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Straightforward approach to efficient oxidative DNA cleaving agents based on Cu(II) complexes of heterosubstituted cyclens†

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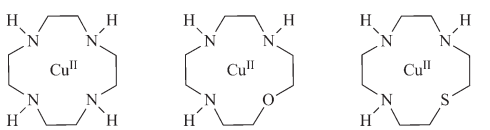
The Cu(II) complexes of cyclen and two of its heterosubstituted analogues were shown to be efficient oxidative DNA cleavers. The reactivity strongly depends on the heteroatom inserted into the macrocycle (O > S > N).

The key to success of organic life is the stability of deoxyribonucleic acid (DNA). The half-life of its phosphodiester bond under physiological conditions is estimated to be in between ten to one hundred billion years.[1] This stability is due to the repulsion of potential nucleophiles by its negatively charged backbone. Nevertheless, cleavage of DNA is of high importance: during replication and transcription of DNA the enzyme topoisomerase cleaves one or both of the DNA strands, reversing supercoiling and enabling the reading process of DNA.[2] Restriction enzymes guard the genome against viral DNA by cutting the alien DNA out.[3] Manipulation of DNA by mankind remains a big challenge, though. Up to the present developed artificial nucleases can by far not catch up with naturally occurring enzymes, although DNA cleaving agents promise numerous applications in medicine and biotechnology.[4] Hydrolytically and oxidatively cleaving nucleases can be distinguished. Whereas the first group may be used to design restriction agents in biotechnology that act *via* hydrolysis of the phosphodiester bond, the latter ones would be suitable as chemotherapeutic agents cleaving nuclear DNA by catalysing oxidation reactions that break down the sugar moiety of DNA.[4,5]

Since their first synthesis in 1961 macrocyclic polyamines such as 1,4,7,10-tetraazacyclododecane, the so called cyclen ligand or [12]aneN$_4$, have been widely used in bioinorganic and medicinal chemistry due to their ability to form stable complexes with transition metals and rare earth metals.[6] Different concepts were used to develop DNA-cleaving agents based on the cyclen ligand. Hydrolytically cleaving complexes based on Zn(II) and Co(III) as well as oxidatively cleaving complexes based on Cu(II) were synthesised.[4,7–9] It is well known that Cu(II) cyclen complexes are able to cleave DNA *via* an oxidative pathway, thus they may represent potential anticancer drug candidates.[10] Recent efforts have focused on further derivatisation of the simple Cu(II) cyclen complex to increase the affinity to DNA. This was achieved by introducing DNA-intercalating agents, adding positively charged groups or synthesising multinuclear complexes.[7–9] The straightforward approach of changing the tetraaza ligand itself, however, by replacing the donor atoms and its impact on DNA cutting activity has never been studied to our knowledge. This is in contrast to applications in protein and RNA cleavers where the oxygen analogue of cyclen is a well established scaffold.[11] Our approach would possibly lead to changes in the redox behaviour of the copper centre, thus resulting in a change of cutting activity depending on the introduced heteroatom.

The three ligands [12]aneN$_4$, [12]aneN$_3$O and [12]aneN$_3$S were synthesised using previously described protocols.[12] The corresponding Cu(II) complexes **1**, **2** and **3** (Fig. 1) were synthesised and characterised (*cf.* S-1, S-2†). While the crystal structure of [Cu([12]aneN$_4$)(NO$_3$)](NO$_3$) **1** has already been published,[13] the crystal structures of the nitrato complexes **2** and **3** are presented in this communication (Fig. 2). All three complexes show a distorted square-pyramidal environment with Cu–N bond lengths of nearly 2 Å. The Cu–O bond (2.231 Å) of complex **2** and the Cu–S bond (2.328 Å) of complex **3** are, however, elongated. Yet another difference exists in the C–N,



Cu([12]aneN$_4$) (1) Cu([12]aneN$_3$O) (2) Cu([12]aneN$_3$S) (3)

Fig. 1 Structures of complexes **1**, **2** and **3**.

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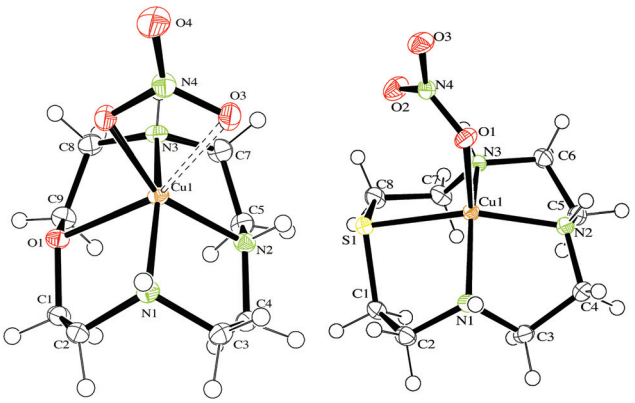

