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N-Methyl Threonine Analogues of Deglycobleomycin A_2: Synthesis and Evaluation

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Abstract—The synthesis of **5** and its D-*allo*-threonine epimer **6** and the comparison of their DNA cleavage efficiency and selectivity with that of deglycobleomycin A_2 (**3**) are detailed. The studies illustrate that *N*-methylation of the L-threonine subunit within deglycobleomycin A_2 dramatically reduces the DNA cleavage efficiency (10–15-fold), weakens and nearly abolishes the inherent DNA cleavage selectivity, but has little effect on the inherent oxidation capabilities of the activated Fe(III) complexes. The results are consistent with a previously unrecognized prominent role for the threonine NH and the potential importance of a hydrogen bond to the Fe(III) hydroperoxide complex of bleomycin or a subsequent activated complex implicated in recent structural models.

Bleomycin A_2 (**1**, Fig. 1),[1–11] the major naturally occurring constituent of the clinical antitumor drug Blenoxane, is thought to derive its therapeutic effects from the ability to mediate the oxidative cleavage of double-stranded DNA or RNA by a process that is metal ion and oxygen dependent.[12–18] Extensive studies employing derivatives of the natural product,[19,20] its degradation products or semisynthetic analogues,[21–30] as well as closely related or substantially simplified analogues[31–34] have contributed to an emerging model of the structural features responsible for the sequence selective cleavage of duplex DNA. Recent structural studies have demonstrated the Co(III) hydroperoxide complexes of bleomycin A_2 and deglycobleomycin A_2 (**3**) bind to an oligonucleotide at a cleavage site in essentially identical manners albeit with **3** exhibiting a slightly lower affinity.[35–37] Both **1** and **3** exhibit comparable DNA cleavage selectivities but with **3** exhibiting lower efficiencies (two to six times). The comparable behavior of **3** and **4**[38] and the further demonstration that both the DNA cleavage selectivity and efficiency of **2**,[39] lacking only the terminal mannose, were nearly indistinguishable from **1** suggests the full disaccharide only contributes to the cleavage efficiency of **1** but not its DNA cleavage selectivity and that it may do so simply by increasing DNA binding affinity. Regardless of the role, the studies illustrate that deglycobleomycin A_2 analogues may provide important and relevant information on the nature of interaction of **1** with duplex DNA. In our own efforts,[38–48] this has entailed single point changes in the structure of deglycobleomycin A_2 conducted with the intention of defining the role of each subunit, functional group, or substituent. These studies, carried out in conjunction with structural studies, have begun to unravel many of the subtle structural features contributing to the properties of the natural product.

Here, we report the synthesis and evaluation of **5** and its epimer **6** in which the threonine secondary amide has

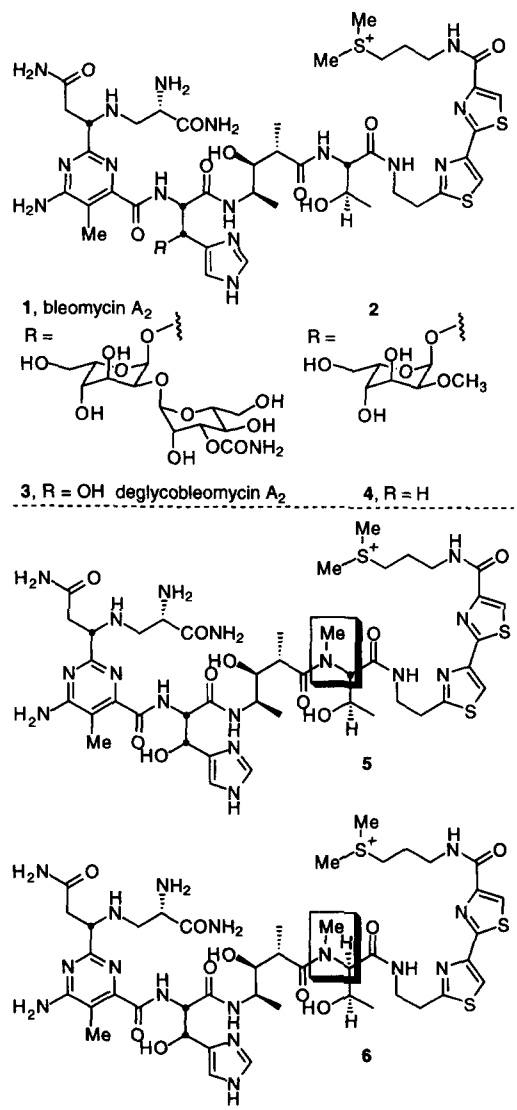


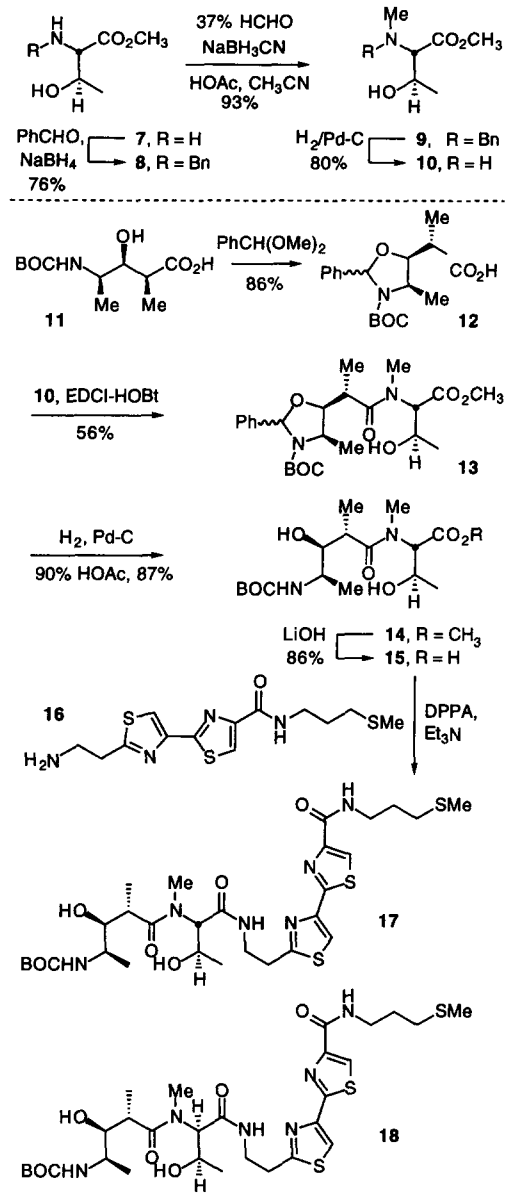
Figure 1.

been replaced with a tertiary *N*-methyl amide. In addition to the impact this may have on the preferred or accessible conformations of **3**, its examination also allows the functional assessment of the consequences of preventing formation of a potentially key hydrogen bond defined in the Stubbe structural studies.[35,36]

Synthesis of 5 and 6

The synthesis of **5** was accomplished through preparation of the tetrapeptide **17** incorporating the *N*-methyl-L-threonine subunit (Scheme 1). *N*-methyl-L-threonine methyl ester (**10**), $[\alpha]^{25}_{D}$ −18.7 (*c* 1.0, CH_3OH), was prepared in three steps from L-threonine methyl ester (**7**) by sequential *N*-benzylation and *N*-methylation by reductive alkylation and subsequent reductive debenzylation. The direct coupling of **10** with **11**[42] failed to provide a useful approach to **14** and the subunit **11** preferentially closed to the corresponding five-membered *N*-BOC lactam rather than couple with the secondary *N*-methyl amine. Consequently, the coupling was successfully accomplished employing the cyclic *N,O*-acetal **12**. Thus, treatment of **11** with benzaldehyde dimethyl acetal in the presence of cat. *p*-TsOH (toluene, 25 °C) cleanly provided **12** (86%). Coupling of **12** with **10** (1.1 equiv, 1.2 equiv EDCI, 0.2 equiv HOBt, DMF, 25 °C, 12 h, 56%) effectively provided **13**. Immediate cleavage of the *N,O*-acetal by hydrogenolysis provided the key dipeptide **14**. Methyl ester hydrolysis of **14** to provide **15** (86%) proved to be best carried out with only a slight excess of LiOH (1.2 equiv) in 35% H_2O–*t*BuOH at 4 °C. Under these conditions, little racemization of the sensitive α-center was observed (10%) while exposure to more conventional conditions (excess LiOH, H_2O–CH_3OH, 25 °C) provided more substantial epimerization (1:1.2 mixture). Coupling of **15** with **16**[42] was most effectively accomplished with activation by DPPA (1.2 equiv) in the presence of Et_3N (1.5 equiv, 25 °C, 40 min) and provided **17** (77%) and small amounts of the separable diastereomer **18**. Alternative attempts to couple **15** with **16** on activation with EDCI–HOBt (DMF, 25 °C) under a variety of conditions or BOPCl–*i*Pr_2NEt (DMF, 25 °C) resulted in more substantial epimerization to provide additional amounts of **18**. In the development of the optimized protocol for preparing **17**, substantial amounts of **18** were collected and ultimately employed to prepare the diastereomer **6**.

The final agent **5** was assembled by sequential couplings to introduce the erythro β-hydroxy-L-histidine and pyrimidoblamic acid subunits following formation of the sulfonium salt (Scheme 2). Thus, treatment of **17** with excess CH_3I (EtOH, 25 °C, 44 h) followed by acid-catalyzed *N*-BOC deprotection (3 N HCl–EtOAc, 25 °C, 1 h) and liberation of the free amine (NH_4OH, CH_3OH, 25 °C, 1 h) cleanly provided the tetrapeptide S analogue **20**. Direct coupling of **20** with **21**[42] activated by treatment with benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP reagent) in the presence of *i*Pr_2NEt (DMF, 25 °C, 5 h) provided **22**



Scheme 1.

