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P-3A and (-)-Desacetamido P-3A: Demonstration and Study of Their Effective Functional Cleavage of Duplex DNA

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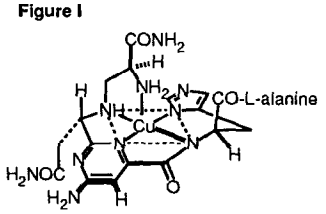
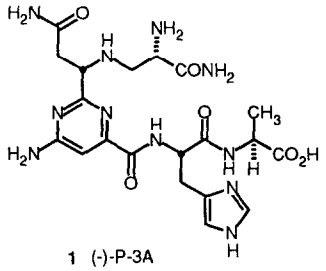
Abstract. A study of the Fe(II) complexes of P-3A (**1**) and (-)-desacetamido P-3A (**2**) abilities to cleave duplex DNA was conducted through examination of single-strand and double-strand cleavage of supercoiled ϕX174 RFI DNA (Form I) in the presence of O_2 to produce relaxed (Form II) and linear (Form III) DNA, respectively. Like Fe(II)-bleomycin A_2 and deglycobleomycin A_2, Fe(II)-**1** and **2** effectively produced both single- and double-strand cleavage of supercoiled ϕX174 DNA. Unlike Fe(II)-bleomycin A_2 or deglycobleomycin A_2, Fe(II)-**1** and **2** were found to cleave duplex w794 DNA with no discernible sequence selectively suggesting that the polynucleotide recognition of the C-terminus tetrapeptide S subunit of the bleomycins including the bithiazole may dominate the bleomycin A_2 DNA cleavage selectivity.

P-3A (**1**),[1] a peptide derived natural product isolated in the conduct of biosynthetic studies of the bleomycins and whose structure was unambiguously established in a single-crystal X-ray structure determination of its copper(II) complex, represents the simplest member of the class of agents related to the clinically important bleomycin antitumor antibiotics.[2] The latter agents are thought to exhibit their biological properties through the metal-dependent oxidative cleavage of duplex DNA. Thus, the timely identification of P-3A and the structural characterization of its copper(II) complex established the functionality responsible for metal chelation and provided the initial indication that the C2-acetamido side chain of P-3A and the related bleomycins may not be intimately involved in the metal coordination. Herein, we provide details of the first demonstration of the duplex DNA cleavage properties of the Fe(II) complex of synthetic P-3A (**1**)[3] and its comparison with the DNA cleavage properties of the Fe(II) complex of (-)-desacetamido P-3A (**2**)[3,4] which establishes a functional role for the C2-acetamido side chain. The pyrimidine nucleus of the agents in conjunction with the linked L-histidine constitutes the core iron(II) chelation subunit required for oxygen activation and the subsequent double-stranded DNA cleavage thought to be responsible for the therapeutic action of the bleomycins.

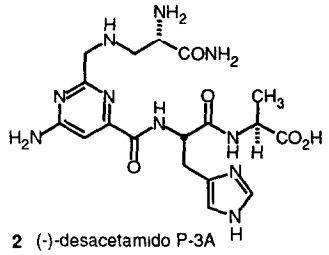
Figure I



P-3A Cu^{+2}



1 (-)-P-3A



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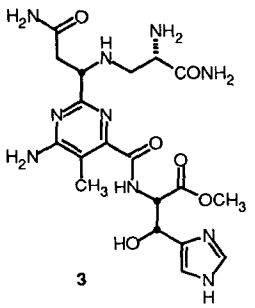
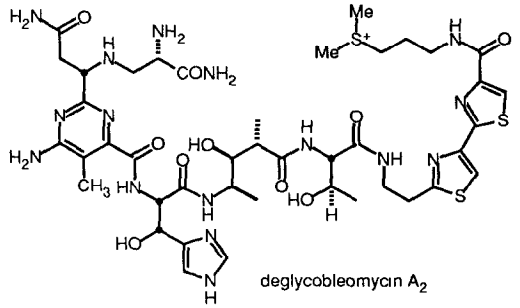


