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Bioorganic & Medicinal Chemistry Letters 18 (2008) 3286–3290
Bioorganic & Medicinal Chemistry Letters

Copper-mediated DNA photocleavage by a tetrapyridoacridine (tpac) ligand

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Received 14 March 2008; revised 15 April 2008; accepted 16 April 2008
Available online 22 April 2008

Abstract—We have focused our interest on the tetrapyridoacridine ligand tetrapyrido[3,2-*a*:2′,3′-*c*:3′′,2′′-*h*: 2′′′,3′′′-*j*]acridine (tpac), as a model system for the preparation of novel copper-based artificial nucleases. The complex of copper(II)–tpac cleaves supercoiled pUC18 plasmid DNA in an oxidative manner by photoactivation with visible light, exhibiting maximum cleaving efficiency at 1:2 metal–ligand stoichiometric ratio. We propose an interaction of the copper–tpac complex with DNA through both major and minor grooves and a photocleavage mechanism via the formation of hydroxyl radicals and singlet oxygen or singlet oxygen-like species.

The design and synthesis of small molecules that bind to DNA and efficiently cleave it remains a major challenge in current research. Artificial nucleases have important applications, for example, as tools for molecular biology and as potential therapeutic agents for the treatment of some cancers and viral diseases.

Oxidative strand scission of nucleic acids can occur through deoxyribose oxidation via hydrogen atom extraction or by nucleobase oxidation via electron abstraction. Because of their diverse structures and reactions, transition–metal complexes have proven to be very useful as chemical nucleases.[1]

Naturally occurring[2,3] and synthetic[4–6] metal complexes that activate molecular oxygen to induce nucleic acid modification are of particular interest given the physiological availability of molecular oxygen. Copper complexes are able to produce oxidative double strand DNA (dsDNA) cleavage. Chemical agents that preferentially cause dsDNA cleavage create lesions that are considered much more difficult for the cell to repair than damage resulted from single strand DNA (ssDNA) breakage. Moreover, unlike hydrolytic DNA cleavage, fragments generated through this pathway cannot be rejoined by the action of ligases.

In typical DNA cleavage experiments the reaction of Cu(II) with a reducing agent generates Cu(I), which then can react with molecular oxygen and/or hydrogen peroxide to give rise to hydroxyl radicals ultimately responsible for DNA breakage. Alternatively, a transient Cu(II)-oxo intermediate may be formed. In previous papers, we have reported the nuclease activity of copper complexes of *N*-substituted sulfonamides[7] and copper complex-intercalator conjugates.[8]

Although studies on copper-mediated DNA cleavage processes are well documented, less attention has been paid to the investigation of DNA binding and cleaving properties of extended aromatic heterocyclic–copper complexes. Chakravarty et al.[9] have recently described the oxidative and hydrolytic DNA cleavage with a bis(dipyridophenazine)copper(II) complex. The authors reported that when mercaptopropionic acid is used as an additive, the oxidative cleavage of plasmid DNA by the copper complex is more efficient than that observed with traditional bis(phen)copper(II) complexes.

In an attempt to research new potent artificial nuclease agents we planned to use a tetrapyridoacridine ligand

Keywords: Tetrapyridoacridine; Copper; DNA; Photonuclease.
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doi:10.1016/j.bmcl.2008.04.040

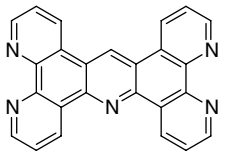

Figure 1. Molecular structure of ligand *tpac*.

(tpac) as a potentially effective metallonuclease model. Whereas the large planar ligand structure could insert into DNA as a classical intercalator, its coordination with the metal would locate the metal centers in the grooves.