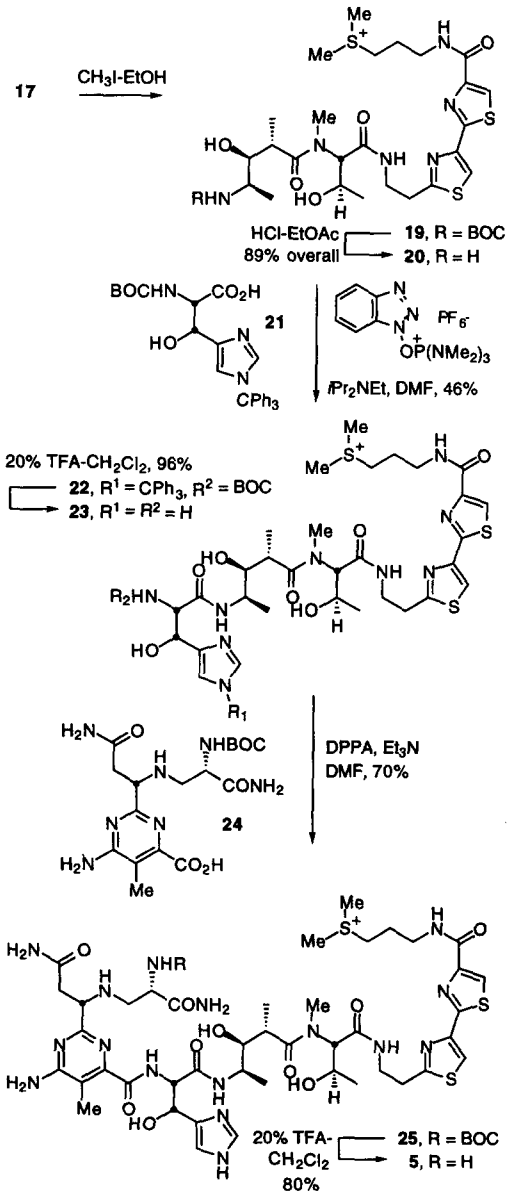
(46%). Alternative coupling procedures including activation by DCC–HOBt proved less satisfactory. Acid-catalyzed deprotection of **22** (20% TFA–CH_2Cl_2, 4 °C, 2 h) and liberation of the free amine (NH_4OH, CH_3OH, 25 °C, 1 h) provided the pentapeptide S analogue **23** (96%) which was coupled with N^{α}-BOC-pyrimidoblamic acid (**24**)[43] on activation with DPPA in the presence of Et_3N (DMF, 25 °C, 15 h, 70%) to provide **25**. That the desired coupling was observed and not that of the potentially competitive free imidazole of **23** was established with the observation of the diagnostic ^1H NMR downfield shift of the His α-proton (δ 4.00 for **23** versus δ 4.73 for **25**) and the unperturbed ^1H NMR chemical shifts of the aromatic imidazole protons (δ 7.71 and 7.11 for **23** versus δ 7.71 and 7.14 for **15**). In preceding studies with bleomycin A_2 itself,[45] we reported that a related coupling of the unprotected imidazole of peptapeptide S complete with the linked disaccharide

effected by activation with DCC–HOBt (DMF, 25 °C, 36 h) provided predominately imidazole versus primary amine coupling. Whether these observations are unique to bleomycin A_2 and result from the more hindered primary amine or whether they are related to the specific activation conditions (DPPA versus DCC–HOBt) and the deliberate use of the liberated free amine are not yet established. It is even plausible that the acylated imidazole serves as an intermediate in route to **25**. An analogous coupling of pentapeptide S with **24** (DPPA, iPr$_2$NEt, DMF) has provided N^{α}-BOC deglycobleomycin A_2 (68–71%).[49] This not only provides an alternative order for the subunit assemblages we reported in our total synthesis of deglycobleomycin A_2,[44] but established the viability of the approach by providing material identical to authentic N^{α}-BOC-deglycobleomycin A_2. Final acid-catalyzed deprotection of **25** provided **5** (80%). Notably, the conversion of **19** to **25** and its deprotection was accomplished on intermediates bearing virtually no protecting groups and with the installed sulfonium salt. This did not result in detectable competitive side reactions but did substantially simplify the synthesis especially with regard to sulfonium salt formation. Moreover, each of the intermediates proved amenable to chromatographic purification.

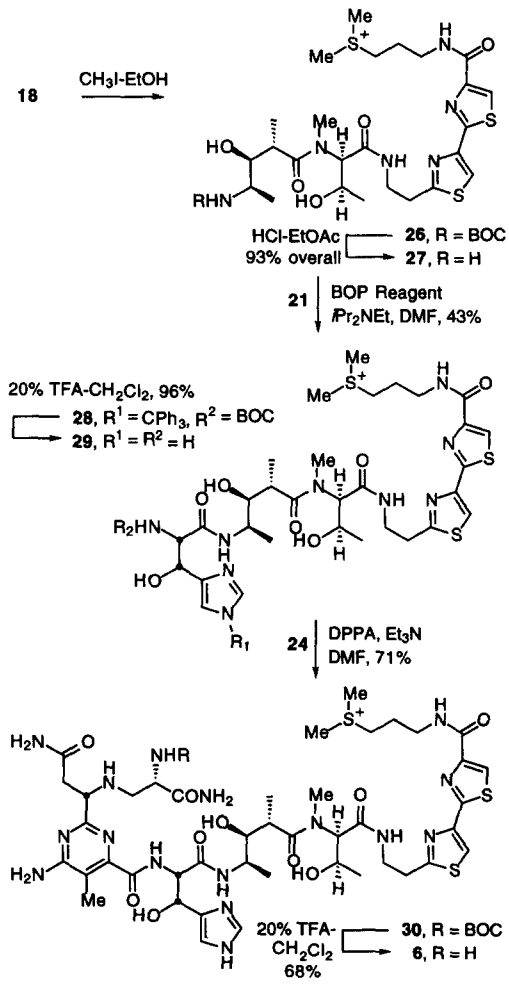
Conducting the same sequence of reactions employing **18** provided **6** in comparable conversions (Scheme 3).

DNA cleavage properties of 5 and 6

Three assays were used to examine the DNA cleavage properties of **5** and **6**. The initial study of the relative efficiency of DNA cleavage was conducted with the Fe(II) complexes and supercoiled ΦX174 RFI DNA in the presence of O_2 and 2-mercaptoethanol. Like Fe(II)-bleomycin A_2 and deglycobleomycin A_2, the Fe(II) complexes of **5** and **6** produced single and double strand cleavage to afford relaxed (form II) and linear (form III) DNA, respectively (Fig. 2 and Table 1). However, both agents were found to be substantially less effective



Scheme 2.



Scheme 3.

Table 1. Summary of DNA cleavage properties

Agent	Relative efficiency of DNA cleavage[a] ΦX174[a]	w794[b]	Ratio of double to single-strand cleavage[c]	DNA cleavage selectivity[b]
1, bleomycin A_2	2–5	5.8	1:6	5′-GC, 5′-GT > 5′-GA
3, deglycobleomycin A_2	1.0	1.0	1:12	5′-GC, 5′-GT > 5′-GA
5	0.10	0.08	1:58	5′-GC, 5′-GT > 5′-GA (very weak)
6	0.10	0.08	1:53	None
$Fe^{a,b}$	0.04	0.03	1:98	None

[a]Relative efficiency of supercoiled ΦX174 RFI DNA cleavage, Fe(II)-O_2, 2-mercaptoethanol. The results are the average of six experiments.
[b]Examined within 5′ ^{32}P-end-labeled w794, Fe(III)–H_2O_2. The results are the average of four experiments.
[c]Ratio of double- to single-stranded cleavage of supercoiled ΦX174 DNA calculated as $F(III) = n_2\exp(-n_2)$, $F(I) = \exp[-(n_1 + n_2)]$.

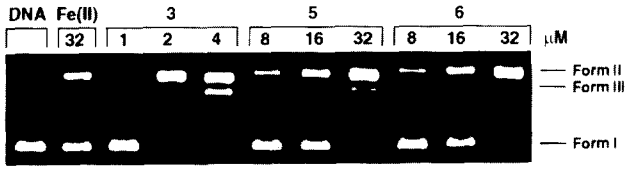


Figure 2. Agarose gel illustrating the cleavage reactions of supercoiled ΦX174 RFI DNA by Fe(II)-agents at 25 °C for 1 h in buffer solutions containing 2-mercaptoethanol. After electrophoresis on a 1% agarose gel, the gel was stained with 0.1 μg/mL ethidium bromide and visualized on a UV transilluminator and quantified on a Millipore BioImage 60S RFLP system. The results are tabulated in Table 1.

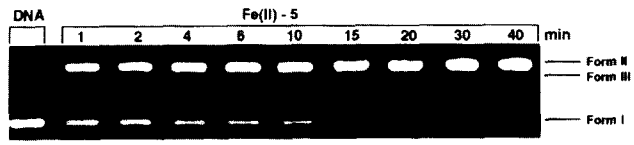


Figure 3. Representative kinetics of supercoiled ΦX174 RFI DNA cleavage by Fe(II)-5 (24 μM) in buffer solution containing 2-mercaptoethanol. The DNA cleavage reactions were run at 25 °C for various lengths of time, and electrophoresis was conducted on a 1% agarose gel. Direct fluorescence quantitation of the percentage of Forms I-III DNA present at each time point was conducted using a Millipore BioImage 60S RFLP system visualized on a UV (312 nm) transilluminator in the presence of 0.1 μg/mL ethidium bromide taking into account the relative fluorescence intensities of forms I-III ΦX174 RFI DNA (forms II and III have fluorescence intensities that are 0.7 times that of form I).

(0.1 ×) at cleaving ΦX174 RFI DNA than deglycobleomycin A_2 and were only two to three times more effective than Fe(II) itself. The lack of DNA cleavage by **5** and **6** in the absence of Fe(II) in control studies was consistent with expectations that they were cleaving DNA by a metal-dependent process.

