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A new phenanthroline–oxazine ligand: synthesis, coordination chemistry and atypical DNA binding interaction†

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1,10-Phenanthroline-5,6-dione and L-tyrosine methyl ester react to form phenanthroline–oxazine (PDT) from which $[Cu(PDT)_2](ClO_4)_2$ and $[Ag(PDT)_2]ClO_4{\cdot}2MeOH$ are obtained. Binding to calf-thymus DNA by Ag(I) and Cu(II) PDT complexes exceed bis-1,10-phenanthroline analogues and the minor groove binding drugs, pentamidine and netropsin. Furthermore, unlike the artificial metallonuclease, $[Cu(phen)_2]^{2+}$, the $[Cu(PDT)_2]^{2+}$ complex does not cleave DNA in the presence of added reductant indicating unique interaction with DNA.

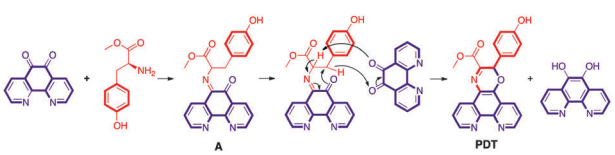
1,10-Phenanthroline (phen), its organic derivatives and the plethora of metal complexes containing these *N,N′*-chelating heterocycles, find use as optical devices,[1,2] catalysts[3] and as integral components of supramolecular structures.[4] In addition, these compounds have found application in the biological field as antimicrobial and anti-cancer agents,[5] DNA intercalators,[6] and as nucleoside constituents for incorporation into the DNA backbone.[7]

The quest to make new and tailored phenanthrolines is ongoing, and methods include synthesising the bases from elementary building blocks[1,8] and extending existing, functionalised phenanthrolines.[1] In the latter context, the quinone, 1,10-phenanthroline-5,6-dione (phendio), has been used as the starting material for grafting appendages onto the phenanthroline framework, *via* simple Schiff base condensation reactions with primary amines.[7,9–14] However, not all reactions of phendio with primary amines have resulted in the expected Schiff base product. For example, combining phendio with urea[15] and *N,N′*-bis(2-aminophenyl)ethylenediamine[16] unexpectedly produced a glycoluril and a gem-*cis*-bis(aminal), respectively.

Generally, phendio and its Ag(I) and Cu(II) complexes are considerably more biologically active than phen and its corresponding metal complexes.[5a,b] With this in mind, we sought to improve biological activity, selectivity and compatibility by attempting to prepare a double Schiff base, phen-type ligand by reacting phendio with two equivalents of (*S*)-methyl 2-amino-3-(4-hydroxyphenyl)-propanoate (L-tyrosine methyl ester).

Phendio was initially treated with L-tyrosine methyl ester (1 : 2 mol ratio) in anticipation of forming a double Schiff base condensation product (Scheme 1). Unexpectedly, this combination produced 1,10-phenanthroline-5,6-diol and a relatively low yield of the new orange-yellow tetracyclic oxazine, **PDT**. The structure of **PDT**·MeOH (Fig. 1) showed that chirality within the compound is retained, with C13 being the stereogenic centre (originally it was C20). The molecule crystallises in the centrosymmetric space group, $P2_1/c$, and so is a racemic mixture. Interestingly, the phenol ring is almost orthogonal to the oxazine ring (bond angle 89.12(2)°) forming a 'chair' profile. The pdtme molecules are linked into zig-zag chains *via* hydrogen bonding through the methanol solvate; the phenol group makes a H-bond to the oxygen of the methanol (2.6288(18) Å), and the methanol OH makes an unsymmetric, bifurcated H-bond to the phenanthroline nitrogen atoms of a second molecule (3.028(2) and 2.907(2) Å to N1 and N2, respectively, under symmetry operation $-x + 2, y - 1/2, -z + 3/2$).

