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# 9-AMINOACRIDINE-EDTA CONJUGATES AS HYDROXY RADICAL FOOTPRINTING REAGENTS WITH NO INTRINSIC CUTTING SPECIFICITY

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**Abbreviations:** Acr, acridine; Boc, *t*-butoxycarbonyl; BOP, benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate; Dap(Ac4), 2,3-diaminopropanoic acid $N^2$,$N^2$,$N^3$,$N^3$-tetraacetic acid; DiPEA, diisopropylethylamine; DMF, N,N-dimethylformamide; DMS dimethylsulphide; EDT, 1,2-ethylenedithiol; EDTA, ethylenediamine tetraacetic acid; ESMS, electrospray mass spectrometry; Fmoc, 9-flurenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; TFA, trifluroacetic acid.

**Abstract** 9-Aminoacridine-EDTA conjugates were prepared as DNA footprinting reagents in conjunction with ferrous ion. The complexes were found to have no significant DNA cutting preferences and are more effective than free EDTA. The application was demonstrated with the footprinting of the *lac* repressor bound specifically to the *lac* operon.

## Introduction

Footprinting techniques based on oxygen radicals generated by transitional metal chelates have been widely used in the study of the interaction between DNA and DNA binding ligands. Methidium-EDTA-iron[1-3], 1,10-phenanthroline-copper[4-6] and EDTA-Fe(II)[7,8] complexes, the commonly used systems, have been widely employed. However, the interpretation of the ligand binding site from the "footprint" is often hindered by the inherent DNA cutting selectivities due to the preferential affinity of these complexes for certain DNA sequences.

Neither 9-aminoacridine, a potent DNA intercalator, nor EDTA, a strong metal chelator, have been reported to have DNA binding selectivity. A combination of these two reagents would yield a conjugate with high DNA affinity and DNA cutting activity. In this report we describe the design and synthesis of a series of acridine-EDTA DNA cutting reagent (**8**,**9**) that showed no preferential DNA cutting specificity, making it ideal for footprinting experiments.

## Results and Discussion

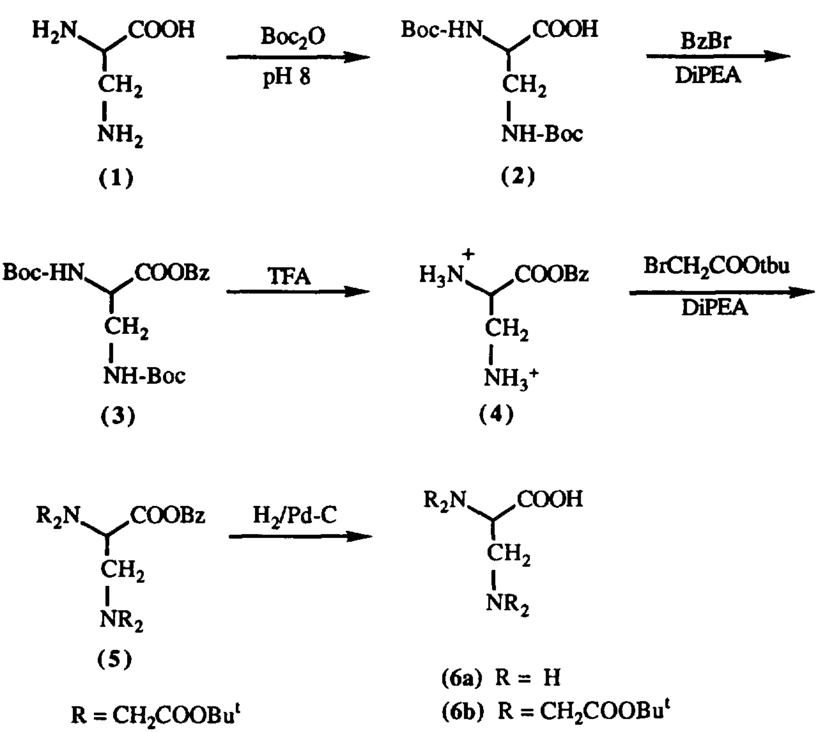
Both phenanthroline-copper and methidium-EDTA-iron complexes are effective DNA cutting reagents because of the combination of the metal chelation stability and high DNA affinity. The site of action of phenanthroline-copper has been suggested to be located near or in the DNA minor groove.[9] Depending on the structure of the phenanthroline, the preferential DNA binding of the complexes produced DNA background cutting patterns with large variation in intensity.[4-6] Methidium-EDTA-iron complexes bind to DNA via intercalation of the methidium moiety. However, a small but substantial variation in the intensity of the background cleavage remains.[1-3] In order to design a DNA cutting reagent with a more even background, we chose to explore the combination of the strong metal chelator, EDTA, and the potent DNA intercalator, 9-aminoacridine, since neither compounds have any reported DNA sequence dependent affinity.