The relative extent of double-strand to single-strand DNA cleavage was established in a study of the kinetics of supercoiled ΦX174 RFI DNA cleavage to produce linear and circular DNA in a manner analogous to that detailed for **1** and **3**. The reactions exhibit initial fast kinetics in the first 1–5 min and the subsequent decreasing rate may reflect conversion to a less active or inactive agent or the kinetics of metal complex reactivation. We assumed a Poisson distribution for the formation of single-strand and double-strand breaks to calculate the average number of double- and single-strand cuts per DNA molecule using the Freifelder–Trumbo equation.[50] The ratio of double- to single-strand cuts observed with the Fe(II) complexes is illustrated in Figure 3 for **5** and the full set of results is summarized in Table 1. The ratio of double to single strand DNA cleavage for **5** and **6** was established to be 1:58 and 1:53, respectively, which was substantially lower than bleomycin A_2 (1:6) or deglycobleomycin A_2 (1:12) and is similar to the ratio derived from uncomplexed Fe(II) cleavage (1:98). 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 RFI DNA assuming that sequential cleavage on the complementary strands within 15 base-pairs is required to permit formation of linear DNA from the hybridized duplex DNA.

Most revealing was the comparison of the DNA cleavage selectivity of **5** and **6**. The selectivity of DNA cleavage along with an additional assessment of the relative efficiency of DNA cleavage were examined within duplex w794 DNA[51,52] by monitoring strand cleavage of singly ^{32}P 5′-end-labeled double-stranded DNA after exposure to the Fe(III)-complex followed by activation with H_2O_2[53] in 10 mM phosphate buffer (pH 7.0). This protocol has proven to be much more sensitive to the distinctions in the relative efficiency of DNA cleavage by related agents than the ΦX174 RFI supercoiled DNA cleavage assays, but both have always provided the same trends in our hands. Thus, incubation of the labeled duplex DNA with the agents in the presence of equimolar $FeCl_3$ and excess H_2O_2 led to DNA cleavage. Following a quench of the reaction with the addition of glycerol, 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. A typical comparison is illustrated in Figure 4.

Under all conditions examined, **5** and **6** were found to cleave DNA only slightly above background Fe(III) (Fig. 4 and Table 1). The Fe(III) complexes of both agents were three times more effective than Fe(III) itself and 13 times less effective than deglycobleomycin A_2. While **5** showed weak, but observable, sequence-

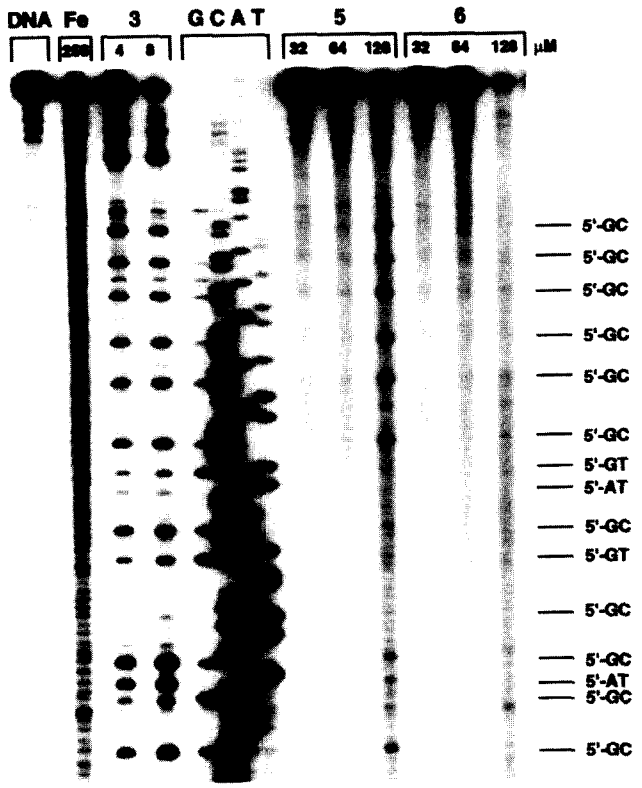


Figure 4. Cleavage of double-stranded DNA by Fe(III)-agents (SV40 DNA fragment, 144 base pairs, nucleotide no. 5238-138, clone w794) in phosphate/KCl buffer containing H_2O_2. The DNA cleavage reactions were run for 90 min at 4 °C, and electrophoresis was run on an 8% denaturing PAGE and visualized by autoradiography.

selective DNA cleavage characteristic of bleomycin A_2, the sequence selectivity for its epimer **6** was not observed under a range of experimental conditions. Comparisons alongside the Fe(III) complexes of bleomycin A_2 and deglycobleomycin A_2 assured that the protocols employed would permit detection of the characteristic sequence selective DNA cleavage reaction. Although it is not surprising that **6** fails to exhibit the DNA cleavage efficiency or selectivity of bleomycin A_2, the substantially reduced and nearly abolished properties of **5** is especially noteworthy.

Oxidation capabilities of 5 and 6

In final efforts to fully characterize the properties of Fe(III)–**5** and **6**, their ability to mediate the oxidation of styrene was investigated.[54] The oxidation of styrene by deglycobleomycin A_2 produces both styrene epoxide and phenylacetaldehyde. A solution of 500 μM Fe(III)–**3**, 50 mM styrene, 30 mM H_2O_2 (0 °C, 1.5 h) produced 1.80 mM styrene epoxide and 1.32 mM phenylacetaldehyde constituting slightly over six oxidations for each Fe(III)–**3** utilized (Table 2). The same products were observed with Fe(III)–**5** and Fe(III)–**6**. The former was slightly less effective than **3** while the latter was slightly more effective.

Discussion

Three significant observations were made in the examination of **5** and **6**. *N*-methylation of the L-threonine subunit of deglycobleomycin A_2 substantially reduces the DNA cleavage efficiency of the resulting Fe complexes. In addition, the DNA cleavage selectivity of **5** was diminished. Finally, reversal of the threonine stereochemistry of the *N*-methyl-L-threonine subunit with **6** resulted in the loss of the characteristic DNA cleavage selectivity. The studies on the inherent oxidation capabilities of the Fe(III)-complexes of **5** and **6** illustrate that *N*-methylation of the L-threonine subunit as well as the use of the epimer **6** did not substantially effect the metal chelation, H_2O_2 activation, catalytic efficiency, or inherent oxidation capabilities of the complexes. Rather, the results suggest that the substantially diminished DNA cleavage efficiency of **5** stems from either the disruption of the interaction or adoption of the productive bound conformation of the agents with duplex DNA. Although the results may be derived from the former, the latter is especially attractive since the Stubbe ^1H NMR studies of the Co(III)–OOH complex of both free and DNA bound bleomycin identify a hydrogen bond from the threonine NH to the proximal oxygen of the metal bound hydroperoxide (Fig. 5). This hydrogen bond could stabilize the productive bound conformation of the activated agent, fix the position or alignment of the reacting hydroperoxide ligand for C4′ hydrogen abstraction, stabilize the metal bound hydroperoxide, or potentially contribute to catalysis of the reaction by assisting homolytic oxygen–oxygen bond cleavage. In addition, the threonine carbonyl of the bound conformation, but not the free solution conformation, of the Co(III)–bleomycin hydroperoxide complex is positioned to accept a hydrogen bond from the terminal oxygen of the hydroperoxide ligand. *N*-Methylation of

Table 2. Styrene oxidation

Agent	Styrene oxide (mM)	Phenylacetaldehyde (mM)	Total product (mM)	Relative efficiency
Fe(III)-**3**	1.80	1.32	3.12	1.00
Fe(III)-**5**	1.22	0.40	1.62	0.52
Fe(III)-**6**	2.28	1.88	4.16	1.33
Fe(III)	0	0	0	0
Fe(III)-**3**, no H_2O_2	0	0	0	0

500 μM Fe(III)-agent, 50 mM styrene, 30 mM H_2O_2, 0 °C, 1.5 h, 20% H_2O–CH_3OH. Results reported are the average of two runs.

Figure 5. Two views of **3** (top) taken from the Stubbe model of bleomycin A_2 bound to d(CCAGGCTGG)$_2$ (ref 35) highlighting the two threonine–Co(OOH) hydrogen bonds. Two views of **5** (bottom) highlighting the destabilizing steric interactions and disrupted hydrogen bonding when bound in the same conformation as **3**. The green CH center on the pink DNA strand indicates the C-H abstraction site leading to DNA cleavage at 5′-GC.

the threonine subunit sterically precludes formation of this carbonyl hydrogen bond and would be expected to disrupt this highly ordered hydrogen bonding network destabilizing the productive DNA bound conformation of the agent that leads to C4′ hydrogen abstraction and DNA cleavage. Thus, disruption of the hydrogen bonding capabilities of the L-threonine subunit NH through *N*-methylation substantially reduces the DNA cleavage efficiency and results in a near loss of the characteristic cleavage sequence selectivity.

Experimental

***N*-Methyl-L-threonine methyl ester (10).** A solution of **7** (0.26 g, 1.95 mmol) in CH_3OH (3 mL) was treated with benzaldehyde (0.24 mL, 2.34 mmol) at 25 °C, and the mixture was stirred for 1 h at 25 °C. $NaBH_4$ (74 mg, 1.95 mmol) was added to the resulting mixture at 0 °C. After stirring for 15 min at 0 °C, the reaction mixture was acidified with 10% aqueous HCl, and concentrated in vacuo. The residue was dissolved in H_2O (3 mL) and basified with 1 N aqueous NaOH, and extracted with EtOAc (2 × 5 mL). The organic layer was washed with H_2O (5 mL) and saturated aqueous NaCl (5 mL), dried (Na_2SO_4) and concentrated in vacuo. Chromatography (SiO_2, 1.5 cm × 13 cm, 2–5% CH_3OH–CH_2Cl_2 gradient elution) provided **8** (0.33 g, 0.44 g theoretical, 76%) as a colorless oil: R_f 0.5 (SiO_2, 10% CH_3OH–CH_2Cl_2); $[\alpha]^{25}_D$ −34.5 (*c* 1.0, CH_3OH); ^1H NMR ($CDCl_3$, 400 MHz) δ 7.23–7.36 (5H, m), 3.82 (1H, d, *J* = 13.0 Hz), 3.70 (3H, s), 3.68 (1H, d, *J* = 13.0 Hz), 3.65 (1H, dq, *J* = 6.2, 7.6 Hz), 3.47 (1H, brs), 3.02 (1H, d, *J* = 7.6 Hz), 1.59 (1H, brs), 1.18 (3H, d, *J* = 6.2 Hz).

