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# THE UNIQUE DNA CUTTING SEQUENCE SPECIFICITY OF A 4-(N,N-DIMETHYL-2-AMINOETHYL)AMINO-1,10-PHENANTHROLINE AND ITS 9-AMINOACRIDINE CONJUGATE

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**Abbreviations** Acr acridine, BOP benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate, DiPEA diisopropylethylamine, DMF N,N-dimethylformamide, DMS dimethylsulphide, EDT 1,2-ethylenedithiol, EDTA ethylenediamine tetraacetic acid, ESMS electrospray mass spectrometry, HOBt 1-hydroxybenzotriazole, phen 1,10-phenanthroline, TEA triethylamine, TFA trifluroacetic acid.

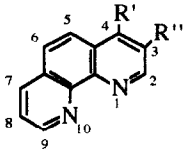
**Abstract** A 1,10-phenanthroline with a 3-carboxamide and a cationic side chain at C4 shows a highly sequence specific DNA cutting activity at 5'-TTAG sites. Conjugation with an 9-aminoacridine produced an additional cleavage site at 5'-TTAC. A binding model involving a 1:1:1 phenanthroline-copper-DNA complex is proposed based on the copper chelation chemistry of several phenanthroline derivatives.

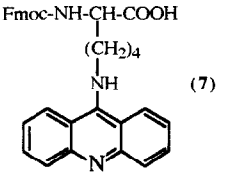
## Introduction

There is a continuing interest in the study of the specific interactions between DNA binding ligands and their binding sites. Oxygen radical mediated DNA cleavage based on EDTA-iron[1-3] or 1,10-phenanthroline-copper chelates[4-6], either as free complexes or covalently attached to the DNA binding ligands, is one of the most widely used techniques for footprinting the sequence specificity of DNA-binding compounds. In the course of our work on a 1,10-phenanthroline-copper system that is suitable for attachment to synthetic peptides, we observed a unique sequence specificity of DNA cleavage due to one of the 4-alkylamino substituted 1,10-phenanthroline derivatives. We present here the synthesis, the DNA cutting specificities and the copper chelation chemistry of these phenanthroline derivatives (**4**) and (**6**). A binding model involving the copper ion is described.

## Results and Discussion

**Chemistry**. The preparation of the 1,10-phenanthroline derivatives (**2, 3, 4**)[7] and Fmoc-lysine(acridine) (**7**)[8] have been reported. The 1,10-phenanthroline-acridine conjugates (**5, 6**) were synthesized with conventional solid phase peptide chemistry[9] from the C-terminal alanine residue. Acid labile amide anchorage,[10] base labile $N^{\alpha}$-Fmoc protection scheme[9] and BOP-HOBt coupling reagents[11] were used. The completed adducts were cleaved from the solid support with a TFA-scavenger mixture, and purified by reversed phase HPLC. The structures of the desired compounds were confirmed by molecular weight determination (ESMS) and $^1$H-nmr.





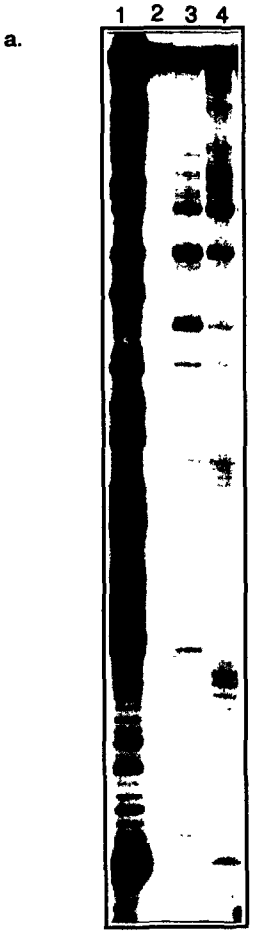
(7)

| | R' | R" |
|---|---|---|
| (1) | H | H |
| (2) | NHMe | $CONHCH_2CH_2COOBu^t$ |
| (3) | H | $CONHCH_2CH_2COOBu^t$ |
| (4) | $NHCH_2CH_2NMe_2$ | $CONHCH_2CH_2COOBu^t$ |
| (5) | H | $CONHCH_2CH_2CO$-Lys(acr)-Ala-$NH_2$ |
| (6) | $NHCH_2CH_2NMe_2$ | $CONHCH_2CH_2CO$-Lys(acr)-Ala-$NH_2$ |

**DNA cleavage experiments.** The DNA cleavage pattern produced by the phenanthroline derivatives were studied using a 188 bp DNA fragment (3'-labelled).[12,13] The test compounds (5 μM) and the DNA were incubated for 30 min at room temperature in the presence of $CuSO_4$ (1:1 molar equiv. phen/Cu) and sodium ascorbate (1 mM), pH 7. The resulting DNA fragments were analysed immediately by denaturing polyacrylamide gel electrophoresis after the samples were diluted with the loading buffer. The sites of cleavage were determined by comparison with the G-sequencing reaction of the labelled DNA analysed on the same gel.

