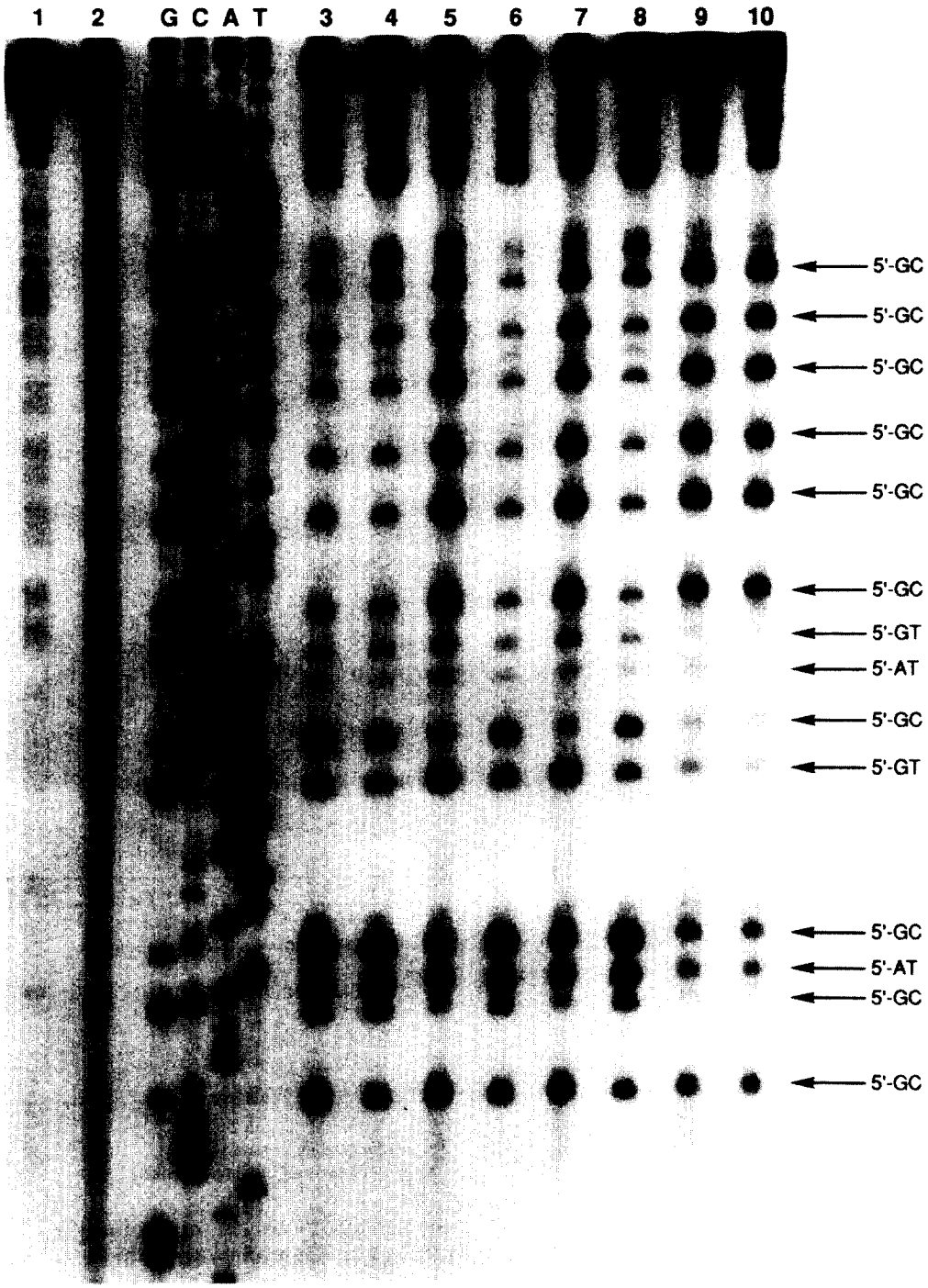


Figure 6. Cleavage of double-stranded DNA (SV40 DNA fragment, 156 base pairs, nucleotide no. 5239-150, clone w794) in 10 mM phosphate (K_2HPO_4–KH_2PO_4)–10mM KCl buffer (pH 7.0) containing H_2O_2 by Fe(III)–bleomycins. The DNA cleavage reactions were run for 30 min at 37 °C, and electrophoresis was conducted at 1100 V (5.5 h) on an 8% denaturing PAGE and visualized by autoradiography. Lane 1, control DNA; lane 2, 64 μM Fe(III) control; lane 3, 0.5 μM Fe(III)–bleomycin A_2 (1); lane 4, 2.0 μM Fe(III)–deglycobleomycin A_2 (**2a**); lane 5, 16 μM Fe(III)–**2b**; lane 6, 16 μM Fe(III)–**2c**; lane 7, 8 μM Fe(III)–**2d**; lane 8, 32 μM Fe(III)–**2e**; lane 9, 64 μM Fe(III)–**2f**; lane 10, 64 μM Fe(III)–**2g**.

events and the magnitude of the effects is exceptionally large. To put the magnitude of this effect into perspective, Figure 7 summarizes the w794 relative efficiency assay results for both the linker domain and other modifications that we have detailed in prior studies.[34,36] Not only is the effect of the L-threonine substituent the largest within the linker domain, but it is also the largest single effect that we have observed to date. This clearly indicates that the functional role of the linker domain exceeds that of simply joining the C- and N-terminus with an appropriately sized or spacing group and is consistent with our prior studies that detected a prominent and stabilizing binding interaction for both the L-threonine subunit as well as its hy-

Table 3. Summary of w794 DNA cleavage properties: Fe(III)-complexes, H_2O_2 activation

agent	relative efficiency of DNA cleavage[a]	DNA cleavage selectivity[b]
1, bleomycin A_2	5.8	5'-GC, 5'-GT > 5'-GA
2a, deglycobleomycin A_2	1.0	5'-GC, 5'-GT > 5'-GA
2b	0.14	5'-GC, 5'-GT > 5'-GA
2c	0.14	5'-GC, 5'-GT > 5'-GA
2d	0.38	5'-GC, 5'-GT > 5'-GA
2e	0.06	5'-GC, 5'-GT > 5'-GA
2f	0.04	5'-GC, 5'-GT > 5'-GA
2g	0.04	5'-GC, 5'-GT > 5'-GA
2h	0.015	5'-GC, 5'-GT > 5'-GA
2i	0.03	5'-GC, 5'-GT > 5'-GA
2j	0.025	nd
2k	0.008	nd
Fe(III)	0.006	none

[a]Relative efficiency of 5'-end-labeled w794 DNA cleavage. The different concentrations of complex employed in the DNA cleavage reaction were taken into account in the quantitation of the consumption of labeled DNA.
[b]5'-GC, 5'-GT > 5'-GA > 5'-AT > 5'-AC > 5'-AA, 5'-TT, 5'-TA, 5'-CT, see Table 2.

