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Metallodesferals as a new class of DNA cleavers: Specificity, mechanism and targetting of DNA scission reactions

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Abstract. Interaction of metal complexes with nucleic acids is currently attracting wide attention due to their potential utility as drugs, regulators of gene expression and tools for molecular biology. Many metal complexes exhibit nucleolytic activity, the most important examples being Cu(II)–OP, Fe(II)–BLM, Fe(II)–EDTA, metalloporphyrins, Ru and Co complexes of 4,7-diphenyl-1,10-phenanthroline and more recently by Ni(II) complexes. Desferal, a well known siderophore and a highly effective drug in chelation therapy of iron overload diseases, forms a stable octahedral co-ordination Fe(III) complex Ferrioxamine B. We have been interested in the DNA damaging properties of metallodesferals and this paper describes the DNA cleaving ability of metallodesferals, metal-dependent base selectively in DNA scission reactions, mechanistic studies on DNA cleavage by CuDFO and targetting of DNA cutting by covalent MDFO conjugates. This paper reports the synthesis of Cu(II), Co(III) and Ni(II) complexes of a siderophore chelating drug desferal, the studies on cleavage of plasmid DNA, the sequence preference of cleavage reactions, and C1′ as the primary site of hydroxyl radical attack in the reactions. Oligonucleotides covalently linked with this molecular scissor can direct the cleavage of either single or double strand DNA's, mediated by duplex or triple helix structures respectively. Such targetting of DNA cleavage reactions, mediated by oligonucleotide-Cu(II)/Co(III) desferal conjugates has demonstrated reasonable site specificity and efficiency

Keywords. DNA cleavers; metallodesferals; molecular scissor.

1. Introduction

Interaction of metal complexes with nucleic acids is currently attracting wide attention due to their potential use as drugs, regulators of gene expression and tools for molecular biology. Many metal complexes exhibit nucleolytic activity, the most important examples being Cu(II)–OP (Sigman 1986), Fe(II)–BLM (Hecht 1986; Stubbe and Kozarich 1987), Fe(II)–EDTA (Dervan 1986), metalloporphyrins (Le Doan *et al* 1986; Wood *et al* 1987), Ru and Co complexes of 4,7-diphenyl-1,10-phenanthroline (Barton 1986; Basile and Barton 1987a; Basile *et al* 1987b) and more recently by Ni(II) complexes (Mack and Dervan 1990; Chen *et al* 1991). These cleave DNA either by oxidative degradation of deoxyribose moiety or by base modification and such chemical methods of nicking DNA have found useful applications in probing sequence dependent conformational variability of DNA (Burkhoff and Tullius 1987; Yoon *et al* 1988), identifying ligand/protein binding sites on DNA (Tullius *et al* 1988;

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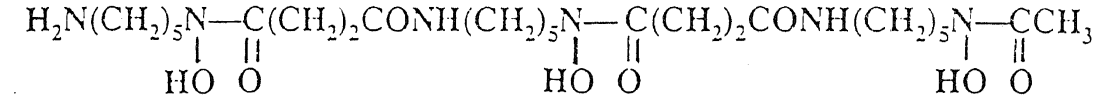
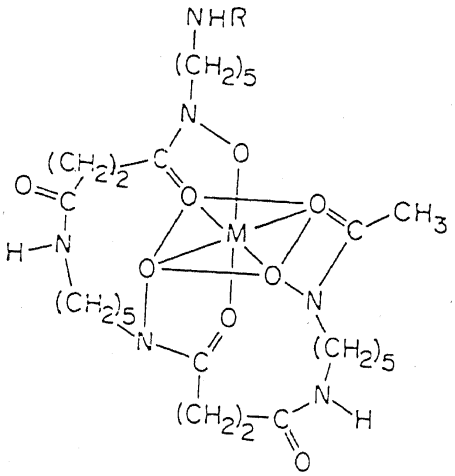


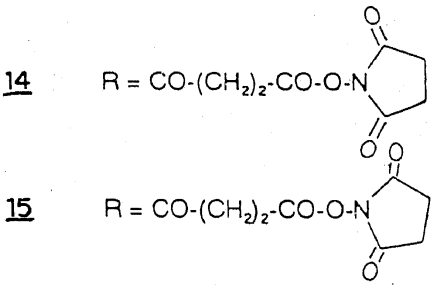
1, DFO



2, M = Fe(III); 3, M = Cu(II); 4, M = Co(III); 5, M = Ni(II).

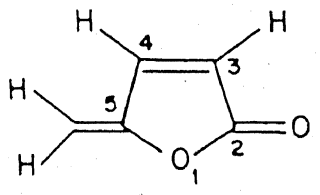
3a R = CO-$(CH_2)_2$-COOH, M = Cu

4a R = CO-$(CH_2)_2$-COOH, M = Co



14 R = CO-$(CH_2)_2$-CO-O-N M = Cu

15 R = CO-$(CH_2)_2$-CO-O-N M = Co



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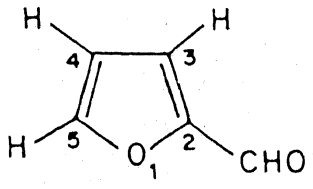

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Tullius 1989) and in the design of artificial sequence-specific nucleases (Hertzberg and Dervan 1984; Dervan 1986; Strobel *et al* 1988).

Desferal 1, a well known siderophore (Keberle 1964; Raymond *et al* 1984, Martell *et al* 1981; Dobbin and Hider 1990) and a highly effective chelation drug in iron overload diseases, forms a stable octahedral co-ordination Fe(III) complex ferrioxamine B 2. In contrast to Fe-EDTA, ferrioxamine cannot undergo a redox cycling, thus preventing iron-catalysed hydroxyl radical formation (Graf *et al* 1984) which is a useful property for its clinical application. Indeed, desferal is employed to arrest hydroxyl radical production in DNA scission reactions caused by Fe(II) complexes (Hertzberg and Dervan 1984, Strobel *et al* 1988). We have been interested in the DNA

damaging properties of metallodesferals (Joshi *et al* 1992, 1994) and this paper describes the DNA cleaving ability of metallodesferals, metal-dependent base selectivity in DNA scission reactions, mechanistic studies on DNA cleavage by CuDFO and targetting of DNA cutting by covalent MDFO conjugates.

2. Results and discussion

The DNA nicking ability of metallodesferals was investigated by plasmid cleavage assay. As can be seen from the results of figure 1, Fe(III)DFO 2 does not cleave the plasmid DNA under any conditions (lane 3), while the respective Cu(II) 3 (lane 4), Co(III) 4 (lane 5) and Ni(II) 5 (lane 6) complexes cleave the plasmid to produce form-II DNA, similar to that observed with Cu(II)–OP (lane 7). The cleavage reaction with Cu(II) complex requires a reducing agent such as 2-mercaptoethanol (ME), dithiothreitol or ascorbate and the reaction is made more efficient by addition of H_2O_2. The cleavage reactions with Co(III) and Ni(II) complexes proceed even in the absence of a reducing agent. The cleavage efficiency is dependent on metal ion concentration, the optimal concentrations for 100% cleavage being 235, 42·5 and 10 μM for Cu(II), Co(III) and Ni(II) complexes respectively. Among the three, the Ni(II) complex showed maximum efficiency at low concentrations and the excess complex did not further degrade the DNA to the linear form. The main product of the scission reactions with Cu(II) ad Ni(II) is the randomly nicked form-II DNA. In case of Co(III), a slight increase in concentration over the optimal value led to extensive degradations, resulting in disappearence of bands on agarose gel. Free desferal in the presence of either an oxidising or a reducing agent or both did not exhibit any DNA cleavage (not shown).

Incubation of plasmid with Cu(II)DFO plus ME in the presence of hydroxyl radical scavengers such as mannitol, glycerol, NaN_3 and catalase significantly inhibited (> 75%) the cleavage, while boiled catalase was ineffective in the inhibition reaction.