Herein we report the DNA cleaving activity of tetrapyridoacridine, tpac (Fig. 1), in the presence of copper(II) and light. The synthesis of tetrapyrido[3,2-*a*:2′,3′-*c*:3″,2″-*h*:2‴,3‴-*j*]acridine was carried out according to the method reported by Demeunynck et al.[10] In this article, the authors also reported tpac chelating ability towards ruthenium(II), describing the formation of mononuclear and dinuclear Ru(II) complexes. Recently, Kirsch-De Mesmaeker et al.[11,12] have reported spectroscopic and spectroelectrochemical properties of mononuclear and dinuclear Ru(II) complexes of the tpac ligand.

The ability to cleave pUC18 plasmid DNA by copper(II)–tpac (1:1) mixtures was first examined and compared with that of the cupric chloride in the presence of ascorbate (2.5-fold excess relative copper(II) concentration) (Fig. 2) under visible light.[13] The copper(II) salt did not present ability to damage DNA plasmid at the concentrations assayed (30–60 μM) (lanes 4–6, Fig. 2). However, mixtures of the copper(II) and the ligand produced complete cleavage of the plasmid (lanes 7–9). Interestingly, the Cu(II)–tpac mixture in the absence of ascorbate also showed plasmid damage (lane 10). This result suggests that ascorbate is not necessary to produce DNA cleavage. We then carried out electrophoretic assays without reducing agent at concentrations ranging from 1.5 to 30 μM (Fig. S1). At the lower concentrations (1.5–12 μM), the Cu(II)–tpac (1:1) mixture was not able to induce DNA cleavage, whereas at concentrations equal or higher than 18 μM a smear appeared in the gel, indicating the production of linearized DNA. Taking into consideration that both

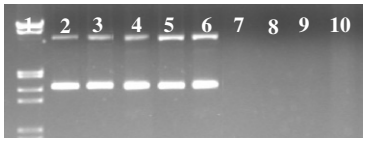

Figure 2. Agarose gel electrophoresis of pUC18 plasmid DNA treated with $CuCl_2$, or $CuCl_2$ + tpac (1:1) mixtures in the presence of 2.5-fold excess of ascorbate. Incubation time 60 min at 37 °C. Gel containing ethidium bromide. Lane 1: λDNA/EcoR1 + HindIII Marker; lane 2: supercoiled DNA; lane 3: supercoiled DNA with ascorbate 150 μM; lane 4: $CuCl_2$ 30 μM; lane 5: $CuCl_2$ 45 μM; lane 6: $CuCl_2$ 60 μM; lane 7: $CuCl_2$ + tpac 30 μM; lane 8: $CuCl_2$ + tpac 45 μM; lane 9: $CuCl_2$ + tpac 60 μM; lane 10: $CuCl_2$ + tpac 60 μM in the absence of ascorbate.

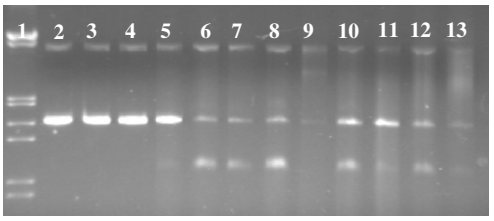

Figure 3. Agarose gel electrophoresis of pUC18 plasmid DNA treated with $CuCl_2$, tpac or $CuCl_2$ + tpac (1:1) mixtures. Incubation time 30 min at 37 °C. Gel containing ethidium bromide. Lane 1: λDNA/EcoR1 + HindIII Marker; lane 2: supercoiled DNA; lane 3: $CuCl_2$ 18 μM; lane 4: tpac 18 μM; lane 5: $CuCl_2$ + tpac 12 μM; lane 6: $CuCl_2$ + tpac 12.75 μM; lane 7: $CuCl_2$ + tpac 13.5 μM; lane 8: $CuCl_2$ + tpac 14.25 μM; lane 9: $CuCl_2$ + tpac 15 μM; lane 10: $CuCl_2$ + tpac 15.75 μM; lane 11: $CuCl_2$ + tpac 16.5 μM; lane 12: $CuCl_2$ + tpac 17.25 μM; lane 13: $CuCl_2$ + tpac 18 μM.