The 4-N-methylamino (**2**) and the 4-unsubstituted phenanthroline derivatives (**3, 5**), as well as $Cu(phen)_2^{2+}$, showed no DNA cutting specificity. However, the phenanthroline derivative incorporating the 4-(N,N-dimethyl-2-aminoethyl)amino side chain showed a strong sequence specificity. Compound (**4**) on its own showed a strong preference for 5'-TTA-G sequence (the dash indicates where cleavage occurred at the adenine site to give 3'-labelled 5'-G fragments). When conjugated with an acridine unit, compound (**6**) showed a slightly modified cutting preference. Additional cleavage at 5'-TTA-C sites as well as 5'-TTA-G sites were observed (**Figure**). All of these strand scission sites were completely abolished by the presence of an equimolar amount of the minor groove binder distamycin.[14]

***In situ* detection of Phenanthroline-copper (II) complexes by ESMS.** Labile metal complexes can be detected *in situ* using electrospray mass spectrometry.[15] Since no external ionisation processes are involved, the different ionic complexes present in solution are often detected intact. In this work ESMS was used to study the chelation chemistry of the phenanthroline derivatives / copper (II) complexes, and its implication on the feasibility of the proposed role of the bis complex in DNA binding and cleavage.[16-18] The phenanthroline-copper mixtures were prepared in 4:1 molar ratio in order to maximise the observation of the bis and tris complexes. One of the phenanthroline-acridine conjugate (**6**) was also analysed at 1:1 molar ratio. The copper (II) chelate formation of the parent unsubstituted 1,10-phenanthroline (**1**) was compared with the 3-CO-β-Ala-O-$Bu^t$ (**3**) and the 4-(N,N-dimethyl-2-aminoethyl)amino-3-CO-β-Ala-O-$Bu^t$ (**4**) substituted phenanthrolines, and their Lys(acridine) conjugates (**5**) and (**6**).




## Figure.

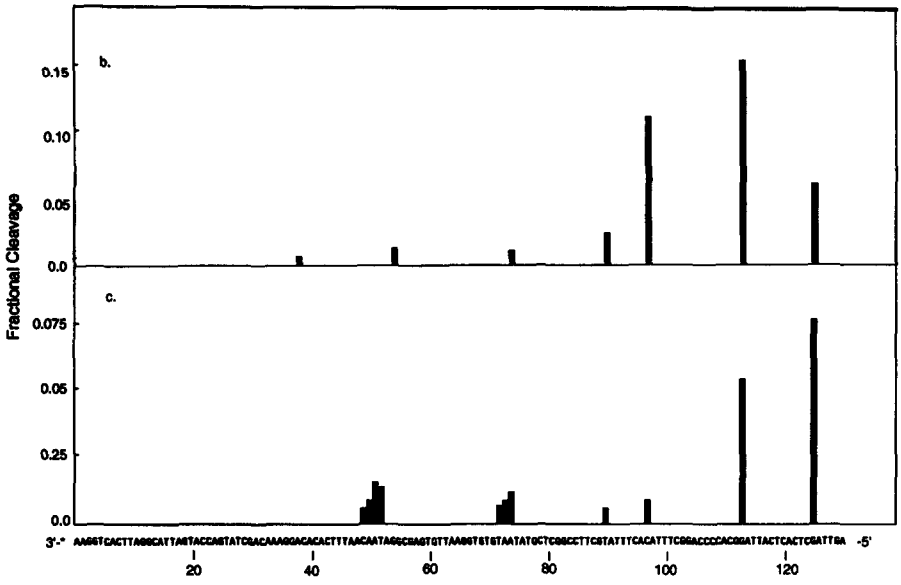
DNA cutting specificity of compound (4) and (6).