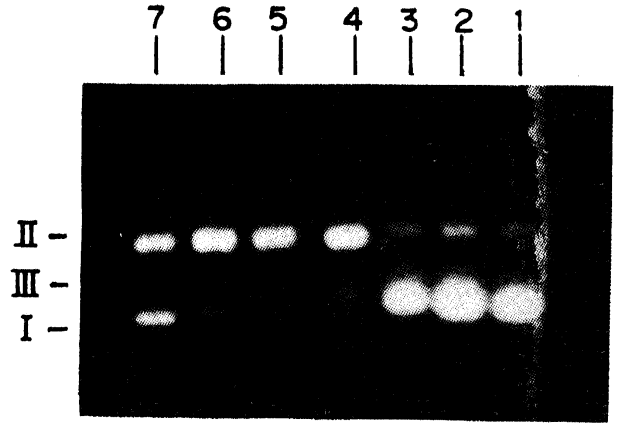


Figure 1. Cleavage of pBR322 with various metal complexes. The reaction mixtures contained (in a total volume of 20 μl) 25 mM *tris*-acetate, *p*H 7·8, 2·5 mM sodium acetate and plasmid pBR322 (final concentration 140 μM) with the following additions: lane 1, none; lane 2, H_2O_2 (4 mM) + ME (0·5 mM); lane 3, H_2O_2 (4 mM) + ME (0·5 mM) + Fe(III)DFO (85 μM); lane 4, H_2O_2 (4 mM) + ME (0·5 mM) + Cu(II)DFO (255 μM); lane 5, H_2O_2 (4 mM) + Co(III)DFO (42·5 μM); lane 6, H_2O_2 (4 mM) + Ni(II)DFO (10 μM); lane 7, H_2O_2 (4 mM) + ME (0·5 mM) + Cu(II)–OP (10 μM).

Recently Cu(II)–thiol induced chemical cleavage of plasmid DNA has been reported (Reed and Douglas 1991) and the suggested mechanistic pathway involves the generation of either a reactive intermediate (probably HO•) in free solution or a cleavage species which is DNA-bound. DNA scission by CuDFO complex may perhaps follow a similar course, since the reaction specificity (redox reagent requirements and inhibitory effects) are identical.

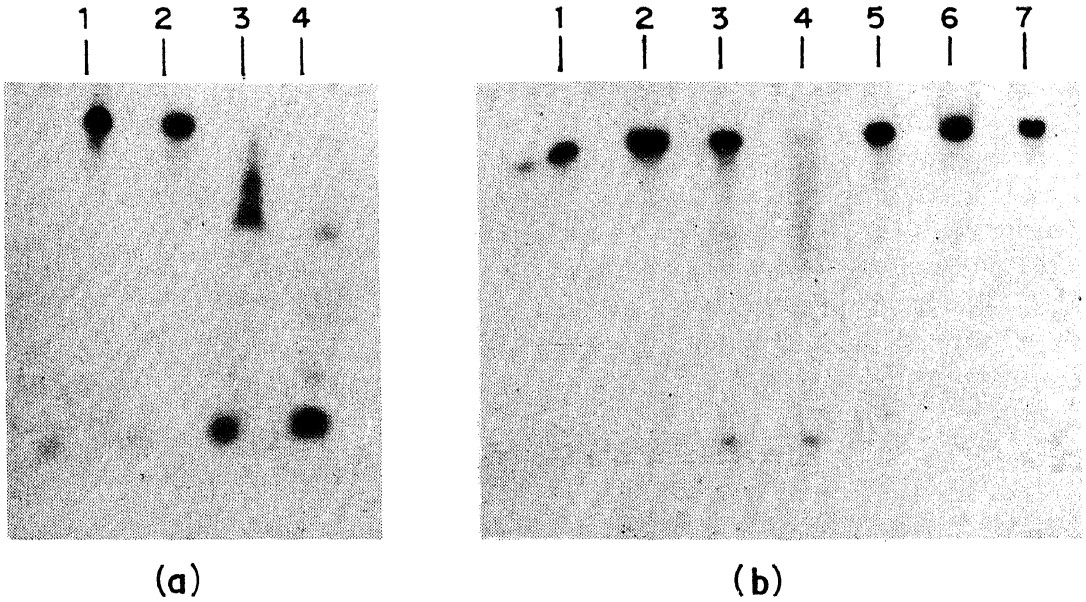


Figure 2. 20% PAGE autoradiogram of cleavage reactions $d(CG)_9$ and $d(AT)_9$ with metallodesferals: (a) Lane 1, $d(CG)_9$; lane 2, $d(CG)_9$ + Pip (1 M); lane 3, $d(CG)_9$ + CuFO (300 μM) + ME (0·5 mM) + H_2O_2 (4 mM); lane 4, 3 + Pip (1 M). (b) Lane 1, $d(AT)_9$; lane 2, $d(AT)_9$ + Pip (1 M); lane 3, $d(AT)_9$ + CuDFO (300 μM) + ME (0·5 mM) + H_2O_2 (4 mM); lane 4, 7 + Pip (1 M); lane 5, $d(AT)_9$ + CoDFO (150 μM) + H_2O_2 (4 mM); lane 6, 9 + Pip (1 M); lane 7, $d(AT)_9$ + NiDFO (150 μM) + H_2O_2 (4 mM) + Pip (1 M).

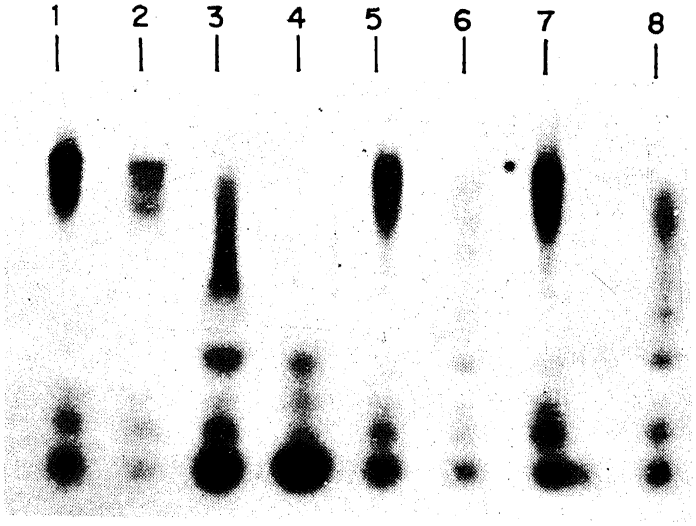


Figure 3. 20% PAGE autoradiogram of cleavage of $d[(CG)_2A_2T_2(CG)_2]$ with metallodesferals: Lane 1, control DNA; lane 2, 1 + Pip (1 M); lane 3, 1 + CuDFO (300 μM) + ME (0·5 mM) + H_2O_2 (4 mM); lane 4, 3 + Pip (1 M). lane 5, 1 + CoDFO (150 μM) + H_2O_2 (4 mM); lane 6, 5 + Pip (1 M); lane 7, 1 + NiDFO (150 μM) + H_2O_2 (4 mM); lane 8, 1 + Cu(II)–OP (170 μM) + ME (0·5 mM) + H_2O_2 (4 mM) + Pip (1 M).

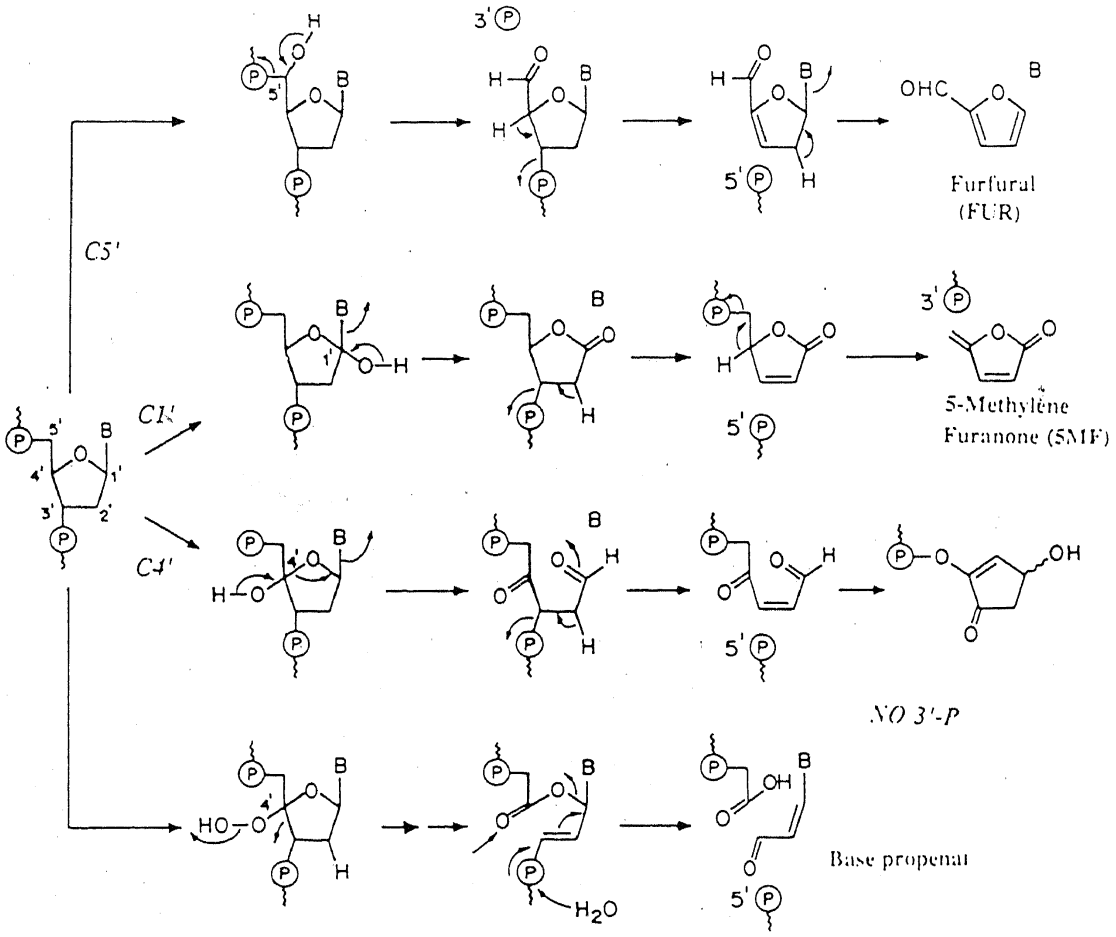
2.1 *Specificity in DNA cleavage by metallodesferals*