What is the mechanism of the cyclization reaction that leads to the formation of the oxazine ring in pdtme? In their paper on the oxidation of α-amino acids by quinones, Lourak and co-workers[17] obtained cyclic derivatives containing oxazine rings. These researchers



Scheme 1 Proposed reaction mechanism of phendio with L-tyrosine methyl ester leading to the formation of **PDT**.

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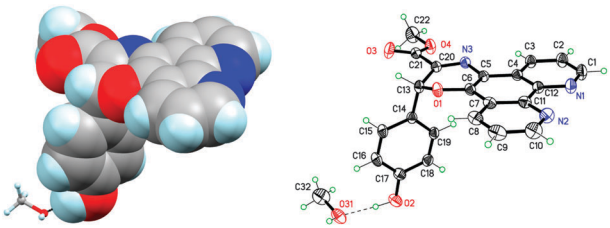

Fig. 1 Space-fill view of pdtme in **PDT**·MeOH (MeOH shown in stick view) (left), and a view of **PDT**·MeOH with 50% ADPs (right).

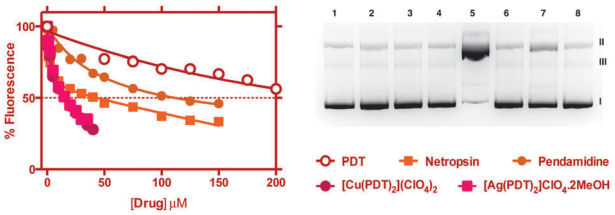

Fig. 2 Competitive EtBr displacement with CT-DNA (left) and DNA cleavage reactions of pBR322 plasmid DNA (400 ng) with 1 mM Na-L-ascorbate analysed on agarose gel electrophoresis (right). Lane 1: DNA control; lane 2: $[Cu(OAc)_2]$; lane 3: $AgNO_3$; lane 4: **PDT**; lane 5: **CuPhen**; lane 6: **AgPhen**; lane 7: **CuPDT**; lane 8: **AgPDT**.

proposed a mechanism for their reaction in which a second quinine molecule acted as a dehydrogenating agent to give the cyclic product. In the present work, the isolation of 1,10-phenanthroline-5,6-diol as a by-product of the reaction suggested that reduction of some phendio was occurring as part of the reaction mechanism. A likely first step is the formation of a Schiff base (molecule **A** in Scheme 1). A plausible, concerted mechanism for formation of the oxazine ring is offered in Scheme 1 and involves the participation of a second phendio molecule as a dehydrogenating agent. Due to steric restraints, resulting from both **A** and phendio, this reaction is expected to be slow, as was observed.

Interestingly, in the preparation of the phendio-amino acid ternary complexes, $[Cu(phendio)(L\text{-}Phe)(H_2O)]ClO_4{\cdot}H_2O$ and $[Ni(phendio)(Gly)(H_2O)]ClO_4{\cdot}H_2O$ (L-PheH = L-phenylalanine, GlyH = glycine), no interaction between the phendio carbonyl functions and the amine group of the amino acid was reported.[18] Reaction of **PDT** with $AgClO_4$ in a *ca.* 2 : 1 molar ratio gave the green solid, $[Ag(\mathbf{PDT})_2]ClO_4{\cdot}2MeOH$ (**AgPDT**), in good yield. The complex cation, $[Ag(\mathbf{PDT})_2]^+$, is thought to be essentially isostructural with the cation in the structurally characterised complex, $[Ag(phendio)_2]ClO_4$,[19] where the metal has approximately tetrahedral geometry. In comparison to the ^1H NMR spectrum of metal-free **PDT**, the spectrum of **AgPDT** showed shifts in the signals associated with the phen part of the pdtme molecule rather than the ester part of the molecule, indicating that the Ag^+ ion is chelated by the two nitrogen atoms. In a similar reaction using $Cu(ClO_4)_2{\cdot}6H_2O$, the green solid, $[Cu(\mathbf{PDT})_2](ClO_4)_2$ (**CuPDT**), was obtained in moderate yield. Again, it is believed that $[Cu(\mathbf{PDT})_2]^{2+}$ is structurally similar to the known tetrahedral cation in $[Cu(phen)_2](ClO_4)_2$ (**CuPhen**).[20]

