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Original article

Synthesis and DNA interaction of a mixed proflavine–phenanthroline Tröger base

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Abstract

We report the synthesis of an asymmetric Tröger base containing the two well characterised DNA binding chromophores, proflavine and phenanthroline. The mode of interaction of the hybrid molecule was investigated by circular and linear dichroism experiments and a biochemical assay using DNA topoisomerase I. The data are compatible with a model in which the proflavine moiety intercalates between DNA base pairs and the phenanthroline ring occupies the DNA groove. DNase I cleavage experiments were carried out to investigate the sequence preference of the hybrid ligand and a well resolved footprint was detected at a site encompassing two adjacent 5′-GTC·5-GAC triplets. The sequence preference of the asymmetric molecule is compared to that of the symmetric analogues.

Keywords: DNA intercalation; sequence recognition; proflavine; phenanthroline; topoisomerase I

1. Introduction

In 1887, J. Tröger synthesised for the first time a methanodibenzo-[1,5]diazocin structure (compound **1** in Fig. 1) from the aromatic electrophilic substitution of *para*-anisidine with formaldehyde [1]. The chiral and rigid structure of this compound, nowadays known as a Tröger base, was elucidated some 50 years later and it is only recently that such molecules were exploited as tools for molecular recognition of specific shapes or conformation of substrates [2,3]. Recently designed Tröger base derivatives include polysubstituted analogues, macrocycles (Trögerophanes) [4,5], crown-ether derivatives [6], heterocyclic analogues [7–10], metal complexes [11] and supramolecular structures [12–14] which can be used as receptors for alicyclic substrates [4,15], aromatic amides and cyclic amides [16–19] as well as chiral solvating agents [20].

Within the past 10 years, a few Tröger bases were developed as chiral probes of nucleic acid structures. We have shown previously that there is a preferential binding of the (−)-isomer of the Tröger base **2** to B-DNA (Fig. 1) [21]. Furthermore we recently demonstrated that the (−)-7*R*,17*R*-isomer of **2** is a sequence-selective agent whereas the (+)-7*S*,17*S*-isomer shows no sequence selectivity [22]. The discovery that proflavine-type Tröger bases can be used for enantiospecific recognition of DNA sequences containing both A·T and G·C base pairs prompted us to extend the strategy via the design of novel Tröger base derivatives.

It has been reported that racemic Tröger bases containing phenanthroline units, such as compound **3** (Fig. 1), can bind to nucleic acids and cleave DNA in the presence of Cu^{2+} ions [23]. But, the mechanism of binding of both acridine- and proflavine-type Tröger bases to DNA remains unknown. The main difficulty to elucidate the DNA interaction process arises from the symmetric structure of the molecules which greatly complicates the analysis of DNA binding data. To

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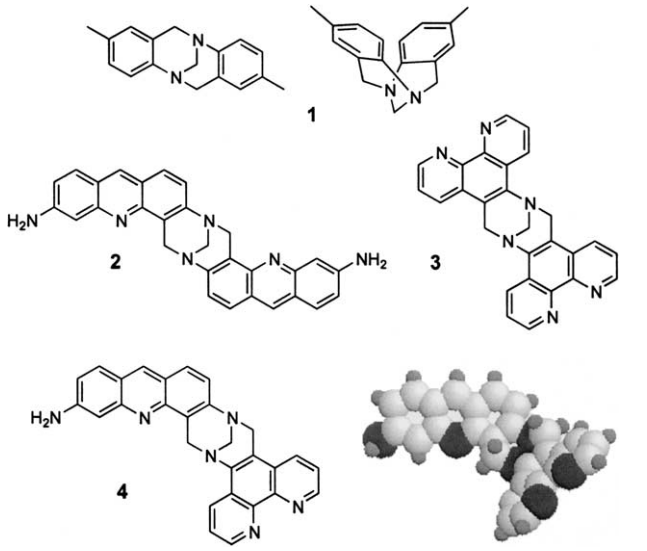

Fig. 1. Structure of the Tröger bases **1**–**4** and synthetic scheme for compound **4**. An energy minimised structure of **4**, constructed with the softwares HyperChem™ 5.01 and Alchemy 2000®, is presented.

solve this problem and to provide further insight into the binding mode of Tröger bases to DNA, we have synthesised an asymmetric acridine–phenanthroline Tröger base and studied its interaction with DNA using complementary biochemical and biophysical methods. The data reported here indicate that the hybrid molecule **4** (Fig. 1) interacts with DNA via intercalation of its aminoacridine ring.

