# Bleomycin mimics. Design and synthesis of an acridine derivative which cleaves DNA in a sequence-neutral manner

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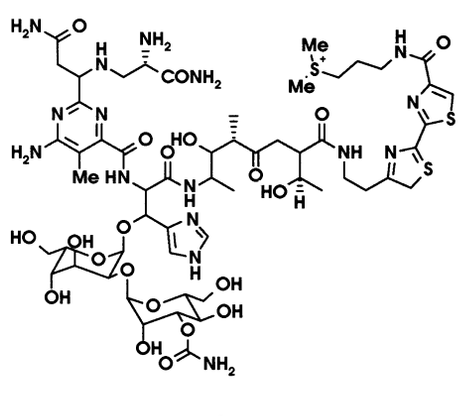
**Bleomycin 1 is a clinically used anticancer drug that cleaves DNA at guanine–pyrimidine dinucleotides by a free-radical mechanism. The compound FTP1 2 was designed as a bleomycin mimic to cleave DNA in a non-sequence selective manner. Compound 2 may provide a route to novel, chemically simple, bleomycin analogues that also have potential as new DNA footprinting agents. FTP1 2 was synthesised from chelidamic acid, 1,3-phenylenediamine and 9-chloroacridine. An untargeted agent 8 was also made. Compound 2 cleaves DNA in a relatively non-sequence selective manner although a number of hotspots of similar sequence were found. It has a 20-fold higher cutting activity than the untargeted agent 8 and has been used to footprint two antibiotics, actinomycin D 9 and a minor groove binder, distamycin A 10. It gives clear, well-defined footprints which compare favourably with those reported by MPE–Fe$^{II}$, DNase I and iron–EDTA and is a useful addition to the range of footprinting agents. Its cytotoxicity against human colon carcinoma cells in culture is 20 times lower than that of bleomycin although its ability to cleave naked DNA is 10 000-fold less, suggesting that on a mole-for-mole basis FTP1-induced DNA damage is more biologically harmful.**

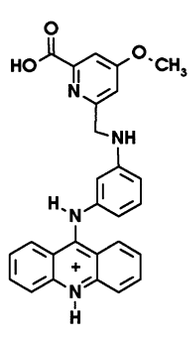


## Introduction

Bleomycins are a family of antitumour antibiotics used in combination chemotherapy against several tumours.[1] Bleomycin $A_2$ **1**, which comprises an iron(II) binding β-aminoalanine–pyrimidine–β-hydroxyhistidine and a DNA-binding bithiazole moiety, is the chief constituent of the clinically used mixture blenoxane. Recently, studies[2] of bleomycin analogues in which the iron chelating portion has been modified to contain a pyridine with an electron-donating substituent in the 4-position, have shown that these compounds, which are targeted to DNA through the usual bithiazole moiety but which lack the disaccharide of the naturally occurring compound, have a DNA cleaving activity about 10% of that of bleomycin. An analogue of the minor groove binding compound distamycin, to which a similar pyridine derived iron (II) chelating moiety was attached, was shown to cleave DNA[3] at recognised distamycin binding sites. A derivative of chelidamic acid has also been appended to an oligonucleotide and shown to cleave DNA in a sequence specific manner.[4] Such 'minimum analogues' of bleomycin are of interest for a number of reasons. The targeting of iron(II) binding pyridine derivatives to DNA by the use of minor groove binders, oligonucleotide analogues or intercalators may lead to compounds with improved antitumour activity compared to the natural compound. It also allows investigation of the sequence preferences of the targeting moiety through determination of DNA cleavage patterns by the compound in the presence of iron(II) and a reducing agent. Similarly, the resulting compounds may in themselves be useful tools in the investigation of drug–DNA interactions by the technique of footprinting.



**1**



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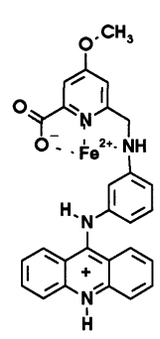


**2a**

There is growing interest in compounds that bind to DNA in a sequence specific manner since they may have therapeutic application in the treatment of cancer, viral and bacterial infections.[5] Investigations into the sequence specificities of minor groove binders,[6] intercalators[7] and oligonucleotide analogues[8] have been carried out using various techniques including those known collectively as footprinting. Footprinting involves the cutting of a strand of $^{32}$P end-labelled DNA of known sequence both in the absence and in the presence of varying concentrations of ligand. From the resulting autoradiograms, information regarding the sequence preference of the DNA binding compound, its binding affinity and even some kinetic data[9] may be obtained. DNA cleavage reactions may be carried out either by enzymes or by chemical agents. Enzymic reactions suffer

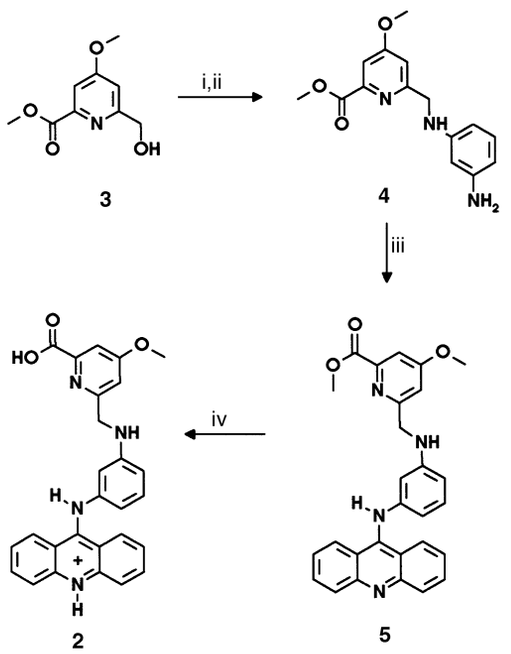
from some inherent sequence preferences. For instance, DNase I preferentially cleaves sequences of mixed nucleotide composition, and cleaves more efficiently in GC regions than AT.[10] Hydroxyl radical cleavage by the $Fe^{II}$–EDTA complex also has limitations, particularly when attempting to footprint intercalating compounds.[11] The preferred chemical agent, known as MPE–$Fe^{II}$ [methidiumpropylethylenediaminetetraacetic acid–iron(II)], is a derivative of methidium and EDTA and its synthesis is not trivial.[7] Until recently, it has not been commercially available.

In order to develop a synthetically more accessible compound which will cleave DNA in a non-sequence specific manner and which has potential as both a new footprinting agent and also as an experimental antitumour 'bleomycin mimic', we have designed the chelidamic acid derivative **2**. The compound is targeted to DNA *via* a 9-anilinoacridine group. 9-Aminoacridines have been shown[12] to bind to DNA with little sequence preference and would be expected to give compounds which cleaved in essentially a non-sequence specific manner. Here, we describe the synthesis of the new compound, its ability to cleave DNA, initial studies on its application to the footprinting of distamycin A and actinomycin D and measurements of its cytotoxicity against human colon carcinoma cell line. DNA cleavage by the new agent, FTP1 **2** was compared to an analogue **8** which is not targeted to DNA through an acridine moiety, although it carries a similar charge and also to the well known footprinting agents MPE–$Fe^{II}$, $Fe^{II}$–EDTA and DNase I. A proposed structure for iron(II) binding is shown by **2a**.