3



deglycobleomycin A_2

Functional DNA Cleavage: Efficiency. A study of the ability of the Fe(II) complex of P-3A (**1**) and (-)-desacetamido P-3A (**2**) to cleave duplex DNA in the presence of O_2 was conducted through examination of single-strand and double-strand cleavage of supercoiled ϕX174 RFI DNA (Form I) to produce relaxed (Form II) and linear (Form III) DNA, respectively. Like Fe(II)-bleomycin A_2 and deglycobleomycin A_2,[5,6] Fe(II)-**1**-**2** effectively produced both single- and double-strand cleavage of ϕX174 DNA, Figure II, and to our knowledge this represents the first demonstration of the functional cleavage of duplex DNA by P-3A.[7] In addition, the comparison of the efficiency of DNA cleavage by Fe(II)-**1** and Fe(II)-**2** permits the direct assessment of the relative importance and functional role of the pyrimidine C2-acetamido side chain. Although the side chain has been shown to not be intimately involved in the metal chelation, it may contribute to the efficiency of DNA cleavage by constituting one side or component of the oxygen binding pocket sterically protecting the reactive iron-oxo intermediate or more subtly by enhancing the agent binding affinity or orientation with duplex DNA. Consistent with such suppositions, Fe(II)-**1** proved to be 3 - 5x more efficient than Fe(II)-**2** in cleaving supercoiled ϕX174 DNA, Figure II. Under the conditions of the assay, Fe(II)-**1** produced little or no cleavage at 0.2 μM, detectable cleavage at 1.0 μM, significant cleavage at 5.0 μM and complete cleavage at 10 0 μM whereas Fe(II)-**2** produced detectable cleavage at 5.0 μM, significant cleavage at 10.0 μM, and complete cleavage at 20.0

