Bioorganic & Medicinal Chemistry Letters 19 (2009) 1614–1617

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Enhancement of DNA cleavage activity of an unnatural ferrocene-amino acid conjugate

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ARTICLE INFO

Article history:
Received 30 December 2008
Revised 30 January 2009
Accepted 3 February 2009
Available online 11 February 2009

Keywords:
Unnatural amino acid
Ferrocene
Nuclease
DNA cleavage

ABSTRACT

The nucleolytic activity of an unnatural ferrocenyl amino acid can be significantly enhanced under reducing or Fenton conditions while exploiting different mechanisms of DNA cleavage. In addition, results from high resolution electrophoresis indicate that the ferrocenyl amino acid exhibits non-sequence specific cleavage of the DNA backbone.

In the field of bioorganometallic chemistry, derivatives of ferrocene exhibit a variety of medicinal properties by serving as antibacterial, antimalarial and antitumor agents.[1] The ability of ferrocenyl compounds to cleave DNA may be responsible for these diverse properties.[1–5] While a multitude of ferrocenyl compounds exist, we are most interested in the design of unnatural ferrocenyl amino acids and peptides which have been developed mainly for use in biomedical studies.[6–9] Selective modification of proteins using similar unnatural amino acids facilitates the study of protein structure, activity, and interaction with other biological molecules. Recently, in vivo techniques to genetically encode for site-specific incorporation of unnatural amino acids into proteins have been developed.[10–12]

Using these new methods, unnatural redox active amino acids (including ferrocenyl groups) have been selectively incorporated into proteins to study electron transfer activity.[13,14] In addition, a metal-binding unnatural amino acid (2,2′-bipyridin-5-yl-alanine) was inserted into the *Escherichia coli* catabolite activator protein (CAP) to generate a site-specific protein nuclease capable of cleaving DNA.[15] Interactions occurring in protein–nucleic acid complexes can be studied with modified protein nucleases when physical techniques such as NMR or crystallography are insufficient. While the majority of synthetic protein nucleases contain an inorganic metal-chelating species, we intend to design a ferrocenyl unnatural amino acid for incorporation into DNA- binding proteins. Efficient production of ferrocenyl site-specific nucleases will further the investigation of protein–nucleic acid interactions.

In previous experiments, we reported the synthesis of a novel unnatural amino acid, N^{α}-N^{ε} -*(ferrocene-1-acetyl)*-L-*lysine* (**1**, Fig. 1) and its ability to cleave DNA.[16] Studies have been continuing to determine the potential mechanisms by which our unnatural amino acid cleaves nucleic acids. Towards this goal, **1** was incubated with DNA in cleavage assays[17] involving the addition of reductants, peroxide/ascorbate and scavengers to determine if the nuclease activity of N^{α}-N^{ε}-*(ferrocene-1-acetyl)*-L-*lysine* resembles that of other iron-chelating chemical nucleases.

Stock solutions of **1** were prepared in 10 mM Tris–HCl pH 8.0 along with 10% THF or DMSO immediately before incubation with DNA. Previous data indicated that at a concentration of 75 μM, N^{α}-N^{ε} -*(ferrocene-1-acetyl)*-L-*lysine* could sufficiently cleave 57% of supercoiled pUC19 plasmid (75 μM bp) upon incubation for 16 h.[16] Since the ferrocene moiety of our compound is easily oxi-

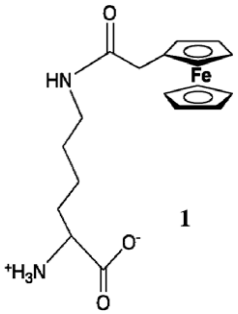

Figure 1. Structure of N^{α}-N^{ε}-(ferrocene-L-acetyl)-L-lysine.

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doi:10.1016/j.bmcl.2009.02.023

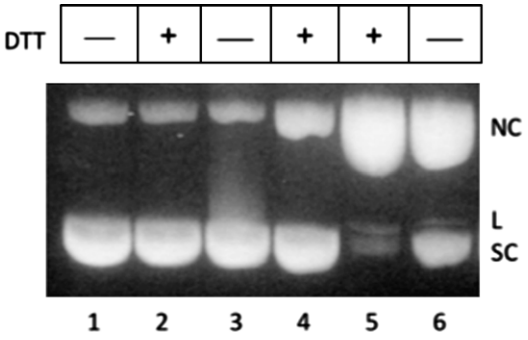

Figure 2. Comparative DNA cleavage assays: **1** versus salen·Fe. A 10 μl reaction containing 1.0 μg of pUC19 DNA (75 μM bp) was incubated in the presence (+) or absence (−) of 200 μM DTT (indicated above each lane) at 25 °C for 3 h along with the following compounds: *lanes 1 and 2*, None; *lanes 3 and 4*, 100 μM of **1**; and *lanes 5 and 6*, 100 μM of salen·Fe(*para*). The super coiled (SC), nicked circular (NC), and linear (L) forms of pUC19 DNA were separated by aganose (1%) gel electro-phoresis in 0.5× TBE.