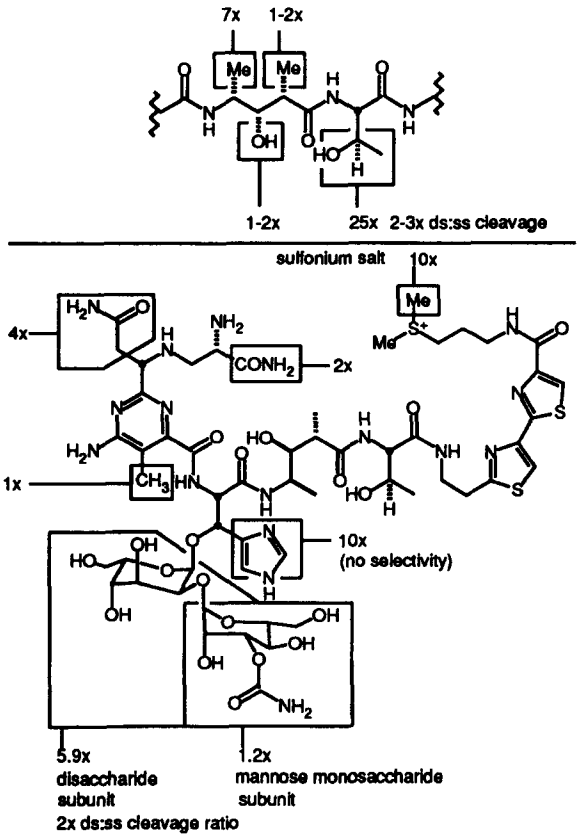


Figure 7.

droxyethyl substituent,[35] Table 4. The effects detected herein suggest that these observations were more important than previously recognized and support the suggestion that it is the tripeptide S subunit that is fully bound to DNA and that the L-threonine substituent significantly affects and stabilizes the productive interaction with duplex DNA. Moreover, the magnitude of the effect suggests that it additionally plays a pivotal role in promoting the adoption of a productive DNA bound conformation or conformations that leads to single or double strand DNA cleavage. Since the series of glycine linked agents **2f–i** would preferentially and nearly exclusively adopt an extended conformation, it seems unlikely that such an extended conformation may be the relevant bound conformation. Alternatively and consistent with past observations and suggestions,[40–42] the agents may adopt a bent bound conformation possessing a turn at the tripeptide S–tetrapeptide S juncture with placement of tripeptide S fully bound to DNA from the minor groove side. Moreover, this juncture may serve as a pivot point that allows the agent to access both strands of duplex DNA through two interrelated, bent bound conformations and facilitate double strand cleavage of duplex DNA.

Table 4. Representative DNA binding constants and binding site sizes for calf thymus DNA[a]

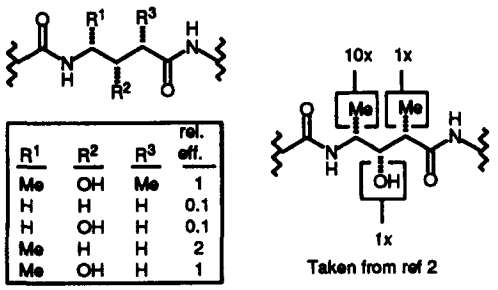
agent	K_B[b] (10^5 M^{-1})	bp size[c]
1, bleomycin A_2	1.0	3.8
2a, deglycobleomycin A_2	1.1	3.9
2h	2.4	3.3
N-BOC-dipeptide S	0.10	2.2
N -BOC-Gly-dipeptide S	0.18	2.7
N -BOC-tripeptide S	0.26	3.6
N -BOC-tetrapeptide S	0.21	3.7
N -BOC-pentapeptide S	0.23	4.2

[a]Taken from Ref. 35 and 36.
[b]Apparent binding constant (K_B).
[c]Apparent base-pair binding site size.

In addition, the Hecht series of $(gly)_n$, n = 0–4, analogs of deglycobleomycin A_2 in which the L-threonine substituent was replaced by $(gly)_n$ were examined initially as demethyldeglycobleomycin A_2 analogs lacking the sulfonium cation.[38] As a consequence, the inherent DNA cleavage efficiency of the deglycobleomycin A_2 agents (0.17 × bleomycin A_2) is further diminished approximately 10 times by removal of the cation and the subsequent magnitude of the direct effect of the further removal of the L-threonine substituent was naturally considerably diminished. Consequently, it is not surprising that the full magnitude of the impact of the L-threonine substituent on the DNA cleavage efficiency was not detected in this work.

The third significant effect detected was the enhancement of DNA cleavage efficiency provided by the

C4-methyl substituent of the (4*R*,3*S*,2*S*)-4-amino-3-hydroxy-2-methylpentanoic acid subunit. Interestingly, this substituent increased DNA cleavage efficiency although not to the same extent as the L-threonine hydroxyethyl substituent and, unlike the L-threonine substituent, it had no apparent effect on the double strand DNA cleavage capabilities of the agent. Nonetheless, this effect was substantial when compared to prior effects (Fig. 7) and clearly indicates that its role in the linker domain is functionally important. Moreover, the observations are remarkably consistent with those of Umezawa, Ohno and coworkers[2] with a complementary but more limited series of bleomycin A_2 analogs containing modifications in the (2*S*,3*S*,4*R*)-4-amino-3-hydroxy-2-methylpentanoic acid subunit where the C4-methyl group was found to increase pBR322 DNA cleavage efficiency 10 times and the C3-hydroxyl and C2-methyl substituents had no effect (Fig. 8).[2] Notably, these observations were made with bleomycin A_2 analogs. Our observation of a comparable magnitude of effect with the deglycobleomycin A_2 analogs **2b** and **d** (Fig. 7) suggests that observations made with the full series **2a–k** are similarly analogous and relevant.



R^1	R^2	R^3	rel. eff.
Me	OH	Me	1
H	H	H	0.1
H	OH	H	0.1
Me	H	H	2
Me	OH	H	1

Figure 8.

Clearly, the studies illustrate an exceptional and important functional role for the L-threonine subunit and substituent, an important role for the C4-methyl substituent within the functionalized 4-aminopentanoic acid subunit, and that the size or length of the linker chain is more important than previously recognized. Consequently, the functional role of the linker domain extends well beyond that of simply joining the C- and N-terminus of bleomycin and clearly contributes to the relative DNA cleavage efficiency and the ratio of double strand to single strand cleavage events. No doubt this may be attributed to orientation effects associated with the agent adopting a productive bent bound conformation that leads to DNA cleavage and, in the case of the pivotal L-threonine substituent, additional direct binding contacts with DNA. Such effects may be used to refine the emerging models of the interaction of bleomycin A_2 with duplex DNA.[40–42]

Experimental

Deglycobleomycin A_2 (**2a**)[19,36] and **2h**[36] were prepared and characterized as disclosed earlier.

General procedure for the deprotection of 3 to provide 4 illustrated with the preparation of 3-[2'-(2"'-((N-(4"-aminobutanoyl)-L-threonyl)amino)ethyl)-2,4'-bithiazole-4-carboxamido]propyldimethylsulfonium chloride hydrochloride (4c)

The solid **3c**[35] (4.0 mg, 0.0052 mmol) was treated with 3M HCl–EtOAc (0.6 mL) under Ar, and the heterogeneous reaction mixture was stirred at 23 °C (1 h). The solvent was evaporated under a N_2 stream and the residue dried *in vacuo*. The crude amine hydrochloride salt was triturated with $CHCl_3$ (3 × 1-mL) and dried *in vacuo* to provide the pure salt **4c** (3.2 mg, 3.2 mg theoretical, 100%) as a film: $[\alpha]_D^{23}$–4 (*c* 0.08, 0.1 N aqueous HCl); ^{1}H NMR (CD_3OD, 400 MHz) δ 8.19 (*s*, 1H), 8.17 (*s*, 1H), 4.26 (*d*, *J* = 4.5 Hz, 1H), 4.11 (*dq*, *J* = 4.5, 6.5 Hz, 1H), 3.69 (*m*, 2H), 3.61 (*t*, *J* = 6.5 Hz, 2H), 3.41 (*t*, *J* = 7.5 Hz, 2H), 3.29 (*m*, 2H), 2.96 (*s*, 6H), 2.95 (*m*, 2H), 2.46 (*t*, *J* = 7.0 Hz, 2H), 2.16 (*tt*, *J* = 6.5, 6.5 Hz, 2H), 1.93 (*tt*, *J* = 7.0, 7.0 Hz, 2H), 1.14 (*d*, *J* = 6.5 Hz, 3H); IR (neat) ν_{max} 3344, 3036, 2923, 1641, 1549, 1431, 1292, 1251, 1118 cm^{-1}; FABHRMS (NBA) m/z 543.1878 (M^+-Cl and HCl, $C_{22}H_{35}N_6O_4S_3$ requires 543.1882).

