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Probing of DNA structure with osmium tetroxide

Effect of ligands

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Fourteen OsO_4 complexes with different ligands were tested as probes of DNA structure. Of these complexes, only OsO_4-2,2′-bipyridine (Os-bipy), OsO_4-bathophenanthrolinedisulfonic acid (Os-bpds) and OsO_4-*N,N,N′,N′*-tetramethylenediamine (Os-TMEN) site-specifically modified the ColE1 cruciform in a supercoiled plasmid pColIR215 at millimolar concentrations. Os-bipy, Os-bpds and Os-TMEN also displayed site-specific modification of the B-Z junctions in the supercoiled plasmid pRW751 containing $(dC\text{-}dG)_n$ inserts.

1. Introduction

After having been introduced as a probe of the structure of DNA [1–4], the osmium tetroxide-pyridine complex (Os-py), has become one of the most frequently used chemical probes of local structures in supercoiled DNAs [5–9] (for a review, see ref. 10). When probing DNA structure with Os-py, osmium tetroxide can be applied at millimolar levels, while the concentration of pyridine must be greater by about two orders of magnitude. Recently, we have shown [11] that replacing pyridine in Os-py by 2,2′-bipyridine (bipy) makes it possible to work with osmium tetroxide and bipy at equimolar concentrations. We have found [12,13] that Os-bipy can be used to probe the DNA structure in *E. coli* cells, and have reported direct evidence of the existence of left-handed DNA in the cell [12].

In this paper, we have attempted to find other osmium tetroxide ligands that are applicable at millimolar concentrations. Such ligands in complexes with osmium tetroxide may improve some of the properties of the osmium probe and be potentially useful in probing DNA structure in various cells. We have investigated 14 different ligands and determined that bathophenanthroline disulfonic acid (bpds) and *N,N,N′,N′*-tetramethylethylenediamine (TMEN) can be applied in complexes with osmium tetroxide to probe DNA structure at concentrations similar to those of Os-bipy.

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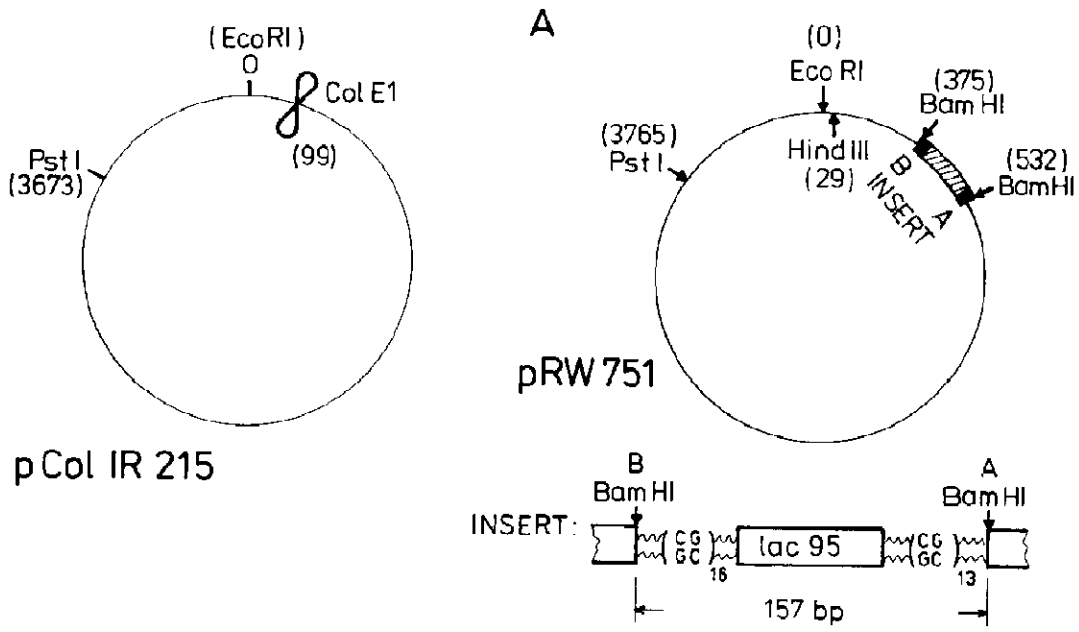
Abbreviations: py, pyridine; bipy, 2,2′-bipyridine; bpds, bathophenanthrolinedisulfonic (4,7-diphenyl-1,10-phenanthroline disulfonic) acid, disodium salt; TMEN, *N,N,N′,N′*-tetramethylenediamine; sc-DNA, supercoiled DNA; lin-DNA, linear DNA; EtBr, ethidium bromide.