the copper salt and the tpac ligand do not exhibit nuclease activity alone, is evident that the tpac and the Cu(II) do contribute to DNA cleavage process in a synergistic rather than in an additive fashion. When intermediate ligand–metal concentrations were tested (12–18 μM) (Fig. 3), a new band with higher mobility than that of supercoiled form was observed in the gels (lanes 6–13). This effect has been previously described[14] and was explained by an interaction of the metal active species with the plasmid through some type of intercalation. As a consequence of this interaction, the ethidium bromide intercalates more easily in the plasmid giving rise to a supercoiled structure recoiled in the opposite direction leading to an increase in its mobility.

In order to avoid the influence of ethidium bromide in our photocleavage experiments we studied the copper(II)-mediated DNA cleavage efficiency of the tpac ligand under similar conditions, but ethidium bromide was added after electrophoresis.[15] Our results (Fig. 4) indicated that the mixture at 12 μM (lane 5) cleaves plasmid DNA to yield a small amount of nicked Form II. At 12.75 μM concentration of tpac–Cu(II) (lane 6), the supercoiled form was partially transformed into nicked DNA and also displayed a progressive shift towards the relaxed form. This effect suggests a change in the superhelicity of DNA as a result of the interaction of an active Cu–tpac complex with the nucleic acid. At

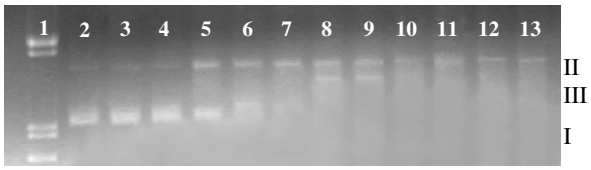

Figure 4. Agarose gel electrophoresis of pUC18 plasmid DNA treated with $CuCl_2$, tpac or $CuCl_2$ + tpac (1:1) mixtures. Incubation time 30 min at 37 °C. Gel stained with ethidium bromide after electrophoresis. Lane 1: λDNA/EcoR1 + HindIII Marker; lane 2: supercoiled DNA; lane 3: $CuCl_2$ 18 μM; lane 4: tpac 18 μM; lane 5: $CuCl_2$ + tpac 12 μM; lane 6: $CuCl_2$ + tpac 12.75 μM; lane 7: $CuCl_2$ + tpac 13.5 μM; lane 8: $CuCl_2$ + tpac 14.25 μM; lane 9: $CuCl_2$ + tpac 15 μM; lane 10: $CuCl_2$ + tpac 15.75 μM; lane 11: $CuCl_2$ + tpac 16.5 μM; lane 12: $CuCl_2$ + tpac 17.25 μM; lane 13: $CuCl_2$ + tpac 18 μM.

14.25 and 15 μM concentrations, a mixture of the plasmid Forms II and III (lanes 8 and 9) was appreciated. Higher concentrations (lanes 10–13) showed complete DNA degradation.

Our next goal was to elucidate the mechanism of the cleavage process. Hydrolytic and redox mechanisms are commonly described in the literature. The latter can originate from an internal oxidation–reduction reaction such as it occurs, for example, between iron or copper with flavones or through a photochemical reaction, as it has been described for ruthenium complexes.[16] To determine if the DNA damage mediated by the copper(II)–tpac system takes place through a hydrolytic pathway, experiments with $ZnCl_2$ instead of $CuCl_2$ were carried out. The Zn(II)–tpac mixtures did not produce a progressive shift of the supercoiled Form I and were not able to induce any cleavage in the large range of concentrations assayed. These findings ruled out a hydrolytic mechanism and indicated that the intrinsic redox properties of copper are necessary to cleave DNA (Fig. S2), suggesting a redox process. A mechanism involving a photochemical reaction could be considered as a plausible possibility. In order to confirm this, a similar experiment to that shown in Figure 4 was carried out in the dark. The results (Fig. S3) indicated that the Cu(II)–tpac in the absence of light does not induce DNA cleavage. Because no DNA breakdown is observed in the dark, we can conclude that the light plays an undisputed role in the DNA cleavage performed by the copper(II)–tpac mixture.