**Chemistry** To simplify the preparation of the 9-aminoacridine-EDTA conjugate, a modular approach was employed based on solid phase peptide synthesis. The 9-aminoacridine component was incorporated into an amino acid unit, Fmoc-lysine(acridine) **(7b)**,[10] and an "EDTA" component was built into a carboxylic acid, Dap($Ac_4$) **(6a)** **(Scheme)**, to be attached as an N-terminal unit. The lysine(acridine) unit **(7a)** was coupled to a C-terminal alanine residue, followed by one or two glycine residues serving as a spacer between the intercalator and the metal chelate. The conjugate was completed with the coupling of the Dap($Ac_4$) unit **(6b)**. The preparation of the Fmoc-lysine(acridine) unit **(7b)** has been reported.[10] The simplest choice for an EDTA-carboxylic acid unit is by the introduction of a carboxylic acid group to the ethylene chain of EDTA, with the N-acetic acid side chains protected as *t*-butyl esters, to give compound **(6b)**. The synthesis of compound **(6b)** was achieved by Boc protection of the amino groups of 2,3-diaminopropanoic acid **(1)** to give compound **(2)**, followed by benzyl ester protection of the carboxylic acid to give compound **(3)**. After acidolytic cleavage of the N-Boc protection of **(3)**, the free amino groups of **(4)** were alkylated to give the four N-acetic acid side chain protected as the acid labile *t*-butyl ester in compound **(5)**. The benzyl protected carboxylic acid was liberated by hydrogenolysis of **(5)** to give the final product **(6b)**, which was used in the synthesis of the conjugates **(Scheme)**. An alternative synthesis of compound **(6b)** was reported recently,[11] but attempts to repeat the reactions were unsuccessful.

The 9-aminoacridine-EDTA conjugates **(8,9)** were synthesized with conventional solid phase peptide chemistry[12] from the C-terminal alanine residue. Acid labile amide anchorage,[13] base labile $N^{\alpha}$-Fmoc protection scheme[12] and BOP-HOBt coupling reagents[14] were used. The completed adducts were cleaved from the solid support with TFA-scavenger mixture, and purified by reversed phase HPLC. The structures of the desired compounds were confirmed by molecular weight determination (ESMS).

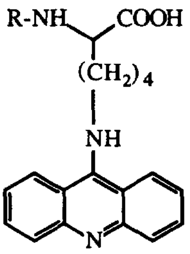
**DNA cleavage and footprinting experiments** The DNA cleavage patterns produced by the 9-aminoacridine-EDTA conjugates **(8,9)** in the presence and absence of the *lac* repressor were studied using a 188 bp DNA fragment containing the *lac* operon (3'-labelled).[15,16] The test compound **(8)** was incubated with DNA in the presence of ammonium ferrous sulphate (1:1 molar equiv. Acr-EDTA/Fe) and sodium ascorbate (1 mM) at pH 7 for 45 min at 10°C. The resulting DNA fragments were analysed immediately by denaturing polyacrylamide (12%) gel electrophoresis after the samples were diluted 1: 1 with the loading buffer. The sites of cleavage and the protected regions were determined by comparison with the G-sequencing reaction of the labelled DNA analysed on the same gel.

From the gel electrophoresis analysis, in the absence of the *lac* repressor, the intensity of the DNA cleavage (background) is remarkably even (**Figure 1, lane 2**). The application of these complexes as footprinting reagents was demonstrated with the location of the *lac* repressor binding site (**Figure 1, lanes 3-7**). A clear 24bp footprint (positions 40 to 65) was revealed with **(8)**, the same as observed by DNase I footprinting.[18]



**Scheme.**

The DNA not protected from hydroxyl radical induced cleavage yielded an even level of strand scission (**Figure 1, lane 4 and Figure 2a**) as compared to other footprinting reagents which are known to exhibit sequence-dependent variation of cleavage efficiency (1-6,17) and this is illustrated in **Figure 2b,c** for MPE-Fe(II) and EDTA-Fe(II) respectively. A similar result was also obtained with (**9**). These acridine-EDTA iron complexes are therefore well suited to use as DNA footprinting reagents.