2. Chemistry

The acridine–phenanthroline Tröger's base analogue **4** was prepared in two steps (Fig. 2). The mono-protected proflavine **5** was reacted with 5-amino-[1,10]phenanthroline **6** in the presence of paraformaldehyde in TFA. In the presence of a slight excess of **6** (2 equiv.), the reaction mainly gives a mixture of symmetrical phenanthroline Tröger's base **3** and the protected form **8** of the desired compound **4**, the acridine Tröger's base **7** being formed as traces as shown by HPLC. Separation on column chromatography affords **8** in 24% yield. Deprotection of the amino group was achieved in basic conditions (NaOH–EtOH–THF) and **4** was obtained in 85% yield.

3. Biophysical and biochemical results

3.1. Absorption measurements

Fig. 3a shows the absorption spectrum of compound **4** at 10 μM in the absence and presence of increasing concentrations of calf thymus DNA. Upon titration, a slight hypochromism is observed in the 290 nm band whereas a significant hyperchromism occurs at 460 nm. This absorption band corresponds to the acridine moiety without contribution from the phenanthroline part which absorbs around 350–390 nm. The interaction with the double helix causes a bathochromic shift of 7 nm, owing to the perturbation of the acridine chromophore upon binding to DNA. The presence of a relatively well defined cross-over point in these spectra at 410 nm suggests that a single mode of binding occurs. Melting temperature experiments provided further evidence that the test molecule binds strongly to DNA. Compound **4** significantly stabilises synthetic polymer poly(dAT)$_2$ against thermal denaturation. A monophasic melting curve with a ΔT_m value ($T_m^{complex} - T_m^{DNA}$) of 3 °C was observed at a drug/DNA ratio of 0.2, but for higher ratio, the melting curves become clearly biphasic and two ΔT_m values of 5.9 and 21.3 °C were measured for a drug/DNA ratio of 0.5 (Fig. 3b). Similar increases in ΔT_m were observed with the

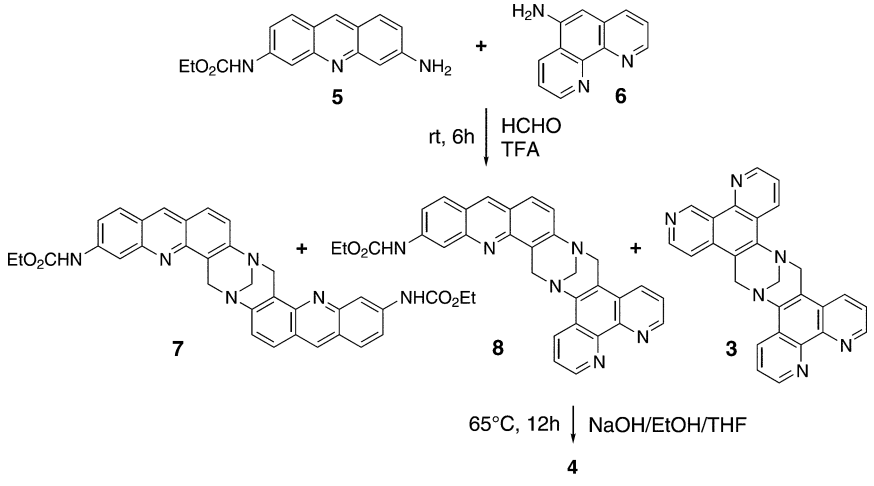

Fig. 2. Synthesis of the Tröger base **4**.