General procedure for the coupling of 4 with 6 to provide 7 illustrated with the preparation of 7c

A solution of **6**[36] (3.0 mg, 0.0037 mmol), HOAt (0.55 mg, 0.0040 mmol), $NaHCO_3$ (0.46 mg, 0.0055 mmol) and **4c** (3.4 mg, 0.0055 mmol) in DMF (46 μL) at 0 °C was treated with DCC (0.83 mg, 0.0040 mmol) under Ar and the reaction mixture was stirred for 1 h (0 °C) after which time it was warmed to 23 °C and stirred for 70 h. The DMF was evaporated in vacuo and the crude residue was dissolved in CH_3OH and the inorganic byproducts were removed by centrifugation and decanting (2 × 0.5-mL). The CH_3OH was evaporated under a N_2 stream and the residue was dried *in vacuo*. The residue was triturated with neutralized (basic alumina) $CHCl_3$ (3 × 0.5-mL) with centrifugation to remove DCC byproducts. The remaining residue was dried *in vacuo* and purified by reversed-phase flash chromatography (C-18, 0.5 × 2.0-cm, 0–70% CH_3OH–H_2O gradient elution) to give **7c** (3.6 mg, 5.0 mg theoretical, 73%) as a white film: R_f 0.38 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{25}$ +7 (*c* 0.1, CH_3OH); ^{1}H NMR (CD_3OD, 400 MHz) δ 8.16 (*s*, 1H), 8.05 (*s*, 1H), 7.49 (*s*, 1H), 7.30 (*m*, 9H), 7.07 (*m*, 6H), 6.95 (*s*, 1H), 5.08 (*d*, *J* = 6.5 Hz, 1H), 4.24 (*d*, *J* = 4.0 Hz, 1H), 4.19 (*dq*, *J* = 4.0, 6.5 Hz, 1H), 4.11 (*m*, 1H), 3.91 (*dd*, *J* = 5.5, 8.5 Hz, 1H), 3.78 (*m*, 1H), 3.61 (*m*, 2H), 3.58 (*m*, 2H), 3.44 (*m*, 2H), 3.37 (*t*, *J* = 7.0 Hz, 2H), 3.24 (*m*, 2H), 2.93 (*s*, 6H), 2.80 (*m*, 1H), 2.75 (*m*, 1H), 2.59 (*m*, 2H), 2.45 (*dd*, *J* = 15.0, 8.5 Hz, 1H), 2.33 (*m*, 1H), 2.28 (*s*, 3H), 2.14 (*tt*, *J* = 7.5, 7.5 Hz, 2H), 1.88 (*m*, 2H), 1.40 (*s*, 9H), 1.12 (*d*, *J* = 6.5 Hz, 3H); IR (neat) ν_{max} 3418, 2946, 1628, 1452, 1375, 1088, 824 cm^{-1}; FABHRMS (NBA) m/z 1345.5440 (M^+, $C_{64}H_{81}N_{16}O_{11}S_3$ requires 1345.5433).

General procedure for the deprotection of 7 to provide 2 illustrated with the preparation of 2c

A solution of **7c** (3.0 mg, 0.0022 mmol) in CH_2Cl_2 (170 μL) was cooled to 0 °C and treated with CF_3CO_2H (50 μL) under Ar. The yellow homogenous reaction mixture was stirred at 0 °C for 4 h and monitored by TLC (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH). The solvents were evaporated under a N_2 stream at 0 °C, and the yellow residue was dried *in vacuo*. The resulting white residue was purified by chromatography (SiO_2, 0.5 × 1.0 cm, 10:9:1 CH_3OH: 10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH) followed by chromatography over Amberlite XAD-2 (H_2O followed by CH_3OH) to remove buffer salts from the sample to afford **7c** (2.1 mg, 2.2 mg theoretical, 95%) as a white film: R_f 0.13 (SiO_2, 10:9:1 CH_3OH: 10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ −8 (*c* 0.05, 0.1 N aqueous HCl); ^1H NMR (D_2O, 400 Mhz) δ 8.54 (*s*, 1H), 8.02 (*s*, 1H), 7.89 (*s*, 1H), 7.11 (*s*, 1H), 5.03 (*d*, *J* = 8.0 Hz, 1H), 4.41 (*m*, 1H), 4.28 (*m*, 1H), 4.17 (*m*, 1H), 3.97 (*d*, *J* = 4.5 Hz, 1H), 3.89 (*m*, 1H), 3.50 (*m*, 1H), 3.39 (*m*, 4H), 3.26 (*m*, 1H), 3.18 (*t*, *J* = 7.5 Hz, 2H), 3.08 (*t*, *J* = 6.5 Hz, 2H), 2.98 (*t*, *J* = 7.0 Hz, 2H), 2.80 (*m*, 2H), 2.70 (*s*, 6H), 2.09 (*m*, 2H), 1.96 (*tt*, *J* = 7.0, 7.0 Hz, 2H), 1.77 (*s*, 3H), 1.54 (*t*, *J* = 7.0 Hz, 2H), 0.88 (*d*, *J* = 6.5 Hz, 3H); IR (neat) ν_{max} 3371, 2927, 1659, 1625, 1596, 1407, 1258, 1091 cm^{-1}; FABHRMS (NBA) m/z 1003.3810 (M^+, $C_{40}H_{59}N_{16}O_9S_3$ requires 1003.3813).

For **4b**: $[\alpha]_D^{23}$ +10 (*c* 0.05, 0.1 N aqueous HCl); ^1H NMR (CD_3OD, 400 MHz) δ 8.22 (*s*, 1H), 8.15 (*s*, 1H), 4.23 (*d*, *J* = 4.5 Hz, 1H), 4.12 (*m*, 1H), 3.83 (*m*, 1H), 3.71 (*m*, 1H), 3.64 (*m*, 1H), 3.60 (*t*, *J* = 6.5 Hz, 2H), 3.38 (*t*, *J* = 7.5 Hz, 2H), 3.29 (*m*, 2H), 3.11 (*m*, 1H), 2.94 (*s*, 6H), 2.90 (*m*, 1H), 2.58 (*m*, 1H), 2.15 (*tt*, *J* = 7.0, 7.0 Hz, 2H), 1.22 (*d*, *J* = 7.0 Hz, 3H), 1.15 (*d*, *J* = 6.5 Hz, 3H); IR (neat) ν_{max} 3343, 3061, 2943, 1643, 1549, 1438, 1291, 1255, 1114, 879 cm^{-1}; FABHRMS (NBA) m/z 573.1980 (M^+-Cl and HCl, $C_{23}H_{37}N_6O_5S_3$ requires 573.1987).