Thus, we propose that the visible light initiates a photochemical reduction of copper(II)–tpac to copper(I)–tpac that in the presence of the dioxygen gives rise to reactive oxygen species (ROS) able to trigger the DNA damage process. Kirsch-De Mesmaeker et al.[11,12] have reported the existence of Ru(II)–tpac complexes with different stoichiometries. In a similar fashion, we envisioned that the study of the nuclease activity of the Cu(II)–tpac system at 1:2 and 2:1 molar ratios would be of interest. The results, shown in Figure 5, clearly demonstrated the Cu(II)–tpac mixtures in 1:2 molar ratio have higher nuclease activity than the 1:1 or 2:1 mixtures.

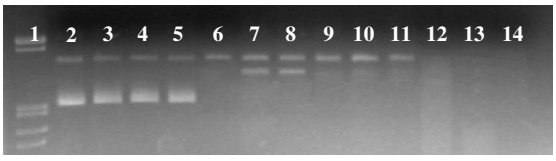

Figure 5. Agarose gel electrophoresis of pUC18 plasmid DNA treated with $CuCl_2$, tpac or $CuCl_2$ + tpac mixtures at different molar ratios. Incubation time 30 min at 37 °C. Gel stained with ethidium bromide after electrophoresis. Lane 1: λDNA/EcoR1 + HindIII Marker; lane 2: supercoiled DNA; lane 3: $CuCl_2$ 30 μM; lane 4: tpac 24 μM; lane 5: tpac 30 μM; lane 6: $CuCl_2$ + tpac (1:1) 12 μM; lane 7: $CuCl_2$ + tpac (1:1) 14.25 μM; lane 8: $CuCl_2$ + tpac (1:1) 15 μM; lane 9: $CuCl_2$ + tpac (2:1) 24 μM:12 μM; lane 10: $CuCl_2$ + tpac (2:1) 28.50 μM:14.25 μM; lane 11: $CuCl_2$ + tpac (2:1) 30 μM:15 μM; lane 12: $CuCl_2$ + tpac (1:2) 12 μM:24 μM; lane 13:$CuCl_2$ + tpac (1:2) 14.25 μM:28.5 μM; lane 14: $CuCl_2$ + tpac (1:2) 15 μM:30 μM.

In order to identify the active metal complex species, MALDI-TOF and ESI spectra of Cu(II)–tpac solutions at different molar ratios were recorded.[17] In MALDI-TOF (Fig. S4), irrespective of the metal to ligand molar ratio assayed (1:2, 1:1, or 2:1), a main peak at m/z = 829 was found, which can be assigned (Fig. 6) to the $Cu(tpac)_2^{1+}$ complex, Calcd for $[C_{50}H_{26}N_{10}Cu]^{1+}$ 829.1638.

Similarly, the ESI spectrum of the Cu(II)–tpac (1:2) presented a main peak at m/z = 864, attributable to a $Cu(tpac)_2Cl^{1+}$ complex, which is in good agreement with the results obtained in the case of a Zn(II)–tpac (1:2) mixture, m/z = 865 for a $Zn(tpac)_2Cl^{1+}$ complex (data not shown). These experiments indicated that the main species in solution under these conditions corresponds to a metallic complex with 1:2 metal to ligand stoichiometry. This observation could account for the higher nuclease activity of the mixtures metal–tpac in a molar ratio 1:2, compared with that of the other two molar ratios assayed.

Moreover, the unwinding of supercoiled DNA that we observed in previous experiments (Fig. 4) can now be explained in terms of a interaction between $Cu(tpac)_2Cl^{1+}$ complex and the DNA plasmid. The interaction of this active species can also account for effect found in Figure 3 when ethidium bromide is added previous to the electrophoretic process.

