JOC The Journal of Organic Chemistry

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Dephosphorylation Reactions with Deferoxamine, A Potential Chemical Nuclease

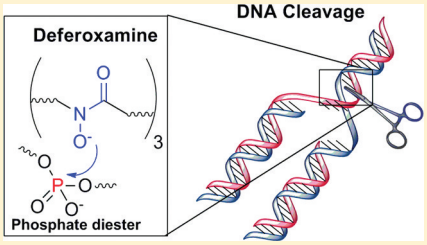
Elisa S. Orth,[†] Michelle Medeiros,[†] Tiago Bortolotto,[‡] Hernán Terenzi,[‡] Anthony J. Kirby,[§] and Faruk Nome*[,†]

[†]INCT-Catálise, Departamento de Química, and [‡]Departamento de Bioquímica, Universidade Federal de Santa Catarina, Campus Universitário − Trindade, 88040-900 Florianópolis, SC, Brasil

[§]University Chemical Laboratory, Cambridge CB2 1EW, U. K.

Supporting Information

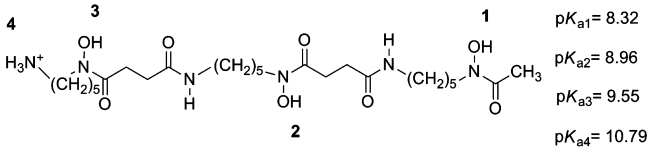
ABSTRACT: We report a detailed kinetic and mechanistic study of the reaction of a widely used therapeutic agent, deferoxamine (DFO), which contains three nucleophilic hydroxamate groups, with the model phosphate diester bis-2,4-dinitrophenylphosphate BDNPP. We clarify the mechanism by detecting important phosphorylated intermediates in the model reaction and show that the mechanism can be extended to the reaction with DNA. The effectiveness of DFO in cleaving DNA was examined over a range of pH in the absence and presence of a biologically available metal (Zn^{2+}). The results inform and complement ongoing studies involving DFO, which can act as a powerful nucleophile toward DNA and other targets susceptible to nucleophilic attack.



Deferoxamine (DFO) is a naturally occurring hydroxamate metal chelator,[1] widely used therapeutically in iron[2] and aluminum[3] overload disorders. For over 40 years, it has been a standard treatment for patients receiving blood transfusions.[4] This treatment is accompanied by numerous side effects,[5] which include the inhibition of DNA synthesis in human lymphocytes.[6] This suggested its potential clinical use as an antiproliferative drug in cancer chemotherapy,[7] and various studies have shown DFO to have antitumor activity in the treatment of neuroblastoma, leukemia, bladder and hepatocellular carcinoma, and brain cancer.[8] DFO can also be used to suppress oxidative degradation of DNA by capturing iron in a nonreactive form.[9]

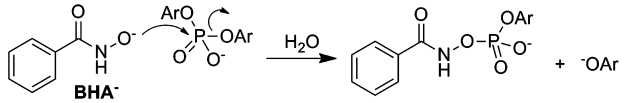
DFO is a bacterial siderophore containing three bidentate oxygen-containing ligands, well-suited for chelating metal cations such as high-spin, octahedral ferric ion. However, these bidentate oxygen-containing ligands are hydroxamic acid groups (Scheme 1) with anions known to be exceptionally powerful nucleophiles, particularly toward the phosphorus centers of phosphate esters.[10]

Scheme 1. DFO Structure and Acid Dissociation Constants of Its Four Ionizing Groups[11]



We have evaluated many reactions of phosphate esters with efficient nucleophiles, including hydroxamates, and have proposed detailed mechanisms and reactivities.[12,13] For example, the reaction of benzohydroxamate (BHA^-, 0.05 mol L^{-1}) with bis(2,4-dinitrophenyl) phosphate (BDNPP) is over 10^5 times faster than the spontaneous hydrolysis of BDNPP ($k_0 = 1.9 \times 10^{-7}$ s^{-1}), Scheme 2.[14]

Scheme 2. Initial Step of the Reaction between Benzohydroxamate Anion BHA^- and a Phosphate Diester



In principle, the hydroxamate groups of DFO, which is used therapeutically in relatively high concentrations (daily dose usually between 20 and 80 mg/kg), could promote the cleavage of any phosphodiester. Indeed, some authors have reported that DFO can damage the DNA strand,[15,16] but no study has focused on the mechanism of action of DFO. We report a complete kinetic and mechanistic study on the reaction of DFO with the model phosphate diester BDNPP. We also present results confirming the effectiveness of DFO in cleaving the DNA strand at different pH levels and in the presence of a biologically available metal (Zn^{2+})

We have compared the reactivities of DFO and the simple hydroxamic acid BHA (benzohydroxamic acid) as nucleophiles toward the reactive model phosphate diester BDNPP (Scheme 2, Ar = 2,4-dinitrophenyl and in Figure 1).