R = H (7a)
= Fmoc (7b)

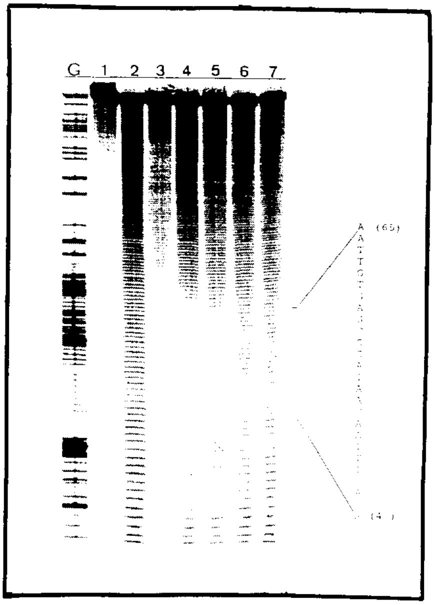


**Figure 1**
Autoradiogram of footprinting of *lac* repressor by (**8**). A 188 bp DNA fragment containing the *lac* operon was 3' end-labelled and then made up to 25μM bp with calf thymus DNA (lane 1). It was then digested with 50μM of (**8**), 50μM ferric chloride and 1mM sodium ascorbate at 10°C in the absence (lane 2) or presence of *lac* repressor (lanes 3-7; 0.64, 0.32, 0.16, 0.08 and 0.04 μM respectively). The sequence numbering is with respect to the 3' end of the DNA fragment.

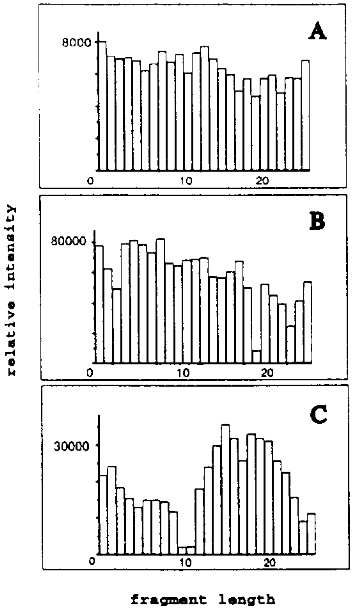


**Figure 2**
Relative intensity of strand scission induced by compound 8,(a), MPE-Fe(II),(b) and EDTA-Fe(II),(c) in a 25 bp region of the 188 bp DNA fragment. Band intensity was obtained using a Molecular Dynamics Model 400B PhosphorImager, with quantitation performed using ImageQuant software.

## Experimental

The synthesis of Fmoc-Lys(acridine)[10] has been reported. Benzyl bromide, *t*-butyl bromoacetate, 2,3-diaminopropanoic acid and TFA were purchased from Aldrich Chemical Co., USA. The Rink amide resin, Fmoc-Gly, Fmoc-Ala and BOP was purchased from Auspep, Australia. Proton and carbon-13 nmr spectroscopy were performed on a Bruker AM300 spectrometer, using $CDCl_3$ and referenced to tetramethyl silane as internal standard (0 ppm) for proton spectra, and to the residual solvent peak at 77.0 ppm for carbon-

13-spectra. Electrospray mass spectral analysis was performed on a VG Biotech Bio-Q spectrometer. The DNA footprinting 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 promotor.[15,16] The results of the denaturing gel electrophoresis analysis of the labelled DNA fragments was analysed using a Molecular Dynamics model 400B PhosphorImager (Molecular Dynamics, CA).

*$N^2$,$N^3$-Bis-t-butoxycarbonyl-2,3-diaminopropanoic acid (2)*

2,3-Diaminopropanoic acid (**1**) (2 g) and excess sodium bicarbonate (10 equiv., 12 g) were dissolved in water (100 ml) and dioxane (100 ml). Di-*t*-butyl-dicarbonate (4 equiv., 12.4 g) was added, and the mixture was stirred at room temperature for 4 h. The mixture was diluted with water (300 ml), washed with dichloromethane (2x100 ml), acidified to pH 2.5 with HCl (5 M), and extracted with dichloromethane (3x150 ml). The combined organic extracts were dried and concentrated under vacuum. The desired product (**2**) was obtained as white prisms from dichloromethane / petroleum ether, yield 3.9 g (89%), m.p. 157°. $^1$H-nmr $\delta$ ($CDCl_3$) 1.47 (s, $CH_3$ $Bu^t$); 3.57 (m, $\beta CH_2$); 4.28 (m, $\alpha$CH); 5.19, 5.92 (m, NH).

*$N^2$,$N^3$-Bis-t-butoxycarbonyl-2,3-diaminopropanoic acid benzyl ester (3)*

Compound (**2**) (4.6 g), benzyl bromide (1.2 equiv., 2.2 ml) and triethylamine (1.4 equiv., 2.9 ml) were refluxed in ethyl acetate (100 ml) for 16 h. The mixture was diluted with ethyl acetate (100 ml) and washed with HCl (1 M, 100 ml), brine (100 ml), $NaCO_3$ (1 M, 100 ml) and brine (100 ml). The organic phase was dried and concentrated under vacuum. The desired product (**3**) was crystallised from dichloromethane / petroleum ether to give a white powder, yield 4.1 g (69%), m.p. 129°. $^1$H-nmr $\delta$ ($CDCl_3$) 1.43 (s, $CH_3$ $Bu^t$); 3.53 (m, $\beta CH_2$); 4.40 (m, $\alpha$CH); 4.76 (m, NH); 5.18 (s, $CH_2$ bzl); 5.92 (m, NH); 7.36 (s, Ar bzl).