2. Materials and methods

2.1. Plasmid DNA

Plasmids pColIR215, pRW751 (fig. 1a) and pUC19 were isolated according to the method



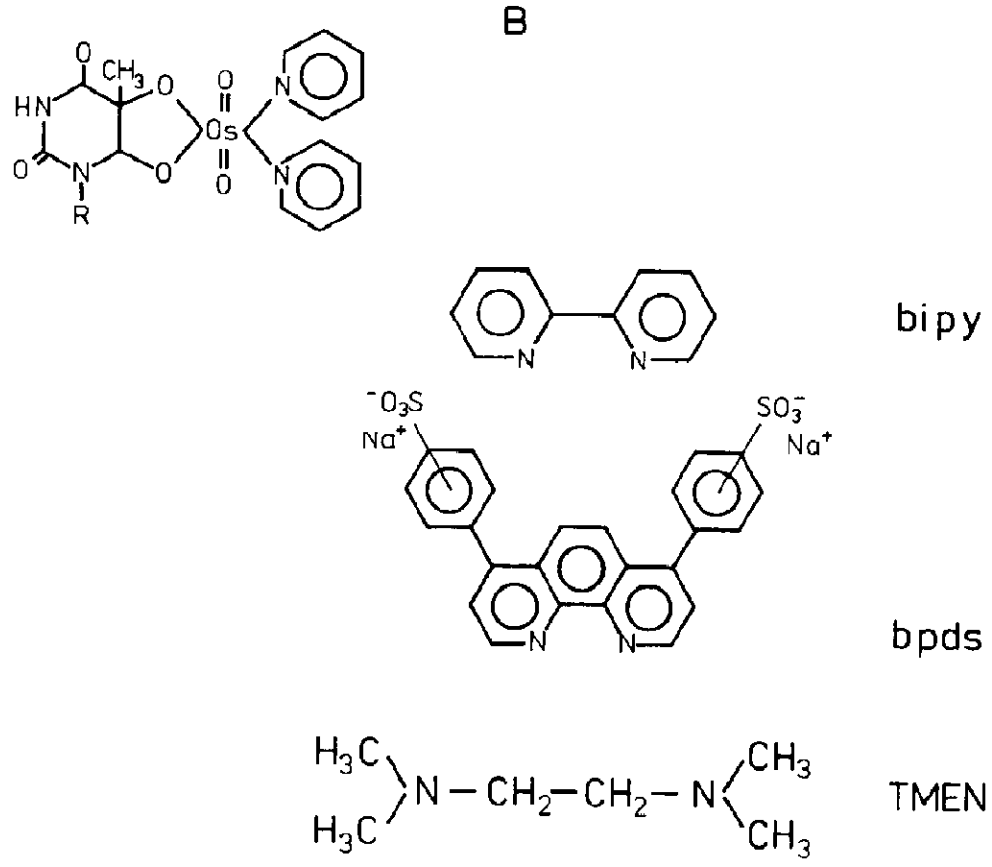


Fig. 1. (a) Maps of plasmids pColIR215 and pRW751. Plasmid pRW751 was constructed [27] by cloning the 157 bp insert into the *Bam*HI site of pBR322; this was done in such a way that the *Bam*HI recognition sequence GGATCC was regenerated at both ends of the insert. Thus, *Bam*HI recognition sites (A, B) lie on the boundary between the $(dC\text{-}dG)_n$ blocks and the pBR322 sequences (the first G of the recognition sequence being the last G of the $(dC\text{-}dG)_{13}$ block and the first C of the $(dC\text{-}dG)_{16}$ block being the last C of the recognition sequence). The segments $(dC\text{-}dG)_{16}$ and $(dC\text{-}dG)_{13}$ at the ends of the insert may (given sufficiently negative superhelical density of the plasmid [28]) be in the form of left-handed Z DNA; the plasmid then contains four B-Z junctions, the 'outer' two reaching the area of the restriction sequences B and A of restriction endonuclease *Bam*HI. (b) The Os-py complex with thymine and other ligands (bipy, 2,2′-bipyridine; bpds, bathophenanthrolinedisulfonic acid; TMEN, *N,N,N′,N′*-tetramethylethylenediamine) which can replace pyridine in the osmium complexes.