Received: October 6, 2011
Published: November 3, 2011

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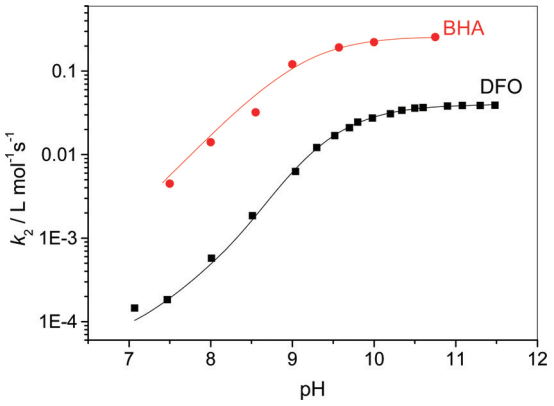


Figure 1. pH/rate profiles for the reactions of BHA and DFO with the model phosphate diester BDNPP at 25 °C and I = 1.0 mol L^{-1} (KCl). The points are experimental, and the curves are calculated. Data for BHA are from the literature.[14]

The pH rate profile for the reaction of BDNPP with DFO was fitted to eq 1, based on the acid dissociation of DFO (Scheme 1).

$$k_{obs} = k_0 + k_{OH}[OH] + (k_1\chi_M + k_2\chi_D + k_3\chi_T) \times [DFO] \quad (1)$$

In eq 1, χ_M, χ_D, and χ_T correspond to the mole fractions of the DFO hydroxamate mono-, di-, and tri-anions, respectively. The derived kinetic parameters are given in Table 1.

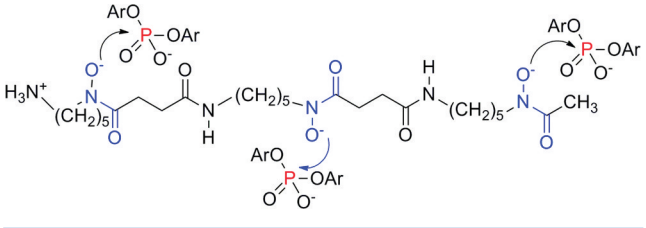
Table 1. Derived Rate Constants for the Reactions of DFO with BDNPP[a]

k_0, s^{-1}	k_{OH}, L mol^{-1} s^{-1}	k_1, L mol^{-1} s^{-1}	k_2, L mol^{-1} s^{-1}	k_3, L mol^{-1} s^{-1}
1.9 × 10^{-7}	3.0 × 10^{-3}	1.15 × 10^{-3}	1.69 × 10^{-3}	3.95 × 10^{-2}

[a]Using acid dissociation constants (Scheme 1) taken from ref 11.

The results are clear-cut and revealing. BHA$^-$ is one of the most powerful known nucleophiles toward phosphorus diesters such as BDNPP (k_{BHA^-} = 0.108 L mol^{-1} s^{-1}).[14] The reaction of DFO with BDNPP is closely similar (Scheme 3), as shown by

Scheme 3. Three Possible Hydroxamate Centers Can Attack the Phosphoryl Group of BDNPP



the pH/rate profiles of Figure 1, just 1 order of magnitude (7–14-fold) slower. The nucleophilic center of rigid benzohydroxamate is exposed, whereas those of DFO are surrounded by an extended conformationally mobile structure that could offer significant steric hindrance to reaction, with the more basic centers reacting faster (Table 1).[17]

The effects of selected metals on the reaction of DFO with BDNPP are shown in Table 2. Addition of Mg^{2+}, Ni^{2+}, and Zn^{2+}, cations readily available in biological systems, potentiates

Table 2. Effects of Metals on the Reaction of DFO with BDNPP[a]

[DFO], mol L^{-1}	[M^{2+}], mol L^{-1}	pH	k_{obs}, s^{-1}	k_{Me-DFO}/k_0
0.01	Mg, 0.01 M	7.5	2.9 × 10^{-6}	15.3
0.01	Ni, 0.01 M	7.5	2.27 × 10^{-6}	11.9
0.01	Zn, 0.01 M	7.5	1.12 × 10^{-5}	58.9

[a]k_{Me-DFO}/k_0 is the ratio between k_{obs} for the reaction of DFO in the presence of metal and the rate constant of the spontaneous reaction.

the phosphorolytic action of DFO at pH 7.5. The same concentrations of the same metals had no effect on the rates of the reaction with BHA.