For **4d**: $[\alpha]_D^{23}$ −13 (*c* 0.15, 0.1 N aqueous HCl); ^1H NMR (CD_3OD, 400 MHz) δ 8.22 (*s*, 1H), 8.18 (*s*, 1H), 4.26 (*d*, *J* = 5.0 Hz, 1H), 4.09 (*dq*, *J* = 4.8, 6.4 Hz, 1H), 3.71 (*m*, 1H), 3.69 (*m*, 2H), 3.65 (*m*, 1H), 3.61 (*t*, *J* = 7.5 Hz, 2H), 3.40 (*t*, *J* = 7.5 Hz, 2H), 3.31 (*t*, *J* = 7.0 Hz, 2H), 2.95 (*s*, 6H), 2.73 (*m*, 1H), 2.16 (*tt*, *J* = 7.0, 7.0 Hz, 2H), 1.30 (*d*, *J* = 7.0 Hz, 3H), 1.18 (*d*, *J* = 7.0 Hz, 3H), 1.16 (*d*, *J* = 6.4 Hz, 3H); IR (neat) ν_{max} 3347, 2927, 1642, 1547, 1427, 1306, 1251, 1131, 1041 cm^{-1}; FABHRMS (NBA) m/z 587.2149 (M^+-Cl and HCl, $C_{29}H_{39}N_6O_5S_3$ requires 587.2144).

For **4e**: $[\alpha]_D^{23}$ −15 (*c* 0.16, 0.1 N aqueous HCl); ^1H NMR (CD_3OD, 400 MHz) δ 8.22 (*s*, 1H), 8.18 (*s*, 1H), 4.26 (*d*, *J* = 4.5 Hz, 1H), 4.09 (*dq*, *J* = 4.7, 6.4 Hz, 1H), 3.85 (*ddd*, *J* = 10.0, 7.0, 3.0 Hz, 1H), 3.71 (*m*, 2H), 3.61 (*t*, *J* = 6.5 Hz, 2H), 3.41 (*t*, *J* = 7.5 Hz, 2H), 3.29 (*m*, 2H), 3.03 (*dd*, *J* = 13.0, 3.0 Hz, 1H), 2.96 (*s*, 6H), 2.87 (*dd*, *J* = 13.0, 10.0 Hz, 1H), 2.58 (*m*, 1H), 2.16 (*tt*, *J* = 7.0, 7.0 Hz, 2H), 1.18 (*d*, *J* = 7.0 Hz, 3H), 1.15 (*d*, *J* = 6.4 Hz, 3H); IR (neat) ν_{max} 3347, 3027, 2947, 1642, 1547, 1432, 1296, 1256, 1126, 1041 cm^{-1}; FABHRMS (NBA) m/z 573.1999 (M^+-Cl and HCl, $C_{23}H_{37}N_6O_5S_3$ requires 573.1988).

For **4f**: $[\alpha]_D^{23}$ +11 (*c* 0.2, 0.1 N aqueous HCl); ^1H NMR (CD_3OD, 400 MHz) δ 8.22 (*s*, 1H), 8.16 (*s*, 1H), 3.94 (*d*, *J* = 16.5 Hz, 1H), 3.75 (*dd*, *J* = 10.0, 2.5 Hz, 1H), 3.70 (*d*, *J* = 16.5 Hz, 1H), 3.65 (*m*, 2H), 3.60 (*t*, *J* = 6.5 Hz, 2H), 3.49 (*m*, 1H), 3.39 (*t*, *J* = 7.5 Hz, 2H), 3.26 (*t*, *J* = 7.0 Hz, 2H), 2.94 (*s*, 6H), 2.44 (*dq*, *J* = 6.8, 10.0 Hz, 1H), 2.15 (*tt*, *J* = 7.0, 7.0 Hz, 2H), 1.28 (*d*, *J* = 6.7 Hz, 3H), 1.26 (*d*, *J* = 6.8 Hz, 3H); IR (neat) ν_{max} 3331, 3073, 2920, 1643, 1543, 1438, 1250, 1144, 1044, 873 cm^{-1}; FABHRMS (NBA) m/z 543.1890 (M^+-Cl and HCl, $C_{22}H_{35}N_6O_4S_3$ requires 543.1882).

For **4g**: $[\alpha]_D^{23}$ +13 (*c* 0.15, 0.1 N aqueous HCl); ^1H NMR (CD_3OD, 400 MHz) δ 8.22 (*s*, 1H), 8.17 (*s*, 1H), 3.95 (*d*, *J* = 16.5 Hz, 1H), 3.82 (*ddd*, *J* = 10.0, 7.5, 3.0 Hz, 1H), 3.73 (*d*, *J* = 16.5 Hz, 1H), 3.65 (*t*, *J* = 7.0 Hz, 2H), 3.60 (*t*, *J* = 6.5 Hz, 2H), 3.40 (*t*, *J* = 7.5 Hz, 2H), 3.27 (*t*, *J* = 7.0 Hz, 2H), 3.16 (*dd*, *J* = 13.0, 3.0 Hz, 1H), 2.95 (*s*, 6H), 2.87 (*dd*, *J* = 13.0, 10.0 Hz, 1H), 2.49 (*m*, 1H), 2.16 (*tt*, *J* = 7.0, 7.0 Hz, 2H), 1.22 (*d*, *J* = 7.0 Hz, 3H); IR (neat) ν_{max} 3284, 3073, 2920, 1649, 1543, 1432, 1291, 1250, 1050, 873 cm^{-1}; FABHRMS (NBA) m/z 529.1730 (M^+-Cl and HCl, $C_{21}H_{33}N_6O_4S_3$ requires 529.1725).

For **4i**: $[\alpha]_D^{23}$ +2 (*c* 0.4, 0.1 N aqueous HCl); ^1H NMR (CD_3OD, 400 MHz) δ 8.27 (*s*, 1H), 8.25 (*s*, 1H), 3.95 (*d*, *J* = 16.5 Hz, 1H), 3.79 (*d*, *J* = 16.5 Hz, 1H), 3.73 (*dd*, *J* = 4.8, 6.4 Hz, 1H), 3.67 (*t*, *J* = 7.0 Hz, 2H), 3.61 (*m*, 1H), 3.61 (*t*, *J* = 6.5 Hz, 2H), 3.44 (*t*, *J* = 7.5 Hz, 2H), 3.38 (*dq*, *J* = 4.8, 6.7 Hz, 1H), 3.32 (*m*, 2H), 2.98 (*s*, 6H), 2.69 (*dq*, *J* = 6.4, 7.0 Hz, 1H), 2.17 (*tt*, *J* = 7.0, 7.0 Hz, 2H), 1.31 (*d*, *J* = 6.7 Hz, 3H), 1.19 (*d*, *J* = 7.0 Hz, 3H); IR (neat) ν_{max} 3307, 3077, 2927, 1642, 1547, 1432, 1366, 1246, 1161, 1041 cm^{-1}; FABHRMS (NBA) m/z 543.1895 (M^+-Cl and HCl, $C_{22}H_{35}N_6O_4S_3$ requires 543.1882).