## Results and discussion

### Synthesis of FTP1 and a non-targeted analogue

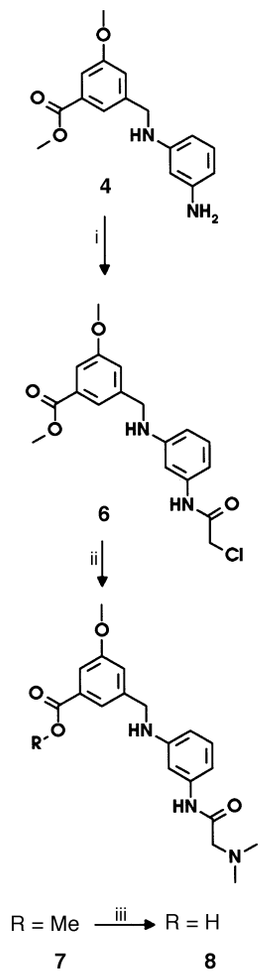
The synthesis of FTP1 is outlined in Scheme 1. Methyl 6-hydroxymethyl-4-methoxypyridine-2-carboxylate[2] was converted to the anilino-derivative **4** *via* mesylation and reaction with 1,3-phenylenediamine. Phenylenediamine was used as the linker, rather than flexible bridges such as propylenediamine, to avoid potential intramolecular reactions of 9-aminoacridines. Reaction *via* the mesylate, rather than by the previously described[2,3] Swern oxidation–reductive alkylation, gave the required product in reasonable yield in a one-pot reaction.




**Scheme 1** (*i*) Methanesulfonyl chloride, $Et_3N$, $CH_2Cl_2$, room temp., 1 h. (*ii*) 1,3-phenylenediamine (3 equiv.), room temp., 16 h. (*iii*) 9-Chloroacridine, anhydrous methanol, room temp., 2 h. (*iv*) 1 M HCl, Δ, 3 h, then precipitated from ethanol–ethyl acetate.

Conversion to the acridine derivative **5** was carried out *via* reaction with freshly prepared 9-chloroacridine and gave, after purification by flash column chromatography,[13] an 84% yield of the acridine methyl ester. Hydrolysis under acid-catalysed conditions followed by precipitation from ethanol with ethyl acetate gave the target compound **2** in 74% yield as the hydrochloride salt.

In order to investigate the degree of activity conferred on the compound by targeting to DNA *via* intercalation of the acridine moiety, and also to explore the sequence preference, if any, of an untargeted compound, the *N*,*N*-dimethylacetylamino analogue **8** was also prepared (Scheme 2). This compound




**Scheme 2** (*i*) Chloroacetic anhydride, $CH_2Cl_2$, room temp., 3.5 h. (*ii*) Dimethylamine, $CH_3CN$, room temp., 21 h. (*iii*) NaOH, $H_2O$–MeOH, room temp., 3 h.

should still have some non-specific interaction with the negatively charged nucleic acid by virtue of its positive charge and should also be reasonably soluble. Its synthesis was straightforward, proceeding directly from the aniline methyl ester **4** to the chloroacetyl derivative **6** *via* reaction with chloroacetic anhydride under relatively mild conditions. Addition of the dimethylamino group *via* alkylation with dimethylamine in acetonitrile gave the methyl ester derivative **7** of the required compound, which was not characterised but immediately hydrolysed to the acid **8**.

### DNA Cleavage by FTP1

The cleavage of both strands of the SV40 early promoter fragment of DNA by 20 μM FTP1 **2**, equimolar iron(II) in the presence of dithiothreitol (DTT, excess) was analysed by scanning laser densitometry (Fig. 1). Investigation of the concentration dependence of the extent of cleavage (data not shown) shows this concentration of FTP1 and iron(II) to be optimal for

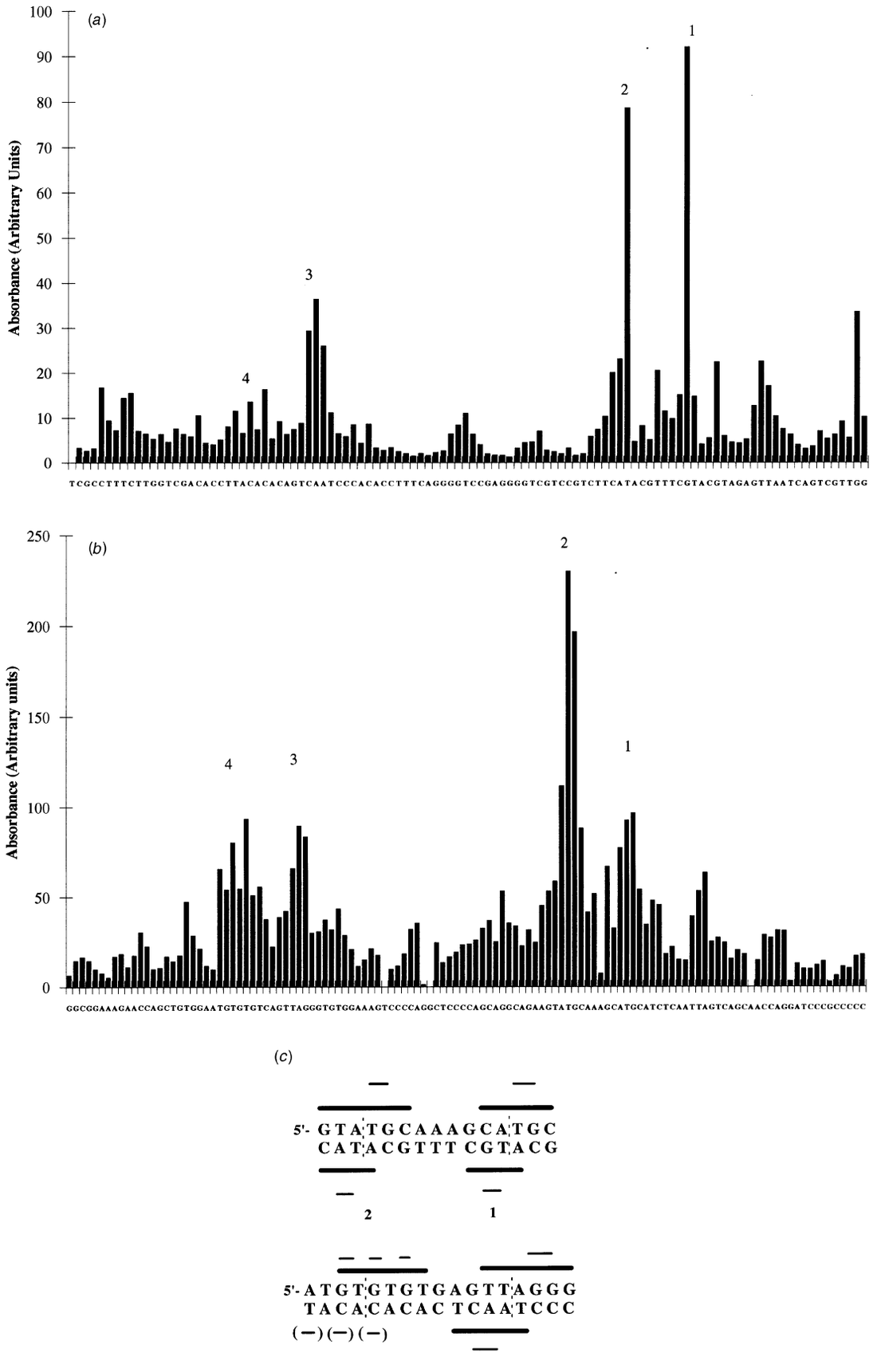



**Fig. 1** DNA cleavage patterns obtained with 20 μM FTP1, 20 μM $Fe(NH_4)_2(SO_4)_2$ and 5 mM dithiothreitol analysed by laser densitometry. (*a*) 3′-Labelled DNA, sequence 5′ to 3′. (*b*) 5′-Labelled DNA, sequence 3′ to 5′. (*c*) Summary of hotspot sequences. Dotted vertical lines represent potential binding sites, bold filled lines represent the extent of the hotspot and smaller lines the site of most intense cutting.