reported in ref. 14, supplemented by double protein depletion of the sample with chloroform; the final purification step involved centrifugation in a CsCl/EtBr gradient.

2.2. Chemicals

OsO_4 was obtained from Fisher, pyridine from Merck, and other ligands (fig. 1b) from Lachema

(Czechoslovakia). Other chemicals were of analytical grade.

2.3. Modification of plasmid DNA

Typically, reaction mixtures contained DNA at a concentration of 40–80 μg/ml in a medium composed of 5 mM Tris-Cl/0.5 mM EDTA (pH 7.5) (pColIR215) or 25 mM Tris-Cl/2.5 mM EDTA (pH 7.5) plus 200 mM NaCl (pRW751), a maximum of 2 mM OsO_4 plus 2% (i.e., about 250 mM) pyridine or 2 mM of the other ligands (fig. 1b). pColIR215 was modified by incubation for 10 min at 37 °C, and pRW751 for 60 min at 26 °C. DNA was precipitated with ethanol, extracted with ether and, after vacuum drying, dissolved in a defined volume of distilled water [11–13,15–18].

2.4. Enzyme cleavage

The modified DNA was cleaved after purification with restriction endonucleases (Institute of Sera and Vaccines, Prague) in a medium salt buffer [19], the concentration of *Bam*HI being at least 10 U/μg DNA or, after precipitation with ethanol, cleaved with S_1 nuclease (isolated according to the method of ref. 20).

3. Results and discussion

The substances chosen as OsO_4 ligands are listed in table 1; the ability of OsO_4 complexes with TMEN and bpds to form adducts with thymine has been previously described [21], however, reactions with nucleic acids have been reported only for the case of Os-bpds [22]. Treatment of supercoiled pColIR215 (at native superhelical density) with the 14 OsO_4 complexes (table 1) (2 mM OsO_4 and 2 mM ligand (2 mM OsO_4, 2% pyridine), for 10 min at 37 °C) revealed that among them only Os-bipy (fig. 2, lane 4), Os-bpds (fig 2, lane 5) and Os-TMEN (fig. 2, lane 6) site-specifically modify the ColE1 cruciform at millimolar (fig. 2) and submillimolar (not shown) concentrations. This modification was detectable (after DNA linearization with *Pst*I) by nuclease S_1, which produced approx. 3570 and approx. 850

Table 1

Ability of ligands in complexes with osmium tetroxide to produce site-specific modification of the ColE1 cruciform in supercoiled plasmid pColIR215

(+) Site-specific modification detected using nuclease S_1 (after DNA linearization with *Pst*I) in the presence of 2 mM OsO_4 and 2 mM ligand (10 min at 37 °C). (−) No site-specific modification observed under the given conditions.

OsO_4-ligand	Site-specific modification
Os-py	+ [a]
-bipy	+
-4,4′-bipyridine	−
-2,2′-dithiobipyridine	−
-1,10-phenanthroline	+ [b]
-bpds	+
-2,9-dimethyl-1,10-phenanthroline	−
-5-nitro-1,10-phenanthroline	−
-TMEN	+
-8-hydroxy-5-quinoline	−
-8-hydroxy-5-quinolinesulfonic acid	−
-8-hydroxy-7-iodo-5-quinolinesulfonic acid	−
$-CN^-$	−
$-SCN^-$	

[a] The presence of at least 0.5% pyridine was necessary.
[b] In addition to cruciform modification, reactions at other sites were also detectable.