Figure 2 shows the electrophoretic gel results of cleavage reactions of $d(CG)_9$ and $d(AT)_9$ with Cu(II), Co(III), and Ni(II) complexes of desferal. The following conclusions emerge from these results: (i) CuDFO cleaves both $d(CG)_9$ and $d(AT)_9$ (figures 2a, b) and (ii) CoDFO and NiDFO do not cleave $d(AT)_9$ (figure 2b). The optimum concentration required for cleavage varies with the metal and was determined by changing the concentration of the metal complexes over a wide range of values (200 μM to 2 mM). Treatment of $d(CG)_9$ (figure 2a) with CuDFO leads to initial cleavage fragments (lane 3), which on treatment with piperidine results in complete degradation (lane 4). Comparison of controls, lanes 1 and 2 of (figures 2a and b) indicate that synthetic DNA is stable to piperidine under these conditions and the degradation seen (lane 4, figure 2a) is due to the fact that the initial products of the reaction are chemically modified and hence susceptible to piperidine action. Both CoDFO and NiDFO behaved similarly in reactions with $d(CG)_9$, leading to a full degradation of DNA after piperidine treatment (not shown). It was observed that with Cu, Co and NiDFO the dinucleotide d(CG) and tetranucleotide $d(CG)_2$ failed to undergo cleavage while $d(CG)_3$ exhibited cleavage reactions. Figure 2b shows reactions of $d(AT)_9$ with Cu, Co and NiDFO complexes under conditions similar to that with $d(CG)_9$. While CuDFO treatment (lanes 3 and 4) led to efficient cleavage, it is seen that Co and Ni complexes (lanes 5–7) do not cleave $d(AT)_9$ sequences even after piperidine treatment of the initial reaction products. Thus Co and Ni complexes are inert to AT sequences and show specificity towards cleavage of CG sequences while Cu complex cleaves both CG and AT containing sequences. It may also be mentioned that while CuDFO reactions necessarily require a reducing agent (e.g. ME, DTT) to promote cleavage, Co and Ni complexes bring about scission under oxidative conditions. These facts may have important bearing on the possible mechanistic differences of cleavage reactions using these complexes.

In view of the characteristic behaviour of Co and Ni complexes in promoting cleavage at CG sites and remaining passive towards AT sites, cleavage reactions were performed on oligonucleotides containing mixed sequences. Figure 3 shows electrophoretic analysis of cleavage reactions of $d[(CG)_2A_2T_2(CG)_2]$ with Cu, Co and NiDFO, complexes. As expected all three complexes were active in cleavage reactions (lanes 3–7). Although the cleavage patterns were similar, differences were noticed in relative intensities of product bands. In each case, it is noticed that piperidine treatment following reaction with metallodesferal leads to further degradation in a way similar to that observed in $d(CG)_n$ and $d(AT)_n$ sequences. The reaction was most efficient in case of CuDFO which led to mostly faster moving products on the gel (lane 4). For comparison, the pattern of products obtained from a similar cleavage reaction with Cu-phenanthroline is shown (lane 8).

2.2 *Oxidative degradation of DNA and mechanism of DNA cleavage by CuDFO*

The general oxidative mechanisms (scheme 1) proposed to account for DNA cleavage by hydroxy radicals via abstraction of a H atom from sugar units predict the release of specific chemical residues arising from transformed sugars, depending on the position from which the H atom is removed (Prativel 1991; Prativel *et al* 1993). A C1′ attack results in generation of MF 6 from the affected sugar while a C5′-route leads to the formation of FUR 7. In both cases, 5′ and 3′-phosphates are produced



Scheme 1. Pathways for DNA cleavage.

with release of free purine and pyrimidine bases. The C4′-route differs from both the above modes: neither FUR nor MF is released and, depending on the nature of the initial attack (·OH vs ·OOH) either sugar is not liberated or part of its structure is fragmented in conjuction with base for release as base propenal. Although 5′/3′-phosphates and the bases are liberated in all reactions, the order in which this occurs differs among these pathways. The base is liberated almost instantaneously in case of both C1′ and C4′ reactions. Upon heating, this is followed by release of the sugar degradation product by a β-elimination process accompanied by formation of 5′ and 3′phosphate termini in case of C1′, but only 5′-phosphate in case of C4′. Thus an unambiguous characterization of the fate of the sugar residue becomes key to determine the mechanistic pathway for the cleavage reaction. Recently, with FUR as a marker, the mechanism of DNA cleavage by Mn-TPyP has been elucidated (Prativel *et al* 1991). In this paper, by using HPLC we analyse the reaction products of DNA cleavage with CuDFO and demonstrate that the major pathway for this reaction proceeds by initial attack at C1′-H of sugar.

2.2a *HPLC identification of cleavage products*: Even under optimal HPLC analytical conditions, the two markers MF and FUR have very close retention times ($\Delta t < 0{\cdot}5$ min) and their unambiguous differentiation requires a careful analysis. We therefore chose to use the technique of peak characterisation by on-line spectral

scanning with photodiode array detector which provides individual UV spectra of various eluants without their actual isolation. Figures 4(a–d) show the HPLC of standard MF and FUR, and their corresponding UV spectra as recorded from the diode array detector. It is seen that although these have nearly close elution times, they differ in their UV spectral maximum (276 nm for FUR and 266 nm for MF), a property that is useful for unambiguous assignment of the HPLC reaction products. Figure 4e shows HPLC analysis of a mixture of FUR and MF which indicates that under the HPLC conditions employed, FUR and MF can be clearly separated with base line resolution even when present as a mixture. No mutual interference in their UV spectral scans was observed in the mixture analysis permitting use of on-line UV spectral scans for unambiguous characterization of FUR and MF in the reaction mixture.

Figure 5 shows HPLC analysis of reaction of CT DNA with CuDFO and the assignment of various peaks was done after comparison with the corresponding standard compounds. It is seen from this figure that while the pyrimidine bases C and T could be easily identified, the purine bases A and G could not be detected. The broad peak around 7 min arises from the CuDFO present in the reaction mixture. Individual bases A and G when subjected to CuDFO reaction under the above conditions, showed formation of products eluting around 7 min and masked by CuDFO peak. Among other peaks, the product at 5·93 min could be clearly identified as due to MF on the basis of its retention time and the UV spectral maximum (266 nm). This product was initially formed in low amounts in the reaction mixture and increased upon simple heating. This is characteristic of C1′ cleavage route. MF could not be seen in HPLC after treatment of the reaction mixture with hot piperidine. The treatment of DNA alone under control conditions without CuDFO or hot piperidine did not result in the formation of MF. These facts clearly established that MF originated solely due to reaction of CuDFO with DNA. This was further confirmed by carrying out analysis of products under identical conditions from an analogous reaction of CT DNA with the well established cleavage agent Cu–OP (figure 6). The reaction yielded the expected MF whose chromatographic and spectral properties resembled that of the product from CuDFO reaction.

As a negative control in CT DNA cleavage by Fe(EDTA), we failed to notice the formation of either MF or FUR by HPLC. This reaction is known to occur via C4′ pathway which does not result in the formation of these products. It may be pointed out that base propenals which are the expected products of C4′ attack, cannot be detected similarly by HPLC as they are prone to decomposition under the basic reaction conditions. On the other hand, they can be assayed spectrophotometrically after reaction with thiobarbituric acid (Burger *et al* 1990). Treatment of the reaction mixture (CuDFO + CTDNA) with TBA indicated the presence of base propenal in minor amounts. Thus, the above analysis conclusively indicated the formation of MF as the major product from sugar degradation in the reaction of CuDFO, with a minor component of base propenal and complete absence of FUR.