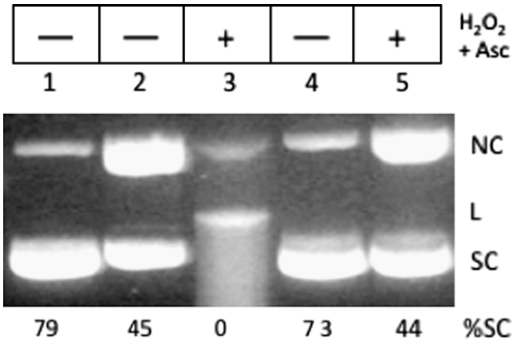

Figure 4. Comparative DNA cleavage assays: **1** versus Fe(II)EDTA with peroxide/ascorbate. Reactions (20 μl) contained pUC-19 (1.0 μg, 75 μM bp) incubated in the presence (+) or absence (−) of 0.003% (88 μM) H_2O_2 and 1.0 mM ascorbate (Asc) at 25 °C for 5 min along with the following compounds: *lane 1*, None; *lanes 2 and 3*, 20 μM Fe(II)EDTA; and *lanes 4 and 5*, 20 μM of **1**.

dized from Fe^{2+} to Fe^{3+} (ferricium ion) in aqueous solutions, cleavage assays containing a reductant (DTT) were performed to assess the effect of regenerating the ferrous ion on the nuclease activity of **1**. Addition of 200 μM DTT increased plasmid cleavage, which was detected after only 3 h of incubation (Fig. 2, *lanes 3 and 4*). However, cleavage by **1** was less significant than that by a *para* salen–Fe complex (Fig. 2, *lanes 5 and 6*) which binds directly to DNA. Binding activates a semi-quinone system within the salen to eventually produce a ligand radical species that cleaves the DNA backbone.[18] Increasing DTT concentrations to 1 or 10 mM and incubation to 16 h resulted in production of the nicked circular and linear form of the plasmid by **1** (Fig. 3 *lanes 3 and 4*) that more closely resemble the DNA damage levels seen with the *para* salen–Fe complex. Clearly, recycling the ferrous ion increases DNA damage by **1**, but other processes may be occurring.

The oxidation of the ferrocene moiety in aqueous solution can produce superoxide radicals ($Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot-}$) that could mediate DNA cleavage. The presence of TEMPOL, a superoxide scavenger,[19] led to a significant decrease in DNA damage (Fig. 3, *lanes 6 and 7*), suggesting that superoxide radicals generated by **1** in aqueous solution play a role in the cleavage of plasmid DNA. In addition, the iron-catalyzed autoxidation of DTT itself produces highly reactive species such as superoxide anions, hydroxyl radicals, hydrogen peroxide and thiyl radicals,[20] all of which can facilitate DNA damage. Further studies to elucidate the contribution of these potential reactions in enhancing the nuclease activity of **1** are underway.

Previous reactions involving organic solvents have also indicated that ferrocene derivatives are capable of producing hydroxyl radicals.[21] To determine if the ferrocene moiety in **1** could generate hydroxyl radicals in aqueous solutions, hydrogen peroxide and ascorbate were included in the DNA assays to induce Fenton chemistry ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + HO-$)[22] and to regenerate the ferrous ion, respectively. The presence of peroxide/ascorbate significantly decreased both the time (5 min vs 16 h) and the necessary concentration of **1** (20 μM vs 100 μM) required to detect the nicked circular form in the DNA cleavage assay (Fig. 4, *lanes 4 and 5*). However, the cleavage by **1** was much less efficient than by Fe(II)EDTA, as the latter chemical nuclease converted the majority of plasmid to the linear form under the same Fenton conditions (Fig. 4, *lane 3*). Hence, hydroxyl radicals are either generated at a slower rate or not at all from Fe^{2+} in the ferrocene moiety.

