NMR Studies of Co·Deglycobleomycin A2 Green and Its Complex with d(CCAGGCCTGG)

Wei Wu,[‡] Dana E. Vanderwall,[§] Shuji Teramoto,[||] Siu Man Lui,[‡] Silvia T. Hoehn,[‡] Xue-Jun Tang,[⊥,£] Christopher J. Turner,[†] Dale L. Boger,*[,||] John W. Kozarich,*[,§] and JoAnne Stubbe*[,‡]

Contribution from the Departments of Chemistry and Biology and Francis Bitter Magnet Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, Merck Research Laboratory, P.O. Box 2000, Rahway, New Jersey 07065-0900, Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, and Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461

Received June 26, 1997. Revised Manuscript Received November 5, 1997

Abstract: NMR studies of the hydroperoxide form of cobalt deglycoBleomycin A2 (HOO−CodGBLM) and its complex with oligonucleotide d(CCAGG*C*CTGG) (**1**, *C* is the site of cleavage) are presented in an effort to establish deglycoBLM as a prototype for studies with BLM analogues, synthesized without the sugar moieties (Boger et al. *Bioorg. Med. Chem.* **1995**, *3*, 1281−1295). The structure determination of free HOO−CodGBLM has been hampered by the lack of NOE or ROE information. By direct comparison of the chemical shifts and coupling constants of HOO−CodGBLM with its glycosylated parent HOO−CoBLM, the former is shown to share many global structural features with the latter, including the nature of axial ligands (a point recently disputed by Caceres-Cortes et al. *Eur. J. Biochem.* **1997**, *244*, 818−828), the screw sense of metal-coordinating ligands, and the folding of the peptide linker region. The structure of HOO−CodGBLM bound to **1** is reported based on molecular modeling using NMR constraints (39 intermolecular and 44 intramolecular NOEs). The intercalative mode of binding of the bithiazole tail 3′ to the cleavage site at C6, the sequence specific recognition of guanine 5′ to the cleavage site by the pyrimidine of HOO−CodGBLM, and the proximity of the hydroperoxide ligand to the 4′ hydrogen of the cleavage site are almost identical to those previously reported for HOO−CoBLM bound to **1** (Wu et al. *J. Am. Chem. Soc.* **1996**, *118*, 1281−1294). The general conformity between the two structures provides strong support for a similar coordination environment of the cobalt and a structural basis for the observed similarity in the DNA cleavage specificity at G*Py* sites by BLM and deglycoBLM. However, observation of minor complex(es), selective broadening of certain protons associated with the A3·T18 base pair, a K_d for **1** of 5.9 μM, 35-fold greater than its parent, and an altered ratio of single strand to double strand cleavage, define the importance of the sugars' nonspecific interactions with DNA to the efficiency and selectivity of cleavage.

Introduction

The bleomycins (BLMs) belong to a family of glycopeptide derived antibiotics that are clinically used in the treatment of squamous cell carcinomas, lymphomas, and testicular cancer.[1] The cytotoxicity of the BLMs is generally believed to be associated with their abilities to inflict both single strand (ss) and double strand (ds) breaks in chromosomal DNA with the latter thought to be more cytotoxic due to the difficulties associated with ds-break repair.[2] Recent studies, however, have also demonstrated that RNAs (t, m, and r) are susceptible to degradation by BLM, albeit at low frequency, requiring that these targets must also be considered as potential contributors to the observed cytotoxic effects.[3] DNA cleavage mediated by BLM requires the presence of a metal and O_2 as cofactors and has been found to be both sequence specific for pyrimidines in d(G*Py*) sequences and site specific with cleavage being initiated at the 4′ position of the deoxyribose of the *Py*.[4,5] Two-dimensional NMR spectroscopy has recently been used to investigate the structural basis for these observations.[6−13] These studies have led to disagreements about the coordination chemistry of

* To whom correspondence should be addressed.
† Francis Bitter Magnet Laboratory, MIT.
‡ Departments of Chemistry and Biology, MIT.
§ Merck Research Laboratory.
⊥ Albert Einstein College of Medicine.
|| The Scripps Research Institute.
£ Present address: Wyeth-Ayerst Research, 401 North Middletown Road, Pearl River, NY 10965.

(1) (a) *Bleomycin Chemotherapy*; Sikic, B. I., Rozencweig, M., Carter, S. K., Ed.; Academic Press: Orlando, FL, 1985. (b) Umezawa, H.; Takita, T. In *Structure and Bonding*; Dunitz, J. D., Hemmerich, P., Jørgensen, C. K., Reiner, D., Goodenough, J. B., Ibers, J. A., Neilands, J. B., Williams, R. J. P., Ed.; Springer-Verlag: New York, 1980; Vol. 40, pp 73−99. (c) Hecht, S. M. *Summary of the bleomycin symposium*; Springer-Verlag: New York, 1979. (d) Lazo, J. S.; Sebi, S. M.; Schellens, J. H. *Cancer Chemother. Biol. Response Modif.* **1996**, *16*, 39−47. (e) Mir, L. M.; Tounekti, O.; Orlowski, S. *Gen. Pharmacol.* **1996**, *27*, 745−748.

(2) (a) Povirk, L. F.; Austin, M. J. F. *Mut. Res.* **1991**, *257*, 127−143. (b) Povirk, L. F. *Mut. Res.* **1996**, *355*, 71−89. (c) Povirk, L. F. in *Molecular Aspects of Anti-Cancer Drug Action*; Neidle Waring Ed.; 1983; Vol. pp 157−181.

(3) Hecht, S. M. *Bioconjugate Chem.* **1994**, *5*, 513−526.

(4) Giloni, L.; Takeshita, M.; Johnson, F.; Iden, C.; Grollman, A. P. *J. Biol. Chem.* **1981**, *256*, 8608−8615.

(5) Stubbe, J.; Kozarich, J. W. *Chem. Rev.* **1987**, *87*, 1107−1136.

(6) Manderville, R. A.; Ellena, J. F.; Hecht, S. M. *J. Am. Chem. Soc.* **1994**, *116*, 10851−10852.

(7) Manderville, R. A.; Ellena, J. F.; Hecht, S. M. *J. Am. Chem. Soc.* **1995**, *117*, 7891−7903.

S0002-7863(97)02125-2 CCC: $15.00
Published on Web 02/26/1998

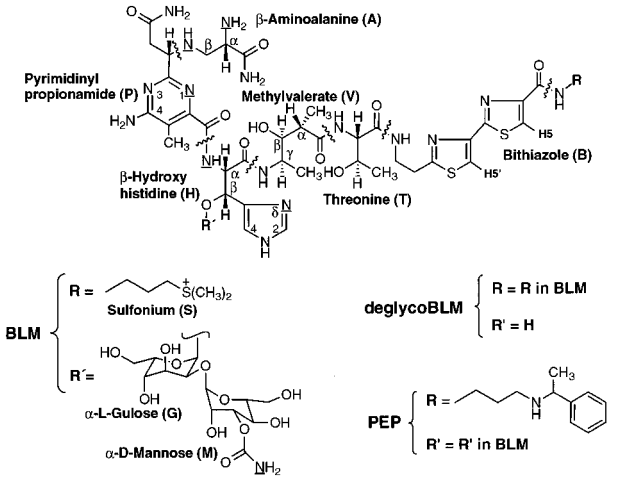

Figure 1. Structure of Bleomycin A2 (BLM), deglycoBLM, and pepleomycin (PEP). Nitrogen ligands coordinating to the metal are underlined.

BLM and deglycoBLM (Figure 1 with R′ = H) and their mode(s) of binding leading to DNA strand scission.[7,10,11,14−20] In an effort to address both of these problems, this paper reports the NMR studies of the cobalt hydroperoxide derivative of deglycoBLM A2 (HOO−CodGBLM) and its complex with d(CCAGGCCTGG) (**1**).

We and others have recently carried out two-dimensional NMR studies of the hydroperoxide form of cobalt BLM A2 (HOO−CoBLM) bound sequence specifically to several oligonucleotides.[8−13,21,22] The HOO−CoBLM was chosen as it was proposed to be a stable analogue of the hydroperoxide form of iron BLM (HOO−FeBLM, activated BLM), the last detectable reactive species in the iron-mediated DNA degradative process.[23−25] The rational for using this metallo-BLM has been previously described.[10,11,21] These NMR studies revealed a picture of the interaction of this metallo-BLM with DNA that accounts for the available chemical and biochemical data on FeBLM's binding, sequence specificity, and cleavage chemistry and the importance of the entire metallo-BLM molecule to these processes. A comprehensive understanding of the interaction of activated BLM with DNA is clearly essential for explaining such complex phenomena like the sequence specific double strand (ds)-cleavage by a single FeBLM.[12,26−29]

One of the most poorly understood components of metallo-BLMs has been the function of their disaccharide moiety consisting of α-L-gulose and 3′-carbamoyl-α-D-mannose connected to the hydroxyl group of the β-hydroxyhistidine moiety (Figure 1). The ability to convert BLM to its aglycon[30] has allowed chemical, biochemical, and in vivo studies of this molecule in an effort to define the function of the sugars.[31−47] A comparison of spectroscopic properties of BLM and deglycoBLMs by NMR and EPR have led to a controversy over whether the carbamoyl group of the mannose in the FeBLM is a ligand to the metal.[15,18,34,35,48] The controversy has most recently been extended to the CoBLMs with a report comparing the cobalt analogues of pepleomycin (HOO−CoPEP and HOO−CodGPEP, Figure 1) using NMR methods.[16] The claim is made that with HOO−CoPEP, the amino group of the carbamoyl moiety is an axial ligand, while with HOO−CodGPEP, the primary amine of β-aminoalanine is the axial ligand.[16] The former results contrast with our recent proposal with HOO−CoBLM, that the primary amine of β-aminoalanine is the axial ligand.[10]

One might expect if the coordination environments of BLM and its aglycon were different, these differences would be reflected in the ability of the cogeners to bind and cleave DNA. However, there is general agreement that deglycoBLMs exhibit