2.2b *Mechanism of DNA cleavage by CuDFO*: DNA cleavage by CuDFO follows a pattern similar to that elucidated for Cu–OP. The reaction proceeds through redox chemistry centred on metal since it requires the addition of a reducing agent such as DTT or ME (Joshi and Ganesh 1992a). The cleavage requires prior binding of CuDFO to DNA since oligonucleotides less than tetramers are not cleaved by this complex

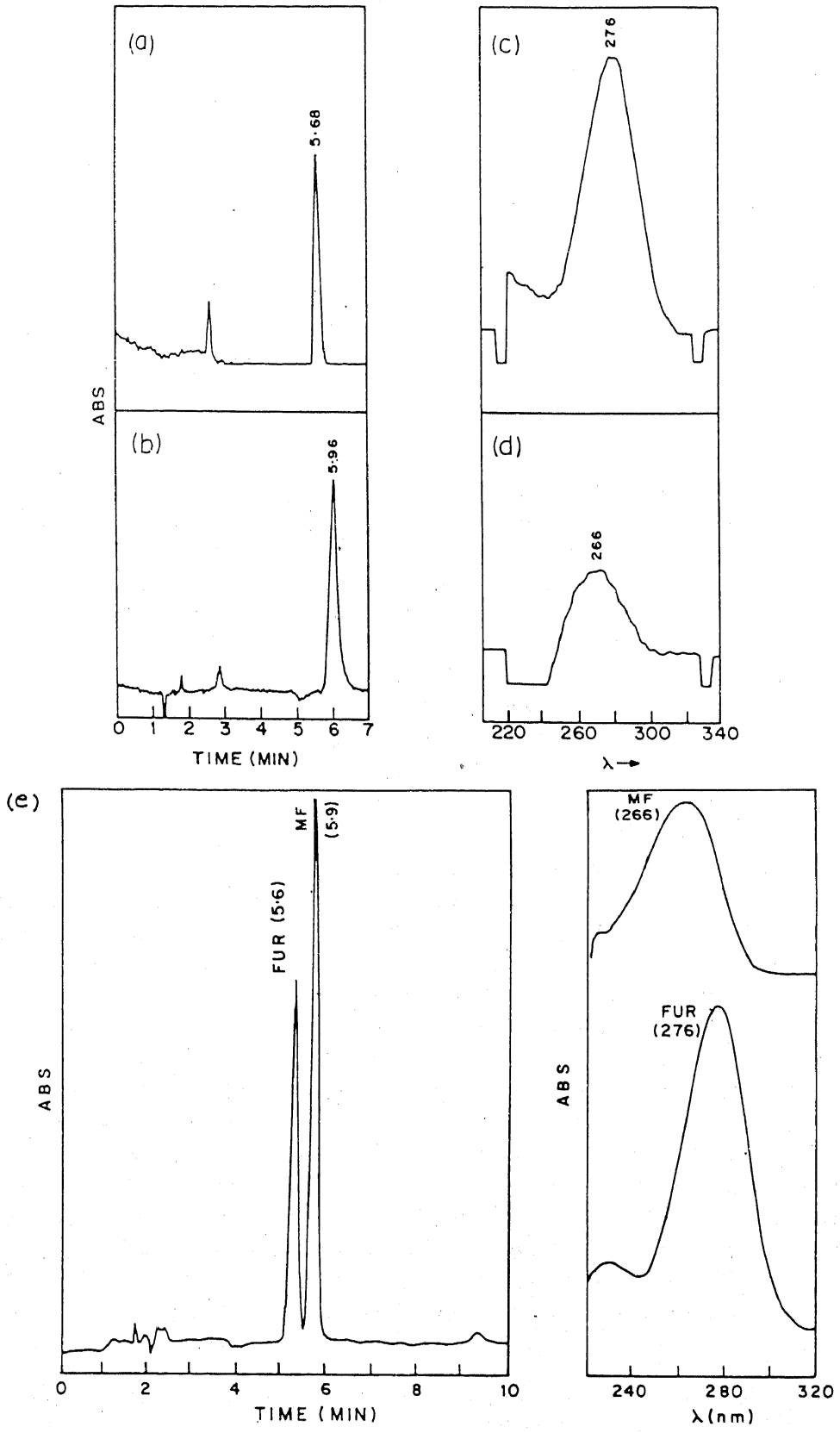


Figure 4. HPLC of (**a**) FUR and (**b**) MF. (**c**) and (**d**) are the UV scans of peaks at 5.68 min (a) and 5·96 min (b). (**e**) HPLC of mixture of FUR and MF and UV scans of respective peaks. For HPLC conditions see §3.

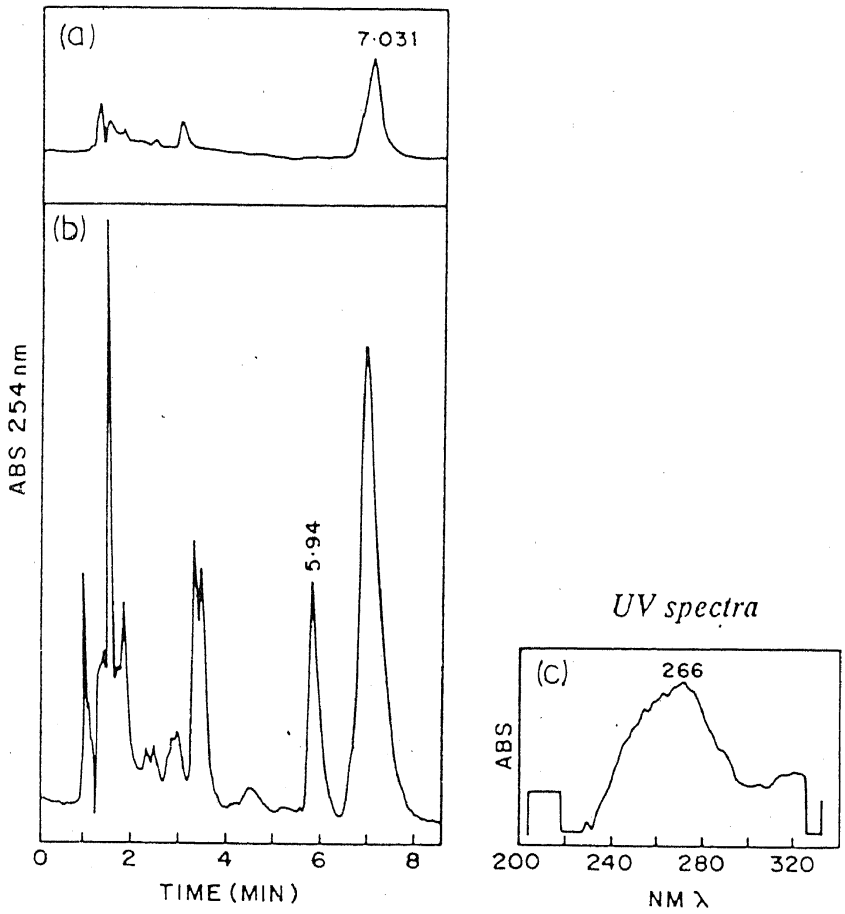


Figure 5. (**a**) HPLC of CT DNA + CuDFO. (**b**) Same as (a) but after heating for 10 min at 90°C. (**c**) UV spectral scan of peak at 5.94 min.

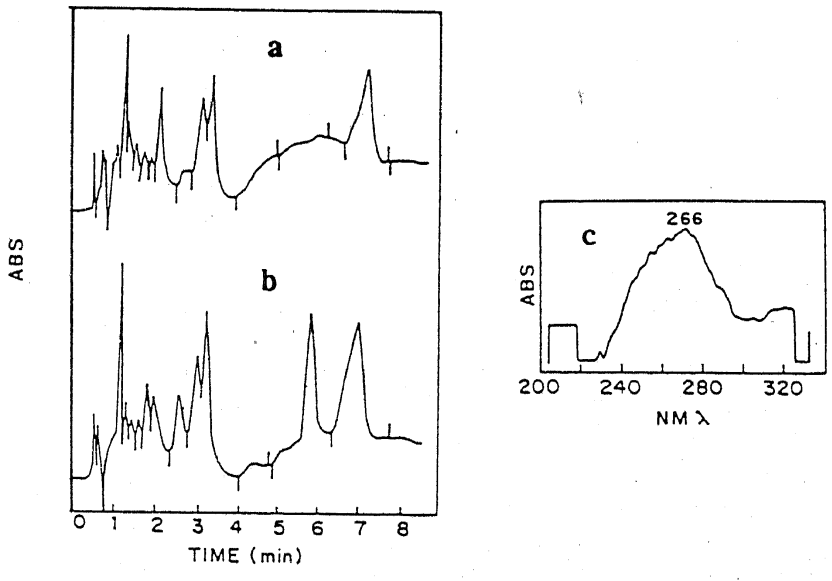


Figure 6. HPLC of (**a**) CT DNA + Cu-OP (**b**) CT DNA + Cu-OP after heating for 10 min at 90°C, (**c**) UV spectral scan of peak at 5·94 min.