Aqueous HCHO (37%, 1.3 mL, 15.4 mmol), $NaBH_3CN$ (0.25 g, 3.7 mmol), and HOAc (0.28 mL, 4.8 mmol) were added sequentially to a solution of **8** (0.37 g, 3.3 mmol) in CH_3CN (10 mL) at 25 °C. After stirring for 30 min at 25 °C, the reaction mixture was basified with 0.1 N aqueous NaOH, and extracted with EtOAc (2 × 10 mL). The organic layer was washed with saturated aqueous NaCl (2 × 10 mL), dried (Na_2SO_4) and concentrated in vacuo. Chromatography (SiO_2, 1.5 × 15 cm, 15–25% EtOAc–hexane gradient elution) provided **9** (0.72 g, 0.78 g theoretical, 93%) as a colorless oil: R_f 0.7 (SiO_2, 5% CH_3OH–CH_2Cl_2); $[\alpha]^{25}_D$ −111.7 (*c* 1.0, CH_3OH); ^1H NMR ($CDCl_3$, 400 MHz) δ 7.25–7.34 (5H, m), 3.96 (1H, dq, *J* = 6.0, 9.9 Hz), 3.80 (1H, d, *J* = 13.2 Hz), 3.76 (3H, s), 3.70 (1H, brs), 3.55 (1H, d, *J* = 13.2 Hz), 3.02 (1H, d, *J* = 9.9 Hz), 2.26 (3H, s), 1.15 (3H, d, *J* = 6.0 Hz); IR (neat) ν_{max} 3446, 2952, 1732, 1455, 1194, 1099, 1026, 746 cm^{-1}.

A solution of **9** (0.54 g, 2.28 mmol) in CH_3OH (10 mL) was stirred with 10% Pd–C (60 mg) under an atmosphere of H_2 (1 atm) at 25 °C for 1.5 h. The reaction mixture was filtered through a Celite pad and washed with CH_3OH (15 mL). After concentration in vacuo, chromatography (SiO_2, 1.5 cm × 15 cm, 5% CH_3OH–CH_2Cl_2) gave **10** (0.27 g, 0.34 g theoretical, 80%) as a colorless oil: R_f 0.3 (SiO_2, 10% CH_3OH–CH_2Cl_2); $[\alpha]^{25}_D$ −18.7 (*c* 1.0, CH_3OH), lit[55] $[\alpha]^{25}_D$ −18.02 (*c* 1.01, CH_3OH); ^1H NMR ($CDCl_3$, 400 MHz) δ 3.75 (3H, s), 3.62 (1H, dq, *J* = 6.2, 7.8 Hz), 3.48 (1H, brs), 2.87 (1H, d, *J* = 7.8 Hz), 2.40 (3H, s), 1.55 (1H, brs), 1.19 (3H, d, *J* = 6.2 Hz).

***N*-[4(*R*)-(*tert*-Butyloxycarbonyl)amino-3(*S*)-hydroxy-2(*S*)-methylpentanoyl]-*N*-methyl-L-threonine methyl ester (14).** A solution of **11** (0.10 g, 0.36 mmol) and benzaldehyde dimethyl acetal (0.30 mL, 2.0 mmol) in toluene (0.5 mL) was treated with *p*-TsOH (cat.) at 25 °C. After stirring for 20 h at 25 °C the reaction mixture was poured into a two-phase solution of EtOAc (5 mL) and 0.5 N aqueous NaOH (2 mL). The organic layer was extracted with 0.5 N aqueous NaOH (1 mL). The combined water layer was acidified with 10% aqueous HCl and extracted with EtOAc (2 × 5 mL). The organic layer was washed with H_2O (2 × 3 mL), saturated aqueous NaCl (5 mL), dried (Na_2SO_4), and concentrated in vacuo. Chromatography (SiO_2, 0.8 cm × 12 cm, 1% CH_3OH–CH_2Cl_2) gave **12** as a white solid (0.10 g, 0.12 g theoretical, 86%): ^1H NMR (CD_3OD, 400 MHz) δ 7.42–7.35 (5H, m), 5.75 (1H, br s), 4.27 (1H, dq, *J* = 5.4, 6.5 Hz), 4.10 (1H, dd, *J* = 5.3, 5.2 Hz), 2.72–2.64 (1H, m), 1.33 (3H, d, *J* = 7.0 Hz), 1.43–1.16 (12H, m).

DMF (1.3 mL) was added to a mixture of the resulting solid (91 mg, 0.27 mmol), **10** (60 mg, 0.41 mmol), EDCI (85 mg, 0.44 mmol), and HOBt (10.8 mg, 0.08 mmol) at 25 °C. After stirring for 12 h at 25 °C, the reaction mixture was poured into a two phase solution of EtOAc (10 mL) and H_2O (6 mL). The water layer was extracted with EtOAc (10 mL) and the combined organic layers were washed with H_2O (2 × 5 mL), saturated aqueous NaCl (10 mL), dried (Na_2SO_4) and concentrated in vacuo. Chromatography (SiO_2, 0.8 cm × 20 cm, 50% EtOAc–hexane) afforded **13** as a white amorphous solid (70 mg, 125 mg theoretical, 56%): ^1H NMR ($CDCl_3$, 400 MHz) δ 7.44–7.36 (5H, m), 5.75 (1H, br s), 5.10 (1H, d, *J* = 4.8 Hz), 4.48 (1H, dq, *J* = 5.1, 6.3 Hz), 4.23 (1H, dd, *J* = 5.4, 5.4 Hz), 4.12 (1H, m), 3.75 (3H, s), 3.25 (3H, s), 3.12 (1H, m), 1.33 (3H, d, *J* = 6.8 Hz), 1.18 (3H, d, *J* = 6.4 Hz), 1.42–1.15 (12H, m); IR (CH_2Cl_2) ν_{max} 3448, 2977, 2935, 1745, 1699, 1640, 1459, 1406, 1365, 1291, 1223, 1169, 1146, 1059, 1015, 893, 757, 698 cm^{-1}.

A solution of **13** (72 mg, 0.15 mmol) in 90% HOAc (1.5 mL) was stirred over 10% Pd–C (7.2 mg) under H_2 (1 atm) at 60 °C for 4 h. The reaction mixture was filtered through a Celite pad and washed with CH_3OH (10 mL) and concentrated in vacuo. Chromatography (SiO_2, 0.8 cm × 10 cm, 50% EtOAc–hexane) gave **14** (49 mg, 56 mg theoretical, 87%) as a white amorphous solid: R_f 0.4 (SiO_2, 10% CH_3OH–CH_2Cl_2); $[\alpha]^{25}_D$ +13 (*c* 0.5, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) mixture of rotamers δ 5.01 (1H, d, *J* = 5.3 Hz), 4.45 (0.87H, dq, *J* = 6.4, 6.4 Hz), 4.31 (0.13H, dq, *J* = 6.2, 6.2 Hz), 3.75 (0.4H, s), 3.37 (2.6H, s), 3.54–3.71 (2H, m), 3.22 (2.6H, s), 2.96 (1H, dq, *J* = 6.7, 6.9 Hz), 2.91 (0.4H, s), 1.42 (9H, s), 1.26 (0.4H, d, *J* = 6.2 Hz), 1.21 (2.6H, d, *J* = 6.9 Hz), 1.18 (2.6H, d, *J* = 6.4 Hz), 1.14 (0.4H, d, *J* = 7.0 Hz), 1.10 (3H, d, *J* = 6.5 Hz); IR (CH_2Cl_2) ν_{max} 3434, 1743, 1684, 1623, 1415, 1173, 1024, 756 cm^{-1}; FABHRMS (NBA–NaI) *m/z* 399.2105 (M + Na^+, $C_{17}H_{32}N_2O_7$ requires 399.2107).

***N*-[4(*R*)-(*tert*-Butyloxycarbonyl)amino-3(*S*)-hydroxy-2(*S*)-methylpentanoyl]-*N*-methyl-L-threonine (15).** A solution of **14** (10 mg, 26.6 μmol) in 35% H_2O–*t*BuOH (0.3 mL) was treated with 1 N aqueous LiOH (32 μL) at 4 °C, and the mixture was stirred for 30 min at 4 °C. The reaction mixture was acidified with the addition of 10% aqueous HCl, and concentrated in vacuo. Chromatography (C-18, 0.5 cm × 3 cm, 0–30% CH_3OH–H_2O gradient elution) provided **15** (8.3 mg, 9.6 mg theoretical, 86%) as a white solid: R_f 0.1 (SiO_2, 10% CH_3OH–CH_2Cl_2); $[\alpha]^{25}_D$ +18 (*c* 0.88, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) mixture of rotamers δ 5.00 (0.8H, d, *J* = 4.4 Hz), 4.45 (0.8H, dq, *J* = 5.8, 6.2 Hz), 4.20–4.36 (0.4H, m), 3.54–3.70 (2H, m), 3.21 (2.4H, s), 2.97 (1H, dq, *J* = 5.8, 6.6 Hz), 2.94 (0.6H, s), 1.42 (9H, s), 1.28 (0.6H, d, *J* = 5.8 Hz), 1.21 (2.4H, d, *J* = 6.9 Hz), 1.18 (3H, d, *J* = 6.4 Hz), 1.11 (0.6H, d, *J* = 6.6 Hz), 1.10 (2.4H, d, *J* = 6.3 Hz); IR (CH_2Cl_2) ν_{max} 3348, 1693, 1682, 1614, 1167, 1077 cm^{-1}; FABHRMS (NBA–NaI) *m/z* 385.1955 (M + Na^+, $C_{16}H_{30}N_2O_7$ requires 385.1951).