We drew on our experience in similar studies[14,18] to probe the mechanism of the reaction of BDNPP with DFO, applying ESI–MS(/MS) in negative ion mode to monitor the course of reaction. After 30 min of reaction in aqueous solution at pH 10 and 25 °C, a characteristic ESI–MS (full spectrum given in the Supporting Information, Figure S2) showed a series of major anions.

We identified two important phosphorylated intermediates of m/z 805 and 885, consistent with the proposed attack on phosphorus by the hydroxamate groups of DFO. ESI–MS/MS was then used to characterize the reactants (data given in the Supporting Information, Figures S4–S5), and these key intermediates via collision-induced dissociation and the resulting tandem mass spectra are shown in Figures 2 and 3.

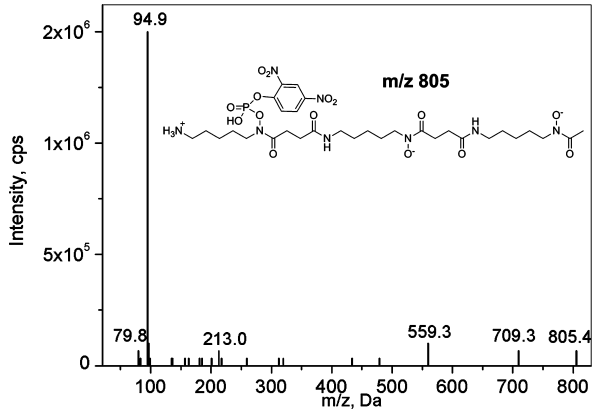


Figure 2. ESI–MS/MS of the phosphorylated intermediate anion of m/z 805.

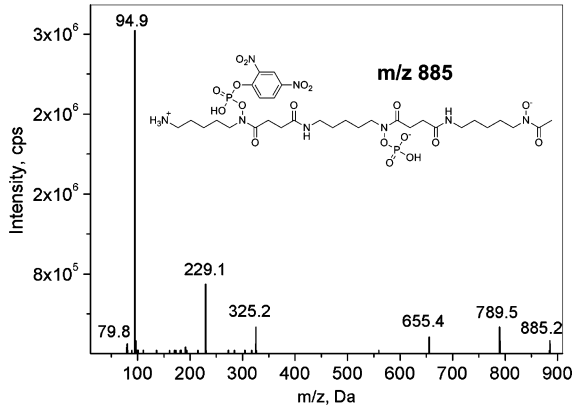


Figure 3. ESI–MS/MS of the phosphorylated intermediate anion of m/z 885.