For **4j**: $[\alpha]_D^{23}$ −10 (*c* 0.2, 0.1 N aqueous HCl); ^1H NMR (CD_3OD, 400 MHz) δ 8.22 (*s*, 1H), 8.18 (*s*, 1H), 3.95 (*d*, *J* = 16.5 Hz, 1H), 3.83 (*ddd*, *J* = 10.0, 7.5, 3.0 Hz, 1H), 3.73 (*d*, *J* = 16.5 Hz, 1H), 3.65 (*t*, *J* = 7.0 Hz, 2H), 3.61 (*t*, *J* = 6.5 Hz, 2H), 3.41 (*t*, *J* = 7.5 Hz, 2H), 3.27 (*t*, *J* = 7.0 Hz, 2H), 3.16 (*dd*, *J* = 13.0, 3.0 Hz, 1H), 2.95 (*s*, 6H), 2.88 (*dd*, *J* = 13.0, 10.0 Hz, 1H), 2.51 (*m*, 1H), 2.16 (*tt*, *J* = 7.0, 7.0 Hz, 2H), 1.21 (*d*, *J* = 7.0 Hz, 3H); IR (neat) ν_{max} 3328, 3057, 2937, 1647, 1547, 1427, 1306, 1251, 1131, 1046 cm^{-1}; FABHRMS (NBA) m/z 529.1738 (M^+-Cl and HCl, $C_{21}H_{33}N_6O_4S_3$ requires 529.1725).

For **4k**: $[\alpha]_D^{23}$ −19 (*c* 0.2, 0.1 N aqueous HCl); ^1H NMR (CD_3OD, 400 MHz) δ 8.22 (*s*, 1H), 8.19 (*s*, 1H), 4.29 (*d*, *J* = 4.0 Hz, 1H), 4.15 (*dq*, *J* = 4.0, 6.5 Hz, 1H), 4.09

(*q*, *J* = 7.0 Hz, 1H), 3.70 (*m*, 2H), 3.60 (*t*, *J* = 6.5 Hz, 2H), 3.41 (*t*, *J* = 7.5 Hz, 2H), 3.29 (*m*, 2H), 2.95 (*s*, 6H), 2.16 (*tt*, *J* = 6.5, 6.5 Hz, 2H), 1.53 (*d*, *J* = 7.0 Hz, 3H), 1.14 (*d*, *J* = 6.5 Hz, 3H); IR (neat) ν_{max} 3421, 1636, 1560, 1491, 1437, 1262, 1085 cm^{-1}; FABHRMS (NBA) m/z 529.1725 (M^+-Cl and HCl, $C_{21}H_{33}N_6O_4S_3$ requires 529.1725).

For **7b**: R_f 0.40 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ +9 (*c* 0.1, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) δ 8.16 (*s*, 1H), 8.12 (*s*, 1H), 7.49 (*s*, 1H), 7.29 (*m*, 9H), 7.07 (*m*, 6H), 6.87 (*s*, 1H), 5.12 (*d*, *J* = 6.5 Hz, 1H), 4.27 (*m*, 1H), 4.21 (*m*, 1H), 4.04 (*m*, 1H), 3.87 (*m*, 1H), 3.74 (*m*, 1H), 3.62 (*m*, 1H), 3.60 (*m*, 2H), 3.42 (*m*, 3H), 3.35 (*m*, 3H), 3.18 (*m*, 2H), 2.92 (*s*, 6H), 2.79 (*m*, 2H), 2.57 (*m*, 2H), 2.43 (*m*, 1H), 2.29 (*s*, 3H), 2.14 (*m*, 2H), 1.40 (*s*, 9H), 1.15 (*d*, *J* = 7.0 Hz, 3H), 1.12 (*d*, *J* = 6.5 Hz, 3H); IR (neat) ν_{max} 3323, 2923, 2841, 1625, 1583, 1437, 1243, 1164, 1075, 849 cm^{-1}; FABHRMS (NBA) m/z 1375.5575 (M^+, $C_{65}H_{83}N_{16}O_{12}S_3$ requires 1375.5539).

For **7d**: R_f 0.42 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ −12 (*c* 0.1, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) δ 8.18 (*s*, 1H), 8.07 (*s*, 1H), 7.61 (*s*, 1H), 7.33 (*m*, 9H), 7.11 (*m*, 6H), 6.80 (*s*, 1H), 4.26 (*d*, *J* = 6.0 Hz, 1H), 4.11 (*m*, 1H), 4.01 (*m*, 2H), 3.82 (*m*, 1H), 3.70 (*m*, 1H), 3.59 (*m*, 6H), 3.35 (*m*, 2H), 3.28 (*m*, 2H), 2.93 (*s*, 6H), 2.82 (*m*, 2H), 2.63 (*m*, 1H), 2.56 (*m*, 1H), 2.44 (*m*, 1H), 2.28 (*s*, 3H), 2.15 (*m*, 2H), 1.40 (*s*, 9H), 1.19 (*d*, *J* = 7.0 Hz, 3H), 1.14 (*d*, *J* = 7.0 Hz, 3H), 1.11 (*d*, *J* = 6.5 Hz, 3H); IR (neat) ν_{max} 3323, 2933, 2851, 1661, 1626, 1544, 1446, 1374, 1164, 1128, 749 cm^{-1}; FABMS (NBA) m/z 1390 (M^+, $C_{66}H_{85}N_{16}O_{12}S_3$).

For **7e**: R_f 0.47 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ −9 (*c* 0.1, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) δ 8.19 (*s*, 1H), 8.08 (*s*, 1H), 7.47 (*s*, 1H), 7.30 (*m*, 9H), 7.09 (*m*, 6H), 6.97 (*s*, 1H), 5.32 (*m*, 1H), 4.25 (*d*, *J* = 4.5 Hz, 1H), 4.11 (*m*, 2H), 3.91 (*m*, 1H), 3.82 (*m*, 1H), 3.59 (*m*, 4H), 3.44 (*m*, 2H), 3.35 (*m*, 3H), 3.26 (*m*, 2H), 2.93 (*s*, 6H), 2.80 (*m*, 2H), 2.59 (*m*, 3H), 2.28 (*s*, 3H), 2.15 (*m*, 2H), 1.41 (*s*, 9H), 1.14 (*d*, *J* = 7.0 Hz, 3H), 1.12 (*d*, *J* = 6.5 Hz, 3H); IR (neat) ν_{max} 3323, 2923, 1636, 1544, 1492, 1441, 1385, 1251, 1164, 1128, 749 cm^{-1}; FABHRMS (NBA) m/z 1375.5559 (M^+, $C_{65}H_{83}N_{16}O_{12}S_3$ requires 1375.5539).

For **7f**: R_f 0.45 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ +6 (*c* 0.13, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) δ 8.17 (*s*, 1H), 8.05 (*s*, 1H), 7.58 (*s*, 1H), 7.33 (*m*, 9H), 7.11 (*m*, 6H), 6.89 (*s*, 1H), 5.65 (*m*, 1H), 4.02 (*m*, 2H), 3.89 (*m*, 1H), 3.88 (*d*, *J* = 16.5 Hz, 1H), 3.80 (*m*, 2H), 3.64 (*m*, 2H), 3.61 (*t*, *J* = 7.5 Hz, 2H), 3.51 (*m*, 1H), 3.35 (*m*, 2H), 3.23 (*m*, 2H), 2.93 (*s*, 6H), 2.79 (*m*, 1H), 2.59 (*m*, 3H), 2.42 (*m*, 1H), 2.28 (*s*, 3H), 2.15 (*m*, 2H), 1.41 (*s*, 9H), 1.20 (*d*, *J* = 7.0 Hz, 3H), 1.18 (*d*, *J* = 7.0 Hz, 3H); IR (neat) ν_{max} 3323, 2923, 2841, 1656, 1626, 1549, 1446, 1251, 1159, 876 cm^{-1}; FABHRMS (NBA) m/z 1345.5470 (M^+, $C_{64}H_{81}N_{16}O_{11}S_3$ requires 1345.5433).