(Joshi and Ganesh 1992b). We have shown earlier that the cleavage is inhibited by radical scavengers, implying that hydroxyl radical or peroxy derivatives mediate the cleavage reaction. Further, the reaction is modulated by a metallo complex bound hydroxyl radical or a peroxo species, generated from the coreactant H_2O_2 or dissolved O_2. This results in oxidative attack on the deoxyribose moiety at C1′ hydrogen leading to a series of elimination reactions that ruptures the phosphodiester backbone and yields 3′ and 5′-phosphomonoester terminii, free bases and MF.

$$\text{Cu(II)DFO} \overset{\text{DTT}}{\rightleftharpoons} \text{Cu(I)DFO}$$
$$\text{Cu(I)DFO} + \text{DNA} \rightleftharpoons \text{Cu(I)DFO} - \text{DNA}$$
$$\text{Cu(I)DFO} - \text{DNA} + H_2O_2 \rightleftharpoons \text{Cu(II)DFO·OH} - \text{DNA}$$
$$\downarrow$$
$$\text{Nicked products}$$

The nature of the actual metalloactive species is not clear at present, since even binuclear peroxo or superoxide species have been suggested to be the possible active species in site specific DNA damage by Cu(II) ion (Yamamoto and Kawanishi 1989; Chikira and Mizukami 1991). However, the preferred attack on C1′ in the minor groove side is perhaps a consequence of binding of the active CuDFO species with DNA prior to initiation of the cleavage chemistry. While Cu-OP does so by an intercalative mechanism, CuDFO may recognise DNA presumably through the non-coordinated peptide groups (Joshi and Ganesh 1992b). Further work is in progress to characterise the structure of the metal complexes and in the identification of the active species.

2.3 *Targetting of DNA cleavage reactions*

Targetted chemical nucleases have been used successfully for single site cleavage in yeast chromosome, SV40 DNA and λ phage DNA (Dervan 1992; Sigman *et al* 1993). The metallodesferal complexes 3 and 4 possess a terminal NH_2 group, that is not involved in complex formation and hence is available for linking to DNA via a suitable spacer chain. Herein we demonstrate the chemical synthesis of oligonucleotide-MDFO conjugates and efficient duplex and triplex directed site-specific targetting of DNA cleavage reactions using these cleaving agents. In addition to targetting, we have also seen enhancement of metal dependent (Cu vs Co) base specificity of cleavage reactions.

2.3a *Chemical synthesis and stability of MDFO conjugates*: The oligonucleotides 8-13 (table 1) were synthesized on a Pharmacia Gene Assembler Plus (0·2 μmole) using β-cyanoethyl phosphoramidite monomers. The oligonucleotides were converted to their 5′-aminoalkylated analogs (scheme 1) by initial activation with CDI (Wachter *et al* 1986) on the solid support in an automated DNA synthesiser. The activation was essentially complete in 30 min after which, the resin was treated with an excess of spacer diamine component (hexamethylenediamine). This resulted in linking of amines to oligonucleotides by urethane linkage leading to DNA containing a primary NH_2 group towards 5′ terminus. No loss of oligonucleotide from polymer support due to reaction of diamine with polymer-succinoyl linkage was observed under these conditions.

Table 1. List of oligonucleotides.

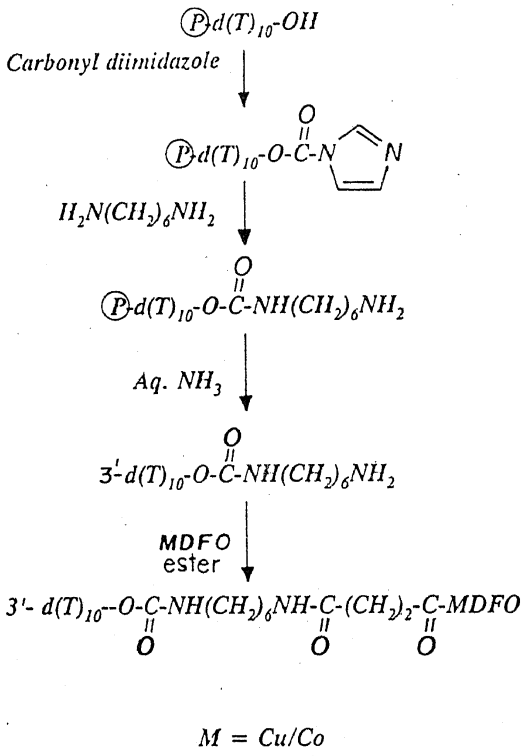
Sequence No.	Sequence
8	5′-TCCTGATAAAGGAGGAGATGAAGAAAAAATGA-3′
9	3′-AGGACTATTTCCTCCTCTACTTCTTTTTTACT-5′
10	5′-TAAAAAAAAAAGCCCGGCG-3′
11	5′-AGTCTGCTAGAAGTAGAGGAGGAAATGAAGATCGTCTGA-3′
12	3′-TTTTTTTTTT–O–C–NH$(CH_2)_6$NH2 ‖ O
13	3′-TTTCCTCCTCT–O–C–NH$(CH_2)_6$NH2 ‖ O
16	3′-TTTTTTTTTT–O–C–NH$(CH_2)_6$NH–C–$(CH_2)_2$–C–X ‖ ‖ ‖ O O O (**a**), X=CuDFO; (**b**), X=CoDFO
17	3′-TTTCCTCCTCT–O–C–NH$(CH_2)_6$NH–C–$(CH_2)_2$–C–X ‖ ‖ ‖ O O O (**a**), X=CuDFO ; (**b**), X=CoDFO

The support-linked 5′-modified oligonucleotides were treated with NH_3 for release of oligonucleotides into solution with concomittant deprotection of phosphate and base protecting groups. The urethane group connecting the spacer diamine to the 5′-OH of oligonucleotide is stable to NH_3 treatment (Wachter *et al* 1986). The amino-alkylated oligonucleotides were purified by gel filtration over Sephadex G-10. The purity and integrity of 5′-aminoalkylated oligonucleotides were checked by HPLC.

The metallodesferals 3 and 4 were reacted individually with succinic anhydride in DMF to obtain corresponding acids 3a and 4a. These were directly converted into their active esters 14 and 15 by treatment with N-hydroxy succinimide and DCCI. Compounds 14 and 15 were condensed with 5′-amino alkylated oligonucleotides to obtain the conjugates 16 and 17. The product conjugate was purified by gel filtration and finally checked by HPLC to test the homogeneity.

The determination of t_m is important to optimize the conditions for targetting reactions. The variations in melting profiles would reflect the differences in stabilities of the duplexes and triplexes formed by different conjugates. The melting temperatures were determined by following UV absorbance change with temperature. Table 2 indicates the results of melting experiments for different duplexes and triplexes constituted by various combinations of oligonucleotide sequences.

The results show that the conjugation of oligonucleotide at 5′-end with MDFO does not significantly affect the melting temperature and hence duplex formation (entry 1,2 and 3). CoDFO conjugate 17b (entry 3) destabilized the helix to a small extent ($\Delta t_m = 3°$) in contrast to CuDFO conjugate 17a which did not show any change (entry 2). The formation of duplex (entry 4) and triple helix (entry 5) involving suitable underivatised oligonucleotide components was indicated by appearance of a duplex



Scheme 2. Conjugation of oligonucleotides.

Table 2. Melting temperatures of duplexes and triplexes†.

Entry	Sequence	t_m°C $T \rightarrow D$	t_m°C $D \rightarrow S$
1	11:13	—	43·7
2	11:17a	—	43·1
3	11:17b	—	40·7
4	8:9	—	65·0
5	8:9:13	16·4	65·0
6	8:9:17a	10·0	65·0
7	8:9:17b	—	65·0

†T→D, triplex→duplex transition, D→S, duplex→single strand transition. For conditions see §3

⇔ single strand transition (65°C) and a low temperature transition at ~16·4°C (8:9:13) (figure 7) and 10°C (8:9:17a) corresponding to triplex ⇔ duplex transition. With probe conjugates as the third strand, it is seen that while the high temperature transition (~65°C, duplex ⇔ single strand) was not affected, the triplex ⇔ duplex transition dropped by 6°C in case of CuDFO (entry 6) and is not seen at all with CoDFO (entry 7).