In an effort to establish how the metal-free **PDT** ligand and its Cu(II) and Ag(I) complexes interact with DNA four distinct assays were conducted. To establish an apparent DNA binding constant (K_{app}) a sample of high-purity calf thymus (CT) DNA is firstly treated with an excess of EtBr and then this highly fluorescent, Et^+-saturated DNA sample is exposed to a range of concentrations of the competitor test compound. A reduction in fluorescence indicates ejection of the bound Et^+ from the DNA backbone and replacement by the test species. Samples are compared based on their K_{app} values, which are calculated from the concentration of sample required to accomplish a 50% reduction of the initial fluorescence (Fig. 2 and Table 1). Metal-free **PDT**, phen and the simple metal salts all showed considerably less DNA binding affinities than **AgPDT** and **CuPDT**, which have almost identical K_{app} values. The Ag(I) and Cu(II) **PDT** complexes also showed a higher binding capacity than the known groove-binding drugs, pentamidine and netropsin. The binding of metal-free **PDT** to DNA is significantly stronger than that of metal-free phen, suggesting that the additional functionalities on the backbone provide secondary binding interactions with the nucleic acid. The contribution of these extra interactions is also apparent when the binding constants of **PDT** and phen metal complexes are compared (K_{app} for **AgPDT** is ~3 times larger than **AgPhen** and **CuPDT** is ~11 times that of **CuPhen**).

Competitive displacement of DNA-bound Hoechst 33258 (minor groove binder) and ethidium cations (Et^+, intercalator) was assessed using fluorescence quenching, which utilises an unsaturated dye–DNA combination in which a limited number of binding spaces on the DNA polymer are occupied at any one time by the interacting dye, which strongly fluoresces once bound with a relatively high number of unoccupied sites remaining for test compound interaction. The introduction of a new compound to the DNA may then displace Hoechst or Et^+ either directly or indirectly (*i.e.* through a conformational change which results in dye ejection) and can give valuable information regarding a binding interaction mode,[21] although, caution must be exercised here considering the binding constant and binding stoichiometry of ethidium bromide and Hoechst to CT-DNA. The minor groove binders, pentamidine and netropsin, displayed high quenching affinity (Q) for Hoechst 33258 bound DNA and were, as expected, an order of magnitude less effective in their quenching of ethidium (Table 1). Both **AgPhen** and **CuPhen** displayed higher Q values toward Et^+ than compared with Hoechst. Interestingly, both **AgPDT** and **CuPDT** displayed higher Q values, along with different profiles,† and were almost identical in their ejection of bound Hoechst and Et^+. From these quenching data it is evident that the

Table 1 Apparent DNA binding constants (K_{app}) of the test compounds and fluorescence quenching (Q) values

Compound	C_{50}[a] (μM)	K_{app}[b]	Q[c] Hoechst 33258 (μM)	Q[c] Ethidium bromide (μM)
Pentamidine	109.41	1.09×10^6	35.86	>150
Netropsin	39.99	4.77×10^6	3.50	35.98
AgPDT	15.75	7.60×10^6	24.65	18.18
CuPDT	15.70	7.62×10^6	18.00	18.59
AgPhen	45.01	2.65×10^6	45.54	27.90
CuPhen	179.21	6.67×10^5	34.96	20.38
1,10-Phen	>300	N/A	—	—
PDT	247.21	4.80×10^5	—	—
$AgClO_4$	>300	N/A	—	—
$[Cu(OAc)_2]$	>300	N/A	—	—

[a] C_{50} = concentration required to reduce fluorescence by 50%. [b] $K_{app} = K_e \times 12.6/C_{50}$ where $K_e = 9.5 \times 10^6$ $M(bp)^{-1}$; N/A = not available. [c] Q = reduction of 50% initial fluorescence from DNA-bound dye.