dx.doi.org/10.1021/jo202074y | J. Org. Chem. 2011, 76, 10345–10348

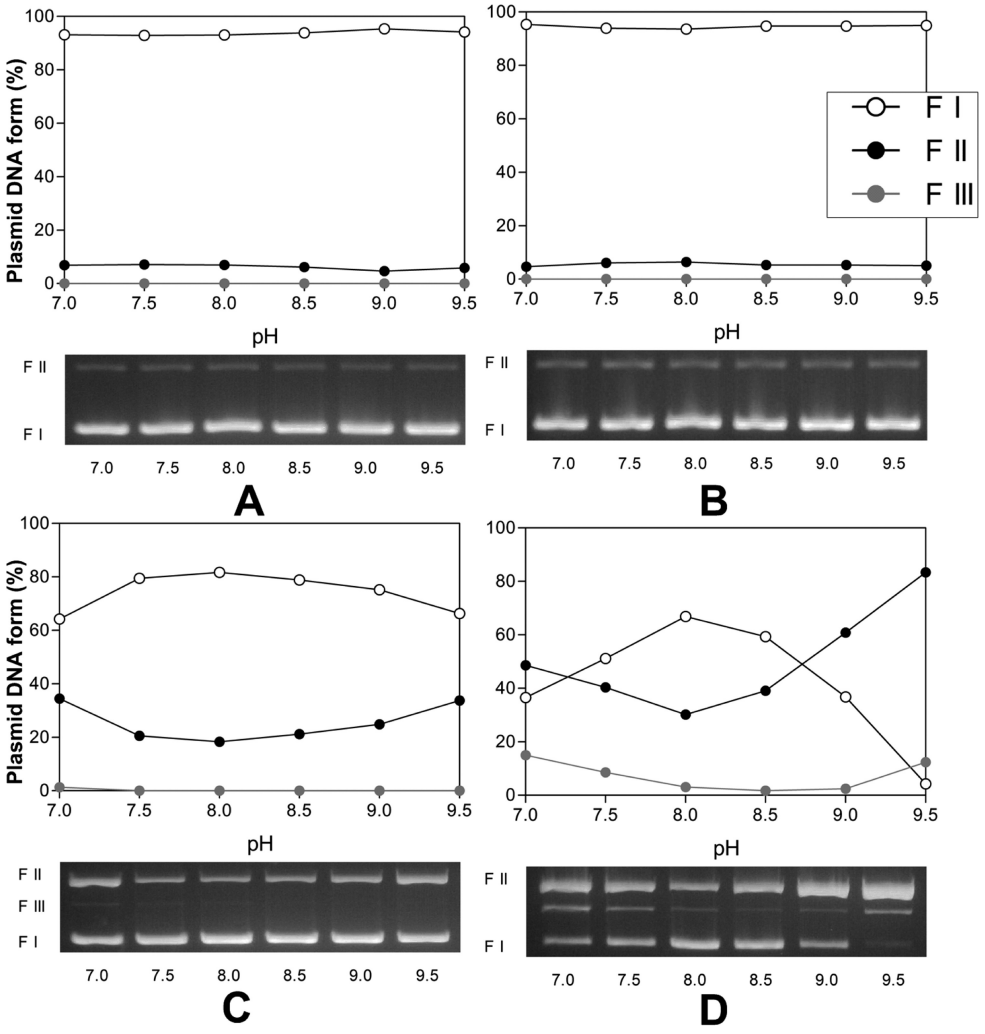


Figure 4. pH/product profiles for the cleavage reactions of DNA after 24 h at 37 °C. (A) Control experiment with buffer only. (B) Control experiment with added 10 mmol L^{-1} Zn^{2+}. (C) Reaction with 10 mM DFO, self-buffered. (D) Reaction with DFO and Zn^{2+}, both 10 mmol L^{-1}, self-buffered. Buffers for control experiments: pH 7–7.5 PIPES, pH 8.0 HEPES, pH 8.5–10 CHES; all at 10 mmol L^{-1}. (F I) supercoiled, (F II) open-circular, and (F III) linear pBSK II DNA forms. Preparation of plasmid DNA and cleavage assays were carried out as previously described[19] and are given in the Supporting Information.

The anions of m/z 885 and 805 both dissociate to the fragment of m/z 95 (Figures 2 and 3), which originates from the aromatic ring cleavage of BDNPP and is not observed for DFO. In addition, the anion of m/z 805 dissociates to 559, which is DFO itself (assignments of the different m/z fragments are given in the Supporting Information, Scheme S3).

Experiments with the far less reactive phosphodiester groups of DNA confirmed the ability of DFO to cleave the DNA strands. We used plasmid DNA as a convenient, sensitive substrate and followed its reactions with DFO in the presence and absence of Zn^{2+} (one of the metals most abundant in the cell) at different pH levels. The data are summarized in Figure 4.

Significantly, there is no detectable reaction between DNA and the chemically more reactive BHA under the same conditions (reactions were run for 24 h at 37 °C over the pH range 7–9.5; data not shown). Under the control conditions (Figure 4A,B), no reaction is observed at any pH. In the presence of 0.01 M DFO (Figure 4C), almost 40% of nicked circular product is formed at pH 7 (and the reaction is close to quantitative at pH 9.5), but with both DFO and Zn^{2+} present (Figure 4D), the half-life of the plasmid at pH 7 is about 16 h with ~65% of cleaved DNA as open circular plus linear forms.

This level of activity implies a significant binding interaction with the anionic polynucleotide chain, which could involve the primary ammonium group and multiple hydrogen bonds as well as the metal cation, so the binding has the potential to show specificity. We intend to examine the binding of DFO to DNA as part of a more detailed investigation.