(8) Mao, Q.; Fulmer, P.; Li, W.; DeRose, E. F.; Petering, D. H. *J. Biol. Chem.* **1996**, *271*, 6185−6191.
(9) Wu, W.; Vanderwall, D. E.; Stubbe, J.; Kozarich, J. W.; Turner, C. J. *J. Am. Chem. Soc.* **1994**, *116*, 10843−10844.
(10) Wu, W.; Vanderwall, D. E.; Lui, S. M.; Tang, X.-J.; Turner, C. J.; Kozarich, J. W.; Stubbe, J. *J. Am. Chem. Soc.* **1996**, *118*, 1268−1280.
(11) Wu, W.; Vanderwall, D. E.; Turner, C. J.; Kozarich, J. W.; Stubbe, J. *J. Am. Chem. Soc.* **1996**, *118*, 1281−1294.
(12) Vanderwall, D. E.; Lui, S.-M.; Wu, W.; Turner, C. J.; Kozarich, J. W.; Stubbe, J. *Chem. Biol.* **1997**, *4*, 373−387.
(13) Lui, S.-M.; Vanderwall, D. E.; Wu, W.; Tang, X.-J.; Tuner, C. J.; Kozarich, J. W.; Stubbe, J. *J. Am. Chem. Soc.* **1997**, *119*, 9603−9613.
(14) Akkerman, M. A. J.; Haasnoot, C. A. G.; Hilbers, C. W. *Eur. J. Biochem.* **1988**, *173*, 211−225.
(15) Akkerman, M. A. J.; Neijman, E. W. J. F.; Wijmenga, S. S.; Hilbers, C. W.; Bermel, W. *J. Am. Chem. Soc.* **1990**, *112*, 7462−7474.
(16) Caceres-Cortes, J.; Sugiyama, H.; Ikudome, K.; Saito, I.; Wang, A. H.-J. *Eur. J. Biochem.* **1997**, *244*, 818−828.
(17) Calafat, A. M.; Won, H.; Marzilli, L. G. *J. Am. Chem. Soc.* **1997**, *119*, 9, 3656−3664.
(18) Lehmann, T. E.; Ming, L.-J.; Rosen, M. E.; Que, L. J. *Biochemistry* **1997**, *36*, 2807−2816.
(19) Otvos, J. D.; Antholine, W. E.; Wehrli, S.; Petering, D. H. *Biochemistry* **1996**, *35*, 1458−1465.
(20) Xu, R. X.; Nettesheim, D.; Otvos, J. D.; Petering, D. H. *Biochemistry* **1994**, *33*, 907−916.
(21) Stubbe, J.; Kozarich, J. W.; Wu, W.; Vanderwall, D. E. *Acc. Chem. Res.* **1996**, *29*, 322−330.
(22) Petering, D. H.; Mao, Q.; Li, W.; DeRose, E.; Antholine, W. E. *Met. Ions. Biol. Syst.* **1996**, *33*, 619−648.
(23) Sausville, E. A.; Peisach, J.; Horwitz, S. B. *Biochem. Biophys. Res. Commun.* **1976**, *73*, 814−822.
(24) Sausville, E. A.; Stein, R. W.; Peisach, J.; Horwitz, S. B. *Biochemistry* **1978**, *17*, 2746−2754.
(25) Sam, J. W.; Tang, X.; Peisach, J. *J. Am. Chem. Soc.* **1994**, *116*, 5250−5256.
(26) Povirk, L. F.; Han, Y.-H.; Steighner, R. J. *Biochemistry* **1989**, *28*, 8, 5808−5814.
(27) Steighner, R. J.; Povirk, L. F. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 8350−8354.
(28) Absalon, M. J.; Stubbe, J.; Kozarich, J. W. *Biochemistry* **1995**, *34*, 4, 2065−2075.
(29) Absalon, M. J.; Wu, W.; Stubbe, J.; Kozarich, J. W. *Biochemistry* **1995**, *34*, 2076−2086.
(30) Muraoka, Y.; Suzuki, M.; Fujii, A.; Umezawa, Y.; Naganawa, H.; Takita, T.; Umezawa, H. *J. Antibiot.* **1981**, *34*, 353−357.
(31) Nakayama, Y.; Kunishima, M.; Omoto, S.; Takita, T.; Umezawa, H. *J. Antibiot.* **1973**, *26*, 400−401.
(32) Hori, M. In *Bleomycin: chemical, biochemical, and biological aspects*; Hecht, S. M., Ed.; Springer-Verlag: New York, 1979; Vol. pp 195−206.
(33) Takahashi, K.; Ekimoto, H.; Aoyagi, S.; Koyu, A.; Kuramochi, H.; Yoshioka, O.; Matsuda, A.; Fujii, A. *J. Antibiot.* **1979**, *32*, 36−42.
(34) Sugiura, Y. *J. Am. Chem. Soc.* **1980**, *102*, 5208−5215.
(35) Sugiura, Y.; Suzuki, T.; Muraoka, Y.; Umezawa, Y.; Takita, T.; Umezawa, H. *J. Antibiot.* **1981**, *34*, 1232−1236.
(36) Oppenheimer, N. J.; Chang, C.; Chang, L. H.; Ehrenfeld, G.; Rodriguez, L. O.; Hecht, S. M. *J. Biol. Chem.* **1982**, *257*, 1606.
(37) Sugiura, Y.; Suzuki, T.; Otsuka, M.; Kobayashi, S.; Ohno, M.; Takita, T.; Umezawa, H. *J. Biol. Chem.* **1983**, *258*, 1328−1336.
(38) Umezawa, H.; Takita, T.; Sugiura, Y.; Otsuka, M.; Kobayashi, S.; Ohno, M. *Tetrahedron* **1984**, *40*, 501−509.
(39) Sugiyama, H.; Ehrenfeld, G. M.; Shipley, J. B.; Kilkuskie, R. E.; Chang, L.-H.; Hecht, S. M. *J. Nat. Products* **1985**, *48*, 869−877.
(40) Sugiyama, H.; Kilkuskie, R. E.; Chang, L.-H.; Ma, L.-T.; Hecht, S. M.; van der Marel, G.; van Boom, J. H. *J. Am. Chem. Soc.* **1986**, *108*, 3852−3854.
(41) Shipley, J. B.; Hecht, S. M. *Chem. Res. Toxicol.* **1988**, *1*, 25−27.
(42) Kénani, A.; Bailly, C.; Helbecque, N.; Catteau, J.-P.; Houssin, R.; Bernier, J.-L.; Hénichart, J.-P. *Biochem. J.* **1988**, *253*, 497−504.
(43) Kénani, A. *Anticancer Drugs* **1994**, *5*, 199−201.
(44) Boger, D. L.; Teramoto, S.; Honda, T.; Zhou, J. *J. Am. Chem. Soc.* **1995**, *117*, 7338−7343.
(45) Boger, D. L.; Teramoto, S.; Zhou, J. *J. Am. Chem. Soc.* **1995**, *117*, 7, 7344−7356.
(46) Bailly, C.; Kénani, A.; Waring, M. J. *FEBS Lett.* **1995**, *372*, 144−147.
(47) Bailly, C. *Nucleic Acids Res.* **1997**, *25*, 1516−1522.

similar cleavage specificity to their parent, although their efficiency of cleavage is reduced by 50%.[36,39−41,44,45] These results seem more consistent with the primary amine of the β-aminoalanine being the axial ligand in deglycoBLM and its parent.

Finally, several groups have proposed that the disaccharide functions to protect the chemically reactive hydroperoxide ligand.[34,35,37,38,42,49] This conclusion is at odds with our recent structural model for HOO−CoBLM in which the disaccharide is positioned on the same face as the axial β-aminoalanine ligand and is not adjacent to the axial hydroperoxide on the opposite face.[10]

In light of the continued interest and controversy on the role of the disaccharide, we report in this paper a model for the structure of the HOO−CodGBLM bound to d(CCAGGCCTGG) based on the constraints derived from NMR studies. This model in comparison with that previously reported for HOO−CoBLM provides further support for the axial ligand being the primary amine of β-aminoalanine and allows further definition of the role of the sugars and the suitability of deglycoBLM analogues, synthetically more accessible than the corresponding BLMs, as probes of the ds cleavage process mediated by BLM.[10,20]

Materials and Methods

Preparation of HOO−CodGBLM. Apo deglycoBLM A2 (4.7 mg) obtained by published procedures[50,51] was dissolved in 100 mM sodium phosphate buffer (3 mL, pH 6.8). $CoCl_2$ (1.1 equiv) was then added to a rapidly stirred solution to ensure oxygenation. The reaction was allowed to proceed for 12 h at room temperature. The mixture of products was separated using a semipreparative reverse phase Alltech Econosil C-18 column (10 mm) and the elution system of 0.1 M ammonium acetate (pH 6.8) as solvent A and acetonitrile as solvent B. The products were eluted at a flow rate of 3 mL/min using a linear gradient from 12 to 16% A over 60 min (compound, retention time in min, yield in mg, yield in percentage): Co-deglycoBLM A2 brown, 18, 1.6, 32; HOO−CodGBLM, 22, 1.4, 27. The lyophilized samples were redissolved in 50 mM sodium phosphate (pH 6.8) and stored at −80 °C.

Characterization of HOO−CodGBLM. The extinction coefficient of HOO−CodGBLM was measured using inductively coupled plasma emission spectroscopy for cobalt quantitation as previously described[10] and was determined to be $2.05 \pm 0.2 \times 10^4$ M^{-1} cm^{-1} at 290 nm. The electrospray mass spectrum of HOO−CodGBLM was obtained as described previously.[10]

Titration and Binding Studies of HOO−CodGBLM with 1. Oligonucleotide **1** was prepared and quantitated as previously described.[10] Aliquots of HOO−CodGBLM (0−1 equiv) were added to the solution of DNA in D_2O and complex formation was followed by monitoring the changes in the ^1H NMR spectrum. The K_d of HOO−CodGBLM to **1** was measured by monitoring fluorescence quenching and Scatchard analysis.[10,13]

Ss- and Ds-Cleavages of a Hairpin Oligonucleotide. Methods are identical to those previously described by Absalon et al.[28,29] except that deglycoBLM (5 μM to 20 μM at 4 °C) replaced BLM. Activated BLM (HOO−FeBLM) was generated ex situ as previously described.[28,29]

NMR Experiments. All NMR experiments were performed on 750 or 500 MHz Varian NMR spectrometers or on 600 or 500 MHz custom-built instruments at the Francis Bitter Magnet Laboratory. Data were then transferred to a Silicon Graphics work station and processed using Felix software (version 2.3, Molecular Simulations Inc., formerly Biosym Technologies, Inc.). ^1H and ^{13}C chemical shifts are referenced to an internal standard, sodium 3-(trimethylsilyl)-1-propanesulfonate at 0.00 ppm.