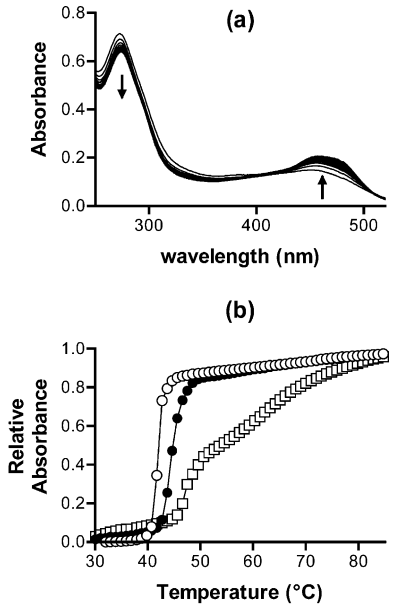

Fig. 3. (a) DNA titrations of compound **4**. The figure contains the absorption spectrum of the free drug (10 μM), intermediate spectra, and final spectra of the drug–DNA complexes, in which the ligand has been sequestered completely by the DNA. To 1 ml of drug solution (10 μM) were added aliquots of a concentrated calf thymus DNA solution, in 1 mM Na cacodylate buffer, pH 7.0. Spectra are referenced against DNA solutions of exactly the same DNA concentration and were adjusted to a common baseline. The phosphate–DNA/drug ratio increased from 0 to 20 (top to bottom curves, at 290 nm). (b) Thermal denaturation curves for poly(dA-dT)$_2$ in the absence (○) and presence of **4** at a drug/DNA ratio of (●) 0.2 and (□) 0.5. T_m measurements were performed in BPE buffer pH 7.1 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA) using 20 μM poly(dA-dT)$_2$ in 1 mL quartz cuvettes at 260 nm with a heating rate of 1 °C min^{-1}. Each drug concentration was tested in triplicate. The T_m values were obtained from first-derivative plots.

polymer poly(dIC)$_2$ and to a lower extent with calf thymus DNA (data not shown). It was not possible to study the effects on poly(dGC)$_2$ due to its high stability even at low ionic strength. Altogether, these absorption measurements indicate clearly that the molecule **4** has significant interaction with DNA.

3.2. Circular and electric linear dichroism measurements

CD and ELD experiments were performed to define the orientation of the compounds with respect to the DNA helix. The compound has no circular dichroism (CD) spectrum when free in solution but has induced CD spectrum in its DNA complex. The CD spectrum of a 10 μM solution of **4** bound to 200 μM calf thymus DNA (Fig. 4a) shows a positive band in the 350–420 nm region and a negative band in the 420–500 nm region which correspond to the phenanthroline and the acridine bands, respectively. A weakly negative CD signal is commonly observed with intercalating agents including ellipticines and acridines whereas a positive CD signal is usually observed with minor groove binders such as netropsin and distamycin. The CD spectrum of the **4**-DNA complex is consistent with a dual binding mode where the acridine ring would be intercalated and the phenanthroline ring would reside in one of the two DNA grooves. Similar CD spectra were previously obtained with hybrid molecules named combilexin (e.g. netropsin–acridine and distamycin–ellipticine), composed of an intercalator and a well-defined minor groove binder [24–26].

The ELD measurements provided additional evidence for the intercalation of the acridine moiety of **4** in DNA. As shown in Fig. 4b, the reduced dichroism ΔA/A is largely negative in the acridine absorption band around 460 nm whereas it is close to zero in the phenanthroline band around 350 nm. The ΔA/A value at 460 nm is almost identical to that measured for DNA alone at 260 nm (-0.317). Moreover, the measurements of ΔA/A at several field strengths showed that the reduced dichroism depends similarly upon the field strength for the **4**-DNA complex at 460 nm and the DNA bases at 260 nm in the absence of ligand (data not shown). The ELD data indicate that the acridine moiety of **4** is oriented parallel to the DNA base pairs, as expected for an intercalative binding. Therefore, the two sets of spectroscopic measurements utilising polarised light, CD and ELD, are mutually consistent and indicate that the drug behaves as a typical intercalating agent.

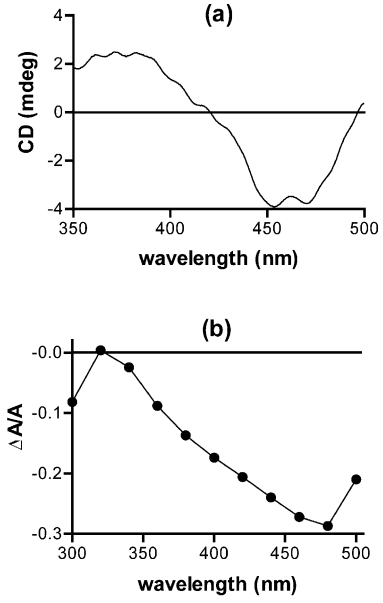

Fig. 4. (a) CD spectrum of the **4**-DNA complex at a DNA/drug ratio of 20 [10 μM drug–200 μM DNA]. The experiments were performed in a 2 cm cell with a 3 mL solution in a 1 mM cacodylate buffer pH 7.0 at room temperature. (b) Electric linear dichroism spectrum of the **4**-DNA complex. Conditions: 13.6 kV cm^{-1}, P/D = 20 (200 μM DNA, 10 μM drug), in 1 mM sodium cacodylate buffer, pH 7.0, at room temperature.