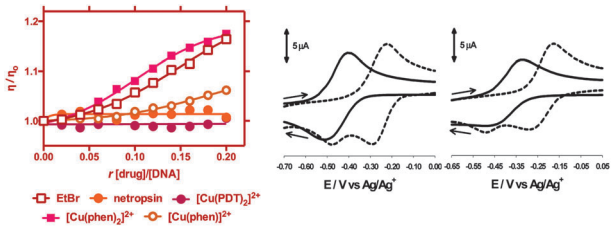

Fig. 3 Relative viscosity changes in salmon testes dsDNA (left) and cyclic voltammograms describing the redox behavior of 1 mM **CuPhen** (centre) and **CuPDT** (right), at a scan rate of 100 mV s^{-1}, in the absence (solid black trace) and presence (dashed black trace) of 2 mM ascorbate.†

PDT complexes have significantly higher binding affinities (K_{app}) and an alternate binding mode compared with their bis-phen analogues.

Viscosity experiments were conducted using salmon testes dsDNA (1 mM in DNAp) (Fig. 3).† Intercalating agents (*e.g.* ethidium) are known to increase viscosity which results from a conformational change induced after accommodation between DNA bases, while surface binding species (*e.g.* netropsin) can typically have only a moderate or diminished effect on viscosity. This expected pattern emerged during our analysis of both these standard agents (Fig. 3). It was also evident that **CuPhen**, containing the $[Cu(phen)_2]^{2+}$ cation, produced an intercalative profile while, significantly, **CuPDT** did not appear to intercalate DNA but had a profile similar to the surface binding drugs, netropsin and pentamide. Furthermore, the viscosity profile for **CuPDT** was distinctive from that of the partial intercalating mono-phen complex, $[Cu(phen)]^{2+}$.

Relaxation of supercoiled pBR322 (Form I) into open circular (Form II) and linear (Form III) conformations was used to quantify the relative DNA cleavage efficiency (nuclease activity) of the test complexes. DNA cleavage by $[Cu(phen)_2]^{2+}$ is known to be dependent on the presence of added oxidant and/or reductant, and the assay was conducted in an aerobic environment (O_2 oxidant) along with the addition of a 1 mM solution of the reductant, sodium L-ascorbate. Samples (5 μM) were incubated for 30 min at 37 °C before being quenched and analysed using gel electrophoreses. The only complex found to be active (Fig. 2) was the known chemical nuclease, $[Cu(phen)_2]^{2+}$.[22] This dication renders almost complete degradation of Form I to linear Form III by interacting at the surface of the minor groove of DNA and in the presence of a reductant, which can gain access to the Cu(II) centre in the complex, DNA oxidation occurs predominantly in this region. It was somewhat surprising that **CuPDT** was almost inactive compared with $[Cu(phen)_2]^{2+}$. Furthermore, any role that the phenol moiety in **PDT** may play in scavenging free radicals to prevent DNA damage looks quite unlikely as **CuPhen**, incubated with up to 100 μM of phenol, produced only a marginal inhibition of pBR322 cleavage (Fig. S3).†

The redox behaviour of **CuPhen** and **CuPDT** was investigated, using cyclic voltammetry, to shed light on the observed differences in their efficacy as DNA nuclease agents (Fig. 3 and Fig. S4–S9).† The Cu(II)/Cu(I) redox couple was centred around −0.46 and −0.39 V for **CuPhen** and **CuPDT**, respectively. Addition of 2 mM ascorbate results in a significant anodic shift in the Cu(II)/Cu(I) redox couple of both complexes, indicative of a more facile electron transfer regime. The original Cu(II) reduction peak for **CuPhen** is observed at −0.47 V but, interestingly, is not evident at scan rates ≤40 mV s^{-1}, which suggests a transient species. While **CuPhen** tends toward reversibility in the presence of ascorbate (ΔE_P = 65 mV), **CuPDT** remains quasi-reversible (ΔE_P = 119 mV, Table S1, ESI†). Thus, the reversibility of the Cu^{2+}/Cu^{+} couple may justify the greater DNA cleavage ability of **CuPhen**.

For concluding remarks please see ESI.†

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