DQF−COSY, TOCSY (MLEV-17 spin lock pulse with 70 ms mixing times) and NOESY (50, 200, and 400 ms mixing times) experiments were recorded at 20 °C in D_2O or H_2O on the DNA/HOO−CodGBLM complex. Data sets with 4096×512 complex points were acquired with sweep widths of 5000 Hz (500 MHz instrument) or 8000 Hz (750 MHz instrument) in both dimensions and 32 scans per t_1 increment. During the relaxation delay period, a 2.0 s presaturation pulse was used for solvent suppression. For the NOESY experiments in H_2O, a WATERGATE gradient pulse sequence[52] was used, and data sets with 4096×512 complex points were acquired with sweep widths of 12 000 Hz (500 MHz instrument) or 15 000 Hz (750 MHz instrument) in both dimensions. The spectra were processed as previously reported.[11]

The HMQC[53] spectrum was recorded at 20 °C in D_2O with a J_{C-H} coupling constant of 190 Hz on the DNA/HOO−CodGBLM complex. Data sets with 2048×256 complex points were acquired with 6000 Hz (^1H) and 25 000 Hz (^{13}C) sweep widths on a 500 MHz instrument. Two hundred fifty-six scans were collected for every t_1 increment. During the relaxation delay period, a 1.5 s presaturation pulse was used for solvent suppression. The spectrum was processed as described previously.[11]

2D NOESY (100, 200, 400 ms mixing times) and DQF−COSY experiments at 20 °C on HOO−CodGBLM in D_2O or 90% H_2O/10% D_2O were similarly recorded and processed.[10] A ROESY experiment[54] at 20 °C was acquired in D_2O with 9 kHz rf field strength on a 500 MHz instrument.

Molecular Modeling. All calculations were carried out with Quanta 4.0/CHARMm 22 (Molecular Simulations Inc.; Waltham, MA) on a Silicon Graphics Indigo2, or a Cray Y-MP, or J-90. The methodology used has been described previously.[10,11] Only notable changes are described here in detail. The calculation of nonbonded van der Waals and electrostatic interactions were truncated at 13.0 Å using the force switching function between 8.0 and 12.0 Å and a distance-dependent dielectric constant. The list of nonbonded terms was updated every 20 steps, except in the final molecular dynamics run, where the list was updated if an atom moved greater than 0.5 Å. The terms for electrostatic interactions and hydrogen bonds were only included in the final 15 ps step of the calculations for the DNA/HOO−CodGBLM complex. Hydrogen bonds were cut off at 5.0 Å and switched between 3.5 and 4.5 Å. Hydrogen bonds were also cut off at 90° and switched between 130° and 110°. Following heating, the temperature was maintained by scaling the velocities of the atoms to keep the temperature at 300 ± 10 K. In the final 15 ps molecular dynamics phase, scaling was only required 2−3 times.

All force constants for bonds and angles, distance constraints, and dihedral constraints were set analogously to those previously described.[10,11]

Distance and Dihedral Angle Constraints. All NOEs were classified as strong, medium, or weak based on visual inspection of the cross-peak intensities in the 200 ms NOESY spectra. In HOO−CodGBLM complex with **1**, 44 intramolecular NOEs within HOO−CodGBLM and 39 intermolecular NOEs were used as constraints. In both cases, these constraints were set at 1.9−3.0, 1.9−4.0, and 1.9−5.0 Å for strong, medium, and weak NOEs, respectively. The 203 intramolecular distance constraints for DNA were set at 1.9−3.0, 2.5−4.0, and 3.5−5.0 Å for strong, medium, and weak NOEs, respectively. An additional 1.0 Å was added to the constraints for the pseudoatoms of methyl and ambiguous methylene protons. For HOO−CodGBLM in the complex with **1,** coupling constants were derived from 2D COSY spectra. The five dihedral angles were qualitatively constrained to a gauche conformation for J coupling values of 0−4 Hz and trans conformation for those >8 Hz.

(48) Oppenheimer, N. J.; Rodrigues, L. O.; Hecht, S. M. *Biochemsitry* **1979**, *18*, 3439−3445.
(49) Arai, T.; Shinozuka, K.; Sawai, H. *Bioorg., Med. Chem. Lett.* **1997**, *7*, 15−18.
(50) Boger, D. L.; Menezes, R. F.; Yang, W. *Bioorg. Med. chem. Lett.* **1992**, *2*, 959.
(51) Kénani, A.; Lamblin, G.; Hénichart, J.-P. *Carbohydrate Res.* **1988**, *177*, 81−89.
(52) Sklenar, V.; Piotto, M.; Leppik, R.; Saudek, V. *J. Magn. Res. Ser. A* **1993**, *102*, 241−245.
(53) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093−2094.
(54) Bothner-By, A. A.; Stephens, R. L.; Warren, C. D.; Jeanloz, R. W. J. *J. Am. Chem. Soc.* **1985**, *106*, 811.

Molecular Dynamics (MD) Simulated Annealing. Eleven calculations of restrained MD simulated annealing were run, following the same protocol described previously.[11] The MD was initiated with the distance constraints applied using a force constant of 0.6 kcal mol^{-1} $Å^{-2}$. The structure was heated and equilibrated over 4 ps, the force constants for the distance constraints were then scaled to 120 kcal mol^{-1} $Å^{-2}$ over 6.5 ps, and the system was allowed to evolve for 10 ps before being cooled to 300 K over 7 ps. The dihedral constraints were applied gradually, starting with a force constant of 5 kcal mol^{-1} rad^{-2} and increasing to 50 kcal mol^{-1} rad^{-2} in 4 stages over 10 ps. The dihedral angle constraints on the DNA backbone angles β, γ, and ϵ at the intercalation site and the adjacent base pairs were applied with force constants of 5 and 10 kcal mol^{-1} rad^{-2}, respectively. The electrostatic and hydrogen bond energy terms were then introduced, and the system was allowed to equilibrate for 4 ps, followed by the final 15 ps MD run. The final structure for each iteration was generated by averaging the coordinates of the final 5 ps of the 15 ps molecular dynamics simulation, followed by 1000 steps of conjugate gradient minimization with the distance constraints and dihedral angle constraints. The DNA backbone constraints were not used in the minimization.

Results and Discussion

Preparation and Characterization of HOO−CodGBLM. HOO−CodGBLM was prepared by a modification of the previously published procedure for HOO−CoBLM.[10,55] In the present case, the pH was maintained by running the reaction in phosphate buffer (pH 6.8, 100 mM), and the number of side products was minimized by extending the reaction to 12 h. The products (a mixture of desired hydroperoxide and aquo or hydroxide forms) were separated by HPLC (see methods), and the UV/vis spectrum of the hydroperoxide analogue and its extinction coefficient ($\epsilon_{290} = 2.0 \times 10^4$ M^{-1} cm^{-1}) was found to be almost identical to HOO−CoBLM ($\epsilon_{290} = 2.1 \times 10^4$ M^{-1} cm^{-1}). The ^{1}H NMR spectrum in the downfield spectrally uncrowded region exhibits four singlets H−C2H, H−C4H, B−C5H, and B−C5′H, indicative of a compound of >95% homogeneity. Examination of the NMR spectrum (20 °C, pH 6.8) over a period of several months revealed no decomposition, a requirement for the acquisition of 2D NMR data.

To establish that one of the axial ligands is in fact a hydroperoxide, electrospray mass spectrometry was used. This method has previously been employed to characterize both activated BLM (HOO−FeBLM) and HOO−CoBLM.[10,25] The electrospray mass spectrum is shown in Figure 2 and exhibits a *m*/*z* of 569.0 with the predicted isotopic distribution (calculated MW of 1138.32). A total charge of +2 associated with the HOO−CodGBLM is consistent with a deprotonated β-hydroxyhistidine amide (−1), a hydroperoxide ligand (−1), Co(+3) and a positively charged sulfonium tail (+1).

Proton Chemical Shifts and Coupling Constants of HOO−CodGBLM: A Comparison with HOO−CoBLM. To examine the structure of HOO−CodGBLM bound to an oligonucleotide, the free HOO−CodGBLM needs to be characterized first. The chemical shifts of both the exchangeable and nonexchangeable protons were assigned through COSY connectivities using strategies previously established for CO−FeBLM, ZnBLM, and HOO−CoBLM[10,14,15] and are reported in Table 1. The uncoupled B−C5H and B−C5′H protons were assigned based on their chemical shift similarities to the corresponding values in HOO−CoBLM (Table 1). In an effort to obtain information about through-space interactions, NOESY experiments at various mixing times (100, 200, and 400 ms) were carried out. They were unsuccessful, however, presumably due to the correlation time of HOO−CodGBLM (MW 1138) falling into the range where the NOEs approach zero.[56,57] The unavailability of concentrated solutions of HOO−CodGBLM hampered attempts to use ROESY methods. No quantifiable interresidue ROEs were observed, although small cross-peaks between spatially close intraresidue protons were identified. The lack of NOE and ROE information has precluded the determination of the structure of the free HOO−CodGBLM.

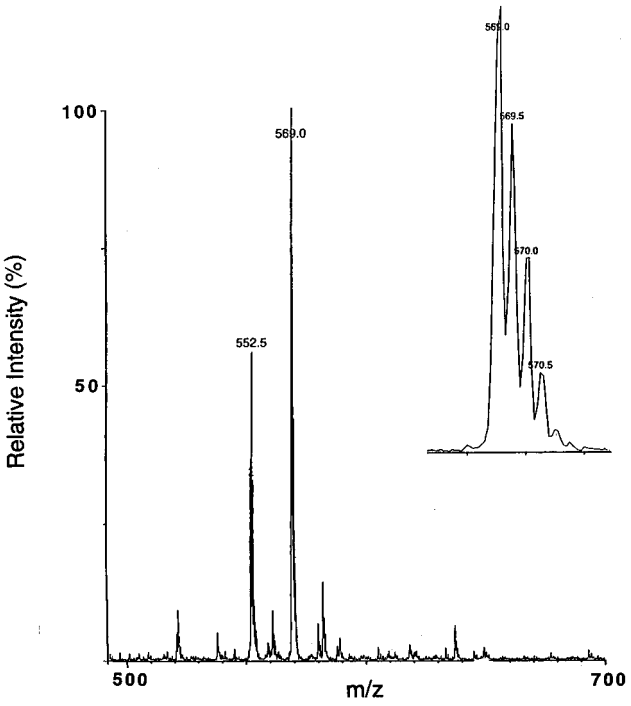

Figure 2. Electrospray mass spectrum of HOO−CodGBLM. The peaks at *m*/*z* of 569.0 and 552.5 represent the intact HOO−CodGBLM and CodGBLM (loss of hydroperoxide ligand), respectively. The inset shows the isotopic distribution of the peak at *m*/*z* of 569.0.

The successful structure determination of the parent HOO−CoBLM,[10] however, has given us insight into the corresponding HOO−CodGBLM structure by direct comparison of the NMR data. The chemical shifts of most of the protons (pH 6.8 and 20 °C) are within 0.1 ppm of the parent compound (pH 6.8 and 5 °C) (Table 1). Of notable exception are the protons associated with the β-aminoalanine moiety, A−CαH and A−NH, which are upfield and downfield shifted by 0.17 and 0.26 ppm, respectively, relative to the parent HOO−CoBLM.