***N*-[4(*R*)-(*tert*-Butyloxycarbonyl)amino-3(*S*)-hydroxy-2(*S*)-methylpentanoyl]-*N*-methyl-1-[(((3-(methylthio)-1-propylamino)carbonyl)-2′,4-bithazol-2-yl)-ethylamino]-L-threonine (17).** DPPA (3.6 μL, 16.5 μmol), and Et_3N (2.9 μL, 20.7 μmol) were added to a solution of **15** (5.0 mg, 13.8 μmol) and **16** (7.1 mg, 20.7 μmol) in DMF (80 μL) at 25 °C, and the mixture was stirred for 40 min at 25 °C. The reaction mixture was quenched with the addition of H_2O (0.5 mL) and extracted with EtOAc (3 × 0.5 mL). The combined organic layer was washed with H_2O (0.5 mL), saturated aqueous NaCl (0.5 mL), and dried (Na_2SO_4). PTLC (SiO_2, 10% CH_3OH–CH_2Cl_2) gave **17** (7.3 mg, 9.5 mg theoretical, 77%) as a white amorphous solid: R_f 0.4 (SiO_2, 10% CH_3OH–CH_2Cl_2); $[\alpha]^{25}_D$ −26 (*c* 0.5, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) mixture of rotamers δ 8.16 (1H, s), 8.14 (1H, s), 4.85 (0.9H, d, *J* = 8.2 Hz), 4.12–4.27 (1.1H, m), 3.54–3.73 (4H, m), 3.51 (2H, t, *J* = 7.0 Hz), 3.28 (2H, t, *J* = 6.8 Hz), 3.12 (2.7H, s), 2.97 (0.3H, s), 2.92 (1H, dq, *J* = 6.2, 6.6 Hz), 2.57 (2H, t, *J* = 7.0 Hz), 2.09 (3H, s), 1.92 (2H, tt, *J* = 7.0, 7.0 Hz), 1.42 (9H, s), 1.17 (3H, d, *J* = 6.9 Hz), 1.14 (2.7H, d, *J* = 6.2 Hz), 1.02–1.10 (3.3H, m); IR (CH_2Cl_2) ν_{max} 3331, 1651, 1538, 1366, 1259, 1166, 1074, 736 cm^{-1}; FABHRMS (NBA–NaI)

m/z 709.2483 (M + Na^+, $C_{29}H_{46}N_6O_7S_3$ requires 709.2488).

***N*-[4(*R*)-(*tert*-Butyloxycarbonyl)amino-3(*S*)-hydroxy-2(*S*)-methylpentanoyl]-*N*-methyl-1-[(((3-(methylthio)-1-propylamino)carbonyl)-2′,4-bithazol-2-yl)-ethylamino]-D-*allo*-threonine (18)**. DMF (0.25 mL) was added to a mixture of **15** (15.1 mg, 41.7 μmol), **16** (17.0 mg, 49.6 μmol), EDCI (14.6 mg, 76.4 μmol), and HOBt (1.6 mg, 11.8 μmol) at 4 °C, and the mixture was stirred for 1 h. The reaction mixture was poured into water (1 mL), and extracted with EtOAc (3 × 2 mL). The organic layer was washed with H_2O (2 × 1 mL), saturated aqueous NaCl (1 mL), and dried (Na_2SO_4). PTLC (SiO_2, 10% CH_3OH–CH_2Cl_2) provided **18** (8.0 mg, 28.6 mg theoretical, 28%) as a white amorphous solid and **17** (10.0 mg, 35%). For **18**: R_f 0.4 (SiO_2, 10% CH_3OH–CH_2Cl_2); $[\alpha]^{25}_D$ +49 (*c* 0.5, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) mixture of rotamers δ 8.16 (1H, s), 8.14 (0.1H, s), 8.13 (0.9H, s), 4.68 (0.9H, d, *J* = 9.1 Hz), 4.15–4.29 (1.1H, m), 3.54–3.79 (4H, m), 3.51 (2H, t, *J* = 7.1 Hz), 3.28 (2H, t, *J* = 6.7 Hz), 2.99 (2.7H, s), 2.84 (1H, dq, *J* = 6.1, 6.3 Hz), 2.80 (0.3H, s), 2.58 (2H, t, *J* = 7.1 Hz), 2.09 (3H, s), 1.92 (2H, tt, *J* = 7.1, 7.1 Hz), 1.41 (9H, s), 1.13–1.20 (3.6H, m), 1.11 (2.7H, d, *J* = 6.8 Hz), 1.07 (2.7H, d, *J* = 6.3 Hz); IR (CH_2Cl_2) ν_{max} 3332, 1660, 1651, 1538, 1366, 1249, 1160, 1074, 735 cm^{-1}; FABHRMS (NBA–NaI) *m/z* 709.2482 (M + Na^+, $C_{29}H_{46}N_6O_7S_3$ requires 709.2488).

***N*-[4(*R*)-Amino-3(*S*)-hydroxy-2(*S*)-methylpentanoyl]-*N*-methyl-1-[(((3-(dimethylsulfonio)-1-propylamino)carbonyl)-2′,4-bithazol-2-yl)-1-ethylamino]-L-threonine (20)**. A solution of **17** (30.8 mg, 44.8 μmol) in CH_3OH (1.0 mL) was treated with CH_3I (140 μL, 2.24 mmol) at 25 °C, and the mixture was stirred for 44 h at 25 °C. After concentration with a N_2 stream, the residue of **19** was treated with 3 N HCl–EtOAc (1.0 mL) at 25 °C. The mixture was stirred for 1 h at 25 °C, and concentrated with a N_2 stream. The residue was triturated with CH_2Cl_2 (0.5 mL), and dried in vacuo. A solution of the residue in CH_3OH (1.0 mL) was treated with 29% aqueous NH_4OH (20 μL) at 25 °C, and stirred for 1 h at 25 °C. After concentration with a N_2 stream, chromatography (C-18, 0.6 cm × 5 cm, 0–15% CH_3OH–H_2O gradient elution) gave **20** (24.1 mg, 27.0 mg theoretical, 89%) as a white amorphous solid: R_f 0.5 (SiO_2, 10:9:1 CH_3OH–10% NH_4OAc–10% NH_4OH); $[\alpha]^{25}_D$ −9.8 (*c* 0.5, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) mixture of rotamers δ 8.23 (0.7H, s), 8.22 (0.7H, s), 8.21 (0.3H, s), 8.20 (0.3H, s), 4.80 (0.7H, d, *J* = 7.5 Hz), 4.26 (0.7H, dq, *J* = 6.3, 7.5 Hz), 4.12–4.20 (0.6H, m), 3.86–3.93 (1H, m), 3.56–3.78 (2H, m), 3.61 (2H, t, *J* = 6.4 Hz), 3.42 (2H, t, *J* = 7.6 Hz), 3.31–3.40 (1H, m), 3.29 (2H, t, *J* = 7.7 Hz), 3.19 (2.1H, s), 3.00 (0.9H, s), 2.97 (6H, s), 2.81–2.94 (1H, m), 2.17 (2H, tt, *J* = 6.4, 7.6 Hz), 1.28 (2.1H, d, *J* = 6.8 Hz), 1.21 (0.9H, d, *J* = 6.8 Hz), 1.11–1.19 (6H, m); IR (KBr) ν_{max} 3406, 1654, 1547, 1294, 1100, 811 cm^{-1}; FABHRMS (NBA) *m/z* 601.2304 (M^+, $C_{25}H_{41}N_6O_5S_3$ requires 601.2300).

***erythro*-*N*-(*tert*-Butyloxycarbonyl)-1-[[4(*S*)-((N-(1(*S*)-(((2-(4′-(((3-(dimethylsulfonio)-1-propyl)amino)carbonyl)-2′,4-bithiazol-2-yl)-1-ethyl)amino)carbonyl)-2(*R*)-hydroxy-1-propyl)-*N*-methylamino)carbonyl)-3-(*S*)-hydroxy-2(*R*)-pentyl]amino]-N^{im}-(triphenylmethyl)-β-hydroxy-L-histidine (22)**. A solution of **20** (4.5 mg, 7.5 μmol), **21** (5.1 mg, 10.0 μmol), and BOP Reagent (6.5 mg, 14.7 μmol) in DMF (100 μL) was treated with iPr_2NEt (3.5 μL, 20.0 μmol) at 0 °C. After stirring for 5 h at 25 °C, the reaction mixture was concentrated in vacuo. The residue was dissolved in CH_3OH (40 μL), and H_2O (160 μL) was added to the solution while stirring. The insoluble material was collected by filtration through a Celite pad. The crude insoluble material was dissolved and eluted in CH_3OH, and concentrated with a N_2 stream. Chromatography (C-18, 0.5 cm × 4 cm, 40–90% CH_3OH–H_2O gradient elution) gave **22** (3.8 mg, 8.2 mg theoretical, 46%) as a white amorphous solid: R_f 0.5 (SiO_2, 10:9:1 CH_3OH–10% NH_4OAc–10% NH_4OH); $[\alpha]^{25}_D$ −4.3 (*c* 0.3, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) mixture of rotamers δ 8.20 (0.15H, s), 8.19 (0.85H, s), 8.14 (0.85H, s), 8.10 (0.15H, s), 7.41 (1H, s), 7.32–7.39 (9H, m), 7.09–7.17 (6H, m), 6.91 (1H, s), 4.84 (0.85H, d, *J* = 8.3 Hz), 4.77 (0.85H, d, *J* = 7.0 Hz), 4.74 (0.15H, d, *J* = 7.0 Hz), 4.39 (0.15H, d, *J* = 8.3 Hz), 4.31 (0.85H, d, *J* = 7.0 Hz), 4.25 (0.15H, d, *J* = 7.0 Hz), 4.21 (1H, dq, *J* = 6.2, 8.3 Hz), 3.92 (0.85H, dq, *J* = 6.5, 6.5 Hz), 3.66–3.85 (0.3H, m), 3.63 (2H, t, *J* = 6.6 Hz), 3.58 (2H, t, *J* = 6.5 Hz), 3.36 (2H, t, *J* = 7.4 Hz), 3.26 (2H, t, *J* = 6.6 Hz), 3.13 (2.55H, s), 2.99 (1H, dq, *J* = 6.0, 6.6 Hz), 2.94 (0.45H, s), 2.93 (0.9H, s), 2.92 (5.1H, s), 2.13 (2H, tt, *J* = 6.5, 7.4 Hz), 1.38 (9H, s), 1.21 (0.45H, d, *J* = 6.0 Hz), 1.13 (2.55H, d, *J* = 6.2 Hz), 1.12 (2.55H, d, *J* = 6.5 Hz), 1.02–1.08 (3H, m), 0.98 (0.45H, d, *J* = 6.8 Hz); IR (KBr) ν_{max} 3416, 1654, 1546, 1161, 1130, 750 cm^{-1}; FABHRMS (NBA) *m/z* 1096.4415 (M^+, $C_{55}H_{70}N_9O_9S_3$ requires 1096.4458).