The implication of ROS (hydroxyl, superoxide, singlet oxygen-like species, and hydrogen peroxide) in the photocleavage mechanism can be inferred by monitoring the quenching of DNA cleavage in the presence of ROS scavengers in solution. To clarify other aspects of the mechanism, the copper(I) chelator (neocuproine) and groove binders such as distamycin (minor-groove binder) or methyl green (major-groove binder) have also being used.[18] Figure 7 shows the results from our experiments with these compounds.

The addition of hydroxyl radical scavengers such as DMSO, *tert*-butyl alcohol or sodium formate (lanes 4–6, respectively) attenuated DNA strand scission. This observation suggests that hydroxyl radicals are reactive species involved in the cleavage process. The presence of 2,2,6,6-tetramethyl-4-piperidone (lane 7), sodium azide (lane 8) or strychnine (lane 9) protected against the DNA strand breakage, which implies that the $^1O_2$ or any other singlet oxygen-like entities also participate in the oxidative cleavage. Lane 10 shows a significant reduction of DNA cleavage in the presence of tiron, indicating that $O_2^{-\cdot}$ is another of the reactive species involved in DNA breakage. The presence of catalase enzyme inhibits the DNA damage (lane 11) suggesting that hydrogen peroxide also participates in the process. As for neocuproine (lanes 12,13), it diminished DNA degradation as well. This attenuation of the cleavage activity can be explained taking into consideration the reduction of the Cu(II) ion in the ROS production process. The addition of methyl green (lane 14) and distamycin (lane 15) produced a decrease in the