A comparison of the coupling constants of these two molecules (Table 2) are also virtually identical with the exception, once again, being associated with β-aminoalanine protons of A−CαH and A−CβHs (13.1 and 3.2 Hz in HOO−CodGBLM vs 4.0 and 3.0 Hz in HOO−CoBLM). These observed differences reflect a significant change in the rotamer population around the Cα−Cβ bond of the β-aminoalanine moiety.

The similarities in the chemical shifts, coupling constants, and the physical properties suggest that HOO−CodGBLM is very similar to its parent, with the exception of the conformation of the putative axial ligand, the primary amine of the β-aminoalanine moiety. This point will be returned to subsequently.

Screw Sense and the Linker Conformation. The solution structure of HOO−CoBLM determined using extensive NOE

(55) Chang, C.-H.; Dallas, J. L.; Meares, C. F. *Biochem. Biophys. Res. Comm.* **1983**, *110*, 959−966.

(56) Neuhaus, D.; Williamson, M. P. *The Nuclear Overhauser Effect in Structural and Conformational Analysis*; VCH Publishers: Inc.: New York City, 1989.

(57) Sanders, J. K. M.; Hunter, B. K. *Modern NMR Spectroscopy: A Guide for Chemists*, 2nd ed; Oxford University Press: New York, 1993.

Table 1. Comparison of Proton Chemical Shifts (ppm) of ApoBLM, Metallo-BLMs, and HOO−CodGBLM

protons		dG[a] 293 K pH 6.8	G[b] 297 K pH 6.7	Apo[c] 278 K pH 6.8	Zn[d] 277 K pH 7	Fe[d] 277 K pH 7	protons		dG[a] 293 K pH 6.8	G[b] 297 K pH 6.7	Apo[c] 278 K pH 6.8	Zn[d] 277 K pH 7	Fe[d] 277 K pH 7
P	CαH	3.22	3.20	2.62	2.87	2.86	V	NH	9.12	8.89		7.55	7.75
	CαH′	3.58	3.51	2.69	3.24	3.08	T	CH_3	1.19	1.19	1.08	1.02	0.99
	Cβ H	5.23	5.10	3.96	4.50	4.34		CαH	4.34	4.39	4.21	4.10	4.03
	CH_3	2.56	2.46	2.01	2.38	2.24		Cβ H	4.20	4.25	4.08	4.00	3.93
	4-NH_2	7.73/7.94		7.02	6.91			NH	8.89	8.92		7.96	8.20
H	CαH	4.85	4.98	5.05	4.85	4.93	B	CαH	3.14	3.06	3.24	3.16	3.08
	Cβ H	5.44	5.53	5.26	5.20	5.19		CαH′	3.29	3.12	3.24	3.26	
	C2H	8.71	8.72	7.79	8.04	7.86		Cβ H	3.49	3.44	3.60	3.51	3.35
	C4H	7.62	7.60	7.26	7.31	7.23		Cβ H′	3.84	3.51	3.60	3.86	
A	CαH	3.24	3.41	3.84	3.72	3.76		C5H	8.21	8.17	8.20	8.21	8.11
	Cβ H	2.85	2.74	2.83	2.46	2.42		C5′H	7.93	7.82	8.00	8.04	7.85
	Cβ H′	3.16	3.22	2.88	3.36	2.88		NH	8.67	8.57		8.36	8.33
	NH	6.27	6.01		4.32	5.38	S	$C\alpha H_2$	3.40	3.36	3.38	3.36	3.31
V	αCH_3	0.65	0.62	1.10	0.98	1.03		Cβ H_2	2.16	2.13	2.16	2.13	2.09
	γCH_3	1.00	0.98	1.12	0.93	0.96		Cγ H	3.57	3.51	3.60	3.57	3.53
	CαH	0.98	0.94	2.45	1.95	2.36		Cγ H′	3.63	3.63	3.60	3.57	3.53
	Cβ H	3.38	3.33	3.70	3.42	3.64		$(CH_3)_2$	2.95	2.94	2.91	2.88	2.83
	Cγ H	3.52	3.50	3.86	3.62	3.58		NH	8.82	8.66		8.94	8.90

[a] HOO-CodGBLM, 50 mM phosphate buffer. [b] HOO-CoBLM, 50 mM phosphate buffer. [c] Akkerman et al.[14] [d] Akkerman et al.[15]

Table 2. Comparison of Coupling Constants (Hz) in Metallo-BLMs and Metallo-dGBLMs[e]

protons	HOO-Co BLM[a]	HOO-CodG BLM[b]	CO-FeBLM[c]	CO-Fe dGBLM[d]	ZnBLM[c]	Apo BLM[c]
G-C1H to G-C2H	3.9 ± 0.3				4.9	
M-C1H to M-C2H	1.2 ± 0.3				1.8	
H-CαH to H-Cβ H	2.7 ± 0.3	3.3 ± 0.3	3.1 ± 0.2	3.4	3.1 ± 0.2	5.8 ± 0.2
P-Cβ H to P-CαH	8.3 ± 0.3	9.2 ± 0.3		8.2		
P-Cβ H to P-CαH′	4.6 ± 0.3	4.3 ± 0.3		6		
T-CαH to T-Cβ H	2.9 ± 0.3	3.4 ± 0.3				
T-NH to T-CαH	8.7 ± 0.3	7.6 ± 0.3				
V-NH to V-Cγ H	7.9 ± 0.3	8.0 ± 0.3				
V-CαH to V-Cβ H	1.8 ± 1.2	1.5 ± 0.3				
V-Cβ H to V-Cγ H	9.5 ± 1.2	9.0 ± 1.2				
A-NH to P-Cβ H	<3	<3				
A-NH to A-Cβ H′	5 ± 2	<3				
A-NH to A-Cβ H	5 ± 2	4 ± 2				
A-Cβ H′ to A-CαH	3.0 ± 0.5	3.2 ± 0.3	4.2 ± 0.2	3.7	2.0 ± 0.2	5.2 ± 0.2
A-Cβ H to A-CαH	4.0 ± 1.2	1.31 ± 0.3	7.2 ± 0.2	13.0	3.8 ± 0.2	7.2 ± 0.2

[a] 50 mM phosphate, pH 6.8 at 5 °C. [b] This study, 50 mM phosphate, pH 6.8 at 20 °C. [c] Akkerman et al.[15] [d] Oppenheimer et al.[37] [e] The dihedral angles that were constrained in the molecular dynamics as described in Materials and Methods are underlined.

and dihedral angle constraints derived from NMR studies in conjunction with molecular modeling revealed that its linker peptide adopts a well defined conformation, and its bithiazole tail folds underneath the equatorial coordination plane on the same face as the hydroperoxide.[10,20] This information is compatible with a single screw sense isomer that is shown in Figure 3A. This model, important with respect to defining the role of the sugars, places the sugars on the same face as the β-aminoalanine moiety whose primary amine functions as an axial ligand. The question then arises as to whether, despite the lack of NOE information, an argument can be made that HOO−CodGBLM has the same ligands and screw sense as its parent. Two lines of argument allow us to answer this question in the affirmative. The first involves the similarities in the conformation of the linker region apparent from comparison of chemical shifts and coupling constants of V and T moieties (Figure 1). The second, and the most convincing, is the structure of HOO−CodGBLM bound to **1** described below.

The constrained conformation of the linker, specifically the V moiety, is apparent from the observed coupling constant (1.5 Hz) between V−CαH and V−Cβ H, indicative of a gauche conformation between these two protons, and the 9.0 Hz coupling constant between V−Cβ H and V−Cγ H, indicative of a trans conformation between these protons (Table 2). In addition, the chemical shifts of V−CαH at 0.98 ppm and V−αCH_3 at 0.65 ppm are dramatically upfield shifted relative to apoBLM (Table 1), and the V−NH and T-NH of the linker region are downfield shifted by ∼1.5 and 0.7 ppm, respectively, relative to other metallo-BLMs. The dramatic upfield shifts are due to shielding by the imidazole ring. The dramatic downfield shifts appear to be associated with hydrogen bonding of these amide hydrogens to the penultimate oxygen of the

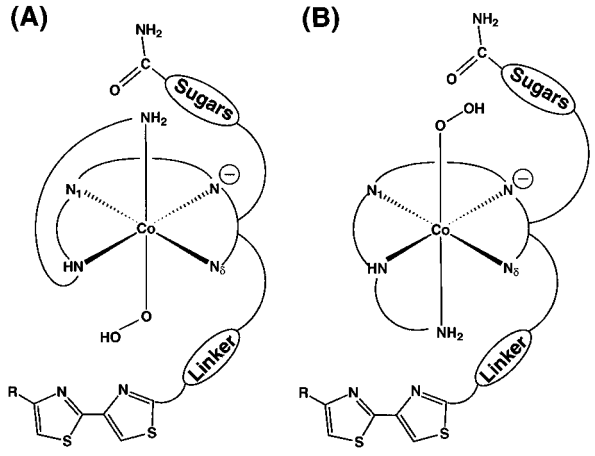

Figure 3. Structures of HOO−CoBLM with the primary amine of A moiety as an axial ligand in two screw senses. Structure **A** is favored based on studies of Wu et al.[11]

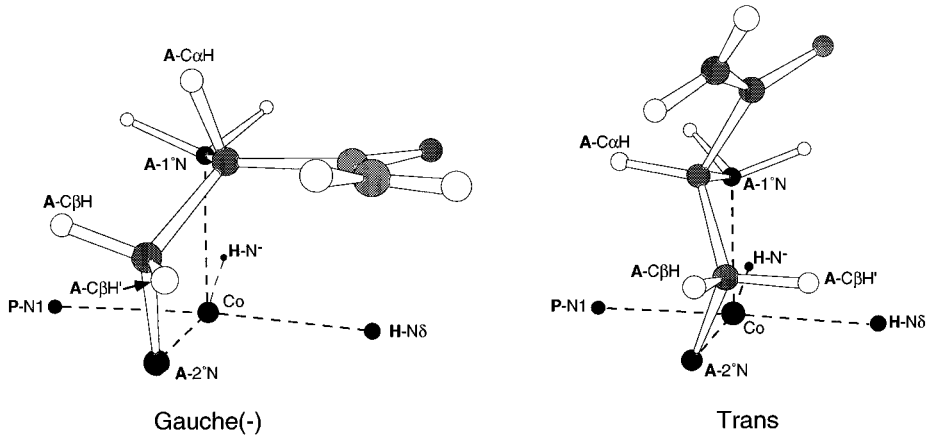

Figure 4. Trans and gauche(−) conformations around the Cα−Cβ bond of the β-aminoalanine (see Figure 1 for nomenclature and trans and gauche(−) conformations refer to the nomenclature by Akkerman et al.[14,15]).

hydroperoxide in the parent compound and by inference in the deglycosylated congener. The similarities in both the coupling constants and unusual chemical shifts within the V and T moieties between HOO−CodGBLM and its glycosylated parent provide strong support for assignment of a similar screw sense arrangement of the ligands (Figure 3A).