For **7g**: R_f 0.40 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ +10 (*c* 0.1, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) δ 8.18 (*s*, 1H), 8.13 (*s*, 1H), 7.59 (*s*, 1H), 7.31 (*m*, 9H), 7.08 (*m*, 6H), 6.96 (*s*, 1H), 5.10 (*d*, *J* = 7.0 Hz, 1H), 4.60 (*s*, 1H), 3.98 (*d*, *J* = 14.0 Hz, 1H), 3.96 (*m*, 1H), 3.82 (*m*, 3H), 3.63 (*m*, 2H), 3.59 (*m*, 4H), 3.37 (*t*, *J* = 7.0 Hz, 2H), 3.22 (*m*, 2H), 2.93 (*s*, 6H), 2.85 (*m*, 1H), 2.77 (*m*, 1H), 2.56 (*m*, 2H), 2.42 (*m*, 1H), 2.28 (*s*, 3H), 2.14 (*tt*, *J* = 7.0, 7.0 Hz, 2H), 1.40 (*s*, 9H), 1.19 (*d*, *J* = 7.0 Hz, 3H); IR (neat) ν_{max} 3333, 2923, 2862, 1656, 1549, 1492, 1441, 1246, 1159, 1036, 850 cm^{-1}; FABHRMS (NBA) m/z 1331.5245 (M^+, $C_{63}H_{79}N_{16}O_{11}S_3$ requires 1331.5276).

For **7i**: R_f 0.43 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ −7 (c 0.09, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) δ 8.14 (*s*, 1H), 7.95 (*s*, 1H), 7.63 (*s*, 1H), 7.35 (*m*, 9H), 7.13 (*m*, 6H), 6.74 (*s*, 1H), 5.10 (*m*, 1H), 4.10 (*m*, 1H), 4.08 (*d*, *J* = 17.0 Hz, 1H), 4.00 (*m*, 2H), 3.82 (*m*, 1H), 3.63 (*m*, 3H), 3.55 (*m*, 3H), 3.42 (*m*, 2H), 3.25 (*m*, 2H), 2.93 (*s*, 6H), 2.82 (*m*, 1H), 2.61 (*m*, 3H), 2.40 (*m*, 1H), 2.29 (*s*, 3H), 2.15 (*m*, 2H), 1.40 (*s*, 9H), 1.35 (*d*, *J* = 7.0 Hz, 3H), 1.20 (*d*, *J* = 7.0 Hz, 3H); IR (neat) ν_{max} 3333, 2923, 2841, 1651, 1615, 1544, 1441, 1369, 1246, 1159, 1128, 1041, 744 cm^{-1}; FABMS (NBA) m/z 1327 (M^+-18, $C_{64}H_{81}N_{16}O_{11}S_3$).

For **7j**: R_f 0.41 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ −11 (*c* 0.09, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) δ 8.15 (*s*, 1H), 8.04 (*s*, 1H), 7.47 (*s*, 1H), 7.29 (*m*, 9H), 7.08 (*m*, 6H), 6.98 (*s*, 1H), 5.13 (*d*, *J* = 7.0 Hz, 1H), 4.58 (*s*, 1H), 4.09 (*m*, 1H), 4.02 (*m*, 1H), 3.94 (*d*, *J* = 16.5 Hz, 1H), 3.88 (*m*, 1H), 3.80 (*m*, 3H), 3.59 (*m*, 4H), 3.34 (*m*, 2H), 3.20 (*m*, 2H), 2.93 (*s*, 6H), 2.78 (*m*, 2H), 2.57 (*m*, 3H), 2.28 (*s*, 3H), 2.14 (*m*, 2H), 1.41 (*s*, 9H), 1.15 (*d*, *J* = 7.0 Hz, 3H); IR (neat) ν_{max} 3313, 2923, 2851, 1656, 1549, 1492, 1441, 1164, 1128, 1041, 749 cm^{-1}; FABHRMS (NBA) m/z 1331.5297 (M^+, $C_{63}H_{79}N_{16}O_{11}S_3$ requires 1331.5276).

For **7k**: R_f 0.46 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ +18 (*c* 0.1, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) δ 8.16 (*s*, 1H), 8.12 (*s*, 1H), 7.62 (*s*, 1H), 7.32 (*m*, 9H), 7.09 (*m*, 6H), 6.84 (*s*, 1H), 4.59 (*m*, 1H), 4.27 (*m*, 2H), 4.22 (*m*, 1H), 4.05 (*m*, 1H), 3.85 (*m*, 1H), 3.67 (*m*, 1H), 3.60 (*m*, 2H), 3.51 (*t*, *J* = 7.0 Hz, 2H), 3.47 (*t*, *J* = 7.5 Hz, 2H), 3.26 (*m*, 2H), 2.93 (*s*, 6H), 2.81 (*m*, 2H), 2.56 (*m*, 1H), 2.44 (*m*, 1H), 2.29 (*s*, 3H), 2.14 (*m*, 2H), 1.40 (*s*, 9H), 1.34 (*d*, *J* = 7.0 Hz, 3H), 1.13 (*d*, *J* = 6.5 Hz, 3H); IR (neat) ν_{max} 3385, 3118, 2933, 1621, 1574, 1405, 1354, 1103, 821 cm^{-1}; FABHRMS (NBA) m/z 1331.5297 (M^+, $C_{63}H_{79}N_{16}O_{12}S_3$ requires 1331.5276).

For **2b**: R_f 0.11 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ −45 (*c* 0.01, 0.1 N aqueous HCl); ^1H NMR (D_2O, 400 MHz) δ 7.97

(*s*, 1H), 7.88 (*s*, 1H), 7.79 (*s*, 1H), 7.15 (*s*, 1H), 4.98 (*d*, *J* = 6.5 Hz, 1H), 4.20 (*s*, 1H), 4.03 (*m*, 2H), 3.91 (*m*, 2H), 3.47 (*m*, 1H), 3.40 (*m*, 4H), 3.31 (*m*, 2H), 3.18 (*t*, *J* = 7.5 Hz, 2H), 3.07 (*t*, *J* = 7.0 Hz, 2H), 2.95 (*m*, 2H), 2.70 (*s*, 6H), 2.52 (*m*, 2H), 2.31 (*m*, 1H), 1.97 (*s*, 3H), 1.96 (*m*, 2H), 0.95 (*d*, *J* = 6.5 Hz, 3H), 0.91 (*d*, *J* = 6.0 Hz, 3H); IR (neat) ν_{max} 3415, 1646, 1410, 1139 cm^{-1}; FABHRMS (NBA) m/z 1033.3950 (M^+, $C_{41}H_{61}N_{16}O_{10}S_3$ requires 1033.3919).