2.3b *Duplex directed site specific cleavage reactions*: Figure 8a depicts cleavage reactions of DNA 10 with free CuDFO 3 and its conjugates with DNA. The identity

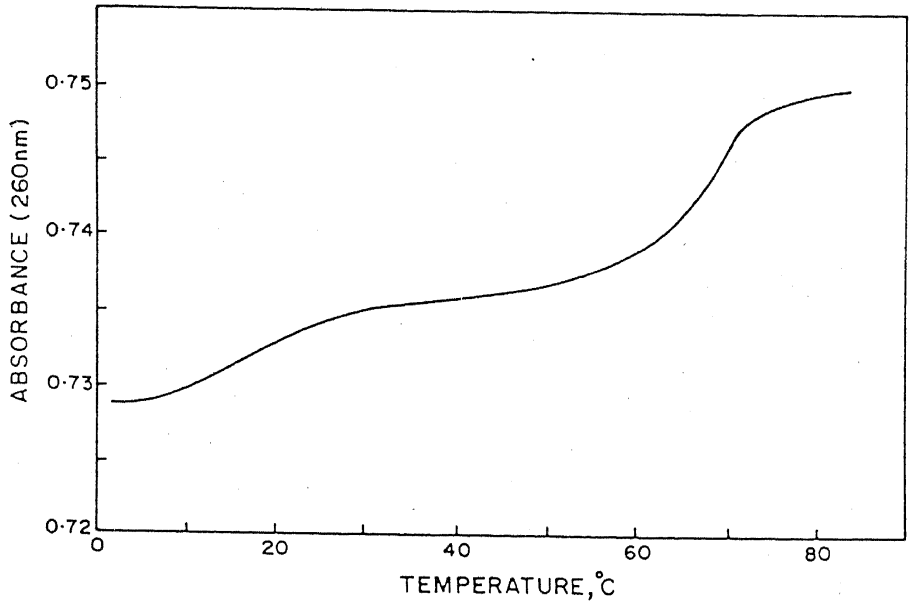


Figure 7. UV melting profile of triplex 8:9:13.

of bands was established by Maxam-Gilbert G reaction products (lane 4). The pattern of intensities of cleavage product bands indicated a preference for cleavage at G sites (G12,G16,G17,G19) over C sites (C14,C15,C16) with poor efficiency for A sites. Upon hybridization of 10 with CuDFO-d$(T)_{10}$ conjugate 16a, followed by cleavage reactions (lane 3), the bands corresponding to bases 16-19 became relatively more intense. The results are more prominent in the bar diagram depiction which shows the effect of targetting. The bands due to G12, G16 and G17 in targetted reactions (figure 9a) are significantly enhanced over that from untargetted reaction (figure 9b). The preference for G sites was also clearly enhanced over C sites with almost negligible cleavage at A sites. Even among the various G sites, the relative intensity patterns in lane 3 was different compared to that in lane 2. The above results implied that (i) CuDFO showed an inherent cleavage selectivity for CG sites over AT sites confirming our previous results and (ii) duplex formation of target DNA 10 with 16a enhanced cleavage specificity at 3′ end of DNA 10 arising from a site-targetting of scission reactions.

Figure 8b shows analogous reaction of target DNA 10 carried out with CoDFO 4 and its oligonucleotide conjugate 16b. In contrast to reaction with CuDFO 3, under comparable conditions free CoDFO 4 did not show any cleavage of target DNA (lane 5). On the other hand, oligonucleotide conjugate 16b, upon duplexation with target DNA 10, effected a highly remarkable G-site specific scission of 10 (lane 6). Not only the bands corresponding to cleavage at non-G sites were completely absent, but also, among the four G's (G12,G16,G17 and G19), a well seen preference was indicated towards G16 (~ 55%); an almost equal specificity (~ 15% each) was observed with other three G's. These results indicated that exclusive G-site specificity for CoDFO and targetting of cleavage of 10 was achieved by its duplex formation with CoDFO conjugate 16b.

The cleavage variation noticed between CuDFO and CoDFO conjugates may arise due to mechanistic differences. The pattern of broader specificity in cleavage reaction

employing CuDFO (lane 3) points to generation of hydroxyl radicals effecting several sites (Joshi *et al* 1994a). This is also assisted by a flexible spacer chain, which enables scission moiety (Cu/CoDFO) to reach nucleotides over a wide range (from G12 and G19). In case of CoDFO, the observed G specific cuts with a maximum at G16 may indicate a preferential binding of CoDFO to G via coordination of metal centre perhaps to N7.

2.3c *Triplex mediated site specific cleavage reactions*: The targetting and cleavage of duplex DNA via third strand was followed by radiolabelling of one of the strands, carrying out hybridization and cleavage by the probes. Figure 9 shows attempts at site directed cleavage of synthetic duplex 8:9, corresponding to the polypurine: polypyrimidine stretch spanning positions 5007 to 5038 of SV40 DNA. The cleavage was targetted by 17a and comparison of lanes 1 and 2 indicate that although the targetting is only modest, a relatively intense band at G5 was noticed pointing to a cleavage preference at this site. The bar diagram depiction (figure 9b and c) reiterates this point: G5 intensity becomes maximum upon targetting. This arises from the hybridization of 17a to the duplex. placing the conjugated CuDFO in the vicinity of G5. The cleavage at adjacent sites to G5 indicates that the tether allows a fairly long and flexible reach and perhaps can be improved by a more rigid spacer and a longer third strand to better the triplex stability.

3. Materials and methods

Desferal was obtained as a generous gift from Hindustan Ciba Geigy. The nucleoside bases A, G, T and C and ME were from Sigma. FUR 7 was obtained from Fluka and MF 6 was synthesised according to literature procedure (Helberger *et al* 1949; Grundmann *et al* 1955) and characterized by HPLC, ^{1}H NMR and mass spectra. HPLC analysis were done using Perkin–Elmer pumps (PE410) equipped with Hewlett Packard photodiode array detector (1050MWD). HPLC: Solvent: 0·5% CH_3CN in TEAA 0·1 M, *p*H 6·8 under isocratic conditions; R_t(FUR) = 5·68 min, R_t(MF) = 5·96 min; λmax(FUR) = 276 nm, λmax(MF) = 266 nm. Carbonyl diimidazole (CDI), succinic anhydride, dicyclohexylcarbodiimide (DCCI) were obtained from Aldrich (USA). Radioactive γ-^{32}P ATP was obtained from the Bhabha Atomic Research Centre, India. The electrophoresis chemicals, namely, acrylamide, *bis* acrylamide, urea, sigmacote etc. were obtained from Sigma (USA). Melting studies were carried out on Perkin–Elmer Lambda 15 UV/Vis spectrophotometer attached with a temperature programmer having a Peltier heating assembly. Oligonucleotides (table 1) were synthesised on a Pharmacia Gene Assembler Plus (0·2 μmole) using phosphoramidite chemistry followed by deprotection with NH_3 and purified by HPLC. The Cu(II) 3, Co(III) 4 and Ni(II) 5 complexes were synthesised, by stirring desferal (1 eq) separately with 10 eq. of $CuCl_2$, $CoCl_2$ and $NiCl_2$ in H_2O. The complexes were purified by chromatography over Sephadex G10 and their homogeneity checked by HPLC. Column: C18 Reverse phase; Solvent system; 30% CH3CN in triethylammonium acetate buffer (0·05M), *p*H 7·0; 2, R_t = 5·80, λmax = 429 nm (ε = 2200); 3, R_t = 5·97, λmax = 427 nm (ε = 1200); 4, R_t = 6·10, λmax = 511 nm (ε = 268); 5, R_t = 6·55, λmax = 394 nm. Among the complexes 2–5, only 3 showed a reversible