***erythro*-1-[[4(*S*)-((*N*-(1(*S*)-(((2-(4′-(((3-(Dimethylsulfonio)-1-propyl)amino)carbonyl)-2′,4-bithiazol-2-yl)-1-ethyl)amino)carbonyl)-2(*R*)-hydroxy-1-propyl)-*N*-methylamino)carbonyl)-3(*S*)-hydroxy-2(*R*)-pentyl]-amino]-β-hydroxy-L-histidine (23)**. A sample of **22** (5.2 mg, 4.7 μmol) was treated with 20% CF_3CO_2H–CH_2Cl_2 (0.5 mL) at 4 °C, and the mixture was stirred for 2 h at 4 °C. After concentration with a N_2 stream, the residue was treated with a solution of 29% aqueous NH_4OH (10 μL) in CH_3OH (0.2 mL). After stirring for 1 h at 25 °C, the mixture was concentrated with a N_2 stream. Chromatography (C-18, 0.5 cm × 4 cm, 0–20% CH_3OH–H_2O gradient elution) provided **23** (3.4 mg, 3.5 mg theoretical, 96%) as a white amorphous solid: R_f 0.1 (SiO_2, 10:9:1 CH_3OH–10% NH_4OAc–10% NH_4OH); $[\alpha]^{25}_D$ −8.1 (*c* 0.16, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) mixture of rotamers δ 8.22 (0.85H, s), 8.21 (0.15H, s), 8.14 (0.85H, s), 8.12 (0.15H, s), 7.71 (1H, d, *J* = 2.3 Hz), 7.11 (1H, d, *J* = 2.3 Hz), 4.98 (0.85H, d, *J* = 5.8 Hz), 4.92 (0.15H, d, *J* = 6.0 Hz), 4.85 (0.85H, d, *J* = 8.2 Hz), 4.15–4.26 (1H, m), 4.00 (0.85H, d, *J* = 5.8 Hz),

3.97 (0.15H, d, J = 6.0 Hz), 3.83–3.94 (1.15H, m), 3.64–3.77 (3H, m), 3.59 (2H, t, J = 6.4 Hz), 3.37 (2H, t, J = 7.2 Hz), 3.27 (2H, t, J = 6.9 Hz), 3.13 (2.55H, s), 2.99 (0.45H, s), 2.93 (6H, s), 2.86 (1H, dq, J = 6.8, 7.2 Hz), 2.14 (2H, tt, J = 6.4, 7.2 Hz), 1.22 (0.45H, d, J = 6.4 Hz), 1.18 (2.55H, d, J = 6.8 Hz), 1.13 (2.55H, d, J = 6.2 Hz), 1.12 (0.45H, d, J = 6.6 Hz), 1.03 (0.45H, d, J = 6.8 Hz), 1.00 (2.55H, d, J = 6.8 Hz); IR (KBr) ν_{max} 3372, 1654, 1550, 1401, 1101, 748 cm^{-1}; ESMS m/z 754 (M^+, $C_{31}H_{48}N_9O_7S_3$).

N^{α}-(*tert*-Butyloxycarbonyl)-N^{β}-[3(*S*)-[4-amino-6-[[[1(*S*)-(((4(*S*)-((N-(1(*S*)-(((2-(4′-(((3-(dimethylsulfonio)-1-propyl)amino)carbonyl)-27prime;,4-bithiazol-2-yl)-1-ethyl)amino)carbonyl)-2(*R*)-hydroxy-1-propyl)-*N*-methylamino)carbonyl)-3(*S*)-hydroxy-2-(*R*)-pentyl)amino)carbonyl)-2(*R*)-(4-imidazolyl)-2(*R*)-hydroxy-1-ethyl]amino]carbonyl]-5-methylpyrimidin-2-yl]-1-amino-1-oxo-3-propyl]-(*S*)-β-aminoalanine amide (25). DPPA (1.0 μL, 4.8 μmol) and Et_3N (1.1 μL, 7.9 μmol) were added to a suspension of **24** (2.0 mg, 4.7 μmol) and **23** (2.5 mg, 3.3 μmol) in DMF (50 μL) at 25 °C, and the mixture was stirred for 15 h at 25 °C. After concentration in vacuo, chromatography (C-18, 0.5 cm × 3 cm, 0–60% CH_3OH–H_2O gradient elution) provided **25** (2.7 mg, 3.8 mg theoretical, 70%) as a white amorphous solid: R_f 0.4 (SiO_2, 10:9:1 CH_3OH–10% NH_4OAc–10% NH_4OH); $[\alpha]^{25}_D$ −3.3 (c 0.15, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) mixture of rotamers δ 8.19 (0.75H, s), 8.17 (0.25H, s), 8.14 (0.75H, s), 7.98 (0.25H, s), 7.71 (0.25H, s), 7.69 (0.75H, s), 7.14 (0.75H, s), 7.12 (0.25H, s), 5.17 (0.75H, d, J = 6.5 Hz), 5.08 (0.25H, d, J = 7.0 Hz), 4.83 (0.75H, d, J = 8.2 Hz), 4.73 (0.75H, d, J = 6.5 Hz), 4.56 (0.25H, d, J = 7.0 Hz), 4.51 (0.25H, d, J = 7.6 Hz), 4.20 (0.75H, dq, J = 6.1, 8.2 Hz), 4.07–4.25 (1.25H, m), 3.82–3.91 (2H, m), 3.58 (2H, t, J = 6.2 Hz), 3.54–3.74 (3H, m), 3.35 (2H, t, J = 7.2 Hz), 3.28 (2H, t, J = 6.4 Hz), 3.11 (2.25H, s), 3.00 (0.75H, s), 2.93 (1.5H, s), 2.92 (4.5H, s), 2.71–2.88 (3H, m), 2.62 (1H, dd, J = 5.2, 14.4 Hz), 2.51 (1H, dd, J = 8.8, 14.4 Hz), 2.26 (2.25H, s), 2.17 (0.75H, s), 2.15 (2H, tt, J = 6.2, 7.2 Hz), 1.43 (9H, s), 1.24 (0.75H, d, J = 6.2 Hz), 1.09–1.17 (5.25H, m), 1.06 (2.25H, d, J = 6.4 Hz), 0.94 (0.75H, d, J = 6.9 Hz); IR (KBr) ν_{max} 3393, 1654, 1490, 1255, 1098, 778 cm^{-1}; FABHRMS (NBA) m/z 1161.4689 (M^+, $C_{48}H_{73}N_{16}O_{12}S_3$ requires 1161.4756).

N^{β}-[3(*S*)-[4-Amino-6-[[[1(*S*)-(((4(*S*)-((N-(1(*S*)-(((2-(4′-(((3-(dimethylsulfonio)-1-propyl)amino)carbonyl)-2′,4-bithiazol-2-yl)-1-ethyl)amino)carbonyl)-2(*R*)-hydroxy-1-propyl)-*N*-methylamino)carbonyl)-3(*S*)-hydroxy-2(*R*)-pentyl)amino)carbonyl)-2(*R*)-(4-imidazolyl)-2(*R*)-hydroxy-1-ethyl]amino]carbonyl]-5-methylpyrimidin-2-yl]-1-amino-1-oxo-3-propyl]-(*S*)-β-aminoalanine amide (5). A sample of **25** (1.5 mg, 1.3 μmol) was treated with 20% CF_3CO_2H–CH_2Cl_2 (0.2 mL) at 4 °C, and the mixture was stirred for 2 h at 4 °C. After concentration with a N_2 stream, chromatography (C-18, 0.5 cm × 3 cm, 0–30% CH_3OH–H_2O gradient elution) provided **5** (1.1 mg, 1.4 mg theoretical, 80%) as a white amorphous solid: R_f 0.1 (SiO_2, 10:9:1 CH_3OH–10% NH_4OAc–10% NH_4OH); $[\alpha]^{25}_D$ −12 (c 0.05, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) mixture of rotamers δ 8.20 (0.7H, s), 8.18 (0.3H, s), 8.14 (0.7H, s), 8.00 (0.3H, s), 7.70 (0.3H, d, J = 1.1 Hz), 7.68 (0.7H, d, J = 1.1 Hz), 7.13 (0.7H, brs), 7.11 (0.3H, brs), 5.15 (0.7H, d, J = 6.7 Hz), 5.07 (0.3H, d, J = 6.8 Hz), 4.83 (0.7H, d, J = 8.4 Hz), 4.75 (0.7H, d, J = 6.7 Hz), 4.58 (0.3H, d, J = 6.8 Hz), 4.49 (0.3H, d, J = 8.4 Hz), 4.20 (0.7H, dq, J = 6.3, 8.4 Hz), 4.18–4.27 (0.3H, m), 3.83–3.99 (2H, m), 3.59–3.73 (4H, m), 3.58 (2H, t, J = 6.5 Hz), 3.34–3.39 (2H, m), 3.27 (2H, t, J = 6.6 Hz), 3.11 (2.1H, s), 2.99 (0.9H, s), 2.93 (1.8H, s), 2.92 (4.2H, s), 2.63 (1H, dd, J = 5.1, 14.8 Hz), 2.58–2.75 (3H, m), 2.51 (1H, dd, J = 8.8, 14.8 Hz), 2.26 (2.1H, s), 2.18 (0.9H, s), 2.10–2.17 (2H, m), 1.23 (0.9H, d, J = 6.3 Hz), 1.19 (0.9H, d, J = 6.5 Hz), 1.11–1.16 (4.2H, m), 1.05 (2.1H, d, J = 6.8 Hz), 0.95 (0.9H, d, J = 6.7 Hz); IR (KBr) ν_{max} 3355, 1664, 1562, 1207, 1095, 779 cm^{-1}; FABHRMS (NBA) m/z 1061.4220 (M^+, $C_{43}H_{65}N_{16}O_{10}S_3$) requires 1061.4231.