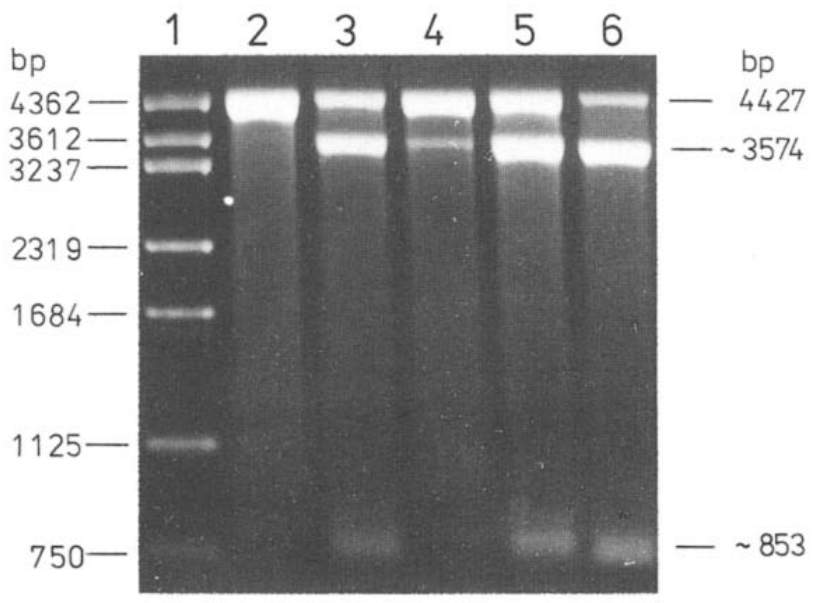


Fig. 2. Site-specific modification of the cruciform in pColIR215 with various osmium tetroxide complexes as detected by nuclease S_1 cleavage. Supercoiled DNA was reacted (10 min at 37 °C) with 2 mM OsO_4 plus 2% py (lane 3), 2 mM Os-bipy (lane 4), 2 mM Os-bpds (lane 5) or 2 mM Os-TMEN (lane 6) and after purification linearized with *Pst*I and cleaved with nuclease S_1. For comparison, lane 2 contains unmodified control cleaved with *Pst*I plus nuclease S_1 and lane 1 shows molecular weight markers. Positions of linear DNA (4427 bp) and fragment lengths are denoted.

Table 2

Fragments arising after osmium tetroxide modification and restriction enzyme cleavage of pRW751 (cf. fig. 1a)

RS, *Bam*HI restriction site (cf. fig. 1a); A, RS adjacent to the $(dC\text{-}dG)_{13}$ segment; B, RS adjacent to the $(dC\text{-}dG)_{16}$ segment.

Modification	Cleavage	Fragments
–	*Bam*HI	4362, 157 bp
RS A		lin-DNA (4519 bp)
RS B		lin-DNA (4519 bp)
RS B+A		sc-DNA
–	*Eco*RI/*Bam*HI	3987, 375, 157 bp
RS A		4144, 375 bp
RS B		3987, 532 bp
RS B+A		lin-DNA (4519 bp)

bp fragments (fig. 1a), consistent with cleavage at the cruciform loop (observed previously [23] in the same plasmid after treatment with Os-py (fig. 2, lane 3)). Under identical conditions, 1,10-phenanthroline, in addition to cruciform modification, also underwent reactions at other sites (not shown). The other ligands (table 1) displayed no detectable modification by nuclease S_1 in pColIR215 DNA under the same conditions (not shown).

It has been shown earlier [15,24] that the structural distortions which occur at the boundary between the left-handed Z and right-handed B DNA conformations are recognized by Os-py. Site-specific modification of the B-Z junction can be detected in various ways [10,25]. The most sensitive and simplest means of detection is based on inhibition of restriction enzyme cleavage [11–13,15,18,26], provided a restriction site forms part of the B-Z junction. In pRW751 (ref. 27, fig. 1a) the first G of the *Bam*HI recognition sequence GGATCC is the last G of the $(dC\text{-}dG)_{13}$ segment and the first C of the $(dC\text{-}dG)_{16}$ is the last C in GGATCC, and, therefore, investigation of *Bam*HI inhibition can yield information concerning modification of the B-Z junction (table 2).