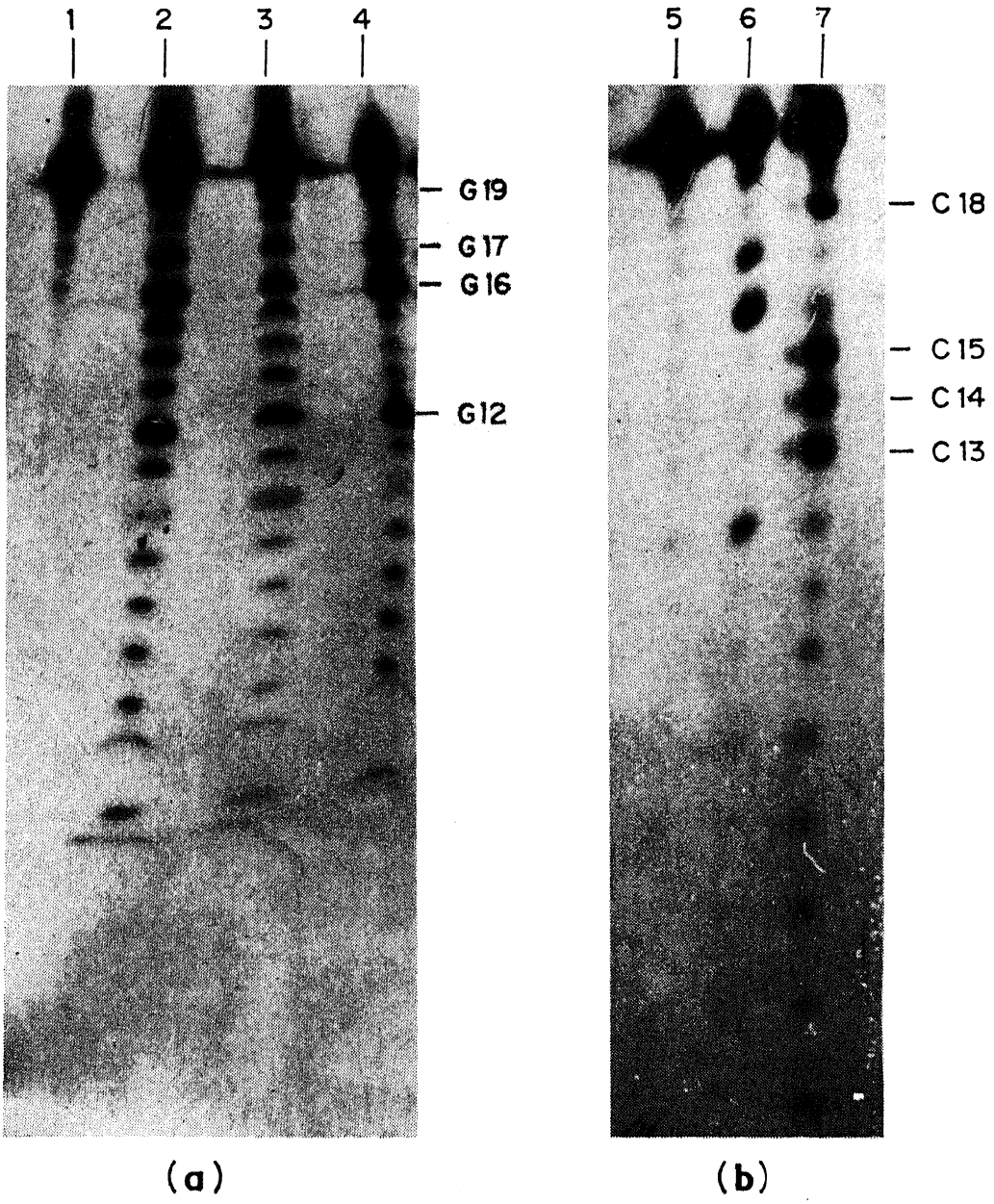


Figure 8. Polyacrylamide gel electrophoresis of DNA cleavage reactions. (a) Lane 1, control 10; lane 2, 10 + 3 (1 mM) + ME (0·5 mM) + H_2O_2 (4 mM); lane 3, 10 + 16a (17·8 μM) + ME (0·5 mM) + H_2O_2 (4 mM); lane 4, MG sequencing of 10, G reaction; (b) lane 5, 10 + 4 (1 mM) + H_2O_2 (4 mM); lane 6, 10 + 16b (27·1 μM) + H_2O_2 (4 mM); lane 7, MG sequencing of 10, C + T reaction.

signal in cyclic voltammogram (range: + 0·6 V to − 0·5 V) corresponding to an $E^0_{1/2}$ of + 0·24 V.

The oligonucleotides 8, 9 and 10 were 5′-end labelled using T4 polynucleotide kinase (10 units/200 pmole) and (γ-^{32}P)-ATP (specific activity 3000 μci/mmol) (Sambrook *et al* 1989). The labelled DNA sequences were precipitated by ethanol with the help of carrier calf thymus DNA (5 μg) and the purity of the labelled sequences checked by gel electrophoresis. Maxam Gilbert sequencing (G and C + T reactions) of γ-^{32}P labelled 10 was carried out by standard procedures (Maxam and Gilbert

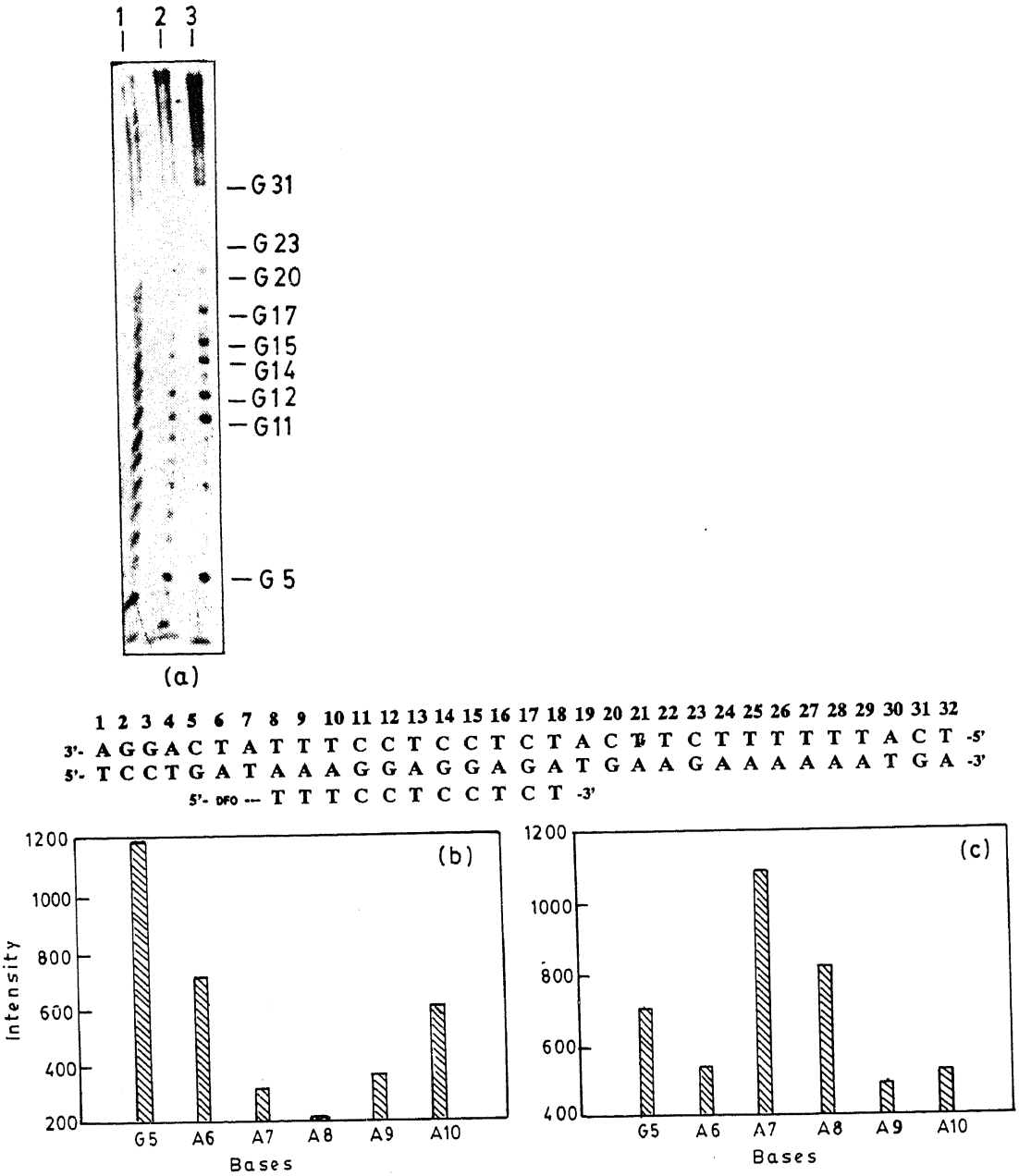


Figure 9. (**a**) Polyacrylamide gel electrophoresis of triplex targetted DNA cleavage reactions. Lane 1, Control 8 + 9; lane 2, 8 + 9 + CuDFO 3 (1 mM) + ME (0·5 mM) + H_2O_2 (4 mM); lane 3, 8 + 9 + 17a (17·8 μM)) + ME (0·5 mM) + H_2O_2 (4 mM). (**b**) Bar diagram indicating the intensity of the targetted cleavage at the bases on the target double strand DNA 8:9. (**c**) that of untargetted.

1980) and the product analysed on 20% polyacrylamide gel (0·4 mm, 38 cm × 15 cm, 1200 V, 30mA) followed by autoradiography overnight at −20°C.

3.1 *Plasmid DNA cleavage reactions*

The DNA cleavage reactions were carried out on double stranded plasmid DNA pBR322 (> 90% form-I, Bangalore Genei) by incubation for 30 min at 37°C with

various reagents (see legend Figure 1). It was followed by addition of bromophenol blue and loaded directly into different wells on a 1% agarose gel for analysis by electrophoresis at 100 V, 25mA, till the dye reached about 75% of the gel length. The gel was stained with ethidium bromide for visualisation on a transilluminator followed by photography.