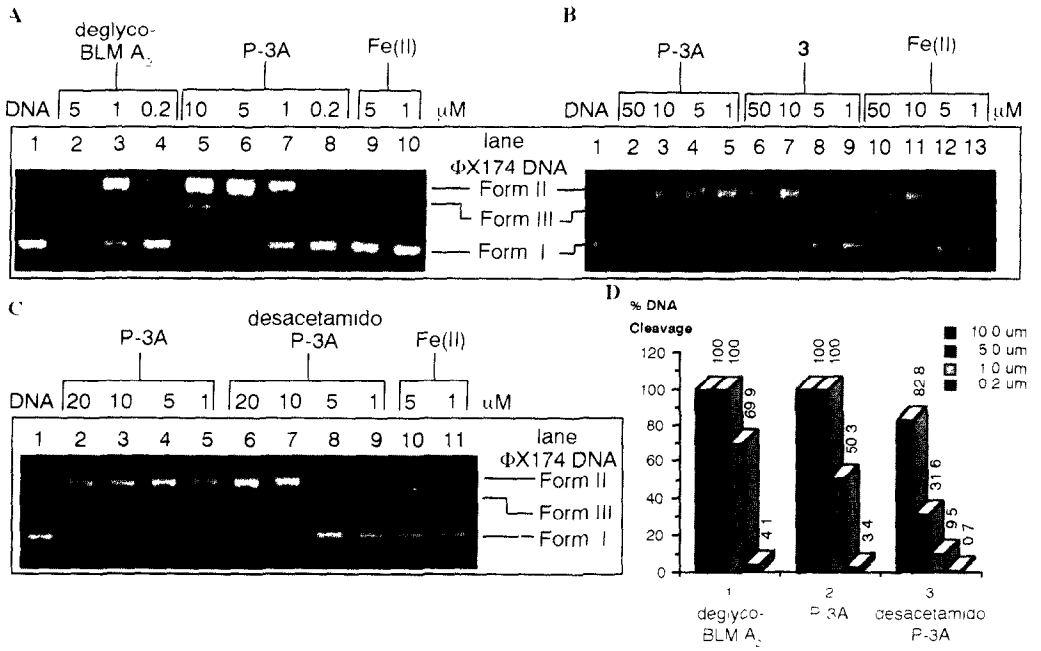


Figure II. Cleavage of supercoiled ϕX174 DNA by Fe(II)-**1**, Fe(II)-**2** and Fe(II)-deglycobleomycin A_2. Solutions contained 0 25 μg supercoiled ϕX174 DNA (1.4×10^{-8} M) in 50 mM Tris-HCl, pH 8 containing 10 mM 2-mercaptoethanol. The DNA cleavage reactions were run for 60 min at 25°C and electrophoresis was conducted at 50 V (2.5 h) on a 1.0% agarose gel containing 0 1 μg/mL ethidium bromide. A) Lane 1, control ϕX174 DNA 95% Form I (supercoiled), 5% Form II (relaxed), lane 2-4, 5 μM, 1 μM and 0.2 μM Fe(II)-deglycobleomycin A_2; lane 5-8, 10 μM, 5 μM, 1 μM and 0.2 μM Fe(II)-**1**; lane 9-10, 5 μM and 1μ M Fe(II) control. B) Lane 1, control ϕX174 DNA 95% Form I (supercoiled), 5% Form II (relaxed), lane 2-5, 50 μM, 10 μM, 5 μM and 1 μM Fe(II)-**1**; lane 6-9, 50 μM, 10 μM, 5 μM and 1 μM Fe(II)-**3**, lane 10-13, 50 μM, 10 μM, 5 μM and 1 μM Fe(II) control. C) Lane 1, control ϕX174 DNA 95% Form I (supercoiled), 5% Form II (relaxed); lane 2-5, 20 μM, 10 μM, 5 μM and 1 μM Fe(II)-**1**, lane 6-9, 20 μM, 10 μM, 5 μM and 1 μM Fe(II)-**2**; lane 10-11, 5 μM and 1 μM Fe(II) control Form I = supercoiled DNA, Form II = relaxed DNA (single-strand cleavage), Form III= linear DNA (double-strand cleavage). D) Comparison of the relative efficiency of cleavage of supercoiled ϕX174 RFI DNA by Fe(II)-deglycobleomycin A_2, Fe(II)-**1** and Fe(II)-**2**. Direct fluorescence quantification of the DNA in the presence of ethidium bromide was conducted using a Millipore Bio Image 60S RFLP system visualized on a UV (312nm) transilluminator

μM. These results proved to be precisely analogous to than those made in our recent comparison of Fe(II)-deglycobleomycin A_2 and Fe(II)-deglyco desacetamidobleomycin A_2 in which the agent lacking the pyrimidoblamic acid C2-acetamido side chain proved to be 3 - 5x less effective at cleaving ϕX174 RFI DNA.[8] The lack of DNA cleavage in control studies in which the agents alone in the absence of Fe(II) (data not shown) or Fe(II) alone in the absence of agent at identical concentrations assure that the DNA cleavage reactions are derived from the Fe(II) complexes of **1-2** presumably in a manner analogous to that observed with bleomycin A_2. Importantly, the two agents proved to be only slightly less efficient than Fe(II)-deglycobleomycin A_2 in cleaving the supercoiled ϕX174 DNA (rel. efficiency 1:0.8-0.5, deglycobleomycin A_2:**1**) and surprisingly more effective than Fe(II)-**3** which proved indistinguisable from Fe(II) itself.

Functional DNA Cleavage: Quantification of Double-Stranded and Single-Stranded Cleavage. While both single-strand and double-strand DNA lesions result from the radical-mediated oxidative cleavage of DNA by bleomycin