Second, the screw sense of the metallo-BLM obviously has a profound effect on its ability to bind to DNA. With HOO−CoBLM, given the extensive NOE constraints for its binding to several oligonucleotides,[8,11,12] only a single screw sense (Figure 3A) is compatible. As described in detail below, the HOO−CodGBLM binds to DNA in almost identical fashion to the parent. Based on these considerations, we feel confident that the HOO−CodGBLM and its parent have the same chiral ligand organization.

Primary Amine Axial Ligand. The nature of the axial ligand has been one of contention for many of the metallo-BLMs.[10,14−20] Our recent 2D NMR studies with HOO−CoBLM in conjunction with chemical and biochemical studies strongly support the proposal in this case that the primary amine of β-aminoalanine and not the carbamoyl N or O of the mannose is the second axial ligand.[10] Since our screw sense isomer (Figure 3A) requires both of these ligands to be on the same face, the distinction between these options is structurally, but we believe not chemically, a subtle one.

The major arguments concerning the primary amine ligation for a number of metalloBLMs have been associated with the examination of the coupling constants between the A−CαH and A−Cβ Hs of the β-aminoalanine moiety.[10,14,15] It has been proposed that if the primary amine of β-aminoalanine is coordinated to a metal as an axial ligand, then predominately a single rotamer population around Cα−Cβ bond of β-aminoalanine should be observed. This conformation is for example gauche(−) for both ZnBLM and HOO−CoBLM in Figure 4.[10,14] If, on the other hand, the primary amine is not an axial ligand, as in the case of free apoBLM, the coupling constants would reflect a mixture of rotamer populations.[14,15] In the case of HOO−CodGBLM, the ability of the carbamoyl moiety to serve as an axial ligand, of course, has been eliminated. Thus, the only possibilities for the axial ligand are the favored primary amine, a H_2O molecule, or no ligand at all. In support of the primary amine, consider the following arguments. First, the *J* values between CαH and Cβ Hs are 13.1 and 3.2 Hz, indicative of a predominately trans conformation around the Cα−Cβ bond of the β-aminoalanine moiety (Figure 4). This conformation can be easily generated from the gauche(−) conformation, while the primary amino group of β-aminoalanine remains ligated to the cobalt. The factors that govern which conformation is favored are, at present, unclear. Second, a sensitive probe of electronic environment is EPR spectroscopy and in fact extensive analysis of O_2−Co(II) BLM in comparison with the O_2−Co(II)-dGBLM suggest that their environments are strikingly similar.[35,37,38] Third, crystallographic precedence for the primary amine as an axial ligand has been well documented in BLM analogues, Cu−P3A, a biosynthetic precursor of BLM lacking both the sugar and the linker domains, and several model compounds.[58,59] Thus, we believe that the axial ligand for HOO−CodGBLM is also the primary amine of β-aminoalanine.

Very recently Caceres-Cortes et al. reported a solution structure of the HOO−CoPEP and HOO−CodGPEP.[16] These analogues differ from the corresponding BLM A2 analogues only in the bithiazole tail, by a mass of 58 Da (Figure 1). This difference allowed them with both compounds to obtain NOE information and as a consequence models of both structures. They assigned the mannose carbamoyl group as the axial ligand to cobalt in HOO−CoPEP, while in HOO−CodGPEP, the primary amine of β-aminoalanine was the proposed axial ligand. The authors' analysis, however, is heavily dependent upon the accurate assignment of the exchangeable protons of the primary amine of β-aminoalanine (A−NH_2) and of the carbamoyl amino group (M−NH_2). For the following reasons, these assignments, in contrast to the claims made by these authors, have not been unambiguously established. First, the assignments of all exchangeable protons have not been possible in their system or for any other metallo-BLMs. Missing in their analysis are the V−OH, T−OH, secondary amine of the C-terminus, and the G−OHs and M−OHs (for HOO−CoPEP only). Furthermore, their method of assignment for the remaining exchangeable protons was not described and appears to be based on the previous work of Akkerman et al. for the OC−FeBLM.[14,15] The Akkerman et al. paper unfortunately also has ambiguous assignments for the chemical shifts of the key exchangeable protons, a difficult problem, as no through bond connectivities are observed.[14,15] Two protons were detected for the amides of A and P and the M−NH_2 and showed only cross-peaks to each other in all three cases.[60] While the carbamoyl NH_2

(58) Iitaka, Y.; Nakamura, H.; Nakatani, T.; Muraoka, Y.; Fujii, A.; Takita, T.; Umezawa, H. *J. Antibiot.* **1978**, *31*, 1070−1072.

(59) Tan, J. D.; Hudson, S. E.; Brown, S. J.; Olmstead, M. M.; Mascharak, P. K. *J. Am. Chem. Soc.* **1992**, *114*, 3841−3853.

(60) If the carbamoyl nitrogen, as claimed,[15,16] is ligated to cobalt, one might expect that it would be bonded to a single proton and not two protons proposed. Coordination to the metal would be analogous to protonation of an amide. Rapid loss of a proton would be expected upon coordination. This is actually observed in the equitorial amide ligand of BLM.

protons were assigned chemical shifts of 6.19 and 6.43 ppm, Akkerman et al. clearly stated that "this assignment was somewhat ambiguous since in the 5.5−6.5 ppm region in the spectrum several broad resonances are observed".[15] In addition, despite the fact that A−NH_2 is proposed not to be a ligand in the OC−FeBLM complex, two protons were also detected at 6.77 and 7.75 ppm and assigned to A−NH_2 based on a single NOE to A−CαH. If in fact the primary amine is neither a ligand to the metal nor hydrogen bonded, a single downfield shifted proton due to the protonated primary amine (NH_3^+) would be expected or exchange broadened to such an extent that it would not be detectable.[61] Thus, the Akkerman assignments are unfortunately not unambiguous and as a consequence neither are those of Caceres-Cortes et al.[16]

The one interesting and unique report of Caceres-Cortes et al. is the observation of two exchangeable protons at 3.97 and 4.09 ppm which exhibit NOEs to A−CαH and Cβ H protons of HOO−CodGPEP and have consequently been assigned to a coordinated A−NH_2. However, there is no compelling connection of these protons to those assigned to be A−NH_2 in HOO−CoPEP (5.93 and 6.58 ppm). In fact in the case of this parent compound, neither through bond couplings nor any NOEs are reported, and thus their assignment to the primary amine of A is ambiguous. (It should be noted that for some reason the pH of the NMR studies on the two PEP analogues differed by 0.5, making chemical shift arguments even less compelling.)

Several additional points need to be considered regarding the likelihood of a carbamoylamine vs the primary amine of β-aminoalanine as a ligand. First, there is little doubt that the primary amine, given an identical environment to a carbamoyl amino group, is more basic and hence a better ligand. Second and more compelling is the ability of this primary amine to form a five-membered ring chelate structure which many previous studies have shown to drive ligation.[62] There is no compelling steric reason the carbamoyl amino group should ligate based on similar considerations. Thus, chemical arguments strongly favor a coordinated primary amine of β-aminoalanine.

In the absence of a thorough assignment of all exchangeable protons by NMR methods, an impossible task with the metallo-BLMs, we believe that the case for the primary amine ligand of β-aminoalanine as the axial ligand still remains compelling. As described in our original paper,[10] while we cannot unambiguously assign the primary amine as the axial ligand, we believe from detailed arguments in our previous publications,[10] that this is the case. Because of the persistent controversy over this point, we are in the process of synthesizing BLM A2, specifically 15-N labeled in the primary amine of β-aminoalanine. NMR, EPR, and ENDOR studies with this compound should lay this controversy to rest.

Binding Affinity of HOO−CodGBLM to Oligonucleotide 1. Previous fluorescence quenching studies have shown that oligonucleotide **1** contains a single binding site for HOO−CoBLM with a $K_{d\ apparent}$ of 0.17 ± 0.7 μM.[10,11] A similar measurement has been made with HOO−CodGBLM in an effort to examine the contribution of the sugar domain to DNA binding. Under identical conditions, a $K_{d\ apparent}$ of 5.9 ± 0.7 μM was obtained.

1D ^{1}H NMR Titration of 1 with HOO−CodGBLM. The previous NMR titration of **1** with HOO−CoBLM exhibited the formation of a 1:1 complex in slow exchange on the NMR time scale, which allowed the detection of intermolecular (60) and intramolecular (65) NOEs and the subsequent structure determination.[11] In an effort to delineate the role of the sugars in DNA binding and cleavage, the solution structure of HOO−CodGBLM bound to **1** has been determined.

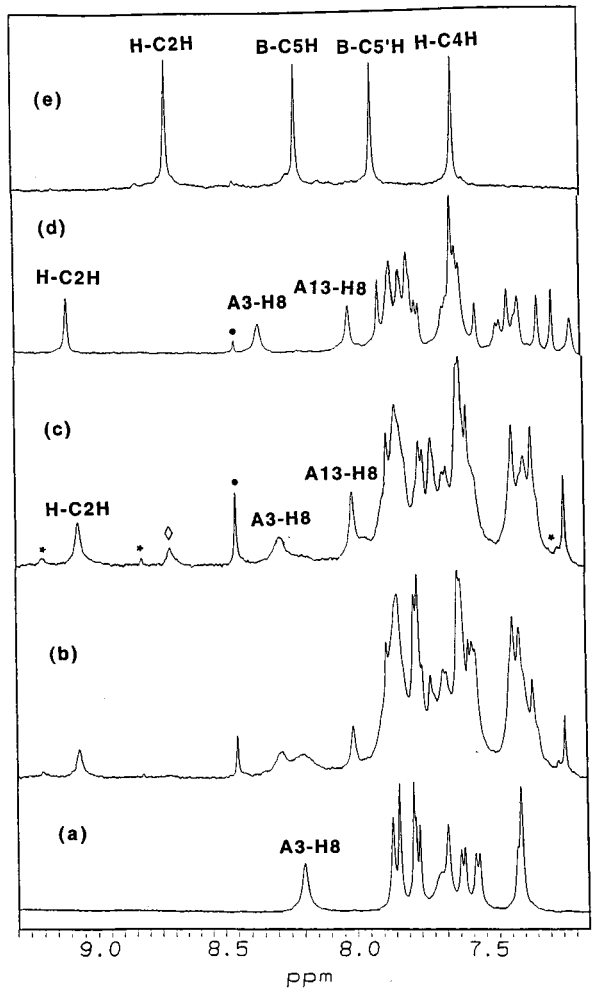

Figure 5. Titration of d(CCAGGCCTGG) with HOO−CodGBLM at 20 °C. Downfield region of the ^{1}H NMR (500 MHz): (a), (b), (c) decameric duplex DNA (1.0 mM) in 50 mM sodium phosphate (pH 6.8) with 0, 0.5, and 1 equiv of HOO−CodGBLM A2 green added respectively; (d) decameric duplex DNA in 50 mM sodium phosphate (pH 6.8) with 1 equiv of HOO−CoBLM added. (e) Free HOO−CodGBLM in 50 mM sodium phosphate (pH 6.8). Asterisks indicate the presence of minor complexes. The feature (·) comes from an impurity in sample handling. The broad feature (◊) at 8.70 ppm represents the coalescence of several resonances, one of which appears to be the H−C2H of free HOO−CodGBLM based on the observed NOE pattern. The reason for the appearance of residual free HOO−CodGBLM is probably the result of overtitration.