'single hit kinetics'.[14] Bleomycin cleaves DNA to a similar extent at a concentration of 2 nM.[15] At 20 μM, FTP1 **2** cuts DNA at all nucleotides with a general five-fold variation in cutting intensity. In addition, there are four hotspots which show an increased, up to 15-fold, variation in cleavage, labelled 1–4 in Fig. 1(*a*) and (*b*) and shown in Fig. 1(*c*). Three of the four

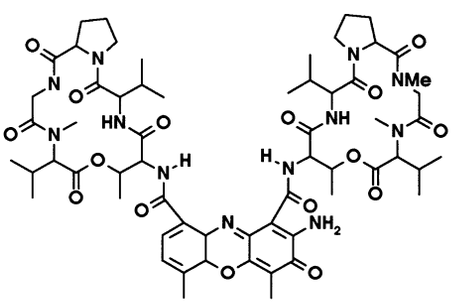
hotspots comprise alternating purine–pyrimidine sequences with the two sites of highest cleavage intensity (1 and 2) containing a similar GpyATGC motif. The cutting pattern at these sites suggests that the intercalator is positioned between the base pairs of the central ApT step. Site 3 appears to be centred around a TpA step in the sequence 5′-TTAGGG. Site 4 is similar to 1 and 2 in being a purine–pyrimidine sequence but is of the form $(GT)_n$. The acridine may be intercalating at any of the GpT steps, although as cleavage appears to be asymmetric with cutting only clearly visible on the 5′-strand, it is not possible to identify a precise binding site. Both MPE–$Fe^{II}$ and $Fe^{II}$–EDTA also show the hotspot at position 3 (data not shown), but not at the other three sites, which suggests that enhanced cleavage by hydroxyl radical at this position is a consequence of sequence rather than being attributable to the nature of the cleaving agent.

**DNA cleavage by the untargeted compound 8**

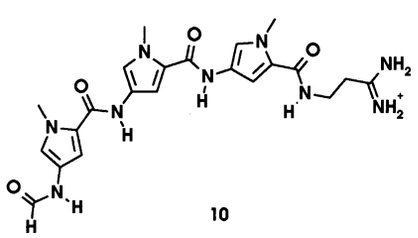
Comparison of cleavage by FTP1 **2** and the untargeted agent **8** in the presence of equimolar iron(II) showed that the untargeted compound cleaves to a similar extent to **2** only at a concentration of 200 μM (data not shown), indicating that targeting of the chelidamic acid moiety to DNA *via* the acridine chromophore leads to a compound which is at least 20 times more effective. It should also be noted that iron(II) cleaves DNA in the absence of added ligand to a similar extent to compound **8** at comparable concentrations.

**Footprinting of actinomycin D**

In order to investigate the suitability of FTP1 **2** as a footprinting agent, its ability to reveal the sequence selectivities of two known DNA binding antibiotics was assessed, namely the GC-selective intercalator actinomycin D **9** and the AT-selective minor groove binder distamycin A **10**. Resulting autoradiographs were analysed using scanning laser densitometry.



**9**



**10**

Actinomycin D **9** is an antitumour antibiotic which binds to DNA by intercalation, positioning its two cyclic depsipeptides in the minor groove. Previous footprinting studies have revealed a selectivity for binding to 5′-GpC steps with some secondary affinity for GpG and CpC sequences.[16]

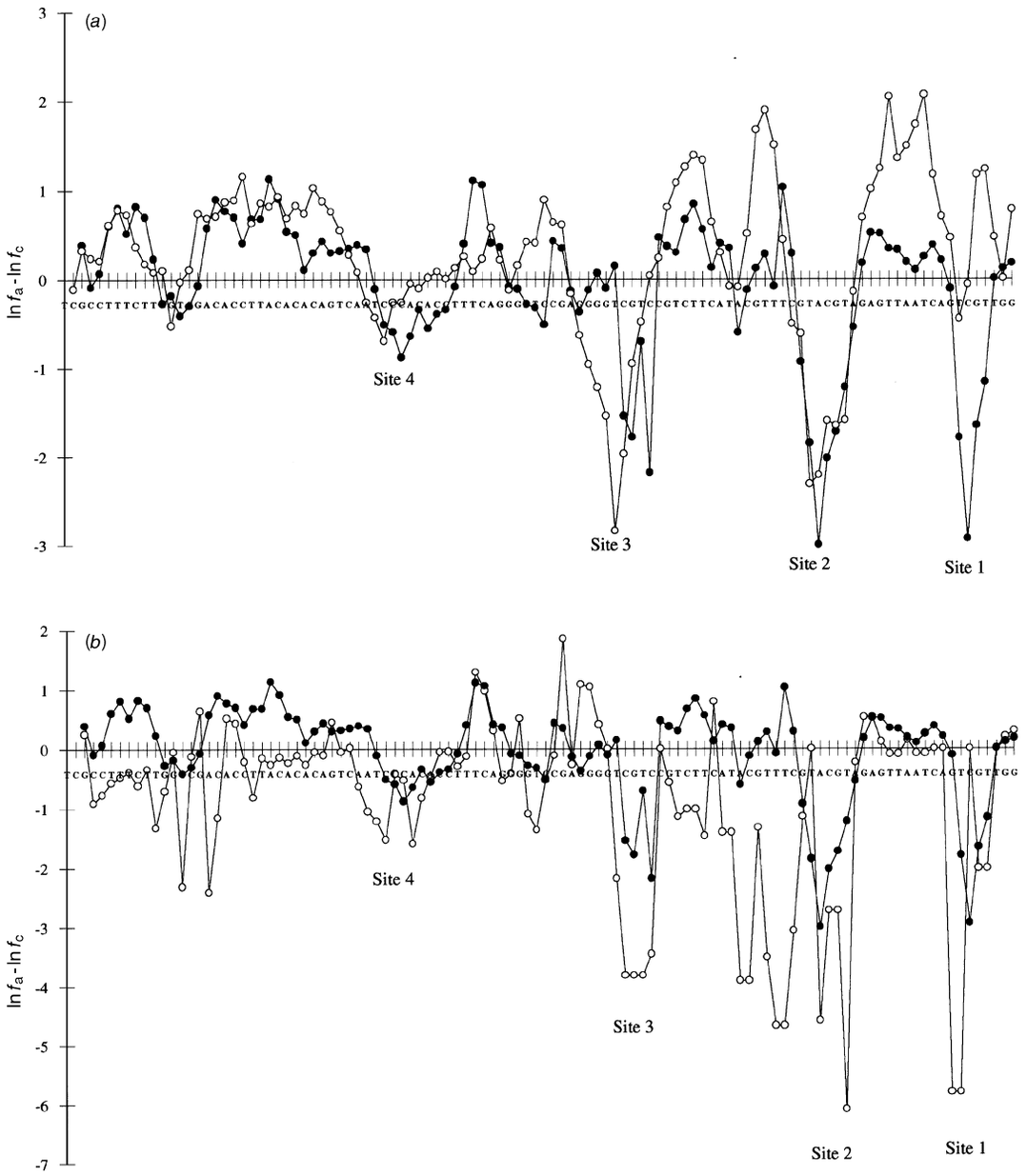
Footprinting studies of actinomycin D using FTP1 **2** as the cleavage agent show protection from cleavage at four sites on the the 3′-labelled strand, a fifth site being revealed on the 5′-labelled strand (Fig. 2). Four of the five sites contain a GpC step while the fifth (labelled site 4) contains two GpG sequences, potential secondary sites for actinomycin D. Sites 1, 2 and 4 clearly display a 3′-overhang which suggests that cleavage is taking place in the minor groove, similar to observations for DNase I, an enzyme known to cut the sugar phosphate backbone from this groove.[10] Site 3 is extended on the 5′-strand, perhaps suggesting some binding to the secondary CpC and GpG sites to the 5′- and 3′-sides respectively of the main site. Site 5, which is not revealed on the 3′-strand, is a poor binding site for actinomycin D. Its sequence 5′-TATGCAAA contains a GC step in the centre of an AT rich region which suggests a narrow minor groove, perhaps too narrow to accommodate the cyclic depsipeptides. It has previously been shown that actinomycin D dissociates rapidly from the sequence $T_9GCA_9$, suggesting that a GC step preceded by thymine and followed by a run of adenines is a poor binding site for the drug.[17] Sites 1, 2 and 5 extend over 4–6 base pairs, which concurs with the number of base pairs physically occluded by actinomycin D as revealed by crystallography.[18]

