# Mechanism of DNA Strand Scission Induced by (1,10-Phenanthroline)copper Complex: Major Direct DNA Cleavage Is Not through 1′,2′-Dehydronucleotide Intermediate nor β-Elimination of Forming Ribonolactone

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Since Sigman et al. first reported the DNA cleavage activity of the (1,10-phenanthroline)copper complex ($(OP)_2Cu$) with thiol or $H_2O_2$ in 1979, the nuclease activity of $(OP)_2Cu$ has been widely applied in structural studies of nucleic acids and nucleic acid−protein complexes.[1] Based on the formation of considerable 5-methylenefuranone during heating of $(OP)_2Cu$-treated DNA, the major reaction pathway induced by the agents has been deduced as C1′ oxidation via a 2′-deoxyribonolactone intermediate.[2] However, because direct DNA strand cleavage by this agent was readily detected by gel electrophoresis, Sigman et al. recently proposed an alternative mechanism involving a 1′,2′-dehydronucleotide intermediate which is also consistent with the incorporation of $^{18}O$ into 5-methylenefuranone from $H_2{}^{18}O$ (Scheme 1, path a).[3] Subsequently, Chen and Greenberg demonstrated that the mononucleotide analogue of 1′,2′-dehydronucleotide is quite stable under the reaction conditions of DNA cleavage, and they proposed that $(OP)_2Cu$ catalyzes β-elimination of the 2′-deoxyribonolactone residue in DNA (Scheme 1, path b).[4] In addition to our results, other groups have found that the 2′-deoxyribonolactone-containing site is relatively stable at neutral pH. Furthermore, our preliminary results indicated that deoxyribonolactone-containing hexamer is stable even in the presence of $(OP)_2Cu$. To better understand the DNA cleavage mechanism, we performed a detailed analysis of the degradation products of $d(CGTACG)_2$ and calf thymus DNA by $(OP)_2Cu$. Herein we report experimental evidence suggesting that the major direct cleavage occurs due to C4′ and C5′ H abstraction, neither through 1′,2′-dehydronulceotide intermediate nor from β-elimination during formation of 2′-deoxyribonolactone.

Figure 1 shows the HPLC profile of the reaction mixture containing d(CGTACG) and $(OP)_2Cu$ in the presence of mercaptopropionic acid (MPA).[1h,5] Interestingly, an analogous single-strand hexamer d(CTACGC) was not digested at all by $(OP)_2Cu$ under the reaction conditions and was digested efficiently in the presence of the complementary strand, confirming that the reaction we observed was derived from duplex DNA substrate. Major

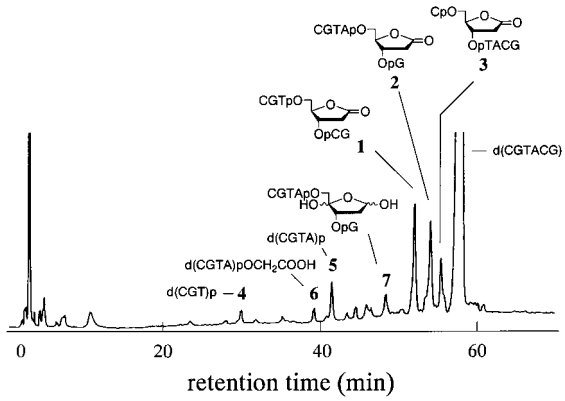
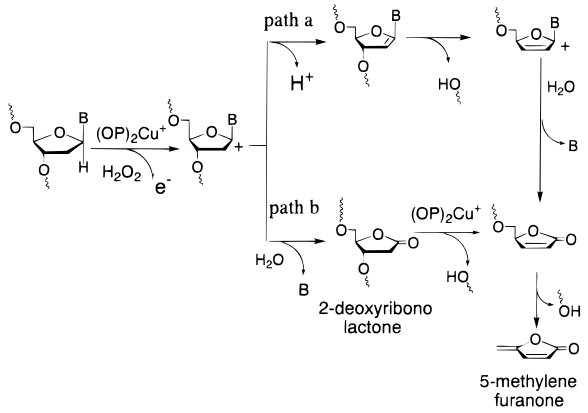