In conclusion, this work elucidates the mechanism for the reaction of a widely used therapeutic agent, DFO, with the phosphate diester BDNPP, which involves nucleophilic attack on phosphorus by the hydroxamate groups of DFO and the formation of phosphorylated intermediates. DFO shows moderate chemical nuclease activity, over and above that expected for a simple hydroxamic acid, and this activity is enhanced by Zn^{2+} cations. It seems clear that our mechanistic proposal for the reaction of DFO with BDNPP can be extended to the reaction with DNA. The results presented here inform and complement several ongoing studies involving DFO, which can act as a powerful nucleophile toward DNA and other targets susceptible to nucleophilic attack.[10,12–14]

dx.doi.org/10.1021/jo202074y | J. Org. Chem. 2011, 76, 10345–10348

EXPERIMENTAL SECTION

Materials. Inorganic salts were of analytical grade and were used without further purification. Distilled water was used throughout these experiments. DFO was obtained commercially. Solutions were prepared immediately before use. BDNPP was prepared according to the procedure described in the literature.[14]

Kinetics. Reactions were followed spectrophotometrically by monitoring the appearance of 2,4-dinitrophenolate (DNP) at 400 nm, under first order conditions. Reactions were started by adding 10 μL of 4 mmol L^{-1} stock solutions of the substrate in acetonitrile into 3 mL of aqueous solutions to give a final concentration of the substrate of 13.33 μ mol L^{-1}. The temperatures of reaction solutions in quartz cuvettes were controlled with a thermostatted water-jacketed cell holder, and ionic strengths were kept constant at 1.0 mol L^{-1} with KCl. Absorbance versus time data were stored directly on a microcomputer, and observed first-order rate constants, k_{obs}, were calculated from linear plots of $\ln(A_\infty - A_t)$ against time for at least 90% of the reaction by using an iterative least-squares program; correlation coefficients were >0.999 for all kinetic runs. Solutions were self-buffered by DFO in the pH range of 7−11.5.

Mass Spectrometry. The mass spectrometer system consisted of a hybrid triplequadrupole/linear ion trap mass spectrometer QTrap 3200 (Applied Biosystems/MDS Sciex, Concord, Canada) coupled to a Harvard Pump 11 Plus (Harvard Apparatus, Holliston, MA) for sample infusion. The mass spectrometry was tuned in the negative and positive modes by infusion of polypropylene glycol (PPG) solution. The experiments were performed using the Turbo VTM source (electrospray-ESI) in positive and negative ion mode. The capillary needle was maintained at 5500 V, and the declustering potential (DP) was set to 35 V. Synthetic air was used as nebulizer gas (GS1) at a pressure of 15 psi, and nitrogen was used at 10 psi as Curtain GasTM in the interface as well as collision gas (CAD GasTM) at 6 arbitrary units in the LINAC collision cell. The sample solution was diluted to 1.0 mg L^{-1} in water solution and infused at a rate of 10 μL min^{-1} using the built-in infusion pump. Then, electrospray mass and tandem mass experiments (MS2) were performed.

Preparation of Plasmid DNA and Screening. The plasmid pBSK II (2961 bp), used for DNA cleavage assays, was purchased from Stratagene, transformed into DH5α *Escherichia coli* competent cells, and amplified as previously described.[20] The plasmid DNA was extracted from *E. coli* and purified using Qiagen Plasmid Maxi KitTM protocol (Quiagen). The DNA cleavage ability of the DFO in absence or presence of Zn^{2+} was examined following the conversion of pBSK II supercoiled DNA (F I) to the open circular (F II) and linear DNA (F III) using agarose gel electrophoresis to separate the cleavage products. In general, 400 ng of pBSK II DNA (~30 μmol L^{-1} in bp) were treated with DFO (0.01 mol L^{-1}), self-buffered in the pH range 7−10 in the absence or presence of Zn^{2+} (0.01 mol L^{-1}) for 24 h at 37 °C; we also included control reactions without DFO, buffered with PIPES, 7.0−7.5; HEPES, 8.0; and CHES, 8.5−9.5 with or without Zn^{2+} at 0.01 mol L^{-1}. After the given time, the reaction was quenched by adding 5 μL of buffer solution (50 mmol L^{-1} Tris-HCl pH 7.5, 0.01% bromophenol blue, 50% glycerol, and 250 mmol L^{-1} EDTA) and then subjected to electrophoresis in a 0.8% agarose gel containing 0.3 μg mL^{-1} of ethidium bromide in 0.5 × TBE buffer (44.5 mM Tris, pH 8.0, 44.5 mmol L^{-1} boric acid, and 1 mmol L^{-1} EDTA) at 90 V for 1.5 h. The resulting gels were visualized and digitized using a DigiDoc-It gel documentation system (UVP). The proportion of plasmid DNA in each band was quantified using KODAK Molecular Imaging software 5.0 (Carestream Health). The quantification of supercoiled DNA (F I) was corrected by a factor of 1.47 because the ability of ethidium bromide to intercalate into this DNA topoisomeric form is decreased relative to open circular and linear DNA.[21]