***N*-[4(*R*)-Amino-3(*S*)-hydroxy-2(*S*)-methylpentanoyl]-*N*-methyl-1-[(((3-(dimethylsulfonio)-1-propylamino)carbonyl)-2′,4-bithazol-2-yl)-1-ethylamino]-D-*allo*-threonine (27).** A solution of **18** (22.9 mg, 33.3 μmol) in CH_3OH (0.7 mL) was treated with CH_3I (104 μL, 1.7 mmol) at 25 °C and the mixture was stirred for 44 h at 25 °C. After concentration with a N_2 stream, the residue was treated with 3 N HCl–EtOAc (0.7 mL) at 25 °C. The mixture was stirred for 1 h at 25 °C, and concentrated with a N_2 stream. The residue was triturated with CH_2Cl_2 (0.5 mL), and dried in vacuo. A solution of the residue in CH_3OH (1.0 mL) was treated with 29% aqueous NH_4OH (20 μL) at 25 °C, and stirred for 1 h at 25 °C. After concentration with a N_2 stream, chromatography (C-18, 0.6 cm × 5 cm, 0–15% CH_3OH–H_2O gradient elution) gave **27** (18.7 mg, 20.0 mg theoretical, 93%) as a white amorphous solid: R_f 0.4 (SiO_2, 10:9:1 CH_3OH–10% NH_4OAc–10% NH_4OH); $[\alpha]^{25}_D$ +39 (c 0.5, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) mixture of rotamers δ 8.22 (0.2H, s), 8.215 (0.8H, s), 8.17 (0.2H, s), 8.15 (0.8H, s), 4.39 (0.8H, d, J = 9.1 Hz), 4.33 (0.8H, dq, J = 6.1, 9.1 Hz), 4.14–4.27 (0.4H, m), 3.84 (0.8H, dd, J = 2.6, 9.4 Hz), 3.63–3.93 (2.2H, m), 3.60 (2H, t, J = 6.5 Hz), 3.38 (2H, t, J = 7.4 Hz), 3.31–3.36 (1H, m), 3.28 (2H, t, J = 7.4 Hz), 3.07 (2.4H, s), 2.94 (6H, s), 2.76 (0.6H, s), 2.72–2.83 (1H, m), 2.15 (2H, tt, J = 6.5, 7.4 Hz), 1.27 (0.6H, d, J = 6.9 Hz), 1.24 (2.4H, d, J = 6.8 Hz), 1.21 (0.6H, d, J = 6.0 Hz), 1.18 (2.4H, d, J = 6.8 Hz), 1.13 (3H, d, J = 6.1 Hz); IR (KBr) ν_{max} 3415, 1647, 1546, 1294, 1100 cm^{-1}; FABHRMS (NBA) m/z 601.2302 (M^+, $C_{25}H_{41}N_6O_5S_3$ requires 601.2300).

***erythro*-N^{α}-(*tert*-Butyloxycarbonyl)-1-[[4(*S*)-((N-(1(*R*)-(((2-(4′-(((3-(dimethylsulfonio)-1-propyl)amino)carbonyl)-2′,4-bithiazol-2-yl)-1-ethyl)amino)-carbonyl)-2(*R*)-hydroxy-1-propyl)-*N*-methylamino)-carbonyl)-3(*S*)-hydroxy-2(*R*)-pentyl]amino]-N^{im}-(triphenylmethyl)-β-hydroxy-L-histidine (28).** A solution

of **27** (16.5 mg, 27.4 μmol), **21** (16.5 mg, 32.1 μmol), and BOP reagent (21.9 mg, 49.5 μmol) in DMF (250 μL) was treated with iPr$_2$NEt (10.5 μL, 60.3 μmol) at 0 °C. After stirring for 9 h at 25 °C, the reaction mixture was concentrated in vacuo. The residue was dissolved in CH$_3$OH (0.3 mL), and H$_2$O (1.2 mL) was added to the solution while stirring. The insoluble material was collected by filtration through a Celite pad. The insoluble material was dissolved and eluted in CH$_3$OH, and concentrated with a N$_2$ stream. Chromatography (C-18, 1 cm × 2.5 cm, 40–90% CH$_3$OH–H$_2$O gradient elution) gave **28** (13.0 mg, 30.1 mg theoretical, 43%) as a white amorphous solid: R_f 0.5 (SiO$_2$, 10:9:1 CH$_3$OH–10% NH$_4$OAc–10% NH$_4$OH); $[\alpha]^{25}_D$ +36 (*c* 0.3, CH$_3$OH); ^{1}H NMR (CD$_3$OD, 400 MHz) mixture of rotamers δ 8.20 (0.8H, s), 8.17 (0.2H, s), 8.11 (0.8H, s), 8.09 (0.2H, s), 7.40 (1H, s), 7.32–7.39 (9H, m), 7.08–7.17 (6H, m), 6.90 (1H, s), 4.72–4.77 (1H, m), 4.62 (0.8H, d, J = 9.2 Hz), 4.21–4.31 (2.2H, m), 3.91 (1H, dq, J = 6.6, 6.8 Hz), 3.62–3.77 (3H, m), 3.59 (2H, t, J = 6.4 Hz), 3.37 (2H, t, J = 7.5 Hz), 3.27 (2H, t, J = 6.6 Hz), 3.00 (2.4H, s), 2.93 (6H, s), 2.90–2.97 (1H, m), 2.82 (0.6H, s), 2.15 (2H, tt, J = 6.4, 7.5 Hz), 1.40 (1.8H, s), 1.37 (7.2H, s), 1.13 (3H, d, J = 6.3 Hz), 1.04–1.10 (3.6H, m), 1.02 (2.4H, d, J = 6.8 Hz); IR (KBr) ν_{max} 3414, 1655, 1543, 1160, 1128, 750 cm^{-1}; FABHRMS (NBA) m/z 1096.4499 (M$^+$, $C_{55}H_{70}N_9O_9S_3$ requires 1096.4458).

***erythro*-1-[[4(*S*)-((*N*-(1(*R*)-(((2-(4′-(((3-(Dimethylsulfonio)-1-propyl)amino)carbonyl)-2′,4-bithiazol-2-yl)-1-ethyl)amino)carbonyl)-2(*R*)-hydroxy-1-propyl)-*N*-methylamino)carbonyl)-3(*S*)-hydroxy-2(*R*)-pentyl]-amino]-β-hydroxy-L-histidine (29)**. A sample of **28** (6.0 mg, 5.5 μmol) was treated with 20% CF$_3$CO$_2$H–CH$_2$Cl$_2$ (0.5 mL) at 4 °C, and the mixture was stirred for 2 h. After concentration with a N$_2$ stream, the residue was treated with a solution of 29% aqueous NH$_4$OH (15 μL) in CH$_3$OH (0.3 mL). After stirring for 1 h at 25 °C, the mixture was concentrated with a N$_2$ stream. Chromatography (C-18, 0.5 cm × 4 cm, 0–20% CH$_3$OH–H$_2$O gradient elution) provided **29** (4.0 mg, 4.2 mg theoretical, 96%) as a white amorphous solid: R_f 0.1 (SiO$_2$, 10:9:1 CH$_3$OH–10% NH$_4$OAc–10% NH$_4$OH); $[\alpha]^{25}_D$ +43 (*c* 0.2, CH$_3$OH); ^{1}H NMR (CD$_3$OD, 400 MHz) mixture of rotamers δ 8.21 (1H, s), 8.13 (0.17H, s), 8.12 (0.83H, s), 7.69 (1H, s), 7.07 (1H,s), 4.65 (0.83H, d, J = 9.2 Hz), 4.25 (0.83H, dq, J = 6.3, 9.2 Hz), 4.14–4.25 (0.34H, m), 3.91 (0.83H, dq, J = 5.1, 6.6 Hz), 3.81 (0.83H, d, J = 6.2 Hz), 3.63–3.79 (3.34H, m), 3.60 (2H, t, J = 6.4 Hz), 3.37 (2H, t, J = 7.5 Hz), 3.28 (2H, t, J = 6.6 Hz), 3.00 (2.49H, s), 2.93 (6H, s), 2.83 (0.51H, s), 2.28 (1H, dq, J = 6.2, 6.7 Hz), 2.15 (2H, tt, J = 6.4, 7.5 Hz), 1.12–1.16 (3.51H, m), 1.11 (2.49H, d, J = 6.8 Hz), 1.05 (0.51H, d, J = 6.5 Hz), 1.01 (2.49H, d, J = 6.7 Hz); IR (KBr) ν_{max} 3415, 1654, 1570, 1421, 1124 cm^{-1}; ESMS m/z 754 (M$^+$, $C_{31}H_{48}N_9O_7S_3$).