The results of titration of **1** with HOO−CodGBLM monitored by 1D NMR spectroscopy are shown in Figure 5a−c and are similar to that previously reported for identical experiments with HOO−CoBLM (Figure 5d). The disappearance of A3−H8 in free DNA upon addition of HOO−CodGBLM is accompanied by the generation A3−H8 and A13−H8 associated with the 1:1 complex (Figure 5b,c) in slow exchange on the NMR time scale. The appearance of many minor features (* in Figure 5c) is also evident, in contrast to the titration with HOO−CoBLM (Figure 5d), as is the selective broadening of A3−H8 (Figure 5c). These subtle but discernible differences in DNA binding by the two congeners will be the subject of further discussion.

(61) Even if the primary amine was unprotonated, rapid inversion of the nitrogen should make these protons equivalent. Thus the nonequivalent protons assigned to the putative primary amine of β-aminoalanine,[15,16] strongly suggest that it is coordinated to the metal.

(62) Sigel, H.; Martin, R. B. *Chem. Rev.* **1982**, *82*, 385−426.

Table 3. Proton Chemical Shifts (ppm) of HOO−CodGBLM and HOO−CoBLM Complexed with 1 at 20 °C

		HOO-CodGBLM pH 6.8	HOO-CoBLM pH 6.8
P	CαH	3.06	2.78
	CαH′	3.61	3.69
	Cβ H	5.25	5.16
	CH_3	2.59	2.61
	4-NH_2	6.86, 10.23	7.14, 10.36
H	CαH	4.86	5.01
	Cβ H	5.45	5.48
	C2H	9.06	9.10
	C4H	7.55	7.60
A	CαH	2.96	3.37
	Cβ H	3.08	2.46
	Cβ H′	3.50	3.24
	NH	5.71	5.69
V	αCH_3	0.67	0.65
	γCH_3	0.95	0.96
	CαH	1.23	1.21
	Cβ H	3.79	3.73
	Cγ H	3.47	3.51
	NH	9.07	8.78
	OH	6.71	6.79
T	CH_3	1.25	1.23
	CαH	4.53	4.53
	Cβ H	4.51	4.51
	NH	9.56	9.36
B	CαH	2.82	2.83
	CαH′	2.90	2.76
	Cβ H	2.95	2.93
	Cβ H′	3.69	3.76
	C5H	7.32	7.26
	C5′H	7.18	7.21
	NH	8.62	8.62
S	C$αH_2$	3.54, 3.67	3.56, 3.46
	CβH_2	2.05, 2.15	2.07, 2.16
	Cγ H	3.34	3.43
	Cγ H′	3.44	3.43
	$(CH_3)_2$	2.97	2.97, 3.00
	NH	7.73	7.81
	CoOOH	9.02	8.89

Proton Assignment of HOO-dGBLM Bound to 1. Proton assignments for HOO−CodGBLM in the complex (Table 3) have been made following the strategy used previously for HOO−CoBLM complexed with the same DNA.[11] Limited availability of HOO−CodGBLM (∼1 mM) and the presence of minor species, however, have made these assignments more challenging. Highlighted below are the assignments for protons of particular interest and those in which difficulties were encountered.

One of the hallmarks of the intercalative mode of binding by the CoBLMs has been the upfield shift of the protons associated with the bithiazole rings, B−C5H and B−C5′H, when bound to DNA.[9,10,12,13] The successful assignment of these two protons, in the absence of any COSY connectivities, utilized the relatively invariant carbon chemical shifts of thiazolium rings of both free HOO−CoBLM and its complex with DNA obtained from HMQC experiments.[9,11−13] A similar strategy has been used in assigning the corresponding protons in HOO−CodGBLM bound to DNA. The HMQC spectrum of this complex (supporting Figure 1) displays two C/H cross-peaks at the signature carbon chemical shifts of B−C5 (126.9 ppm) and B−C5′ (117.4 ppm), assigned by comparison to 126.8 and 117.5 ppm, respectively, in HOO−CoBLM complex. In turn, the proton chemical shifts for B−C5H and B−C5′H in the complex with DNA are assigned to 7.32 and 7.18 ppm, respectively, in the HMQC spectrum (supporting Figure 1). Compared to the chemical shifts of B−C5H and B−C5′H in free HOO−CodGBLM (8.21 and 7.93 ppm respectively), both bithiazole protons are upfield shifted, consistent with an intercalative mode of binding.

Table 4. Intermolecular NOEs between HOO−CodGBLM and 1 at 20 °C

5′ end	strand 1	HOO-dGBLM residues	3′ end	strand 2	HOO-dGBLM residues
G5	H4′	P-CH_3/w	C16		
		P-NH_2(1)[a]/w			
	H1′	P-CH_3/w			
		P-NH_2(1)/w			
		P-NH_2(2)/m			
C6	H5″	P-CH_3/w	G15	NH	B-C5H′/m
		V-γCH_3/m			B-Cβ H′/w
	H5′	V-γCH_3/w			B-Cβ H/w
	H4′	CoOOH/w		H8	B-C5H/m
		P-CH_3/w			
		V-γCH_3/m			
	H2″	CoOOH/w			
	H2′	CoOOH/w			
	H1′	CoOOH/m			
		P-CH_3/w			
		P-Cβ H/m			
	NH_2[h]	B-C5H′/m			
	NH_2[e]	B-C5H′/w			
C7	H5″	T-CαH/w	G14	H1′	B-C5H/s
	H5′	CoOOH/w		H2″	B-C5H/m
		T-Cβ H/w		H2′	B-C5H/m
		T-CαH/w		H4′	B-C5H/w
	H1′	B-CαH′/w		NH	B-C5H′/w
		B-CαH/w		H8	B-C5H/m
					S-Cβ H′/w
					S-Cβ H/w
					S-Cγ H/w
					S-Cγ H′/w
T8			A13	H8	S-CH_3/w
3′ end	strand 1		5′ end	strand 2	

[a] P-NH_2 (1) and P-NH_2 (2) are the hydrogens at 10.23 and 6.86 ppm, respectively. h = hydrogen bonded; e = external.

The NMR studies of the HOO−CoBLM complex have previously revealed that the sequence specificity of DNA cleavage by BLM is derived from its pyrimidine moiety.[11−13] More specifically, one of the 4-amino protons and N3 of the pyrimidine are within hydrogen bond distances of the N3 and one of the 2-amino protons of guanine, 5′ to the cleavage site in d(GPy).[11−13] The NMR evidence for this hydrogen bonding network included the observation of a downfield shifted 4-amino proton of the pyrimidine (10.36 ppm) and its NOE interactions with its own P−CH_3, and the G5-H1′, G5-H4′ and the imino protons of G5 and G4 (both are weak) in the DNA.[11] In the HOO−CodGBLM complex, an exchangeable proton is observed at 10.23 ppm that shows NOEs to P−CH_3, G5-H1′, and G5-H4′, and this has also been assigned to one of the 4-amino protons of the pyrimidine.

The fortuitous observation of the hydroperoxide proton in HOO−CoBLM bound to DNA previously provided the opportunity to examine the orientation of the metal-bound hydrogen peroxide ligand relative to the 4′ hydrogen of C6, the site of cleavage.[11,12] This assignment (8.89 ppm) was supported by NOEs to the protons of the linker region of BLM and the deoxyribose protons of the cleavage site, as well as by molecular dynamics calculations.[11,12] By analogy, an exchangeable proton at 9.02 ppm in HOO−CodGBLM complex has been assigned to the hydroperoxide proton of HOO−CodGBLM. This resonance shows many NOEs to the minor groove protons of the cleavage site C6 (Table 4) and protons of the linker region (Supporting Information Table 1) in a pattern similar to that observed with the HOO−CoBLM complex. To ensure an unbiased assignment, the initial restrained molecular dynamics

calculation described below was performed without the NOEs associated with the hydroperoxide proton. The distances between the hydroperoxide proton and the protons of the linker region and the C6 deoxyribose, derived from the resulting structural model were found to match well with the intramolecular and intermolecular NOEs observed by NMR spectroscopy.

Of the protons associated with the β-aminoalanine, the putative second axial ligand, the assignments of A−CαH and A−CβHs in HOO−CodGBLM bound to DNA have been most challenging. Their tentative assignments are deduced from the following observations. The A−NH proton was first identified based on its NOE to H−C2H. This NOE is ubiquitous among metallo-BLMs as a result of the spatial arrangement of four equatorial ligands (Figure 3) and is readily identifiable in an uncrowded region of the NOESY spectrum. The COSY spectrum in H_2O does not exhibit any cross-peaks associated with the A−NH, largely due to its broad line width and S/N constraints. Inspection of the TOCSY spectrum in H_2O, however, reveals a small cross-peak between A−NH and a resonance at 3.08 ppm. Corroboratively, the NOESY spectrum in H_2O also contains a NOE from A−NH to this resonance at 3.08 ppm. The resonance at 3.08 ppm is thus assigned to one of the A−CβHs. The COSY spectrum in D_2O shows a cross-peak between the resonance at 3.08 and 3.50 ppm, affording the assignment of the other A−CβH. Subsequent analysis of the NOESY spectrum in D_2O reveals NOEs from both A−CβHs to a resonance at 2.96 ppm, tentatively assigned to A−CαH. No COSY cross-peaks can be discerned between A−CβHs and A−CαH.

These results are reminiscent of the studies with HOO−CoBLM bound to **1**.[11] The assignments in this case were difficult as well due to broadening of the signals associated with each hydrogen and the small coupling constants. In this case, however, extensive NOE information about their interactions with the surrounding environment confirmed the assignments.

In the deglyco case, the resonances associated with the α and β protons of A are even broader and again the coupling constants are small. These problems, the presence of at least one minor species, and the spectral crowding in the 2−4 ppm region have exacerbated the situation and minimized the number of detectable intermolecular NOEs between these protons and DNA.