ASSOCIATED CONTENT

Supporting Information

Kinetic data and further ESI−MS spectra and fragment assignments are included in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: faruk@qmc.ufsc.br. Fax: 55-48-3721-6850. Tel.: 55-48-3721-6849.

ACKNOWLEDGMENTS

We are grateful to INCT-Catálise, CNPq, Capes, PRONEX, and FAPESC for support of this work.

REFERENCES

(1) Kiss, T.; Farkas, E. *J. Inclusion Phenom. Mol. Recognit. Chem.* **1998**, *32*, 385.
(2) Maggio, A. *Br. J. Haematol.* **2007**, *138*, 407.
(3) Kontoghiorghes, G. J. *Toxicol. Lett.* **1995**, *80*, 1.
(4) Cappellini, M. D.; Pattoneri, P. *Annu. Rev. Med.* **2009**, *60*, 25.
(5) Abetz, L.; Baladi, J. F.; Jones, P.; Rofail, D. *Health Qual. Life Outcomes* **2006**, 4.
(6) Lederman, H. M.; Cohen, A.; Lee, J. W. W.; Freedman, M. H.; Gelfand, E. W. *Blood* **1984**, *64*, 748.
(7) Becton, D. L.; Roberts, B. *Cancer Res.* **1989**, *49*, 4809.
(8) Dayani, P. N.; Bishop, M. C.; Black, K.; Zeltzer, P. M. *J. Neurooncol.* **2004**, *67*, 367.
(9) Daniels, J. S.; Gates, K. S. *J. Am. Chem. Soc.* **1996**, *118*, 3380.
(10) Miller, M. J. *Chem. Rev.* **1989**, *89*, 1563.
(11) Farkas, E.; Enyedy, E. A.; Csoka, H. *Polyhedron* **1999**, *18*, 2391.
(12) Kirby, A. J.; Tondo, D. W.; Medeiros, M.; Souza, B. S.; Priebe, J. P.; Lima, M. F.; Nome, F. *J. Am. Chem. Soc.* **2009**, *131*, 2023.
(13) Kirby, A. J.; Manfredi, A. M.; Souza, B. S.; Medeiros, M.; Priebe, J. P.; Brandao, T. A. S.; Nome, F. *ARKIVOC* **2009**, 28.
(14) Orth, E. S.; da Silva, P. L. F.; Mello, R. S.; Bunton, C. A.; Milagre, H. M. S.; Eberlin, M. N.; Fiedler, H. D.; Nome, F. *J. Org. Chem.* **2009**, *74*, 5011.
(15) Kaplinsky, C.; Estrov, Z.; Freedman, M. H.; Gelfand, E. W.; Cohen, A. *Leukemia* **1987**, *1*, 437.
(16) Joshi, R. R.; Ganesh, K. N. *FEBS J.* **1992**, *313*, 303.
(17) Dhungana, S.; White, P. S.; Crumbliss, A. L. *J. Biol. Inorg. Chem.* **2001**, *6*, 810.
(18) Orth, E. S.; Brandao, T. A. S.; Milagre, H. M. S.; Eberlin, M. N.; Nome, F. *J. Am. Chem. Soc.* **2008**, *130*, 2436.
(19) Fernandes, L.; Fischer, F. L.; Ribeiro, C. W.; Silveira, G. P.; Sa, M. M.; Nome, F.; Terenzi, H. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4499.
(20) Ausubel, F. M.; Brent, R.; Kingston, R. E.; Moore, D. D.; Seidman, J. G.; Smith, J. A.; Struhl, K. *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*; Wiley: New York, 1999.
(21) Bernadou, J.; Pratviel, G.; Bennis, F.; Girardet, M.; Meunier, B. *Biochemistry* **1989**, *28*, 7268.