Experiments were performed using a 3'-end labelled ($\alpha$-$^{32}$P) 188-bp *Pvu* II / EcoR1 restriction fragment of a modified pBR322 vector containing the *lac* UV5 promoter. After electrophoresis the gel was dried and exposed to XAR-5 X-rays film. The intensity of the bands was quantitated using a Molecular Dynamics model 300S Computing Densitometer (Molecular Dynamics, CA), and the intensity of each band was expressed as a fraction of the total intensity of the lane.

a) Autoradiogram.
Lane 1, G-sequencing reaction;
Lane 2, uncut 3'-end labelled 188 bp-DNA;
Lane 3, phenanthroline-acridine conjugate (6), 5 $\mu$M;
Lane 4, phenanthroline (4), 5 $\mu$M.

b) Relative intensity of DNA cleavage of (6), lane 3.

c) Relative intensity of DNA cleavage of (4), lane 4.



It was observed that the 4-unsubstituted phenanthrolines (**1**) and (**3**) exist predominantly as bis and tris phenanthroline-copper (II) complexes. For compound (**4**), which has the cationic side chain at 4, the tris complex was not detected. In all cases, the 1:1 complex was not observed. However, under similar conditions, the phenanthroline-acridine conjugates (**5, 6**) exist exclusively as 1:1 complexes (the bis-aqua-phen-copper complexes were also observed), and the bis and tris complexes were not found. Presumably, one of the acridinyl nitrogen atoms and solvent water molecules serve as the remaining ligands, and formation of the bis and tris complexes is not feasible.

**Phenanthroline-copper DNA binding model.** The DNA minor groove binding and nuclease activity of 1,10-phenanthroline has recently been reviewed.[19] For compounds that bind to the DNA minor groove, it has been proposed that there is a contribution from H-bonding and polar-polar interactions between the polar functional groups of these compounds with those exposed groups in the DNA minor groove. However for the phenanthroline-copper complex additional stabilisation is provided by hydrophobic interactions between the aromatic nucleus and the C1' and C5' side of the sugar residues in the minor groove. This also accounts for the preference of these compounds for the narrower minor groove in A/T rich regions.[20]

It has been suggested that 1,10-phenanthroline-copper complexes bind in or near the minor groove of B-DNA.[17] Oxygen radicals generated by the oxidation-reduction cycle of the metal centre leads to ring opening of the riboside ring, followed by cleavage of the sugar-phosphate backbone. The bis-phenanthroline-copper complex has been proposed to be the active DNA cutting species.[16,17] Using ESMS, we have shown that although phenanthroline derivatives exist predominantly as bis or tris complexes with copper (II), phenanthroline-acridine conjugates only occur as 1:1 complexes with copper (II) ion. Furthermore, since 5-substituted phenanthroline-copper complexes are active in DNA cutting and also show some selectivity,[4,5,6,16,17] it seems unlikely that phenanthroline-copper complexes can produce DNA cutting selectivity through the interaction of the non-chelation side (C3 to C8) of the phenanthroline nucleus with the minor groove of DNA.

Based on the DNA cutting specificity and the copper chelation chemistry of this series of 4-(N,N-dimethyl-2-aminoethyl)amino-1,10-phenanthroline derivatives (**4, 6**), the evidence that binding occurs in the minor groove (abolition of cutting by the minor groove binder distamycin) and computer aided modelling, an alternative binding model involving the formation of a 1:1:1 phenanthroline-copper-DNA complexes can be proposed. The carbonyl (O2) of thymine and the ring nitrogen (N3) of adenine are accessible from the minor groove. These electronegative functional groups can act as ligands for metal chelation. It is feasible to envisage the formation of a transient tetradentate copper complex with N1 and N10 of a phenanthroline molecule and two other ligands from the adenine and thymine bases on the same DNA strand. This binding mode is more favourable for A/T rich regions as additional hydrophobic interactions with the inherently narrower minor groove are possible.[19] In the presence of a cationic side chain, additional charge-charge interaction with the anionic phosphate backbone should produce a very stable phenanthroline-copper-DNA complex. The reason for the cutting preference to favour 5'-TTA-G over 5'-TTA-C can be accounted for by the extra hydrogen bonding interaction between the nitrogen atom of the 3-carboxamide side chain with the 2-amino group of the guanine base. This is not possible with the cytosine base in the 5'-TTAC sequence.

