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A Minor Groove Binding Copper-Phenanthroline Conjugate Produces Direct Strand Breaks via β-Elimination of 2-Deoxyribonolactone

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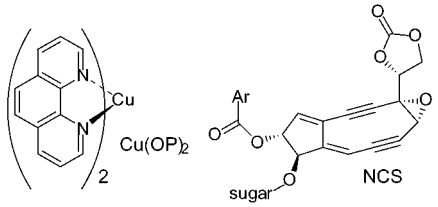
In 1979, Sigman discovered that copper ion complexed to 1,-10-phenanthroline ($Cu(OP)_2$) cleaves DNA.[1] Over the past two decades, copper-phenanthroline complexes and their respective conjugates have been used to footprint DNA binding molecules, map macromolecular interactions, and prepare sequence specific cleaving molecules.[2−4] $Cu(OP)_2$ exhibits a strong, and under some conditions exclusive, preference for oxidative cleavage of B-DNA over single-stranded biopolymers.[5] The mechanism(s) by which copper-phenanthroline complexes damage DNA is complicated, and a number of issues remain unresolved.

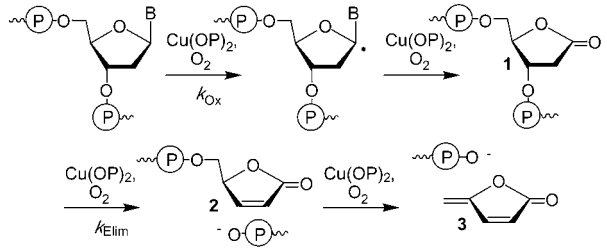


Most data indicate that the major pathway for direct strand scission involves C1′-oxidation, with a minor amount of DNA cleavage resulting from C4′-oxidation.[2,6] Recent product analyses revealed that oxidation occurs at C5′ as well.[4a,7] Support for direct strand scission resulting from C1′-oxidation was derived from product studies in which 5-methylenefuranone (**3**) was identified unequivocally and **2** was ascribed to a labile product observed during denaturing gel electrophoresis (Scheme 1).[6b] It was proposed that oxidation is initiated via hydrogen atom abstraction from the respective deoxyribose positions by an activated copper complex of uncertain structure. The copper complex is believed to play a role in product formation after C1′-radical formation.[6b,c,8]

Product studies carried out by Sigman indicate $Cu(OP)_2$ produces direct strand breaks from the C1′-radical, but other damaging agents, such as the neocarzinostatin chromophore (NCS), produce the alkali-labile 2-deoxyribonolactone (**1**) lesion.[9] Recently, the bifurcation in the reactivity of this reactive intermediate was explained by model studies, which indicated that **1** undergoes β-elimination in the presence of $Cu(OP)_2$.[8]

This explanation and the proposal that C1′-oxidation is the major source of $Cu(OP)_2$-induced direct strand breaks have been questioned.[7] LC-MS analysis of a palindromic hexanucleotide (**4**) damaged by $Cu(OP)_2$ showed that **1** was stable under these conditions and underwent β-elimination only upon further alkaline treatment. The observation of appropriate strand scission products

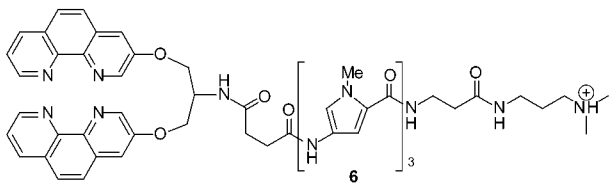
Scheme 1



led these investigators to propose that $Cu(OP)_2$ produces direct strand scission via C4′- and C5′-oxidation, but not C1′-hydrogen atom abstraction.

5′-d(CGT ACG)	5′-d(CGT XGC)
3′-d(GCA TGC)	3′-d(GCA ACG)
4	**5a** X = T
	5b X = **1**

We were concerned that the hexanucleotide duplex (**4**) employed in the LC-MS studies may be an inappropriate DNA substrate to test whether $Cu(OP)_2$ generates direct strand breaks via C1′-oxidation. Abasic sites lower the melting temperature (T_m) of 13-mer duplexes by 12−20 °C depending upon base sequence.[10] We considered the possibility that hexameric duplexes containing **1** might melt under the low salt conditions in which the cleavage studies were carried out (50 mM Tris·HCl, 0 °C). A nonpalindromic sequence, which would enable analysis of the effect of a single molecule of **1** on the thermal stability of duplex DNA, was required to examine this possibility. Hexanucleotide **5a** contains the same overall base content as **4**. In our hands, **5a** melted at a higher temperature than **4** under the cleavage conditions employed in the recent LC-MS studies.[7,11] The oligonucleotide containing **1** was prepared as previously described, purified by reverse phase HPLC, and characterized by ESI-MS.[11,12] Examination by UV spectroscopy provided no evidence for duplex formation (**5b**) at 2 °C upon attempted hybridization of this oligonucleotide with the appropriate complement.[11] Given the inability of $Cu(OP)_2$ to cleave single-stranded DNA,[5] the absence of direct strand scission resulting from C1′-oxidation in the hexanucleotide studied by LC-MS is not unexpected.