**Figure 1.** HPLC profile of $(OP)_2Cu$-treated $d(CGTACG)_2$. The reaction mixture (50 μL) was analyzed by HPLC on a CHEMCOBOND 5-ODS-H column (4.6 × 150 mm) detected at 254 nm; elution was with 0.05 M ammonium formate (pH 6.5) isocratic, 10 min, containing 0−6% acetonitrile, linear gradient, 50 min, 6% acetonitrile isocratic, 10 min at a flow rate of 1.0 mL/min, at 40 °C. The identification of each peak (**1**−**7**) was confirmed by electrospray MS (ESMS) and enzymatic digestion.

**Scheme 1**




peaks 1, 2, and 3 formed from $(OP)_2Cu$-treated $d(C_1G_2T_3A_4C_5G_6)$ were found to contain 2′-deoxyribonolactone at the $A_4$, $C_5$, and $G_2$ sites which were characterized as **1**, **2**, and **3**, respectively, by electrospray MS (ESMS) together with heat degradation.[6] We further confirmed that $^{18}O$ was incorporated into **1** and **2** from $H_2{}^{18}O$ in the DNA degradation experiment in $H_2{}^{18}O$ (Figure 2S). In accordance with previous observations, 2′-deoxyribonolactone-containing oligonucleotides were quite stable at neutral pH at room temperature.[7] However, under mild alkaline conditions (pH 9.9, room temperature, 1 h), purified **1** and **2** underwent β-elimination to provide butenolide **1a** and **2a** accompanied by the release of the 5′-phosphate fragment in ca. 70% yield (Scheme 2, Figure 3S).[8]

(1) (a) For reviews, see: Sigman, D. S.; Chen C.-h. B. *Annu. Rev. Biochem.* **1990**, *59*, 207. (b) Sigman, D. S.; Mazumder, A.; Perrin, P. M. *Chem. Rev.* **1993**, *93*, 2295. (c) Pratviel, G.; Bernadou, J.; Meunier, B. *Angew. Chem., Int Ed. Engl.* **1995**, *34*, 746. (d) Pogozelski, W. K.; Tullius, D. *Chem. Rev.* **1998**, *98*, 1089. (e) Sigman, D. S.; Graham, D. R.; D'Aurora, V.; Stern, A. M. *J. Biol. Chem.* **1979**, *254*, 12269. (f) Veal, J.; Rill, R. L. *Biochemistry* **1988**, *27*, 1822. (g) Veal, J.; Rill, R. L. *Biochemistry* **1989**, *28*, 3243. (h) Kuwabara, M.; Yoon, C.; Goyane, T.; Thederahn, T.; Sigman, D. S. *Biochemistry* **1986**, *25*, 7401. (i) Pan, C. Q.; Johnson, R. C.; Sigman, D. S. *Biochemistry* **1996**, *35*, 4326. (j) Perrin, D. M.; Chen, C.-H. B.; Xu, Y.; Pearson, L.; Sigman, D. S. *J. Am. Chem. Soc.* **1997**, *119*, 5746. (k) Bullard, J. M.; van Waes, M. A.; Bucklin, D. J.; Hill W. E. *EMBO J.* **1995**, *252*, 572. (l) Hill, W. E.; Bucklin, D. J.; Bullard, J. M.; Galbraith, A. L.; Jammi, N. V. Rettberg, C. C.; Sawyer, B. S.; van Waes, M. A. *Biochem. Cell Biol.* **1995**, *73*, 1033. (m) Bullard, J. M.; van Waes, M. A.; Bucklin, D. J.; Rice, M. J.; Hill, W. E. *Biochemistry* **1998**, *37*, 1350. (n) Muth, G. W.; Thompson, C. M.; Hill, W. E. *Nucleic Acids Res.* **1999**, *27*, 1906.