The cutting preference persisted even when peptide chains were coupled to the side chain at C3 of compound (**4**). This observation mirrors those reported for a series of phenanthroline derivatives attached via the C5 position to several classes of DNA binding ligands. The DNA cutting patterns produced by these adducts were dominated by the inherent cutting preferences due to the phenanthroline nucleus alone.[6]

The inherent cutting preferences of (**4**) was modified slightly when a 9-aminoacridine was coupled to give (**6**). 5'-TTA-G cut sites were retained but 5'-TTA-C sites were also cleaved. This may be due to the effect of intercalation of the acridine in a nearby site on the local geometry to allow a stable complex to be formed.

**Summary**

While most phenanthroline derivatives showed preference for A/T rich regions, compound (**4**), a phenanthroline with a cationic side chain at the 4 position showed exceptional DNA cutting specificity (5'-TTA-G). Its acridine conjugate (**6**) also selectively cuts DNA at 5'-TTA-G and 5'-TTA-C sites. From ESMS, it was observed that a 1:1 phenanthroline-copper (II) complex was the predominant solution species for the acridine conjugates (**5, 6**). Thus, the probable active DNA cutting species can not be the bis or tris phenanthroline-copper (II) complex. An alternative DNA-Cu-phenanthroline model was proposed. Phenanthroline binds to A/T rich regions of the DNA minor groove through mixed chelation with the central copper (II) ion. Additional ionic interaction from the 4-cationic side chain, and hydrogen bonding of the 3-carboxamide group impart the selectivity for 5'-TTA-G sites.

**Experimental**

The synthesis of the phenanthroline derivatives[7] and Fmoc-Lys(acridine) (**7**)[8] has been reported. The Rink amide resin and BOP was purchased from Auspep, Australia. Proton nmr spectroscopy was performed on a Bruker AM300 spectrometer, using methanol-$d_4$ and referenced to residual solvent peak at 3.300 ppm. Electrospray mass spectral analysis was performed on a VG Biotech Bio-Q spectrometer.

*Solid phase synthesis of the phenanthroline-acridine conjugates (5) and (6)*
A standard protocol of Fmoc peptide chemistry was employed. Fmoc deprotection of amino groups was performed with piperidine in DMF (1:2 v/v, 1x5 min, 1x10 min). Coupling reactions were performed in the presence of the carboxylic acid component (2.5 equiv.), BOP (2.7 equiv.), HOBt (2.7 equiv.) and DiPEA (5.4 equiv.). The final product was cleaved from the solid support with a solution of 5% anisole, 5% EDT and 5% DMS in TFA (20 v/w of resin) for 1.5 h at room temperature. The product was purified by reverse phase HPLC (Whatman Partisil 10 C8 M9/25 column, 9x250 mm, solvent A 0.1% TFA in water, solvent B 0.07% TFA in acetonitrile-water (9:1 v/v), linear gradient 20 to 40% B in A over 30 min, at 3 ml/min), and an orange powder was obtained after freeze-drying.

*Phen-3-β-Ala-Lys(acr)-Ala-$NH_2$* (**5**), HPLC $t_R$ 19.8 min; UV-Vis $\lambda^{max}$ 230, 274, 318, 396, 419, 439 nm; ESMS 336.1 $[M+2H]^{2+}$ (100%), 671.3 $[M+H]^+$ (45%), $C_{38}H_{38}N_8O_4$ calc. 670.31. Yield as bis-TFA salt 52 mg (72%) from 0.2 g resin at 0.4 mmol/g initial loading. $^1$H-nmr δ 1.355 (d, J 7.2, βCH$_3$ Ala), 1.548 (m, γCH$_2$ Lys), 1.782 (m, βCH Lys), 1.80-2.00 (m, β'CH + δCH$_2$ Lys), 2.644 (t, J 6.3, CH$_2$CO βAla), 3.65-3.86 (m, NHCH$_2$ βAla), 3.969 (t, J 7.2, εCH$_2$ Lys), 4.30-4.42 (m, αCH Ala + αCH Lys), 7.487 (t, J 7.8, H2,2' Acr), 7.697 (d, J 7.8, H4,4' Acr), 7.899 (t, J 7.7, H3,3' Acr), 7.99-8.11 (m, H5,6,8 Phen), 8.345 (d, J 8.9, H1,1' Acr), 8.783 (dd, J 1.1, 8.2, H7 Phen), 8.946 (d, J 1.5, H4 Phen), 8.080 (dd, J 1.1, 4.0, H9 Phen), 9.465 (d, J 1.5, H2 Phen) ppm.