A number of interesting features are revealed by comparing FTP1-derived actinomycin D footprints with those generated by MPE–$Fe^{II}$ and DNase I (Fig. 2). No inhibition of cleavage is seen at site 1 for MPE–$Fe^{II}$ whereas site 2 is almost identical to that reported by FTP1. Site 5 is again not seen on the 3′-strand while site 3 is longer, including the sequence five base pairs to the 3′-side of the site given by FTP1. Site 4, which includes the two secondary GpG steps is smaller and misses much of the 3′-end given by the new reagent. A number of studies with MPE[11,19] have suggested that the strong affinity of methidium for DNA may lead to displacement of the ligand under investigation from some weaker, secondary sites. It is possible that this is also occurring at sites in this study. Comparing the FTP1 and DNase I footprints reveals that the sequence selectivity of the enzyme leads to profound variations in cutting frequency, making site size difficult to define. However, FTP1 and DNase I clearly display the same five sites, although sites 2, 3 and 5 appear to coalesce into one large footprint on the DNase I-cleaved fragment. Iron–EDTA shows no footprints for actinomycin D.

**Footprinting of distamycin A**

Distamycin A **10** is a member of the family of oligopyrrole peptide antibiotics. It binds to DNA by displacing the spine of hydration from the minor groove of AT-rich sequences where its curved planar structure is isohelical with the duplex. It makes hydrophobic contacts with the walls of the groove and electrostatic, hydrophobic and hydrogen bonding interactions in the base of the groove. Previous studies[20] have shown that the precise ordering of the bases in the site modifies the width of the groove and determines the binding mode for distamycin. Monomeric binding predominates for the narrow sequence AAAAA while the sequence AAATT, with a slightly wider minor groove, accommodates side-by-side dimers. When distamycin binds to the SV40 early promoter fragment, three sites are clearly protected from cleavage by FTP1 **2**, as revealed by scanning laser densitometry (see Fig. 3). Site 1 is a classical distamycin binding site with an AATTA sequence flanked by GC. Sites 2 and 3 show an acceptance of GC base pairs in the body of an AT tract. Again, all three sites have a 3′-overhang, suggesting that cleavage by FTP1 is in the minor groove.

MPE reveals similar distamycin footprints to FTP1, there being a number of qualitative differences. For instance, inhibition of cleavage by MPE at site 1 is weaker, the site is longer and displaced to the 3′-end. Site 2, while covering essentially the same sequence, is also weaker in intensity. By contrast, site 3 is longer, inhibition of cleavage is enhanced and the footprint is displaced to the 5′-end. As reported by DNase I, site 1 becomes more complex and is difficult to define. It is displaced to the 3′-end and an enhancement of cleavage occurs within the site defined by FTP1. Site 2 is comparable with that given by FTP1 but, due to the sequence selectivity of DNase I, no cutting




(*c*)

5' AGGCGGAAA GAACCAGCTG TGGAATGTGT GTCAGTTAGG
3' TCCGCCTTT CTTGGTCGAC ACCTTACACA CAGTCAATCC

5

GTGTGGAAAG TCCCCAGGCT CCCCAGCAGG CAGAAGTATG
CACACCTTTC AGGGGTCCGA GGGGTCGTCC GTCTTCATAC

4 3

CAAAGCATGC ATCTCAATTA GTCAGCAACC AGGATCCCGC
GTTTCGTACG TAGAGTTAAT CAGTCGTTGG TCCTAGGGCG

2 1

CCCTAAC
GGGATTGAGGCGC

**Fig. 2** Comparison of differential cleavage plots for actinomycin D-induced protection from cleavage of 3′ end-labelled SV40 early promoter DNA by FTP1 with those obtained by cleavage with (*a*) MPE, (*b*) DNase I. FTP1 = filled circles, other agents open circles. Sites 1–4 are labelled for clarity. Vertical scales are in units of ln $f_a$ − ln $f_c$, where $f_a$ is the fractional cleavage at any bond in the presence of the drug (0.1 molecule /bp) and $f_c$ is the fractional cleavage of the same bond in the control. Positive values indicate enhancement, while negative values indicate protection. (*c*) Sequence summary showing sites of footprints obtained in the presence of actinomycin D using FTP1 as the cleaving agent.