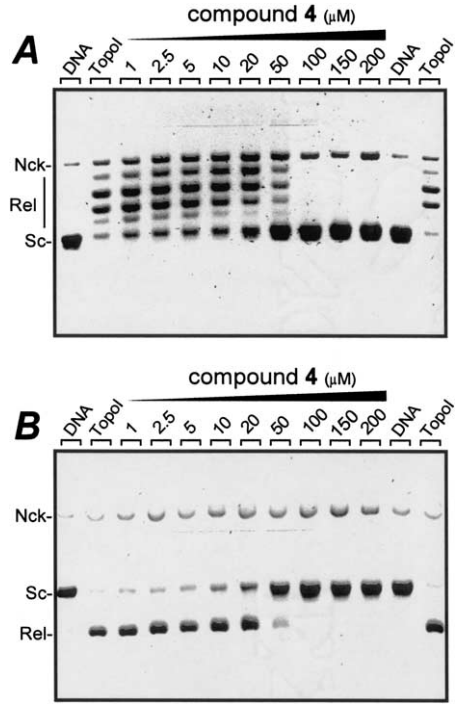

Fig. 5. Effect of increasing concentrations of **4** on the relaxation of plasmid DNA by human topoisomerase I in the absence (A) or presence (B) of ethidium bromide. Native supercoiled pKMp27 DNA (0.5 μg) (lane DNA) was incubated with 4 units topoisomerase in the absence (lane Topo) or presence of the drug at the indicated concentration (μM). Reactions were stopped with sodium dodecylsulphate and treatment with proteinase K. DNA samples were separated by electrophoresis on a 1% agarose gel. In panel A, after the electrophoresis the gel was stained with ethidium bromide (1 μg/ml), washed with water and then photographed under UV light. Nck, nicked; Lin, linear; Rel, relaxed; Sc, supercoiled.

3.3. Topoisomerase I inhibition

A DNA relaxation assay was used to investigate the effects of the test drug on the catalytic activity of human topoisomerase I. Closed circular DNA was treated with the enzyme in the presence of increasing concentrations of **4** and the DNA relaxation and cleavage products were analysed by agarose gel electrophoresis. Fig. 5 shows that at a high drug concentration the compound totally inhibits the relaxation of supercoiled plasmid DNA by topoisomerase I. The inhibition can be associated with the intercalative binding of the drug into DNA. But in addition to the inhibition of DNA relaxation, we observed that the drug increases the production of nicked DNA molecules by topoisomerase I. This is also observed in the experiments performed with ethidium bromide in the gels during the electrophoresis (Fig. 5B). Under these experimental conditions, it can be seen more clearly that the band intensity of nicked DNA is enhanced in the presence of the Tröger base, suggesting that the drug can act as a poison for the enzyme, either by stimulating the cleavage step or by inhibiting the religation step in the catalytic cycle of the enzyme. It is conceivable that in the presence of **4**, the enzyme may stay longer on DNA, thereby producing more single strand breaks. On this gel, the inhibition of DNA relaxation detected for drug concentrations ≥ 10 μM is most likely a consequence of the intercalative binding of the drug into DNA. It should be mentioned that parallel experiments were performed with human topoisomerase II (p170 isoform from TopoGen) but only a non-specific inhibition was detected without formation of linear DNA molecules (data not shown).