We tested the inhibition of *Bam*HI cleavage in order to ascertain the ability of osmium tetroxide complexes with py, bipy, bpds and TMEN to modify site-specifically the B-Z junction in pRW751 DNA. Treatment (60 min at 26 °C) of supercoiled DNA with 2 mM Os-bpds (fig. 3, lane 7) and Os-TMEN (fig. 3, lane 8) as well as with 2 mM Os-bipy (ref. 11; fig. 3, lane 6) and 2 mM OsO_4-py (2%) (refs. 11 and 18; fig. 3, lane 5) resulted in strong inhibition of *Bam*HI cleavage (table 2) manifested by the appearance of linear DNA (4519 bp, cleavage inhibition at one site) and supercoiled DNA (inhibition of cleavage at both sites) while complete cleavage in an unmodified control was observed (fig. 3, lane 4). pUC19 DNA, which was added to each sample prior to modification with osmium tetroxide, was com-

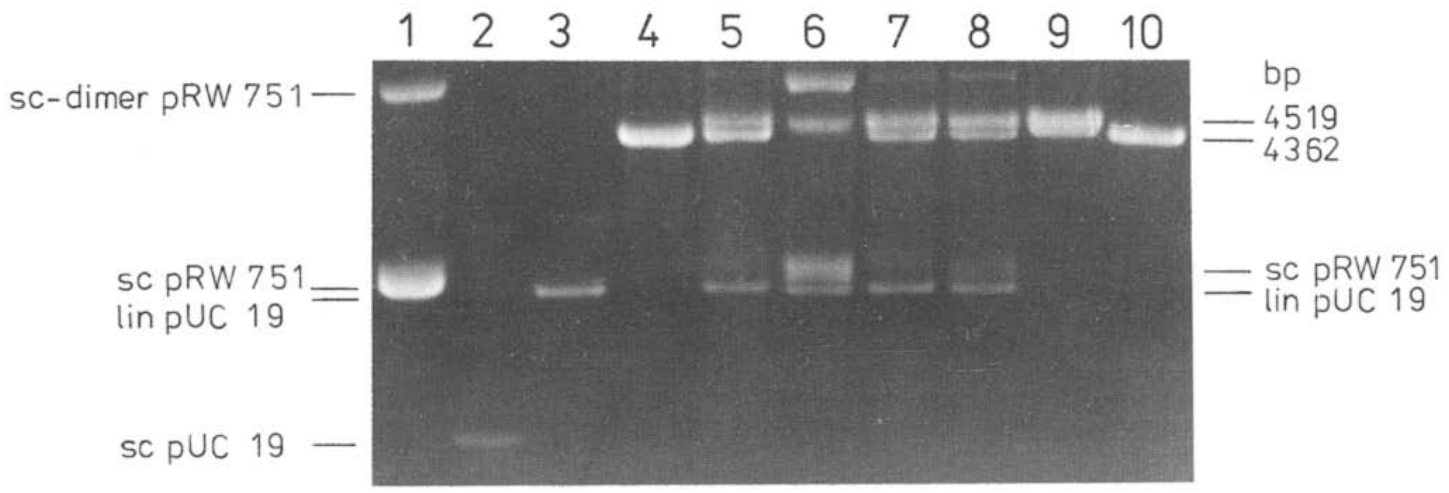


Fig. 3. Site-specific modification of the B-Z junctions in pRW751 with various osmium tetroxide complexes as detected by inhibition of *Bam*HI cleavage. A mixture of supercoiled DNAs pRW751 and pUC19 was reacted (60 min at 26 °C) with 2 mM OsO_4 plus 2% py (lane 5), 2 mM Os-bipy (lane 6), 2 mM Os-bpds (lane 7) or 2 mM Os-TMEN (lane 8) and after purification cleaved with *Bam*HI. For comparison, lane 4 contains unmodified pRW751 cleaved with *Bam*HI, lane 3 pUC19 cleaved with *Bam*HI, lane 1 supercoiled (uncleaved) pRW751 and lane 2 superhelical pUC19. Lane 9 contains pRW751 cleaved with *Eco*RI (linear DNA, 4519 bp) and lane 10 pBR322 cleaved with *Bam*HI (4362 bp). Positions of supercoiled pRW751, supercoiled and linear pUC19 DNAs and fragment lengths are denoted.