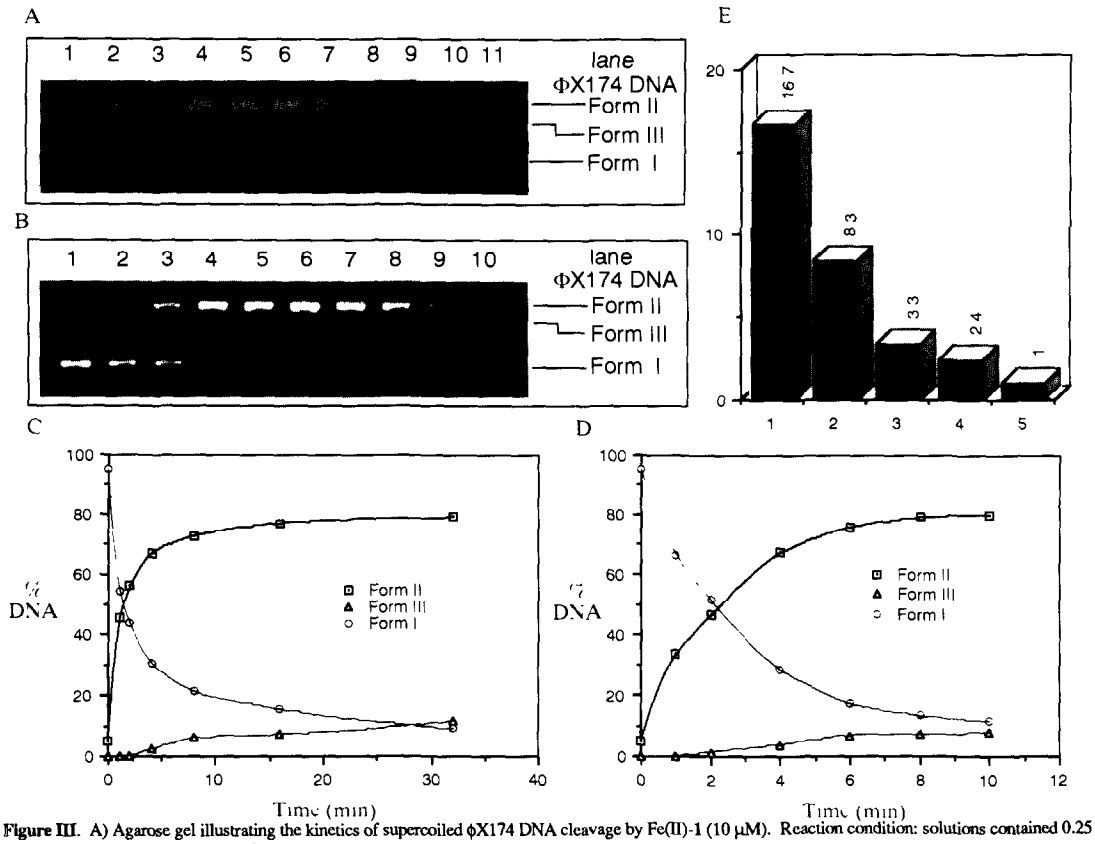


Figure III. A) Agarose gel illustrating the kinetics of supercoiled ϕX174 DNA cleavage by Fe(II)-**1** (10 μM). Reaction condition: solutions contained 0.25 μg supercoiled ϕX174 DNA (1.4 x 10^{-8}M) in 50 mM Tris-HCl, pH 8 containing 10 mM 2-mercaptoethanol. The DNA cleavage reactions were run at 25°C and electrophoresis was conducted at 50 V (2.5 h) on a 1.0% agaraose gel containing 0.1 μg/mL ethidium bromide. Lane 1, untreated DNA; lane 2-11, extent of the reaction at time = 1, 2, 4, 8, 16, 32, 48, 64, 96, 128 min. B) Agarose gel illustrating the kinetics of supercoiled ϕX174 DNA cleavage by Fe(II)-**2** (20 μM). Reaction condition: solutions contained 0.25 μg supercoiled ϕX174 DNA (1.4 x 10^{-8}M) in 50 mM Tris-HCl, pH 8 containing 10 mM 2-mercaptoethanol. The DNA cleavage reactions were run at 25°C and electrophoresis was conducted at 50 V (2.5 h) on a 1.0% agarose gel containing 0.1 μg/mL ethidium bromide. Lane 1, untreated DNA; lane 2-10, extent of the reaction at time = 1, 2, 4, 6, 8, 10, 15, 20, 30, 40 min. C) The quantification of percentage of Forms I, II, and III present at each time point of Fe(II)-**1**. D) The quantification of percentage of Forms I, II, and III present at each time point of Fe(II)-**2**. E) Comparison of the relative efficiency of double-strand to single-strand cleavage of supercoiled ϕX174 RFI DNA by 1) Fe(II)-bleomycin A_2, 2) Fe(II)-deglycobleomycin A_2, 3) Fe(II)-P-3A, 4) Fe(II)-desacetamido P-3A, and 5) Fe(II). Direct fluorescence quantification of the DNA in the presence of ethidium bromide was conducted using a Millipore Bio Image 60S RFLP system visualized on a UV (312nm) transilluminator.