***N*$^{\alpha}$-(*tert*-Butyloxycarbonyl)-*N*$^{\beta}$-[3(*S*)-[4-amino-6-[[[1(*S*)-(((4(*S*)-((*N*-(1(*R*)-(((2-(4′-(((3-(dimethylsulfonio)-1-propyl)amino)carbonyl)-2′,4-bithiazol-2-yl)-1-ethyl)amino)carbonyl)-2(*R*)-hydroxy-1-propyl)-*N*-methylamino)carbonyl)-3(*S*)-hydroxy-2(*R*)-pentyl)amino)carbonyl)-2(*R*)-(4-imidazolyl)-2(*R*)-hydroxy-1-ethyl]amino]carbonyl]-5-methylpyrimidin-2-yl]-1-amino-1-oxo-3-propyl]-(*S*)-β-aminoalanine amide (30)**. DPPA (2.1 μL, 9.5 μmol), and Et$_3$N (2.7 μL, 19.1 μmol) were added to a suspension of **24** (2.7 mg, 6.4 μmol) and **23** (4.0 mg, 5.3 μmol) in DMF (50 μL) at 0 °C, and the mixture was stirred for 15 h at 25 °C. After concentration in vacuo, chromatography (C-18, 0.5 cm × 4 cm, 0–50% CH$_3$OH–H$_2$O gradient elution) provided **30** (4.4 mg, 6.2 mg theoretical, 71%) as a white amorphous solid: R_f 0.4 (SiO$_2$, 10:9:1 CH$_3$OH–10% NH$_4$OAc–10% NH$_4$OH); $[\alpha]^{25}_D$ +18.5 (*c* 0.2, CH$_3$OH); ^{1}H NMR (CD$_3$OD, 400 MHz) mixture of rotamers δ 8.19 (0.2H, s), 8.18 (0.8H, s), 8.08 (0.2H, s), 8.04 (0.8H, s), 7.75 (0.2H, s), 7.72 (0.8H, s), 7.16 (0.2H, s), 7.14 (0.8H, s), 5.07–5.15 (1H, m), 4.69 (0.8H, d, J = 7.2 Hz), 4.67 (0.8H, d, J = 9.3 Hz), 3.94 (1H, dq, J = 6.5, 6.8 Hz), 3.52–3.85 (6H, m), 3.36 (2H, t, J = 7.2 Hz), 3.23–3.29 (2H, m), 3.02 (2.4H,s), 2.92 (4.8H, s), 2.90 (1.2H, s), 2.83 (0.6H, s), 2.81–3.01 (3H, m), 2.73 (1H, dd, J = 4.8, 15.4 Hz), 2.58 (1H, dd, J = 8.7, 15.4 Hz), 2.26 (2.4H, s), 2.21 (0.6H, s), 2.14 (2H, tt, J = 6.6, 7.2 Hz), 1.44 (9H, s), 1.13 (3H, d, J = 6.2 Hz), 1.09 (3H, d, J = 6.5 Hz), 1.03 (3H, d, J = 6.8 Hz); IR (KBr) ν_{max} 3358, 1670, 1558, 1407, 1251, 1165 cm^{-1}; FABHRMS (NBA) m/z 1161.4692 (M$^+$, $C_{48}H_{73}N_{16}O_{12}S_3$ requires 1161.4756).

***N*$^{\beta}$-[3(*S*)-[4-Amino-6-[[[1(*S*)-(((4(*S*)-((*N*-(1(*S*)-(((2-(4′-(((3-(dimethylsulfonio)-1-propyl)amino)carbonyl)-2′,4-bithiazol-2-yl)-1-ethyl)amino)carbonyl)-2(*R*)-hydroxy-1-propyl)-*N*-methylamino)carbonyl)-3-(*S*)-hydroxy-2(*R*)-pentyl)amino)carbonyl)-2(*R*)-(4-imidazolyl)-2(*R*)-hydroxy-1-ethyl]amino]carbonyl]-5-methylpyrimidin-2-yl]-1-amino-1-oxo-3-propyl]-(*S*)-β-aminoalanine amide (6)**. A sample of **30** (4.2 mg, 3.6 μmol) was treated with 20% CF$_3$CO$_2$H–CH$_2$Cl$_2$ (0.2 mL) at 4 °C, and the mixture was stirred for 2 h. After concentration with a N$_2$ stream, chromatography (C-18, 0.5 cm × 3 cm, 0–40% CH$_3$OH–H$_2$O gradient elution) provided **6** (2.6 mg, 3.8 mg theoretical, 68%) as a white amorphous solid: R_f 0.1 (SiO$_2$, 10:9:1 CH$_3$OH–10% NH$_4$OAc–10% NH$_4$OH); $[\alpha]^{25}_D$ +8.3 (*c* 0.06, CH$_3$OH); ^{1}H NMR (CD$_3$OD, 400 MHz) mixture of rotamers δ 8.19 (0.8H, s), 8.18 (0.2H, s), 8.10 (0.8H, s), 8.03 (0.2H, s), 7.70 (0.2H, d, J = 1.1 Hz), 7.68 (0.8H, d, J = 1.1 Hz), 7.13 (0.2H, d, J = 1.1 Hz),), 7.11 (0.8H, d, J = 1.1 Hz), 5.08 (1H, d, J = 6.9 Hz), 4.71 (0.8H, d, J = 6.9 Hz), 4.64 (0.8H, d, J = 9.3 Hz), 4.63 (0.2H, d, J = 6.5 Hz), 4.25 (0.8H, dq, J = 6.2, 9.3 Hz), 4.20–4.31 (0.4H, m), 3.62–4.01 (6H, m), 3.59 (2H, t, J = 6.4 Hz), 3.37 (2H, t, J = 7.5 Hz), 3.27 (2H, t, J = 6.2 Hz), 3.03 (2.4H, s), 2.93 (6H, s), 2.86–3.01 (3H, m), 2.93 (1.8H, s), 2.81 (0.6H, s), 2.72 (1H, dd, J = 4.1, 15.4 Hz), 2.49 (1H, dd, J = 10.1, 15.4 Hz), 2.26 (2.4H, s), 2.23 (0.6H, s), 2.14 (2H, tt, J = 6.4, 7.5 Hz), 1.14 (3H, d, J = 6.2 Hz), 1.07 (3H, d, J = 6.7 Hz), 1.06 (3H, d, J = 7.0 Hz); IR (KBr) ν_{max} 3370, 1663, 1490, 1207, 1095, 778 cm^{-1};

FABHRMS (NBA) *m/z* 1061.4239 (M^+, $C_{43}H_{65}N_{16}O_{10}S_3$ requires 1061.4231).

General procedure for the supercoiled ΦX174 RFI DNA cleavage reactions: 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 the 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 supercoiled 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 32 μM Fe(II) control, 1.0, 2.0 and 4.0 μM deglycobleomycin A_2, 8.0, 16.0 and 32.0 μM **5**, and 8.0, 16.0 and 32.0 μM **6**. The DNA reaction solutions were 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 at 50 V for 3 h, and the gel was stained with 0.1 μg/mL ethidium bromide, visualized on a UV transilluminator and photographed using Polaroid T667 black-and-white instant film (Figure 2). Direct fluorescence quantitation of DNA in the presence of ethidium bromide was conducted using a Millipore BioImage 60S RFLP system visualized on a UV (312 nm) transilluminator taking into account the relative fluorescence intensities of forms I–III (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 RFI DNA cleavage

The Fe(II) complex was formed by mixing 15 μL of aqueous 240 μM **5** or **6** solution with 15 μL of a freshly prepared 240 μM aqueous $Fe(NH_4)_2(SO_4)_2$ solution, respectively. Analogous to studies with **1** and **3**, the mixture was incubated at 25 °C for 30 min. The DNA cleavage reaction was initiated by adding to the Fe(II) complex 120 μL of DNA–thiol mixture containing 105 μL of a buffered supercoiled ΦX174 RFI DNA (1.4×10^{-8} M) in 50 mM Tris–HCl buffer solution (pH 8) and 15 μL of aqueous 10 mM 2-mercaptoethanol. The final concentration of **5** and **6** employed in the study was both 24 μM. The solution was incubated at 25 °C, quenched by pipetting out a 10 μL aliquot and adding it into 5 μL of loading buffer at 1, 2, 4, 6, 10, 15, 20, 30, and 40 min, and electrophoresis was run on a 1% agarose gel at 50 V for 3 h. Direct fluorescence quantitation of the DNA in the presence of ethidium bromide was conducted using a Millipore BioImage 60S RFLP system taking into account the relative fluorescence intensities of forms I–III ΦX174 RFI DNA (forms II and III fluorescence intensities are 0.7 times that of form I). The ratio of double to single strand DNA cleavage was calculated with use of the Freifelder–Trumbo equation[50] assuming a Poisson distribution and the results are summarized in Table 1. For **5**, the ratio was established to be 1:58 at 24 μM. For **6**, the ratio was established to be 1:53 at 24 μM.

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

All reactions were run with freshly prepared Fe(III) complexes. The Fe(III) complexes were prepared by combining 1 μL of a H_2O solution of the agent at 10 times the specified concentration with 1 μL of a freshly prepared equimolar aqueous $FeCl_3$ solution. Each of the Fe(III) complex solutions were treated with 7 μL of a buffered DNA solution (10 mM Na_2HPO_4–NaH_2PO_4, pH 7.0 containing 10 mM KCl) containing the ^{32}P 5′-end-labeled w794 DNA.[52] The final concentrations of the agents employed in the study were 256 μM control Fe(III), 4.0 and 8.0 μM deglycobleomycin A_2, 32, 64, and 128 μM **5** and **6**. The DNA cleavage reactions were initiated by adding 1 μL of 50% aqueous H_2O_2. The DNA reaction solutions were incubated at 4 °C for 90 min. The reactions were quenched with the addition of 1 μL of 50% aqueous glycerol followed by EtOH precipitation and isolation of the DNA. The DNA was resuspended in 6 μL of TE buffer (pH 8.0), and formamide dye (6 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*-methylenebisacrylamide, 8 M urea) at 40 W for 3 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. Autoradiography of the 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 BioImage 60S RFLP system measuring the remaining uncleaved DNA and the values recorded in Table 1 are the average of four experiments.

General procedure for the oxidation of styrene

A solution of 25 μL of CH_3OH at 0 °C was treated with 5 μL of a 28 mM methanolic solution of ethyl benzoate (internal standard), 10 μL of a 0.25 M methanolic solution of styrene, and 5 μL of a 5 mM aqueous solution of Fe(III)-**3**, **5**, or **6**. The reaction was initiated

by the addition of 5 μL of 0.3 M aqueous H_2O_2. The reaction mixture was stirred at 0 °C for 1.5 h, diluted with 400 μL of H_2O, and extracted with 100 μL of $CHCl_3$. The $CHCl_3$ extract was analyzed by GC using the following temperature program at a He gas flow of 15 psi: initial temperature 45 °C/2 min; rate of 5 °C/min; final temperature of 150 °C/5 min. Under these conditions, the observed retention times were as follows: styrene, 7.06 min; phenylacetaldehyde, 13.33 min; styrene oxide, 13.58 min; ethyl benzoate, 16.36 min. A control was run under the same conditions with Fe(III)-**3** or with Fe(III)/H_2O_2 but no agent and no reaction occurred. GC was performed on a Hewlett Packard 5890 gas chromatograph equipped with J & W Scientific DB-1, 0.25 μm capillary column equipped with a flame ionization detector. The reactions were run in duplicate, quantitated by comparison with the internal standard, and the average is recorded in Table 2.

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