3.4. Sequence selectivity

The footprinting study was performed with bovine pancreatic DNase I which is a sensitive endonuclease for mapping DNA-binding sites of small molecules. A ^{32}P-radiolabeled DNA restriction fragment of 117 base pairs was incubated with **4** at different concentrations and after equilibrium; the complexes were subjected to limited cleavage by DNase I. The autoradiograph and the corresponding differential cleavage plot presented in Fig. 6 reveal that the drug exhibits a moderate level of sequence selectivity. From the densitometric analysis of the 5 μM lane, we concluded that the ligand protects the sequence 5′-GTCACGACG from cutting by the enzyme (Fig. 6). Interestingly, this binding sequence is composed of two (underlined) adjacent and antiparallel 5′-GTC·5′-GAC triplets which may be viewed as a preferential target sequence for the asymmetric Tröger's base. The sequences 5′-GGGTTTT and 5′-AAAACGAC located on the 3′ and 5′ side of the GTC-containing binding site are rendered much more susceptible to attack by DNase I than in the control. These enhancements of DNase I cleavage at flanking the binding region may be attributed to intercalation-induced local structural changes which facilitate cutting by the enzyme at these sequences. At low concentrations (< 5 μM), compound **4** binds preferentially to the sequence around nucleotide positions 50–58 but at a higher concentration (> 10 μM) the cleavage by the enzyme is totally inhibited, suggesting that at high drug/DNA ratios the drugs bind non-specifically to all type of DNA sequences. Such a non-specific effect is commonly observed with DNA intercalators.

4. Discussion

The interaction of different types of Tröger bases has been reported in several studies but thus far their mode of binding to DNA remains unknown essentially because their symmetric structures complicate the analysis of the DNA interaction process. To circumvent this problem, we undertook the synthesis of an asymmetric

acridine–phenanthroline Tröger's base and the use of spectroscopic methods enabled us to estimate the orientation of the acridine ring with respect to the phenanthroline ring in the DNA complex. CD and ELD measurements provide information that suggests that the acridine ring of compound **4** inserts between DNA base pairs. The biochemical results are also consistent with an intercalation process. Considering that the acridine is intercalated, on the one hand, and that the two aromatic moieties of the hybrid molecules are oriented at a right angle to delimit a V-structure (Fig. 1), on the other hand, it seems logical to believe that the phenanthroline ring resides in one groove of DNA. We therefore propose a model in which the asymmetric Tröger base **4** would bind to DNA via a bimodal process implicating intercalation of the acridine moiety coupled with a groove binding of the phenanthroline part.

The sequence recognition aspect is also quite interesting. We have previously shown that the symmetric proflavine-type Tröger base **2** recognises preferentially certain DNA sequences containing both A·T and G·C base pairs, such as the motifs 5′-GTT·AAC and 5′-ATGA·TCAT [22]. The central sequence of the 117-mer fragment used for the footprinting experiments provides different binding sites for the three Tröger bases. As illustrated in Fig. 6C, we found that **2** binds preferentially to the 3′ side of the sequence to recognise GACGTTGT whereas **3** prefers the 5′ side to interact with AGTCA. The footprint detected with the hybrid ligand **4** is closer to that of the phenanthroline analogue **3** than to the acridine parent **2**. Triplets 5′-GTC·5′-GAC may provide optimal binding site for compound **4**. We suspect that it is the fraction of the molecule occupying the DNA groove, i.e. the phenanthroline moiety, that is responsible for the footprint. High resolution studies, NMR or X-ray crystallography, are required to define the exact configuration of the complexes between the Tröger base **4** and DNA but the present data provide the first set of evidences suggesting