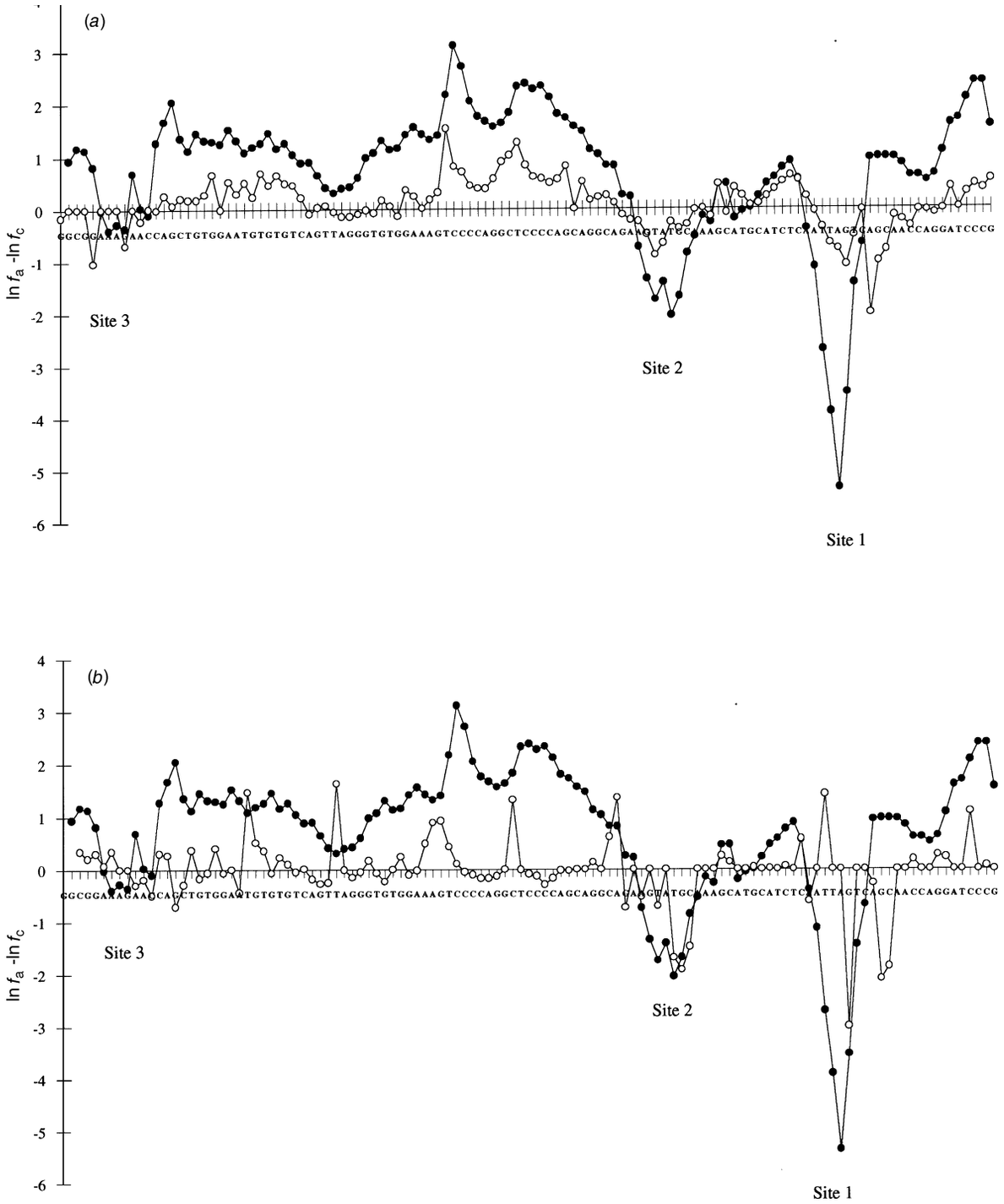
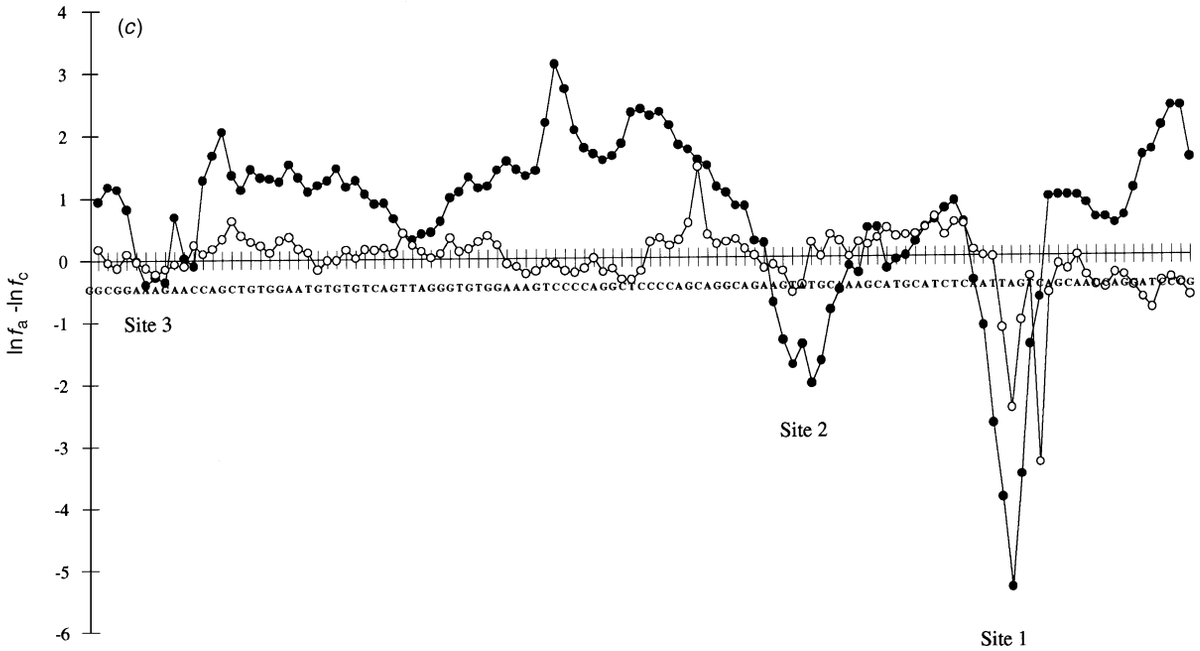



**Fig. 3** Comparison of differential cleavage plots for distamycin-induced protection from cleavage of 3′ end-labelled SV40 early promoter DNA by FTP1 with those obtained by cleavage with (*a*) MPE, (*b*) $Fe^{II}$–EDTA or (*c*) DNase I. FTP1 = filled circles, other agents open circles. Vertical scales are in units of ln $f_a$ − ln $f_c$, where $f_a$ is the fractional cleavage at any bond in the presence of the drug (0.1 molecule/bp) and $f_c$ is the fractional cleavage of the same bond in the control. Positive values indicate enhancement, while negative values indicate protection. Sites 1–3 are shown for clarity. (*d*) Sequence summary showing sites of footprints obtained in the presence of actinomycin D using FTP1 as the cleaving agent.

occurs at certain bases in either control or drug-treated lanes. Site 3 is not discernible with DNase I. Iron(II)–EDTA shows very weak footprints, with only site 1 clearly discernible.