*4-(N,N-Dimethyl-2-aminoethyl)amino-phen-3-β-Ala-Lys(acr)-Ala-NH$_2$* **(6)**, HPLC $t_R$ 17.0 min: UV-Vis $\lambda^{max}$ 229, 274, 335, 399, 419, 439 nm; ESMS 379.2 $[M+2H]^{2+}$ (100%), 757.3 $[M+H]^+$ (1%), $C_{42}H_{48}N_{10}O_4$ calc. 756.39. Yield as tris-TFA salt 75 mg (85%) from 0.2 g resin at 0.4 mmol/g initial loading. $^1$H-nmr δ 1.330 (d, J 7.2, $\beta CH_3$ Ala), 1.628 (m, $\gamma CH_2$ Lys), 1.75-1.96 (m, β,β'CH Lys), 2.014 (m, $\delta CH_2$ Lys), 2.653 (m, $CH_2CO$ βAla), 2.986 (s, $NMe_2$ Phen), 3.637 (t, J 6.3, $CH_2$-$NMe_2$ Phen), 3.750 (q, J 6.6, $NHCH_2$ βAla), 4.066 (t, J 6.2, 4-$NHCH_2$ Phen), 4.143 (t, J 7.4, $\varepsilon CH_2$ Lys), 4.29-4.36 (m, αCH Ala + αCH Lys), 7.543 (t, J 7.9, H2,2' Acr), 7.754 (d, J 8.4, H4,4' Acr), 7.87-7.97 (m, H3,3' Acr + H8 Phen), 8.114 (d, J 9.4, H6 Phen), 8.398 (d, J 9.4, H5 Phen), 8.471 (d, J 8.8, H1,1' Acr), 8.567 (dd, J 1.5, 8.3, H7 Phen), 8.691 (s, H2 Phen), 9.134 (dd, J 1.5, 4.4, H9 Phen) ppm.

*Electrospray mass spectrometry detection of copper (II) chelates.*
The phenanthroline-copper (II) chelates were prepared by mixing a solution of the phenanthroline derivative (10 µl, 1 mM in methanol) with the required volume of copper sulphate solution (1 mM in water) and diluted to 50 µl with a 1:1 mixture of methanol and water to give a final phenanthroline concentration of 0.2 mM, with a molar ratio of phen/Cu of 4:1 for all compounds, and also at 1:1 for compound **(6)**. The samples were analysed by ESMS after 30 min.

*1,10-Phenanthroline* ***(1)***, $C_{12}H_8N_2$ calc. 180.07, obs. 181.1 $[M+H]^+$ (100%), 211.6 $[M_2+Cu]^{2+}$ (28%), 302.1 $[M_3+Cu]^{2+}$ (45%).

*Phen-3-CO-β-Ala-O-Bu$^t$* ***(3)***, $C_{20}H_{21}N_3O_3$ calc. 351.16, obs. 352.2 $[M+H]^+$ (66%), 382.8 $[M_2+Cu]^{2+}$ (26%), 558.5 $[M_3+Cu]^{2+}$ (100%).

*4-(N,N-Dimethyl-2-aminoethyl)amino-phen-3-β-Ala-O-Bu$^t$* ***(4)***, $C_{24}H_{31}N_5O_3$ calc. 437.24, obs. 219.6 $[M+2H]^{2+}$ (21%), 438.4 $[M+H]^+$ (100%), 469.0 $[M_2+Cu]^{2+}$ (12%).

*Phen-3-β-Ala-Lys(acr)-Ala-NH$_2$* ***(5)***, $C_{38}H_{38}N_8O_4$ calc. 670.31, obs. 336.1 $[M+2H]^{2+}$ (100%), 367.1 $[M+Cu]^{2+}$ (87%), 382.9 $[M+Cu+2H_2O]^{2+}$ (7%), 671.3 $[M+H]^+$ (58%).

*4-(N,N-Dimethyl-2-aminoethyl)amino-phen-3-β-Ala-Lys(acr)-Ala-NH$_2$* ***(6)***, $C_{42}H_{48}N_{10}O_4$ calc. 756.39, 1:1 mixture obs. 409.9 $[M+Cu]^{2+}$ (100%), 427.9 $[M+Cu+2H_2O]^{2+}$ (23%); 4:1 mixture obs. 379.5 $[M+2H]^{2+}$ (100%), 410.0 $[M+Cu]^{2+}$ (28%), 757.4 $[M+H]^+$ (3%).

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