To further determine the involvement of hydroxyl radicals in DNA cleavage by **1**, assays were performed in the presence of mannitol, a hydroxyl radical scavenger. Mannitol had little effect on the nuclease activity of **1** (Fig. 5, *lanes 6–8*). Previous reports suggest that a readily available coordination site is necessary for Fe^{2+} to catalytically generate hydroxyl radicals via the Fenton reaction.[23] The two cyclopentadiene rings that contribute multiple bonds to the ferrous ion may prevent such catalytic activity by the ferrocene moiety. Similarly, no change in nuclease activity was detected upon addition of azide (a singlet oxygen scavenger) to the DNA cleavage assays (Fig. 5, *lanes 3–5*). Together, these results suggest that diffusible reactive oxygen species, such as hydroxyl radicals and singlet oxygen, are not responsible for the nuclease activity of **1** in the presence of peroxide/ascorbate in aqueous solutions. We surmise that a non-diffusible, high valent iron-oxo species (such as the ferryl ion, $[Fe(IV)O]^{2+}$)[24] may be produced directly on the ferrocene moiety of **1** which then leads to the DNA cleavage under these Fenton conditions.

To examine the sequence specificity of the nuclease activity of *N^{α}-N^{ε}-(ferrocene-1-acetyl)-L-lysine,* a ^{32}P-end labeled 5S rRNA gene[25] replaced the pUC19 plasmid in the DNA assays and the

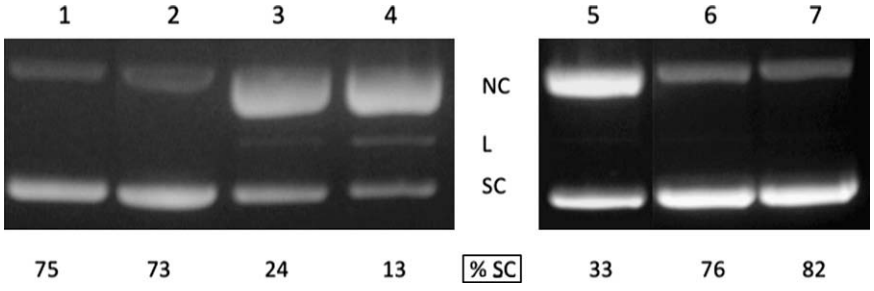

Figure 3. Effect of DTT and TEMPOL on DNA cleavage by **1**. A 10 μl reaction containing 0.5 μg of pUC19 DNA (75 μM bp) was incubated at 25 °C for 16 h alone (*lane 1*), with 10 μM DTT(*lane 2*) or with 100 μM of **1** (*lanes 3–7*). In addition, reactions in *lanes 3 and 4* contained 1 mM and 10 mM DTT (respectively) while reactions in *lanes 5–7* contained 0, 50, or 100 mM TEMPOL, respectively.

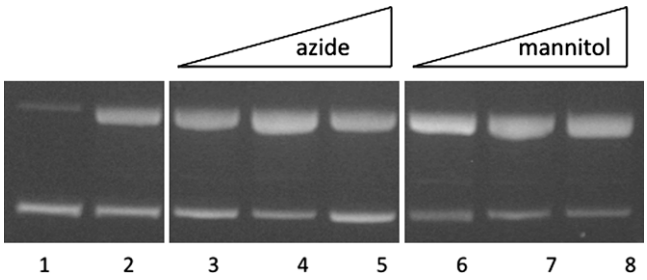

Figure 5. Effect of azide and mannitol on DNA cleavage by **1** in the presence of peroxide/ascorbate. A 10 μl reaction containing 0.5 μg of pUC19 DNA (75 μM bp) was incubated at 25 °C for 5 min alone (*lane 1*) or with 100 μM of **1** in the presence of 0.003% (88 μM) H_2O_2 and 1.0 mM ascorbate (Asc) (*lanes 2–8*). Reactions also contained 2, 5, or 10 mM sodium azide (*lanes 3–5*, respectively) or 2, 5, or 10 mM mannitol (*lanes 6–8*, respectively).

reaction products were separated using high resolution denaturing gel electrophoresis.[26] As seen in Figure 6A, **1** generates a ladder of linear DNA fragments in the presence of DTT (*lane 3*). The non-specific pattern suggests that the damaging species has no base preference during attack of the DNA molecule. Diffusible reactive species tend to exhibit this pattern, which supports the idea of superoxide molecules (among potentially other species) generated by **1** are facilitating the DNA cleavage under these conditions. In Figure 6B, a comparable non-specific ladder of linear DNA fragments is produced by the Fe(II)EDTA complex in the presence of peroxide/ascorbate (*lane 6*). The exact identity of a diffusible species generated by this complex to cleave the DNA backbone has been widely investigated and argued in the literature.[24] Regardless, under equal conditions, the *N^{α}-N^{ε}-(ferrocene-1-acetyl)-L-lysine* did not generate any significant amount of linear DNA fragments (*lanes 3–5*). This result supports our previous speculation that a diffusible reactive oxygen species is not likely to be responsible for DNA cleavage by **1** under aqueous Fenton conditions.