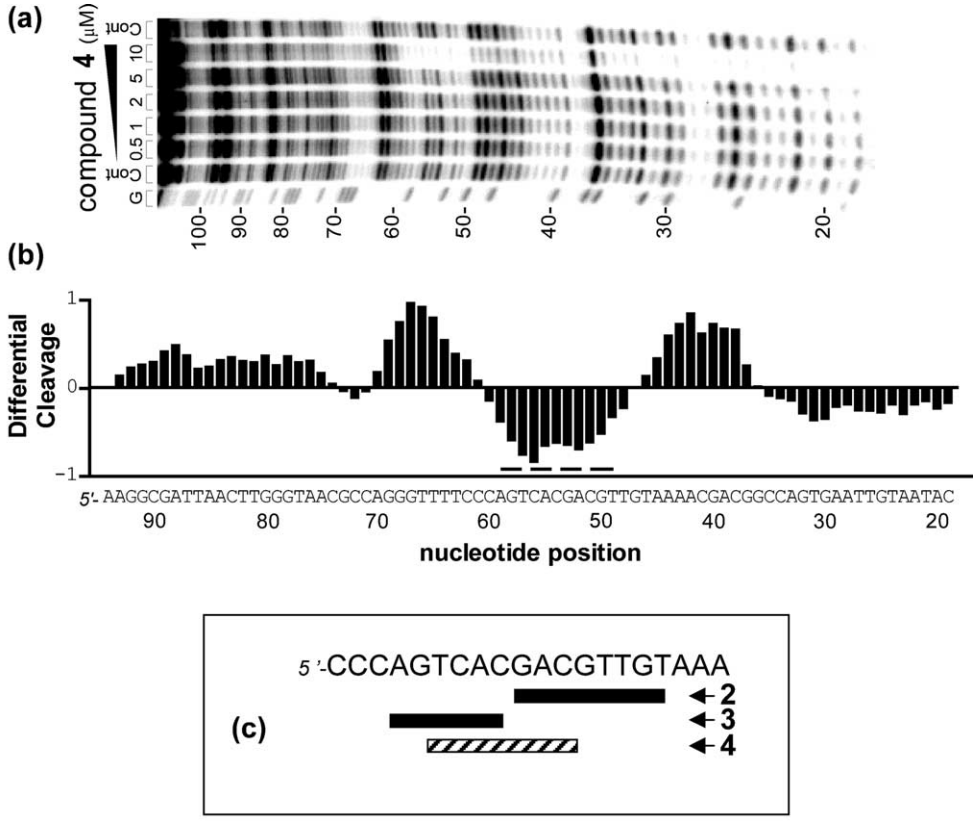

Fig. 6. (a) DNase I footprinting with the 117-mer *Pvu*II-*Eco*RI restriction fragment cut from the plasmid pBS in the presence of **4** at concentrations increasing from 0.5 to 10 μM. The DNA was 3′-end labelled at the *Eco*RI site with [α-^{32}P]dATP in the presence of AMV reverse transcriptase. The products of nuclease digestion were resolved on an 8% polyacrylamide gel containing 7 M urea. Control tracks (Cont) contained no drug. Guanine-specific sequence markers obtained by treatment of the DNA with dimethylsulphate followed by piperidine were run in the lane marked G. Numbers below the gel refer to the standard numbering scheme for the nucleotide sequence of the DNA fragment. (b) Differential cleavage histogram comparing the susceptibility of the fragment to DNase I cutting in the presence of 5 μM **4**. Negative values correspond to a ligand-protected site and positive values represent enhanced cleavage. Vertical scales are in units of ln(fa)-ln(fc), where fa is the fractional cleavage at any bond in the presence of the drug and fc is the fractional cleavage of the same bond in the control, given closely similar extents of overall digestion. Only the region of the restriction fragment analysed by densitometry is shown. (c) Diagrammatic representation of the sequence recognition by the symmetric Tröger bases **2** and **3** and the unsymmetric hybrid **4**, inferred from footprinting experiments.

that, on the one hand, the binding involves the intercalation of the acridine ring coupled with minor groove binding of the phenanthroline moiety and, on the other hand, preferential recognition of GTC·GAC triplet sequences.

5. Experimental protocols

5.1. Chemistry

5.1.1. 3-Ethoxycarbonylamino-6H,16H-5,15-methano-[1,5]-diazocino[2,3-c;6,7-c′]-[1,10]phenanthroline-acridine (8)

A mixture of 6-amino-3-ethoxycarbonylamino–acridine **5** (0.2 g, 0.71 mmol) [27], paraformaldehyde (0.095 g, 3.15 mmol) and 5-amino[1,10]phenanthroline **6** (0.276 g, 1.4 mmol) in 10 mL of TFA was stirred at room temperature for 6 h. The reaction was then neutralised by slow addition of aqueous 4N NaOH. The resulting solution was extracted three times with ethyl acetate. The organic layers were collected and the solvent was removed under reduced pressure. The crude product was chromatographed on silica gel column. We used a CH_2Cl_2–MeOH mixture. The Tröger's base **7** was first eluted in 98/2 mixture, and was isolated as traces. The polarity of the mixture was increased to 95/5 to elute the desired compound **8**. Compound **8** (0.088 g, 0.17 mmol) was thus obtained in 24% yield.