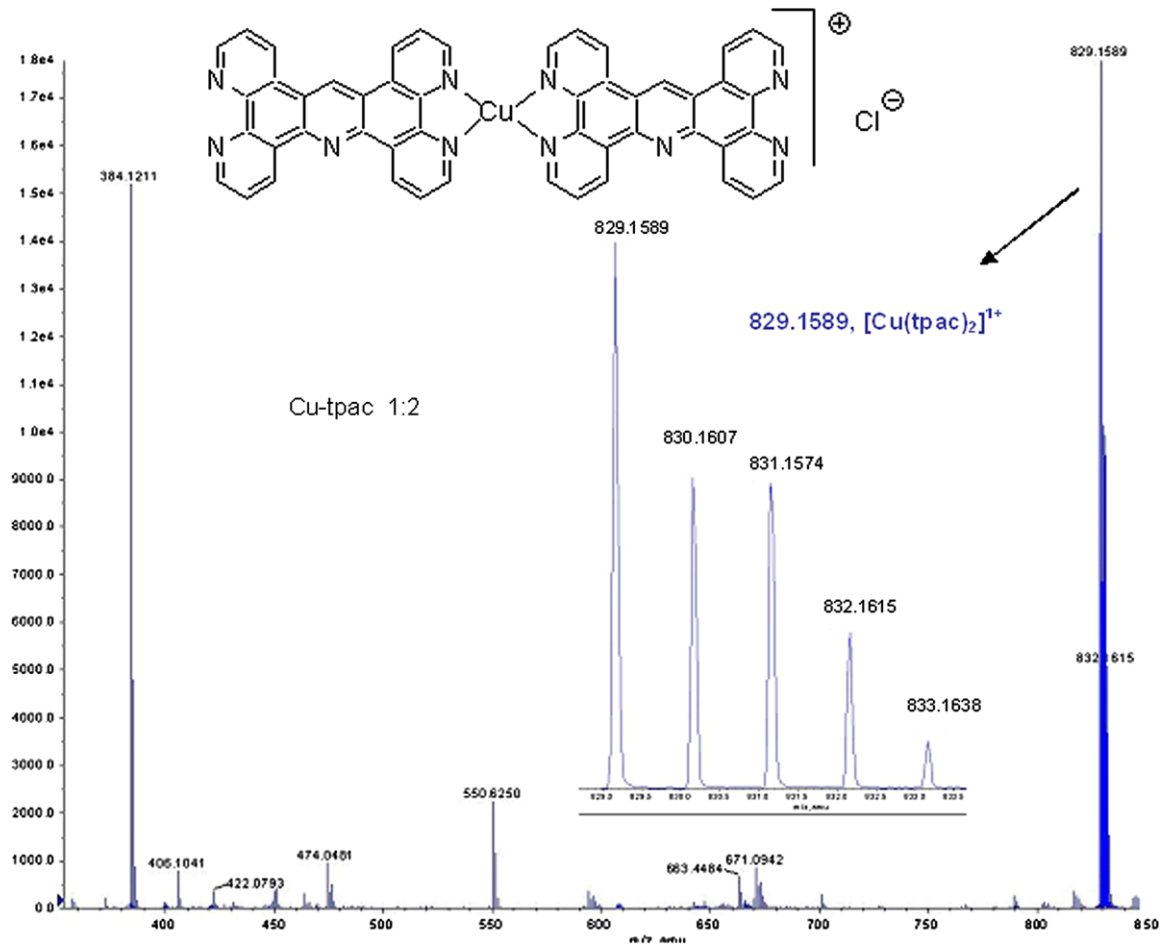

Figure 6. MALDI-TOF spectrum showing the complex formed between the ligand tpac and $CuCl_2$ in HPLC-grade methanol, at a metal to ligand ratio of 1:2.

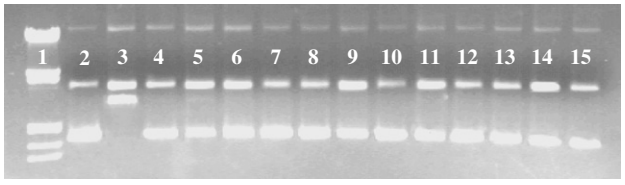

Figure 7. Agarose gel electrophoresis of pUC18 plasmid DNA treated with 9 μM $CuCl_2$ + 18 μM tpac [Cu(II):tpac (1:2)] and potential inhibiting agents. Incubation time 30 min at 37 °C. Gel stained with ethidium bromide after electrophoresis. Lane 1: λDNA/EcoR1 + HindIII Marker; lane 2: supercoiled DNA; lane 3: 9 μM $CuCl_2$ + 18 μM tpac; lane 4: 9 μM $CuCl_2$ + 18 μM tpac + DMSO (0.4 M); lane 5: 9 μM $CuCl_2$ + 18 μM tpac + *tert*-butyl alcohol (0.4 M); lane 6: 9 μM $CuCl_2$ + 18 μM tpac + sodium formate (0.4 M); lane 7: 9 μM $CuCl_2$ + 18 μM tpac + 2,2,6,6-tetramethylpiperidone (0.4 M); lane 8: 9 μM $CuCl_2$ + 18 μM tpac + NaN_3 (20 mM); lane 9: 9 μM $CuCl_2$ + 18 μM tpac + strichnine (0.4 M); lane 10: 9 μM $CuCl_2$ + 18 μM tpac + tiron (10 mM); lane 11: 9 μM $CuCl_2$ + 18 μM tpac + catalase (10 μg/mL)(650 U/mL); lane 12: 9 μM $CuCl_2$ + 18 μM tpac + neocuproine (60 μM); lane 13: 9 μM $CuCl_2$ + 18 μM tpac + neocuproine (120 μM); lane 14: 9 μM $CuCl_2$ + 18 μM tpac + methyl green (2.5 μL of a 0.01 mg/mL solution); lane 15: 9 μM $CuCl_2$ + 18 μM tpac + distamycin (8 μM).

cleaving ability of the copper(II)–tpac system. Thus, a major- and a minor-groove interaction can be inferred.