### Cytotoxicity studies

The toxicity of FTP1 **2** was measured in the human colon carcinoma cell line HT-29. The concentration required to inhibit growth by 50% ($IC_{50}$) was found to be 57 μM. In the same cell line, bleomycin was found to have an $IC_{50}$ of 3.1 μM. Although the ability of FTP1 to cleave naked DNA is 10 000-fold lower than that for bleomycin, its cytotoxicity in this cell line is only 20-fold less. If FTP1 kills cells by a free radical mechanism, this indicates that on a mole-for-mole basis FTP1 induces strand breaks 500-fold more cytotoxic than those caused by bleomycin. This may be due to differences in intracellular uptake and/or due to the number and type of strand breaks induced. For instance, differences may derive from the ratio of double to single strand breaks generated by the two compounds or from the sequence selectivity of bleomycin for GpC and GpT sequences compared to the sequence neutrality of FTP1. Analogues of FTP1 which cleave DNA in a sequence-selective fashion are currently under investigation.




(*d*)

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5'  AGGCGGAAA  GAACCAGCTG  TGGAATGTGT GTCAGTTAGG
3'  TCCG CCTTT  CTTGGTCGAC  ACCTTACACA CAGTCAATCC
        3

GTGTGGAAAG TCCCCAGGCT  CCCCAGCAGG CAGAAGTATG
CACACCTTTC AGGGGTCCGA  GGGGTCGTCC GTCTTCATAC
                                        2

CAAAGCATGC ATCTCAATTA  GTCAGCAACC AGGATCCCGC
GTTTCGTACG TAGAGTTAAT  CAGTCGTTGG TCCTAGGGCG
           1

CCCTAAC
GGGATTGAGGCGC
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However, FTP1 also shares many structural characteristics with the antitumour agent amsacrine, which is also a 9-anilinoacridine derivative substituted at the 2′- and 4′-positions. The mechanism of action of amsacrine involves inhibition of the DNA processing enzyme topoisomerase II and its $IC_{50}$ in the Ht-29 cell line was found to be 170 nM. This suggests that if the activity of FTP1 stems from something other than DNA cleavage, that it is a poor inhibitor of topoisomerase II.

**Sequence selectivity and binding orientation of FTP1**

The broad sequence neutrality of intercalation by 9-anilinoacridines was first established by the results of binding studies using natural DNAs of varying sequence composition and synthetic oligonucleotides such as poly(dA–dT) and poly (dG–dC).[21] In recent times, NMR and footprinting studies have failed to give any more detailed information as regards the sequence preference of 9-anilinoacridines for mixed sequence DNA. This is presumably due to the kinetics of binding of the 9-anilinoacridines where the residence time on the duplex has been shown to be of the order of milliseconds.[22] Similarly, the orientation of the 9-substituent when the chromophore is intercalated in the DNA has been a matter of some debate. Models have been suggested for amsacrine, based on computational calculations, in which the anilino-portion resides in the minor groove,[23] whereas a kinetic study suggested that a major groove interaction could be feasible.[22] Once again, NMR studies fail to resolve this problem and no crystal structures have been reported of 9-anilinoacridine–oligonucleotide complexes.

The studies reported here provide some insights into the resolution of these issues. By tethering a chelidamic acid derivative to 9-anilinoacridine, which, as a bleomycin mimic, has the ability to cleave DNA in the presence of iron(II) and a reducing agent, it is possible to ascertain whether the acridine displays any sequence selectivity. By using the compound as a footprinting agent, it is possible to analyse the overhang generated by inhibition of cleavage on the 3′- and 5′-labelled strands and to find the orientation of binding of the cleavage agent. We find that the sequence selectivity of FTP1 **2** appears to be slight, with some preference for an alternating purine–pyrimidine sequence, in particular the sequence GpyATGC. The overhang generated by both actinomycin D and distamycin A in the footprinting experiments is directed towards the 3′-end of the DNA

fragment, suggesting that FTP1 binds to DNA with the 9-substituent located in the minor groove.

Whether these conclusions can be extended to other acridines is not altogether clear. It is probable that the 9-substituent in FTP1 is orientated orthogonally to the chromophore, as previously described for amsacrine,[24] and as such it is likely that both compounds can bind in a similar groove orientation. The 9-substituent of FTP1 contains a number of potential hydrogen bond donors and acceptors which could endow a sequence recognition different to that for other 9-anilinoacridines. However, compound **8** does not appear to generate the same cleavage hotspots as FTP1, supporting the inference that it is the acridine and not the 9-substituent which endows the sequence selectivity on DNA.

## Experimental

### Synthesis of FTP1

**Materials.** With the exception of chelidamic acid (Fluka Chemical Ltd., Dorset, UK), all other chemicals were from Aldrich (Dorset, UK). Anhydrous solvents were from Aldrich and were used without further purification. Chromatography was carried out using Silica gel 60 TLC plates (Merck 5554 and 13792) and silica gel 60 for flash chromatography (Merck particle size 0.040–0.063 mm). Methyl 6-hydroxymethyl-4-methoxypyridine-2-carboxylate **3** was made *via* esterification of chelidamic acid with acidic methanol,[4] alkylation of the 4-hydroxy group with methyl iodide and reduction with sodium borohydride.[2]

**Methods.** TLC plates were visualised by UV (254 nm) and were also developed using cerium(IV) sulfate (5% mass/vol in 4 M sulfuric acid) followed by heating. Flash column chromatography was carried out by the method of Still *et al.*[13] NMR spectra were recorded using a JEOL 270 MHz spectrometer and were referenced to $SiMe_4$ or to residual $Me_2SO$. $\delta$ values are given in ppm; $J$ values are given in Hz. Microanalyses were provided by the Chemical Services Unit of University College Dublin. Fast Atom Bombardment Mass Spectra using 3-nitrobenzyl alcohol as a matrix were obtained from the Mass Spectroscopy Service of University College London, UK.

**Methyl 6-[*N*-(3-aminophenyl)aminomethyl]-4-methoxypyridine-2-carboxylate 4.** Methyl 6-hydroxymethyl-4-methoxypyridine-2-carboxylate **3** (475 mg, 2.41 mmol) was stirred in dry dichloromethane (5 ml). Methanesulfonyl chloride (205 ml, 2.65 mmol), triethylamine (369 ml, 2.65 mmol) and 4-dimethylaminopyridine (32 mg, 0.27 mmol) were added and the solution stirred for 30 min. 1,3-Phenylenediamine (783 mg, 7.32 mmol) was dissolved in anhydrous dichloromethane (5 ml) and added to the above solution in one portion. The resulting solution was stirred at room temp. for 16 h. Water (10 ml) and dichloromethane (10 ml) were then added. The layers were separated and the organic layer washed with water (10 ml), then extracted with hydrochloric acid (1 M, 2 × 10 ml). The pH was adjusted to 4–5 (1 M $NaHCO_3$) and the resulting solution extracted with chloroform (2 × 20 ml). The organic layers were dried ($MgSO_4$) and volatiles removed under reduced pressure to give a brown oil, pure by TLC ($R_f$ 0.30, 10% MeOH in $CHCl_3$), 495 mg (72%), which was used without further purification.

$\delta_H(CDCl_3)$ 7.54 (d, 1 H, Py*H*, *J* 2.25), 7.08 (d, 1 H, Py*H*, *J* 2.54), 6.93 (t, 1 H, Ar*H*, *J* 8.16, 7.87), 6.07 (dd, 2 H, Ar*H*, *J* 1.97, 7.88, 1.97), 4.80 (s, 2 H, $CH_2$), 4.00 (s, 3 H, $OCH_3$) and 3.84 (s, 3 H, $CO_2CH_3$).