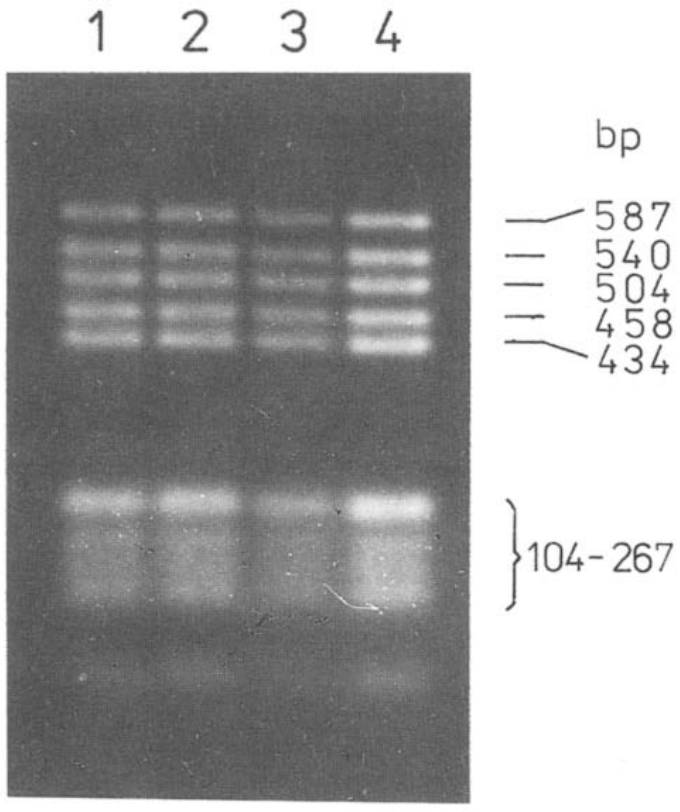


Fig. 4. Effect of modification of pRW751 with various osmium tetroxide complexes on cleavage with restriction endonuclease *Bsp*RI. Supercoiled DNA was reacted (60 min at 26 °C) with 2 mM Os-bipy (lane 2), 2 mM Os-bpds (lane 3) or 2 mM Os-TMEN (lane 4) and after purification cleaved with *Bsp*RI. For comparison, lane 1 contains unmodified pRW751 cleaved with *Bsp*RI. Fragment lengths are denoted.

pletely cleaved with *Bam*HI (fig. 3, lanes 5–8). Similarly, no inhibition of *Bsp*RI cleavage of supercoiled pRW751 modified with 2 mM Os-bipy (fig. 4, lane 2), Os-bpds (fig. 4, lane 3) or Os-TMEN (fig. 4, lane 4) and at multiple restriction sites was observed. These results suggest that inhibition of *Bam*HI cleavage observed in modified pRW751 samples (fig. 3, lanes 5–8) was not due to the presence of impurities in the samples or to random DNA modification, but was the result of site-specific modification of the B-Z junctions.

In agreement with previous results obtained with Os-py and Os-bipy [11–13,15,18], inhibition of *Bam*HI cleavage due to modification of supercoiled pW751 DNA with Os-TMEN (fig. 5) and Os-bpds (not shown) was also observed when *Bam*HI cleavage was tested after DNA linearization with *Eco*RI (table 2). Treatment (60 min at 26 °C) with 0.5 mM Os-TMEN resulted in the appearance of a 4144 bp fragment corresponding to *Bam*HI cleavage inhibition at site A (fig. 5, lane 4); under the same conditions, 1.0 and 1.5 mM Os-TMEN induced stronger inhibition at site A; this treatment also resulted in the appearance of linear (4519 bp) DNA (fig. 5, lanes 5 and 6), corresponding to the inhibition of *Bam*HI cleavage at both restriction sites (A and B). No inhibition of *Bam*HI cleavage was observed in an unmodified control (fig. 5, lane 3) and in samples which were first linearized with *Eco*RI and then treated (60 min at 26 °C) with either 2 mM Os-bipy (fig. 5, lane 7), Os-bpds (fig. 5, lane 8) or Os-TMEN (fig. 5, lane 9).