According to these observations we propose the following mechanism:

1. The copper(II) complex of tpac is reduced to copper(I) by visible light.
2. The copper(I)–$(tpac)_2$ compound interacts with the DNA through the major and minor grooves.
3. Then, copper(I)–$(tpac)_2$ reacts with dioxygen giving rise to copper(II) and superoxide ion:

$$\text{Cu(I)–(tpac)}_2 + \text{O}_2 \rightarrow \text{Cu(II)–(tpac)}_2 + \text{O}_2^{-\cdot}$$

4. The superoxide anion dismutates and copper(I)–$(tpac)_2$ reacts with hydrogen peroxide producing copper-peroxide-type complex of tpac or hydroxyl radicals:

$$2\text{O}_2^{-\cdot} + 2\text{H}^+ + 1\text{e}^- \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$$
$$\text{Cu(I)–(tpac)}_2 + \text{H}_2\text{O}_2 \rightarrow \text{(tpac)}_2\text{–Cu(I)OOH} + \text{H}^+$$
$$\text{Cu(I)–(tpac)}_2 + \text{H}_2\text{O}_2 \rightarrow \text{Cu(II)–(tpac)}_2 + \text{OH}^- + \text{OH}^{\cdot}$$

5. The ROS damage DNA.

In conclusion, in this paper we report for the first time the nuclease activity of the copper(II)–tpac system induced by visible light. A mechanism involving the tpac-sensitized photoreduction of copper(II) and the

subsequent reaction of the low oxidation state of copper with dioxygen generating reactive oxygen species is proposed. The efficient photonuclease activity of the Cu–tpac complex at low concentrations is a significant result within the context of the chemistry of copper-based artificial nucleases, with potential applications in photodynamic therapy.

Acknowledgments

We thank the CICYT (project CTQ2007-63690) and University of Alcalá (project UAH GC2007-003) for financial support.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.04.040.

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13. The nuclease activity experiments of tpac in the presence of copper(II) were carried out by mixing 6 μL of 5–100 μM ligand + $CuCl_2$ solutions in DMF, 1 μL of pUC18 (750 μM in nucleotides) and 10 μL of cacodylate buffer 0.1 M pH 6.0. Assays were performed in the absence or in the presence of ascorbate. Samples were incubated at 37 °C for 60 min and then 3 μL of a quench buffer solution (0.25% bromophenol blue, 0.25% xylene cyanide and 30% glycerol) were added. Agarose gel electrophoresis was performed using 0.8% gel in 0.5× TBE buffer (0.045 M Tris, 0.045 M boric acid and 1 mM EDTA) containing 2 μL/100 mL of a solution of ethidium bromide (EB) (10 mg/mL) for 2 h at 80 V. The gel was photographed on a capturing system gel printer plus TDI.
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15. The influence of EB in the cleavage process was determined by post-staining experiments. Agarose gels were prepared in the same manner as previous experiments with the exclusion of the EB. After electrophoresis the gels were stained in water containing EB (10 mg/L).
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17. Electrospray ionization (ESI) mass spectra were generated using an Automass Multi Finnigan spectrometer, while MALDI-TOF MS were recorded on an Agilent 6210 Time-of-Flight mass spectrometer. We prepared the samples by mixing different volumes of 0.4 mM stock solutions of tpac and $CuCl_2$ (or $ZnCl_2$) in pure HPLC-grade methanol. The samples were then allowed to equilibrate at room temperature for 45 min, after which mass spectra were recorded.
18. Mechanistic aspects of DNA cleavage reaction were investigated by testing the cleavage ability of the copper(II)–tpac system in the dark or using inhibiting reagents such as hydroxyl radical scavengers (DMSO, *tert*-butyl alcohol), singlet oxygen scavengers (2,2,6,6-tetramethyl-4-piperidone, sodium azide, and strychnine), superoxide anion scavenger (tiron), H_2O_2 scavenger (catalase), chelating agents (neocuproine) and the minor- and major-groove binders distamycin and methyl green, respectively. Samples were treated as described above.