Proton Assignment of 1 in its Complex with HOO−CodGBLM. Assignment of the exchangeable imino protons (Supporting Information Table 2) of the HOO−CodGBLM complexed with **1** was initially based on their assignment in the parent compound with **1** (compare Figure 6 (parts a and b)). They were then verified by NOESY experiments. For example, the two upfield shifted imino protons were assigned to C6·G15 and C7·G14. These assignments were verified by NOEs from the C−H6 and C-amino protons to the imino in each case. In addition, both iminos show NOEs to B−C5′H (supporting Figure 2). The upfield shift of these imino protons relative to free DNA and the NOEs to B−C5′H are consistent with an intercalative mode of binding. Corroboratively, no NOE is observed between these imino protons as insertion of the bithiazole increases their distance from one another.

While the spectra of the imino protons of HOO−CodGBLM and its parent are very similar (Figure 6 (parts a and b)), the imino proton from base pair A3·T18 stands out. It is upfield shifted by 0.51 ppm relative to its parent, and the NOE cross-peaks associated with many protons of the A3·T18 base pair are broadened (data not shown). Recall that in our 1D titration (Figure 5c), the H8 proton of A3 was also selectively broadened.

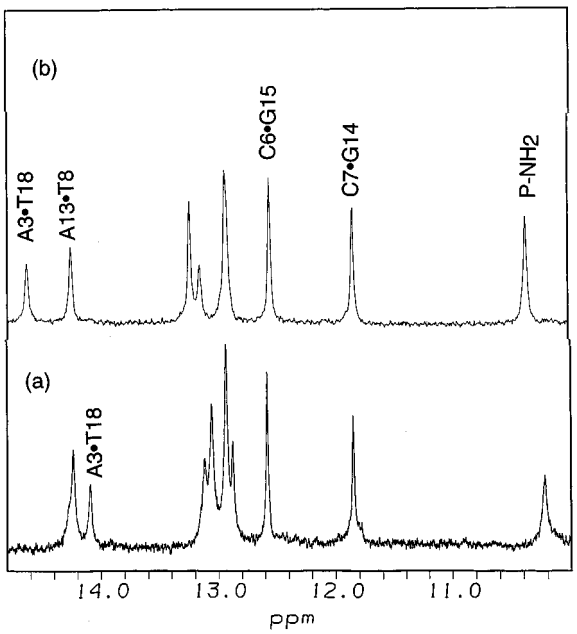

Figure 6. 1D imino proton comparisons between DNA/HOO−CodGBLM (a) and DNA/HOO−CoBLM (b).

These observations suggest that the A3·T18 base pair is conformationally dynamic. The consequences of these observations will be discussed subsequently. The remainder of the exchangeable protons have been assigned as previously described for the parent complex.[11]

Assignments of the nonexchangeable protons were achieved by analyzing the NOESY, TOCSY, and DQF−COSY spectra by standard procedures.[63] Patterns of sequential connectivity between H1′ and base protons are similar to those reported for the parent complex for both oligonucleotide strands (a representative 2D spectrum is shown in supporting Figure 3). Once again, a region of particular uncertainty is associated with the deoxyribose protons of both A3 and T18, due to their line widths. All other sugar protons, with the exception of several H5′, H5"s, have been assigned (Supporting Information Table 2) and indicate that with the exception of the deoxyribose of C6, all sugars experience a C2′-endo conformation. At C6, as with the parent, a C3′-endo conformation is observed.

Restrained Molecular Dynamics Studies. Thirty-nine intermolecular NOEs between **1** and HOO−CodGBLM (Table 4) and forty-four intramolecular NOEs within HOO−CodGBLM (Supporting Information Table 1) have been used as constraints in molecular modeling. The initial starting structure was constructed by positioning the bithiazole moiety of HOO−CodGBLM between the C6·G15 and C7·G14 base pairs. The orientation was based on the NOE data showing specific interactions of B−H5 and B−H5′ with residues of G14, G15, and C6 (Table 4). This initial structure was then submitted to minimizations and molecular dynamics simulated annealing calculations. The final distances are in very good agreement with distance constraints giving a rms deviation of 0.025 ± 0.002, and no constraint errors over 0.2 Å (Supporting Information Table 3). A final structure was obtained by averaging 11 separately determined structures, followed by minimization of the averaged coordinates. This model is the basis for the following discussion.

Mode of Binding and Sequence Specificity. The minimized structure of HOO−CodGBLM bound to **1** is shown in Figure

(63) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; John Wiley & Sons, Inc: New York, 1986.

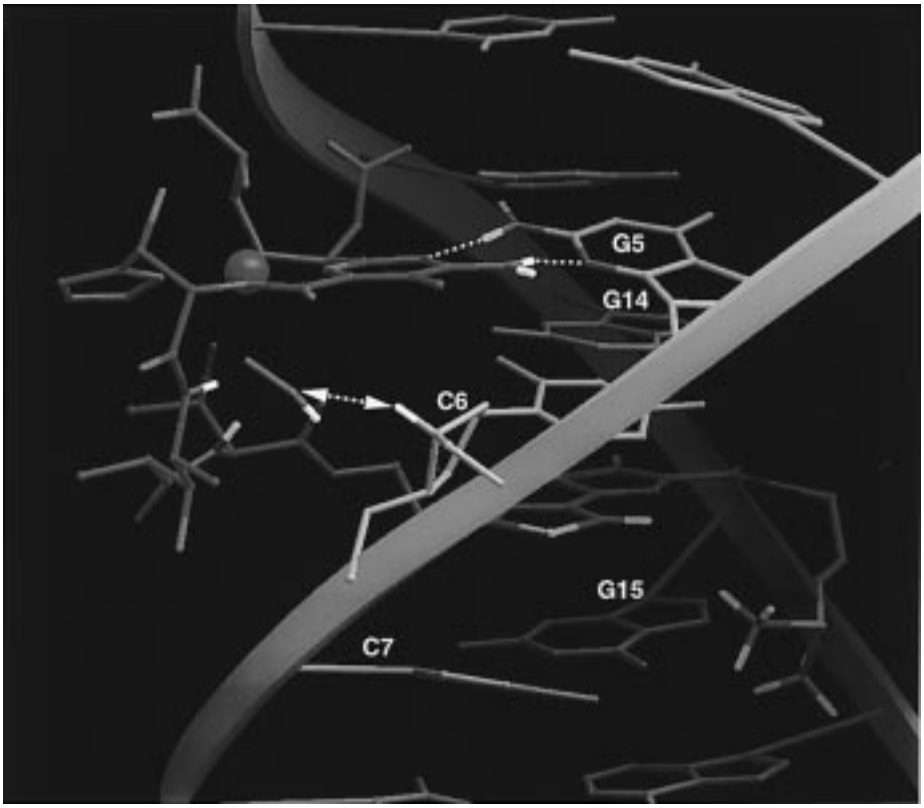

Figure 7. The structure of HOO−CodGBLM (atoms colored by element, C = green, O = red, N = blue, S = yellow) bound to DNA (white ribbon). The strand where cleavage occurs is in the foreground, running 5′→3′ from the upper right to lower left corner. The dotted lines indicate the H-bond interactions between the P moiety of HOO−CodGBLM and the G5 of the DNA. Also indicated is the proximity of the distal oxygen of the hydroperoxide ligand to the C6−H4′ (2.5 Å).

7 and is very similar to that previously reported for HOO−CoBLM. The bithiazole rings in HOO−CodGBLM are inserted between base pairs C6•G15 and C7•G14, 3′ to the cleavage site C6 (Figure 7). The trans orientation of their protons is almost identical to that in HOO−CoBLM bound to **1**. The terminal thiazolium ring is completely stacked between the bases of G14 and G15, and the penultimate thiazolium ring is partially stacked between the bases of C7 and C6. The intercalation of the thiazolium rings results in the unwinding of DNA by a total of 12° over the three steps, (G5•C16)∼(C7•G14)∼(T8•A13). The NOE between the sulfonium methyl protons and A13−H8 of DNA (Table 4) unambiguously locates the C-terminus of BLM in the major groove near A13 as observed in the case of HOO−CoBLM bound to DNA. The electrostatic interaction between this positively charged moiety and the DNA plays an important role in providing binding energy and anchoring the bithiazole rings.

The pyrimidine moiety of HOO−CodGBLM is within H-bonding distance of G5 in the minor groove forming a base triple and providing an explanation for the observed sequence specificity of cleavage. One of the putative hydrogen bonds is between one of the 4-amino protons of the pyrimidine in HOO−CodGBLM and the N3 of G5 at a distance of 1.9 Å and an angle of 170°. The other is between one of the 2-amino protons of G5 and the N3 of the pyrimidine in HOO−CodGBLM at a distance of 2.2 Å and an angle of 173°. The orientation of the metal binding domain as well as the distances and angles of the two putative hydrogen bonds are similar to those observed in the DNA/HOO−CoBLM complex.

The observation of the proton of the hydroperoxide in the complex between the HOO−CoBLM and DNA provides a remarkable perspective on how the alignment of the metal-bound hydroperoxide toward the minor groove protons contributes to the site specific hydrogen atom abstraction at the 4′ position.[11,12] Not surprisingly, the hydroperoxide proton in HOO−CodGBLM bound to DNA is also observed with a similar chemical shift and analogous sets of inter- and intramolecular NOEs (Table 4 and Supporting Information Table 1). The final structure reveals that the distal oxygen of the hydrogen peroxide is ∼2.5 Å from the H4′ proton of C6. The orientation of the hydrogen peroxide toward the minor groove of C6 is almost superimposable on the arrangement observed in HOO−CoBLM bound to DNA, suggesting a similar DNA cleavage mechanism by deglycoBLM. The similarities in model structures between HOO−CoBLM and its corresponding aglycon, and the lack of a carbamoyl amino group as a potential axial ligand in the latter case, provide further support for our proposal that the primary amine of β-aminoalanine is a ligand in the parent compound.

The similarities suggest that deglycoBLM analogues, available by synthetic methods,[44,45,64−66] will provide a good model to examine the ds cleavage process. Recent studies,[67] using the hairpin methodology described by Absalon et al.[28,29] and FeBLM to quantitatively evaluate the ratio of ss:ds cleavage, indicate that the ratio for a G*T*AC containing hairpin changes from 3:1 with the parent to 6:1 with the sugars removed (data not shown).

(64) Boger, D. L.; Teramoto, S.; Cai, H. *Bioorg., Med. Chem.* **1996**, *4*, 179−93.
(65) Boger, D. L.; Ramsey, T. M.; Cai, H. *Bioorg., Med. Chem.* **1996**, *4*, 195−207.
(66) Boger, D. L.; Honda, T.; Menezes, R. F.; Colletti, S. L. *J. Am. Chem. Soc.* **1994**, *116*, 5631−5646.
(67) Hoehn, S.; Stubbe, J. **1997**, unpublished results.