Our results show that in addition to Os-bipy [11–13], Os-bpds and Os-TMEN represent valuable new probes of DNA structure which, under the conditions usually employed, can be applied at millimolar (figs. 2 and 3) or submillimolar (fig. 5) concentrations. The negative results obtained with

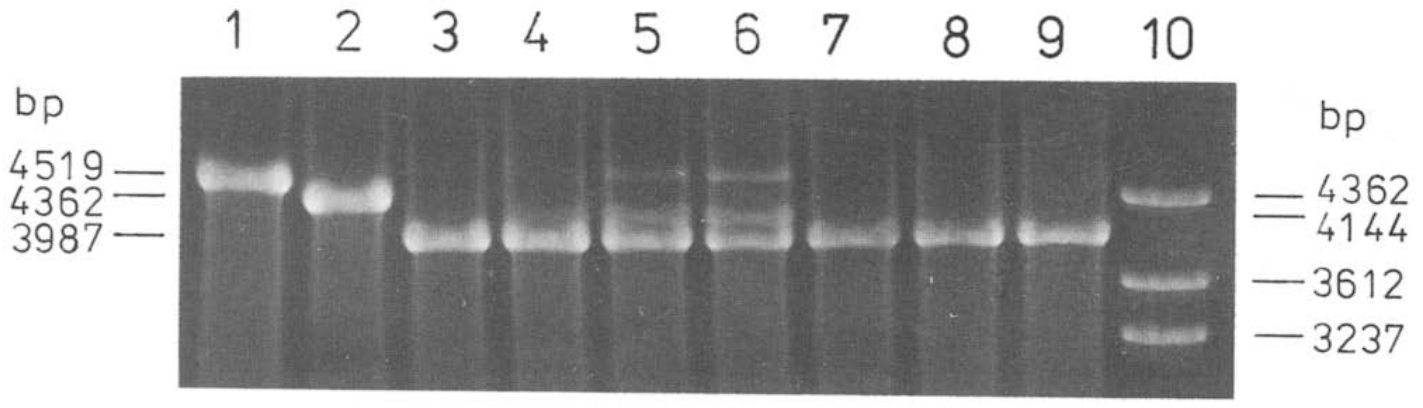


Fig. 5. Dependence of Os-TMEN concentration on site-specific modification of the B-Z junctions in pRW751. Modification of linearized pRW751. Supercoiled DNA was reacted (60 min at 26 °C) with 0.5 mM (lane 4), 1.0 mM (lane 5) or 1.5 mM (lane 6) Os-TMEN and after purification linearized with *Eco*RI and cleaved with *Bam*HI. Superhelical DNA was linearized with *Eco*RI, modified with 2 mM Os-bipy (lane 7), 2 mM Os-bpds (lane 8) or 2 mM Os-TMEN (lane 9) and after purification cleaved with *Bam*HI. For comparison, lanes 1–3 contain unmodified DNA cleaved with *Eco*RI, *Bam*HI or *Eco*RI plus *Bam*HI, respectively. Lane 10 contains molecular weight markers. Positions of linear DNA (4519 bp) and fragment lengths are denoted.

several compounds presented in table 1 do not necessarily mean that the given substance does not react at all with DNA; in fact, some gave rise to positive results (P. Boublikova, unpublished data) at substantially higher concentrations. Os-bipy, Os-bpds and Os-TMEN complexes (fig. 1b) differ greatly in size and other properties and one may expect that such differences might come into play in the selectivity of the probes for specific structures as well as probe penetration and interactions in the cell when applied in studies of DNA structure in situ. On comparison of the efficiency of Os-py, Os-bipy, Os-bpds and Os-TMEN in our experiments, one can see that the effect of Os-bipy is weakest with the cruciform (fig. 2, lane 4) and strongest in the case of the B-Z junction (fig. 3, lane 6). Further work, including piperidine cleavage of modified sites and sequencing is required in order to explain this difference.

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