A_2, the latter have been considered the more significant biological event.[9] Bleomycin A_2 produces sequence specific double-strand DNA cleavage above that which may be attributable to random or independent, closely placed cleavage of the two complementary strands of duplex DNA.[9] Consequently in efforts to establish the efficiency of double-stranded to single-stranded DNA cleavage, the kinetics of cleavage to produce linear and circular DNA were examined for Fe(II)-**1** and Fe(II)-**2** with supercoiled ϕX174 DNA. Typical results are illustrated in Figure III. Consistent in each of the multiple runs, Fe(II)-**1** proved more effective than Fe(II)-**2** in producing linear versus circular DNA resulting from double-strand DNA cleavage and both agents proved less efficient at doing so than deglycobleomycin A_2. However, a statistical treatment of the time dependence of relative amount of circular versus linear DNA generated in the DNA cleavage suggests that the linear DNA generated with Fe(II)-**1** and Fe(II)-**2** is not the consequence of random, unrelated single-strand DNA cleavage events that eventually leads to multiple DNA cleavage with linearlization of the DNA.[10] The reactions show initial fast kinetics in the first 5-10 min depending on the substrate and the decreasing rate of DNA cleavage may reflect the ultimate conversion of the agent to a less active or inactive form through the time couse of the assay or metal complex reactivation kinetics. We assumed a Poisson distribution for the formation of single-stranded and double-stranded breaks in order to calculate the average number of single- and double-strand cuts per DNA molecule using the Freifelder-Trumbo equation.[10] The data from the first 5 - 10 minutes could be fitted to a linear equation with a ratio of 1:20 to 1:40 double-stranded to single-stranded cuts observed for Fe(II)-**1**, and 1:40 to 1:45 for Fe(II)-**2**. The theoretical ratio of approximately 1:100 is required in order for the linear DNA to be the result of accumulation of single-strand breaks within the 5386 base-pair size of ϕX174 DNA assuming that sequential cleavage in the complementary strands within 15 base-pairs is required to permit linearlization of the hybridized DNA. Experimentally, it was determined that Fe(II) alone produced a ratio of 1:98 double:single-strand breaks under our conditions fully consistent with the theoretical ratio. In contrast, Fe(II)-bleomycin A_2 and Fe(II)-deglycobleomycin A_2 produce the double-strand DNA cleavage with the greater frequency of 1:5 - 1:7 and 1:12, respectively, under our experimental conditions illustrating that complementary strand cleavage within 15 base-pairs occurs more often and, as previously detailed, may be related by a single binding event. This result compares nicely with the previously established value of 1:9 for bleomycin A_2[10] and the reported values of 1:2[11] for calicheamicin γ^1 or 1:6 - 1:41 for neocarzinostatin.[12]

Functional DNA Cleavage: Selectivity. The selectivity of DNA cleavage was examined within duplex w794 DNA[13] by monitoring strand cleavage of singly ^{32}P 5′-end-labeled double-stranded DNA after exposure to the Fe(II)-complexes of **1**-**2** in the presence of O_2 following past protocols.[14] Thus, incubation of the labeled duplex DNA with the agents (50 mM Tris-HCl, pH 8 containing 10 mM 2-mercaptoethanol, incubation at 37°C for 30 min) in the presence of equimolar $Fe(NH_4)_2(SO_4)_2$ and O_2 led to DNA cleavage. Removal of the agent by EtOH precipitation of the DNA, resuspension of the cleaved DNA in aqueous buffer, gel electrophoresis of the resultant DNA under denaturing conditions adjacent to Sanger sequencing standards permitted the identification of the sites of DNA cleavage, Figure IV. Similar to the trends observed with supercoiled ϕX174 DNA, the relative efficiency of w794 DNA cleavage was found to be : bleomycin A_2 > deglycobleomycin A_2 > P-3A > (-)-desacetamido P-3A. In contrast to bleomycin A_2 (data not shown) or deglycobleomycin A_2 which exhibited the characteristic 5′-GC and 5′-GT DNA cleavage selectivity,[15] Fe(II)-**1**-**2** exhibited DNA cleavage with little or no discernible selectivity. Notably, the observed DNA cleavage with Fe(II)-**1** occurs under conditions and concentrations for which Fe(II) alone in the absence of agent fails to significantly cleave DNA