*2,3-Diaminopropanoic acid benzyl ester bis-trifluroacetate (4)*

The benzyl ester (**3**) (4.1 g) was treated with a solution of trifluoroacetic acid (36 ml) and water (4 ml) at room temperature for 1 h. The mixture was concentrated under vacuum to a viscous oil and the product (**4**) was crystallised from ethyl acetate and ether to give a white powder, yield 4.2 g (91%), m.p. 149°. $^1$H-nmr $\delta$ (DMSO-$d_6$) 3.27, 3.34 (d-ABq, J 6.4, 13.6, $\beta CH_2$); 4.36 (t, J 6.4, $\alpha$CH); 5.20, 5.28 (ABq, J 12.4, $CH_2$ bzl); 7.40 (m, Ar bzl).

*$N^2$,$N^2$,$N^3$,$N^3$-Tetrakis-(t-butoxycarbomethylene)-2,3-diaminopropanoic acid benzyl ester (5)*

Compound (**4**) (3.6 g) was treated with *t*-butyl bromoacetate (6 equiv., 8.5 ml) and diisopropylethylamine (6 equiv., 9.3 ml) in DMF (20 ml) at 70° for 16 h. The mixture was concentrated under vacuum, diluted with ethyl acetate (200 ml) and washed with $NaCO_3$ (1 M, 100 ml). The organic phase was dried, concentrated and purified by silica column chromatography (eluting with 10 to 25% ethyl acetate in petroleum ether) to give the desired product (**5**) as an oil (4.2 g, 77%). $^1$H-nmr $\delta$ ($CDCl_3$) 1.43 (s, $CH_3$ $Bu^t$); 3.06-3.14 (m, $\beta CH_2$); 3.47, 3.56 (s, $NCH_2CO$); 3.66 (m, $\alpha$CH); 5.12 (ABq, J 12.5, $CH_2$ bzl); 7.28-7.41 (m, Ar bzl).

*$N^2$,$N^2$,$N^3$,$N^3$-Tetrakis-(t-butoxycarbomethylene)-2,3-diaminopropanoic acid (6b)*

Compound (**5**) (4 g) was hydrogenated under an atmosphere of hydrogen (200 psi) over 10% Pd-charcoal catalyst (300 mg) in methanol (100 ml) at room temperature for 16 h. The mixture was filtered, concentrated under vacuum, and the product (**6b**) purified on silica column (eluting with 5 to 10% ethyl acetate in dichloromethane) to give a gummy material, yield 2.1 g (60%). $^1$H-NMR $\delta$ ($CDCl_3$) 1.46 (s, $CH_3$, $Bu^t$); 3.16

(d, J 7.4, $\beta CH_2$); 3.52, 3.56 (s, $NCH_2CO$); 3.75 (t, J 7.4, $\alpha$CH). $^{13}$C-nmr $\delta$ ($CDCl_3$) 27.86 ($CH_3$ Bu$^t$); 54.45, 54.36 ($NCH_2CO$); 56.42 ($\beta CH_2$); 63.03 ($\alpha$CH); 81.44 (qC, Bu$^t$); 169.92 (COOH); 171.30, 173.10 (COOBu$^t$).

*Solid phase synthesis of the 9-aminoacridine-EDTA conjugates (8) and (9)*

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 by treatment 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 15 to 40% B in A over 30 min, at 3ml/min), and a yellow powder was obtained after freeze-drying.

*Dap(Ac$_4$)-Gly-Lys(Acr)-Ala-NH$_2$ (8)* HPLC $t_R$ 17.9 min; UV-Vis $\lambda^{max}$ 226, 272, 395, 418, 438 nm; ES-MS 385.5 $[M+H_2]^{2+}$, 769.4 $[M+H]^+$, calc. 769.8 $[M+H]^+$. Yield 31 mg (67%) from 0.1 g resin at an initial loading of 0.6 mmol/g.

*Dap(Ac$_4$)-Gly-Gly-Lys(Acr)-Ala-NH$_2$ (9)* HPLC $t_R$ 17.8 m; UV-Vis $\lambda^{max}$ 226, 272, 395, 418, 438 nm; ES-MS 413.9 $[M+H_2]^{2+}$, 826.4 $[M+H]^+$, calc. 826.8 $[M+H]^+$. Yield 32 mg (64%) from 0.1 g resin at an initial loading of 0.6 mmol/g.

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