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(5) A reaction mixture (50 μL) containing 5′-d(CGTACG)-3′ (540 μM base concentration), 1,10-phenanthroline (90 μM), $CuSO_4$ (20 μM), and mercaptopropionic acid (MPA, 200 μM) in 50 mM Tris HCl buffer (pH 7.5) was incubated under aerobic conditions at 0 °C for 15 min. Then 50 μL of 2,9-dimethylphenanthroline (4 mM) was added to the solution, which was then directly analyzed by HPLC. The $T_m$ value for 0.1 mM (base concentration) $d(CGTACG)_2$ in 50 mM Tris HCl buffer (pH 7.5) was 20.5 °C, indicating that we observe the reaction between duplex and $(OP)_2Cu$. CD spectra of 0.1 mM (base concentration) of $d(CGTACG)_2$ in 50 mM Tris HCl (pH 7.5) showed typical B-form conformation under reaction conditions (Figure 1S).

(6) Structures of **1**, **2**, and **3** were characterized by ion spray MS and heat treatment. ESMS (negative): **1**, calcd 1673.1, found 1673.0; **2**, calcd 1697.1, found 1696.8 (Figure 2S); **3**, calcd 1657.1, found 1656.8 (Figure 6S(a)). Heat treatment of **1**, **2**, and **3** provided corresponding fragments with phosphate termini and 5-methylenefuranone.

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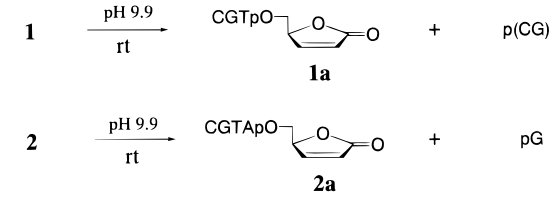
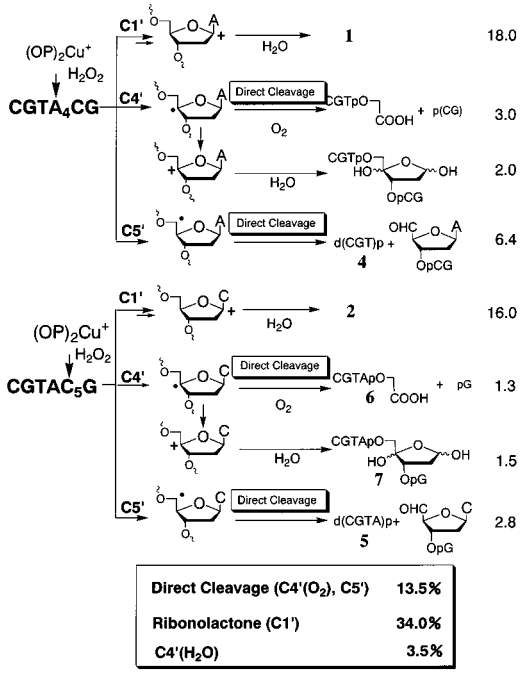
**Scheme 2**




Of note, the isolated ribonolactones **1** and **2** and also butenolide **1a** and **2a** were found to be fairly stable under the DNA cleavage conditions,[9] and neither **1a** nor **2a** was detected in the reaction mixture of DNA cleavage (Figure 4S). These results indicate that 1′,2′-dehydronucleotide intermediate is not involved in direct cleavage by $(OP)_2Cu$ and strongly suggest that $(OP)_2Cu$ does not considerably catalyze $\beta$-elimination of ribonolactone residue in DNA via $\beta$-elimination, as proposed by Chen and Greenberg.[4]