For **2d**: R_f 0.11 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ −26 (*c* 0.04, 0.1 N aqueous HCl); ^1H NMR (D_2O, 400 MHz) δ 8.05 (*s*, 1H), 7.92 (*s*, 1H), 7.17 (*s*, 1H), 6.77 (*s*, 1H), 4.06 (*d*, *J* = 5.5 Hz, 1H), 3.98 (*m*, 1H), 3.89 (*m*, 1H), 3.72 (*m*, 1H), 3.62 (*m*, 2H), 3.51 (*m*, 2H), 3.43 (*t*, *J* = 7.5 Hz, 2H), 3.21 (*t*, *J* = 7.0 Hz, 2H), 3.13 (*t*, *J* = 6.5 Hz, 2H), 2.95 (*m*, 2H), 2.73 (*s*, 6H), 2.40 (*m*, 2H), 2.22 (*m*, 1H), 1.99 (*tt*, *J* = 7.0, 7.0 Hz, 2H), 1.80 (*s*, 3H), 0.98 (*d*, *J* = 7.0 Hz, 3H), 0.95 (*d*, *J* = 7.0 Hz, 3H), 0.90 (*d*, *J* = 6.0 Hz, 3H); IR (neat) ν_{max} 3382, 1654 cm^{-1}; FABHRMS (NBA) m/z 1047.4036 (M^+, $C_{42}H_{63}N_{16}O_{10}S_3$ requires 1047.4075).

For **2e**: R_f 0.10 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ −6 (*c* 0.05, 0.1 N aqueous HCl); ^1H NMR (D_2O, 400 MHz) δ 8.37 (*s*, 1H), 8.27 (*s*, 1H), 8.08 (*s*, 1H), 7.11 (*s*, 1H), 4.95 (*m*, 1H), 4.05 (*m*, 1H), 3.95 (*m*, 4H), 3.52 (*m*, 1H), 3.45 (*m*, 4H), 3.33 (*m*, 2H), 3.22 (*m*, 2H), 3.10 (*m*, 2H), 2.89 (*m*, 2H), 2.73 (*s*, 6H), 2.53 (*m*, 2H), 2.34 (*m*, 1H), 2.00 (*s*, 3H), 1.99 (*m*, 2H), 0.74 (*d*, *J* = 6.5 Hz, 3H), 0.70 (*d*, *J* = 6.5 Hz, 3H); IR (neat) ν_{max} 3373, 1638, 1432, 1145, 1083 cm^{-1}; FABHRMS (NBA) m/z 1033.3950 (M^+, $C_{41}H_{61}N_{16}O_{10}S_3$ requires 1033.3919).

For **2f**: R_f 0.10 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ +14 (*c* 0.04, 0.1 N aqueous HCl); ^1H NMR (D_2O, 400 MHz) δ 7.99 (*s*, 1H), 7.87 (*s*, 1H), 7.73 (*s*, 1H), 7.17 (*s*, 1H), 5.10 (*m*, 1H), 4.21 (*m*, 1H), 3.93 (*m*, 3H), 3.64 (*m*, 3H), 3.41 (*m*, 4H), 3.31 (*m*, 2H), 3.19 (*t*, *J* = 7.5 Hz, 2H), 3.07 (*m*, 2H), 2.71 (*s*, 6H), 2.53 (*m*, 2H), 2.37 (*m*, 1H), 2.24 (*s*, 3H), 1.97 (*m*, 2H), 1.01 (*d*, *J* = 6.5 Hz, 3H), 0.90 (*d*, *J* = 7.0 Hz, 3H); IR (neat) ν_{max} 3451, 1643, 1256, 1094, 1021 cm^{-1}; FABMS (NBA) m/z 1003 (M^+, $C_{40}H_{59}N_{16}O_9S_3$).

For **2g**: R_f 0.10 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ −50 (*c* 0.01, 0.1 N aqueous HCl); ^1H NMR (D_2O, 400 MHz) δ 7.98 (*s*, 1H), 7.87 (*s*, 1H), 7.14 (*s*, 1H), 5.03 (*d*, *J* = 6.5 Hz, 1H), 4.25 (*d*, *J* = 6.5 Hz, 1H), 3.89 (*m*, 2H), 3.83 (*m*, 1H), 3.68 (*m*, 3H), 3.60 (*m*, 1H), 3.39 (*t*, *J* = 6.5 Hz, 2H), 3.36 (*t*, *J* = 7.0 Hz, 2H), 3.29 (*m*, 2H), 3.18 (*t*, *J* = 6.5 Hz, 2H), 3.04 (*m*, 2H), 2.70 (*s*, 6H), 2.52 (*m*, 2H), 2.26 (*m*, 1H), 1.97 (*s*, 3H), 1.96 (*tt*, *J* = 7.0, 7.0 Hz, 2H), 0.89 (*d*, *J* = 7.0 Hz, 3H); IR (neat) ν_{max} 3221, 1636, 1410, 1103, 1046 cm^{-1}; FABHRMS (NBA) m/z 989.3630 (M^+, $C_{39}H_{57}N_{16}O_9S_3$ requires 989.3657).

For **2i**: R_f 0.09 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ −5 (*c* 0.04, 0.1 N aqueous HCl); ^1H NMR (D_2O, 400 MHz) δ 7.97 (*s*, 1H), 7.63 (*s*, 1H), 7.85 (*s*, 1H), 7.56 (*s*, 1H), 7.12 (*s*, 1H), 5.40 (*m*, 1H), 4.18 (*m*, 1H), 3.93 (*m*, 3H), 3.69 (*m*, 3H), 3.41 (*m*, 4H), 3.32 (*m*, 2H), 3.19 (*m*, 4H), 2.73 (*s*, 6H), 2.59 (*m*, 2H), 2.41 (*m*, 1H), 2.15 (*s*, 3H), 1.99 (*m*, 2H), 1.14 (*d*, *J* = 7.0 Hz, 3H), 1.02 (*d*, *J* = 7.0 Hz, 3H); IR (neat) ν_{max} 3355, 1638, 1409, 1083 cm^{-1}; FABMS (NBA) m/z 1003 (M^+, $C_{40}H_{59}N_{16}O_9S_3$).

For **2j**: R_f 0.09 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ −11 (*c* 0.05, 0.1 N aqueous HCl); ^1H NMR (D_2O, 400 MHz) δ 8.06 (*s*, 1H), 7.93 (*s*, 1H), 7.91 (*s*, 1H), 7.20 (*s*, 1H), 5.12 (*d*, *J* = 6.5 Hz, 1H), 4.16 (*m*, 1H), 3.95 (*m*, 1H), 3.88 (*m*, 1H), 3.77 (*d*, *J* = 16.0 Hz, 1H), 3.55 (*d*, *J* = 16.0 Hz, 1H), 3.44 (*m*, 4H), 3.39 (*m*, 2H), 3.21 (*m*, 3H), 3.08 (*m*, 2H), 2.94 (*m*, 2H), 2.73 (*s*, 6H), 2.53 (*m*, 2H), 2.33 (*s*, 3H), 2.19 (*m*, 1H), 1.99 (*m*, 2H), 0.96 (*d*, *J* = 6.5 Hz, 3H); IR (neat) ν_{max} 3390, 1638, 1428, 1141, 1078 cm^{-1}; FABMS (NBA) m/z 990 (M^+, $C_{39}H_{57}N_{16}O_9S_3$).