**Methyl 6-{*N*-[3-(acridin-9-yl)aminophenyl]aminomethyl}-4-methoxypyridine-2-carboxylate (5).** 9-Chloroacridine (95 mg, 0.44 mmol) was dissolved in dry methanol (2 ml) and the above product (**4**) (127 mg, 0.44 mmol) was added. The mixture was stirred at room temp. for 16 h, volatiles removed under reduced pressure and the residue dissolved in water (1 ml). The solution was basified (2 M NaOH) and extracted with chloroform (4 × 10 ml). The organic layers were dried ($MgSO_4$), and evaporated to give an orange oily solid. This was purified by flash column chromatography (gradient elution : chloroform–5% methanol in chloroform) to give pure product as an orange solid ($R_f$ 0.20, 10% methanol in chloroform), 172 mg (84%).

$\delta_H(CDCl_3)$ 8.02 (d, 2 H, *J* 9), 7.98 (d, 2 H, *J* 11), 7.57 (t, 2 H, *J* 7.63, 8.16), 7.52 (d, 1 H, Py*H*, *J* 2.54), 7.20 (t, 2 H, *J* 7.03, 8.16), 7.06 (t, 1 H, *J* 8.15, 7.88), 7.01 (d, 1 H, Py*H* *J* 2.25), 6.36 (t, 2 H, Ar*H*, *J* 6.75, 7.87), 6.28 (s, 1 H, Ar*H*), 4.40 (s, 2 H, $CH_2$), 3.98 (2, 3 H, $OCH_3$) and 3.86 (s, 3 H, $CO_2CH_3$).

**6-{*N*-[3-(acridin-9-yl)aminophenyl]aminomethyl}-4-methoxypyridine-2-carboxylic acid (2) (FTP1).** The methyl ester **5** (406 mg, 1.58 mmol) was dissolved in hydrochloric acid (1 M, 20 ml) and heated under reflux for 3 h. At the end of this period the solvent was removed under reduced pressure and the residue azeotroped with toluene (2 × 25 ml). The residue was then dissolved in ethanol (10 ml) and precipitated with excess ethyl acetate. The resulting solid was filtered and immediately dried over $P_2O_5$ *in vacuo*. The yield was 283 mg (74%).

Microanalysis. $C_{27}H_{22}N_4O_3{\cdot}3HCl{\cdot}4H_2O$ requires C 51.31 H 5.23 N 8.87 Cl 16.86, found C 51.56 H 5.03 N 8.72 Cl 17.31.

$\delta_H[(CD_3)_2SO]$ 11.64 (s, 1 H, O*H*), 8.26 (d, 2 H, *J* 8.72), 8.17 (d, 2 H, *J* 8.72), 7.93 (t, 2 H, *J* 7.60, 7.87), 7.46 (d, 1 H, Py*H*, *J* 2.25), 7.32 (t, 2 H, *J* 7.32, 8.15), 7.21 [m (d overlapping t), 1 H, *J* 8.15, 7.88], 6.73 (d, 1 H, *J* 9), 6.66 (s, 1 H), 6.62 (d, 1 H, Ar*H*, *J* 9), 4.40 (s, 2 H, $CH_2$) and 3.89 (s, 3 H, $OCH_3$). Mass Spectrum ($FAB^+$). 451 (M + 1), 407, 329, 274, 176, 136, 107, 89, 77, 63, 51 and 39.

**6-{*N*-[3-(*N*,*N*-dimethylaminoacetyl)aminophenyl]aminomethyl}-4-methoxypyridine-2-carboxylic acid (8).** The aniline derivative (**4**) (90 mg, 0.31 mmol) was stirred in dry dichloromethane (1 ml). Chloroacetic anhydride (90%, 60 mg, 0.31 mmol) was added and the solution stirred for 3.5 h. The solvent was removed under reduced pressure, the residue redissolved in chloroform (10 ml) and washed with water (2 × 5 ml), sodium hydrogen carbonate (1 M, 2 × 5 ml) and water (2 × 5 ml). The organic solution was dried ($MgSO_4$) and evaporated to give the chloroacetyl derivative **6** as a green oil, 91 mg (81%) which was unstable over long periods at room temp.

$\delta_H(CDCl_3)$ 8.16 (s, 1 H, N*H*), 7.57 (d, 1 H, Py*H*, *J* 2.53), 7.06 (m, 3 H, Ar*H*), 6.78 (d, 1 H, Ar*H*, *J* 7.87), 6.43 (d, 1 H, Ar*H*, *J* 7.59), 4.51 (s, 2 H, $CH_2$), 4.15 (s, 2 H, $CH_2$), 4.01 (s, 3 H, $CH_3$) and 3.87 (s, 3 H, $CH_3$).

This compound **6** (43 mg, 0.12 mmol) was dissolved in acetonitrile (1 ml) and dimethylamine (33% in ethanol, 19 ml, 0.14 mmol) was added. The solution was stirred for 16 h, after which time the reaction was still incomplete (TLC, 10% methanol in chloroform, $R_f$ starting material 0.50, $R_f$ product 0.20). 1 equiv. of dimethylamine (33% in ethanol, 19 ml, 0.14 mmol) was added, the mixture stirred for 3 h, 1 equiv. of dimethylamine (33% in ethanol, 19 ml, 0.14 mmol) added and the mixture again stirred for 3 h. At the end of this period, the solvents were removed *in vacuo* and water (2 ml) and chloroform (1 ml) added. The organic fraction was separated, and the water layer extracted with chloroform (3 × 2 ml). The organic layers were combined, dried ($MgSO_4$) and evaporated. The resulting oil was purified by preparative TLC (Silica gel, eluting solvent 10% methanol in chloroform, $R_f$ 0.20) to give the methyl ester **7** as an oil, 37 mg (84%). This compound was immediately dissolved in methanol (1 ml) and sodium hydroxide (4 mg, 0.1 mmol) in water (0.5 ml) was added. After 3 h at room temp., the solvents were removed under reduced pressure and the resulting oil azeotroped with toluene (1 × 5 ml). It was redissolved in dry methanol (1 ml) which was then saturated with dry hydrogen chloride. Addition of ethyl acetate precipitated a product **8** (24 mg, 69%) which, although correct by NMR analysis, contained 1.5 equiv. of sodium chloride by microanalysis. It was used without further purification for the DNA cleavage experiments.