Peaks 4 and 5 were found to be d(CGT)p (**4**) and d(CGTA)p (**5**), respectively, by ESMS and enzymatic dephosphorylation.[10] These results indicate the existence of direct DNA strand cleavage processes in the $(OP)_2Cu$-mediated reaction. Moreover, $NaBH_4$ reduction of the reaction mixture gave d(CG) and d(ACG), indicating that $(OP)_2Cu$ oxidized at the C5′ position of the $A_4$ and $C_5$ sites.[11] Peaks 6 and 7 were characterized as d(CGTA)-phosphoroglycolate (**6**) and the C4′-hydroxyabasic site at the C5 site (**7**), respectively, resulting from abstraction of C4′ H at $C_5$ as observed in neocarzinostatin- and BLM-mediated DNA cleavage.[12,13] The product distribution formed at the $A_4$ and $C_5$ sites in $d(CGTACG)_2$, based on the quantitative analysis of the degradation products, is summarized in Scheme 3. At the $A_4$ site, reaction at C1′, C4′($O_2$), C4′($H_2O$), and C5′ sites occurred in 18.0%, 3.0%, 2.0%, and 6.4% contribution, respectively, indicating that at least 32% of the reaction at the $A_4$ site causes direct cleavage.[14] Recently, Greenberg et al. proposed the existence of direct DNA strand cleavage derived from the C1′ radical. Even though the structures of the products are not identified, possibly 1.8% of cleavage that is 10% of 2′-deoxyribonolactone is assumed to be derived from the C1′ radical on the basis of their observation.[15] Similarly, at least 19% of the reaction of the $C_5$ site induces direct cleavage. Formation of C4′ oxidation products leading to direct strand cleavage was also confirmed in $(OP)_2Cu$-treated calf thymus DNA.[16] Present HPLC analysis covered about 50% of degradation products based on the consumed d(CGTACG). The remaining products are assumed to be various types of base modifications, which do not induce direct DNA cleavage as reported previously.[17]

**Scheme 3**




The present results clearly demonstrate that direct DNA scission induced by $(OP)_2Cu$ occurs primarily due to C4′ and C5′ oxidations, neither through 1′,2′-dehydronucleotide intermediate nor from $\beta$-elimination of ribonolactone residue catalyzed by $(OP)_2Cu$.

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**Supporting Information Available:** Figures 1S−7S, showing the CD spectrum of d(CGTACG), ESMS of **1**−**7**, **1a**, and **2a**, HPLC profiles of degradation of **1** and **2** under mild alkaline conditions, and HPLC analysis of the reaction mixture with co-injection of authentic **1a** and **2a** (PDF). This material is available via the Internet at http://pubs.acs.org.

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(8) Structures of **1a** and **2a** were characterized by ESMS and heat treatment. ESMS (negative): **1a**, calcd 1036.7, found 1036.6; **2a**, calcd 1349.9, found 1349.8. Heat treatment (90 °C, 30 min) of **1a** and **2a** provided d(CGT)p and d(CGTA)p, respectively, together with 5-methylenefuranone.

(9) HPLC analysis indicate that **1**, **2**, **1a**, and **2a** were fairly stable at 0 °C and were recovered more than 95% under incubation in a solution containing 1,10-phenanthroline (90 $\mu$M), $CuSO_4$ (20 $\mu$M), and MPA (200 $\mu$M) in 50 mM Tris HCl buffer (pH 7.5) at 37 °C for 15 min. The half-lives of ribonolactones **1** and **2** under pH 7.5 at 37 °C with $(OP)_2Cu$ and MPA were 4.2 and 6.2 h, respectively.

(10) Structures of **4** and **5** were characterized by ESMS as well as by comparison with authentic materials after dephosphorylation with alkaline phosphatase. ESMS (negative): **4**, calcd 940.6, found 940.4; **5**, calcd 1253.8, found 1253.4 (Figure 6S).

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(12) (a) Structures of **6** and **7** were characterized by ESMS. ESMS (negative): **6**, calcd 1311.9, found 1311.6; **7**, calcd 1697.1 and 1715.1 (hydrate), found 1696.8 and 1714.8 (Figure 7S). The amounts of C4′($O_2$) and C4′($H_2O$) were estimated by quantification of monomeric products after enzymatic digestion and our previously developed chemical and enzymatic detection method,[13] respectively. Snake venom phosphodiesterase and alkaline phosphatase treatment gave dC-, dG-, dT-, and dA-phosphoroglycolate in a ratio of 1:1:1:1. Hydrazine treatment and successive enzymatic digestion gave dC-, dG-, dT-, and dA-pyridazine in a ratio of 1:2:1:1.

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(16) A C4′ oxidation process providing 3′-phosphoroglycolates contributes approximate 3.7% to total modification of calf thymus DNA.[13a] The contributions of A-, G-, C-, and T-phosphoroglycolate are 1.1%, 0.6%, 1.0%, and 1.0%, respectively.

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