3.2 *Cleavage reactions of oligonucleotides*

The cleavage reactions of synthetic oligonucleotides $d(CG)_9$, $d(AT)_9$, $d(A_3T_3)$ and $d[(CG)_nA_nT_n(CG)_n]$ were carried out using oligonucleotides (8000 cpm) in *tris* buffer (10 mM) and sodium acetate (1·2 mM) and additional reactants depended on metal complex, with the following effective concentrations. (a) CuDFO (300 μM), ME (0·5 mM), H_2O_2(4 mM). (b) CoDFO (150 μM), H_2O_2(4 mM). (c) NiDFO (150 μM), H_2O_2 (4 mM). The reactions were carried out by incubating the above mixture at 37°C for 30 min followed by freezing at −20°C and dried on a Savant SpeedVac concentrator. This was then loaded in formamide: bromophenol blue (5 μl), either directly or after piperidine treatment (100 μl, 1 M, 90°C, 30 min), into different wells on 20% polyacrylamide gel for analysis by electrophoresis at 15 mA, 400 V till the dye reached about 75% of the gel length.

3.3 *Cleavage reactions on calf thymus DNA*

Calf thymus DNA (10 OD units) in *tris* buffer (25 mM, pH 6·8) containing NaOAc (2·5 mM) was treated with CuDFO 3 (250 μM), ME (500 μM) and H_2O_2 (4 mM) in a total reaction volume of 200 μl. The cleavage reactions were effected by heating the mixture at 37°C for 30 min followed by heating at 90°C for 10 min (for HPLC analysis). The cleavage reactions with Cu-OP (85 μM) were done similarly but at 37°C for 30 min.

3.4 *Analysis of cleavage products*

The reaction samples (10–20 μl) were directly injected into HPLC column for analysis which were done on a reverse phase C18 column and eluted under isocratic conditions with 0·5% CH_3CN in TEAA (0·1 M, *p*H 6·8) with a flow rate of 2·5 ml/min. All products were detected using photodiode array detector at 254 nm. The UV spectral scan of individual peaks was recorded on-line for identification of FUR and MF.

3·5 *Detection of base propenal*

The formation of base propenals in the reaction mixture was qualitatively detected by the reagent TBA (Burger *et al* 1990). An aqueous solution of TBA (0·6%, 0·9 ml) was added to the reaction sample (100 μl) and heated for 20 min at 80°C to decompose base propenal into malondialdehyde and base. The former was analysed by its reaction with TBA to obtain a coloured complex characterised by a visible spectral band at 532 nm.

3.6 *Synthesis of CuDFO-oligonucleotide conjugates*

The synthesis of 5′-aminoalkylated oligonucleodites was carried out according to scheme 1 (Wachter *et al* 1986) using procedures of Pharmacia GA plus. After

aminoalkylation the cassette was subjected to aqueous ammonia treatment (60°C, 24 h), to deprotect base and phosphate protecting groups. Evaporation of ammonia yielded a residue that was dissolved in water and subjected to NAP (G-25) gel filtration column. The appropriate fractions were pooled, concentrated and purified by FPLC.

3·6a *Synthesis of succinoyl-MDFO and its active ester*: MDFO 3/4 (18 mg) dissolved in dry DMF (2·5 ml) was reacted with succinic anhydride (24 mg) for 24 h at 37°C followed by concentration to give the product 3a/4a. This product (33 μmol) was taken in dry DMF (1 ml) into which DCCl (6·7 mg, 0·03 mmol) and N-hydroxy-succinimide (4·9 mg, 0·04 mmol) were added. The reaction was stirred for 24 h at 37°C and the product 14/15 was isolated by concentration of the reaction mixture.

3·6b *Conjugation of MDFO to 5′ aminoalkylated oligonucleotides*: The purified 5′-aminoalkylated oligomer (12 and 13) was resuspended in 200 μl HEPES buffer (0·2 M, *p*H 7·7), to which was added a solution of the active ester of MDFO 14/15 (Cu/Co) (2 mg in 100 μl DMF). After 24 h the reaction mixture containing products 16/17 was applied to a Sephadex G-15 column (15 ml) and eluted with 10 mM triethylammonium bicarbonate (*p*H 7·0). Fractions (0·5 ml) were monitored by absorbance at 260 nm, pooled and checked by RP–HPLC.

3·7 *Melting studies on triple helix forming oligonucleotides*

The DNA melting experiments were done using the buffer *tris*-sodium acetate (2 ml, 10 mM, *p*H 7·0) containing spermine (10 μl, 20 mM) and sodium chloride (20 μl, 1 M). For duplex melting. appropriate complementary strands were taken in the ratio 1:1 based on UV absorbance and molar extinction coefficients at 260 nm (A = 15·4, T = 8·8, C = 7·3 and G = 11·7 $cm^2/\mu mol$) and for triplex melting, corresponding DNA strands were taken in the ratio 1:1:1. The oligonucleotides solutions were heated at 90°C for 10 min and annealed by slow cooling to room temperature. The melting experiment was carried out by heating the sample from 5° to 85°C at the rate of 0·5°/min while recording the absorbance every minute. For experiments below 20°C, dry nitrogen gas was flushed in the spectrometer sample to prevent moisture condensation. A plot of absorbance against temperature was sigmoidal and *tm* was obtained from the midpoint of transition (table 2).

3.8 *Duplex mediated cleavage*

The ^{32}P-labelled target DNA 10 (12000 cpm) was hybridized with unlabelled 16a (17·8 μM) or 16b (27·1 μM) in *tris*-sodium acetate (10 mM, pH 7·0), NaCl (100 mM) containing spermine (1 mM) by heating at 60°C for 3 min followed by slow cooling to 0°C. The cleavage reaction was initiated by addition of H_2O_2 (4 mM) and ME (0·5 mM) in case of 16a and only H_2O_2 (4 mM) in case of 16b and continued for 16 h at 0°C, following which it was frozen and dried on a Savant Speedvac concentrator. This resultant was treated with piperidine (1 M, 100 μl), at 90°C for 30 min and dried. All samples were denatured by heating at 90°C for 3 min and cooled immediately in ice before loading on a 20% PAGE sequencing gel (0·4 mm thick, 38 cm long). The electrophoresis was done at 1500 V and 15 mA till the dye front migrated to three-fourths of the total length of the gel.

3.9 *Triplex directed cleavage of synthetic oligonucleotides*

The labelled oligonucleotide 8 (12000 cpm) was hybridized with unlabelled 9 (0·02 OD units) in presence of 17a (17·8 μM). The hybridization was carried out in *tris* sodium acetate (10 mM, *p*H 7·0), NaCl (100 mM) containing spermine (1 mM), by heating at 60°C for 3 min followed by a slow cooling to 0°C. The cleavage reaction was initiated by addition of H_2O_2 (4 mM) and ME (0·5 mM) and continued for 16 h at 0°C, following which it was frozen and dried in a Savant Speedvac concentrator. The samples were heated with piperidine (1 M, 100 μl) at 90°C for 30 min followed by drying. The denatured samples (90°C for 3 min and cooled immediately) were analysed on a 20% polyacrylamide sequencing gel (0·4 mm, 38 cm × 15 cm) run at 1500 V and 15 mA.

4. Conclusions

It has been shown in this paper that (i) Cu(II), Co(III) and Ni(II) complexes of a siderophore chelating drug desferal cleave plasmid DNA, while Fe(II) complex is inert, (ii) CuDFO had marginal sequence preference for CG over AT for cleavage reactions, while the corresponding Co and Ni complexes nicked DNA only at CG sites and ineffective at AT sites, (iii) the Cu complex of desferal cleaves DNA, the primary site of hydroxyl radical attack being the sugar C1′ in the minor groove, (iv) the metallocomplexes of desferal can be sucessfully linked to oligonucleotides at the 5′- end by derivatisation with aminoalkyl spacer chain, and (v) oligonucleotides covalently linked with this molecular scissor can direct the cleavage of either single or double strand DNA's, mediated by duplex or triple helix structures respectively. Such targetting of DNA cleavage reactions, mediated by oligonucleotide- Cu(II)/ Co(III) desferal conjugates was possible with reasonable site specificity and efficiency. Further work is focussed on use of shorter linkers and longer dinucleotide conjugates to direct specific cleavages in large target sequences.

Desferal is the only drug used for clinical treatment of thalassemia, despite the associated cerebral and ocular toxicity observed during desferal therapy (Niehaus 1981; Davies *et al* 1983; Hershko 1993). In this context, the present results assume importance since complexation of Cu by desferal in vivo may lead to DNA cleavage and, perhaps, protein oxidation by CuDFO. The oxidative damage of DNA observed with metallodesferal complexes may also have implications in the emerging applications of microbial chelators as drug delivery agents (Miller and Malouin 1983) and antimalarials (Hershko *et al* 1988; Hallaway *et al* 1989; Traore *et al* 1991; Sullivan *et al* 1992).

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