$\delta_H[(CD_3)_2SO]$ 10.91 (s, 1 H, O*H*), 7.57 (d, 1 H, Py*H*, *J* 2.53), 7.35 (s, 1 H, Py*H*), 7.10 (m, 2 H, Ar*H*), 7.01 (d, 1 H, Ar*H*, *J* 8),

**Table 1**

| | | | | |
|---|---|---|---|---|
| | 10 | 20 | 30 | 40 |
| 5'- | GGTACCTTCT | GAGGCGGAAA | GAACCAGCTG | TGGAATGTGT |
| 3'- | CCATGGAAGA | CTCCGCCTTT | CTTGGTCGAC | ACCTTACACA |
| | 50 | 60 | 70 | 80 |
| | GTCAGTTAGG | GTGTGGAAAG | TCCCCAGGCT | CCCCAGCAGG |
| | CAGTCAATCC | CACACCTTTC | AGGGGTCCGA | GGGGTCGTCC |
| | 90 | 100 | 110 | 120 |
| | CAGAAGTATG | CAAAGCATGC | ATCTCAATTA | GTCAGCAACC |
| | GTCTTCATAC | GTTTCGTACG | TAGAGTTAAT | CAGTCGTTGG |
| | 130 | 140 | 150 | 160 |
| | AGGATCCCGC | CCCTAACTCC | GCCCATCCCG | CCCCTAACTC |
| | TCCTAGGGCG | GGGATTGAGG | CGGGTAGGGC | GGGGATTGAG |
| | 170 | 180 | 190 | 200 |
| | CGCCCAGTTC | CGCCCATTCT | CCGCCCCATG | GTCGACTAAT |
| | GCGGGTCAAG | GCGGGTAAGA | GGCGGGGTAC | CAGCTGATTA |
| | 210 | 220 | 230 | 240 |
| | TTTTTTTATT | TATGCAGAGG | CCGAGGCCGC | CTCGGCCTCT |
| | AAAAAATAA | ATACGTCTCC | GGCTCCGGCG | GAGCCGGAGA |
| | 250 | 260 | 270 | 280 |
| | GAGCTATTCC | AGAAGTAGTG | AAGAGGCTTT | TTTGGAGGCC |
| | CTCGATAAGG | TCTTCATCAC | TTCTCCGAAA | AAACCTCCGG |
| | 290 | 300 | | |
| | TAGGCTTTTG | CAAAAAGCTT | | |
| | ATCCGAAAAC | GTTTTTCGAA | | |

6.48 (d, 1 H, Ar*H*, *J* 8.16), 4.49 (s, 2 H, C$H_2$), 4.13 (d, 2 H, C$H_2$, *J* 4), 3.93 (s, 3 H, C$H_3$) and 2.84 [d, 6 H, N(C$H_3$)$_2$, *J* 3.94].

**DNA cleavage reactions.** The plasmid pSP72 containing the SV40 early promoter was linearised with Eco R1. The SV40 early promoter sequence is given in Table 1.

To label the DNA at the 5′-end, the plasmid was dephosphorylated with calf intestinal phosphatase, and subsequently labelled with [$\gamma^{32}$P]ATP and T4 polynucleotide kinase. Alternatively the linearised plasmid was 3′-end labelled with [$\alpha^{32}$P]dATP and Klenow fragment. The labelled DNA was then cut with Hind III and the 300 bp SV40 early promoter fragment purified on a 4% agarose gel.

The cleavage reactions were carried out with 30 000 cpm of labelled SV40 early promoter DNA (approx 3 nM bp) plus 1 μg μl$^{-1}$ sonicated calf thymus DNA in 20 mM Tris-HCl (pH 7.6). In a final volume of 10 μl, the DNA was preincubated in the presence of Tris-HCl (pH 7.6) containing 50 mM NaCl with increasing concentrations of FTP1, before addition of an equimolar concentration of [Fe(NH$_4$)$_2$(SO$_4$)$_2$] followed by addition of 1 μl DTT (50 mM). The reactions were incubated for 15 min, stopped with 5 μl thiourea (100 mM), the DNA precipitated in absolute ethanol, and washed twice in 70% ethanol. The dried DNA was loaded on 8% polyacrylamide sequencing gels and cleavage products separated at 55 °C at constant power of 60 W. The gels were fixed in 5% acetic acid, 5% methanol, dried and exposed to autoradiographic film.

### Footprinting experiments

**Footprinting with FTP1.** Distamycin A (**9**) or actinomycin D (**10**) was incubated with DNA (30 000 cpm SV40 early promoter fragment plus 1 μg μl$^{-1}$ calf thymus DNA, in 20 mM Tris HCl, pH 7.6, 50 mM NaCl) for 30 min at room temp., before addition of 1μl FTP1 (200 μM), 1 μl Fe$^{II}$ (200 μM) and 1μl DTT (50 mM) in the order stated. After a further 15 min, the reactions were stopped with thiourea and the DNA processed as outlined above.

**Footprinting with MPE, Fe$^{II}$–EDTA and DNase I.** To footprint ligand binding sites with MPE, Fe$^{II}$–EDTA or DNase I, the labelled DNA was incubated as outlined above with either of the two ligands for 30 min. Then the cleaving agents were added under conditions which resulted in 10–20% cleavage of the DNA. For MPE footprinting, a 1:1 mix of MPE and [Fe(NH$_4$)$_2$(SO$_4$)$_2$] was added at a final concentration of 2 μM followed by 1 μl DTT (50 mM), reactions proceeded for 10 min. For generating Fe$^{II}$–EDTA footprints, a mixture of reagents was prepared by adding the following in the order listed: [Fe(NH$_4$)$_2$(SO$_4$)$_2$] (200 μM) EDTA (4 μM), ascorbic acid (10 mM) and 0.3% $H_2O_2$ in the ratio of (1:1:2:2). An aliquot of 1 μl of this mix was added to each reaction and the cleavage allowed to proceed for 3 min. DNase I footprinting was carried out by incubating DNA–ligand complexes with DNase I (1/2000 dilution of 1 μg ml$^{-1}$ stock) for 1 min on ice. Reactions were stopped by addition of thiourea (100 mM) in the case of MPE and Fe$^{II}$–EDTA, or addition of DNase I stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS, 100 μg ml$^{-1}$ yeast tRNA) and the DNA processed as outlined above.

**Densitometry.** Densitometric analysis of autoradiographs was carried out using a Molecular Dynamics scanning laser densitometer and the scanned images subsequently analysed using the PHORETIX 1D software package (Non-linear Dynamics). The lanes analysed were corrected for the counts loaded and for the extent of strand breaks in the untreated DNA control.

**Growth inhibition assays.** Human colon carcinoma HT29/219 cells were obtained from the American Type Cell Culture Collection and grown at 37 °C in the presence of 5% $CO_2$ in minimum essential medium with Earle's salts (EMEM) supplemented with 10% heat inactivated foetal calf serum, 2 mM glutamine, 0.1 mM (MEM) non-essential amino acids, 10 mM HEPES, pH 7.3, 50 U ml$^{-1}$ penicillin and 50 mg ml$^{-1}$ streptomycin. Cells were routinely seeded at a density of $2 \times 10^4$ cells ml$^{-1}$ in 6 well plates, 48 h prior to drug addition.

All drug stock solutions, with the exception of bleomycin, were in $Me_2SO$ at a concentration of 10 μM and stored at −20 °C until use. Subsequent dilutions were made at the time of drug treatment using sterile distilled $H_2O$. Bleomycin was dissolved in sterile distilled $H_2O$ at a concentration of 10 μM and stored at −20 °C until use.

For each drug treatment the test compound was added to the cells in concentrations of 0.01, 0.1, 1.0 and 10 μM. Cells were exposed continuously to drug for 72 h after which time the cells were trypsinized and counted using a Coulter Counter (ZM model). $IC_{50}$ values were determined by calculating the drug concentration required to reduce cell growth to 50% of the control cell values.

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*Paper* 6/06218K
*Received* 9*th September* 1996
*Accepted* 6*th November* 1996