For **2k**: R_f 0.2 (SiO_2, 10:9:1 CH_3OH:10% aqueous $CH_3CO_2NH_4$:10% aqueous NH_4OH); $[\alpha]_D^{23}$ +60 (*c* 0.03, 0.1 N aqueous HCl); ^1H NMR (D_2O, 400 MHz) δ 8.00 (*s*, 1H), 7.82 (*s*, 1H), 7.73 (*s*, 1H), 7.54 (*s*, 1H), 4.81 (*m*, 1H), 4.40 (*m*, 1H), 4.13 (*m*, 2H), 4.10 (*m*, 1H), 3.93 (*m*, 1H), 3.64 (*m*, 1H), 3.39 (*t*, *J* = 7.0 Hz, 2H), 3.30 (*m*, 2H), 3.17 (*t*, *J* = 7.5 Hz, 2H), 3.07 (*t*, *J* = 7.0 Hz, 2H), 3.05 (*m*, 2H), 2.71 (*s*, 6H), 2.21 (*m*, 2H), 2.09 (*s*, 3H), 1.96 (*m*, 2H), 1.26 (*d*, *J* = 6.5 Hz, 3H), 1.06 (*d*, *J* = 6.0 Hz, 3H); IR (neat) ν_{max} 3385, 2933, 1662, 1589, 1405, 1262, 949, 877 cm^{-1}; FABMS (NBA) m/z 972 (M^+-18, $C_{39}H_{57}N_{16}O_9S_3$).

General procedure for the DNA cleavage reactions: supercoiled ΦX174 DNA relative efficiency study

All reactions were run with freshly prepared Fe(II) complexes. The Fe(II) complexes were prepared by combining 1 μL of a H_2O solution of agent at the 10 times specified concentration with 1 μL of a freshly prepared equimolar aqueous $Fe(NH_4)_2(SO_4)_2$ solution followed by vortex mixing and centrifugation. Each of the Fe(II) complex solutions was treated with 7 μL of a buffered DNA solution containing 0.25 μg of a supercoiled ΦX174 RFI DNA (1.4×10^{-8} M) in 50 mM Tris–HCl buffer solution (pH 8). The DNA cleavage reactions were initiated by adding 1 μL of aqueous 10 mM 2-mercaptoethanol. The final concentrations of the agents employed in the study were 2.0, 4.0, 8.0 and 16.0 μM Fe(II) control, 0.1, 0.2, 0.5 and 1.0 μM bleomycin A_2 (1), 1.0, 2.0 and 4.0 μM deglycobleomycin A_2 (**2a**) and 4.0, 8.0 and 16.0 μM **2b–k**. The DNA reaction solution was incubated at 25 °C for 1 h. The reactions were quenched with the addition of 5 μL of loading buffer formed by mixing Keller buffer (0.4 M Tris–HCl, 0.05 M NaOAc, 0.0125 M EDTA, pH 7.9) with glycerol (40%), sodium dodecyl sulfate (0.4%), and bromophenol blue (0.3%). Electrophoresis was conducted on a

1% agarose gel containing 0.1 μg·mL^{-1} ethidium bromide at 50 V for 3 h, and the gel was immediately visualized on a UV transilluminator and photographed using Polaroid T667 black and white instant film. Direct fluorescence quantitation of DNA in the presence of ethidium bromide was conducted using a Millipore Bio Image 60S RFLP system visualized on a UV (312 nm) transilluminator taking into account the relative fluorescence intensities of Forms I–III ΦX174 DNA (Form II and III fluorescence intensities are 0.7 times that of Form I).

General procedure for quantitation of double strand and single strand supercoiled ΦX174 DNA cleavage

The Fe(II) complexes were formed by mixing 1 μL of an aqueous solution of **2a–2k** at 10 times the specified concentration with 1 μL of a freshly prepared equimolar aqueous $Fe(NH_4)_2(SO_4)_2$ solution. Eight microliters of a buffered DNA solution containing 0.25 μg of supercoiled ΦX174 RFI DNA (1.4×10^{-8} M) in 50 mM Tris–HCl buffer solution (pH 8) containing 10 mM 2-mercaptoethanol was added to each of the complex solutions. The final concentrations of the complexes employed in the study were 1 μM bleomycin A_2 (**1**), 2 μM deglycobleomycin A_2 (**2a**), 8 μM **2c**, 3μM **2d**, 8 μM **2e**, 8 μM **2f**, 24 μM **2g**, 30 μM **2h**, 26 μM **2i**, 50 μM **2j**, and 60 μM **2k**. The solutions were thoroughly mixed and incubated at 25 °C for 60, 40, 30, 20, 15, 10, 8, 6, 4, 2 and 1 min, respectively. The reactions were quenched with the addition of 5 μL of loading buffer, and electrophoresis was run on a 1% agarose gel containing 0.1 μg·mL^{-1} ethidium bromide at 50V for 3 h. Direct fluorescence quantitation of the DNA in the presence of ethidium bromide was conducted using a Millipore Bio Image 60S RFLP system taking into account the relative fluorescence intensities of Forms I–III ΦX174 DNA (Forms II and III fluorescence intensities are 0.7 times that of Form I). The ratio of the double to single strand DNA cleavage was calculated with use of the Freifelder–Trumbo equation[43] by assuming a Poisson distribution.

General procedure for cleavage of 5' end-labeled w794 and w836 DNA: relative efficiency and selectivity

All reactions were run with freshly prepared Fe(III) complexes. The Fe(III) complexes were prepared by combining 2 μL of an aqueous solution of agent at the 4 times specified concentration with 2 μL of a freshly prepared equimolar aqueous $FeCl_3$ solution. Each of the Fe(III) complex solutions were treated with 2 μL of a buffered DNA solution containing the ^{32}P 5' end-labeled w794 or w836 DNA[45] in 10 mM phosphate buffer (K_2HPO_4–KH_2PO_4, pH 7.0) containing 10 mM KCl. The final concentrations of the agents employed in the study were 128 and 64 μM control Fe(III), 0.2, 0.3 and 0.5 μM bleomycin A_2 (**1**), 2.0 and 4.0 μM deglycobleomycin A_2 (**2a**), 8.0 and 16.0 μM **2b** and **2c**, 4.0 and 8.0 μM **2d**, 16.0 and 32.0 μM **2e**, 32.0 and 64.0 μM **2f**, **2g**, and **2i**, 64.0 and 128 μM **2h**, **2j**, and **2k** and Fe(III) control. The DNA cleavage reactions were initiated by adding 2 μL of 50% aqueous H_2O_2. The DNA reaction solutions were incubated at 37 °C for 30 min. The reactions were quenched with the additions of 2 μL of 50% aqueous glycerol followed by EtOH precipitation and isolation of the DNA. The DNA was resuspended in 4 μL of TE buffer (pH 8.0), and formamide dye (4 μL) was added to the supernatant. Prior to electrophoresis, the samples were warmed at 100 °C for 5 min, placed in an ice bath, centrifuged, and the supernatant (3 μL) was loaded onto the gel. Sanger dideoxynucleotide sequencing reactions were run as standards adjacent to the agent-treated DNA. Gel electrophoresis was conducted using a denaturing 8% sequencing gel (19:1 acrylamide-N,N-methylenebis-acrylamide, 8 M urea) at 1100 V for 5.5 h. Formamide dye contained xylene cyanol FF (0.03%), bromophenol blue (0.3%), and aqueous Na_2EDTA (8.7%, 250 mM). Electrophoresis running buffer (TBE) contained Tris base (100 mM), boric acid (100 mM), and Na_2EDTA–H_2O (0.2 mM). Gels were prerun for 30 min with formamide dye prior to loading the samples. Auto-radiography of dried gel was carried out at –78 °C using Kodak X-Omat AR film and a Picker spectra intensifying screen. Quantitation of the DNA cleavage reaction was conducted on a Millipore Bio Image 60S RFLP system measuring the remaining uncleaved w794 or w836 DNA.

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