Fig. 2 ORTEP[14] diagrams of the molecular structure of **2** (left) and **3** (right). Thermal ellipsoids are drawn at a probability level of 50%. The counter ions are omitted for clarity.‡

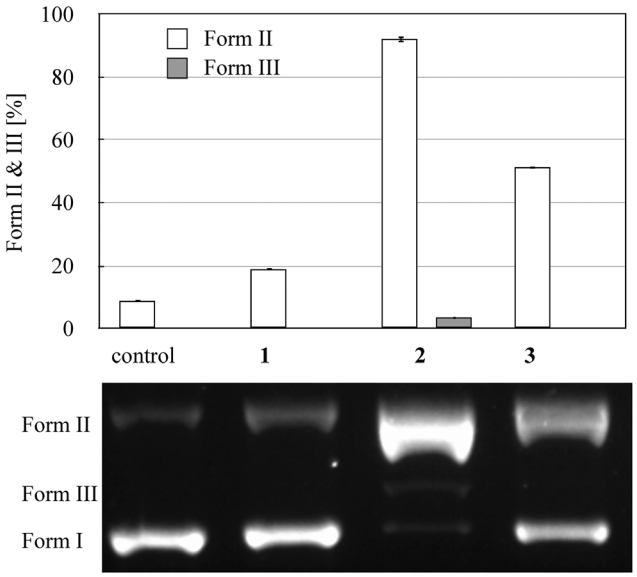

Fig. 3 Cleavage activities of complexes **1**, **2** and **3** (0.04 mM) on pBR322 (0.025 μg μL^{-1}) in Tris-HCl buffer (100 mM, pH 7.4) and ascorbic acid (0.32 mM) at 37 °C for 2 h. Illustrated is the average of two measurements, the standard deviation is shown as error bars.

C–O and the C–S bond lengths. While the C–N bond length of complex **1** (1.453 Å) and the C–O bond length of complex **2** (1.435 Å) differ only slightly, the C–S bond length of complex **3** (1.818 Å) is considerably longer. The hapticity of the nitrato ligand of complexes **1** and **3**, where it coordinates in η^1-fashion with a typical apical Cu–O bond length[15] of 2.183 and 2.160 Å, respectively, differs from complex **2**, where it shows η^2-like coordination with Cu–O bond lengths of 2.031 and 2.470 Å. Such an anisodentate chelation of nitrate in Cu(II) cyclen analogues has been observed by Spiccia *et al.*[15] before. They have assumed that the long Cu–O bond indicates an electrostatic rather than a coordinative interaction.

The cleavage activities of **1**, **2** and **3** toward pBR322 supercoiled plasmid DNA were studied under approximate physiological conditions at 37 °C and ascorbic acid at 0.32 mM concentration as a reducing agent. Agarose gel electrophoresis was used to monitor the conversion of supercoiled (I) pBR322 into its nicked (II) and linear (III) form. Fig. 3 shows a comparison between the DNA cleavage activities of the complexes **1**, **2** and **3**.

After two hours incubation and at a complex concentration of 0.04 mM gel electrophoresis shows that complex **2** was more reactive than complexes **1** and **3**. Complex **2** cleaved supercoiled pBR322 plasmid DNA almost completely to form II and additionally it was able to generate about 4% of form III. Compared to complex **1**, the DNA cleavage efficiency of complex **3** was two times and of complex **2** even four times higher. Further investigations of the cleaving ability were carried out performing a series of optimisation experiments including variations of concentration, pH value and reaction time. The concentration dependent investigation shows that complex **2** exhibited an approximately linear relationship between complex concentration and DNA cleavage activity (Fig. 4). At 0.08 mM concentration form I DNA was cleaved into the nicked form almost completely. As opposed to that complexes **1** and **3** did not cleave plasmid DNA to that extent and did not show a linear relationship between complex concentration and cleavage activity. In contrast the Cu([12]aneN$_4$) complex **1** shows a maximum in cutting activity at 0.02 mM and the Cu([12]aneN$_3$S) complex **3** at 0.16 mM. After passing these concentrations cleavage activities of **1** and **3** decrease. A similar behaviour has been described for Cu([9]aneN$_3$) complexes prior to our study by Burstyn *et al.* and was attributed to the formation of bis-(μ-hydroxo)-bridged dimers.[16] As hydroxo-bridged dimer formation should also be favoured by a higher hydroxide concentration we conducted a pH dependent study, proving that the DNA cleavage activity decreases with increasing pH values (*cf.* S-3,2†).

Furthermore, the effect of reaction time and temperature on the cleavage of pBR322 plasmid DNA was studied. Increasing DNA cleavage activity was observed when both temperature and reaction time were raised (*cf.* S-3,3 and S-3,4†).