As such, this observation represents the first demonstration that agents related to the iron chelation subunit of bleomycin A_2 may effectively cleave DNA above a control Fe(II) background and that they do so with no discernible sequence selectivity. Consistent with past studies, this suggests that the polynucleotide recognition of the C-terminus of bleomycin A_2 including bithiazole and tri- or tetrapeptide subunits may dominate the bleomycin A_2 duplex DNA cleavage selectivity.[16-18] In addition, the comparison of the DNA cleavage properties of Fe(II)-**1** and Fe(II)-**2** suggest a prominant role a for the C-2 acetamido side chain of **1**. Like observations made in the comparison of deglycobleomycin A_2 and deglyco desacetamidobleomycin A_2,[8] both the efficiency of DNA cleavage and the ratio of double to single strand DNA cleavage events are reduced significantly by the removal of the C-2 acetamido side chain.

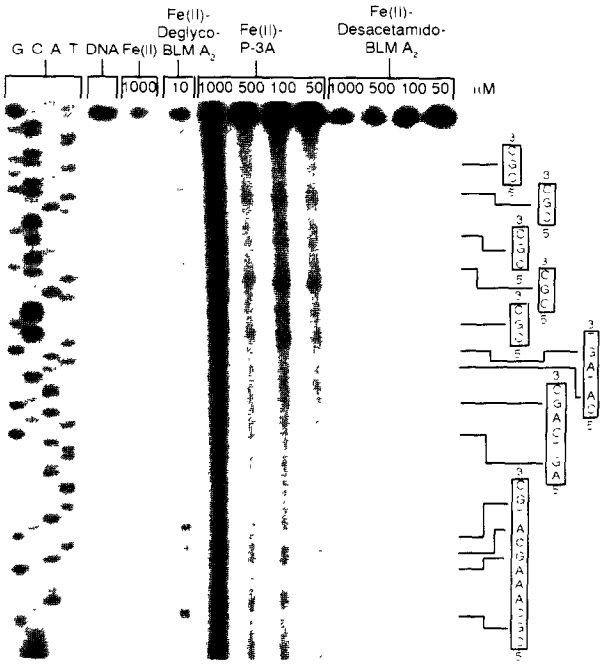


Figure IV Cleavage of double-strand DNA (SV40 DNA fragment, 144 b.p.; nucleotide no. 5238-138, clone w794) in 50 mM Tris-HCl, pH 8 containing 10 mM 2-mercaptoethanol) by Fe(II)-deglycobleomycin A_2, Fe(II)-P-3A, and Fe(II)-desacetamido P-3A. The DNA cleavage reactions were run for 30 min at 37°C and electrophoresis was conducted at 1100 V (5.5 h) on an 8% denaturing polyacrylamide gel and visualized by autoradiography. Lane 1, control DNA; lane 2, 1000 μM Fe(II) control, lane 3, 10 μM, Fe(II)-deglycobleomycin A_2, lane 4-7, 1000, 500, 100 and 50 μM Fe(II)-P-3A, lane 8-11, 1000, 500, 100 and 50 μM Fe(II)-desacetamido P-3A. No discernible cleavage by 100 μM Fe(II) was detected (data not shown).

Table I. Summary of DNA Cleavage Properties of **1**-**2**.

agent	relative efficiency of DNA cleavage[a]	ratio of double- to single-stranded DNA cleavage[b]	DNA cleavage selectivity[c]
bleomycin A_2	4 -10	1:6	5′-GC, 5′-GT
deglycobleomycin A_2	1.25 - 2	1:12	5′-GC, 5′-GT
P-3A (**1**)	1	1:30	none
(-)-desacetamido P-3A (**2**)	0.33 - 0.2	1:40	none
Fe(II)	0.1 - 0.05	1:98	none

(a) Relative efficiency of supercoiled ϕX174 DNA cleavage, Figure II.

(b) Ratio of double- to single-stranded supercoiled ϕX174 DNA cleavage, Figure III, calculated as $F_{III} = n_2 exp(-n_2)$, $F_I = exp-(n_1 + n_2)$

(c) Examined within singly 5′-end labeled w794 DNA, Figure IV.

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