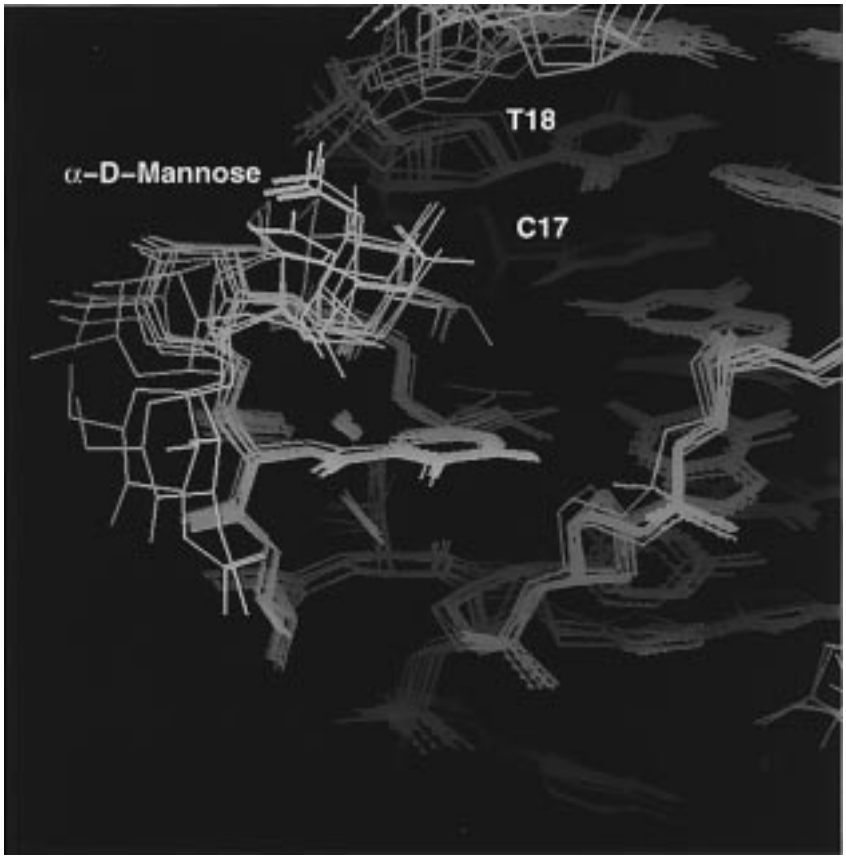

Figure 8. An overlay of the final eight structures of HOO−CoBLM complexed with **1**, looking into the minor groove. The mannose moiety (yellow) is within the minor groove and is within van der Waals contact with the deoxyribose of either C17 or T18 in six of the eight structures.

The Subtle Differences between the Deglycosylated and Parent Bound to DNA. We now begin to focus on the differences between the two complexes in an effort to understand how the lack of the sugar domain can lead to the subtle changes observed in sequence specificity at secondary cleavage sites[41,46,47] as well as diminished binding. Already revealed in the 1D titration spectra (Figure 5c) are the presence of minor species and the selective broadening of the A3·T18 protons in HOO−CodGBLM bound to DNA. Each of these two points and their implications will be discussed in turn.

The Minor Complexes. Under identical conditions, the binding of HOO−CodGBLM to **1** displays many small additional features (Figure 5c) that are absent in the complex between HOO−CoBLM and the same oligonucleotide. Attempts to unambiguously assign these protons have been unsuccessful because of their low abundance. However, chemical shift comparisons with associated protons and the analysis of NOESY spectra at lower contour levels have been informative. Some of these resonances in the downfield region appear to represent a minor complex(es) formed between HOO−CodGBLM and DNA, based on the resemblance of their NOEs to the intramolecular NOEs shown in the major complex (Supporting Information Table 1). For example, the resonance at 9.21 ppm (Figure 5c) displays NOE cross-peaks to resonances at 7.58, 4.22, 3.09, and 1.29 ppm (Supporting Information Figure 4). Based on the chemical shift similarities, the NOE between protons at 9.21 and 7.58 ppm resembles that observed between H−C2H (9.06 ppm) and H−C4H (7.55 ppm) and the NOE between protons at 9.21 and 1.29 ppm to that between H−C2H (9.06 ppm) and T-CH_3 (1.25 ppm) (Supporting Information Table 1). Thus, protons at 9.21, 7.58, and 1.29 ppm can be tentatively assigned to another complex containing H−C2H, H−C4H, and T-CH_3 protons, respectively. Unfortunately, no NOEs were detected between this putative complex and **1**. These signals may be associated with a minor and distinct complex between HOO−CodGBLM and DNA.

Although the mode of binding in this minor complex is not discernible from the available information, a few possibilities can be ruled out. First, the molecular modeling studies show that it is unlikely that two molecules of metallo-BLM could occupy the minor groove of this decamer at the same time. Second, the purity of both HOO−CodGBLM and DNA have been analyzed by NMR prior to the titration, and thus it is also unlikely that this minor complex originates from contaminates or from a metalloBLM with the opposite chirality.

The most plausible model for this minor complex therefore is one in which the same HOO−CodGBLM binds to a different site on the DNA. Integration of the two respective H−C2H protons at 9.21 and 9.06 ppm shows that the minor complex accounts for 5% of the predominant form. Because both complexes are in slow exchange on the NMR time scale, this ratio reflects the equilibrium concentration of the two complexes and hence the differences in binding affinity. Finally, additional minor resonances appear in the titration spectrum (Figure 5c) that may not be associated with this minor complex. Thus, the HOO−CodGBLM has an additional secondary binding site(s) in comparison to its parent. It is however not known whether this minor binding event leads to cleavage. It is also not clear what structural changes resulting from the removal of the sugar domain are responsible for alternative binding sites in **1**. Our hypothesis at present suggests that local structural changes such as the movement of the amide group of the pyrimidine and the β-aminoalanine moiety may alter the binding complementarity between metallo-deglycoBLM and DNA.

Dynamic Characteristics of the A3·T18 Base Pair. Selective broadening of the base and sugar protons of A3 and T18 residues is observed in the complex of HOO−CodGBLM with **1**.[68] The line width of A3−H8, for example, is 22 Hz at 20 °C in comparison with A13−H8 which is 11 Hz. Interestingly, this conformational flexibility is also apparent in the uncomplexed **1** in solution and from a crystallographic structure determination of **1**.[69] NMR studies of free DNA reveal that the line width of A−H8 is 13 Hz in comparison with the average line width of 8 Hz for other base protons. The crystal structure reveals that the stacking between the base pairs A·T and 5′ C·G is disrupted.[69] The conformational flexibility observed with HOO-dGBLM complexed to **1** is even greater than that observed with its parent. These results suggest that despite the fact that this base pair is two steps removed from the metal binding domain, the sugars confer some conformational stability to this region. The structure in Figure 8 reveals that the sugars, in six of eight structures, are nuzzled adjacent to the deoxyribose protons of C17 and T18, which then constrains the flexibility of T18·A3. These changes in DNA conformation removed from the immediate binding site of HOO−CodGBLM could lead to reduction in binding affinity and as a consequence increase cleavage at secondary sites.

Differences in Binding Affinity. With oligonucleotide **1**, the Kd for HOO-dGBLM is 35-fold less than for its parent. The loss in binding energy may be related to loss of nonspecific van der Waals interactions between the sugar domain and DNA and/or altered specific hydrogen bonding interactions between the drug and **1**. In six of the eight final structures of HOO−CoBLM bound to DNA, the mannose moiety (e.g., M−C6H2) is within van der Waals distance of the DNA backbone and the deoxyribose protons of C17 and T18 (Figure 8). The modeling also reveals consistent differences in the conformation in HOO−CodGBLM relative to HOO−CoBLM when bound to **1**. A hydrogen bond interaction appears to be present between a proton of the amide moiety of β-aminoalanine and O3′ of C17 in the latter, which does not appear to be present in the former. It should be noted, however, that the above putative hydrogen bond as well as the nonspecific interactions between the sugar domain and DNA are thus far deduced only from the structures of eight restrained molecular dynamics calculations and require additional confirmatory evidence.

The studies described here strongly support a common structural motif for HOO−CodGBLM and HOO−CoBLM both free in solution and bound to DNA. Although our NMR analysis of free HOO−CodGBLM is limited somewhat by the lack of NOEs, the conformation of the linker region and the highly refined structure of HOO−CodGBLM to DNA support the comparison to HOO−CoBLM.

On the basis of intercalation into DNA, recognition by hydrogen bonding of the 5′-guanine residue, and the chemistry of cleavage, it is clear that HOO−CodGBLM and HOO−CoBLM behave in a highly similar, if not identical, manner. Two points require further resolution. First, HOO−CodGBLM binds to DNA 35-fold less tightly than HOO−CoBLM. The molecular basis for this difference is not understood although the contribution of the disaccharide moiety to the binding is an obvious candidate. To date the inability to assign NOEs between sugars protons of HOO−CoBLM and DNA in the complex has made it impossible to accurately position the disaccharide with respect to the DNA backbone and thereby define specific interactions which contribute to the additional binding energy. Since the energetic difference between the HOO−CodGBLM/DNA and HOO−CoBLM/DNA complexes is only ∼2 kcal/mol, individual energetic contributions of portions of the disaccharide to the total binding may be quite subtle.

Second, the controversy over the issue of the axial ligand demands a definitive experiment. We believe that the high degree of conservation of structure and reactivity between the two complexes and a simple set of intuitive chemical and energetic arguments strongly favors the primary amine over the carbamoyl group as the axial ligand. Isotope labeling studies are needed to ultimately resolve this issue.

The complexes of HOO-dGBLM and HOO−CoBLM to DNA have provided the field a unique opportunity to structurally define a model for the "activated" FeBLM/DNA complex. These models will be crucial to future studies directed toward understanding the interplay of structure and chemistry in the unusual double strand cleavage activity of BLM.

Acknowledgment. We gratefully acknowledge NIH Grants, GM 34454 to J.S., CA 42056 to D.L.B., and RR0095 to the NMR facility for the support of this research.

Supporting Information Available: Three tables listing the nontrivial intramolecular NOEs within HOO−CodGBLM, proton chemical shifts of DNA in the complex, and structural statistics of HOO−CodGBLM bound to **1** and four figures showing a HMQC spectrum, and three NOESY spectra of the complex between **1** and HOO−CodGBLM (7 pages). See any current masthead page for ordering and Web access instructions.

JA972125I

(68) This broadening is apparent from examining the NOE cross-peaks associated with many protons of A3·T18 base pair.

(69) Heinenmann, U.; Alings, C. *J. Mol. Biol.* **1989**, *210*, 369−381.