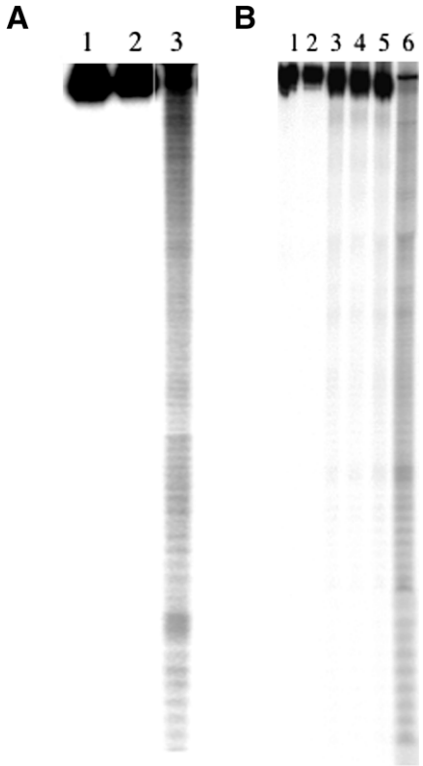

Figure 6. Cleavage of ^{32}P end-labeled 5S rRNA gene by **1**. **A**) A 20 μ1 reaction containing ^{32}P-end- labeled 5S rRNA gene (≈8000 cpm) was incubated at 25 °C for 16 h alone (*lane 1*), with 5 mM of DTT (*lanes 2 and 3*), or with 100 μM of **1** (*lane 3*). **B**) A 20 μl reaction containing ^{32}P-end-labeled 5S rRNA gene (14,000 cpm) was incubated at 25 °C for 5 min alone (*lane 1*) or with 100 μM of **1** (*lane 2*). Reactions in *lanes 3–6* also contained 0.003% (88 μM) H_2O_2 and 1.0 mM ascorbate and the following compounds: 20, 40, or 100 μM of **1** (*lanes 3–5*, respectively) or 20 μM of Fe (II)/EDTA (*lane 6*). All reactions were run on a 10% denaturing acrylamide gel and bands were detected upon exposure to a phosphor screen and scanning laser densitometry.

In conclusion, superoxide molecules produced by *N^{α}-N^{ε}-(ferrocene-1-acetyl)-L-lysine* (**1**) under aqueous reducing conditions lead to significant, non-sequence specific cleavage of the DNA backbone. The presence of Fenton reagents increased the efficiency of DNA cleavage by **1**, however, the generation of a high valent iron-oxo species directly on the compound, rather than a diffusible reactive oxygen species, mostly likely accounts for the DNA damage under these conditions. Studies continue to incorporate **1** into DNA-binding proteins to replace native lysine residues (they are commonly involved in contacting nucleic acids to form complexes). In addition, investigation of this compound for use in biomedical applications due to its nuclease activity are also underway.

Acknowledgements

This work was financially supported by Dickinson College Research and Development sabbatical support grant and an award to Dickinson College from the Howard Hughes Medical Institute under the Undergraduate Biological Science Education Program.

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17. DNA cleavage assays involved the incubation of pUC19 vector DNA (New England Biolabs) along with noted concentrations of **1** (dissolved in 10% THF or DMSO in 10 mM Tris–HCl, pH 8.0), Fe(II)/EDTA (prepared by mixing equal volumes of 200 mM ferrous ammonium sulfate and 200 mM EDTA, pH 8.0) or *para*-salen Fe (prepared as per Routier et al.[18]). After incubation at 25 °C for the times noted, the cleavage products of each reaction were separated by gel electrophoresis (1% agarose, 0.5 mg/mL ethidium bromide) in 0.5× TBE buffer at 90 V for 90 min, visualized by UV light, and captured on a digital image. To calculate the percentage of supercoiled (SC) DNA remaining after a reaction, the intensity of each visible band was measured using ImageAide Band Matching software (Spectronics Corporation) and incorporated into the equation: SC/(SC + NC + L) × 100. A correction factor of 1.1 was applied to compensate for the low affinity of supercoiled (SC) DNA in comparison to the nicked circular (NC) and linear (L) forms of DNA.
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26. High resolution denaturing electrophoresis was performed on a 10% acrylamide:bis acrylamide (19:1) gel containing 8 M urea. After incubation, DNA cleavage assays were precipitated with ethanol/acetate, re-suspended in urea loading dye, and incubated at 55 °C before loading onto the gel. Samples were run at 1700 V for 2 h in 1× TBE buffer before exposure to a storage phosphor screen (Amersham Biosciences) and subsequent scanning laser densitometry (Storm 860 Molecular Imager).