To elucidate the mechanism of DNA cleavage promoted by the complexes **1**, **2** and **3** the incubation was conducted under an argon atmosphere and under addition of scavengers for reactive oxygen species (ROS) like hydroxyl radicals (*t*-BuOH and DMSO),[17] singlet oxygen (NaN$_3$),[18] hydrogen peroxide (catalase)[19] and superoxide (superoxide dismutase, SOD).[18] Under anaerobic conditions cleavage was reduced, indicating that oxygen plays a role in the cleavage mechanism (Fig. 5A). In the case of **2** even traces of oxygen are enough to promote DNA cleavage indicating a high catalytic activity of **2**. As shown exemplarily for complex **3** in part B of Fig. 5 DMSO and catalase show a strong and NaN$_3$ a weak inhibition effect on DNA cleavage. These results prove the mechanism of DNA cleavage to be oxidative involving hydroxyl radicals, hydrogen peroxide and to some extent singlet oxygen as ROS.

To gain further insight into the redox reactions involved electrochemical studies were carried out. Complexes **1–3** display a one-electron reduction wave in their cyclic

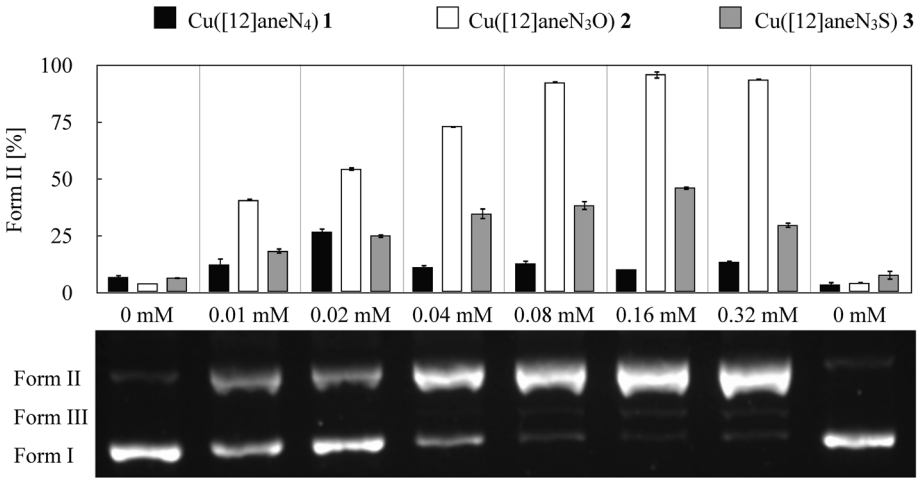

Fig. 4 Effect of different concentrations of complexes **1**, **2** and **3** on pBR322 (0.025 μg μL^{-1}) cleavage activity in Tris-HCl buffer (100 mM, pH 7.4) and ascorbic acid (0.32 mM) at 37 °C for 2 h. Illustrated is the average of two measurements, the standard deviation is shown as error bars (top). As an example the agarose gel of complex **2** is shown (bottom). See ESI† for gels of complexes **1** and **3** (*cf.* S-3,1).