M.p.: >300 °C. ^{1}H-NMR (200 MHz, $CDCl_3$): δ ppm = 9.11 (1H, dd, $J = 4.1$ Hz and $J = 1.4$ Hz, H-Ar); 9.0 (2H, m, $J = 7.8$ Hz, H-Ar); 8.43 (1H, s, H-Ar); 8.15 (1H, d, $J = 2.0$ Hz, H-Ar); 8.07 (1H, dd, $J = 8.2$ Hz and $J = 1.4$ Hz, H-Ar); 7.83–7.67 (3H, m, H-Ar); 7.55–7.42 (2H, m, H-Ar); 7.35 (1H, d, H-Ar); 6.85 (1H, s, NH); 5.32–5.00 (3H, m, Ar-CH_2–N(phen-acr)); 4.81–4.65 (3H, m, acr-CH_2-phen and N–CH_2–N)) 4.26 (2H, q, $J = 6.9$ Hz, CH_3–CH_2–O); 1.33 (3H, t, $J = 6.9$ Hz, CH_3–CH_2–O). MS (FAB (+) glycerol): M = 512, m/z: 513 $[M + H]^+$.

5.1.2. 3-Amino-6H,16H-5,15-methano[1,5]-diazocino-[2,3-c;6,7-c′]-[1,10]phenanthroline-acridine (4)

The starting product **8** (0.04 g, 0.078 mmol) was dissolved in a 10N NaOH–EtOH–THF (3/5/1, v/v/v) mixture. The reaction was stirred for 12 h at 65 °C. After removal of the organic solvents under reduced pressure, the resulting aqueous phase was diluted with water (50 mL), then extracted three times with ethyl acetate. The organic layers were concentrated until the product precipitated. Precipitation was achieved by slow addition of pentane. Compound **4** (0.029 g, 0.078 mmol) was obtained as a yellow powder in 85% of yield.

M.p.: >300 °C. ^{1}H-NMR (200 MHz, $CDCl_3$): δ ppm = 9.20–8.85 (3H, m, H-Ar); 8.34 (1H, s, H-Ar); 8.08 (1H, dd, $J = 8.2$ Hz and $J = 1.4$ Hz, H-Ar); 7.80–7.64 (2H, m, H-Ar); 7.53 (1H, m, H-Ar); 7.27 (1H, m, H-Ar); 7.43 (1H, d, $J = 1.4$ Hz, H-Ar); 6.91 (1H, dd, $J = 8.9$ Hz and $J = 2.0$ Hz, H-Ar); 5.32–5.00 (3H, m, Ar-CH_2–N(phen-acr)); 4.82–4.61 (3H, m, acr-CH_2-phen and N–CH_2–N); 4.11 (2H, s large, NH_2). MS (FAB (+) glycerol): M = 440, m/z: 441 $[M + H]^+$.

5.2. Chemicals and biochemicals

Calf thymus DNA and the double-stranded polymers poly(dAT)$_2$ and poly(dIC)$_2$ were obtained from Pharmacia (Uppsala, Sweden). Calf thymus DNA was deproteinised with sodium dodecyl sulphate (protein content $<0.2\%$). The nucleoside triphosphate labelled with [^{32}P](α-dATP) was obtained from Amersham (Buckinghamshire, England) (3000 Ci mmol^{-1}). Restriction endonucleases and AMV reverse transcriptase were purchased from Boehringer (Mannheim, Germany) and used according to the supplier's recommended protocol in the activity buffer provided. All other chemicals were analytical grade reagents.

5.3. Absorption spectra and melting temperature studies

Melting curves were measured using an Uvikon 943 spectrophotometer coupled to a Neslab RTE111 cryostat. For each series of measurements, 12 samples were placed in a thermostatically controlled cell-holder, and the quartz cuvettes (10 mm pathlength) were heated by circulating water. The measurements were performed in BPE buffer pH 7.1 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA). The temperature inside the cuvette was measured with a platinum probe; it was increased over the range 20–100 °C with a heating rate of 1 °C min^{-1}. The "melting" temperature T_m was taken as the mid-point of the hyperchromic transition. The Uvikon 943 spectrophotometer was also used to record the absorption spectra. Titrations of the drug with DNA, covering a large range of DNA phosphate/drug ratios (P/D), were performed by adding aliquots of a concentrated DNA solution to a drug solution at constant ligand concentration (20 μM). DNA blanks at the same nucleotide concentrations were prepared concomitantly and used as a reference in the recording of absorption spectra.