Although the thermal melting experiments indicate that hexanucleotides are not suitable substrates for testing $Cu(OP)_2$ C1′-strand

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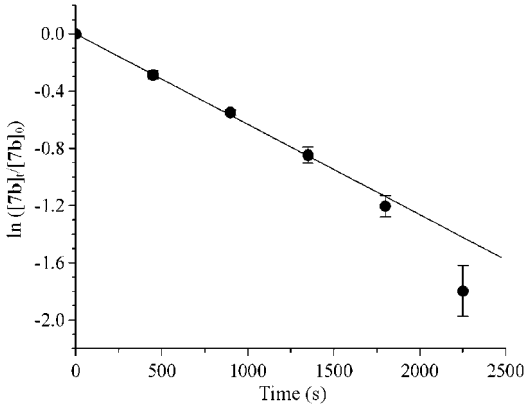

Figure 1. Disappearance of **7b** as a function of time. Conditions: [**7b**] = 1 μM, [**6**] = 20 μM, 10 mM Tris (pH 7.0), NaCl 50 mM, 2 mM 3-mercaptopropionic acid.

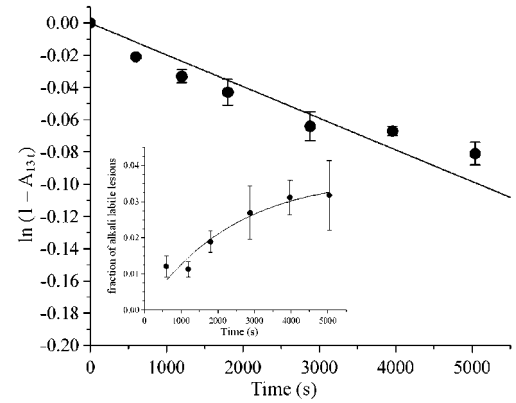

Figure 2. Total cleavage (direct strand breaks + alkali labile lesions) of **7a** by **6** as a function of time. Conditions: [**7a**] = 1 μM, [**6**] = 20 μM, 10 mM Tris (pH 7.0), NaCl 50 mM, 2 mM 3-mercaptopropionic acid. Inset: Mole fraction of alkali labile lesions as a function of time.

cleavage mechanisms, such proposals require further substantiation.[2,6,8] The possibility that $Cu(OP)_2$ creates strand breaks from **1** was investigated by taking advantage of the ability to independently generate the lactone (**1**) at a defined site in an oligonucleotide and to deliver $Cu(OP)_2$ to this position using a DNA binding molecule.[4,12] In the presence of copper and reductant, distamycin

5'-d(GTA GTT TAT CAC XGT TAA ATT GCT AAC GCA GTC AG)
(CAT CAA ATA GTG TCA ATT TAA CGA TTG CGT CAG TC)

7a X = A_{13}
7b X = **1**

conjugate **6** oxidatively damages **7a** at nucleotides (e.g., A_{13}) flanking a seven base pair binding region.[4a] The rate of strand scission was measured in the respective duplex (**7b**) in which **1** was substituted for A_{13} in the presence of **6** (Figure 1).[12] The decay followed first-order kinetics ($k_{elim} = 5.6 \pm 0.7 \times 10^{-4}$ s^{-1}, $t_{1/2}$ = 20.6 min) and was ~100 times faster than the rate of cleavage in the absence of the conjugate.[11] The cleavage product consisted solely of 3′-phosphate termini. Using the rate constant for β-elimination ($k = 1.5 \times 10^{-3}$ M^{-1} s^{-1}) in the previously reported model study indicates that formation of an intramolecular complex via DNA binding achieves an effective molarity that is 0.38 M.[8]

The total rate of formation of direct strand breaks and alkali labile lesions at A_{13} in **7a** ($k_{ox} = 1.9 \pm 0.6 \times 10^{-5}$ s^{-1}) was also measured (Figure 2) and indicated that β-elimination of **1** by **6** is faster than overall DNA oxidation. Measuring the amount of alkali labile lesions present in the reaction between **6** and **7a** as a function of time suggests that such lesions build up and decay over time (Figure 2, inset). Fitting the quantity of alkali labile lesions as a function of time to two sequential unimolecular processes enables us to extract rate constants for their growth and decay (eq 1). The rate constants for the growth ($k_G = 1.8 \pm 0.4 \times 10^{-5}$ s^{-1}) and decay ($k_D = 4.7 \pm 0.9 \times 10^{-4}$ s^{-1}) are remarkably close to the rate constants reported above that describe the overall rate of DNA oxidation (k_{ox}) and β-elimination (k_{elim}) of **1**, respectively.

$$\underset{\textbf{(6·7a)}}{Cu(OP)_2\cdot DNA} \xrightarrow{k_G} \underset{\text{lesion}}{\text{alkali labile}} \xrightarrow{k_D} \text{strand break} \qquad (1)$$

Barring a coincidence that two different alkali labile lesions cleave in the presence of **6** at comparable rates, these data suggest that the major alkali labile lesion produced by the $Cu(OP)_2$ conjugate is 2-deoxyribonolactone (**1**, $k_D \approx k_{elim}$). Likewise, the similarity in the rate constants for overall formation of alkali labile lesions and direct strand breaks (k_{ox}) and that which describes the growth of the former (k_G) suggests that C1′-oxidation is the major pathway for direct strand breaks produced by **6**.

In conclusion, these kinetic studies support Sigman's proposal that $Cu(OP)_2$ cleaves DNA via a mechanism in which C1′-oxidation is a major pathway.[2,6a] Furthermore, direct strand breaks resulting from C1′-oxidation are due to the instability of 2-deoxyribonolactone (**1**) in the presence of $Cu(OP)_2$.[8]

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Supporting Information Available: ESI-MS of hexanucleotide containing **1** and UV-melts of hexamers. Plot of [**7b**] as a function of time in the absence of **6**. Autoradiogram showing cleavage of **7a** by **6**. Experimental procedures for producing DNA containing **1**. Experimental procedures for DNA cleavage and extracting rate constants from measurements of alkali labile lesions as a function of time (Figure 2, inset) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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