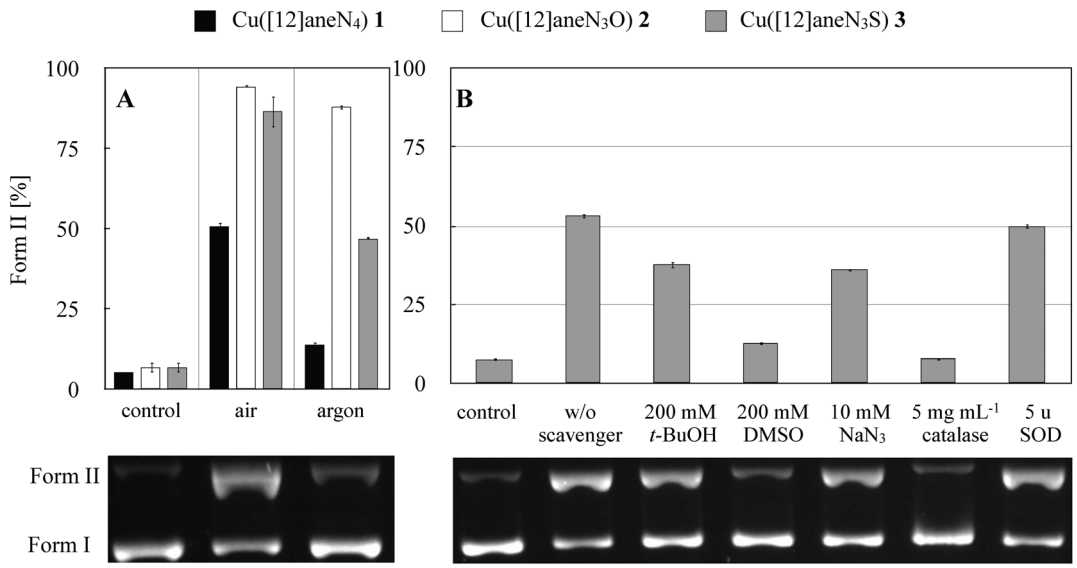

Fig. 5 Cleavage of pBR322 plasmid DNA (0.025 μg μL^{-1}) in Tris-HCl buffer (100 mM, pH 7.4) and ascorbic acid (0.32 mM) for 2 h at 37 °C. The average of two measurements is illustrated, the standard deviation is shown as error bars. (A) Cleavage by complexes **1**, **2** and **3** (0.04 mM) under aerobic and anaerobic conditions. As an example the agarose gel of complex **1** is shown (bottom). (B) Cleavage by complex **3** in the presence of ROS scavengers. See ESI† for a complete overview of gels (*cf.* S-3,5 and S-3,6).

voltammogram (*cf.* S-4,1†). This process is attributed to the reduction of Cu(II) to Cu(I). Whereas this wave is reversible for **3**, it is electrochemically irreversible for **1** and **2**. The irreversibility of this wave is attributed to the release of Cu(I) from the cyclen ligands after reduction. This phenomenon has been observed earlier[20] and in the present case, proof comes from scanning the cyclic voltammograms multiple times, which shows an increase in the intensity of the wave attributed to redox processes at the released copper centre (*cf.* S-4,2†). The reversible nature of the reduction step for **3** is readily explained by the higher affinity of Cu(I) for a sulphur donor as for an oxygen or a nitrogen donor. As would be expected, **2** is reduced at a lower negative potential compared to **1**, an observation that is related to the higher electronegativity of "O" compared to "N". The reduction of **3** at even lower negative potential than **2** is attributed to the better π-donor ability of oxygen compared to sulphur. Such a trend has precedence in the literature.[21]

The release of Cu(I) from macrocycles **1** and **2** as suggested by the cyclovoltammetric results implies that the free Cu(I)/Cu(II) could be the reactive species for the generation of ROS.[22] This might explain the high reactivity of complex **2** despite the unfavourable potential for reduction in comparison to complexes **1** and **3**. Electrochemistry, however, displays an outer-sphere electron transfer process, whereas the reduction of O_2 generating ROS must be attributed to inner sphere electron

transfer.[23] Therefore, activity in DNA cleavage does not necessarily correlate with redox potentials. The attenuated cleavage activity of **1** might be explained by hydroxo-bridged dimer formation[16] which blocks the coordination sites of Cu(II) leaving no space for coordination of reducing agents, thus inhibiting the reduction process and the release of Cu(I).

In conclusion, we present a series of simple macrocyclic ligands for Cu(II)-based DNA cleavers. In contrast to other cyclen-based systems, where activity is increased by conjugation with moieties enhancing DNA affinity, activity increase is achieved here by simply changing one out of four donor atoms in the system. Cu(II) oxacyclen is the most efficient complex that exceeds the literature-known system Cu(II) cyclen by a factor of four to ten depending on the concentration. The high reactivity of this derivative might be a good starting point for developing more efficient DNA cleavers for potential medicinal applications.

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Notes and references

‡Crystal data for **2**: $C_8H_{19}CuN_5O_7$, M = 360.82, monoclinic, a = 7.921(19) Å, b = 11.979(3) Å, c = 14.757(3) Å, α = 90°, β = 92.587(5)°, γ = 90°, V = 1398.9(6) Å^3, T = 133(2) K, space group $P2_1/n$, Z = 4, μ(MoK$_\alpha$) = 1.604 mm^{-1}, 17 378 reflections measured, 4238 independent reflections (R_{int} = 0.0253). The final R_1 value was 0.0290 ($I > 2\sigma(I)$). The final wR_2 value was 0.0683 ($I > 2\sigma(I)$). The final R_1 value was 0.0399 (all data). The final wR_2 value was 0.0731 (all data). The goodness of fit on F^2 was 1.089.

Crystal data for **3**: $C_8H_{19}CuN_5O_6S$, M = 376.88, monoclinic, a = 8.532(16) Å, b = 11.903(2) Å, c = 14.168(3) Å, α = 90°, β = 94.368 (4)°, γ = 90°, V = 1434.7(5) Å^3, T = 133(2) K, space group $P2_1/c$, Z = 4, μ(MoK$_\alpha$) = 1.703 mm^{-1}, 17 105 reflections measured, 4381 independent reflections (R_{int} = 0.0143). The final R_1 value was 0.0241 ($I > 2\sigma(I)$). The final wR_2 value was 0.0639 ($I > 2\sigma(I)$). The final R_1 value was 0.0264 (all data). The final wR_2 value was 0.0649 (all data). The goodness of fit on F^2 was 1.129.

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