5.4. Circular dichroism (CD)

CD measurements were recorded on a Jobin-Yvon CD6 dichrograph. Solutions of drugs and/or nucleic acids were scanned in a 2 cm-quartz cuvette, in 1 mM sodium cacodylate buffer, pH 7.0. Measurements were made by progressive addition of DNA to a pure ligand solution to obtain the desired drug/nucleic acid ratios. Three scans were accumulated and automatically averaged.

5.5. Electric linear dichroism (ELD)

Measurements were performed with a computerised optical measurement system using the procedures previously outlined [28]. All experiments were conducted with a 10 mm pathlength Kerr cell having 1.5 mm electrode separation. The samples were oriented under an electric field strength varying from 1 to 13 kV cm^{-1}. The drug under test was present at 10 μM concentration together with the DNA at 200 μM concentration unless otherwise stated. This electro-optical method has proved most useful to determine the orientation of the drugs bound to DNA. It has the additional advantage that it senses only the orientation of the polymer-bound ligand: free ligand is isotropic and does not contribute to the signal [29].

5.6. Purification and radiolabelling of the DNA substrate

The 117-mer fragment was rendered radioactive by 3′-[^{32}P]-end labelling of the *Eco*RI–*Pvu*II double digest of the plasmid pBS (Stratagene) using [α-^{32}P]dATP (3000 Ci $mmol^{-1}$) and avian myeloblastosis virus reverse transcriptase. The labelled digestion products were separated on a 6% polyacrylamide gel under non-denaturing conditions in TBE buffer (89 mM Tris-borate pH 8.3, 1 mM EDTA). After autoradiography, the requisite band of DNA was excised, crushed and soaked in elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate) overnight at 37 °C. This suspension was filtered through a Millipore 0.22 μM filter and the DNA was precipitated with ethanol. Following washing with 70% ethanol and vacuum drying of the precipitate, the labelled DNA was resuspended in 10 mM Tris adjusted to pH 7.0 containing 10 mM NaCl.

5.7. DNase I footprinting

Experiments were performed essentially as previously described [30]. Briefly, reactions were conducted in a total volume of 10 μl. Samples (3 μl) of the labelled DNA fragments were incubated with 5 μl of the buffered solution containing the ligand at appropriate concentration. After 30 min incubation at 37 °C to ensure equilibration of the binding reaction, the digestion was initiated by the addition of 2 μl of a DNase I solution whose concentration was adjusted to yield a final enzyme concentration of about 0.01 unit ml^{-1} in the reaction mixture. After 3 min, the reaction was stopped by freeze drying. Samples were lyophilised and resuspended in 5 μl of an 80% formamide solution containing tracking dyes. The DNA samples were then heated at 90 °C for 4 min and chilled in ice for 4 min prior to electrophoresis.

5.8. Electrophoresis and quantitation by storage phosphor imaging

DNA cleavage products were resolved by polyacrylamide gel electrophoresis under denaturating conditions (0.3 mm thick, 8% acrylamide containing 8 M urea). After electrophoresis (about 2.5 h at 60 W, 1600 V in Tris-Borate-EDTA buffered solution, BRL sequencer model S2), gels were soaked in 10% acetic acid for 10 min, transferred to Whatman 3 mm paper, and dried under vacuum at 80 °C. A Molecular Dynamics 425E PhosphorImager was used to collect data from the storage screens exposed to dried gels overnight at room temperature. Base line-corrected scans were analysed by integrating all the densities between two selected boundaries using ImageQuant version 3.3 software. Each resolved band was assigned to a particular bond within the DNA fragments by comparison of its position relative to sequencing standards generated by treatment of the DNA with dimethylsulphate followed by piperidine-induced cleavage at the modified guanine bases in DNA (G-track).

5.9. DNA relaxation experiments

Supercoiled pKMp27 DNA (0.5 μg) was incubated with 4 units human topoisomerase I (TopoGen Inc.) at 37 °C for 1 h in relaxation buffer (50 mM Tris pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA) in the presence of varying concentrations of the test drug. Reactions were terminated by adding SDS to 0.25% and proteinase K to 250 μg ml^{-1}. DNA samples were then added to the electrophoresis dye mixture (3 μl) and electrophoresed in a 1% agarose gel at room temperature for 2 h at 120 V. Gels were stained with ethidium bromide (1 μg ml^{-1}), washed and photographed under UV light. Similar experiments were performed using ethidium-containing agarose gels.

Acknowledgements

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